School of Public Health

Increasing the Gelling Functionality of Lupin Protein Using Ultrasound Treatment

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Declaration

To the best of my knowledge and belief, this thesis titled “Increasing the Gelling Functionality of Lupin Protein Using Ultrasound Treatment” contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

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Abstract

Food markets predict that the demand for plant protein will increase because of the increasing number of consumers who are looking for healthier food options and sustainable food production. Plant protein sources are capable to provide food with required nutritional value and texture with low environmental footprint. Lupin is considered to be an abundant source of protein with high nutritional value. However, its weak gelation and thickening properties limit its use in a wide range of food applications. Protein gelation depends on unfolding of the protein structure by heat, chemical or enzymatic treatment to create a three-dimensional protein network. This network can entrap water, oil, flavour compounds and other food ingredients. Few studies have used emerging technologies such as ultrasound to focused on modifying lupin protein-gelation properties. This thesis focused on improving lupin protein-gelation properties using ultrasound treatment and uncovered a link between lupin protein structural limitations and its gel quality.

In this thesis, an experimental program was conducted using a cold-set gel system to examine the link between changes in lupin protein physicochemical properties triggered by ultrasound treatment and lupin protein gel quality. This research was performed in three main stages.

In the first stage, a screening study of lupin variety was conducted to understand the effect of the *Lupinus angustifolius* variety and growth location and their interaction on kernels and lupin protein concentrate (LPC) and protein techno-functionality (solubility, foaming, emulsifying, viscosity and gelation) of LPC.

In the second stage, the effects of ultrasound treatment time and power on lupin protein physicochemical properties were investigated. The parameters measured included particle size by dynamic light scattering, zeta potential, solubility, differential scanning calorimetry, Fourier-transform infrared spectroscopy (FTIR) and electrophoresis. Gel strength (Gs), water-holding capacity (WHC) and gel storage modulus (G′) were measured to evaluate lupin protein gel quality.

In the third stage, response surface methodology was performed in two steps to optimise lupin protein gel quality. The first step was factorial screening of the ultrasound

treatment time (min), ultrasound treatment power (W/cm^2), heat treatment temperature (°C), heat treatment time (min) and pH to identify the most significant factors controlling lupin protein-gelation properties. In the second step, central composite rotatable designs (CCRD) were used to identify the optimal levels of independent factors to optimise LPC gel quality. The results show that the variety and location and their interaction have limited effect on lupin protein gel quality. This finding highlights the need for modifying lupin protein to improve its weak functionalities to increase its use as a human food ingredient. The use of ultrasound treatment as a physical technique for protein modification shows its ability to modify lupin protein physiochemical properties. Ultrasound treatment reduced protein particle size, zeta potential and denaturation enthalpy of LPC. The FTIR results indicated that unfolding and aggregation of lupin protein occurred and presented as broader β-sheet peak formation at 1618 cm^{-1}. Lupin protein gelation properties GS, WHC and G′ improved significantly after ultrasound treatment.

The factorial screening study revealed a significant influence of ultrasound time, ultrasound power and heat treatment temperature on lupin protein gel properties. The CCRD model results showed that independent factors create a synergetic effect on lupin protein, which improved its gel rheological properties significantly. The model indicated that ultrasound treatment time (19.8 min), power (~38 W/cm^2) and heat treatment temperature (94°C) were the optimal levels of these independent factors for maximising LPC acid-induced Gs, WHC and gel yield. In addition, the validated model could produce a wide range of gel quality (soft-like to solid-like) by using the desirability function. This versatility may be helpful for producing modified lupin protein with different gel quality to suit a wide range of food applications.

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Awards, Grants and Publications

Awards

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Statement of Contribution

To Whom It May Concern，

I, Hayder A. Al-Ali, am the major contributor to this thesis titled “Increasing the Gelling Functionality of Lupin Protein Using Ultrasound Treatment”. The thesis and all manuscripts that will be submitted for publication from the thesis were primarily conducted, interpreted, discussed, written and edited by me. This was under the main supervision and guidance of A/Prof Stuart K. Johnson and co-supervision of Dr Mark Hackett, Dr Mohammad Gulzar and Dr Emmanuel Karakyriakos.

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A/Prof Stuart K. Johnson (Lead supervisor)

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List of Abbreviations

AA	Amino acid
Abs_{500}	Ultraviolet light bsorption at 500 nm
ASL	Australian sweet lupin
ATR	Attenuated Total Reflectance
ANOVA	Analysis of varianc
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CCRD	Central composite rotatable design
D	Desirability function
db	Dry basis
DSC	Differential scanning calorimetry
EAI	Emulsifying activity index
EC	Effect of contribution
ER	Eradu
ESI	Emulsifying stability index
FAO	Food and Agriculture Organization of the United Nations
FC	Foaming capacity
FS	Foaming stability
FTIR	Fourier-transform infrared spectroscopy
G′	Storage modulus
G″	Loss modulus
G × E	Genotype by environment
GDL	Glucono-δ-lactone
GM	Genetically modified
Gs	Gel strength
Gy	Gel yield
HIU	High-intensity ultrasound
HIUp	High-intensity ultrasound power

HIUt	High-intensity ultrasound time
HTt	Heat treatment time
HTT	Heat treatment temperature
IP	Isoelectric precipitation point
LPC	Lupin protein concentrate
LPI	Lupin protein isolate
Mw	Molecular weight
ND	Not determined
PC	Protein concentrate
P	Power
PPI	Pea protein isolate
RSM	Response surface methodology
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SH	Sulfhydryl
SPI	Soybean protein isolate
S-S	Disulphide bond
T_{onset}	The temperature when the denaturation start
T_{peak}	Denaturation temperature
tan δ	Tangent δ
UF	Ultrafiltration
UV	Ultraviolet light
WH	Wongan Hills
WHC	Water-holding capacity
ΔH	Denaturation enthalpy
ζ	Zeta potential

CHAPTER 1

General Introduction

1.1. Background

The ever-expanding world population, food security challenges, drought and increased number of consumers looking for sustainable, healthier and cheaper food options are factors that encourage the exploration for alternative protein sources (Rizzo & Baroni, 2018; Stone et al., 2018). In the past few years, the demand for plant protein has increased given the proven feasibility of replacing animal protein in terms of techno-functionality and nutritional value (González-Pérez & Arellano, 2009; Sá et al., 2020). There is growing interest in new plant protein sources within the food industry, especially those with higher nutritional value, fewer anti- nutritional factors, and sustainable and cheaper production than the currently dominating sources such as soybean (Sá et al., 2019).

Lupins are protein-rich legumes belonging to the genus *Lupinus* (family of Fabaceae), with around several hundred species. The most cultivated species are *L. angustifolius, L. albus, L. atlanticus, L. consentinii* and *L. luteus* (Foley et al., 2015). Given the intrinsic nutritional, economic and environmental benefits of lupin seed, there is a global interest in lupin as a potential new source of plant protein that may contribute to food security (Gao et al., 2011; Johnson, Clements, Villarino, & Coorey, 2017). The ability of lupin plants to fix nitrogen has led to improved cereal yields and grain quality by using lupin as a rotation crop in the wheatbelt farming system of Western Australia, which has in turn reduced the cost of nitrogen fertiliser (David, 1998). The reduced use of chemical fertiliser can add environmental benefits though reduced water pollution and greenhouse gas emissions (McMichael, Powles, Butler, & Uauy, 2007; Association International Fertilizer Industry, 2009).

Global lupin production was 1,160,867 tonnes in 2018 of which Australia produced around 61% of the worldwide production (FAO-STAT, 2018). In terms of lupin species, *L. angustifolius* (narrow-leaved lupin) comprises 95% of Australian lupin production and is grown mainly in Western Australia. Fifty per cent of lupin seeds produced in Western Australia in 2017 were exported (Grains Research And

Development Corporation, 2017). *L. angustifolius* is known as Australian sweet lupin (ASL) because of its low content of bitter and potentially toxic alkaloids (Gladstones et al., 1998). ASL seed is high in protein and dietary fibre, but lower in fat and potentially toxic phytoestrogens compared with soybean (Berger et al., 2013; Gladstones et al., 1998; Sirtori, Arnoldi, & Johnson, 2005).

Lupin is considered to be a non-genetically modified plant (ISAAA, 2016). Similar to other legumes, lupin protein contains high levels of lysine, which is deficient in most cereal proteins (Drakos, Doxastakis, & Kiosseoglou, 2007; Gulewicz et al., 2008). In addition, despite its higher content of sulphur amino acids (AA) such as methionine and cysteine compared with other legumes, the sulphur amino acids content remains below the level of human nutritional needs (Anna Arnoldi, 2011). Lupin protein has been reported to have higher digestibility than other legumes, possibly because of its low content of anti-nutritional factors, such as lectins and protease inhibitors, which inhibit digestion of other legumes (Chew et al., 2003; El-Adawy et al., 2000).

The main fraction of lupin seed protein contains globulins, which account for 87% of the total protein. The remaining lupin protein consists of albumins (Duranti et al., 2008; Petterson, 1998; van de Noort, 2016). The globulin proteins fractions are α-conglutin (35-37%), which appears as multiple sub-units of molecular weight, 50-80 kDa; β-conglutin (44-45%), which appears as multiple sub-units of 20-60 kDa; γ-conglutin (4-5%0, which appears as two subunits of 17 and 30 kDa; and δ-conglutin (10-12%), which appears as two subunits of 4 and 9 kDa (Blagrove & Gillespie, 1975; Duranti et al., 2008; Foley et al., 2015). The α, β-conglutins are rich in glutamic acid, in contrast to γ-conglutin, which contains a high content of essential sulphur AAs.

Lupin protein isolation and fractionation are conducted using conventional alkaline extraction followed by isoelectric precipitation (Chew, Casey, & Johnson, 2003). The major isoelectric precipitate (pH 4.5) lupin protein fraction α- and β-conglutin exhibits excellent emulsifying properties, and the acid-soluble fraction (γ-conglutin) shows good foaming properties (Wong et al., 2013). Commercially, lupin protein fractions are classified according to their techno-functionality as the E (emulsifying)

fraction ((α- and β-conglutin) and F (foaming) fraction (γ-conglutin) (Sironi et al., 2005; Wasche et al., 2001). However, there is no report that these lupin protein fractions exhibit any thickening or gelling ability.

Semi-solid gel texture is one of the most desirable protein techno-functional properties for food processing because it gives sensory and textural acceptability to a wide range of processed foods (Valim et al., 2009). The ability of a protein gel to retain a substantial amount of water with a stable semi-solid structure is considered to be a viable method for producing low-energy foods (Aguilera & Rademacher, 2004). It has been reported that native lupin protein has weak gelation properties compared with other legumes, which limits its usability in a wide range of food applications (Berghout, Boom, et al., 2015; Vogelsang-O'Dwyer et al., 2020). In the literature, there is a lack of studies have focused on improving lupin protein gelation properties using novel technologies such as ultrasound.

Several studies have highlighted the potential of ultrasound treatment for improving plant protein (soy and pea) gel quality by changing the protein physiochemical properties to facilitate new intermolecular interactions through disulphide, hydrophobic, electrostatic and hydrogen bonding. It has been reported that ultrasound treatment improves protein gel strength, water-holding capacity (WHC), gel yield and gel storage modulus (G′) (Hu, Fan, et al., 2013; Hu, Wu, et al., 2013; Chuan He Tang et al., 2009; Zhang et al., 2016). Ultrasound can be used to modify protein structure by facilitating protein unfolding and structural conformational changes, which lead to improved gelling properties of plant proteins. However, there is no published research on whether ultrasound can improve the intrinsically poor gelation properties of lupin protein.

1.2. Aims

This study aimed to provide new knowledge about the techno-functional properties of ASL protein. This new knowledge may lead to increased utilisation of this sustainable pulse crop as a plant protein source by the food manufacturing industry and thus increase its value and returns for lupin farmers. The specific aims were as follows.

1- Identification of the ASL variety with the most useful protein techno-functional properties. This knowledge will help lupin breeding program to develop further new varieties with greater protein techno-functionality and to direct lupin farmers to grow the best variety for food processing applications.

2- Increase and optimise lupin protein gel quality. This may encourage the food industry sector to investigate the potential use of lupin protein as a texturising agent in a range of other products.

1.3.Objectives

The objectives of this thesis research were to:

1- Investigate the effects of *Lupinus angustifolius* genotype and production environment on the functional properties of its seed protein.

2- Investigate the potential of ultrasound treatment for modifying lupin protein physiochemical structure and improving gel quality.

3- Optimise the effects of ultrasound treatment on lupin protein gelation properties using statistical predictive modelling and response surface methodology.

CHAPTER 2

Review of Literature

Abstract

There is growing focus worldwide on lupin protein as a healthy and sustainable plant protein source compared with other plant proteins such as soybean and pea. Despite lupin's good nutritional value, it is still underutilised because of deficiencies in its protein techno-functionality. This reduces the use of lipid protein as a functional food ingredient. However, efforts to improve lupin techno-functionality are lacking, especially using emerging technologies such as ultrasound. This review discusses the possible relationship between the structural properties of the main lupin protein globulin fractions (α- and β-globulin) and lupin protein gelation properties. It also highlights the possibility of improving lupin protein gelation properties using ultrasound treatment, which is an emerging physical, non-thermal and non-chemical protein-modification technique by discussing its use with other proteins such as soybean protein.

2.1. Introduction

The Latin word *legume* means 'seeds harvested in pods' (Uebersax & Occeña, 2003). The second common name for legumes is pulse, which is derived from Latin *puls* and refers to mature seeds or grains that can make pottage, a thick soup (Uebersax & Occeña, 2003). Legumes comprise around 13,000 species under the Fabaceae (Leguminosae) family. However, only around 20 species have been adopted for global commercial production (Uebersax & Occeña, 2003; Zhou et al., 2013). Commercially, the USA, Brazil and Argentina are the biggest legume producers. Soybean, lupin, chickpea, fava-broad bean, field pea, lentil, and mung bean are the main legume crops produced globally (FAO-STAT, 2018).

Legumes are an abundant source of protein and are a potential alternative to animal protein. For example, the average protein content is around 20% in pea and 38-40% in soybean and lupin (Duranti, 2006; Raikos, Neacsu, Russell, & Duthie, 2014; Chardigny & Walrand, 2016). Protein concentrates and isolates are widely produced from legumes (Foschia, Horstmann, Arendt, & Zannini, 2017). An estimated 1.7 million tonnes of

isolated plant protein is consumed as a food ingredient, and soybean protein dominates the market by 99% (Chardigny & Walrand, 2016).

Lupin protein has recently gained commercial interest because of its valuable nutritional properties, but its lack of thickening and gelling functionality has restricted its use as a human food ingredient. To overcome the techno-functional limitations of plant proteins, various modification techniques have been used including physical methods such as extrusion, high pressure, ultrasound, microwave energy, osmotic pressure, pulsed electric field, moist heat treatment and gamma irradiation; chemical methods such as crosslinking and oxidation; enzymatic methods such as genetic modification; addition of additives; or a combination of techniques (Speroni, Beaumal, de Lamballerie, Anton, Anon, et al., 2009). However, few studies have investigated the use of these methods to improve lupin protein functionality. These modification methods may improve techno-functionality (e.g. solubility, viscosity, gelling, foaming, emulsifying) as well as bio-functionality (e.g. nutritional, digestibility and hypoallergenic properties) by altering the structure of the proteins at all levels of conformation (Mirmoghtadaie et al., 2016).

Compared with the technological approaches, chemical modifications have some drawbacks such as the potential toxicity, impaired nutritional value and complexity. Enzymatic methods may not be cost-effective (Klompong et al., 2007). Therefore, most of the current research focuses on innovative and novel physical techniques, which are highly efficient at altering protein functionality (Mirmoghtadaie et al., 2016). Herein, this chapter provide insight into the investigation of lupin protein structural functionality by much emphasising the properties related to gelation. In addition, factors controlling the gelation of plant proteins are discussed in detail. Finally, innovative and emerging technology (ultrasound energy) that may improve lupin protein gelation are evaluated critically.

2.2. Lupins

Lupins are legumes of the *Lupinus* sp. and includes several hundred species (Foley et al., 2015). However, only five are fully domesticated: *L. angustifolius, L. albus, L. atlanticus, L. consentinii* and *L. luteus* (Gladstones, 1998, van Barneveld, 1999). There is global interest in lupin seed and its production because of its intrinsic nutritional, economic and environmental benefits (Gao et al. 2011, Johnson et al., 2017). One example is the ability

of lupin plants to fix nitrogen. This has led to improved cereal yields and quality by using them as a rotation crop in the wheatbelt of Western Australia, which has in turn reduced the amount of nitrogen fertiliser required (David, 1998). In addition to the reduced use of chemical fertiliser, there are other environmental benefits including reduced water pollution and greenhouse gas emissions (Association International Fertilizer Industry, 2009; McMichael et al., 2007). From a nutritional point of view, lupin seed is higher in protein than most legumes and has low levels of anti-nutritional factors (Chew et al., 2003; Petterson, 1998). Global lupin production was estimated to be around 1.1 million tonnes in 2018 of which Australia produced around 50% of the world production followed by the Russian Federation and Poland at 12% and 11%, respectively (FAO-STAT, 2018).

2.2.1. Australian sweet lupin (*Lupinus angustifolius*)

L. angustifolius (narrow-leaved lupin) comprises 95% of Australian lupin and is grown mainly in Western Australia (White, French, McLarty, & Grains Research and Development Corporation, 2008). There are around 25 *L. angustifolius* genotypes planted in Australia at the commercial scale (e.g. *PBA Gunyidi*, *PBA Barlock*, *PBA Jurien*, *Jenbillup*, *Mandelup* and *Coromup*) (Department of Agriculture and Food, 2016). Fifty per cent of lupin seeds produced in Western Australia are exported (Grains Research And Development Corporation, 2017). Most lupin produced in Australia is used as low-value animal feed. However, lupin production holds promise for greater economic advantages for farmers given the increasing demand for plant proteins as human food ingredients (Duranti, Consonni, Magni, Sessa, & Scarafoni, 2008).

Modern commercially grown varieties of *L. angustifolius* are known as Australian sweet lupin (ASL) because of their low content of bitter and potentially toxic alkaloids (Chew, Casey, & Johnson, 2003; Taylor, 2017). ASL seed is high in protein and dietary fibre, but lower in fat and potentially toxic phytoestrogens compared with soybean (Sirtori, Arnoldi, & Johnson, 2005, Berger et al., 2013). There is an enormous genotypic diversity of ASL seeds (White et al., 2008, Department of Agriculture and Food, 2016) with wide variation in protein composition (Kim, Mullan, Heo, Hernandez, & Pluske, 2009). Commercially, the demand for plant protein has increased (White et al., 2008). Lupin is considered to be an excellent alternative protein source to soybean because of its high protein content (Table 2.1), high protein digestibility, phytoestrogen free, non-

genetically modified (GM) and low content of anti-nutritional factors (Department of Agriculture and Food, 2016; El-Adawy et al., 2001). However, the use of ASL protein as a food ingredient at the industrial scale remains limited, mainly because of the lack of techno-functionality of the main lupin protein fraction compared with soybean. This may be because of the lack of understanding about how to maximise the techno-functionality of the lupin main protein fraction compared with soybean isolates, which have been investigated extensively (Hu, Fan, et al., 2013; Kinsella, 1998; Min et al., 2005; Tang & Ma, 2009).

2.2.2. Composition of Australian sweet lupin seeds

ASL seeds contain high protein content (32.1 g/100 g) in the whole seed and around 40 g/100 g of protein in dehulled kernels (Petterson, 1998). Lupin also has the highest content of dietary fibre among legumes, which is around 40 g/100g in whole seeds (Lucas et al., 2015). ASL kernel flour contains around 37.8 g/100 g dietary fibres, which can be divided into 29.5 g/100 g insoluble and 8.3 g/100 g soluble dietary fibres (Table 2.1) (Coorey, Chao, Kumar, & Jayasena, 2013). ASL kernels have 6.9 g/100 g fat and around 2.8 g/100 g minerals (Petterson, 2004, Coorey et al., 2013). Lupin seeds are also considered to be a good source of calcium, copper, iron, manganese, phosphorus and zinc (Petterson, 1998) and vitamins such as α, γ and δ tocopherols, thiamine, riboflavin and vitamin C (Martínez-Villaluenga, Frías, & Vidal-Valverde, 2006).

Table 2. 1 Chemical composition of ASL kernels.

Component	Content g/100g db
Protein	39.0
Lipid	7.6
Ash	3.6
Total dietary fibre	37.8

(Coorey, Chao, Kumar, & Jayasena, 2013; White et al., 2008; Wong et al., 2013)
db, dry weight basis

2.2.3. Lupin protein nutritional quality

Lupin seeds are an important potential new source of plant protein that may contribute to food security (Multari, Stewart, & Russell, 2015) and are a rich source of protein (Duranti et al., 2008, White et al., 2008), although the protein level can vary according to genotype (Villarino et al., 2015). Lupin protein has a good balance of essential amino acids (AA) (Drakos, Doxastakis, & Kiosseoglou, 2007) and has high levels of lysine to meet human nutritional needs (Table 2.2). However, like most other legumes, lupin is deficient in the sulphur AAs methionine and cysteine (Gulewicz et al., 2008, Arnoldi, 2011). Lupin has similar content of sulphur AAs cysteine and methionine (3.58g/16 g N) as other legume seed proteins such as pea (1.79 g/16 g N) and soybean (2.43 g/16 g N) (Table 2.2) (Chin et al., 2019; Denke, 2001; Leterme, Monmart, & Baudart, 1990; Petterson, 1998; Sujak, Kotlarz, & Strobel, 2006). In addition, lupin protein has been reported to have similar digestibility to soybean of around 98% (Chew et al., 2003; Hughes et al., 2011), which may be related in part to its low anti-nutritional factor level (Cerletti & Duranti, 1979, Rodriguez-Ambriz, Martinez-Ayala, Millin, & Davila-Ortiz, 2005). Lupin protein has been considered as an allergen. Food Standard Australia New Zealand (FSANZ) has included lupin protein in the allergenic food list (peanuts, tree nuts, milk, egg, sesame seeds, fish, shellfish, soy, wheat and lupin) since 2017 (Australasian Society of Clinical Immunology and Allergy, 2019). The lupin proteins α- and β-conglutin were considered as the most allergic proteins followed by δ-conglutin (Holden et al., 2008). As a result, food products containing lupin must carry an allergen warning (Food Standards Australia New zealand, 2017).

Lupin is a valuable potential protein source for human nutrition and may represent a viable alternative protein source to that from animal sources and soybean, especially with the global trend in consumers looking for sustainable and non-GM protein sources.

Table 2. 2 Amino acid composition (g AA per 16 g N) for some legume globulin fractions.

Amino acid	Pea	Soybean	Lupin
Non-essential AA*			
Aspartic acid	11.40	9.4	9.61
Tyrosine	3.40	4.2	3.54
Serine	4.87	3.7	4.99
Glutamic acid	16.94	17.8	20.90
Proline	4.47	5.49	4.00
Glycine	3.84	4.0	3.92
Alanine	4.00	3.9	3.08
Cysteine + Methionine	1.79	2.43	3.58
Essential AA			
Threonine	3.24	4.0	3.11
Valine	4.86	4.80	3.85
Isoleucine	4.74	5.3	4.30
Leucine	8.94	5.1	6.46
Phenylalanine	5.49	5.1	3.71
Tryptophan	0.97	1.28	ND
Lysine	7.01	2.7	4.36
Histidine	2.23	2.6	2.73
Arginine	8.08	6.2	11.31
Acidic/basic amino acids%	1.5	1.9	2.4
Polar %	15	15.4	15.1
Non-polar%	37.4	38.3	30.8

(Boye et al., 2010; Chin et al., 2019; Fernández-Quintela et al., 1997; Leterme et al., 1990)
AA, amino acid

2.2.4. Lupin protein structure

Proteins can be classified into four groups according to their solubility properties: albumins, globulins, glutelins and prolamins. Albumins are proteins that are soluble in water, whereas globulins require salt solution to be solubilised, the glutelin group of proteins is soluble in dilute acid or base solutions and prolamins require an aqueous alcoholic solution to be solubilised (Li-Chan 2004).

The main fraction of lupin seed protein is globulin, which comprises 87% of the total protein. The remaining lupin protein fraction is albumins (Petterson, 1998, Duranti,

Consonni, Magni, Sessa, & Scarafoni, 2008). The globulins can be divided into four major fractions (Table 2.3): α-conglutin (35-37%), in the form of multiple subunits of molecular weight, 50-80 kDa); β- conglutin (44-45%), in the form of multiple subunits of 20-60 kDa; γ-conglutin (4-5%), in the form of two subunits of 17 and 30 kDa; and δ-conglutin (10-12%), in the form of two subunits of 4 and 9 kDa (Blagrove & Gillespie, 1975; Duranti et al., 2008; Foley et al., 2011; Petterson, 1998). For example, the main lupin protein fraction (α- and β-conglutin) is rich in glutamic acid, whereas γ-conglutin lacks glutamic acid but has a high content of sulphur AAs. This variation in the AA composition between lupin protein fractions, hence their higher-order structure, results in variation in protein functional properties. For instance, the γ-conglutin fraction exhibits good foaming properties, whereas the major α, β-conglutin fraction has poor foaming but excellent emulsifying properties (Sironi, Sessa, & Duranti, 2005). Commercially, lupin protein fractions have been named according to their functionality as the E (emulsifying) fraction (α, β-conglutin), which is isolated using isoelectric precipitation and F (foaming) fraction (γ-conglutin), which is separated using ultrafiltration (Sironi et al., 2005; Wasche et al., 2001). However, the lupin genotype may affect its protein content, structure and protein techno-functionality, which are discussed in detail in the following section.

Table 2. 3 Comparison between some legumes (lupin, soybean and pea) protein properties.

Protein type	Main protein fractions	Fraction %	Non-RC kDa	RC kDa	IP	S-S µmol/g	Free SH µmol/g	(T_d) C°	Sh $H_o \times 10^4$	References
Lupin protein	α-conglutin (legumins)	35-37	330-430	42-45 and 20-22	5.1-5.8	6	19.5 without heat treatment (increase with heating)	87.00-91.48	20.2 bh 34.4 ah	(Benjamin et al., 2014; Berghout et al., 2015; Duranti et al., 2008; Foley et al., 2015; Sirtori, Resta, Brambilla, Zacherl, & Arnoldi, 2010; Villarino, Jayasena, Coorey, Chakrabarti-Bell, Foley, et al., 2015)
	β-conglutin (vicilins)	44-45	143-260	53-64 and 25-46 and 17-20	5.0-6.6	0		71.49		
	γ-conglutin	4-5	200	29-17	7.9	2		ND		
	δ-conglutin	10-12	13	4 and 9	Acidic	4		ND		
Soybean	Glycinin	52	300-360	ND	ND	2	10.6 decrease with heating	80-88	27.3 bh 28.1 ah	(Berghout et al., 2015; Bos, Martin, Bikker, & Vliet, 2001; Renkema, 2001)
	β conglycinin	33-35	150-200	63.5-67.2 and 47.8	ND	0		63-68		
	2S	15	8-22	ND	ND	0		ND		
Pea	Legumin	20-28	56-58	18-24 38-42	6	1	2.1 increase with heating to 7.1	76.6	31.2 bh 32.5 ah	(O'Kane, Vereijken, Gruppen, & Boekel, 2005, Mession, Chihi, Sok, & Saurel, 2015, Chihi et al., 2016; Mession, Assifaoui, Cayot, & Saurel, 2012)
	Vicilin	61-67	200	48-52 30-37 14.2-22 60	5.7	0		68.5		
	Convicilin	6.1-10	70	ND	ND	ND		ND		

RC, reducing condition; T_d, denaturation temperature; Sh, Surface hydrophobicity; bh: before heat treatment; ah, after heat treatment; ND, not determined.

2.2.5. Effect of genotype on the lupin protein profile

The protein content and structure of lupin seed are affected by genotype. For example, Coromup has the highest protein content among *L. angustifolius* genotypes (Villarino, Jayasena, Coorey, Chakrabarti-Bell, & Johnson, 2015). Islam et al. (2011) reported that the 81% variation in protein molecular weight was related to the genotype effect. Vaz, Pinheiro, Martins, and Ricardo (2004) reported variation in the ratios of the main protein species α, β, δ and γ between *L. albus* genotypes. The lupin genotype also influences the AA sequence of each protein fraction (Krochko & Bewley, 2000). For instance, the sulphur AAs (methionine and cysteine) content is affected by genotype, which may influence their protein techno-functional properties (Krochko & Bewley, 2000). Thiol groups are involved in the formation of disulphide (S-S) bonds, which have an essential role in gel stabilisation (Aguilera & Rademacher, 2004; Alting, Hamer, De Kruif, & Visschers, 2000; Li-Chan, 2004). However, few studies have focused on the effects of ASL variety on protein structure and functional properties compared with the larger number of studies on soybean and pea.

2.2.6. Lupin protein isolation and fractionation

Lupin protein isolation and separation from the other seed kernel components (e.g. dietary fibre) depends on the different level of solubility of each fraction at various pH values (Duranti et al., 2008; Wong, Pitts, Jayasena, & Johnson, 2013). The standard method of isolation of the main lupin globulins (α, β and δ) is alkaline extraction of the milled kernel followed by acid precipitation (Wong et al., 2013). The precipitate is the (α, β and δ-conglutin-rich fraction, whereas γ-conglutin remains in solution but can be recovered by ultrafiltration (Wasche et al., 2001).

2.3. Protein techno-functional properties

The techno functionality of a protein is dependent mainly on its ability to interact with other food ingredients (i.e. protein, starch, oil and water) (Li-Chan, 2004; Tolstoguzov, 1992). Generally, the techno-functionality of a protein depends on both its AA sequence (primary structure), which in turn influences the higher-level structures involved in polypeptide folding (secondary and tertiary structures), and folded polypeptide aggregation (quaternary structure) (Berghout, Venema, et al., 2015; Luyten et al., 2000).

The AA sequence affects the techno functional properties because of its influence on the distribution of hydrophobic and hydrophilic R-groups and, thus, the covalent and noncovalent interactions between these groups (Li-Chan, 2004). Accordingly, proteins can vary dramatically in their ability to behave as gelling, emulsifying and foaming agents (Li-Chan, 2004, Foegeding, 2015). Table 2.4 presents the diversity of food systems and the target functionality required by proteins in each system (Luyten et al., 2000, Owusu-Apenten, 2004). The demand for plant proteins as functional ingredients has increased tremendously, and ongoing research into understanding and improving plant protein functional properties for economic, nutritional and technological reasons is crucial (Makri et al., 2005).

Table 2.4 Different food system and required protein functionality.

Food system	Functionality	Protein type
Beverage	Solubility, colour, grittiness	Whey, Soy
Baked goods	Emulsifying, foaming, gelation	Caseinate, egg white, whey, Lupin
Dairy substitutes	Emulsifying, foaming, gelation	Caseinate, egg white, whey, Soy
Egg substitutes	Foaming, gelation	Egg white, whey, Soy, Pea
Meat emulsions	Emulsifying, foaming, gelation	Caseinate, egg white, whey, Soy, Pea
Soups and gravies	Viscosity, emulsifying, water adsorption	Caseinate, whey, Soy, Pea
Topping	Emulsifying, foaming	Caseinate, egg white, Soy, Pea
Whipped dessert	Emulsifying, foaming, gelation	Caseinate, egg white, Whey Pea, Soy
Low fat dairy products	Thickening, gelation	Gelatine

(Anonymous 2011; Boland 2011; Burger & Zhang, (2019; Ladjevardi, Gharibzahedi, & Mousavi 2015; Luyten et al. 2000; Owusu-Apenten 2004; Schreuders et al., 2019 ; Wang et al., 2018)

2.3.1. Solubility

Increasing the use of plant proteins as food ingredients will depend on the ability of their techno-functional properties to be perceived as equivalent to those of animal proteins (Boire et al., 2018; Chardigny & Walrand, 2016a). High protein solubility is considered

to be the most important techno-functional property because it beneficially influences many other functional properties (Morr, 1990). Lupin protein concentrates produced by isoelectric precipitation have 63% solubility at neutral pH, which is similar to pea protein solubility (59%) but higher than of Soybean protein (45%) (Hojilla-Evangelista et al., 2004; Shand et al., 2007; Wasche et al., 2001). However, the protein preparation and drying methods can influence the protein solubility profile of the final product (Chew et al., 2003). For instance, freeze-dried lupin protein isolate (LPI) produced using the isoelectric precipitation method has a protein solubility of about 95% at pH 7, whereas spray-drying lupin protein concentrate produced using isoelectric precipitation has only 57% solubility at the same pH (Berghout et al., 2014; Chew et al., 2003). The difference may reflect protein denaturation during the spray-drying step.

2.3.2. Gelation mechanisms

Food gels can be considered as high-moisture, three-dimensional polymeric networks that resist flow and retain distinct structural shape upon deformation (Berghout, Pelgrom, et al., 2015). Semi-solid gel texture is one of the most desirable protein techno-functional properties for food processing because it provides sensory and textural acceptability to a wide range of processed foods (Nicolai & Chassenieux, 2019). The factors that control the gel-forming process include protein type and concentration, pH, ionic strength and temperature (Gosal & Ross-Murphy, 2000). In addition, the ability of a protein gel to retain a substantial amount of water with a stable semi-solid structure is considered to be a viable solution for the production of low energy (low-fat) foods (Aguilera & Rademacher, 2004), such as low-fat dairy products, desserts and meat analogues. These food categories depend mainly on the gelation properties of their ingredients for their desirability and textural stability (Aguilera & Rademacher, 2004; Kilara & Vaghela, 2004). Commercially, the gel in these foods is often formed from globular proteins, such as whey protein and egg albumin, because these types of proteins can provide a wide range of crosslinking (covalent and non-covalent) during processing to form a stable gel network (Williams et al., 2000; Yanniotis et al., 2013). The two main protein gelation approaches used in the food industry are heat-set gels and cold-set gels.

2.3.2.1. Heat-set gels

Heat-induced gels are formed from proteins by two main steps: 1) heating a protein solution to denature it by unfolding its native structure and aggregation of unfolded protein strands, and 2) formation of a three-dimensional cross-linked (junction zone) network (Vilgis, 2015). Gel network formation occur at high temperature through non-covalent bonding, which is then stabilised via accessible covalent bonding at a lower temperature (Alting, Hamer, De Kruif, & Visschers, 2003). Factors such as protein concentration, temperature and ionic strength affect a gel's rheological properties (Burey, Bhandari, Howes, & Gidley, 2008; Chen, Deckinson, Lee, & Lee, 2001). For instance, crosslinking density and stability correlate positively with gel elastic properties (Loveday et al., 2012; Uruakpa & Arntfield, 2004).

A heat-set gel is the most common form of protein gel used in food-processing systems (Clark et al., 2001). However, heat-set gels require a higher protein concentration compared with cold-set gels because not all polypeptides in the denatured protein solution (step 1 above) participate in the gel network formation given the high repulsion forces between some proteins (Alting, Hamer, De Kruif, & Visschers, 2003). Heat-set gels are not ideal for the inclusion of heat-sensitive components (e.g. vitamins, polyphenols, flavours and colours) (Wan & Yang, 2015; Wen et al., 2017) because of high temperature environment during gel formation stage.

2.3.2.2.Cold-set gels

The process of creating a cold-set gel (otherwise known as an acid or salt-induced gel) first involves heating of a protein solution in alkaline conditions to facilitate protein unfolding and exposure of hydrophobic AA R-groups. To avoid heat-set gelation, the critical protein concentration should be low in cold-set gels, otherwise the gel will form during the cooling process (Maltais, Remondetto, Gonzalez, & Subirade, 2005). Once the protein unfolding occurs, peptides aggregate with each other through hydrophobic interaction, although the level of aggregation is too low to form the gel network. The aggregated solution is then cooled to ambient temperature and no gelation occurs during that time. Gelation is then triggered in the ambient temperature solution by two commonly used approaches. (1) Acid-induced gelation is triggered adding acid to lower the pH to

around the isoelectric point (IP) of the protein (Hu, Fan, et al., 2013). At the IP, the neutral protein net charge reduces the repulsion forces and increases the attraction between protein molecules. This increased affinity allows very close association of polypeptides, which permits the formation of more covalent and non-covalent bonds to give the stable gel network (Alting, Hamer, De Kruif, & Visschers, 2000). (2) Salt-induced gelation is triggered by adding salts such as $CaCl_2$ (Lu et al., 2010). The calcium ions can build salt bridges between negatively charged protein aggregates, which facilitates the formation of the gel network without the need to adjust the pH (Maltais, Remondetto, Gonzalez, & Subirade, 2005). In cold-set gels, the repulsion between protein molecules is much lower because of the lower pH or high salt content used.

Cold-set gelation results in the formation of a continuous three-dimensional crosslinked protein network that traps water as a discontinuous phase and forms a gel (Bryant & Julian McClements, 1998). In cold-set gel systems, lowering the pH near the IP or adding salt at ambient temperature is the trigger for gelation. Thus, the gelation step can be separated from the heat treatment step (Alting et al., 2003). Cold-set gels are particularly useful for inclusion of heat-sensitive ingredients (Desai & Jin Park, 2005; Mortensen, 2006) because the ingredient can be mixed into the cold but still liquid aggregated protein solution before gel formation is activated (Valim et al., 2009).

2.3.2.3. Evaluation of gel quality

Characterisation of gel rheological properties is important to understanding and improving the gelling of plant proteins. One of the most important gel quality attributes, gel strength (Gs) or stiffness is defined as the maximum force (N or g) needed to rupture the gel (Hongsprabhas & Barbut, 1996). Gs can be determined using a texture analyser and provides a comprehensive understanding of gel quality (Saha & Bhattacharya, 2010). It has been reported that the increase in the number of S-S bonds and hydrophobic interactions in legume protein gels improve their Gs (Campbell et al., 2009; Hu, Fan, et al., 2013; O'Kane et al., 2005; Riblett et al., 2001).

Water-holding capacity (WHC) is an essential gel functionality that has a direct effect on final food texture. WHC is defined as the ability of a gel to maintain the entrapment of water after being subjected to the effects of pressure such as centrifugal

force (Gyawali & Ibrahim, 2016). The ability of protein gels to hold water usually depends on the gel network microstructure such as the network pore size, gel crosslinking strength, protein aggregate size, pH, ionic strength and protein hydrophobicity (Kinsella, 1979; Saha & Bhattacharya, 2010; Urbonaite et al., 2014).

Rheometry, in particular, small-strain dynamic rheology, is a useful technique for evaluating protein gelling properties (Burey et al., 2008). Rheometry can be used to determine the storage modulus (G′), loss modulus (G″) and tangent δ (tan δ) (Tunick, 2011). These are defined as follows.

1. G′ is a measure of the ability of a material to store energy that is applied to it and to return to its original shape after removal of the stress.

2. G″ is a measure of the ability of a material to dissipate energy that is applied to it. After removal of the stress, the material does not return to its original shape.

3. tan δ is the tangent of the phase angle (δ) and represents the ratio of the loss modulus to the storage modulus (G″/G′).

A high G′ value represents a strong, high-elasticity gel whose properties are associated with a high level of protein crosslinking in the gel network. A high G″ value refers to a highly viscous, weak gel (Nicolai & Chassenieux, 2019; Uruakpa & Arntfield, 2004). A tan δ value <1 indicates solid-like behaviour and values >1 indicate liquid-like behaviour for the material. Rheology measurements can play an important role in predicting the sensory quality of food products because they correlate with mouth feel (Burey et al., 2008).

2.3.3 Factors controlling legume protein gelation properties

The ability of polypeptides to interact with each other after heat treatment by forming covalent bonds such as disulphide and dityrosine bonding, hydrogen bonding, electrostatic attractions, and hydrophobic interactions are crucial factors controlling gelation properties (Aguilera & Rademacher, 2004; Berghout, et al., 2015; Gosal & Ross-Murphy, 2000; Liu & Hsieh, 2008; Nguyen et al., 2017; Owusu-Apenten, 2004). The protein AA sequence and composition, and higher-order structure (secondary, tertiary and

quaternary) strongly affect their gelation properties (Beck, Knoerzer, Sellahewa, Emin, & Arcot, 2017; Wang, Li, Jiang, Qi, & Zhou, 2014). In addition to protein structure, various gelation conditions (protein concentration, pH and heat treatment) affecting gelation properties are discussed in detail in the following sections.

2.3.3.1. Amino acid composition

The AA composition and sequence within a polypeptide determine the higher-order structure of a protein and, in turn, its functional properties such as its gelation and chemical properties including the hydrophobicity/hydrophilicity, net charge and the ability of the protein to interact with other proteins or food components in food systems (Chardigny & Walrand, 2016a; Nakai, 1983). A deeper understanding of legume protein structures will help to reveal their structure–function relationships and the most appropriate ways to process these proteins to modify and improve their gelation (Hua et al., 2005; Schmitt et al., 2019).

Hydrogen bonds and hydrophobic interactions play a major role in the initial formation of a network during the formation of a legume protein gel. Next, a balance of covalent interactions, primarily S-S bonds, will increase gel strength through the availability of cysteine and cystine (Tables 2.3 and 2.6) (Benjamin, Silcock, Beauchamp, Buettner, & Everett, 2014). For example, the native 11S glycinin fraction of soy protein has two S-S bonds and 10.6 μmol/g free sulfhydryl groups, which allow it to unfold during heating and then to form a stabilised gel network (Adachi et al., 2004; Berghout, et al., 2015; Hua et al., 2005). However, in the case of native lupin protein, a higher number of S-S bonds increases its thermal stability, which reduces its gel-formation ability. For example, lupin α-conglutin has 10 S-S bonds and 19.5 μmol/g free sulfhydryl groups. The S-S bonds hinder protein unfolding during heat treatment, which leads to lupin protein forming a weaker gel than soybean protein under the same conditions (Batista et al., 2005; Berghout, Boom, et al., 2015; Sousa et al., 1995).

By contrast, Khatib, Herald, Aramouni, MacRitchie, & Schapaugh, (2002) reported that difference in AA composition between soybean genotypes influences protein techno-function properties. They found that genotypes with a higher level of sulphur

(methionine and cysteine) and hydrophobic AAs exhibited a more solid-like gel structure (high G′ value) compared with those with a lower content of these AAs. However, they did not report the number of S-S bonds initially available in soybean protein, which is the case in terms of lupin protein thermal stability. In another study, lupin (*Lupinus angustifolius*) required a 23% protein concentration to start forming a gel compared with 7% for *L. albus* (Vogelsang-O'Dwyer et al., 2020).

These differences may reflect slight differences in AA content and protein profile (protein subunits) between the two species, which highlights the importance of AA content to determining protein functionality (gelation). For instance, heterogeneity in the protein profile and AA composition between soybean genotypes leads to significant differences in protein denaturation temperature, which influence the protein unfolding and gelation properties (Bainy et al., 2008).

2.3.3.2. Protein concentration

The ability of proteins to form a gel is a techno-functionality that is demanded in a wide range of food and non-food applications (Burey et al., 2008). Some plant proteins are replacing animal proteins, such as gelatine, egg white, casein and whey protein, in these applications because of their good gel-like texturising properties (Kolakowska, 2003; Sá et al., 2020). For efficient use of plant proteins for gelling application, it is important to understand the minimum protein concentration (critical concentration) required for gelation; that is whether it can form a stable gel after heating and cooling treatments (Carvajal-Larenas, Linnemann, Nout, Koziol, & van Boekel, 2016; Sathe, Deshpande, & Salunkhe, 1982). The critical protein concentration depends on the protein properties (i.e. type of AA groups on the surface of protein molecules, protein subunit molecular weight and disulphide content) and on gelation conditions such as the protein concentration, ionic strength, pH and gelation system used (cold-set or heat-set) (Table 5) (Alting et al., 2003; Clark, Kavanagh, & Ross-Murphy, 2001).

Soybean isoelectric precipitated protein isolate requires a minimum protein concentration of 10% (w/w) for gelation at neutral pH, which is lower than for pea (12.5% (w/w)) and lupin (16% (w/w) protein isolate (Batista et al., 2005). Lupin protein shows a

weak gel quality compared with soybean and pea protein even at higher protein concentration (Batista et al., 2005). Similarly, Berghout, et al., (2015) observed that lupin protein has a lower storage modulus (G′) than soybean protein isolate. Low G′ values represent weak intermolecular network formation between lupin protein molecules. Proteins with a higher critical protein concentration for gelation demonstrate thermostable native protein structure that is resistant to unfolding and high electrostatic repulsion forces that inhibit aggregation and, thus, limited crosslinking ability (Lee & Lucey, 2010; Mantovani, Cavallieri, & Cunha, 2016).

As mentioned earlier, using a cold-set gel system can reduce the critical protein concentration required for gelation and improve gel network properties (Maltais, Remondetto, & Subirade, 2008). In a cold-set gel system using calcium salt, soybean protein isolate starts forming a stable gel at 6% protein concentration, which is a lower concentration than the 10% in a heat-set gel system (Maltais et al., 2008; Maltais, Remondetto, Gonzalez, & Subirade, 2005). In addition, it has been reported that pea isoelectric precipitate protein can form a cold-set gel at 10% (w/w) protein concentration (Klost & Drusch, 2019). In terms of lupin protein, no published studies have investigated the minimum protein concentration, pH and salt concentration needed for gelation in a cold-set gel system.

Increasing protein concentration above the critical limits will influence the gel quality properties significantly. For instance, the gel microstructure can change in a compact gel network with smaller pores between the protein molecules in the gel caused by a higher density of crosslinking (Alting et al., 2003, Cramp et al., 2008; van der Linden & Foegeding, 2009). Increasing the protein concentration can significantly affect the gel rheological properties (Langton, Hermansson, & Box, 2004). For example, in soybean protein gels, increasing the protein concentration leads to a higher G′, which indicates increased elastic properties of the gel network (Campbell et al., 2009; Maltais, Remondetto, Gonzalez, & Subirade 2005).

Table 2.5 Studies on the effects of pH, protein concentration and heat treatment on gelation properties in legumes

Legume	Species	Protein purity g/100 g	Gelation condition				conc	pH	WHC	G′	G″	T.A	Reference
			Gm	T	TTt	GT							
Soybean	ND	DF, IP 82.4 (N x 6.25)	H	90	30	24-48	8-14g	2.75 and 3.5(IP)	80	ND	ND	ND	(Puppo, Lupano, & Añón, 1995)
Soybean	ND	DF, IP 94 (N x 6.25) dry basis	CS	105	30	24	6-9%(w/w)	7	ND	850 at 20mM $CaCl_2$ & 8% sample	ND	ND	(Maltais et al., 2008)
Soybean	*Glycin emax* L.	DF	H	95	180	24	8%	6.8	ND	ND	ND	ND	(Cramp et al., 2008)
Soybean	ND	DF, IP 94	CS	105	30	24	9.5% (w/w)	7	80.83	2191 at 9% p.	ND		(Maltais et al., 2005)
Soybean	ND	DF	H				10% (w/w)		ND	ND	ND	1.23	(Batista et al., 2005)
Soybean	*Glycin emax* L.	DF 93.11	CS	80,10 0, 120	15,45,75	ND	5%, 6%, 7%, 8% (w/w)	7	ND	~1100 at 8% and 100ºC	ND	ND	(Lu, Lu, Yin, Cheng, & Li, 2010)
Soybean	ND	DF 92.6	H	80	30	ND	6.5-12% (w/v)	7	ND	ND	ND	ND	(Wang et al., 2017)
Soybean	ND	90	CA	95	10	18	4%, 6% and 8% (w/v)	3.5 - 5.5	62.6 at 6.9 pH	119 at 8%	18.7 at 8%	ND	(Campbell et al., 2009)
Soybean	ND	DF	CS	65-90	5, 10 and 15	ND	7.25% (w/v)	ND	90 at 90C°	ND	ND	ND	(Zhao, Li, Qin, & Chen, 2016)
soybean	ND	DF, SPI	H	90	30	24	12%(w/v)	7	ND	150 at 25C°	ND	ND	(Jian et al., 2014)

Legume	Species	Protein purity g/100 g	Gelation condition				conc	pH	WHC	G′	G″	T.A	Reference
			Gm	T	TTt	GT							
Soybean	ND	DF, SPI 90 (N x 6.25)	H	95	30		12,15,18 and 24% (w/v)	7.1-7.2	ND	ND	ND	ND	(Berghout et al., 2015)
Pea	ND	81.9 (N x 5.7)	H	82.2-104.6	ND	ND	14.5% (w/v)	5.7-6	ND	0.35-14560	ND	ND	(Sun & Arntfield, 2012)
Pea	ND	85-86%	H	100	30	24	18% (w/v) LGC 16%	7.6	ND	10 at 98 C°&1000 at 25 C°	ND	ND	(O'Kane et al., 2005)
Pea	ND	IP isolate	H	90	15	24	16% (w/w)	5.5	ND	ND	ND	1.34	(Batista et al., 2005)
Pea	ND	81.9 (N x 5.7)	H	25-95	ND	ND	10.5%	5.65 - 5.70.	ND	291.6	48.8	ND	(Sun & Arntfield, 2011)
Pea	*Pisum sativum* L.	IP isolate 60.7	H	90	45	24	20	6.5	ND	ND	ND	ND	(Makri et al., 2005)
Lupin	LL	IP isolate 79.2	H	90	45	24	20	6.5	ND	ND	ND	ND	(Makri et al., 2005)
Lupin	LL	IP isolate	H	95	60	24	15(w/w)	7	ND	ND	ND	0.15	(Bader et al., 2011)
Lupin	LL	DF, LPI 86.1 (N*5.7)	H	90	30	24	7%	7	ND	ND	ND	ND	(Vogelsan et al., 2020)
Lupin	LA	IP isolate	H	95	60	24	15 (w/w)	7	No gel	No gel	No gel	No gel	(Bader et al., 2011)

Legume	Species	Protein purity g/100 g	Gelation condition				conc	pH	WHC	G′	G″	T.A	Reference
			Gm	T	TTt	GT							
Lupin	ND	IP isolate	H	90	15	24	16% (w/w)	5.5	ND	ND	ND	0.43	(Batista et al., 2005)
Lupin	LL	DF, LPI 87.4	H	100	60	2	2,4,6-20% 12%	7	ND	ND	ND	ND	(Lqari et al., 2002)
Lupin	LA	DF, LPI 84.5 (N*5.7)	H	90	30	24	23%	7	ND	ND	ND	ND	(Vogelsan et al., 2020)

conc, concentration; CS, cold-set gel using salt; CA, cold-set gel using acid; DF, defatted; GT, gel curing time; Gm, gelation method; G″, loss modulus; G′, storage modulus; G, using GDL to trigger cold-set gelation; H, heat-set gel; IP, isoelectric precipitation; LPI, lupin protein isolate; LL, *Lupinus albus*; LA, *Lupinus angustifolius*; ND, not determined; N, nitrogen conversation factor; R, room temperature; S, using salt to trigger gelation; SPI, soy protein isolate; T, temperature(ºC); TTt, thermal treatment time (min); T.A, texture analysis; WHC, water holding capacity.

2.3.3.3. pH effect

The pH is crucial to the gelation process and subsequent gel properties because of its effect on protein net charge and intermolecular interactions (Puppo, Lupano, & Añón, 1995, Rao, 2007, Stokes, 2012). At a pH near the IP, the protein net charge is neutral, and the repulsive forces between the molecules are minimised. Under this condition, hydrophobic interactions dominate, which results in protein–protein crosslinking and gel formation (Lawal, 2004). In addition, in a cold-set gel system having a pH near the IP can reduce the critical protein concentration required to form a stable gel (Alting et al., 2003). This is because the low repulsion forces at the IP allow participation in the three-dimensional protein network involving a higher proportion of the protein molecules (Mantovani et al., 2016; Yanez et al., 1999). Akintayo, Oshodi, & Esuoso, (1999) reported that acidic pH influenced pea protein concentrate (72%) significantly. They found that pea protein formed a gel at 6% (w/v) protein at an acidic pH (2–6,around IP 4.5), whereas at an alkaline pH (pH 8), pea protein starts to form a gel at 12% (w/v). Similarly, Lakemond et al., (2003) reported that, at a neutral pH (pH 7.6), 51–69% of total soybean protein participated in building the gel network, whereas reducing the pH to 3.8 resulted in 100% of the protein incorporated into the gel network. This resulted in improved soybean gel elastic properties such as G′ because of the increased possibility of association under the low-repulsion force condition (pH near the IP).

In terms of gel quality, Akintayo, Oshodi, & Esuoso, (1999) reported that acidic pH values influenced pea protein concentrate (72%) significantly. They found that pea protein (6%) formed a stable gel network at an acidic pH (pH 2–6, around IP 4.5), whereas at an alkaline pH (pH 8), pea protein showed a viscous texture at the same protein concentration because of the high repulsion forces between protein molecules. In addition, the pH of the gel affects its physical appearance. For example, at the pH of the IP, a less transparent gel forms because of the very low repulsion forces between the protein molecules and small amount of entrapped water; however at a pH further from the IP, a transparent gel forms because of the higher repulsion forces between protein molecules, which gives a fine-stranded gel with a higher water-holding capacity (Maltais, Remondetto, Gonzalez & Subirade, 2005; Puppo et al., 1995; Rao, 2007). Lakemond et al. (2003), examined the

WHC of soybean protein gels at alkaline and acidic pH. They found that soybean protein was able to hold more water at pH 7.6 than at 3.8. Murekatete, Hua, Chamba, Djakpo, & Zhang (2014) found that acid-induced soybean protein tofu produced a higher gel strength than salt-induced tofu. However, increasing the gel strength led to more synereses (water leaking from the gel matrix) compare with a lower gel strength (salt-induced tofu).

2.3.3.4. Heat treatment

Heat treatment is considered to be the trigger step for the aggregation of globular proteins required for gelation (Mitchell & Ledward, 1986). Depending on their composition, proteins can have different denaturation temperatures, which in turn can influence their gelation properties (Cai et al., 2002; Damodaran & Paraf, 1997). Thermally induced protein denaturation increases protein hydrophobicity by unfolding the higher-level structure of protein. This exposes hydrophobic groups, which increases hydrophobic interactions and the formation of a gel network (Table 2.3) (Bainy et al., 2008; van der Linden & Foegeding, 2009).

The thermal stability of a protein is influenced by the level of covalent crosslinking (e.g. Disulphide bonds) within the intrinsic structure of protein molecules (Fennema et al., 2008). disulphide bonds require more energy to break during the heat treatment step of gelation compared with other intraprotein interactions (e.g. hydrogen bonds, hydrophobic interactions) (Table 2.3). The globular proteins of legumes such as pea, soybean and lupin each have different AA composition, sulphur AA content, molecular weight, protein subunits and surface hydrophobicity, all of which affect their thermal stability and thus gel-formation ability (Table 2.6) (Berghout et al., 2015; Sousa et al., 1995). Table 2.3 shows that lupin protein contains 10 S-S bonds (six in α-conglutin and four in δ-conglutin), which is significantly more than in the analogous protein of soybean (two S-S bonds) and pea (one S-S bond). This affects Differential Scanning Calorimetry (DSC) profile of these proteins. Lupin protein α-conglutin fraction has much higher denaturation temperature compared soybean (glycinin) and pea (legumin) fractions.

Berghout et al., (2015) examined the gelling properties of lupin protein isolate (LPI) and soybean protein isolate. They found that LPI functioned differently than soybean

protein isolate in that it formed a weaker and more deformable gel. They also found that LPI had more free sulfhydryl groups than soybean protein isolate and, upon heating, their number increased, which highlighted the lack of S-S bond formation in LPI. By contrast, soybean protein isolate, in which the number of free sulfhydryl groups decreased because of the formation of S-S bonds. Batista et al., (2005) reported that, high denaturation temperature of lupin protein reduced the protein's ability to unfold upon heat treatment, which is limited stable gel network formation through a wide range of interactions within a gel matrix. Similarly, Berghout et al., (2015) reported that even prolonged heat treatment (8 h at 80 °C) did not change the LPI particle size compared with swelled particles of soybean protein, which may represent unfolding and aggregation.

In another study, soybean protein isolate showed no gelation ability under S-S bond-reducing conditions, which highlights the importance of S-S crosslinking to soybean protein gelation ability (Cramp, Kwanyuen, & Daubert ,2008; Jian et al., 2014; Van Vliet, Martin, & Bos, 2002). By contrast, pea protein, having a low number of S-S bonds (one) and low denaturation temperature (Table 2.3) facilitates protein unfolding and stable gel network formation through mainly hydrophobic, hydrogen and ionic strength bonding (Chihi, Mession, Sok, & Saurel, 2016; Sun & Arntfield, 2012,). A proposed mechanism to explain soybean and lupin protein gelation based on the evidence available in the literature is presented in Figure 1A and B. In terms of soybean protein, having less thermostability (Table 2.3) leads to effective protein unfolding followed by aggregation and gel network formation through covalent and non-covalent interactions. However, the effects of suppression of lupin protein unfolding and S-S bond formation because of compact heat-stable protein particle properties leads to weak gel network formation with limited non-covalent interactions (Batista et al., 2005; Berghout, Boom, et al., 2015).

Given the crucial nature of the denaturation temperature of a protein in the formation of the gel network, a range of physical pretreatment steps have been investigated to lower this temperature and help to induce gel formation. Recent studies have reported that chemical-free, high-energy physical treatments, such as ultrasonication, and high pressure can modify protein structure and may reduce the temperature needed for the protein denaturation required for effective gelation (Arzeni et al., 2012; Gülseren et al., 2007; Sun

& Arntfield 2011; Tang & Ma 2009). The next section describes in further detail some of the novel and effective chemical-free protein-modification techniques that have been shown to improve lupin protein gelling and thickening properties.

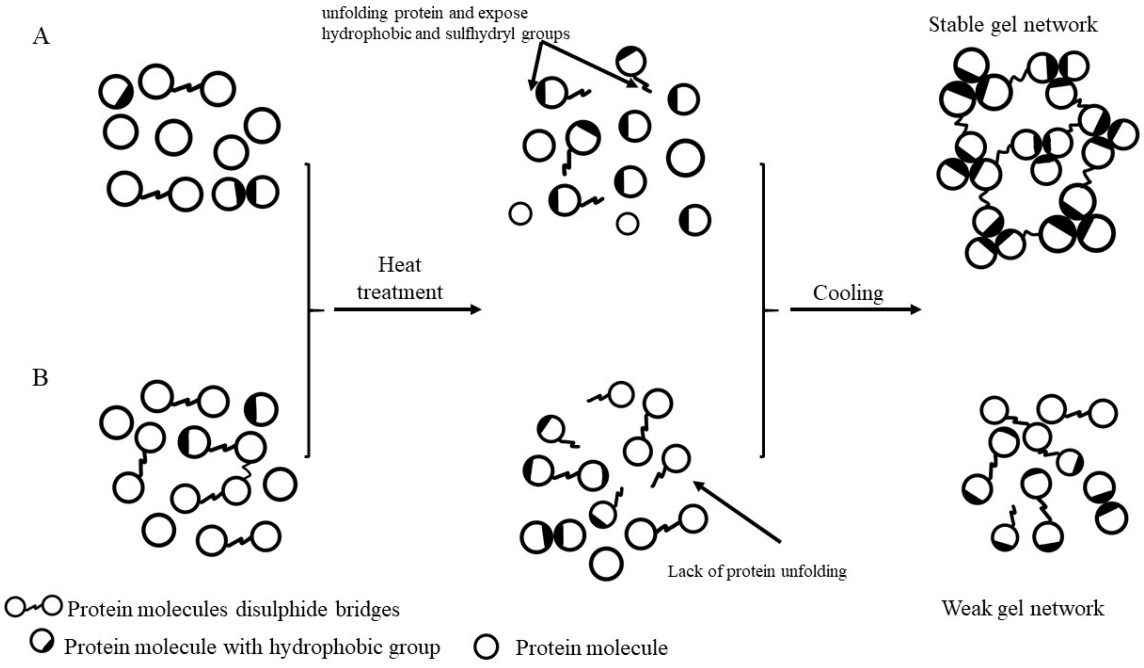


Figure 2. 1 A comparison between proposed gelation mechanism of (A) soybean protein and (B) lupin protein

2.4.Ultrasound

Several physical energy-dependent techniques have potential for modifying plant protein and improving its functionality. Ultrasound is one of these technologies (Chemat et al., 2011; Ozuna et al., 2015). Ultrasound is considered to be a sustainable technology because of its efficient use of energy and time (Chemat et al., 2011; Knorr et al., 2011). Ultrasound technology depends on using mechanical waves above the threshold of human hearing (>16 kHz) that are classified as either high frequency (100 kHz to 1 MHz) or low frequency (16-100 kHz). High-frequency waves with low intensity (<1 W/cm^2) is used for quality assessments such as the determination of physicochemical properties of food, including firmness, ripeness, sugar content and acidity(O'Sullivan, Park, Beevers, et al., 2016). Low frequency (16-100 kHz) high-intensity waves (10-1000W/cm^2) are used to

modify chemical and physical food properties such as protein techno-functionality (Awad, Moharram, Shaltout, Asker, & Youssef, 2012; Soria & Villamiel, 2010).

The effects of ultrasound depend on the cavitation phenomenon, by which gas bubbles are formed in a solution in response to the effect of sound wave pressure. These newly formed bubbles pass through continuous cycles of compression and decompression (aquatic mechanical waves) (Awad et al., 2012; Povey & Mason, 1998). In these cycles, the gas bubbles expand and burst, which releases high energy, creates extreme temperatures of 2000-5000 K and pressures of 1000 1000 atm in the cavitation zone (Gallo, Ferrara, & Naviglio, 2018; Kentish & Feng, 2014) and causes molecular changes in molecules within the solution. Ultrasonic treatment can also divide water molecules to create reactive free radicals (hydrogen and hydroxyl) and nonradical compounds (hydrogen peroxide), which can modify protein structure through oxidation reactions (Ozuna et al., 2015; Rahman, Byanju, Grewell, & Lamsal, 2020). Therefore, the extreme energy in the cavitation zone and the reactive free radicals can promote large changes in protein structure, which can modify protein functionality. Ultrasound has a complex effect on globular proteins by unfolding them and exposing their hydrophobic groups (Jambrak, Lelas, Mason, Krešić, & Badanjak, 2009; Jambrak, Mason, Lelas, Herceg, & Herceg, 2008). It can also break down globular protein quaternary complexes into their subunits, and this effect can be irreversible when oxidation occurs (Chemat et al., 2011). Free radicals produced by sonication can react with protein to increase the degree of crosslinking, for instance, by creating new S-S bonds within and between protein molecules, which can stabilise newly formed structures (Arzeni et al., 2012; Cavalieri, Ashokkumar, Grieser, & Caruso, 2008; Gülseren, Güzey, Bruce, & Weiss, 2007). Gülseren et al. (2007) reported a reduction in the number of free sulfhydryls and increase in disulphide bond numbers of bovine serum albumin (BSA) after sonication. This suggests that ultrasound can lead to the formation of new protein networks that may assist in gel formation. Dissociation of agglomerated legume proteins into their individual subunits by the ultrasound cavitation phenomenon has been reported (Morales, Martínez, Pizones Ruiz-Henestrosa, & Pilosof, 2015; Zhou et al., 2016). For example, the application of high-intensity ultrasound (HIU) to soybean isolate protein suspension for a short time can reduce particle size significantly (Hu, Fan, et al., 2013; Morales et al., 2015;

O'Sullivan, Murray, Flynn, & Norton, 2014). However, in some cases, ultrasound treatment may increase protein particle size because of the high temperatures generated, which facilitates protein unfolding followed by aggregation of the unfolded protein (Gulseren et al., 2007).

Many authors have mentioned that ultrasound treatment can also alter protein secondary structure; for instance, by reducing the percentage of α-helices and increasing β-sheet structure of soybean protein isolates when treated with low-power ultrasound treatment (75-83 W/cm^2). By contrast, high-power treatment (105-110 W/cm^2 and 131-138 W/cm^2) can increase the percentage of α-helix structure (Hu, Wu, et al., 2013). However, Zhou et al. (2016) observed that treating soybean glycinin (11S) protein fraction with 20 kHz at 80 W/cm^2 intensity for 40 min did not have an effect on protein secondary structure. The β-sheet structure appears to be more susceptible to modification by ultrasound because of the stabilisation of the α-helical structure by both hydrogen and S-S bonds (Hu, Cheung, Pan, & Li-Chan, 2015).

Stathopulos et al. (2004) noted that the sonication effect is more noticeable for the tertiary structure than the secondary of protein. Aguilar-Acosta, Serna-Saldivar, Rodríguez-Rodríguez, Escalante-Aburto, and Chuck-Hernández (2020) investigated the effect of ultrasound treatment (85 W/cm^2 for 10 and 15 min) on *L. angustifolius* and *L. mutabili* protein secondary structure. They found that HIU treatment triggered changes in the secondary structure of *L. mutabili* by increasing the unordered structures proportion and decreasing in α-helix after 15 min treatment, whereas they found no changes in the *L. angustifolius* secondary structure. These observations demonstrate the necessity to optimise sonication treatment carefully to achieve the desired modification of protein structure and to improve its gelation properties. Several studies have highlighted the potential of ultrasound treatment to significantly increase plant protein gel quality through changes in protein secondary structure that facilitate a variety of new intermolecular interactions through disulphide, hydrophobic, electrostatic and hydrogen bonding. For instance, Gs, WHC and gel yield increased after ultrasound treatment of soybean protein isolate at 105-110 W/cm^2 for 5-40 min in a cold-set gel system (Zhang et al., 2016). In addition, HIU treatment improves acid- or salt-induced gelation of soybean protein isolate by increasing the Gs and WHC (Hu, Fan, et al., 2013; Hu, Wu et al., 2013). The G′ of

soybean protein isolate gel can also be increased using ultrasound treatment at 200 W and 15 kHz for 10 min (Tang et al., 2009). By contrast, treating soybean protein isolate with 4.27 W at 20% amplitude for 20 min leads to a slight decrease in G′ (Arzeni et al., 2012), which highlights the need to optimise ultrasound exposure.

In soybean protein isolates, Fourier-transform (FT)-Raman spectroscopy and circular dichroism show that ultrasound treatment creates protein conformational changes by increasing hydrophobic interactions, which play a major role in stabilising the gel network (Hu et al., 2015; Hu, Li-Chan, Wan, Tian, & Pan, 2013; Zhou et al., 2016). The free sulfhydryl groups number are increased after ultrasound treatment and their number decreases after gel formation because of formation of new disulphide bonds, which tend to stabilise the gel network (Hu, Fan, et al., 2013; Hu, Li-Chan, et al., 2013; Zhang et al., 2016). Furthermore, ultrasonic treatment can create a denser gel by decreasing the particle size (Zhou et al., 2016). Hu, Li-Chan, et al. (2013) treating soybean protein suspension with 400 W for 40 min and found that this reduced the protein particle size from 176 μm to 97 μm and produced a more uniform and denser gel network. Ultrasound has an ability to modify protein structure by facilitating protein unfolding and structural conformational changes, which should lead to improved gelling properties of plant proteins. However, no research has been published on whether ultrasound treatment of lupin protein can improve its intrinsically poor gelation properties.

Table 2.6 Molecular forces involved in gel matrix crosslinking of some legume proteins

Type	Energy kJ/mol	Functional groups	Examples of amino acids
Covalent bond	330-380	Disulphide	Cysteine
	-	Dityrosine	Tyrosin
Hydrogen bonds	8-40	Amide, hydroxyl, phenolic groups (hydrophilic)	Asparagine, Glutamine (Serine, threonine, and tyrosine)
Hydrophobic interaction	4-14	Aliphatic and aromatic side chains	Tyrosine, Tryptophan, phenylalanine, valine, leucine, isoleucine
Electrostatic interactions	42-84	Carboxylic acids and amino groups (hydrophilic)	Aspartic, glutamic (Lysine, Arginine, Histidine)
Van der Waals	1-9	Permanent, induced and instantaneous dipoles	Glycine, Alanine, leucine, isoleucine, valine, Phenylalanine, Tryptophan, Methionine, Proline

(Li-Chan., 2004; Nguyen et al., 2017)

2.5.Conclusion

There is a growing demand for more sustainable alternatives to animal protein. Lupin protein has potential as a successful plant protein source given its environmental, health and economic benefits compared with both animal and soybean protein sources. In terms of health benefits, lupin protein has a high protein content, good balance of AA, low alkaloid content (sweet lupin varieties), no phytoestrogens and is a non-GM plant protein source. Lupin protein composition can vary according to species, genotype and production environment, which in turn can influence its techno-functionality. Developing lupin protein as an effective gelling agent for use in the food industry remains a difficult task partly because of its high thermal stability, which prevents the required unfolding of the structure for aggregation during gelation. Few studies have focused on improving lupin protein functionality. Improving lupin protein gelation properties may help to increase the demand of this legume by industry and consumers. Therefore, the application of novel technologies to improve lupin protein techno-functionality through the modification of the protein structure is required. Previous research has shown the ability of ultrasound treatment to modify the protein structure and improve the gel quality. The

use of ultrasound treatment to improve lupin protein gelation has great potential, although careful optimisation of the ultrasound conditions will be vital for its success.

CHAPTER 3
Effect of Variety and Growing Location on the Composition of Australian Sweet Lupin Kernels and the Composition and Techno-functionality of Protein Concentrates

Abstract

Australian sweet lupin (ASL, *Lupinus angustifolius*) seed protein has potential for use as a technologically functional food ingredient. The variety (V) (genotype) and location (L) (environment) and their interaction (V × L) may influence kernel protein content and structure, which in turn can affect the protein techno-functional properties. The aim of the work described in this chapter was to identify differences in the nutritional content and variation in protein concentrate (PC) composition and techno-functional properties in ASL kernels from six commonly grown varieties (*PBA Gunyidi*, *PBA Barlock*, *PBA Jurien*, *Jenabillup*, *Mandelup* and *Coromup*), each produced in two contrasting locations in Western Australia (Eradu (ER) and Wongan Hills (WH)) in the same growing season. V, L and V × L had significant effects on lupin kernel flour composition and PC techno-functional properties. Samples from WH exhibited higher protein content and slightly increased protein solubility, foaming stability, and emulsifying and viscosity properties compared with varieties grown in ER. Electrophoretic analysis of lupin protein identified differences in protein profile triggered by the effect of V. However, all lupin varieties showed weak foaming, thickening and gelation properties, which may limit the use of lupin protein as a human food ingredient.

3.1. Introduction

Lupins are legumes of the *Lupinus* sp. and include 267 species (Drummond et al., 2012; Foley et al., 2015). However, only five are fully domesticated: *Lupinus angustifolius, L. albus, L. atlanticus, L. consentinii* and *L. luteus* (Gladstones, 1998; van Barneveld, 1999). Global lupin production was 1,160,867 tonnes in 2018, of which Australia produced around 61% of the worldwide production (FAO-STAT, 2018). *L. angustifolius* (narrow-leaved lupin) comprises 95% of Australian lupin production and is grown mainly in Western Australia. Fifty per cent of lupin seeds produced in Western Australia in 2017 were exported (Grains Research and

Development Corporation, 2017). Lupin is planted widely to improve soil fertility because of its high atmospheric nitrogen-fixing ability compared with other legumes (Chew et al., 2003). In addition, the use of lupin as a rotation crop in the wheatbelt farming system of Western Australia has led to improved cereal yields and grain quality (Seymour et al., 2012; White et al., 2008). Rotational planting of lupin has also reduced the use of nitrogen fertiliser, which contributes to lowering environmental pollution and greenhouse gas emissions (Association International Fertilizer Industry, 2009; David, 1998; McMichael et al., 2007).

L. angustifolius is known as Australian sweet lupin (ASL) because of its low content of bitter and potentially toxic alkaloids (Gladstones et al., 1998). The lupin seed is considered as a high protein source with high protein digestibility given the low levels of anti-nutritional factors such as lectins and protease inhibitors, which inhibit digestion of other legume seeds (Chew et al., 2003; El-Adawy et al., 2000). Lupin is considered to be a non-genetically modified plant (ISAAA, 2016). Lupin contains 38–40% (on a dry weight basis (db)) of protein, which is similar to soybean and higher than pea (20%) (Duranti, 2006; Raikos et al., 2014). Similar to other legumes, lupin protein contains high levels of lysine, which is deficient in most cereal proteins (Drakos, Doxastakis, & Kiosseoglou, 2007; Gulewicz et al., 2008). Despite its higher content of sulphur amino acids (AA) such as methionine and cysteine compared with other legumes, the protein content remains below the level of human nutritional needs (Arnoldi, 2011).

High-protein food products are produced widely from legumes (Foschia, Horstmann, Arendt, & Zannini, 2017). The demand for plant protein has increased given the proven feasibility of replacing animal protein in terms of techno-functionality and nutritional value (Manners et al., 2020). Protein techno-functionality refers to non-nutritional applications of protein in food systems such as solubility, water- and oil-binding capacity, foaming capacity and stability, emulsion capacity and stability, viscosity and gelation (Moure et al., 2006). According to their purity, protein food ingredients can be classified into two main categories: protein concentrate with minimum 65% (db) and protein isolate with 90% protein db (Berghout et al., 2014; H. Wang et al., 2004). To achieve high protein purity, several purification steps such as defatting (using organic solvents), desalting (washing with water or ultrafiltration) and removing unwanted carbohydrate are used (Berghout et al., 2014; Wong et al., 2013).

Increasing number purification steps may increase the production cost, influence the techno-functionality and lead to unsustainable production because of the excessive use of water and chemicals during the extraction and purification processes (Bader et al., 2011; Hojilla-Evangelista et al., 2004; Russin et al., 2011).

The PC can be considered as a viable protein ingredient and can have similar techno-functionality, lower production cost and more ecofriendly production compared with protein isolate (Berghout et al., 2014; Berghout, Venema, et al., 2015). An estimated 1.7 million tonnes of plant PC/isolate are consumed as a functional food ingredient, and soybean protein ingredients dominate the plant protein market by accounting for 99% of such ingredients (Chardigny & Walrand, 2016). Despite the high lupin protein content and nutritional value, less than 4% of lupin production is consumed as human food (DPIRD, 2018). One factor limiting the use of PC and isolates from lupin is the lack of some protein techno-functionalities (Batista et al., 2005; Chew et al., 2003). The main lupin protein fraction (α,β-conglutin), which is produced by isoelectric point precipitation show weak foaming, thickening and gelation properties (Berghout, Boom, et al., 2015; Chew et al., 2003). The lack of these techno-functional properties limits the useability of lupin protein as a food ingredient for humans in many food systems. The quality of a protein's techno-functionality is directly related to its structure, molecular weight and AA type and distribution in polypeptide chain (Cramp et al., 2008; Nicolai & Chassenieux, 2019).

Even within a lupin species, the variety and growing location can influence the seed protein content and structure (Cowling & Tarr, 2004; Fehr et al., 2003). For instance, *Coromup* and *Mandelup* varieties had the highest protein compared to other *L. angustifolius* varieties in a study by Villarino, Jayasena, Coorey, Chakrabarti-Bell, and Johnson (2015). Variety diversity was reported to be responsible for 81% of the protein molecular weight variation between *L. angustifolius* cultivars (Islam et al., 2011). In addition, the ratio of main lupin protein fractions (α-, β-, δ-, and γ-conglutins) in *L. albus and L. angustifolius* protein is affected by both the variety and growing location and their interaction (Islam et al., 2013; Vaz et al., 2004). It has also been demonstrated that the AA sequence in the lupin protein fraction is also influenced by variety within a lupin species (Krochko & Bewley, 2000).

Variation in the protein profile, sequence and content of AAs of *L. angustifolius* varieties may have consequences for the techno-functional properties of

the lupin protein made from these seeds (Krochko & Bewley, 2000; Villarino, Jayasena, Coorey, Chakrabarti-Bell, & Johnson, 2015). Protein gel quality can be affected by variety because of its effect on the content of disulphide (S-S) bonds, which have an essential role in gel stabilisation (Aguilera & Rademacher, 2004; Alting, Hamer, De Kruif, & Visschers, 2000; Li-Chan, 2004). However, more studies are required to investigate the protein structure and the techno-functional properties of the protein concentrate of different *L. angustifolius* varieties.

The work described in this chapter evaluated the seed PC of six of the most recent ASL genotypes, namely *PBA Gunyidi*, *PBA Barlock*, *PBA Jurien*, *Jenabillup*, *Mandelup* and *Coromup* grown in two locations in Western Australia and the techno-functional properties of their PC.

3.2. Methods and materials

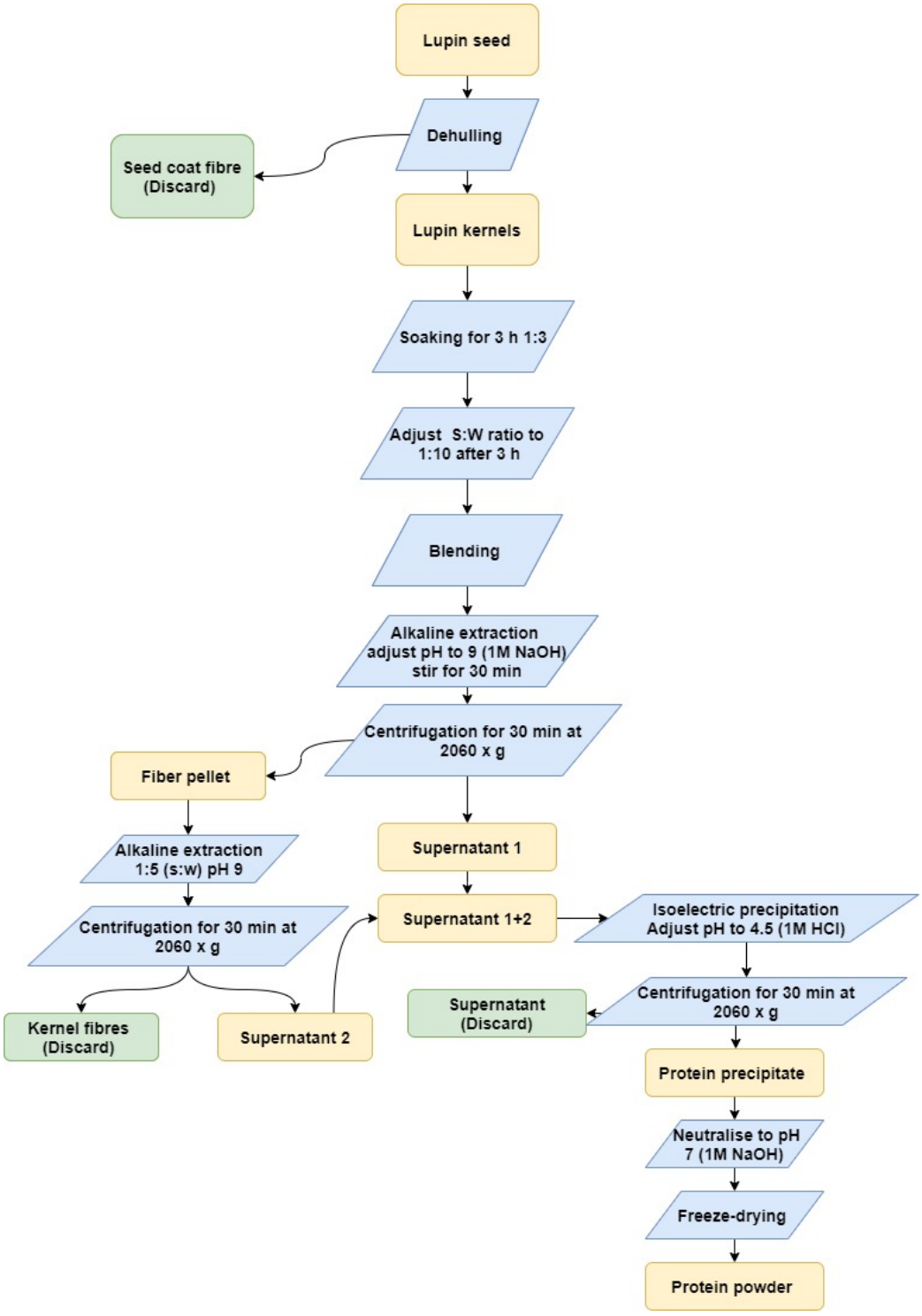
3.2.1. Lupin seeds

The lupin seeds were kindly supplied by the Department of Primary Industries and Regional Development - Agriculture and Food (Kensington, WA, Australia). The samples consisted of six varieties (genotypes) of *L. angustifolius* namely *PBA Gunyidi*, *PBA Barlock*, *PBA Jurien*, *Jenabillup*, *Mandelup* and *Coromup* harvested in the 2015 growing season (April–December), all of which grown ontwo locations (environments) in Western Australia. The selected locations have different environmental conditions. The first location is Eradu (ER) (28.70°S, 115.05°E), which belongs to Agriculture Zone 2 (Corporation Grains Research and Development, 2017) and has a high monthly mean annual rainfall of 450.4 mm and temperature variation of 14.4–24.7°C with 108.1 clear days (BOM, 2018). The second location, Wongan Hills (WH), belongs to Agriculture Zone 5 (Corporation Grains Research and Development, 2017) and has a lower monthly mean annual rainfall of 388.4 mm and temperature variation of 12–27.74 °C with 153.50 clear days (BOM, 2018). All seeds were dehulled using a lab-scale AMAR dehuller (NSIC.SSI, Mumbai, India). The kernels were then separated from the hull using a vacuum separator (Kimseed, Perth, Australia). Any remaining hull fragments were separated by hand, and the cleaned kernels were vacuum packed and kept at 4 °C.

3.2.2. Preparation of lupin protein concentrate

Duplicate lupin protein concentrates (LPCs) were produced from each sample of kernels according to the method of Chew et al. (2003) as presented in Figure 3.1.

Firstly, the lupin kernels were soaked in distilled water 1:3 (w/v) for 3 h at room temperature. After soaking, the ratio of the kernels to water was adjusted to 1:10 (w/v), which was followed by blending for 1 min at high speed using a Waring blender (Model 32BL80, Torrington, CT, USA). The pH of the slurry was adjusted to 9 using 1 M NaOH, and the slurry was homogenised at maximum speed for 30 min using an Ingenieurburo CAT model R50D homogeniser (Hamburg, Germany). The sample was separated by centrifugation for 30 min at 2060 *g* and 4 °C using an Eppendorf centrifuge (model 5810R, Hamburg, Germany). The resulting supernatant (protein extract) was removed by decantation from the fibre pellet. The extraction was repeated on the fibre pellet with five volumes of liquid to the original kernel weight. The supernatants from the two extractions were then combined. The supernatant pH was adjusted to 4.5 using 1 M HCl to induce protein isoelectric precipitation. Next, the sample was centrifuged at 2060 *g* for 30 min at 4 °C to separate the protein precipitate from the supernatant (acid-soluble fraction) by decantation. The pH of the precipitate was adjusted to 7±0.1 using 1 M NaOH. This neutralised precipitate was freeze-dried (model ALPHA 1-2 LO, Christ, Osterode am Harz, Germany) to give the final LPC that was vacuum-packed and stored at 4 °C until future use.



(Chew et al., 2003)

Figure 3.1 Lupin protein concentrate production process

3.2.3. Compositional analysis of lupin kernels and protein concentrates

All compositional analyses of kernels and protein concentrate were conducted in duplicate following standard methods (AOAC, 2011) and the concentration is expressed in db as g/100 g. Moisture content was determined by drying at 105 ℃ in a

fan-forced oven to constant weight. The protein content was measure using the Kjeldahl digestion distillation method (Model Kjeltec™ 2100 Distillation unit, Foss, Hillerod, Denmark) using the N × 5.5 protein conversion factor (Hudson, 1994). The fat content was measured using the Soxhlet extraction distillation method (Buchi E-816, Flawil, Switzerland) using petroleum ether as the solvent. The ash content was determined by furnace combustion at 550 °C overnight. The carbohydrate content of kernels and isolates was calculated based on the equation of Johnson et al., (2003):

Carbohydrate% = 100 – moisture% – protein% – lipid% – ash%.

The carbohydrate content was considered as dietary fibres given the negligible content of starch in *L. angustifolius* kernels (Johnson et al., 2003).

3.2.4. Techno-functional properties

3.2.4.1.Solubility

The solubility of each LPC sample was determined in duplicate according to the method described by Smith et al., (1985). A 3% (w/v) solution of LPC was prepared by dissolving 1.5 g of LPC in 50 mL of 0.1 M phosphate buffer at pH 7. The protein suspension was mixed using a vortex mixer for 2 min before heat treatment at 40 °C for 30 min in a shaking water bath (OLS 200, Grant, UK) using a 40 rpm shaking speed and then kept at 4 °C for ~12 h to equilibrate. Following this step, 1 mL of each sample (duplicate) was centrifuged at 15,000 *g* for 25 min at 15 °C using an Eppendorf centrifuge (Model 5810 R). The resulting supernatant was diluted 50 times by mixing 100 μL with 4900 μL of deionised water. Duplicate 25 μL aliquots of the diluted supernatant were mixed with 200 μL of BCA reagent (Sigma-Aldrich Co., Sydney, Australia) in a 96-well microplate. The microplate was incubated at 37 °C for 30 min and then left at room temperature for 15 min to cool. The UV absorbance of the samples was measured at a wavelength of 562 nm using a Bio-Tek microplate spectrophotometer (Model Synergy HT, Winooski, VT, USA). The protein was

determined using a calibration curve of 200, 400, 600, 800 and 1000 μg/mL of bovine serum albumin (BSA, 1000 μg/mL).

Protein solubility was calculated according to the formula:

$$Solubility\ \% = \frac{Protein\ in\ supernatant\ mg/mL}{Total\ initial\ protein\ \ mg/mL} \times 100$$

3.2.4.2.Foaming capacity and stability

Foaming capacity (FC) was measured according to Wong, Pitts, Jayasena, and Johnson (2013) by preparing duplicate 50 mL samples of a 1% (w/w) of each LPC in deionised water adjusted to pH 7 using 0.1 M NaOH or HCl where necessary and stirring using an MR Hei-Standard stirrer (Schwabach, Germany) at 500 rpm for 2 h at room temperature for hydration. The LPC dispersion was whipped at room temperature using a domestic cake mixer (Sunbeam, Boca Raton, the USA) at maximum speed for 5 min. Any resulting foam and all remaining suspension were transferred carefully to a 500 mL graduated cylinder. Foam volume (mL) and total volume (foam + suspension in mL) were immediately recorded, and these values were recorded as time 0 min. The total foam volume was then recorded at 15, 30, 45, 60, 75, 90 and 120 min.

FC was calculated based on the formula:

$$FC\ \% = \frac{V1 - V2}{V2} \times 100$$

Where V1 is foam volume (mL) immediately after whipping and V2 is the - volume (mL) before whipping.

Foaming stability (FS) was calculated at each time interval according to the formula:

$$Fs\ \% = \frac{Vt - V1}{V1} \times 100$$

Where Vt is thefoam volume (mL) at a time interval and V1 foam volume (mL) immediately after whipping. ×

The FS after 120 min was include in the analyses and is presented in this chapter.

3.2.4.3.Emulsifying activity and stability index

The emulsifying activity index (EAI) and emulsifying stability index (ESI) were determined based on the method of Hu et al., (2009) and Pearce & Kinsella, (1978). In duplicate samples, 10 mL of canola oil was added to 30 mL of a 3% (w/w) suspension of each LPC in deionised water. The pH was adjusted to 7 with 0.1M HCl or NaOH. The emulsion was dispersed by mixing in a shear homogenizer (Model IKA-ULTRA- TURRAX T25 Basic, IKA Works, Inc., Wilmington, NC) at 12,000 rpm for 1 min at 25 °C. Then, 50 μL of the emulsion was taken from the bottom and diluted with 5 mL of phosphate buffer (0.1 M) containing 0.1% (w/v) sodium dodecyl sulphate (SDS). The emulsion absorbance was determined in 1 cm path length cuvette analysed at 500 nm in a Shimadzu UV spectrophotometer (Model UV-1800, Kyoto, Japan). The EAI is expressed as the absorbance at 500 nm at 0 h and 1 h.

The ESI represents the time required for the emulsion to break down. It was determined after keeping the emulsion for 24 h at 4 °C and then measuring the absorbance as described above. The ESI was calculated using the following formula:

$$ESI\ (h) = (A_0/(\Delta A_{0-24})) \times \Delta t$$

Where: A_0 is the absorbance at 0 h, ΔA_{0-24} is the change in absorbance during the storage period, and Δt is the time interval.

The mean of duplicates is presented.

3.2.4.4.Gelation

The gelation properties of LPC were investigated according to the method described by Piornos et al. (2015). Duplicate 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20% (w/w) suspensions of each LPC were made in deionised water. The sample pH was adjusted to 7 using NaOH or HCl as required. Samples were stirred for 2 h using an MR Hei-Standard stirrer at 500 rpm for 2 h at room temperature and then kept at 4 °C for 24 h to complete hydration. Ten millilitres of each protein solution was transferred to a new 10 mL test tube (16 mm × 100 mm outer dimensions) and the sample was heat treated for 1 h at 95 ºC in a water bath. The samples were cooled to room temperature, and the test tubes were stored at 4 °C for 20 h. The least gelation concentration is expressed as the minimum protein concentration at which a gel forms and does not fall or slip out of the inverted test tube.

3.2.4.5.Viscosity

The apparent viscosity of LPC dispersions was measured based on the method described by Arzeni et al. (2012). The LPC dispersions (50 mL) were prepared in deionised water at 10% (w/w) at pH 7. The samples were stirred for 2 h at room temperature and kept at 4 ℃ overnight to reach maximum hydration. The pH was adjusted to 7 using 0.1 M NaOH or HCl as required. The samples underwent heat treatment at 95 ℃ for 0, 30, 60, 90 and 120 min. This was followed by cooling in an ice bath for 10 min and then equilibration at 25 ℃ in a water bath for 1 h to facilitate protein aggregation. The apparent viscosity was then measured using a Brookfield DV-1-*prime* viscometer (Brookfield Engineering, Stoughton, MA, USA) with Spindle 62 and a spindle speed of 100 rpm to give torque in the range 10–100% and is expressed as mPa/s. The mean of triplicate samples was used in the analyses and is presented.

3.2.5. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

The protein profile of LPC was investigated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE), under reducing (by adding 2-mercaptoethanol to sample buffer) and non-reducing conditions based on the method described by Villarino et al. (2015). The LPC samples were dissolved in NuPAGE sample buffer (Invitrogen, Sydney, Australia) to give 7 μg of protein in 10 μL of the final solution, which was loaded into NuPAGE Novex 4–12% Bis-Tris gels (Invitrogen). Electrophoresis was run for 30 min at 200 V with MES SDS running buffer (Invitrogen). Bio-Safe Coomassie G-250 stain (Bio-Rad Laboratories, Carlsbad, CA, USA) was used for protein staining. Gel destaining was performed using deionised water. Protein markers (Unstained Mark 12^{TM} unstained protein standard, Invitrogen, Life Technologies Corp, Sydney, Australia) were used as the molecular weight reference. Image Lab software (V 6.0.1) (Bio-Rad Laboratories) was used to image the gels. The molecular weight of protein bands in the samples was estimated by the distance travelled on the gel compared with the standards and their identity by comparing their molecular weight with that of α-, β-, γ- and δ- conglutins in the literature.

3.2.6. Statistical analysis

All data are reported as mean ± standard deviation of duplicate samples unless stated. The data were analysed using IBM SPSS software (V 26, IBM Corp., Armonk, NY,

USA). Two-way analysis of variance (ANOVA) was used to identify the main effects of variety and growing location, and their interaction (V × L). If a significant main effect or interaction was observed, one-way ANOVA with the Tukey post hoc test was used to isolate the differences. Pearson correlations between functionalities were analysed using SPSS Statistics (V 26). For all analysis, $P < 0.05$ was considered to be significant.

3.3. Results and discussion

3.3.1. Chemical composition of lupin kernels

Protein: The protein of the lupin kernels is presented in Table 3.1. Protein values ranged from 39.98 to 44.16 g/100 g db. Villarino, Jayasena, Coorey, Chakrabarti-Bell, and Johnson(2015) reported that *L. angustifolius* the protein content of kernel protein was 39.50–42.42 g/100 g (db), which this study results agree with. Two-way ANOVA showed that protein was significantly ($P < 0.05$) affected by V and L but not V × L (Table 3.2).-*Coromup* and *Mandelup* varieties grown in the low-rainfall location (WH) contained significantly ($P < 0.05$) higher protein compared with the same varieties grown in the ER location (Table 3.1). Cowling & Tarr (2004) found that both V and L influence protein in *L. angustifolius* seed; however, they concluded that high-rainfall locations in some cases produce seed with lower protein content compared with mediumand low-rainfall locations. Similarly, Arslanoglu et al. (2011) reported that soybean varieties produced in a low-rainfall location produced higher protein than those from a high-rainfall location. However, there is no clear evidence about the relationship between rainfall and protein content (Arslanoglu et al., 2011; Cowling & Tarr, 2004). Maharjan et al., (2019) find that environment conditions variations did not influence pea seeds protein content.

Oil: Oil content in lupin kernels ranged from 5.49 to 8.04 g/100 g db (Table 3.1). The oil content of *L. angustifolius* kernels has been reported as 7–8 g/100 g db (Wäsche et al., 2001; Wong et al., 2013), which the results of this thesis are similar to. The univariate analysis showed that the effects of V and L and V × L had a significant ($P < 0.05$) effect on lupin kernel oil content (Table 3.2).

In both locations, *PBA Barlock* had the highest ($P < 0.05$) oil content of all the varieties. In addition, *PBA Barlock* from the higher-rainfall location (ER) had higher oil content than that from the moderate-rainfall location (WH). Cowling and Tarr

(2004) found that *L. angustifolius* planted in high-rainfall locations had increased seed weight, which was positively associated with the oil content. Lupin varieties grown in the ER location in Western Australia (2015) have been reported to have significantly higher seed weight compared with those grown in the WH location (Zhong et al., 2020). These authors also found that the oil content of the lupin seed coat in most varieties grown in the ER location has a higher oil content than that grown in WH. The findings of this thesis report support these earlier findings. Similarly, Arslanoglu et al. (2011) reported that soybean genotypes planted in a high-rainfall location had a higher oil content compared with those from a lower-rainfall environment. However, Annicchiarico et al. (2014) reported the opposite trend, in that the oil content of *L. albus* grown in a subcontinental climate with high rainfall had lower oil content compared with the lower-rainfall Mediterranean climate.

Ash: content ranged from 2.64 to 3.45 g/100 g db (Table 3.1). Ash content in *L. angustifolius* varieties has been reported as 2.7–3.6 g/100 g db (Villarino, 2015; Wong et al., 2013), and the results here are consistent with this range. Two-way ANOVA showed that ash content was significantly ($P < 0.05$) affected by V, L, and V × L (Table 3.2).

In present study, ash content followed the same pattern as oil content, being higher in the ER location than the WH location (Table 3.1). The *Jenabillup* type had the highest ash content compared with all other varieties in both the ER and WH locations. Bhardwaj et al. (1998) reported that the mineral content (equivalent to ash content) in lupin seed is significantly influenced by V, L and V × L, and the results presented here are consistent with this previous finding. Similarly, Bellaloui et al. (2011) found that V, L and V × L has significant effects on soybean seed mineral content.

Carbohydrate: The level of total carbohydrate in the lupin kernels ranged from 48.05 to 50.95 g/100 g db (Table 3.1). It has been reported that total carbohydrate content (dietary fibre + available carbohydrates) in *L. angustifolius* varieties is 46.4–48.6 g/100 g db (Villarino, Jayasena, Coorey, Chakrabarti-Bell, & Johnson, 2015), and the results of this thesis are consistent with this range. V and L had a significant ($P < 0.05$) effect on lupin kernel carbohydrate content (Table 3.2). However, V × L was not significant. *PBA Jurien* had the highest level of carbohydrate compared with other

varieties in the ER and WH locations. *Coromup* had the lowest carbohydrate content compared with other varieties harvested from WH. Maharjan et al. (2019), found that the effect of V and L have significant effects on pea seed starch content. However, they found that the effect of V × L was not significant, which was also found in this thesis research. Sharma et al. (2014) reported that total carbohydrate (starch + sugar) is significantly influenced by the soybean genotype.

3.3.2. Chemical composition of lupin protein concentrate

Protein: The protein content of LPC is presented in Table 3.3. The protein ranged from 62.87 g/100 db in *PBA Barlock* (ER) to 69.97 g/100g db in *Jenabillup* (WH). Previous work by Chew et al., (2003) on *L. angustifolius* (*Gungurru*) found that the protein concentrate had 67.1 g/100 g db protein, which supports current study findings. Similarly, protein concentrate extracted from soy by IP precipitation has a protein content of 65–70 g/100 g db (Foschia et al., 2017; Hojilla-Evangelista et al., 2004; Sathe et al., 1982). Alu'datt et al. (2017) found a protein content of 58.75 g/100 g db in their LPC, which is slightly lower than that in the current study and may reflect the single extraction step in their method compared with the two- step extraction used in this thesis. Two-way ANOVA showed that protein content of the LPC was significantly ($P < 0.05$) influenced by V, L and V × L (Table 3.2). Five varieties grown in WH location show significantly ($P < 0.05$) higher protein content in their concentrate compared with varieties grown in ER location(Table 3.1). *PBA Gunyidi* planted in the ER location show significantly ($P < 0.05$) higher protein content in it concentrates compared with other varieties grown in the same location. *Jenabillup* PC showed a significant ($P < 0.05$) increase in protein content from both growing locations.

Oil: Oil content of all LPC was similar to that of *L. angustifolius* concentrates reported by Mittermaier (2013), which ranged from 6.3 to 10.8 g/100 g db. ANOVA showed that the oil content in LPC significantly affected ($P < 0.05$) V, L and V × L. Oil content of the LPC was significantly ($P < 0.05$) higher in seeds from the ER location compared with the WH location, which may reflect the higher initial fat content in the kernels from the ER location (Section 3.3.1). LPC from *PBA Barlock* grown in the ER location had the highest oil content compared with the other five varieties ($P < 0.05$). *PBA Jurien* and *Jenabillup* harvested from the WH location had the significantly ($P < 0.05$) lowest fat content among the six genotypes by 9.32 and –9.37 g/100 g db, respectively.

Ash: Ash content in LPCs ranged from 3.37 to 5.60 g/100 g db (Table 3). In previous studies, mineral content was in the range of 3.2 g/100 g to 4 g/100 (Bader et al., 2011; Berghout et al., 2014), which is supported by the values in the present study. Two-way ANOVA showed that the ash content of LPC was significantly influenced ($P < 0.05$) by V, L, and V × L. *PBA Gunyidi* grown in the ER location had the significantly ($P< 0.05$) highest ash content compared with all other varieties grown in both locations. *PBA Barlock*, *Mandelup* and *Coromup* varieties grown in the WH location had the significantly ($P< 0.05$) lowest ash content compared with other varieties. *Total carbohydrate*: Total carbohydrate content of the LPCs ranged from 13.4 to 18.67 g/100 g (Table 3.3). Univariate analysis showed that the carbohydrate content in LPCs followed the same trend as in lupin kernel content, in that V and L had significant ($P< 0.05$) effects on total carbohydrate content of LPCs, although V × L was not significant.

Compositional analysis of IP-precipitated LPCs showed that their protein purity were comparable to those in previous studies (Chew et al., 2003; Hojilla-Evangelista et al., 2004). High levels of impurities such as oil, ash and total carbohydrate in protein concentrate are caused by minimum purification (i.e. defatting and desalting) steps used during production compared with protein isolate (Berghout et al., 2014; Wäsche et al., 2001). Having high levels of oil, salt and carbohydrate in the protein concentrate may be considered a desirable value add in food processing, especially in manufacturing complex food systems (e.g. meat analogues), which demand a substantial amount of raw materials (oil, salt and carbohydrate) (Chiang et al., 2019; Ribeiro et al., 2016).

Table 3.1 Effects of variety and growing location on the chemical composition (g/100 g db) of lupin

Composition	Location	Variety					
		PBA Gunyidi	***PBA Barlock***	***PBA Jurien***	***Jenabillup***	***Mandelup***	***Coromup***
Protein	ER	41.05 ± 0.47^{aA}	39.98 ± 0.9^{aA}	40.50 ± 0.7^{aA}	41.30 ± 0.53^{aA}	40.19 ± 0.01^{aA}	42.01 ± 0.07^{aA}
	WH	42.33 ± 0.43^{abA}	41.36 ± 0.45^{aA}	42.05 ± 0.7^{abB}	41.98 ± 0.7^{abA}	42.56 ± 0.19^{abB}	44.16 ± 0.66^{bB}
Oil	ER	6.73 ± 0.0^{cA}	8.04 ± 0.02^{eA}	5.67 ± 0.02^{aA}	6.68 ± 0.04^{cA}	6.55 ± 0.01^{bA}	6.81 ± 0.0^{dA}
	WH	5.54 ± 0.01^{aB}	7.09 ± 0.01^{eB}	5.49 ± 0.04^{aB}	6.93 ± 0.03^{dB}	6.36 ± 0.04^{bB}	6.74 ± 0.04^{cA}
Ash	ER	3.16 ± 0.01^{bA}	2.89 ± 0.02^{aA}	2.88 ± 0.07^{aA}	3.45 ± 0.09^{cA}	3.03 ± 0.06^{abA}	3.13 ± 0.01^{bA}
	WH	2.85 ± 0.01^{bcB}	2.72 ± 0.09^{abA}	2.76 ± 0.06^{abA}	2.98 ± 0.01^{cB}	2.64 ± 0.01^{aB}	2.93 ± 0.03^{cB}
Total carbohydrate	ER	49.06 ± 0.46^{abcA}	49.1 ± 0.86^{abcA}	50.95 ± 0.65^{cA}	48.56 ± 0.58^{abA}	50.22 ± 0.06^{bcA}	48.05 ± 0.06^{aA}
	WH	49.28 ± 0.42^{bA}	48.83 ± 0.54^{bA}	49.7 ± 0.72^{bA}	48.11 ± 0.77^{abA}	48.45 ± 0.22^{abB}	46.18 ± 0.64^{aA}

Data presented as mean ± standard deviation, n = 2
a, b, c, d, e Means with different letters in the same row are significantly different ($P < 0.05$).
A, B Means with different letters in the same column are significantly different ($P < 0.05$).
ER, Eradu; WH, Wongan Hills.

Table 3.2 Effects of variety, growing location and their interaction (V × L) presented as *P* values from analysis of variance

	P value							
	Kernels composition				Concentrate composition			
	Protein	Oil	Ash	Total carbohydrate	Protein	Oil	Ash	Total carbohydrate
Variety	<0.0010	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Location	< 0.0001	< 0.0001	< 0.0001	0.003	< 0.0001	< 0.0001	< 0.0001	< 0.0001
V × L	0.368	0.0001	0.002	0.125	< 0.0001	< 0.0001	< 0.0001	0.054

V × L, Variety and location interaction.

Table 3. 2 Effects of variety and growing location on chemical composition (g/100 g db) of lupin protein concentrates

Composition	Location	Variety					
		PBA Gunyidi	***PBA Barlock***	***PBA Jurien***	***Jenabillup***	***Mandelup***	***Coromup***
Protein	ER	68.61 ± 0.09^{dA}	62.87 ± 0.04^{aA}	68.65 ± 0.18^{dA}	69.26 ± 0.03^{eA}	66.85 ± 0.07^{bA}	67.52 ± 0.12^{cA}
	WH	68.03 ± 0.01^{aB}	67.77 ± 0.16^{aB}	68.81 ± 0.20^{bA}	69.97 ± 0.13^{cB}	67.99 ± 0.05^{aB}	68.79 ± 0.21^{bB}
Oil	ER	12.23 ± 0.04^{cA}	13.39 ± 0.09^{dA}	11.01 ± 0.03^{aA}	10.70 ± 0.14^{aA}	11.37 ± 0.02^{bA}	12.13 ± 0.07^{cA}
	WH	11.38 ± 0.10^{cB}	11.76 ± 0.10^{dB}	9.32 ± 0.01^{aB}	9.37 ± 0.04^{aB}	10.31 ± 0.02^{bB}	11.43 ± 0.10^{cB}
Ash	ER	5.60 ± 0.07^{dA}	4.5 ± 0.05^{cA}	4.22 ± 0.11^{aA}	4.35 ± 0.07^{bcA}	4.48 ± 0.03^{cA}	4.33 ± 0.04^{bcA}
	WH	4.45 ± 0.09^{dB}	3.37 ± 0.06^{aB}	3.70 ± 0.02^{bB}	3.90 ± 0.02^{cB}	3.44 ± 0.02^{aB}	3.55 ± 0.01^{abB}
Total carbohydrate	ER	13.4 ± 0.47^{aA}	16.65 ± 1.98^{aA}	15.16 ± 0.41^{aA}	14.01 ± 1.41^{aA}	16.47 ± 0.81^{aA}	15.71 ± 2.04^{aA}
	WH	14.78 ± 0.28^{aA}	16.41 ± 0.05^{bcA}	18.67 ± 3.98^{dB}	15.41 ± 1.32^{abB}	18.28 ± 2.97^{dB}	17.18 ± 2.95^{cdB}

Data presented as mean ± standard deviation, n = 2

a, b, c, d, e Means with different letters in the same row are significantly different ($P < 0.05$).

A, B Means with different letters in the same column are significantly different ($P < 0.05$).

ER, Eradu; WH, Wongan Hills.

3.3.3. Lupin protein profile

Images of the electrophoretic separation of the LPCs (α- and β-conglutin fractions) of the six varieties from the two locations ER and WH under both non-reducing (N) and reducing (R) conditions are presented in Figures 3.5 and 3.6. that the molecular weights of the protein monomers and subunits of α- and β-conglutin separated under N conditions have been reported as 80–50 kDa and 60–20 kDa, respectively (Wong et al., 2013). All varieties from both locations showed similar lupin protein profiles under N conditions with molecular weights range 97–20 kDa (Berghout et al., 2014; Duranti et al., 1992, 2008; Sironi et al., 2005). The LPCs samples differed in the intensity of one band at a molecular weight around 31 kDa, which corresponds to one of the β-conglutin fraction subunits (Duranti, Consonni, Magni, Sessa, & Scarafoni, 2008; Muranyi et al., 2016). This band was strong in samples of *PBA Barlock*, *PBA Jurien* and *Jenabillup* harvested from the two locations but very faint in samples of *PBA Gunyidi*, *Mandelup* and *Coromup* from both environments.

These differences suggest a genotypic effect on the protein profile. It has been reported that the molecular weights of proteins and the protein profile can differ between genotypes (Islam et al., 2012). Similarly, Fehr et al. (2003) observed that the protein profiles of 14 soybean (*Glycine max* L.) varieties was significantly influenced by genotypic diversity, but the effect of growing location was not significant. Bainy et al., (2008) found that genetic diversity among soybean varieties influences their glycinin and β-conglycinin subunit percentages and their glycinin:β-conglycinin ratios. They found that these differences in protein profile led to changes in protein denaturation temperatures between varieties. They noted that the decreased glycinin ratio in some varieties led to a decrease in denaturation enthalpy, which significantly improved the gel quality of those varieties compared with varieties with a high glycinin:β-conglycinin ratio. Thermal studies of lupin protein have reported that the α-conglutin fraction has greater thermal stability than β-conglutin because of the high number of disulphide bonds in α-conglutin compared with β-conglutin (Section 2.3.3.4). Taken together, these findings suggest that changes in the β-conglutin profile in some lupin varieties alters the protein profile and may have limited effects on some of the techno-functional properties of LPC.

Under the R condition, lupin protein exhibited greater band separation and the protein bands appeared at a lower molecular weight because of the cleavage of S-S bonds (Blagrove & Gillespie, 1975; Rumiyati et al., 2012). However, changes in the protein profile seemed to involve α-conglutin because of the absence of S-S bonds in the β-conglutin fraction (Duranti et al., 2008).

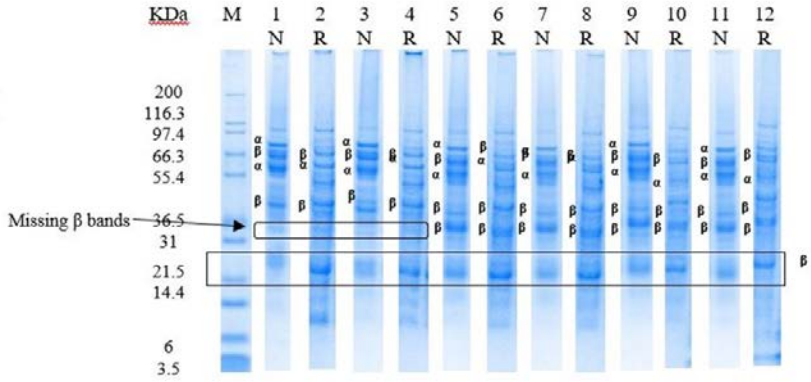


Figure 3. 2 Electrophoresis patterns under non-reducing and reducing conditions of three lupin varieties grown in two locations. M: protein marker, lanes 1 and 2 *PBA Gunyidi* ER, lanes 3 and 4 *PBA Gunyidi* WH, lanes 5 and 6 *PBA Barlock* ER, lanes 7 and 8 *PBA Barlock* WH, lanes 9 and10 *PBA Jurien* ER, lanes 11 and 12 *PBA Jurien WH*

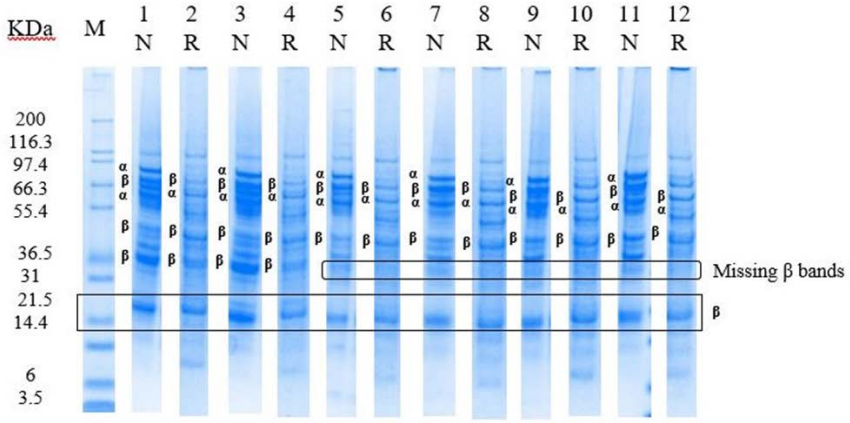


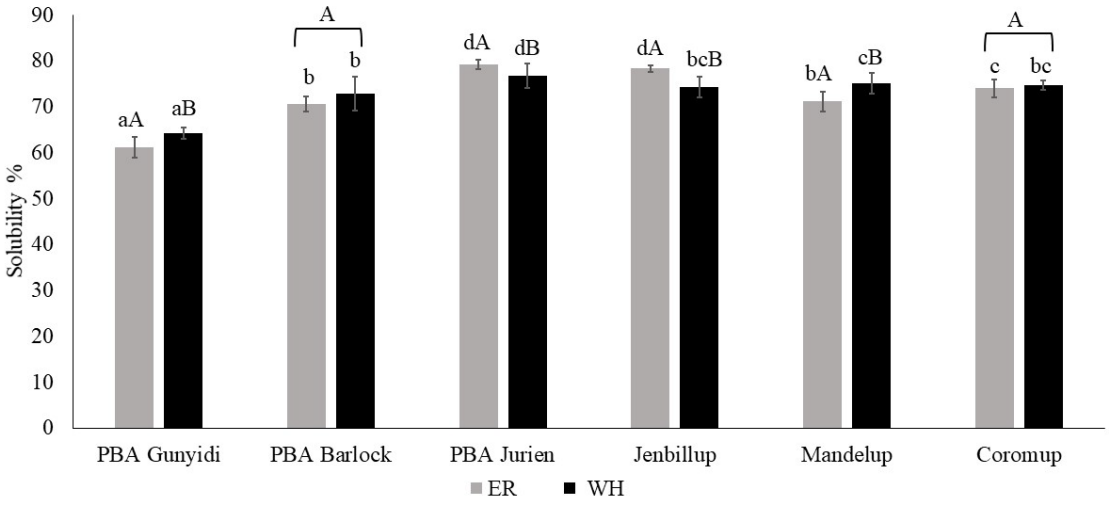
Figure 3. 3 Electrophoresis patterns under reduced and non-reduced conditions of three lupin varieties grown in two locations. M: protein marker, lane 1&2 *Jenabillup* ER, lane 3&4 *Jenabillup* WH, 5&6 *Mandelup* ER, 7&8 *Mandelup* WH, 9&10 *Coromup* ER, 11&12 *Coromup* WH

3.3.4. Lupin protein concentrate techno-functional properties

3.3.4.1. Protein solubility

The protein solubility values at pH 7 of LPC made from the six varieties grown in the two locations are presented in Figure 3.4. The two-way ANOVA indicated that V, L, and V × L had significant effects on the LPC protein solubility ($P < 0.05$) (Table 3.4).

There was a wide range of variation in LPC solubility. LPC from *PBA Jurien* had the highest ($P < 0.05$) protein solubility of the different varieties grown in both locations, whereas *PBA Gunyidi* had the significantly lowest protein solubility. These differences in protein solubility reflect differences in the protein profile between some of the lupin genotypes (Section 3.3.3). It has been reported that the V, L and V × L a significant effects on lupin protein AA composition (Bhardwaj et al., 1998). Khatib et al. (2002) found that differences in soybean genomic diversity between varieties affect the protein content, ratios of protein fractions (β-conglycinin and glycinin) and changes in AA profile. These differences may be the source of variation in protein solubility and other protein techno-functionalities. It has been reported that LPC solubility is around ~70% at pH 8 (Chew et al., 2003), and the current study result of pH 7 is near this value. However, the higher pH (8) used by Chew et al. may have increased the protein solubility because legumes are known for having higher protein solubility at alkaline pH (Bartkiene et al., 2018; Ghribi et al., 2015). Vogelsang-O'Dwyer et al. (2020) reported that a lupin protein isolate had 76.9% solubility, which is a slightly higher than that recorded in this study. This is relate to the defatting step that they used before protein extraction, which may have resulted in the low fat content in their samples of 0.97 g/100 g db. Low fat content can improve protein solubility by reducing phospholipid content (Boatright & Hettiarachchy, 1995). Similarly, in the current study, Pearson correlational analysis showed a significant negative correlation ($r = –0.482$, $P < 0.05$) between fat content and protein solubility. The lupin protein solubility results in the current study are comparable to those for pea (59%) but are higher than the 45% for soybean protein concentrate at neutral pH (Fernández-Quintela et al., 1997; Hojilla-Evangelista et al., 2004).



Mean ± standard deviation, n = 2
a, b, c, d, Means with different letters in the same location are significantly different ($P < 0.05$).
A, B, Means with different letters in the same variety are significantly different ($P < 0.05$).
ER, Eradu; WH, Wongan Hills.

Figure 3.4 Effects of variety and growing location on lupin protein concentrate solubility at pH 7.0

Table 3. 3 Effects of variety, growing location and their interaction (V × L) presented as *P* values from analysis of variance

	P value						
	Solubility	Foaming properties		Emulsifying properties		Viscosity	
Techno-functionality		FC	FS	EAI 0 h	ESI 24 h	0 min HTt	120 min HTt
Variety	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Location	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.006	< 0.0001	< 0.0001
V × L	< 0.0001	0.032	0.449	< 0.0001	< 0.0001	< 0.0001	< 0.0001

FC, foaming capacity (%); FS, foaming stability (%); EAI, emulsifying activity index (h); ESI, emulsifying stability index (h); HTt, heat treatment time (°C); V × L, variety and location interaction.

3.3.4.2. Foaming properties

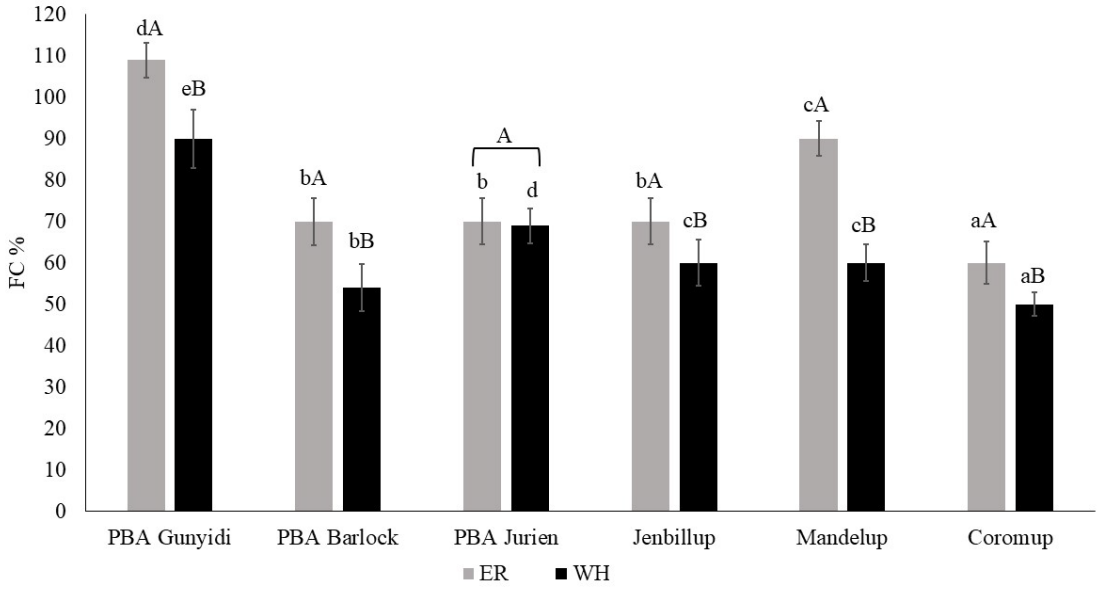
FC and FS of the IP-precipitated LPC from the six varieties grown in the two locations are presented in Figures 3.5 and 3.6. Foaming quality analysis showed that FC ranged from 50% to 110%. V, L and V × L had significant ($P < 0.05$) effects on foaming properties (Table 3.4). *PBA Gunyidi* significantly ($P < 0.05$) higher FC (110%) compared with the other varieties in the ER and WH locations. Genotype difference can influence protein component (11S and 7S) and their ratios, which can affect techno-functional properties. Fehr et al., (2003) report that soy genotype difference affects globulins protein subunits ratio significantly. The 7S of soy protein shows lower solubility, higher hydrophobicity and higher FC compare to 11S (Sirison et al., 2021). Similarly, low solubility of LPC extracted from *PBA Gunyidi* maybe due to high 7S ratio, which can improve its FC.

Lqari, Vioque, Pedroche, Milla, et al. (2002) reported that their *L. angustifolius* IP protein had 119% FC, which the current study's results agree with. Lqari et al. used a higher sample of 3% (w/v) with higher protein concentration sample 86.3 g/100 g db (5.8 conversion factor) compared with 1% protein in the current study. However, the FC of 166.7% for LPC reported by Wong, Pitts, Jayasena, and Johnson (2013) is higher than that of all varieties examined in this thesis. This maybe reflects the use of different lupin genotypes, which can influence foaming properties significantly.

The FS values of LPCs are presented in Figure 3.4. The FS was significantly ($P < 0.05$) influenced by variety, location and their V x L (Table 3.4). The LPC from the *PBA Barlock* variety grown in the WH location had significantly higher ($P < 0.05$) FS compared with LPCs from other varieties grown in both locations. The protein solubility significantly influences other techno-functional properties such as foaming (Damodaran & Paraf, 1997). Pearson correlational analysis showed a significant positive correlation ($r = 0.615$, $P < 0.01$) between solubility and FS. Jayasena, Chih, Naser-Abbas (2010) reported that LPC has a 70–71% FS at pH 8. The current study findings are comparable to the findings of Jayasena et al. and Lqari, Vioque, Pedroche, Milla, and Millán (2002), who reported an FS of 94.8% for lupin IP-precipitated protein. However, the main lupin protein fractions in LPC (α- and β-conglutin) are known to have weak foaming properties compared with some IP-precipitated protein fractions of other legumes such as soybean concentrate, as described by Hojilla-Evangelista et al. (2004). Hojilla-Evangelista et al. (2004) reported that the IP-

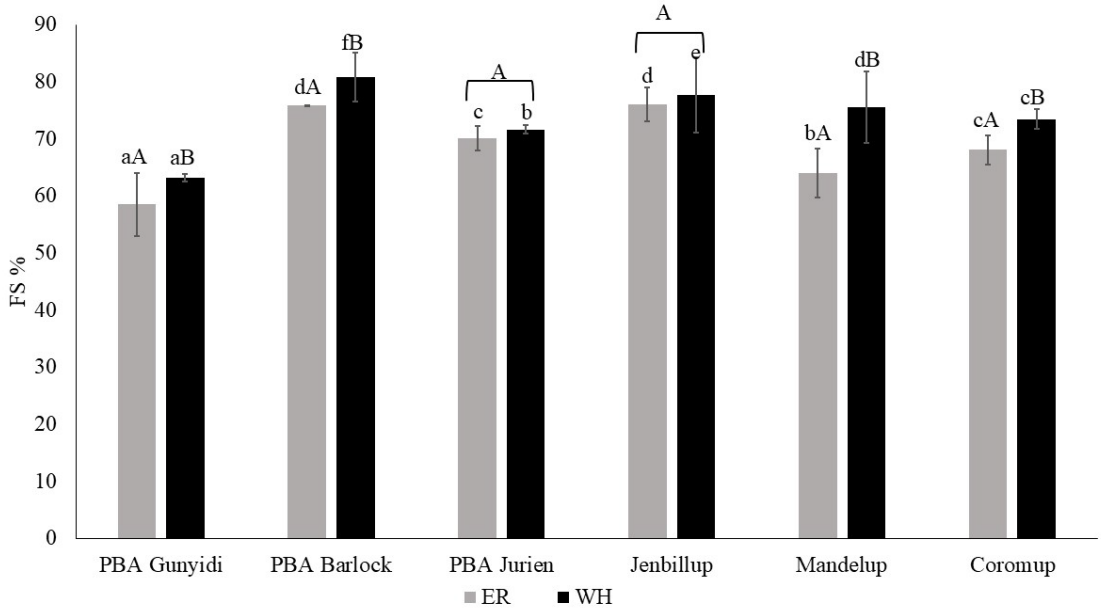
precipitated PC of soybean has a significantly higher FS (95%) than the 16.8% for LPC. This difference may reflect the use of defatted soybean meal to extract soybean protein, whereas full-fat lupin meal is used to extract lupin protein; the latter can interfere with the protein-foaming properties. Alu'datt et al. (2017) examined the foaming properties of chickpea, broad bean and lupin protein concentrate. They found that the FS was lower in lupin protein (50%) than broad bean (70.8%) but was similar to that of chickpea protein concentrate (50%).

The use of other protein extraction technique such as ultrafiltration (UF) may influence the foaming properties compared with the IP-precipitated extraction used in the current study. Hojilla-Evangelista et al. (2004) reported that IP-precipitated LPC has a higher FS (16.8%) than that of UF extract FS (2.6%). Chew et al. (2003) found that both UF and IP-precipitated LPCs had similar weak foaming properties and that there were no significant difference between them. Even though the UF method can enrich lupin LPC (α-and β-conglutin) by adding γ-conglutin fraction (the so-called F(foam) fraction), which has excellent foaming properties similar to those of egg white protein (Wäsche et al., 2001; Wong et al., 2013), the UF extract cannot enhance the foaming properties of LPC. However, D'Agostina et al. (2006) developed a process to maximise the so-called γ-conglutin ratio in LPC using IP precipitation and UF combined. This increase in the γ-conglutin fraction content led to improve foaming properties of the new extract compared with extracts prepared with IP precipitation or UF alone. This suggests the importance of the extraction technique to the protein extract composition and functionality.



Mean ± standard deviation, n = 2
a, b, c, d, Means with different letters in the same location are significantly different ($P < 0.05$).
A, B, Means with different letters in the same variety are significantly different ($P < 0.05$).
FC, Foaming capacity; ER, Eradu; WH, Wongan Hills.

Figure 3. 5 Effects of variety and growing location on lupin protein concentrate foaming capacity



Mean ± standard deviation, n=2
a, b, c, d, e, f Means with different letters in the same environment are significantly different ($P < 0.05$).
A, B, Means with different letters in the same genotype are significantly different ($P < 0.05$).
FS, Foaming stability. ER, Eradu; WH, Wongan Hills.

Figure 3. 6 Effects of variety and growing location on lupin protein concentrate foaming stability.

3.3.4.3. Emulsifying properties

The emulsifying properties of the LPCs are presented in Table 3.5. Emulsifying activity is the maximum ability of a protein to emulsify oil in protein solution (Pearce & Kinsella, 1978). It estimated as the emulsion turbidity absorbance at 500 nm (Abs_{500}). Univariate analysis showed that the EAI of LPC was significantly affected by V, L and L × V ($P < 0.05$) (Table 3.4). At 0 h, the EAI was the highest (0.88 Abs_{500}) for the PC of *PBA Jurien* grown at WH ($P < 0.05$) and the lowest for *Jenabillup* (WH (0.53 Abs_{500}). All LPCs showed comparable results to those reported for soybean protein isolates of 0.588–0.625 Abs_{500} (Hu et al., 2009), even though the soybean protein isolate had a higher protein concentration (90 g/100 g db) than the LPCs in the current study. Similarly, Jayasena, Chih, & Naser-Abbas (2010) reported that their LPC had a higher EAI than their soy protein isolate. The IP-precipitated fraction (α- and β-conglutin) of lupin protein has been named the emulsifying (E) fraction because of its superior emulsion capacity compared with the acid-soluble fraction (F-fraction, γ-conglutin) (Wäsche et al., 2001). Chew et al. (2003) reported that the IP-precipitated fraction had higher EAI than the UF fraction (enriched with γ-conglutin) at all pH values except 2, for which the UF fraction was higher.

The ESI represents the time required for the emulsified layer to separate back into the oil and the aqueous phase. The ESI was significantly ($P < 0.05$) influenced by V, K and V × L) (Table 3.4). The *Coromup* variety grown in WH location had a significantly ($P < 0.05$) higher ESI of 75.12 h compared with the LPCs from the other varieties grown in either location (Table 3.5). By contrast, the LPC from *PBA Gunyidi* grown in WH had the lowest ESI (44.28 h) ($P < 0.05$). Hojilla-Evangelista et al. (2004) demonstrated that LPC has a superior ESI compared with that from soybean. Hu et al., (2009) reported that the ESI of soybean protein isolates ranged from 59.28 to 84.86 min, which is lower than that for the LPCs in the current study. It has been reported LPC has a similar ESI to those from chickpea and broad bean. The findings in the current study suggest that LPCs of *L. angustifolius* demonstrate high-quality emulsifying properties (EAI and ESI), which support the potential use of LPC as an emulsifying agent in range of plant-based product such as mayonnaise and meat and dairy analogues.

Table 3. 4 Effects of variety and growing location on the emulsifying properties of lupin protein concentrate

Emulsifying properties	**Location**	**Variety**					
		PBA Gunyidi	***PBA Barlock***	***PBA Jurien***	***Jenabillup***	***Mandelup***	***Coromup***
EAI at 0 h (Abs_{500nm})	ER	0.58 ± 0.01^{cA}	0.56 ± 0.01^{bA}	0.55 ± 0.11^{aA}	0.69 ± 0.11^{eA}	0.69 ± 0.12^{eA}	0.64 ± 0.10^{dA}
	WH	0.66 ± 0.01^{dB}	0.66 ± 0.20^{cB}	0.88 ± 0.20^{fB}	0.53 ± 0.10^{aB}	0.68 ± 0.21^{eB}	0.64 ± 0.01^{bA}
ESI at 24 h	ER	51.29 ± 0.37^{bA}	45.42 ± 0.52^{aA}	74.82 ± 1.53^{eA}	68.95 ± 0.07^{dA}	65.6 ± 0.17^{cA}	63.97 ± 0.05^{cA}
	WH	44.28 ± 0.05^{aB}	64.21 ± 0.31^{bB}	57.44 ± 0.16^{bB}	56.37 ± 0.13^{bB}	68.42 ± 0.34^{dB}	75.12 ± 0.60^{eB}

Data presented as mean ± standard deviation, n = 2

a, b, c, d, e, f Means with different letters in the same row are significantly different ($P < 0.05$).

A, B, C, D Means with different letters in the same column are significantly different ($P < 0.05$).

Abs, Absorbance. ER, Eradu; WH, Wongan Hills. EAI, emulsifying activity index; ESI, emulsifying stability index.

3.3.4.4. Viscosity and gelation

Viscosity: Table 3.6 presents the apparent viscosity of the LPC. Viscosity was measured (mPa.s) using 10% (w/w) as the PC concentration at 95 ℃ for 0, 30, 60, 90 and 120 min of heat treatment to evaluate the thickening ability, which is considered to be the first stage of gelation (Kessler & Beyer, 1991). The apparent viscosity of unheated LPCs was 8.88–12.77 mPa.s. Two-way ANOVA showed that viscosity was influenced significantly ($P < 0.05$) by the V, L, and V × L (Table 3.6). The LPC viscosity was higher in varieties grown in the WH location ($P < 0.05$) than in those from the ER location at 0 min of heat treatment. This difference may reflect the higher protein content of the PC of varieties from WH location. Pearson correlational analysis showed a significant positive correlation ($r = 0.459$, $P < 0.05$) between the protein content in LPCs and apparent viscosity, which supports the viscosity results. A higher protein concentration will lead to more protein–protein and protein–water interactions, which can increase viscosity (Philipp et al., 2018). The LPC viscosities reported in the current study are higher than the 6 mPa.s for IP-precipitated samples reported by Chew et al. (2003), which is maybe due to different varieties that used in the current study.

The viscosity of all LPCs increased significantly ($P < 0.05$) with heat treatment time (Table 3.6). Heat treatment (95 °C) for 120 min almost doubled the viscosity of all LPCs compared with their unheated samples. Berghout et al. (2015) reported that heat treatment to 120 °C for 5 min increased lupin protein viscosity significantly compared with 60 °C and 80 °C for 30 min. Heat treatment may lead to physical and chemical changes in the protein structure, which would then increase viscosity (Berghout et al., 2015). However, lupin protein exhibit lower viscosity than that of soy protein concentrate and isolate reported by Berghout, Boom, et al. (2015) and Lamsal et al. (2007). Berghout, Venema, et al. (2015) reported that lupin protein isolate had lower viscosity than soybean protein isolate even after prolonged heat treatment at 80 °C for 8 h. They also noted that lupin protein required a higher protein isolate concentration (30% w/v) to exhibit similar viscosity to that of soybean at a lower concentration (20% w/v).

Gelation: The assessment of LPC gel quality used a heat-set gel system and minimum gelling concentration technique. The gelling ability of LPCs was examined under the range of PC concentrations (total solids) of 2–22% w/w. All LPC varieties from the two location were unable to form a self-standing gel at all concentrations studied.

Berghout, Boom, and van der Goot (2015) reported that soybean protein isolates (PC 90 g/100 g db) can form a stable gel at 15% (w/v) protein, whereas in this thesis research *L. angustifolius* protein isolates (protein 90 g/100 g, N × 6.25 db) formed a gel at 18% protein. This finding of the gelation ability of the lupin protein isolate may reflect the higher protein concentration in the isolate used by Berghout, Boom and van der Goot compared with the LPCs used in this thesis. In a recent study, Vogelsang-O'Dwyer et al. (2020) reported that spray-dried *L. angustifolius* protein concentrate (89.44 g/100 g, N × 5.7 (db)) forms a weak gel at a high protein concentration (23%). By contrast, Lqari, Vioque, Pedroche, and Milla (2002) reported that *L. angustifolius* protein concentrate (90.47 g/100 g N × 5.8 db) produced by the same IP precipitation method as used in the present study had a minimum gelation protein isolate concentration of 12% (w/w), which is the lowest minimum protein required for gelation reported for *L. angustifolius* protein in the literature.

Weak lupin protein viscosity and gel quality compared with those of other legume proteins might relate to the physicochemical limitations of lupin protein given its high denaturation temperature and high repulsion forces between protein molecules (Batista et al., 2005; Berghout, Boom, et al., 2015), which may limit its thickening and gelling abilities. It has been reported that α-conglutin has a high denaturation temperature of 90.92 °C (Fontanari et al., 2012). A high denaturation temperature will limit lupin protein unfolding during the gelation process, which can lead to weaker thickening and gelation properties. This may highlight the need to modify lupin protein using novel techniques such as ultrasound to enhance the lupin gelation properties.

Table 3. 5 Effects of variety and growing location on lupin protein concentrate viscosity

HTT (°C)	HTt (min)	Location	Variety					
			LPC viscosity (mPa/s)					
			PBA Gunyidi	***PBA Barlock***	***PBA Jurien***	***Jenbillup***	***Mandelup***	***Coromup***
95	**0**	ER	9.32±0.04^{aA}	8.94±0.42^{aA}	10.83±0.64^{bA}	8.88±0.01^{aA}	11.63±0.07^{cA}	9.28±0.04^{aA}
		WH	12.63±0.07^{cB}	11.07±0.01^{aB}	12.41±0.21^{cB}	12.57±0.07^{cB}	12.01±0.14^{bB}	12.77±0.07^{cB}
	30	ER	10.90±0.21^{aA}	11.40±0.01^{aA}	16.53±0.22^{eA}	12.63±0.01^{bA}	14.53±0.21^{dA}	13.81±0.07^{cA}
		WH	18.67±0.07^{eB}	16.67±0.21^{cB}	16.57±0.01^{cA}	17.33±0.07^{dB}	12.07±0.07^{aB}	16.33±0.01^{bB}
	60	ER	12.67±0.21^{bA}	11.60±0.49^{aA}	17.80±0.28^{eA}	13.47±0.07^{cA}	14.50±0.14^{dA}	13.80±0.07^{cA}
		WH	19.10±0.07^{dB}	16.63±0.07^{bB}	16.63±0.21^{bB}	18.83±0.14^{dB}	12.46±0.52^{aB}	17.51±0.14^{cB}
	90	ER	16.57±0.14^{dA}	14.23±0.35^{abA}	17.73±0.42^{eA}	13.80±0.07^{aA}	15.67±0.07^{cA}	14.50±0.07^{bA}
		WH	20.30±0.14^{dB}	17.80±0.28^{bB}	17.73±0.42^{bA}	19.47±0.14^{cB}	12.47±0.07^{aB}	17.81±0.07^{bB}
	120	ER	17.90±0.14^{cA}	14.33±0.07^{aA}	20.11±0.01^{eA}	16.13±0.49^{bA}	19.10±0.14^{dA}	18.4±0.14^{cA}
		WH	20.27±0.07^{bB}	19.73±0.14^{bB}	20.13±0.01^{bA}	19.64±0.29^{bB}	13.77±0.07^{aB}	22.67±0.07^{cB}

Data presented as mean ± standard deviation, n = 2

a, b, c, d, e, f Values with different letters in the same row are significantly different ($P < 0.05$).

A, B, C, D Values with different letters in the same column are significantly different ($P < 0.05$).

ER, Eradu; WH, Wongan Hills. HTt, heat treatment time; HTT, heat treatment temperature; LPC, lupin protein concentrate.

3.4. Conclusion

This chapter has presented the effects of variety, location and V × L on the chemical composition of *L. angustifolius* kernel flour and the composition and techno-functional properties (solubility, foaming, emulsifying viscosity and gelation) of LPCs. The effects of variety, location and V × L significantly influenced the chemical composition of most kernels and LPCs. The electrophoretic profile of LPCs showed some differences in the β-conglutin profile between varieties regardless of the growing location. Some LPC varieties showed acceptable protein solubility and high emulsifying properties. However, all LPCs showed weak foaming, viscosity and gelation properties. The knowledge gained through this study may benefit several groups: (1) lupin breeders and farmers by focusing on varieties with high potential for human food ingredient manufacturing based on examined lupin protein functionality profile through the current study, (2) food manufacturing companies that use lupin as an emulsifying agent in a range of food systems due to outstanding emulsifying properties of lupin protein (3) food technologist to focus on improve weak lupin protein functionalities, which may open new horizons for the use of lupin protein as a food ingredient for humans consumption, especially with increasing demand for plant protein on food manufacturing market.

In conclusion, given the lack of effects of variety, location and V × L on lupin protein gel quality, *L. angustifolius Coromup* was selected for further protein modification studies. *Coromup* grown in the WH location showed high protein content and viscosity compared with other varieties. The following chapters describe experiments used to study and optimise the effects of ultrasound treatment time and power on LPC physicochemical and gelation properties.

CHAPTER 4

Effect of High-Intensity Ultrasound Pre-Treatments on Acid-Induced Gelation of Lupin Protein Concentrate

Abstract

High-intensity ultrasound (HIU) can modify protein structure and improve its techno-functional properties via a green and chemical-free process. The aim of the research described in this chapter was to investigate the effects of HIU on lupin protein concentrate (LPC) acid-induced gelation properties. LPC dispersions (10%, w/w) were treated with HIU at power 11, 17 and 38 W/cm^2 for 0, 2, 10, 15, 20 and 40 min using an ultrasound probe. Particle size measured by dynamic light scattering, zeta (ζ) potential, solubility, Fourier-transform infrared spectroscopy, differential scanning calorimetry and electrophoresis were used to evaluate the physiochemical changes triggered by HIU. Gel strength (Gs), water holding capacity (WHC) and gel storage modulus (G′) were used to evaluate LPC gel quality. HIU treatment at 38 W/cm^2 power for 20 min produced the highest Gs (195.33 g), WHC (79%) and G′ (3820 Pa) versus 28.33 g, 29.46% and 1600 Pa, respectively, in untreated samples. We hypothesise that HIU modified the lupin protein structure by exposing active hydrophobic amino acid R-groups and reducing the repulsion forces (reduced ζ potential), which improved the crosslinking ability between polypeptides. These changes enabled lupin protein to build a firm three-dimensional gel network. HIU may have potential to increase the use of lupin protein as a novel gelling agent in a wide range of plant-based food products.

4.1. Introduction

There is a burgeoning demand for plant protein as a food ingredient in the food manufacturing sector because of the increasing world population and consumers adopting healthier diet options such as plant-based diets (Faber, Castellanos-Feijoó, Van de Sompel, Davydova, & Perez-Cueto, 2020; Graça, Godinho, & Truninger, 2019). However, the viability of plant protein sources in food manufacturing depends on their nutritional value and techno-functional properties (Sá, Moreno, & Carciofi, 2020).

There is global interest in lupin seed and its production because of its intrinsic nutritional benefits and important role in sustainable agriculture (Gladstones et al., 1998). Lupin kernels have a high content of protein (40 g/100 g on a dry basis (db)) and dietary fibre (40 g/100 g db) but are lower in fat and potentially toxic phytoestrogens compared with soybean (Chew et al., 2003). Lupin protein isolation and fractionation conventionally use alkaline extraction followed by isoelectric point (IP) precipitation (Chew et al., 2003; Wong et al., 2013). The precipitate is the main lupin protein fraction and contains primarily α- and β-conglutin (Wäsche et al., 2001). α-Conglutin accounts for 35-37% of lupin protein, and β-conglutin contributes 44-45% (Blagrove & Gillespie, 1975, Petterson, 1998; Duranti et al. 2008; Foley et al., 2011). Studies have shown that the α and β fractions of lupin protein have a more thermostable structure than the equivalent soybean and pea protein fractions because of the high number of disulphide (S-S) bonds in α-conglutin (six S-S bridges) (Batista et al., 2005; Berghout, Boom, et al., 2015; Sousa et al., 1995). This thermostability contributes to the poor gelation properties of the lupin α- and β-conglutin fraction by hindering the protein unfolding step required for gelation. However, no studies have reported on approaches for improving lupin protein gelation.

The ability of plant proteins to form gels is one of the most demanded functionalities in food processing because this functionality gives sensory and textural acceptability in a wide range of food applications such as dairy and meat analogues and gluten-free cereal-based products (Rees Clayton et al., 2019; Valim et al., 2009). Protein gels can be formed by two key methods: (1) heat-induced protein unfolding at neutral pH followed by aggregation and formation of a three-dimensional crosslinked network (heat-set gel) (Vilgis, 2015); (2) addition of coagulants such as $CaSO_4$ (salt-induced gel) or glucono-δ-lactone (GDL) (acid-induced gel) at ambient temperature to neutralise the negative charge of protein molecules and thus allow three-dimensional gel network formation (cold-set gel) (Zhang et al., 2016). The cold-set gel system is ideal for incorporating heat-sensitive materials such as vitamins, probiotics, food colours and flavour compounds (de Vos et al., 2010; Desai & Jin Park, 2005; Mortensen, 2006) due to the separation of the heat treatment step from that of gel-formation step. So, heat-sensitive materials can be mixed before triggering gel formation at ambient temperature. A cold-set gel can provide a more stable gel network than a heat-induced gel because of

the low repulsion forces between protein molecules, which increases the intermolecular crosslinking ability of the proteins (Alting, Hamer, De Kruif, & Visschers, 2003).

Native lupin protein has poor gelation ability; however, the use of processing methods to modify its physicochemical properties to improve this important functionality has not been reported. High-intensity ultrasound (HIU) treatment is one such method that shows great potential for improving the protein physiochemical properties and thus the functionality of lupin protein (Ozuna et al., 2015). Because of its efficient use of energy and time, ultrasound is considered to be a sustainable technology compared with conventional thermal processing methods (Chemat et al., 2011; Knorr et al., 2011). This technology uses mechanical waves above the threshold of human hearing (>16 kHz) (Soria & Villamiel, 2010). Its effects are caused by the cavitation phenomenon, through which gas bubbles are formed and pass through continuous cycles of compression and decompression until they implode, releasing high energy (Gallo et al., 2018). This creates extreme temperatures (5000 K) and pressures (1000 atm), which can modify protein structure by triggering changes in protein particle size and exposing buried hydrophobic groups to the surface of the protein molecule (Awad et al., 2012; Povey & Mason, 1998). In addition, HIU can cleave water molecules, creating the free radicals ˙H and ˙OH, which can form hydrogen peroxide in the gas phase of the cavitation zone (Ozuna et al., 2015). Hydrogen peroxide may oxidise functional groups on protein molecules, such as sulfhydryl to S-S, which induces conformational changes in protein molecules (Gülseren et al., 2007).

These changes in protein structure can alter protein functionality significantly. For instance, HIU treatment reduces soybean protein particle size by dissociating agglomerated protein into individual subunits, which improves soybean protein crosslinking ability and foaming properties (Morales, Martínez, Pizones Ruiz-Henestrosa, & Pilosof, 2015). Hu, Li-Chan, Wan, Tian, and Pan, (2013) reported that HIU 105-110 W/cm^2 during the first 20 min reduced soybean protein isolate particle size and sulfhydryl (SH) groups significantly because of the formation S-S bonds. These changes resulted in the formation of a more uniform protein network with high gel strength (Gs) and water-holding capacity (WHC). Similarly, treating soybean protein isolate with HIU (105-110 W/cm^2 for 5 or 40 min) reduces SH groups number and improves Gs, WHC and gel yield

(Zhang et al., 2016). These authors also found that HIU increases protein surface hydrophobicity and protein secondary structure, as detected by Raman spectroscopy.

Despite the considerable amount of research focused on using HIU treatment to improve plant and animal protein, no published work has reported on the effects of HIU on lupin protein structural properties and the possible changes on techno-functionality.

The research presented in this chapter examined the effects of protein physiochemical changes triggered by HIU treatment on the quality of lupin protein concentrate (LPC) acid-induced gels.

4.2. Materials and methods

4.2.1. Materials

Lupin seed from *Lupinus angustifolius*, *Coromup* variety, was kindly supplied by the Department of Primary Industries and Regional Development - Agriculture and Food (Kensington, WA, Australia). The seed coats were removed using a laboratory scale seed AMAR dehuller (NSIC.SSI, Mumbai, India,) and the lupin kernels were separated from any residual hull using a vacuum separator (Kimseed, Perth, Australia). The lupin kernels were vacuum packed and kept at 4 °C for future use. GDL was purchased from Sigma Aldrich (Sydney, Australia).

4.2.2. Preparation of lupin protein concentrate (α and β conglutin fraction)

LPC was extracted as described in Section 3.2.2.

4.2.3. Compositional analysis of lupin protein concentrate

The LPC proximate composition was analysed as described in Section 3.2.3. Based on these analyses, the LPC used in this study had (all measures on db) 68.79±0.21 g/100 g protein, 11.43±0.10 g/100 g fat, 3.55±0.10 g/100 g ash and 16.23±0.32 g/100 g total fibre and carbohydrate (by difference) (Chapter 3).

4.2.4. Preparation of lupin protein concentrate solutions for ultrasound treatment

Dispersions of 10% (w/w) LPC were prepared by adding freeze-dried LPC to deionised water and stirring for 2 h at 750 rpm using an MR Hei-Standard stirrer (Schwabach, Germany) at room temperature. The resulting protein suspensions were kept at 4 °C

overnight to complete protein hydration, after which the pH was readjusted to 7±0.1 using 0.1 M NaOH/HCl before HIU treatment.

4.2.5. High-intensity ultrasound treatment

The HIU treatment was performed using an ultrasonic processor model VCX 600 (Sonics & Materials Inc, Danbury, CT, USA) equipped with a converter model CV26 and 13 mm titanium probe threaded to a 3 mm tapered microtip. Duplicate 20 mL samples of the 10% (w/w) LPC solutions were treated in a special 60 mL double wall glass vessel for 0, 2, 10, 15, 20 and 40 min using different ultrasound amplitudes of 10%, 20% and 40%. A chiller was connected to the sonication glass vessel to maintain the sample temperature below 35 °C during the treatment. The sample was used directly for acid-induced gelation or freeze-dried for further analysis.

4.2.5.1.Determination of high-intensity ultrasound power

The applied ultrasound power (P) was calculated according to the calorimetric technique (Jambrak et al. 2009). Ultrasound P was calculated following the formula:

$$P = MC_P\,(dT/dt)$$

where P (W) is the ultrasound P, M is the sample mass (g), Cp is the specific heat of the medium (kJ/gK) and *dT/dt* is the rate of temperature change (T) with time (t). The ultrasound intensity (W/cm^2) is the ultrasound P (W)/unit area (cm^2) of the emitting surface.

The calculated power intensity was 11 W/cm^2, 17 W/cm^2 and 38 W/cm^2 at 10%, 20% and 40% amplitude, respectively.

4.2.6. Investigation of the effects of high-intensity ultrasound on the physiochemical properties of freeze-dried lupin protein concentrate dispersions

4.2.6.1.Particle size distribution

The particle size distributions of the untreated and HIU-treated LPC dispersions (10%, w/w) at 38 W/cm^2 power at 0, 2, 10, 15, 20 and 40 min were determined in triplicate using a Mastersizer laser light-scattering analyser (Mastersizer 2000, Malvern Instruments Ltd., Malvern, UK). The sample was diluted 10-fold using deionised Milli-Q water before the

analysis. The analysis was conducted by transferring 1 mL of diluted sample to the measuring cell. The particle size was expressed as a volume- mean diameter (D4,3).

4.2.6.2.Protein solubility

Protein solubility of the LPC was determined based on method described by Hu, Cheung, Pan, and Li-Chan (2015). In brief, duplicate samples from each treatment at 2 mg/mL were solubilised in phosphate buffer (0.1 M) pH 7, stirred for 2 h and then kept at 4 °C overnight to complete hydration. The suspensions (1 mL) pH 7 were centrifuged at 20000 *g* for 15 min at room temperature using a Heraeus centrifuge (model Pico17, Hesse, Germany). The protein content was measured against a calibration curve using bovine serum albumin (BSA) diluted as described in the bicinchoninic acid (BCA) protein assay kit manual (Sigma-Aldrich). Protein solubility (%) was calculated as (protein content of the supernatant (g)/protein content of the original suspensions (g) × 100.

4.2.6.3.Zeta potential

The zeta (ζ)potential was measured according to the method of Nazari, Mohammadifar, Shojaee-Aliabadi, Feizollahi, and Mirmoghtadaie (2018). HIU-treated and -untreated LPC freeze-dried powder was solubilised at 0.5% (w/w) in Milli-Q water at room temperature. The dispersions were prepared 2 h before the analysis. The ζ potential was measured using a Zetasizer Nano ZS (Malvern Instrument Ltd., and the results are expressed in mV. The average value of two replicates were analysed and presented.

4.2.6.4.Differential scanning calorimetry

Differential scanning calorimetry (DSC) was used to investigate changes in onset temperature (T_{onset}) °C, peak temperature (T_{peak}) °C and denaturation enthalpy (ΔH) J/g of HIU-treated and -untreated freeze-dried LPC. A TA Instruments DSC analyser (model 2910, New Castle, the USA) equipped with universal analysis 2000 software (V4.5A) was used. The DSC analyser was calibrated with indium, and the empty pan was used as a reference. The LPC thermal properties were evaluated by heating about 5 mg from 25 °C to 160 °C at a 5 °C/min heating rate under a nitrogen atmosphere in hermetically sealed aluminium pans. The average of two replicate values was used in the analyses.

4.2.6.5.Lupin protein electrophoretic profile

The lupin protein profile of HIU-treated and -untreated LPC freeze-dried powder was described in Section 3.2.2.

4.2.6.6.Fourier-transform infrared spectroscopy

To investigate the lupin protein higher-order structural changes generated by ultrasound treatment, HIU-treated and -untreated freeze-dried LPC samples were analysed using a Thermo Scientific Nicolet iS50 Fourier-transform infrared spectroscopy (FTIR) spectrometer coupled to a Smart iTR Attenuated Total Reflectance (ATR) sampling accessory (Thermo Scientific, Madison, WI, USA). FTIR spectra were recorded in the range of 4000-400 cm^{-1} at a spectral resolution of 4 cm^{-1} with the co-addition of 64 scans. A background spectrum was recorded from a clean diamond ATR crystal before each sample, with the co-addition of 64 scans.

Second-derivative spectra analysis of amide I (1700-1600 cm^{-1}) (protein secondary structure α-helix, β-sheet and unordered structure) were calculated from the vector-normalised row spectra using a thirteen-point smoothing Savitzky-Golay algorithm using OPUS (V7.0, Bruker, Ettlingen, Germany). Data for HIU-untreated and -treated samples at 38 W/cm^2 power for 20 and 40 min are presented and discussed in the following sections.

4.2.7. The effect of high-intensity ultrasound on the gelation properties of lupin protein concentrate

4.2.7.1.Determination of glucono-δ-lactone level

To reach the required final pH of ~4.5 during gelation, the amount of GDL required needed to be identified because its level of acidification as it slowly hydrolyses to gluconic acid and reduces the pH depends on the protein type and concentration (Alting, Hamer, De Kruif, & Visschers, 2003; de Kruif, 1997). Different amounts (0.20, 0.22, 0.25, 0.27, 0.30, 0.40, 0.50, 0.60, 0.70, 0.75, 0.80, 0.90, 1.0, 1.1, 1.2, 1.5, 1.7, 1.8 and 1.9% (w/v)) of GDL were added to 20 g aliquots of the LPC suspensions (see Section 4.2.4.), and the samples were mixed using a vortex mixer for 30 s at room temperature and then stored at 4 ℃ for 24 h. The sample pH was measured at room temperature. All measurements were done in triplicate. GDL addition at 1% (w/v) gave a pH of ~4.5 (Figure C1).

4.2.7.2.Acid-induced gelation

GDL was added to 20 g of 10% (w/w) LPC dispersion (see Section 4.2.4) after ultrasound treatment to give 1% (w/w) a final concentration (pH~4.5). Samples were mixed using a vortex mixer for 20 s and then heated at 95 °C for 60 min to induce lupin protein aggregates as a pre-gelation step. The heat treatment step was conducted in 50 mL glass containers (40 mm width × 52 mm height) as gelation vessels in a tuneable water bath. After heat treatment, the gel samples were cooled to room temperature and stored at 4 ℃ for ~24 h before the analysis of Gs.

4.2.7.3.Gel strength

Gs was measured according to published methods (Hu, Li-Chan, et al., 2013; Zisu, Bhaskaracharya, Kentish, & Ashokkumar, 2010). Gs was measured at 5 ℃ using a TVT texture analyser (model 6700, Perten Instruments, Sydney, Australia) fitted with a 5 kg load cell and a P/0.5 cylinder probe attachment. Gel compression was performed at 0.5 mm/s speed and 5 g trigger force. Gs was measured in triplicate gels, and the average of these tests is reported as Gs expressed in g.

4.2.7.4.Water-holding capacity

WHC was analysed using the method of Zhang et al., (2016). The samples were prepared as mentioned in Section 4.2.7.2 and placed in 50 mL centrifuge tubes for measurement of the WHC. LPC gel samples were centrifuged at 1811 *g* for 20 min at room temperature using an Eppendorf centrifuge model 5810R (Hamburg, Germany). After centrifugation, free water released was removed by inverting the tube to drain it and the remaining free water on the tube walls was removed with filter paper. WHC% was calculated as follows:

$$W_t = W_a - W_u$$

$$\text{WHC} = (W_t - W_r / W_t) \times 100\%$$

where W_t the weight of entrapped water in the gel matrix, W_a represents the total weight of water added during the gel sample preparation, W_u is the removed free water weight, and W_r is the water released from the gel network after centrifugation.

4.2.7.5.Small deformation measurements to investigate the effects of HIU on the LPC gelation mechanism

The HIU-treated (38 W/cm^2 power for 20 min) and untreated LPC dispersions were prepared as described in Section 4.2.4., after which 1 g/100 g of GDL was added to the dispersion, and the sample was mixed using a vortex for 2 min and then loaded onto a rheometer. Dynamic rheological properties during the gel-formation process were examined by transferring 1 mL of the sample to the parallel plates (40 mm diameter and 1 mm gap) of a TA Instruments AR-G2 controlled-stress rheometer (TA Instruments, Leatherhead, UK). To minimise any sample evaporation during the heating steps the outer edge of the parallel plates had a thin layer of high vacuum grease applied to them and a plate cover was also placed over the parallel plates. The measurements were performed at a constant strain of 0.05%, which was within the linear region (reversible elastic deformation) and at 1 Hz frequency. In the rheometer, to trigger the gelation, the LPC dispersions were heated from 25 °C to 95 °C at a heating rate of 2 °C/min, kept at 95 °C for 20 min, and then cooled to 25 °C at a cooling rate of 2 °C/min. The data are presented as storage modulus (G′). All measurements were conducted in triplicate.

4.2.8. Statistical analysis

All analyses were performed in triplicate unless stated. The data are presented as a mean ± standard deviation. The data were analysed using IBM SPSS Statistics (V26, IBM Corp. Armonk, NY, USA). Two-way analysis of variance (ANOVA) was used to investigate the main effects of HIU time and power and their interaction on gel attributes. If a significant main effect or interaction was observed, one-way ANOVA with the Tukey post hoc test was used to separate individual means. For all analyses, $P < 0.05$) was considered to be significant.

4.3. Results and discussion

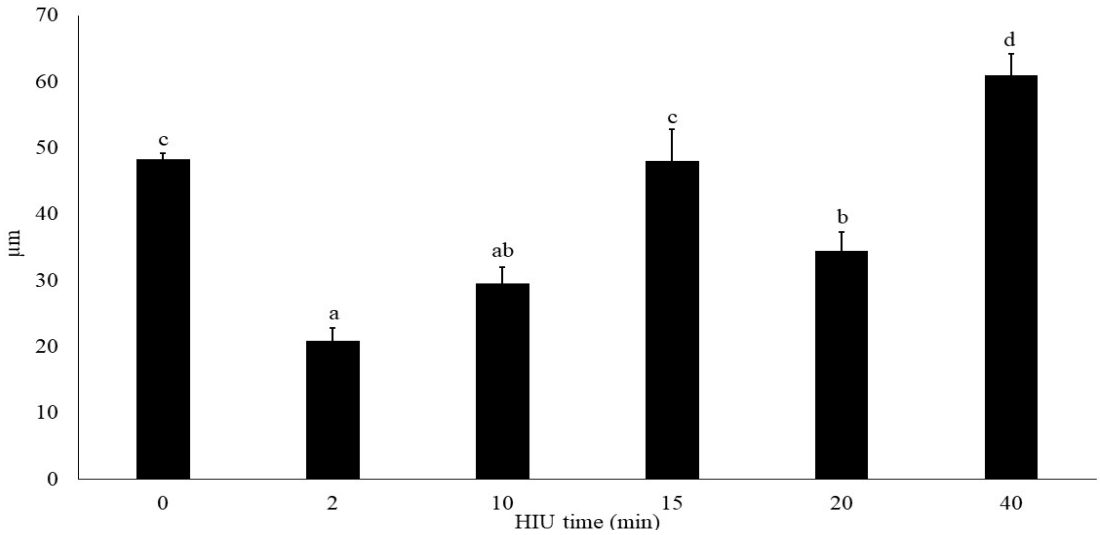
4.3.1. Effects of high-intensity ultrasound on the properties of lupin protein concentrate dispersions.

4.3.1.1. Particle size

The D_{43} represents the volume of the particles, which is sensitive to protein agglomeration and dissociation (Jambrak et al., 2009; Jiang et al., 2014). The effects of HIU treatment

on the D_{43} of LPC dispersions at 38 W/cm^2 for 0, 2, 10, 15, 20 and 40 min are presented in Figure 4.1. One HIU treatment provoked significant ($P < 0.05$, ANOVA) fluctuation in LPC particle size with time (Figure 4.1). Particle size was reduced after the 2, 10 and 20 min HIU treatments compared with 0 min. Ultrasound treatment can reduce protein particle size by inducing dissociation of protein subunits via the extreme local heat, pressure, high shear energy and turbulence created by the cavitation forces (Ozuna et al., 2015). These results are consistent with those of O'Sullivan, Park, and Beevers (2016), who found that treating soybean and wheat protein isolate dispersions with HIU at ~34 W/cm^2 for 2 min reduced protein particle size significantly.

By contrast, HIU treatment for 15 min increased LPC particle size to almost its original size, and the largest particle size was obtained after 40 min of treatment. This phenomenon fluctuation in particle size with HIU treatment time suggests that lupin protein particles aggregated through non-covalent interactions such as hydrophobic and electrostatic, which can be easily disrupted by HIU treatment (O'Sullivan, Park, & Beevers, 2016; Shen, Fang, et al., 2017). HIU treatment can thus create unstable small protein particles with exposed hydrophobic groups, which then can interact to create large agglomerated protein particles (Arzeni et al., 2012; Hu, Fan, et al., 2013; Jambrak et al., 2009). Zheng et al. (2019) reported a similar fluctuation pattern when they sonicated soybean protein isolate (8% w/v) for 10 and 25 min at 80 W/ cm^2. They found that the 10 min treatment increased particle size and that particle size was decreased by treatment for 25 min. The pattern observed here differed from that of the study of Zheng et al., possibly because of their use of higher HIUp and a more highly purified sample; sample impurities, such as fibres and fat, can protect protein particles from ultrasound energy (Jambrak et al., 2009).



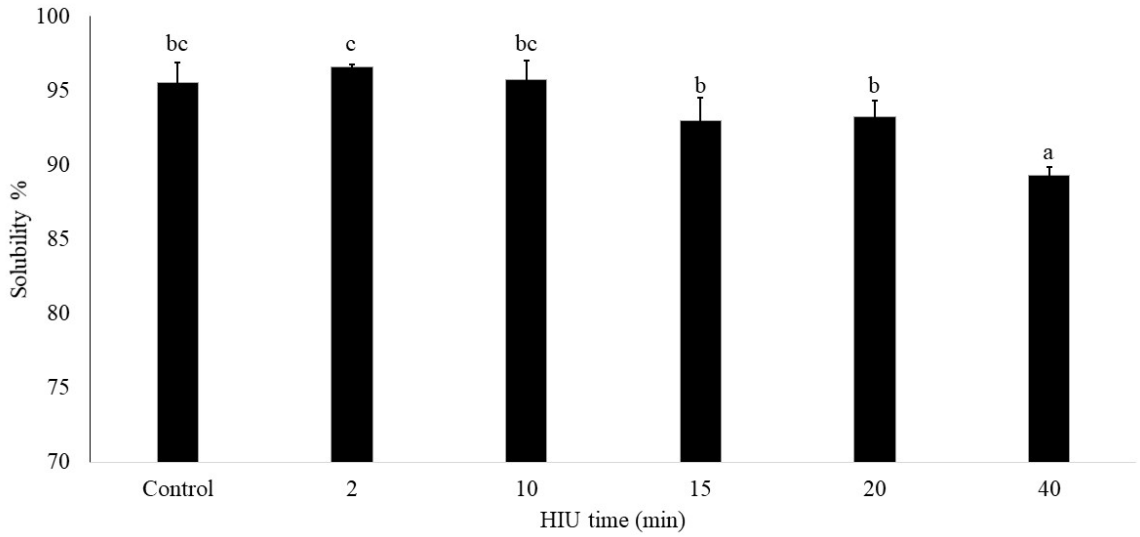
a, b, c, d Means with different letters are significantly different ($P < 0.05$).
HIU, High intensity ultrasound.

Figure 4.1 Effect of ultrasound treatment time (0-40 min) at 38 W/cm^2 power on particle size (D 4,3) of LPC dispersions

4.3.1.2. Determination of solubility

Protein solubility is defined as the protein content in the supernatant after centrifugation at 20000 *g*. The effect of HIU treatment at 38 W/cm^2 power for 0, 2, 10, 15, 20 and 40 min on solubility is illustrated in Figure 4.2. Lupin protein solubility was slightly but significantly higher ($P < 0.05$) after HIU treatment for 2 min compared with prolonged sonication for 15, 20 and 40 min. Ultrasound energy can change the protein conformation by causing the unfolding of protein molecules and even hydrolysation of polypeptides, which increases protein particle surface area (Jambrak et al., 2009). These changes in protein surface chemistry facilitate protein-water interactions and increase protein solubility (Hu et al., 2015). This result is consistent with the findings of Jambrak et al. (2009), who reported that HIU treatment at 45-52 W/cm^2 for 15 min increased the surface area of soybean protein isolate and concentrate significantly and that this improved the protein solubility. However, increasing the ultrasound treatment exposure time (>15 min) significantly reduced lupin protein solubility ($P < 0.05$), especially for the 40 min treatment. These findings have been confirmed by the results for particle size in this thesis showing that ultrasound treatment increased lupin protein D4,3 significantly ($P < 0.05$) (Figure 1) after 40 min. These results are consistent with those of other reports (Hu, Fan,

et al., 2013; Nazari et al., 2018). They noted that long ultrasound treatment reduced protein solubility of soybean and millet protein isolates by exposing the hydrophobic groups of amino acids (AAs) on the surface of the protein molecule. Ultrasound treatment can also reduce protein solubility by triggering the formation of protein aggregates through hydrophobic interactions. These aggregates can precipitate easily during centrifugation because of the increased protein particle size (Gülseren et al., 2007; McCann, Guyon, Fischer, & Day, 2018). Despite the smaller particle size at 20 min (Figure 4.1), protein solubility was similar to control sample (figure 4.2). This phenomenon might be due to exposing more hydrophobic amino acids groups to the surface of the protein particles (Wang et al., 2011).



a, b, c, d, e Means with different letters are significantly different ($P < 0.05$).
HIU, High-intensity ultrasound.

Figure 4.2 Effect of ultrasound treatment time (0-40 min) and 38 W/cm^2 power on solubility of LPC dispersions.

4.3.1.3. Zeta potential

The presence of more negatively charged AAs on the surface of protein molecules at a specific pH results in a negative ζ potential of a protein (Bouzid et al., 2008). The results in Table 4.1 show that the ζ potential was significantly more negative for untreated LPC than after HIU treatment for 20 and 40 min, which indicates the presence of more negative AAs on the protein surface after treatment. This reduction in the negative charge leads to a decrease in repulsion forces by exposing more positively charged groups at the surface,

thus promoting aggregation (Akbari & Wu, 2016) as seen after the 40 min treatment in Figure 4.1. Decreasing in the absolute values of the ζ potential increases the protein's tendency to aggregate (Berghout, Venema, et al., 2015). Similarly, Jiang et al., (2014) found that HIUp treatment (112-120 W/ cm^2) for 12 and 24 min decreased the ζ potential of black bean protein isolate particles. They also found reduction in ζ potential values (~15 mV) compared with ~22 mV after medium-power ultrasonication (300 W - 96-104 W/cm^2). In their experiments, treatment at 450 W for 24 min produced the largest particle size because of aggregation. These changes in the electrical charges on the surface of LPC molecules may, therefore, have a strong influence on the lupin gel viscoelastic properties.

4.3.1.4. Differential scanning calorimetry

Transition temperatures including onset temperature (T_{onset}), peak temperature (T_{peak}) and ΔH for untreated (control) and ultrasound-treated LPC are shown in Table 4.1. T_{onset} and T_{peak} did not differ significantly between ultrasound-treated and untreated LPC ($P > 0.05$). Arzeni et al. (2012) reported that ultrasound at 4.27 W did not change the T_{onse} and T_{peak} of whey and soybean protein isolates after 20 or 30 min. A lower ΔH indicates less thermal stability. Treatment for 20 min reduced the ΔH ($P > 0.05$) compared with the untreated sample. Malik, Sharma, and Saini, (2017) reported that ultrasound treatment at 58-61 W/cm^2 for 20 min reduced sunflower protein isolate ΔH from 6.1 to 3.9 J/g. Nazari et al. (2018) reported a similar trend for the thermal properties of millet protein concentrate. They found that HIU treatment of 73.95 W/cm^2 for 12.5 min reduced ΔH from 31.08 to 23.92 J/g. Protein thermal stability is related to the complexity of its structure at the secondary and tertiary levels; hence, any alteration in thermal properties might related to changes in protein conformation that facilitate denaturation (Morrissey & Shakhnovich, 1996). These results suggest that HIU treatment under specific conditions can reduce the thermal stability of LPC.

Table 4.1 Thermal properties and zeta potential of untreated and ultrasound-treated LPC

HIU Power (%)	HIU time (min)	zeta (mV)	T_{onset} (˚C)	T_{peak} (˚C)	ΔH (J/g)
0	0	-26.85±0.07a	70.4±2.79a	107.28±3.27a	286.65±0.35b
40	20	-18.1 ±1.21 b	70.55±0.63a	110.13±0.61a	245.45±9.12a
40	40	-15.48±0.25c	68.32±5.98a	104.17±2.56a	265.3±7.21ab

a, b, c, Means with different letters in the same column are significantly different ($P < 0.05$)).
HIU, High-intensity ultrasound; $T_{onset,}$ temperature at which denaturation starts; $T_{peak,}$ highest denaturation temperature; ΔH, energy required for denaturation.

4.3.1.5. Lupin protein electrophoretic profile

Non-reducing and reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of HIU-treated and untreated LPC dispersions (Figure 4.3) showed the typical profile of the main lupin protein subunits α-conglutin (11S globulins) and β-conglutin (7S globulins) with no major differences in protein profile when comparing the patterns of untreated and ultrasound treated samples. The results for non-reducing conditions suggest that ultrasound did not trigger the formation or degradation of the protein subunits.

The results for the reducing condition suggest that no hydrolysis of polypeptides had occurred in response to HIU treatment. Similar results were reported by Hu, Wu, et al. (2013), who found that ultrasound-treated soybean protein isolate treated at 105-110 W/cm^2 for 5, 20 or 40 min showed a protein profile similar to that of untreated samples. Jiang et al. (2014) also reported that ultrasound treatment at 0, 150, 300 and 450 W for 12 and 24 min did not provoke any shift in black bean peptide SDS-PAGE pattern. Similarly, Aguilar-Acosta, Serna-Saldivar, Rodríguez-Rodríguez, Escalante-Aburto, and Chuck-Hernández, (2020) found that the use of HIU at 85 W/cm^2 for 10 and 15 min during the protein extraction process did not change the *L. angustifolius* protein profile. By contrast, *L. mutabilis* analysed using non-reducing electrophoreses showed new high molecular bands between 250 and 50 kDa, which indicated that covalent S-S bonds had formed in aggregates during HIU treatment. Ultrasound treatment can modify a protein's net charge,

particle size and hydrophobicity but not protein primary structure (hydrolyses) (Hu et al., 2015; Jambrak et al., 2009; O'Sullivan et al., 2014). In this study, the data suggested that non-covalent bonds such as electrostatic and hydrophobic interactions dominated the newly formed lupin protein aggregates instead of covalent (S-S) bonds.

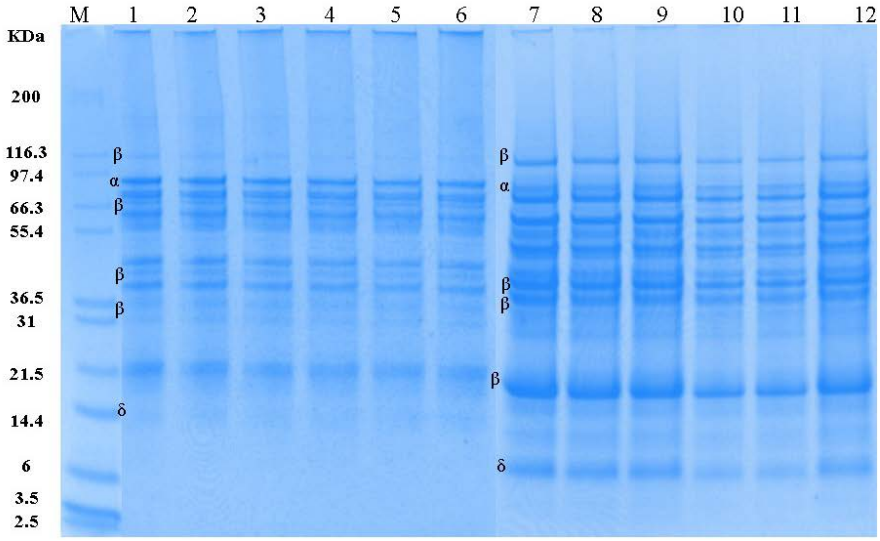


Figure 4. 3 Electrophoresis patterns under non-reduced (lane 1-6) and reduced (lane 7-12) conditions of ultrasound treated 38 W/ cm^2 lupin protein dispersions for 0, 2, 10, 15, 20, 40 min respectively.

4.3.1.6.Fourier-transform infrared spectra

To investigate the effect of ultrasound treatment time and power on changes in the lupin protein structure, the amide I were analysed by monitoring the shift in peak positions using FTIR (Figure 4.3). Absorption on the amide I spectrum reflected the protein secondary structure, which relates to the effect of C=O stretching vibration on the wavenumber range in the 1600-1700 cm^{-1} FTIR spectrum (Rygula et al., 2013). The α-helix and β-sheet are the most important structures on the amide I spectrum at the wave numbers 1662-1655 cm^{-1} and 1618–1640 cm^{-1} respectively. The amide II and III absorption signal is assigned to the stretching vibration of C-N and N-H of the protein peptide side chain in the wavenumber range 1480-1575 cm^{-1} and 1200-1400 cm^{-1},

respectively (Schulz & Baranska, 2007).

The FTIR spectra of amide I band in the sonicated sample B (20 min at 38 W/cm^2 power and C (40 min 38 W/cm^2), showed a slight shifting of the peaks of the wavenumber from 1667 to 1664 cm^{-1} compared with the unsonicated sample (A). The differences in protein secondary between unsonicated and sonicated samples (C) may be related to a change from the α-helix to β-sheet, which can lead to protein aggregation(Kong & Yu, 2007), which is consistent with the data for particle size and zeta potential (section 4.3.1.1 and 4.3.1.3). In addition, β-sheet peak at 1630 cm−1 shifted to lower spectra in ultrasound treated samples (C), which is further evidence of the formation of protein aggregates in the β-sheet structure (Arrondo, 1999). HIU-treated LPC samples exhibited a reduction on α-helix structure at 1656 cm-1 and increased absorption intensity at 1625 cm-1 ,which represent aggregation in β-sheet spectra (Barth, 2007; Hackett et al., 2011). Wang et al., (2011) reports that increase β-sheet formation in soy protein secondary structure positively associate with protein surface hydrophobicity. This may confirm that HIU treatment unfolds and disturb conformation structure of lupin protein and expose hydrophobic groups.

The results of this study are consistent with a previous report that high ultrasound power at 131-138 W/cm^2 for 30 min increases the formation of β-sheet and random coil structures in soybean protein isolate (Hu, Wu et al., 2013). FTIR spectra of amide III in LPC in the region of 1250-1230 cm^{-1} wavenumber showed the formation of new peaks after ultrasound treatment (Figure 4.3B). This finding suggests the formation of new aggregates or creation of larger particles (Nazari et al., 2018). These changes in lupin protein structure support the idea that ultrasound treatment can alter lupin protein secondary structure and thus improve its gelation.

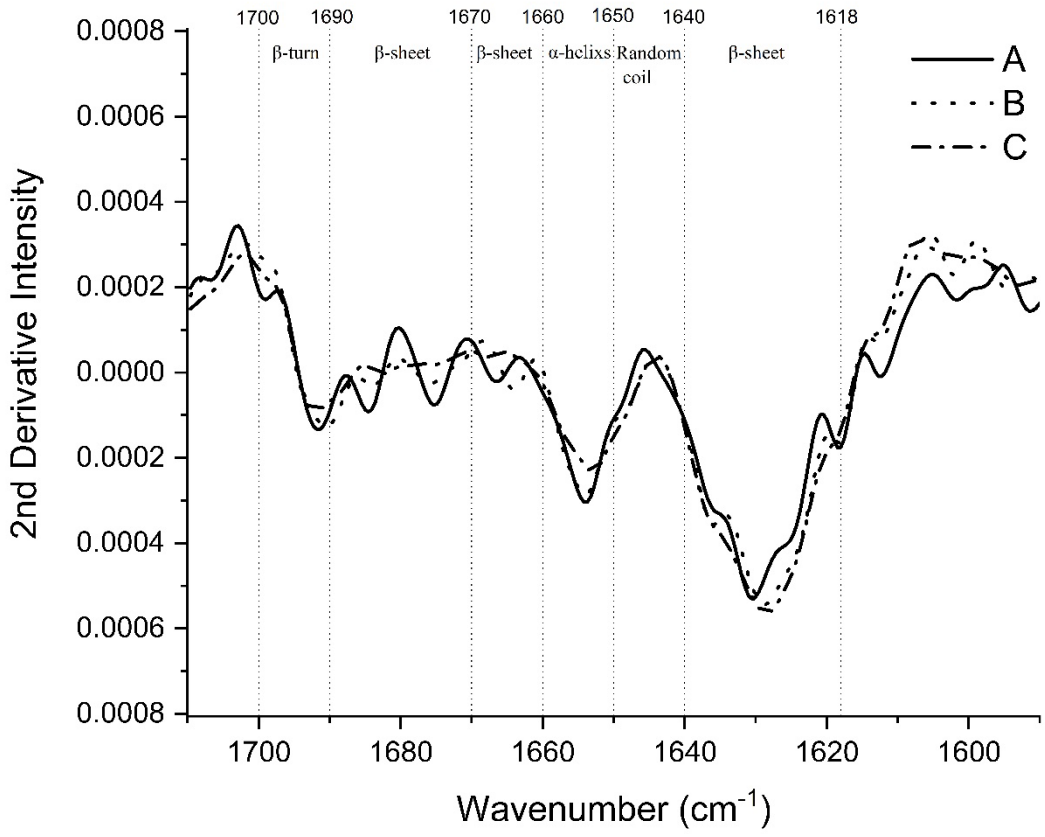


Figure 4.4 FTIR spectroscopy amid I of (A) control (non-sonicated), (B) at 38 w/cm^2 for 20 min for, (C) at 38 w/cm^2 for 40 min of lupin protein.

4.3.2. Quality of acid-induced lupin protein concentrate gels

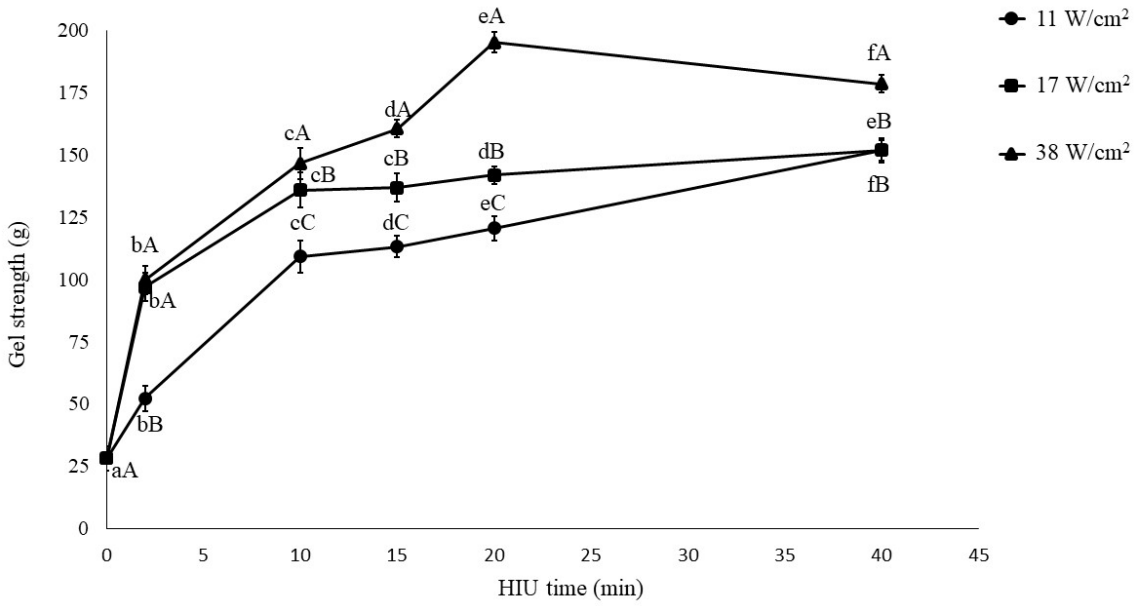
4.3.2.1. Gel strength

Gs is one of the most important attributes in the assessment of protein gel quality. The Gs values for unsonicated and sonicated LPC are shown in Figure 4.4. HIU treatment increased Gs significantly from 28.33 g to 195.33 g. HIU power and time each had a significant main effect ($P < 0.05$) on Gs. The lowest Gs value was recorded for unsonicated LPC gel ($P < 0.05$). Hu, Fan, et al. (2013) reported that ultrasound treatment (105-110 W/cm^2 for 5-40 min) significantly improved the Gs of soybean protein isolate compared with unsonicated isolate. In another study, the HIU (80 W/cm^2 for 10-25 min) increased the Gs of soy protein isolate cold-set gel (Zheng et al., 2019). In the present study, HIU at 38 W/cm^2 produced significantly higher ($P < 0.05$) Gs values compared with lower intensities (11 and 17 W/cm^2) at all treatment times. Power of 38 W/cm^2 for 20 min was found to give the highest Gs (195.33 g). This result is consistent with the results of previous studies on soy and whey protein isolate gels (Hu et al., 2015; Shen, Fang, et al., 2017; Shen, Zhao, Guo, Zhao, & Guo, 2017). These studies found that a moderate ultrasound treatment time (~20 min) can improve Gs significantly.

One possible reason for the best effects at moderate treatment times is that HIU in that time window can unfold the protein tertiary structure. This increases the exposure of hydrophobic groups and provides extra intermolecular crosslinking opportunities for the gel network, which produces a gel structure that is more stabilised against rupture under pressure (Zheng et al., 2019). Lupin protein particles found to be smaller (Figure 4.1) and more hydrophobic (solubility and zeta potential data in Figure 4.2 and Table 4.1), which can facilitate building stronger gel network by the formation dense and highly crosslinked gel network. However, at 38 W/cm^2, increasing the HIU time to >20 min reduced the Gs of LPC slightly. Prolonged HIU treatment (~40 min) can reduce the gel network microstructure uniformity by increasing the protein aggregate size (Figure 1), which hinders strong and uniform gel network formation (Hu, Fan, et al., 2013). Hu, Fan, et al. (2013) reported that HIU treatment at 105-110 W/cm^2 for 40 min reduced the Gs of soy protein isolates to 45.28 g compared with 60.90 g at 20 min. By contrast, Shen, Zhao, et al. (2017) reported that increasing the ultrasound time from 20 min to 40 min at ~107 W/cm^2 did not significantly influence the Gs of whey protein gel. Moderate HIU

treatment time has good potential for improving the Gs of LPC compared with longer treatment time. This information indicates that the HIU treatment time and power are likely to be crucial factors for the optimisation of the gelling properties of LPC.

It has been reported that heat treatment has a limited effect on lupin protein gelation. Berghout et al. (2015) compared changes in lupin protein and soy protein isolate particle size before and after heat treatment. They found that lupin protein isolate particle size remained unchanged after heat treatment at 75, 85 or 95 °C for 30 min compared with a significant increase in particle size of soybean isolate after the same treatment. They noted that the lupin protein particles did not show evidence of association and dissociation even after prolong heat treatment (80 °C for 8 h), which may be responsible for its poor gelation properties.



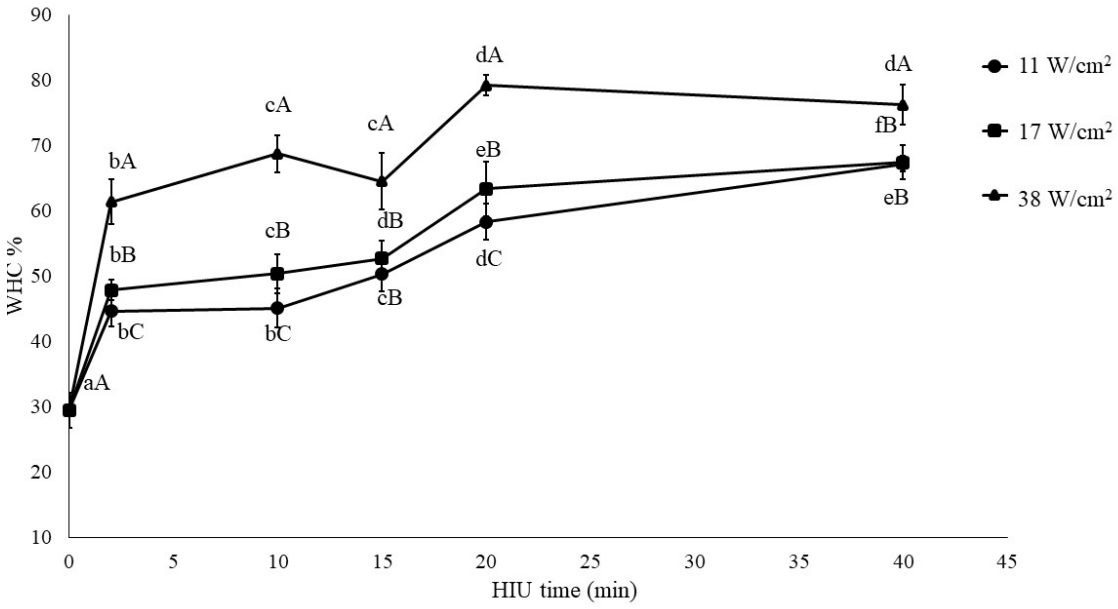
a, b, c, d, e Means with different letters represent a significant difference ($P < 0.05$) between HIU time within each HIU power.
A, B, C, Means with different letters represent a significant difference ($P < 0.05$) between HIU power within each HIU time.
HIU: High-intensity ultrasound

Figure 4.5 Effect of ultrasound treatment time and power on lupin gel strength

4.3.2.2. Water holding capacity

The WHC of LPC gels are presented in Figure 4.5. The HIU-treated samples (11, 17 and 38 W/cm^2 power for 2, 10, 15, 20 and 40 min) had significantly higher values than the unsonicated (control) sample. The results varied from 29% to 79.2% between control and HIU-treated LPC. HIU treatment at 38 W/cm^2 for 20 min gave the maximum WHC (79.2%). LPC gels treated at 38 W/cm^2 ultrasound power had significantly higher WHC values compared with 11 and 17 W/cm^2 for all ultrasound treatments times (2-40 min). Many authors have reported that ultrasound treatment can reduce protein particle size, unfold protein molecules and expose buried hydrophobic groups on the side chain of protein peptides. All of these changes play an essential role in the intermolecular crosslinking ability and thus gelation properties of a protein (Hu et al., 2015; Nazari et al., 2018; O'Sullivan et al., 2014).

The WHC of LPC influenced by GS, samples with High Gs exhibit high WHC. Similarly, Hu, Li-Chan, et al., (2013) reports that WHC and GS of soy protein isolate salt-induced gelation samples are positively associated. A highly crosslinked gel network can build a dense and strong gel network, which can retain more water (Hu, Fan, et al., 2013; Morales et al., 2015; Shen, Fang, et al., 2017). However, overdoing the HIU (time and power) and heat treatment can facilitate the formation of large non-homogeneous protein aggregates linked by hydrophobic interactions, which can increase gel syneresis because of the formation of large pores in the gel network (Kao et al., 2003; Chuan He Tang et al., 2011). Hu, Fan, et al. (2013) found that the WHC was lower for soy protein isolate gels treated for 40 min of HIU (90.5%) than at 20 min (95.53%) at the same power intensity (105-110 W/cm^2). Urbonaite et al. (2016) noted that reducing gel particle size had a significant positive effect on both gel stiffness and WHC of whey protein, whereas increasing protein particle size may have a negative impact on gel WHC.



A, B, C, Means with different letters represent a significant difference ($P < 0.05$) between HIU power within each HIU time.
a, b, c, d, e Means with different letters represent the significant difference ($p<0.05$) between HIU time within each HIU power.
HIU: high-intensity ultrasound; WHC: water-holding capacity

Figure 4.6 Effect of ultrasound treatment time and power on lupin gel water-holding capacity

4.3.2.3. Lupin gel rheological properties

Dynamic small deformation is a useful approach for evaluating the gelling process because of its extreme sensitivity to changes in chemical and physical properties of the sample (Westphalen et al., 2005). Heating and cooling technique were conducted to monitor development of the lupin protein structure during the gelation processes. The G′ was used to monitor the elastic properties of the three-dimensional gel matrix (Sun & Arntfield, 2011). The effect of temperature ramp from 25 °C to 95 °C (at a heating rate of 2 °C/min) on ultrasound-treated LPC at 38 W/cm^2 for 20 min and the untreated sample (control) are presented in Figure 4.6. The G′ was higher in the HIU-treated than the untreated LPC sample. The ultrasound-treated sample started developing viscoelastic texture earlier (after ~375 s) and at a lower temperature (~40° C) compared with the untreated sample, which started at ~1210 s and at a higher temperature (~67 °C). This phenomenon may reflect the ability of HIU treatment to cause changes on the surface of

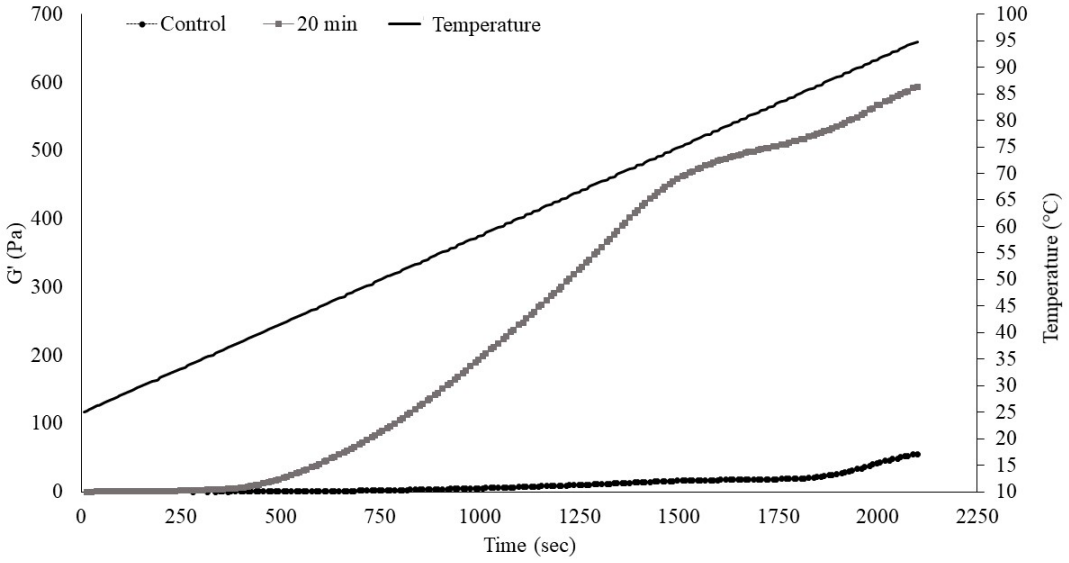
LPC molecules by exposing hydrophobic groups and reducing repulsion forces (as indicated by a reduced zeta potential).

Proteins with a high proportion of hydrophobic groups on the surface can start developing viscoelastic behaviour at lower temperatures (<40 °C) compared with less hydrophobic proteins (Tunick, 2011). Lowering the protein net charge (e.g. lowering the pH and exposing positively charged AAs in case of lupin) will facilitate close association between protein molecules through hydrophobic groups on the surface of the molecules (Madadlou et al., 2010; Westphalen et al., 2005). At ~70 °C, the G′ was significantly higher for the HIU-treated sample (~390 Pa) compared with the untreated (~12.5 Pa) sample ($P < 0.05$). At temperatures >70 °C, the HIU-treated sample enters a small plateau when the temperature reaches the T_{onset} of lupin protein (~70 °C), which is the start of denaturation curve for lupin protein. Then, the G′ value increased significantly at 84 °C and ~1750 s. This increase in G′ may reflect the partial denaturation of α- and β-conglutin at this temperature (84 °C) (Sirtori, Resta, Brambilla, Zacherl, & Arnoldi, 2010), which increases the surface hydrophobicity. At the end of the heat-ramp stage, the G′ of the HIU-treated and the -untreated samples was ~594 Pa and ~55 Pa, respectively.

At the heat-preservation step (95° C for 20 min) (Figure 4.7), the sonicated sample showed some reduction in G′ from 800 Pa to 700 Pa after 1000 s, which may reflect an increase in gel fluidity caused by protein denaturation and disturbance of hydrogen bonding (Qin et al., 2017; Tunick, 2011). By contrast, the G′ of the untreated LPC continued to increase and reached ~182 Pa at the end of this stage. This probably reflects the thermal stability of untreated lupin protein (Section 4.3.1.4).

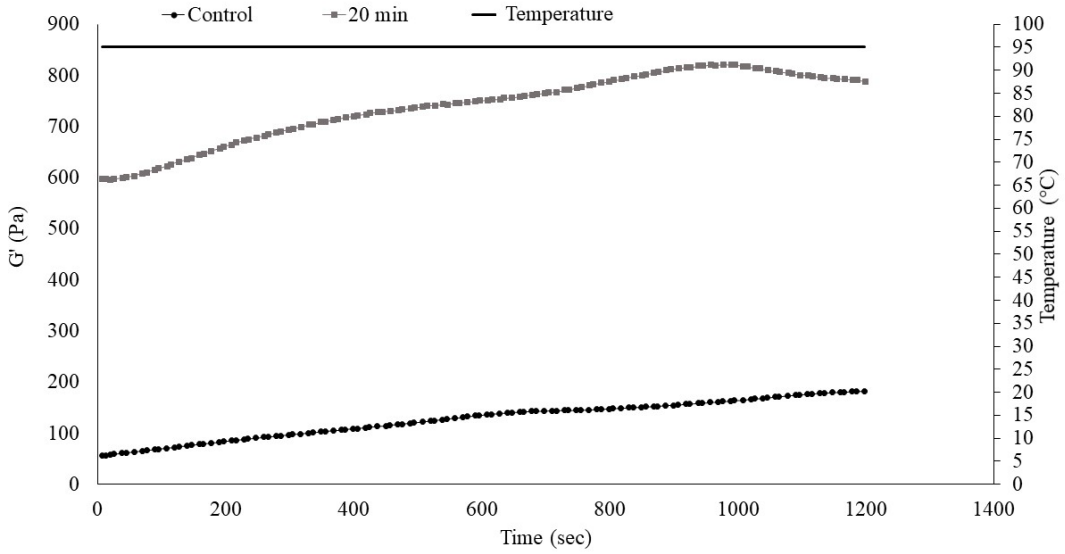
In the cooling step (Figure 4.8), both samples showed a steady increase in G′,which is evidence of the formation of a crosslinked gel network (Hu, Fan, et al., 2013). However, the HIU-treated sample had a significantly higher G′ ($P < 0.05$) than the untreated sample. The maximum G′ values were reached at 25 °C: ~3820 Pa and 1600 Pa for HIU-treated and -untreated LPC gels. These findings suggest that ultrasound, heat treatment and lowering the pH (by adding GDL) during the LPC gelation process significantly improves the viscoelastic properties of lupin protein gels compared with no treatment. HIU seems to improve lupin protein gelation properties by increasing the efficiency of protein unfolding (increased thermal sensitivity) and exposing active groups

to the surface of protein molecules compared with unsonicated lupin protein. In addition, lowering the repulsion forces by reducing the pH seems to facilitate the building of a stable three-dimensional gel network by allowing close protein-protein association, which in turn improves the gel viscoelastic properties (Arzeni et al., 2012).



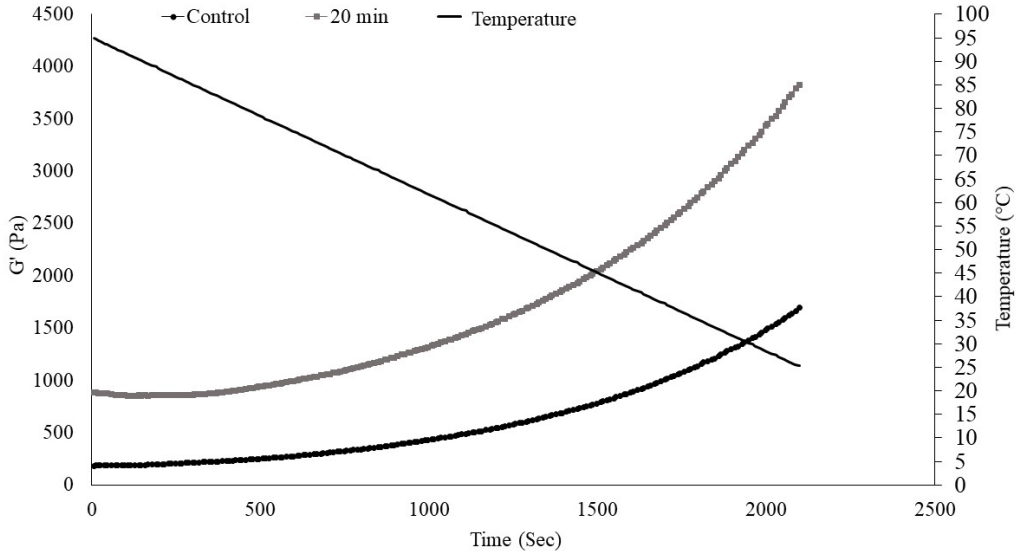
Control: unsonicated; 20 min, 20 min ultrasound treatment at 38 W/cm^2; G′, storage modulus.

Figure 4.7 Effect of ultrasound treatment on gelation properties of acid-induced LPC gelation during heating from 25 °C to 95 °C at a rate of 2°C/min (n = 3).



Control: unsonicated; 20 min; ultrasound treatment for 20 min at 38 W/cm^2; G′, storage modulus.

Figure 4.8 Effect of ultrasound treatment on gelation properties of acid-induced LPC gelation during the heat preservation step at 95 °C at a rate of 20 °C/min (n = 3).



Control: non-sonicated; 20 min: 20 min ultrasound treatment at 38 W/cm^2; G′, storage modulus.

Figure 4.9 Effect of ultrasound treatment on gelation properties of acid-induced LPC gelation during the cooling stage from 95°C to 25 °C at a rate of 2 °C/min (n = 3).

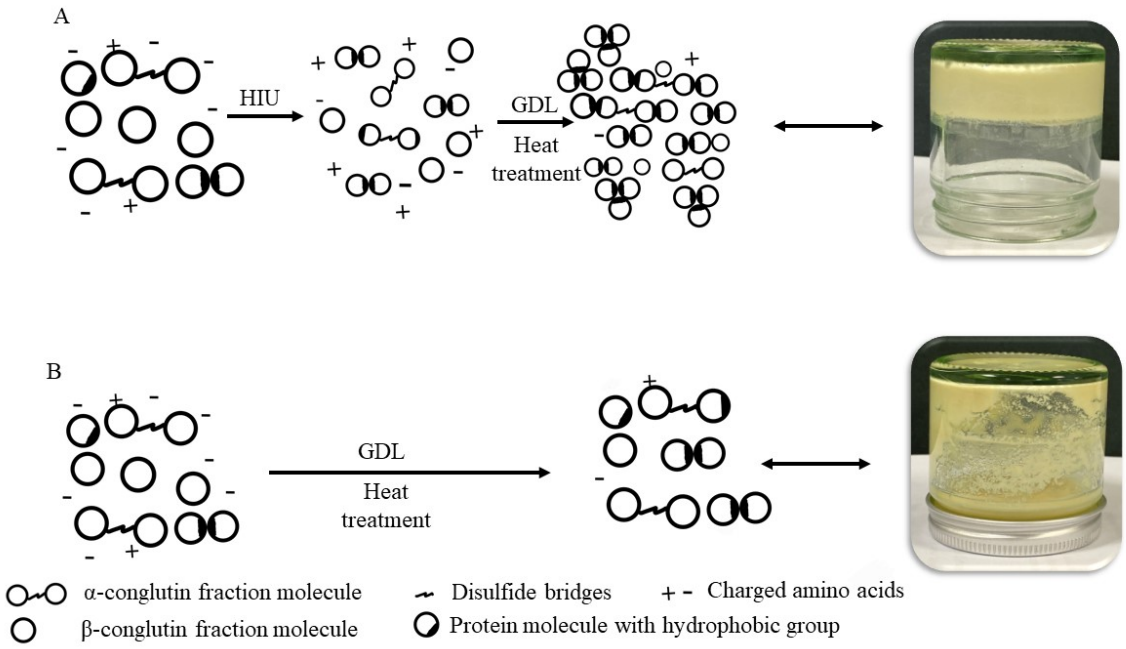
4.3.3. Proposed mechanism to explain the effect of HIU on the acid-induced LPC gelation

A proposed mechanism to explain the acid-induced gelation of ultrasound-treated and unsonicated lupin protein is outlined in Figure 4.9A and B. In this model, HIU treatment generates significant alterations in the LPC particle size. These particles pass through a cycle of dissociation and association (aggregation), which alters their size and exposes their hydrophobic groups to the surface of the protein molecules (Figure 4.5A). The LPC particle size was significantly ($P < 0.05$) smaller after 20 min of HIU treatment (Ssection 4.3.1.1). In addition, HIU treatment for ≥ 20 min at 38 W/cm^2 significantly reduced the zeta potential ($P < 0.05$) (Section 4.3.1.3) because of exposure of positively charged hydrophobic patches on the surface of the protein molecules through unfolding and changes in the protein secondary structure (increase β sheet formation) (Wang et al., 2011). These changes will enable the formation of protein aggregates via hydrophobic and electrostatic interaction (Chandrapala et al., 2013; Salgin et al., 2012).

During the gelation process, adding GDL will neutralise the protein net charge by gradually lowering the pH to around the IP of the major globulins in the protein concentrate (α- and β-conglutins) (reduces the repulsion forces), which will allow close clustering of the protein aggregates formed during HIU treatment. Furthermore, heat treatment will expose more hydrophobic groups at the surface of the protein because of unfolding of the secondary structure. A lower pH and a high level of accessible hydrophobic sites will provoke the formation of a gel network using soluble and insoluble aggregates as the building blocks.

HIU treatment may trigger more changes to the β-conglutin secondary and tertiary structure through unfolding than to α-conglutin. This may because the α-conglutin monomer is stabilised by six intramolecular S-S bridges, whereas β-conglutin has none (Berghout, Boom, et al., 2015). However, the α- and β-conglutins have a high proportion of hydrophobic AAs (leucine, isoleucine and phenylalanine) (Duranti et al., 2008), This may play a major role in the formation of one continuous gel network, especially at the low pH of the acid-induced gel used in the studies in this thesis.

The synergetic effect of HIU and heat treatment alters the lupin protein structure, which produces a firm gel network with significant improvement in its strength, WHC and viscoelastic properties (G′) compared with that formed using an unsonicated sample (Figure 4.9B).



HIU, High-intensity ultrasound; GDL, glucono-δ-lactone

Figure 4.10 Proposed mechanism of (A) ultrasound treated and (B) control (non-sonicated) acid-induced gelation of for lupin protein concentrate

4.4.Conclusion

The HIU treatment has a profound effect on lupin protein gelation properties. This study has demonstrated its ability to trigger significant alterations in lupin protein structure particle size, solubility and zeta potential. HIU treatment significantly increased lupin protein gel quality, Gs, WHC and G′. I hypothesise that HIU triggered structural changes in lupin protein, which created a firm and uniform gel network through hydrophobic interactions. This chapter provides fundamental information that will facilitate understanding of the effects of ultrasound treatment on lupin protein. However, experimental design in which only one factor was changed at a time used in this chapter does not allow identification of the optimal conditions to produce the highest quality gel. To establish this optimisation, the following two chapters present the results of response surface methodology studies based on information gained through the experiments described in this chapter.

CHAPTER 5
The Use of a Fractional Factorial Design to Identify the Significant Factors Controlling Acid-Induced Lupin Protein Concentrate Gel Quality

Abstract

A fractional factorial screening design was used to investigate the effects of the independent factors of high-intensity ultrasound time (min), high-intensity ultrasound power (W/cm^2), heat treatment temperature (HTT,°C), heat treatment time (HTt, min) and pH on lupin protein concentrate acid-induced gel quality (gel strength (Gs g), water holding capacity (WHC%) and gel yield (Gy%). Factorial screening was conducted to identify the most significant independent factors controlling lupin protein gel quality. Independent factors, and their levels in factorial model run influence lupin protein Gs quality(11 g to 215 g) . Statistical analysis of the model showed that Gs was significantly influenced by ultrasound treatment (time and power) and HTT. The WHC ranged from 32.72% to 90.53%. The findings suggest that ultrasound treatment time and power and HTT are the most significant factors controlling the WHC of the gel. In addition, the Gy ranged from 84.04% to 97.67% and was significantly influenced by the HIUt (min) and HIUp (W/cm^2). This factorial screening determined that the HIUt, HIUp and HTT are the most significant factors controlling lupin protein gel properties. These three independent factors were used in the research described in Chapter 6 to predict the optimal level of the combination of these factors to produce the highest quality gel using response surface methodology with a central composite rotatable design.

5.1. Introduction

There is a growing interest in legume protein isolates to use as a food ingredient (Foschia et al., 2017; Martin, 2014). The nutritional value and techno-functional properties are the most important properties for the success as a food ingredient of any plant protein source (Foschia et al., 2017; Luyten, Vereijken, & Buecking, 2000; Taylor, 2017). Lupin protein has great potential as a substitute for animal and other plant protein in the mainstream food industry because of its sustainability, availability, high protein content, high nutritional value and low content of anti-nutritional factors (Carvajal-Larenas, Linnemann, Nout, Koziol, & Van Boekel, 2015; Taylor, 2017). However, lupin protein is underutilised as a food ingredient because of the lack of

some techno-functionalities such as gelation, which prevents its use in many products in which the protein imparts a texturizing effect such as in meat and dairy analogues (Berghout, Boom, et al., 2015; Boye, Zare, & Pletch, 2010; Chardigny & Walrand, 2016; Foegeding & Davis, 2011).

Protein gels are formed by the three steps heating, aggregating and gelation (Saha & Bhattacharya, 2010). These three steps occur simultaneously in heat-set gels but the gelation step can be separated from the preceding two steps in cold-set gels by controlling the gelation conditions such as protein concentration and pH. This allows the gel to form at in cool temperatures with the addition of salt or acid rather than at high temperature, which can be useful for the incorporation of heat-sensitive ingredients such as bioactives compounds (Alting et al., 2003; Can Karaca, Low, & Nickerson, 2015; Foegeding & Davis, 2011).As described in Chapter 4, ultrasound energy has great potential for modifying lupin protein physiochemical properties such as particle size, zeta (ζ) potential, solubility, protein secondary structure and protein thermal properties. In addition, ultrasound can improve lupin protein quality attributes such as gel strength (Gs), water-holding capacity (WHC), and gel storage modulus (G′). However, to achieve best lupin protein gel quality, the ultrasound treatment and gelation process need to be optimised. Optimising the process using one factor at time (univariate) is a time-consuming technique with uncertain results because of the inability to identify significant factors and their interactive effects on the responses. No published work has focused on optimising the quality of lupin protein acid-induced gels. However, incorporating ultrasound into the protein gelation process is a complex task because of the wide range of factors controlling the process and the potential interactions between these factors. These factors include the protein concentration, pH, ionic strength, heat treatment (temperature and time) and ultrasound treatment (time and power) (Gosal & Ross-Murphy, 2000). By identifying a short list of factors that most significantly affect the final product (dependent variables), two-level fractional factorial screening design can be used to reduce the number of factors in studies with many independent factors that may influence the quality attributes of the final product (Britten & Giroux, 2001; Myers, Montgomery, & Anderson-Cook, 2016). Once these most significant factors have been identified, their optimal levels to provide maximum product quality can be predicted using more complex experimental designs such as central composite rotatable design (CCRD) as a response surface

methodology (RSM). RSMs are efficient in time and resources while maintaining high confidence in the results compared with studies in which one factor is changed at a time (Montgomery, 2017). The aim of the research described in this chapter was to use a fractional factorial screening design to investigate the effects of five independent factors (ultrasound time, ultrasound power, heat treatment temperature (HTT), heat treatment time (HTt) and pH) on lupin protein acid-induced gel quality. The quality attributes were Gs, WHC and gel yield (Gy). The three most significant factors were identified and were used in the optimisation study described in Chapter 6.

5.2. Materials and Methods

5.2.1. Materials

All required material (Lupin seed, *Lupinus angustifolius*, Coromup variety and Glucono-delta-lactone (GDL) were as described in Section (4.2.1).

5.2.2. Experimental design

5.2.2.1. Identifying factors controlling lupin protein gel ability using ultrasound treatment under cold-set gel conditions

Five independent factors were chosen based on the knowledge gathered in Chapter 4 of this thesis and information available in the literature on their potential effect on protein gel quality. (Alting et al., 2003; Hu, Li-Chan, Wan, Tian, & Pan, 2013; Hu, Wu, et al., 2013; Shen, Zhao, Guo, Zhao, & Guo, 2017; Tang, Chen, & Foegeding, 2011; Zhou et al., 2016). A two level fractional factorial experimental design 2^{5-1} was generated using Design-Expert software (V11, Stat-Ease, Inc. Minneapolis, MN, USA) to determine the effect of the independent variables, high-intensity ultrasound time (HIUt, min) treatment, high-intensity ultrasound treatment power (HIUp, W/cm^2), HTT (°C), HTt (min) and pH on lupin protein concentrate (LPC) Gs (g), WHC (%) and Gy (%). Design-Expert software was used to generate the experimental runs (Table 5.2) using the minimum and maximum values of each independent factor. The levels of the independent factors were identified based on the previous study described in Chapter 4 and preliminary work to confirm that lupin protein can form a gel under each set of conditions for which the gel quality attributes could be quantified.

Table 5.1 Factorial independent variables with actual and coded values

Factors	Independent variables	Units	Actual values		Coded values	
			Min	Max	Min	Max
A	Ultrasound time	min	2	20	-1	+1
B	Ultrasound power	W/cm^2	11	38	-1	+1
C	Heat treatment temperature	°C	75	95	-1	+1
D	Heat treatment time	min	20	60	-1	+1
E	pH	-	4.5	5.5	-1	+1

5.2.3. Preparation of lupin protein concentrate (α- and β-conglutin fraction)

LPC was extracted using isoelectric precipitation technique as described in Section 3.2.2.

5.2.4. Preparation of lupin protein concentrate solutions for ultrasound treatment

LPC solutions were prepared as described in Section 4.2.4.

5.2.5. High-intensity ultrasound treatment

The HIU treatment was performed as described in Section 4.2.5. LPC solutions were treated with ultrasound at intensities of ~11 W/cm^2 and ~38 W/ cm^2 for 2 or 20 min. Samples were sonicated in a 60 mL double-wall glass vessel connected to a refrigerator (Model 4850, Bio-Rad Laboratories, Hercules, CA, USA) to recirculate the cooling liquid to maintain the sample temperature below 35 °C during the treatment.

Two different ultrasound amplitudes were used in this study to deliver two ultrasound power treatments. Ultrasound power (W) and intensity (W/cm^2) were measured, as mentioned in Section 4.2.6. at 10% and 40% amplitude; the calculated intensities were ~11 W/cm^2, and ~38 W/cm^2, respectively.

5.2.6. Controlled acidification and heat treatment

To induce gelation, 0.5% or 1% (w/v) GDL was added to LPC dispersions to give a final pH of 5.5 and 4.5, respectively. The detailed method is described in Sections

4.2.7.2. The samples were vortexed for 30 s after addition of GDL and then underwent the heat treatment at 75℃ or 95℃ for 20 min or 60 min (Table 2) to facilitate protein unfolding and aggregation, which is essential for gelation of globular proteins (Clark, Kavanagh, & Ross-Murphy, 2001) (Section 4.2.7.2). During the heat treatment, it was expected that the GDL will slowly hydrolyse to gluconic acid and reduce the pH to the required point. After heat treatment, the lupin protein gels were cooled at room temperature for 30 min and then kept at 4℃ for 24 h for gel equilibration before the quality analysis.

5.2.7. Gel quality assessment

The gel quality attributes Gs (g), WHC (%) and Gy (%) were measured in duplicate as follow.

5.2.7.1. *Gel strength*

Gs was determined using a texture analyser method as described in Section 4.2.7.3 (Hu, Li-Chan, et al., 2013; Zisu, Bhaskaracharya, Kentish, & Ashokkumar, 2010).

5.2.7.2.Water holding capacity

WHC was determined by calculating the amount of water entrapped in the gel network after applying centrifugation as previously detailed in Section 4.2.7.4.

5.2.7.3.Gel yield

The gel samples were prepared as described in Section 5.2.7.2. After the gel was formed, unbound (free) water was carefully removed from the gel container using filter paper. Gy (%) was calculated using the following equation:

$$Gel\ yield\ (\%) = (Wg/Wt) \times 100$$

where *Wg* is the gel weight in grams after removing unbounded water, and *Wt* is the weight in grams of the original lupin protein solutions including the weight of the added GDL.

5.2.8. Statistical analysis

Design-Expert software (V11, Minneapolis, MN USA) was used to generate the factorial screening experimental runs and to analyse the data (Montgomery, 2017). Regression analysis generated by the model using Design-Expert software was used to identify the most significant factors affecting lupin protein gel quality (quality attributes). Pearson's correlations between dependent factors were determined using

IBM SPSS Statistics (V25, Armonk, NY, USA). All results are expressed as mean ± standard deviation (n ≥ 2), and $P < 0.05$ was considered to be significant for all analyses.

5.3. Results and discussion

5.3.1. Influence of independent factors on lupin protein gel strength

Lupin protein concentrate Gs was 11-215 g (Table 5.2). The model showed that Gs was significantly ($P > 0.05$) influenced by HIUt, HIUp and HTT (Table 5.3). The highest gel strength was observed when HIUt and HIUp were at their highest levels, 20 min and ~38 W/cm^2 power. It has been reported that improvements in the Gs of soybean and whey proteins by ultrasound treatment was caused by protein denaturation and exposure of active protein groups, which increased intermolecular crosslinking (Hu et al., 2015; Shen, Fang, et al., 2017; Shen, Zhao, et al., 2017). Hu, Fan, et al. (2013) reported that ultrasound treatment for 20 min significantly increased the Gs of soybean protein isolate ($p <0.05$), which the findings of this study support.

Heat treatment also had a strong significant ($P <0.05$) effect on Gs, which had the highest *F*- value (80.97) in the analysis of variance (ANOVA) of factional factorial design and highest effect of contribution (EC) (weight of the significant independent factor effect on the response factor) (Table 5.3). It has been reported that lupin protein particles do not exhibit swelling ability (unfolding) even after high temperature treatment because of the protein thermal properties (Berghout, Boom, et al., 2015). This thermal stability negatively influences lupin protein gelation. However, after appropriate ultrasound treatment (time and power), the Gs of lupin protein was significantly higher ($P <0.05$) in treated than in untreated samples. This finding supports the idea that ultrasound can reduce thermal stability and protein physiochemical properties (i.e. particle size and ζ potential) (Chapter 4).

It has been reported that lowering the pH to near the isoelectric point (IP) of the predominant protein increases Gs because of reduced repulsion forces between protein molecules in response to the decrease in protein net charge at the IP, which facilitates building of a stable gel network by increasing the close interactions between protein molecules (Pang et al., 2019; Verheul & Roefs, 1998).

5.3.2. Influence of independent factors on lupin protein gel water holding capacity

The effects of independent variables on the WHC of the gels are presented in Table 5.2. The WHC ranged from 32.72% to 90.53% and was significantly higher than the 29.46% in the unsonicated gel sample (control) (Table 5.2). ANOVA showed that the WHC was significantly ($P < 0.05$) influenced by HTT, HIUt and HIUp (Table 5.3). It has been reported that ultrasound treatment can modify protein structure and particle size, facilitate protein unfolding and expose hydrophobic groups (Hu et al., 2015; Zhou et al., 2016). These changes in protein structure and particle size facilitate the formation of a uniform and dense gel network with better WHC (Section 4.3.2.2). Similarly, Zhang et al.,(2016) reported that the WHC of a soy protein isolate cold-set gel increased after ultrasound treatment. They claimed that sonication exposes hydrophobic spots on the surface of protein molecules, which facilitates the formation of a compact gel network that remains intact during the centrifugation used to determine the WHC.

Similar to Gs, HTT had the highest EC value for the WHC of 54.96% compared with HIUt and HIUp. Ultrasound treatment seemed to have improved lupin protein performance during the gelation process (heat treatment) by inducing effective protein unfolding and hydrophobic group exposure, which facilitated the formation of a highly crosslinked gel matrix with higher Gs compared with the unsonicated lupin protein (Berghout, Boom, et al., 2015). Pearson's correlational analysis showed a significant positive association ($R = 0.799$, $P = 0.01$) between Gs and WHC. These findings may be explained by the production of a firm and strong gel network, which can entrap and hold more water because of its stable structure, even after centrifugation (Hu, Fan, et al., 2013).

5.3.3. Influence of independent factors on lupin protein gel yield

The effects of the independent factors on Gy are presented in Table 5.3. Gy was influenced by independent factors levels. Gy ranged from 84.6% to 97.17% versus 52.87% for the unsonicated sample. ANOVA showed that HIUt had a significant effect on LPC Gy, which increased with HIUt. This result is consistent with the findings of Zhang et al. (2016), who reported that increasing HIUt from 5 min to 40 min reduced the amount of water leaking (syneresis) from soybean protein gels. This effect of ultrasound may relate to changes in protein particle size. Reducing particle

size during ultrasound treatment increases the protein molecule surface area and facilitates protein-protein and protein-water interactions (Jambrak, Lelas, Mason, Kresic, & Badanjak, 2009). These small particles will build a much denser gel network with smaller pores via the protein-protein interactions and a gel network with increased water-binding ability via protein-water interactions (Hu et al., 2015; Zhang et al., 2016).

Table 5.2 Independent and dependent factors with their actual values obtained from factorial experimental design

Run	Actual values of variables					Response factors		
	A	B	C	D	E	Gs	WHC	Gy
	min	%	℃	time	mol/L	g	%	%
1	20	40	95	20	4.5	215.00±5.66	90.53±1.42	97.29±0.12
2	20	40	95	60	5.5	201.33±6.36	84.95±0.79	96.96±0.06
3	2	10	95	20	4.5	29.00±1.41	56.59±0.14	96.66±0.18
4	2	10	95	60	5.5	50.33±4.24	56.12±0.73	96.08±0.69
5	20	10	75	20	4.5	39.67±2.83	50.26±0.1	95.66±0.33
6	20	40	75	20	5.5	37.33±2.12	50.19±0.75	96.79±0.54
7	2	40	75	60	5.5	25.67±1.41	49.3±1.06	93.34±0.70
8	2	40	95	20	5.5	82.67±2.12	58.06±1.04	97.45±0.50
9	20	10	95	20	5.5	170.67±0.71	63.17±1.31	97.16±0.81
10	20	10	95	60	4.5	119.67±2.12	56.01±0.99	94.17±0.96
11	2	10	75	60	4.5	24.02±0.71	44.46±0.83	96.91±0.86
12	2	40	95	60	4.5	66.01±1.41	61.6±0.88	85.21±0.41
13	2	10	75	20	5.5	11.03±1.41	32.72±0.37	84.04±0.32
14	20	40	75	60	4.5	60.67±1.41	58.77±0.95	97.36±0.47
15	2	40	75	20	4.5	24.00±1.41	38.02±0.97	97.69±0.25
16	20	10	75	60	5.5	56.00±2.12	53.50±1.14	97.33±0.71
control	-	-	95	60	4.5	28.33±1.95	29.46±0.11	52.87±2.18

A, ultrasound treatment time (min); B, ultrasound treatment power (%); C, heat treatment temperature (°C) during the gelation process; D, heat treatment temperature time (min); E, pH ;Gs, gel strength; WHC, water-holding capacity; Gy, gel yield.

Table 5. 3 Analysis of variance of the significant terms in the fractional factorial screening model

	Responses								
	Gs			**WHC**			**Gy**		
Significant terms of the	***F* value**	***P* value**	**EC* %**	***F* value**	***P* value**	**EC* %**	***F* value**	***P* value**	**EC* %**
Model	46.32	<0.0001		14.89	0.0002		24.53	<0.0001	
A	80.97	<0.0001	34.96	13.36	0.0033	29.90	26.85	0.0008	15.63
B	10.70	0.0084	4.62	6.76	0.0232	15.12	-	-	
C	100.76	<0.0001	43.50	24.56	0.0003	54.96	-	-	
D	-	-	-	-	-	-	-	-	
E	-	-	-	-	-	-	-	-	

*EC, Effect of contribution (%), as the sum of squares of each factor in the model divided by the sum squares of the total significant and not significant factors in the model and multiplied by 100. A, Ultrasound treatment time (min); B, ultrasound treatment power (%); C, heat treatment temperature (°C) during the gelation process; D, heat treatment temperature time (min); E, pH ;Gs, gel strength; WHC, water-holding capacity; Gy, gel yield. (-), no significant ($P > 0.05$) effect indicated for the factor by the model.

5.4. Conclusion

A fractional factorial experimental design was used successfully to explore the effects of HIUt (min), HIUp (%), HTT (°C), HTt (min) and pH on LPC acid-induced protein Gs, WHC and Gy. The analyses show that these gel quality attributes (Gs and WHC) are significantly influenced by the independent variables HIUt, HIUp and HTT, whereas the Gy response is influenced by HIUt only. The next chapter describes the study of the optimisation of these three factors using a more robust experimental design of CCRD with the aim of producing an LPC acid-induced gel with the highest possible gel quality.

CHAPTER 6

Optimisation of Acid-Induced Lupin Protein Concentrate Gel Quality Using High-Intensity Ultrasound Treatment

Abstract

The study described in Chapter 5 identified the most significant factors controlling ultrasound-assisted lupin protein acid-induced gelation. This chapter describes a study of the effects of the significant factors (high-intensity ultrasound time (min), high-intensity ultrasound power (W/cm^2) and heat treatment temperature (°C)) on the acid-induced gelation of lupin protein concentrate (LPC) and their optimisation. Response surface methodology using central composite rotatable designs were used to predict the optimal levels of independent factors to maximise the LPC gel properties: gel strength (Gs, g), water-holding capacity (WHC, %) and gel yield (Gy, %) using the model desirability function. The optimal levels of independent factors predicted by the model were validated by comparing the predicted response values to those in the experimental data. At optimal conditions of independent factors, the experimental results of the response (Gs, WHC and Gy) factors increased to 222 g, 93.59% and 97.09%, respectively, compared with 28.33 g, 29.46% and 52.87% in the unsonicated (control) samples. The microstructure of the gel network created under optimal ultrasound conditions was investigated using scanning electron microscopy (SEM), and changes in LPC protein structure were examined using SDS-PAGE and Fourier-transform infrared (FTIR) spectroscopy. SEM of lupin protein gels created under the optimal conditions showed a smooth and dense network. FTIR spectra showed an increase in protein aggregation, which appeared as a shift in β-sheet wavenumber towards a lower position on the spectrum. This new understanding of the optimal conditions for lupin protein gelation may contribute to the development of new food applications using ultrasound-modified LPC as a novel texturising agent.

6.1. Introduction

Green and innovative technologies such as ultrasound are gaining attention in the food manufacturing industry. Ultrasound energy is considered to be a sustainable technology because of its efficient use of energy and time (Chemat et al., 2011; Knorr et al., 2011).

Given its tuneability, ultrasound has wide applications in food processing such as pasteurisation, extraction and quality control processes (Awad et al., 2012). In addition, several studies highlight the potential of ultrasound treatment to significantly increase plant protein gel quality. This increase in gel quality appears to occur through changes in protein secondary structure that facilitate a variety of new intermolecular crosslinking by disulphide, hydrophobic, electrostatic and hydrogen-bonding interactions. For instance, gel strength (Gs), water holding capacity (WHC) and gel yield (Gy) increased after ultrasound treatment of soybean protein isolate at 105-110 W/cm^2 for 5 to 40 min in a cold-set gel system (Zhang et al., 2016). In addition, high-intensity ultrasound (HIU) treatment improves acid- or salt-induced gelation of soybean protein isolate by increasing Gs and WHC (Hu, Fan, et al., 2013; Hu, Wu et al., 2013). The gel storage modulus (G′) of soybean protein isolate can also be increased by ultrasound treatment at 200 W and 15 kHz for 10 min (Tang et al., 2009). By contrast, treating soybean protein isolate with 4.27 W at 20% amplitude for 20 min leads to a slight decrease in G′ (Arzeni et al., 2012), which highlights the need to optimise ultrasound exposure.

Zhou et al. (2016) found that ultrasound treatment creates a denser soy protein gel network by reducing its particle size. Similarly, Hu, Li-Chan, et al. (2013) reported that ultrasound treatment (400 W for 40 min) of soybean protein suspension reduced the protein particle size from 176 μm to 97 μm, which resulted in a more uniform and dense gel network. Fourier transform (FT)-Raman spectroscopy and circular dichroism show that ultrasound treatment creates protein conformational changes in soybean protein structure(Hu et al., 2015; Hu, Li-Chan, Wan, Tian, & Pan, 2013; Zhou et al., 2016). Theses structural changes facilitate gel network stability through newly exposed hydrophobic and disulphide interactions. Ultrasound can modify protein structure by facilitating gel network formation, which improves the gelling properties of plant proteins.

There is a growing demand for plant proteins as food ingredients because of the increased number of consumers looking for sustainable, healthier and cheaper food options (Stone et al., 2018). The successful use of any plant protein source as a food ingredient in the food industry depends mainly on protein functionality and nutritional

value (Gonzalez-Perez & Arellano, 2009). Recently, lupin has gained renewed interest from the food industry because of its high protein content (40 g/100 g as is in kernels), low anti-nutritional factors, high protein digestibility and as a non-genetically modified food (Chew et al., 2003). However, it has been reported that the main lupin protein fraction (α- and β-conglutins, ~87% of total protein) has weak gelation properties compared with soy and pea protein (Batista et al., 2005; Berghout, Boom, et al., 2015; Hojilla-Evangelista et al., 2004). The lack of gelation properties limits the use of lupin protein as a food ingredient in various plant-based food systems such as yoghurt, cheese, meat analogues and gluten-free products where the protein is required to contribute to the texture (Buchert et al., 2010). Native lupin protein has a thermally stable compact structure, which prevents it from unfolding and forming a stable gel network (Batista et al., 2005; Berghout, Boom, et al., 2015). Ultrasound treatment has great potential to modify lupin protein gelation properties (Chapters 4 and 5). However, given the wide range of factors affecting the HIU treatment and protein gelation process, an optimisation study is required to identify the optimal conditions to maximise lupin protein gel quality.

To optimise a process using a one-factor-at-a-time (univariate) method can be a time-consuming task with uncertain results because of the inability to identify vital factors and their interaction effects on the responses. Response surface methodology (RSM) is considered to be the most efficient way to build mathematical models to study the effects of various (multivariate) independent variables that control the response factors simultaneously (Myers, Montgomery, & Anderson-Cook, 2016). This experimental design can be used to identify the most significant factors (factorial screening, Chapter 5) and then to optimise the levels for the desired response (Carlson & Johan, 2005). The aim of the research described in this chapter was to identify the optimal levels of independent factors (high-intensity ultrasound time (HIUt), high-intensity ultrasound power (HIUp) and heat treatment) to give the maximum acid-induced gel quality of lupin protein concentration (LPC).

6.2. Materials and methods

6.2.1. Materials

The materials used in this study are described in Section 4.2.1.

6.2.2. Experimental design

6.2.2.1.Response surface methodology

Optimising the conditions for producing ultrasound-assisted acid-induced gels of LPC with the highest gel quality were conducted by generating a central composite rotatable design (CCRD) using Design-Expert software (V11, Stat-Ease Inc. Minneapolis, MN, USA). Given the many factors controlling the gelation process and ultrasound treatment, a first-order factorial screening (Wass, 2010) study was previously conducted to identify the three most significant factors influencing the gel quality of the HIU-assisted LPC modification (Chapter 5). These factors were HIUt (min), HIUp and thermal treatment temperature (HTT, °C). Two other factors, thermal treatment time (min) and pH, were not significant in the previous study and were fixed at 60 min and 4.5, respectively, for the study described here.

Each independent factor had five levels, as shown in Table 6.1. The independent factors and their levels (maximum and minimum) identified in the factorial secerning study (Chapter 5) and optimisation (this chapter) were chosen based on information gained in the study described in Chapter 4. Three responses factors, GS (g), WHC (%) and Gy (%) were the dependent variables. The CCRD model generated 20 runs in total (14 individual runs and six replicates of the centre points) (Table 6.2). Multiple linear regression analysis was used to evaluate each response factor. Analysis of variance (ANOVA) was used to identify the significant terms in the model and the effects of independent factors on the responses. A polynomial quadric regression equation was used to determine the combined effect of independent factors (HIUt, HIUp and HTT) (Eq. 1):

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum \sum_{i<j=2}^{n} \beta_{ij} X_i X_j \quad (1)$$

where Y represents each response factor in the model (GS, WHC and Gy); β_0, is the constant term or intercept; β_i, β_{ii} and β_{ij} are regression coefficients of the first order, quadratic and interaction teams, respectively; and X_i, X_j represents the independent variables at i and j levels. The model for each response factors was analysed and considered to be significant if the following four criteria were met: the calculated F value was <5% ($P < 0.05$) probability level, the coefficient of determination value R^2 was > 0.7; the model lack of fit was not significant ($P < 0.05$); and the signal-to-noise ratio

(adequate precision) was > 4 (Table 6.3) (Vera Candioti, De Zan, Cámara, & Goicoechea, 2014). Three-dimensional surface plots and two-dimensional contour plots of each response factor were generated using Design-Expert software (V11) to illustrate the relationships between the dependent and independent factors.

Table 6.1 Independent variables with actual and coded values for the central composite design

			Levels				
Factors code	Factors actual	Units	-α	-1	0	+1	+α
A	HIUt	min	1.22**	6	13	20	24.77**
B	HIUp	%	6.47*	15	27.5*	40	48.52*
C	HTT	°C	69.88**	75	82.5*	90	95.11*

HIUt, high-intensity ultrasound time; HIUp, high-intensity ultrasound power; HTT, heat treatment temperature –α, -1, 0, +1 and +α represent the levels of each independent factors.
* adjusted in the actual treatment
** adjusted to one decimal in the actual treatment

6.2.2.2.Multiple response optimisation

The desirability (also named as Derringer) function (*D*) was used to investigate the optimal condition to produce the highest combined level of the lupin protein gel quality attributes (Costa & Lourenço, 2014). *D* can be defined as the combination of importance level (priority) (response factor weight) of each dependent factor (response), which represents the maximum overall (global) *D* of the model (Myers et al., 2016). In multiple response optimisation, Design-Expert software was used to analyse and predict the optimal levels of independent factors dependent on the importance criteria for each response factor, which can be varied from 1 (less important) to 5 (most important) as prioritised by the researcher. In this study, the optimal levels of HIUt (min), HIUp (W/cm^2) and HTT (°C) to produce the best gel quality were investigated by prioritising the responses (identify importance level of the response factor) Gs, WHC and Gy simultaneously using Design-Expert software. The global *D* can be measured as follows:

$$D = (d_1^{r1}.\, d_2^{r2}.\, \ldots\ldots\, d_n^{rn})^{\frac{1}{\sum ri}} = (\prod_{i=1}^{n} d_i^{r1})^{\frac{1}{\sum ri}}$$

where r_i represents the priority score of each response factor (range 1-5), *d* represents the individual *D* function, and *n* is the number of responses in the optimisation model (Vera Candioti et al., 2014).

6.2.2.3.Model validation

The model was validated by choosing an optimal solution (set of independent factor levels) that could be generated based on responses *D* criteria to reach the maximum level of dependent variables (Table 6.5). The validation was conducted by comparing the model predicted levels of responses to the experimental values using independent-sample *t* tests with significance set at $P < 0.05$ (Stat-Ease Inc., 2018). The experimental analysis was performed in duplicate.

6.2.3. Preparation of lupin protein concentrate

LPC was prepared as described in Section 3.2.2.

6.2.4. Compositional analysis of lupin protein concentrate

The LPC proximate composition analysis was described in Section 3.2.3.

6.2.5. Preparation of lupin protein concentrate dispersions

LPC dispersions of 10% (w/w) were prepared as described in Section 4.2.4.

6.2.6. High-intensity ultrasound treatment

The HIU treatment was performed using ultrasound equipment as described in Section 4.2.5. Duplicate samples of 20 mL of 10% (w/w) LPC solutions in special 60 mL double-wall glass vessel (see section 6.2.5.) were treated as per the run conditions given in Table 6.2. A chiller was connected to the sonication glass vessel to maintain the sample temperature below 35 °C during the treatment.

6.2.7. Determination of high-intensity ultrasound power

HIUp was calculated as described in Section 4.2.5.

The calculated power intensity was ~7, 18, 34, 38 and 50 W/cm^2 at ~6.5, 15, 27.5, 40 and 48.5*% amplitude, respectively. The amplitudes of 6.5, 27.5 and 48.5% were adjusted to about one decimal place in the actual ultrasound treatment.

(*) adjusted to ~ one decimal in actual ultrasound treatment.

6.2.8. Acid-induced gelation

Acid-induced gelation was prepared as described in Section 4.2.7.2. Samples were heated as suggested by the model (Table 6.2) for 60 min to induce lupin protein aggregates as a pre-gelation step.

6.2.9. Acid-induced lupin protein concentrate gel quality

6.2.9.1.Gel strength

Gs was measured as described in Section 4.2.7.3.

6.2.9.2.Gel water holding capacity

WHC was analysed as described in Section 4.2.7.4.

6.2.9.3.Gel yield

Gy was measured in gel samples prepared as described in Section 5.2.7.3.

6.2.9.4.Scanning electron microscopy

The microstructure morphology of the network of the lupin gels (unsonicated control), Run 1 (midrange gel quality) and optimised) was investigated using field emission scanning electron microscopy (SEM) on a dual-beam field emission scanning electron microscope (Zeiss Neon 40EsB FIBSEM, Oberkochen, Germany). The samples were dried and then coated with platinum using a sputter coater (208HR, Cressington, Watford, the UK). The samples were placed in the microscope and high-resolution images were captured of samples at an accelerating voltage of 3 kV.

6.2.9.5.Sodium dodecyl sulfate polyacrylamide gel electrophoresis

The protein profile of freeze-dried LPC gel samples was examined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS_PAGE) for unsonicated (control), Run 1 and optimised samples using the method described in Section 3.2.5.

6.2.9.6.Fourier-transform infrared spectroscopy

FT-infrared (FTIR) spectroscopy was used to monitor secondary structure changes of LPC (gel matrix) during acid-induced gel gelation as influenced by the gelation conditions (independent factors). To achieve that, a set of LPC acid-induced gel samples named as unsonicated (control), Run 1 and optimised samples were prepared, freeze-dried and analysed as described in Section 4.2.7.6.

6.2.10. Statistical analysis

The results are expressed as mean ± standard deviation (n=2) unless otherwise stated. One-way ANOVA using Tukey post-hoc test among results of each independent variable (n=2) were analysed using SPSS Statistics (V26, IBM Corp. Chicago, Illinois, USA). ORIGIN (V2018 Pro, OriginLab Corporation, MA, USA) were used for FTIR data visualisation.

6.3.Results and Dissections

6.3.1. Effect of independent factors on lupin protein concentrate acid-induced gel properties

6.3.1.1. Lupin protein concentrate gel strength

Ultrasound treatment at any level produced a higher Gs (36.5-202.5 g) compared with the un sonicated sample (28.33 g) (Table 6.2). These results are consistent with the data from the factorial screening study (Chapter 5). A quadratic model was the best-fit regression model to express the combined influence of the independent factors on Gs. This model was confirmed by the high coefficient of determination (R^2 = 0.9741) and non-significant lack of fit (P > 0.05). ANOVA results for the model are expressed as regression coefficients for the effects on Gs of the independent factors are given in Table 6.3. The significant terms in the model were: HIUt (for model A,P < 0.0001); HIUp (model B, P < 0.0059); HTT (model C, P < 0.0001); interaction (model AC, P < 0.0003). The quadratic effects of A^2, B^2 and C^2 had P values of 0.0299, <0.0001 and 0.0045, respectively.

The predictive mathematical equation of the model is presented in Table 6.4. The interaction between the independent factors HIUt (A) and HTT (C) had a significant effect on LPC Gs. This interaction (AC) may imply that that ultrasound treatment increased lupin protein heat sensitivity, which increased the lupin protein unfolding ability during heat treatment and improved the gel-formation ability (Gs). Berghout et al. (2015) reported that lupin protein has weak gelation properties compared with soy protein, even at higher lupin protein concentration. They found that lupin particles do not exhibit the thermal unfolding ability during heat treatment, which is an essential step in the gelation process. Similarly, Sousa, Mitchell, Ledward, Hill, & Beirão da Costa (1995) investigated

lupin and soy protein thermal properties and found that lupin is more heat stable than soybean protein and that this negatively affected lupin gelling properties. The findings of the research reported in Chapter 4 showed that HIU reduces the heat energy (denaturation enthalpy) required to denature lupin protein.

In the present study, the model showed that HTT is the most significant factor affecting Gs (F = 186.98) followed by HIUt and HIUp (F = 64.09 and F = 12.02, respectively). These findings confirm the notion that ultrasound combined with heat treatment creates a synergetic effect that reduces lupin protein thermostability and improves Gs. HIU can trigger protein unfolding and create conformational changes in protein structure by exposing active amino acid (AA) groups on the surface of protein molecules (Arzeni, Perez, & Pilosof, 2012; Shen, Zhao, Guo, Zhao, & Guo, 2017). The results presented here are consistent with those of Jiang et al. (2017), who reported that HIU increased whey protein Gs significantly compared with unsonicated samples. They also found that the increase in Gs correlates positively with exposure of active AA (hydrophobic) Groups. Three-dimensional surface plots demonstrated the combined effects of independent factors on Gs (Figure 6.1). Figure 6.1A shows the predicted relationship of HIUt and HTT on LPC Gs. Gs increased with HIUt and HTTwhen HIUp was set at the mid-point level. Figure 6.1B shows the interaction effects of HIUp and HTT on Gs. Gs increased significantly with HTT at moderate HIUp. However, when increasing both HIUp and HTT to their maximum levels (within the model range), Gs decreased slightly. Increasing HIUp at a higher temperature may decrease gel Gs because of severe protein denaturation. Cui et al. (2019) examined the Gs of whey and soybean protein isolate (mixture) at different ultrasound power levels (150-450 W). They found that increasing HIUp to 450 W for 30 min and heat treatment at 90 °C reduced Gs significantly compared with 30 W, possibly because of excessive protein denaturation. This highlights the importance of optimising the effects of ultrasound to achieve the highest gel quality.

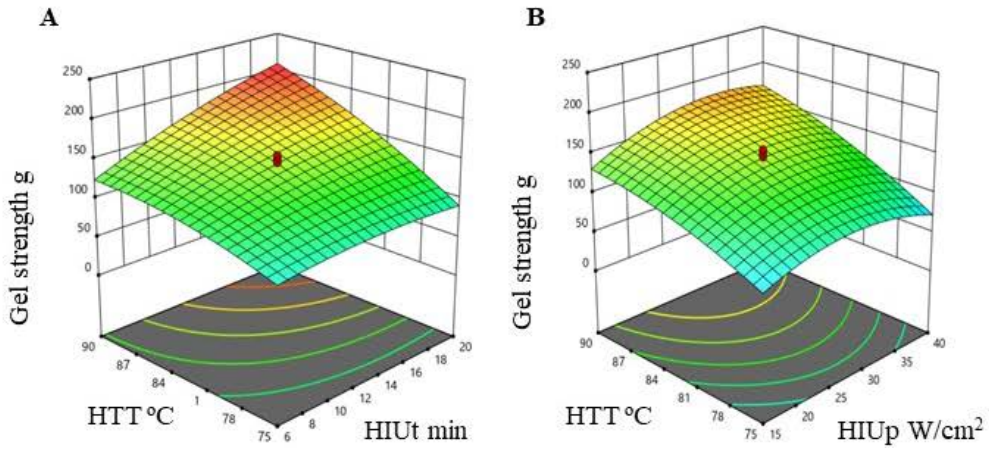
Table 6.2 Generated run of the central composite design with the experimentally determined response factor values

Run	Independent factors			Response factors		
	HIUt (min)	HIUp (%)	HTT (°C)	GS (g)	WHC (%)	Gy (%)
1	13	48.5*	82.5*	87.5±0.71	59.57±1.4	88.74±1.77
2	6	40	90	120.5±2.12	67.8±2.31	90.3±2.18
3	6	15	90	80.±1.41	38.65±2.43	84.32±1.11
4	13	27.5*	95.11*	187.5±3.54	73.12±2.89	95.33±2.41
5	13	27.5*	82.5*	155.3±0.1	73.61±2.18	94.96±0.45
6	20	40	75	71.5±2.12	53.33±3.75	82.28±2.74
7	13	27.5*	82.5*	136.5±0.71	73.32±4.57	92.09±0.56
8	1.22**	27.5*	82.5*	77.±2.83	38.84±1.62	88.38±1.81
9	13	27.5*	69.88*	36.5±0.71	65.44±4.13	77.8±1.43
10	24.77**	27.5*	82.5*	167.0±1.41	76.35±2.17	94.34±1.1
11	13	27.5*	82.5*	148.66±0.71	69.23±2.15	90.63±3.7
12	20	40	90	202.5±2.12	90.2±3.77	97.18±3.03
13	20	15	75	69.0±1.41	51.17±3.33	79.3±1.08
14	20	15	90	160.5±2.12	67.47±4.7	95.05±0.56
15	13	6.47*	82.5	66.0±1.41	34.24±4.5	83.65±6.34
16	13	27.5*	82.5	146.33±0.71	68.59±2.45	93.12±1.94
17	6	15	75	62.5±0.71	45.18±3.34	88.44±1.82
18	13	27.5*	82.5*	129.5±0.71	64.97±2.48	90.42±2.35
19	6	40	85	77.5±2.12	55.3±4.61	87.29±0.85
20	13	27.5*	82.5*	152.5±0.71	68.76±1.81	91.1±3.01
Control	0	0	95	28.33±1.95	29.46±0.11	52.87±2.18

HIUt, high-intensity ultrasound time; HIUp, high-intensity ultrasound power; HTT, heat treatment temperature; Gs, gel strength; WHC, water-holding capacity; Gy, gel yield.

* adjusted to integer in the actual treatment

** adjusted to one decimal place in actual treatment



HIUt, high-intensity ultrasound time; HIUp, high-intensity ultrasound power; HTT, heat treatment temperature

Figure 6.1 Three-dimensional surface plot illustrating the effects of (A) heat treatment temperature (°C) and ultrasound treatment time (min) at the mid-point level of ultrasound treatment power and (C) heat treatment temperature (°C) and ultrasound treatment power at the mid-point level of ultrasound treatment on lupin protein gel strength

Table 6.3 Analysis of variance results of the effect of independent factors on each dependent variable

Source	Gs (g)	WHC (%)	Gy (%)
Type of model	Quadratic	Quadratic	Quadratic
Pvalue			
Model	<0.0001	<0.0001	<0.0001
A	<0.0001	<0.0001	0.0874
B	0.0060	<0.0001	0.0265
C	<0.0001	0.0016	<0.0001
AB	-	-	-
AC	0.0003	0.0043	0.0002
BC	-	0.0117	-
A^2	0.0293	0.0076	-
B^2	<0.0001	<0.0001	0.0026
C^2	0.0035	-	0.0039
Lack of fit	0.3795	0.1339	0.3483
R^2	0.9741	0.9481	0.9341
Adjusted R^2	0.9508	0.9015	0.8747
Adeq precision	20.5860	16.9929	14.6566
F value (model)	41.81	20.31	15.74

A, Ultrasound treatment time (min); B, ultrasound treatment power (%); C, thermal treatment temperature during the gelation process (°C), Gs, Gel strength; WHC, water holding capacity; Gy, gel yield.

Table 6.4 Predictive equation of the CCRD model for each response factor using their actual values

Source	Gs (g)	WHC (%)	Gy (%)
Intercept	-1095.18513	+143.46327	-99.61121
A	-23.85370++	-3.47096++	-
B	+0.956058++	+0.340256++	+0.124491++
C	+27.51303++	-2.38127++	+4.91632++
AB	-	-	-
AC	+0.377778++	+0.093959++	+0.075627++
BC	-	+0.042104++	-
A^2	-0.144643++	-0.087068++	-
B^2	-0.149446++	-0.051655++	-0.012950++
C^2	-0.181521++	-	-0.033658++

A, Ultrasound treatment time (min); B, ultrasound treatment power (%); C, thermal treatment temperature during the gelation process (°C); CCRD, central composite design; Gs, gel strength; WHC, water-holding capacity; Gy, gel yield ;AB,Ac and BC, factors interactions; A2,B2 and C2 , independent factors in their quadratic level.++, Significant coefficients ($P < 0.05$).

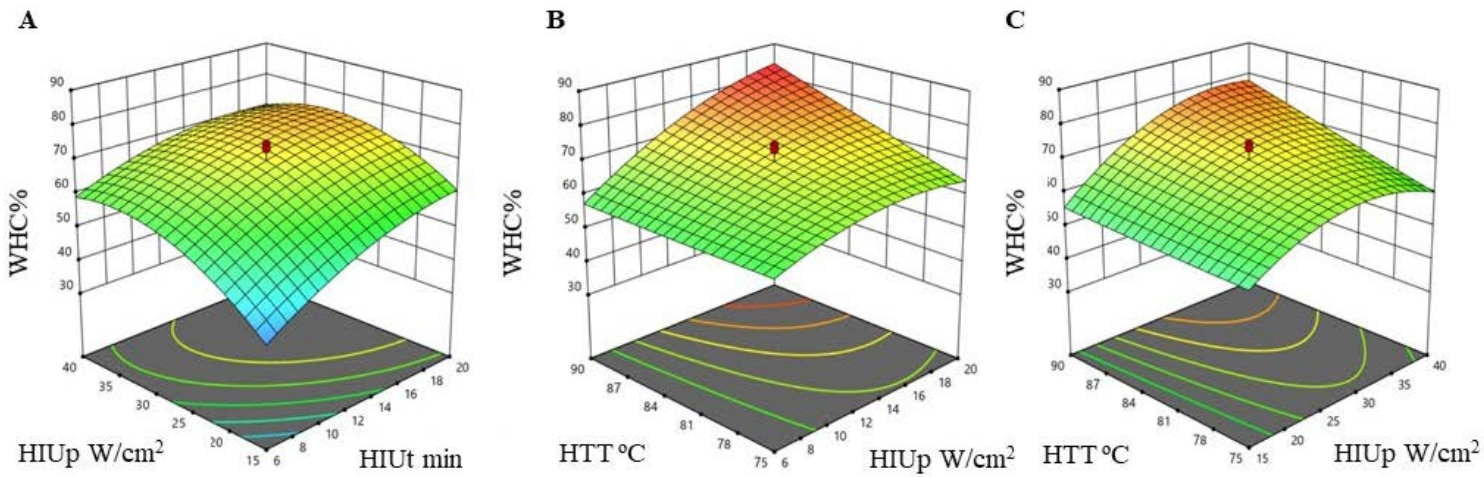
6.3.1.2. Lupin protein concentrate gel water-holding capacity

The WHC represents the ability of a gel to immobilise water within its protein network pores (Wang, Luo, Liu, Adhikari, & Chen, 2019). It is one of the most important gel quality attributes; a higher WHC is better in food manufacturing because loss of water from the food matrix can lead to undesirable changes in texture and quality (Alavi et al., 2020). The WHC of the gels generated from the RSM runs are presented in Table 6.2. A significant quadratic model was generated to predict the combined effect of HIUt (A), HIUp (B) and HTT (C) and their interactions on the gel WHC using their corresponding estimated regression coefficients (Table 6.3). ANOVA showed that the model had a high coefficient of determination ($R^2 = 0.9481$) and nonsignificant lack of fit ($P > 0.05$), which verified its suitability. The model showed that the independent factors linear terms (A, B and C), their interactions (AC and BC) and their quadratic terms (A^2 and B^2) were the significant terms of the model.

A predictive model equation was generated using the significant terms and ignoring the remaining non-significant terms (Table 6.4). Different combinations of independent factors resulted in a wide range of WHC values of 34.24-90.20%, whereas the unsonicated (control) recorded the lowest WHC of 29.46% (Table 6.2). Model analysis showed that HIUt was the most significant factor influencing LPC acid-induced gel WHC ($F = 45.52$, $P < 0.0001$ within the time treatment limits chosen. The WHC of LPC gels increased significantly ($P < 0.05$) after HIU treatment, which was a pattern similar to that for Gs. This positive relationship between Gs and WHC was confirmed by the significant ($P < 0.01$) positive correlation ($r = 0.791$) between the Gs and WHC of LPC gels. These findings are consistent with those of Li et al. (2018), who reported a positive correlation between gel stiffness and WHC of egg white protein. It has been reported that a gel network strength and morphology can influence WHC significantly (Urbonaite et al., 2015). For instance, Hu et al. (2013) demonstrated that soy protein acid-induced Gs and WHC increased with HIUt. They found that an HIUt <20 min at 105-110 W/cm^2) promoted the formation of a firm and smooth gel network because of the reduction in protein particle size and formation of small soluble protein aggregates. Similarly, Shen et al. (2017) found that the WHC of whey protein acid-induced gel increased significantly after treatment at ~107 W/cm^2 for 0-40 min. These findings are in

line with our findings in the current study. It is noteworthy that number of factors such as protein aggregate size, a balance of crosslinking regains (hydrophobic and covalent) and non-association regions (hydrophilic) regions, gel network charge have a critical impact on gel mechanical properties (Gs and high WHC) (Kees de Kruif et al., 2015; Wu et al., 2019).

Three-dimensional surface plots of the quadratic model representing the interactions of pairs of independent factors on WHC are shown in Figure 6.2A-C. Figure 6.2A demonstrates the interaction between HIUt and HTT. The model showed that the predicted WHC increased when HIUt and HTT were at their high levels and HIUp at the mid-point level. Figure 6.2C presents the HIUp and HTT interaction effect on WHC when HIUt was set at the mid-point of 13 min. Keeping both HITp and HTT at their high levels reduced gel WHC, as shown by the U-shaped relationship. Under these harsh treatment conditions, large protein particles can be formed because of protein denaturation and aggregation, which increases the gel network pores size and decreases the WHC (Chen et al., 2016). In the work described in Chapter 4 of this thesis, increasing HIUp to 38 W/cm^2 at 95 °C, especially at a long HIUt (40 min), reduced WHC slightly. This resulted in the formation of large agglomerated particles in the gel network and an increase in water loss from the gel network by increasing the pore sizes between the large protein aggregates (particles) (Wu et al., 2019). In the case of LPC in the current study, the combined effects of ultrasound and heat treatment was necessary to overcome the thermal stability of LPC (Berghout, Boom, et al., 2015). These results show that ultrasound treatment (HIUt and HIUp) and heat treatment created an interactive effect on LPC gel WHC by overcoming the thermal stability issue of lupin protein.



HIUt, high-intensity ultrasound time; HIUp, high-intensity ultrasound power; HTT, heat treatment temperature

Figure 6. 2 Three-dimensional surface plot demonstrating the effect of (A) ultrasound treatment time (min) and ultrasound treatment power at the mid-point level of heat treatment temperature; (B) heat treatment temperature(°C) and ultrasound treatment time (min) at the mid-point level of ultrasound treatment power; (C) heat treatment temperature (°C) and ultrasound treatment power of at the mid-point level of ultrasound treatment time on lupin protein gel water-holding capacity properties.

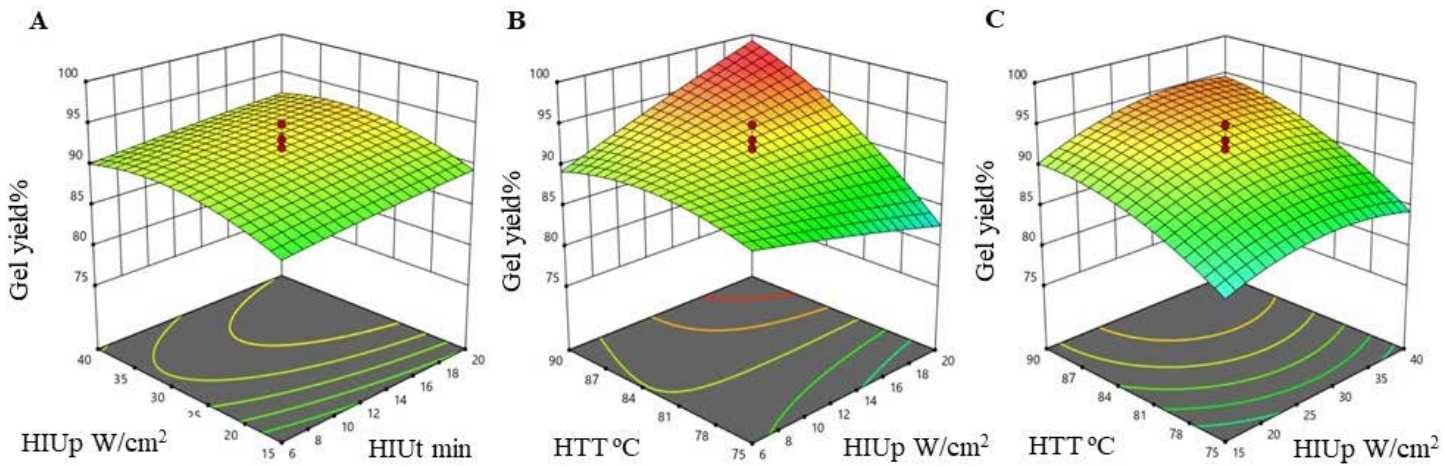
6.3.1.3. Lupin protein concentrate gel yield

Gy is one of most important gel quality attributes for evaluating water leakage (syneresis) of a gel network without application of force (van Vliet et al., 1991). The LPC Gy improved significantly ($P < 0.05$) after ultrasound treatment compared with the unsonicated sample. The Gy values of HIU-assisted LPC acid-induced gel were 77.80-97.18% versus 52.87% in the control (unsonicated) (Table 6.2). A quadratic model was generated to predict the effects of the independent factors and their interactions on Gy (Table 6.3). The model had a high coefficient of determination ($R^2 = 0.9341$) and non-significant lack of fit ($P < 0.05$), which demonstrated its good reliability (Table 6.3). ANOVA predicted that Gy was significantly influenced by B ($P = 0.0265$), C ($P <0.0001$), their quadratic levels (B^2 and C^2) (($P = 0.0026$ and <0.0039, respectively) and AC interaction ($P <0.0002$).

Three-dimensional surface plots illustrate the relationship between the independent factors and Gy (Figure 6.3A-C). Figure 6.3A represents the relationship between HIUt and HUIp with the heat treatment set to the mid-point of 82.5 °C. Under these conditions, the model predicted that Gy will be 91% when the HIUt time is <8 min and with HIUp ~38 W/cm^2. This finding was consistent with the results presented in Chapter 4. HIUp at ~38 W/cm^2 for <10 min reduced LPC particle size. It has been reported that a reduction in particle size increases the surface area for protein-water interactions (Jambrak et al., 2009), which can lead to less gel syneresis. Figure 6.3B represents the effect of the interaction between HIUt and HTT on the LPC Gy when the HIUp was set at the mid-point level. Similar to Gs, the Gy was influenced positively by the HIUt and HTT interaction (AC).

It has been reported that, high Gs can result in less water leakage due to the presence of highly cross-linked network (Urbonaite et al., 2014; Wu et al., 2019). In the current study, the analysis show that, Gy was positively correlated with Gs ($r = 0.862$, $P < 0.0001$) and WHC ($r = 0.636$, $P < 0.0001$). Similar findings were reported by Zhang et al., (2016), who found that soy protein acid-induced gel network leaks less water after HIU treatment compares to untreated gels; which the authors demonstrated was due to a

reduction in protein particle size and the formation of the dense and uniform gel network when ultrasound was used.



HIUt, high-intensity ultrasound time; HIUp, high-intensity ultrasound power; HTT, heat treatment temperature

Figure 6. 3 Three-dimensional surface plots demonstrating the effects of (A) ultrasound treatment time (min) and ultrasound treatment power at the mid-point level of heat treatment temperature; (B) heat treatment temperature(°C) and ultrasound treatment time (min) at the mid-point level of the ultrasound treatment power; (C) heat treatment temperature (°C) and ultrasound treatment power at the mid-point level ultrasound treatment time on lupin protein gel yield

6.3.2. Optimisation and model validation

The optimal levels of independent factors predicted using the desirability function of the multi-response model are presented in Table 6.5. Gs, WHC and Gy were used as response factors to optimise the ultrasound-assisted acid-induced LPC gel quality. Design-Expert software (V11) was used to generated 1-100 solutions using the *D* criteria given in Table 6.5. The goal was to maximise LPC Gs (maximum importance level 5) through its influence on other responses (WHC and Gy), as discussed earlier in Sections 6.3.1.2 and 6.3.1.3., followed by the WHC and Gy with importance levels of 4 and 3 respectively. Optimal solutions that can maximise responses with highest *D* value ($D = 1$) were selected and are presented in Table 6.5. The predicted independent variables values and their experimental results produced using the optimal solutions (independent factors levels) are presented in Table 6.5. All response values were within 95% of the prediction interval limits, which indicates that the model could predict the optimal location (observation range) of the experimental (actual) response values based on selected desirability criteria.

A one-sample *t* test was used to identify any differences between the predicted and actual responses values. No significant ($P > 0.05$) differences between the predicted and actual values were observed, which implies that the RSM model had appropriate robustness to predict (i.e. to validate) the effects of the HIUt, HIUp and HTT on LPC Gs, WHC and Gy. It may also be able to predict the levels of independent factors to give a wide range of gel quality from liquid-like to solid-like texture, depending on the needs of different food applications by modifying the desirability criteria.

Table 6. 5 Verification of the CCRD model using desirability analysis of numerical prediction criteria for the dependent and independent factors in the optimal solution

Factors	Goal	Goal limits	Importance level	Weight	Optimal solution	Actual value	95% PI range
Independent factors							
A (min)	In range	6-20	-	-	19.8		
B (W/cm^2)	In range	7-50	-	-	~38		
C (°C)	In range	75-95*	-	-	94		
Dependent factors							
Gs (g)	Maximise	36.5-202.5	+++++ (5)	1	~ 229	222.±4.24	189.41-259.65
WHC (%)	Maximise	34.49-82.56	++++ (4)	1	92.3	93.59±1.83	78.39-100
Gy (%)	Maximise	77.8-97.18	+++ (3)	1	100	97.09±2.77	95-100
Desirability					1		

A, Ultrasound treatment time (min); B, ultrasound treatment power (W/cm2); C, thermal treatment temperature during the gelation process (°C); Gs, gel strength; WHC, water-holding capacity; Gy, gel yield; CCRD, central composite design; PI, probability interval.

6.3.3. Scanning electron microscopy

The microstructure of the LPC gels was examined using SEM to investigate the influence of treatment conditions (HIUt, HTUp and HTT) on LPC gel network morphology (microstructure). Figure 6.4 presents typical SEM micrographs of the control (unsonicated), non- optimised (Run 1) and optimised gels. The morphology differed noticeably between the unsonicated showed and sonicated samples. Figure 6.4. shows that the control LPC gel sample lacked gel network formation. Control sample (figure 6.4 A) was unable to form gel network due to the lack of protein unfolding, So that most of the protein precipitated as small granules due to the to heat treatment and lowering pH value to near IP. whereas both ultrasound-treated samples (Run 1 and optimised) show porous protein network formation. In addition, SEM images of the optimised gel showed denser and more uniform microstructure than seen in the Run1 sample. The Run1 sample exhibited more irregular pores, which may be due to differences in HIUt between them. Optimised sample sonicated for 19.8 min (Table 6.5), while Run1 sonicated 13 min (Table 6.2). The results presented in Chapter 4 showed that HIUt for 20 min (38 W/cm^2) reduced LPC particle size significantly ($P < 0.05$) compared with the unsonicated sample and 15 min treatment. Reducing the particle size can produce more uniform microstructure. Similarly, Hu, Fan, et al., (2013) observed that HIU for 20 and 40 min (~105 W/cm^2) created a more uniform gel network compared with the 5 min treatment and unsonicated soybean protein gels. Zhang et al. (2016) reported that HIU reduced soybean protein particle size, which a facilitated more uniform and dense gel network formation compared with unsonicated protein. Ultrasound treatment improved the lupin protein gel network microstructure significantly compared with the unsonicated samples, especially when used with optimal gelation conditions. This improvement in LPC gel network properties had a positive impact on lupin protein gel quality, as indicated by the Gs,WHC and Gy.

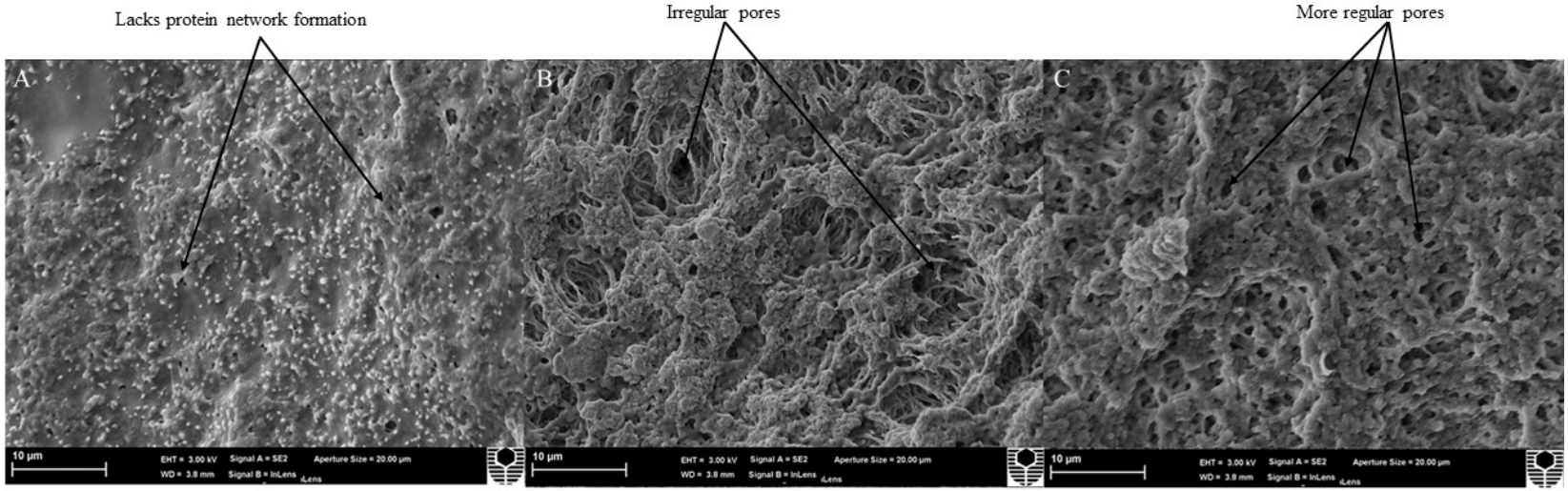


Figure 6. 4 Scanning electron microscopy images of (A) unsonicated (control), (B) Run 1 (mid-range gel quality) and (C) optimised gel sample

6.3.4. Fourier-transform infrared spectroscopy

The effects of HIU on lupin protein secondary structure were investigated using FTIR, and the results are presented in Figure 6.5. To identify changes in protein secondary structures, alterations in the peak positions of amide I (1600-1700 cm^{-1}) were monitored (Schulz & Baranska, 2007). The amide I spectra corresponded mainly to C=O stretching vibration. This was followed by C-N, which represents the secondary structure spectroscopy regions as follows: α-helix (1650-1660 cm^{-1}); β-sheet 1618-1640 cm^{-1}, 1670-1690 cm^{-1} and 1660-1670 cm^{-1}; β-turn 1690-1700 cm^{-1} and; random coil 1640-1650 cm^{-1} (Deng et al., 2020; Shen & Tang, 2012). The FTIR spectra of HIU-treated LPC gels indicated alterations in the secondary structure compared with the unsonicated (control) sample. The control sample (Figure 6.5C) had a large broad peak at ~1631 cm^{-1}, which represents a non-aggregated β-sheet structure (Tidy et al., 2017). Ultrasound-treated samples (Figure 6.5A and B) demonstrated a shift in the β-sheet absorption peak of the amide I to ~1618-1622 cm^{-1}, which implies protein denaturation and aggregation in HIU-treated samples (Figure 6.5A and B). These samples also had an extra peak in the β-sheet spectra region at 1636 cm^{-1}, which was missing in the control sample (Figure 6.5C). In addition, the optimised sample (Figure 6.5A) had a sharp peak in the random coil spectral region (1640-1650 cm^{-1}) compared with a small peak on the unsonicated sample (Figure 6.5C), whereas it was almost missing in medium gel quality sample (Figure 6.5B). The peak positions relating to the α-helix (1650-1660 cm^{-1}) were similar in all samples.

The link between changes in protein structure and improved techno-functionality after HIU treatment are have been reported in the recent literature (Cui et al., 2019; Yang et al., 2017; Zhang et al., 2016). In principles, the effects of HIU depend on the cavitation phenomenon to create high regional heat and high shear pressure in the surface of protein molecules (Mirmoghtadaie et al., 2016). HIU can also cleave water molecules, which releases hydrogen and hydroperoxyl radicals, thus triggering oxidative reactions in protein bonds that lead to structural changes (Miyaji et al., 2017). Rahman, Byanju, Grewell, & Lamsal, (2020) reported that HIU modifies soy protein secondary structure by cavitational forces, which unfold the protein structure and simultaneously trigger protein conformational rearrangement by the oxidative effect of free radicals. These structural

modifications facilitate exposure of hydrophobic groups, which can improve protein crosslinking and aggregation ability and, thus, gelling functionality (Ren et al., 2020).

Interesting findings reported by Wang, Li, Jiang, Qi, & Zhou, (2014) demonstrate that increased β-sheet structure correlates positively with the number of hydrophobic groups on the surface of protein molecules. In studies of gel quality, Cui et al. (2020) reported that, unlike the α-helix structure, the β-sheet has a higher hydration ability, which can produce better gel quality in terms of Gs and WHC. Cui et al. (2019) reported a reduction in α-helix structure proportion of soy, whey and a soy-whey protein isolate blends after ultrasound treatment (300 W for 0, 15, 30 and 45 min), This reduction was positively associated with gel quality properties (Gs, WHC, springiness and cohesiveness). Similarly, Zheng et al. (2019) reported that HIU treatment increased the β-sheet structure aggregation, protein hydrophobicity and Gs of previously aggregated (using heat or alcohol pre-treatments) soy protein isolate with high α-helix content. By contrast, Hu, Wu, et al. (2013) found that HIUp (131-138 W/cm^2) increased α-helix and decreased β-sheet. Chandrapala, Zisu, Palmer, Kentish, and Ashokkumar (2011) highlighted that HIU treatment (31 W for 60 min) resulted in a slight increase in α-helix and decrease β-sheet and surface hydrophobicity of whey protein concentrate, whereas shorter time treatments (5-30 min) showed the opposite effect. In terms of lupin, ultrasound treatment triggered significant changes in lupin protein secondary structure compared with unsonicated (control) gels. These changes reflected the improvement in LPC gel quality attributes after ultrasound treatment.

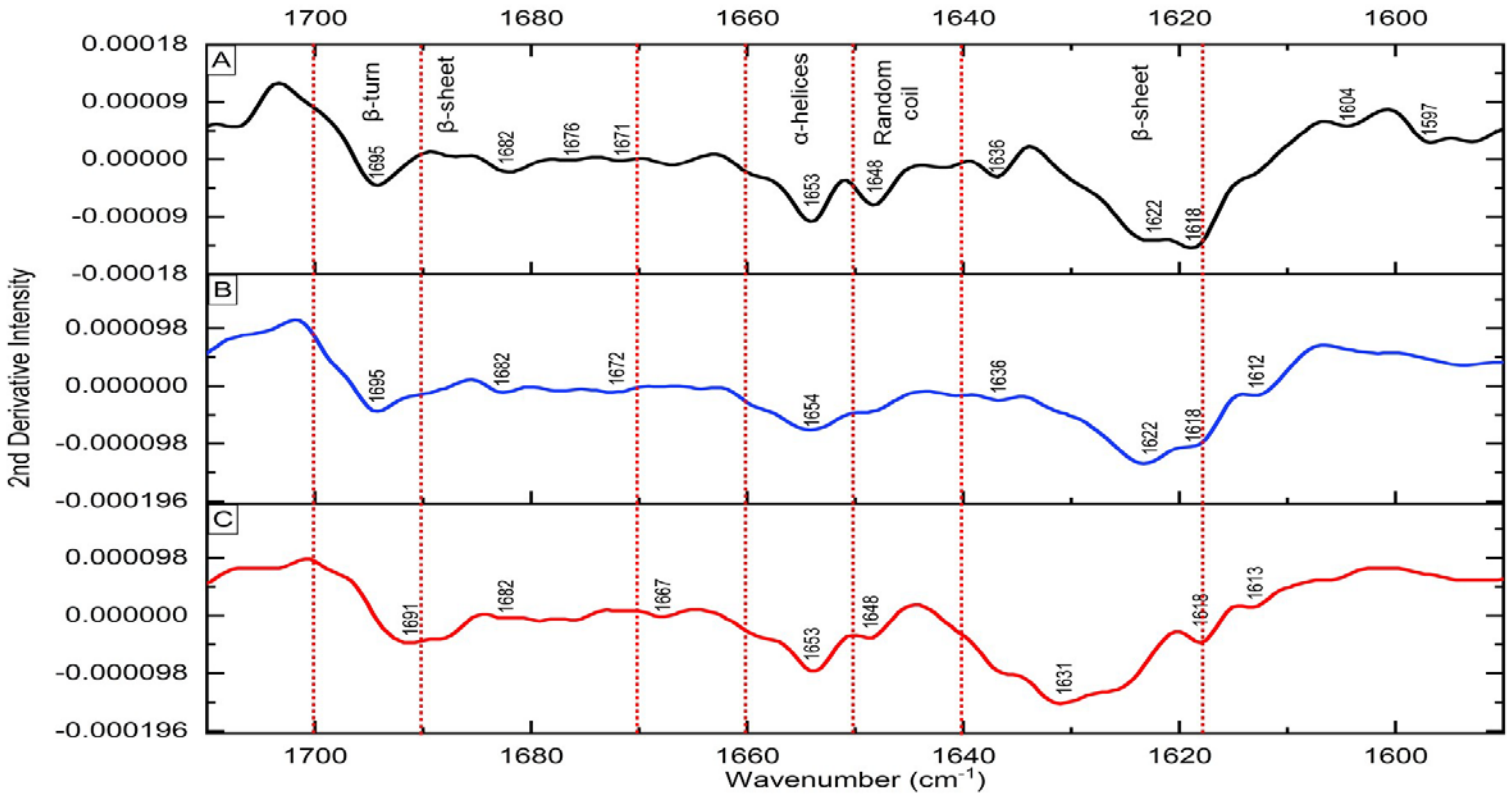


Figure 6. 5 Comparison second-derivative FTIR spectra demonstrating changes in lupin protein secondary structure of (A) (optimised sample), (B) (Run1, representing mid-range gel quality) and (C) unsonicated freeze-dried lupin gel samples (n = 2).

6.3.5. Protein profile

Electrophoretograms images under reducing and non-reducing conditions of unsonicated and sonicated lupin gels are presented in Figure 6.6A and B. The aim of this experiment was to determine whether the combination of HIU and heat treatment would result in the breakdown or formation of protein structures during the gelation process. Figure 6.6A presents the electropherogram under non-reducing conditions (lane 1, control, lane 2, Run 1; lane 3, optimised sample). The ultrasonicated gel samples (lanes 2 and 3) had a similar pattern to those from the unsonicated samples (lane1), but did not have the faint band at ~97 kDa seen in the unsonicated control. In addition, HIU-treated samples have a more intense stain on the top of the stacking gel. This may be due to the formation of large molecular weight complexes, that are unable to enter the gel, due to the combined effect of ultrasound and heat treatment during the gelation process. It has been reported that lupin protein bands in the 90-100 kDa molecular weight range are lipoxygenase (Martínez-Villaluenga et al., 2006; Yoshie-Stark & Wäsche, 2004).

Under reducing conditions (Figure 6.6 B) all samples show very similar protein profiles. The ~97 kDa band (tentatively lipoxygenase) is seen in all samples along with a light staining on the top of each lane. Comparing heat treated sample (control (unsonicated)) to heat treated and sonicated samples (Run1 and optimised sample) analysis after electrophoretic may suggest that the combined effect of ultrasound and heat treatment facilitate the formation of protein-polymer by covalent bonds, resulting in improving lupin protein gelation properties through a verity of interactions(covalent and non-covalent). In contrary to other legumes, the solo effect of heat or HIU treatments on lupin protein has been found to be insufficient to trigger protein aggregate formation through covalent bonds. For instance, Huang, Jia, Zhang, Ma, & Ding, (2020) found that soy protein non-reducing protein electrophoretic pattern showed a new high molecular weight band > 80 kDa after high power ultrasound treatment. In addition, heat-treated pea protein (95 °C for 30 min) resulted in the appearance of high molecular weight protein-polymer on the top of non-reduced electrophoresis gel (Peng et al., 2016). In terms of lupin, the treatment with both HIU and HTT was crucial to unfold lupin protein structure to expose active amino acids groups and enhance crosslinking ability, which result in improving

lupin protein gel quality. However, it is difficult to determine the presence of protein aggregate and type of interactions involved before further exploration.

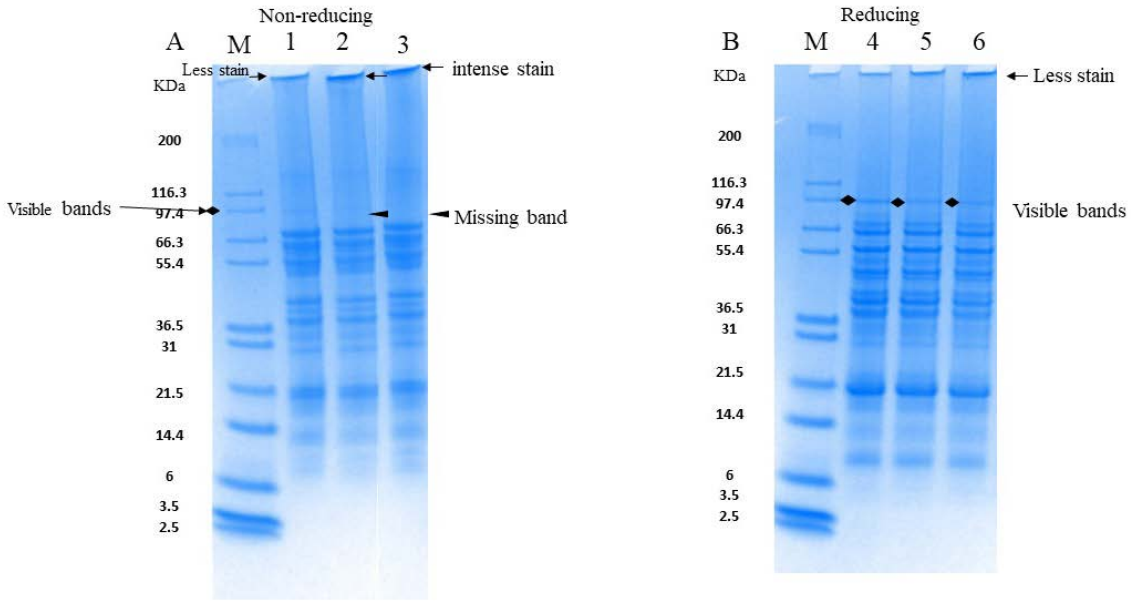


Figure 6. 6 SDS-PAGE of lupin protein concentrate acid-induced gel samples under non-reducing (A) and reducing conditions (B). Lane M, markers; lanes 1 and 4, control unsonicated; lanes 2 and 5, Run 1; lanes 3 and 6, optimised gel sample.

6.4. Conclusion

Lupin protein exhibits very weak gelation properties, which limits it use as food ingredient in food industry. Ultrasound show great potential in improving LPC gel quality (Chapter 4). In this Chapter, A statistical method (central composite rotatable design and desirability technique) was employed to optimise the effect of ultrasound on LPC acid-induced gelation. The model was capable of predicting the combined effects of HIU time, HIU power and HTT on LPC acid-induced gel quality. Optimisation model can be tuned to produce a wide range of gel quality from soft-like to solid-like texture using desirability function. Optimised gel sample show significantly higher Gs, WHC and Gy compared with unsonicated sample (control). Changes in the gel network morphology, protein secondary structure and protein profile in the optimised samples were monitored and compared with other non-optimised samples. The results showed that the independent factors at the optimal levels improved the LPC gel network microstructure and triggered

changes in the lupin protein secondary structure. In addition, the LPC electrophoretic pattern showed changes in molecular weight, which provide evidence of the presence of covalent and non-covalent bonds during lupin gel network formation.

The data presented in this chapter and the tuneable modification process can be considered as a starting point for scaling up lupin protein modification to produce modified lupin protein at the pilot and commercial scales. Modified lupin protein may be used as texturising agent in a wide range of food applications. However, product development studies to examine this new material in a range of food applications will be a crucial part of the development process and first needs to be explored.

CHAPTER 7

General Discussion and Overall Conclusion

7.1.Introduction

It has been predicted that there will be a 30% growth in the world population by 2060 to reach around 11 billion (Goujon, 2019). This increase may outstrip food supply due, in part, to the pressure of climate change on agriculture production (van Meijl et al., 2020). In light of these upcoming challenges, the food industry is in a race to explore novel food resources and technologies to produce alternative food ingredients with the required functional properties (Mirmoghtadaie et al., 2016). Legume seeds are considered to be a valuable plant food source because of their high protein content and sustainable production (Manners et al., 2020). In addition, consumption of legume proteins can improve the health and wellbeing of consumers by preventing or controlling risk factors for diseases such as hypertension (high blood pressure) and type 2 diabetes (Moreno-Valdespino et al., 2020).

7.2. Lupin as an alternative protein source

Lupin is a legume seed containing a high level of protein and dietary fibre, but it is lower in fat and potentially toxic phytoestrogens compared with soybean (Sirtori, Arnoldi, & Johnson, 2005; Berger et al., 2013). Modern commercially grown varieties of *Lupinus angustifolius* are known as Australian sweet lupin (ASL) because of their low content of bitter and potentially toxic alkaloids (Johnson, Clements, Villarino, Coorey, et al., 2017). Lupin protein has a good balance of essential amino acids (Drakos, Doxastakis, & Kiosseoglou, 2007), with high levels of lysine but a deficiency of the sulphur amino acids methionine and cysteine, to meet human nutritional needs (Anna Arnoldi, 2011; Gulewicz et al., 2008). In addition, lupin protein has been reported to have superior digestibility of around 98%, which is similar to soybean digestibility (Cerletti & Duranti, 1979; Chew et al., 2003), which may in part relate to its low level of anti-nutritional factors compared with other legumes (Rodriguez-Ambriz, Martinez-Ayala, Millin, & Davila-Ortiz, 2005). With growing concern about genetically modified food sources, lupin has the advantage

of being considered as a non-genetically modified plant (ISAAA, 2016). In addition, it is one of the cheapest of the high-protein sources and is available at only 25% of the cost of soybean (DPIRD, 2018).

The use of lupin protein as a food ingredient is still not widely recognised within the food industry despite its advantages, and only ~4% of world production is consumed as human food (DPIRD, 2018). This underutilisation is in part attributed to the lack of viscosity and gelation properties of lupin protein, which are required for plant proteins to replace those of animal protein in may food analogues (Johnson et al., 2017). The literature lacks information on lupin protein viscosity and gelation properties, and possible treatments to increase its gel quality. In response, this thesis has presented the findings of investigations of the gelation properties of native lupin protein and the effects of physical protein modification technology on lupin protein gelation. In addition, a statistical model to determine the main effect of independent factors and their interactions on lupin protein concentrate (LPC) acid-induced gel quality attributes has been developed. This model was used and validated to select the optimised combination of factors to produce maximum gel quality. This research has produced several new findings that make a unique contribution to the body of knowledge. The important conclusions that can be drawn from this research are presented in the following sections.

7.2.1. Effect of variety and growing location on the composition of Australian sweet lupin kernels and the composition and techno-functionality of protein concentrates

The aim of this study was to identify whether the variety and growing location can influence lupin protein content, profile and techno-functionality. A series of protein functionality tests were conducted to screen six of most commercially available *L. angustifolius* varieties grown in two locations in Western Australia. Isoelectric precipitate protein concentrate (α- and β-conglutin fraction) were extracted in-house using a lab-scale process. The chemical composition and protein profile of lupin kernel flour and LPC were analysed. Techno-functionality analysis was performed to determine the protein solubility, foaming, emulsifying, viscosity and minimum protein concentration required for gelation (critical concentration) of lupin concentrates. The results showed that, despite the significant effects of variety, variety and their interaction (V x L) on lupin seed

composition and protein concentrate functionality, all genotypes exhibit weak viscosity and gelation properties.

This study concluded that:

1. The effects of variety, location and their V x L influence lupin seed protein and fat content, which may help to direct lupin breeding programs to produce varieties with a higher protein content.
2. The effects of variety and location on protein content and structure were insufficient to influence the initially weak protein functionality such as viscosity and gelation properties.
3. All lupin varieties from the two locations exhibit low viscosity or gelling properties even after prolonged heat treatment (Section 3.3.2.4), which confirms that lupin protein thermal stability persists in all cultivars studied.

In light of the limited variation in lupin protein gelation properties, I chose the highest protein content and viscosity variety (*L. angustifolius Coromup)* for the next phase of the study, in which ultrasound was used to modify lupin protein.

7.2.2. Effects of high-intensity ultrasound on lupin protein concentrate physiochemical and acid induced gelation properties

As discussed in the chapters on the literature review and effects of genotype and environment (Chapters 2 and 3), lupin protein exhibits thermal stability, which limits its viscosity and gel-formation ability after heat treatment. This thermal stability reduces lupin protein molecule unfolding during heat treatment, which inhibits the three-dimensional gel network formation (Berghout, Boom, & van der Goot, 2015; Sirtori, Resta, Brambilla, Zacherl, & Arnoldi, 2010). In Chapter 4, high-intensity ultrasound (HIU) treatment was investigated as a chemical-free and efficient use of time and energy to improve the gelation properties of lupin protein. A comprehensive series of tests was conducted to characterise the effects of HIU treatments (power 11, 17, 38 W/cm^2 and time 0, 2, 10, 15, 20, and 40 min) on LPC physiochemical properties (particle size, solubility, zeta (ζ) potential, thermal properties and protein secondary structure) and gel quality (gel strength (Gs), water-holding capacity (WHC) and gel elastic properties). The study findings and observations are summarised as follows.

A. Protein physiochemical changes in lupin protein after ultrasound treatment

1. Ultrasound treatment successfully triggered changes in lupin protein particle size, solubility, ζpotential, thermal properties and protein secondary structure compared with untreated samples.
2. Ultrasound power at 38 W/cm^2 was the most effective treatment for triggering changes lupin protein physiochemical and gelation properties.
3. Lupin protein particles pass through a series of decreases and increases in size with ultrasound time. This supports the idea that ultrasound can partially modify lupin protein structure.
4. Ultrasound treatment reduced the ζpotential of lupin protein molecules, which suggests that these protein molecules can aggregate more easily because of the reduction in repulsion forces.
5. The protein dissociation and aggregation patterns of lupin protein particles during ultrasound treatment depend mainly on the cleavage or formation non-covalent bonds, which was confirmed by the identical SDS-PAGE profile of unsonicated and sonicated lupin protein.
6. Fourier-transform infrared (FTIR) spectra show that ultrasound treatment changed the lupin protein secondary structure, as shown by lowering β-sheet peak spectrum because of aggregated β-sheet formation.

B. Effects of ultrasound treatment on acid-induced gelation properties of lupin protein concentrate

1. Lupin protein gel quality attributes (Gs, WHC and elastic modulus (G′)) improved significantly after ultrasound treatment, especially at 38 W/cm^2 power for 20 min. The reduction in protein particle size after the 20 min treatment and reduced pH value during gelation, which decreased the repulsive forces, led to a dense gel network formation with higher Gs and WHC.

2. Dynamic small deformation measurement (G″) data showed that ultrasound-treated lupin protein exhibited significantly higher viscoelastic properties compared with unsonicated samples. This may have resulted from changes in lupin protein particle

size, ζpotential, protein secondary structure and thermal properties triggered by ultrasound treatment.

In conclusion, this study showed that sonication triggered changes in lupin protein physiochemical properties and increased gelation properties compared with unsonicated LPC. Given the many factors controlling gelation and lack of information in the literature about the effects of these factors and their interactions on lupin protein acid-induced gelation, an optimisation study using Response surface methodology (RSM) was conducted to investigate the main effects and interaction of factors controlling lupin protein ultrasound-assisted acid-induced gel formation.

7.2.3. Optimising lupin protein concentrate gel quality using response surface methodology

The RSM experimental design has superior efficiency over classical the one factor at a time approach in terms of the ability to identify interactions and the efficiency of time and resources, and is a more robust analysis (Montgomery, 2017). RSM can also predict the optimal levels of significant variables based on the researcher's priority(Vera Candioti et al., 2014). To optimise lupin protein gel quality, the fractional factorial experimental design (Chapter 5) was used to screen five factors (ultrasound time, ultrasound power, heat treatment temperature, heat treatment time and pH) to identify the significant factors influencing lupin protein acid-induced gel quality attributes (Gs, WHC and gel yield (Gy)). Subsequently, the gel quality optimisation experiment was performed using the central composite rotatable design (CCRD) (Chapter 6). The findings can be summarised as follows.

1. Factorial screening identified ultrasound treatment time (min), ultrasound treatment power (W/cm^2) and heat treatment temperature (°C) as the significant factors influencing ultrasound-assisted lupin protein acid-induced gel quality.
2. The CCRD design predicted the optimal levels of independent factors and responses successfully; these were validated by comparing the predicted response values to the experimental results under the suggested optimal conditions (solution).
3. The combination of ultrasound and heat treatment created a synergetic effect on lupin protein unfolding, aggregation and gelation properties, which may reflect increasing

heat sensitivity, as discussed in Chapter 4. At optimal levels of the independent variables, lupin protein gels exhibit superior Gs, WHC and Gy compared with non-optimised and unsonicated variants.

4. FTIR spectra (amide I (1600-1700 cm^{-1})) of optimised lupin gel sample showed a shift in the β-sheet absorption position to ~1618-1622 cm^{-1} wavenumber area compared with ~ 1631 cm^{-1} in the unsonicated sample, which implies that protein denaturation and aggregation occurred in the the sonicated sample.
5. Scanning electron microscopy results demonstrated that optimised lupin protein gel sample clearly formed a fine three-dimensional gel network with homogenised pores size, which was missing on the unsonicated gel sample.

7.3. Potential impact and challenges

Limited research has focused on improving lupin protein techno-functional properties, especially using novel protein modification approaches. The data presented in this thesis are the first on the use of ultrasound technology to improve lupin protein acid-induced gelation properties. Ultrasound is considered to provide clean and sustainable energy because of its low power consumption and fast and chemical-free nature compared with other protein modification techniques (Gharibzahedi & Jafari, 2018; Gharibzahedi & Smith, 2020).

Ultrasound treatment improved lupin protein gelation and viscoelastic properties. The results of these studies provide: (a) fundamental new knowledge on the effects of ultrasound energy on lupin protein physiochemical properties and gel quality; (b) an optimised process for modifying lupin protein and enhance lupin protein gelation properties; (c) a new plant-based protein with valuable texturising properties that may be used in a wide range of food applications. However, up scaling lupin protein may involve several challenges. In the current study batch lab scale process was used to modify lupin protein. Commercial batch scale process potential are limited by the quantity of material that can be process by the ultrasound prob (Chemat et al., 2017). In order to overcome this challenge, a continuous ultrasound treatment needs to be implemented. Continuous ultrasound treatment can treat larger amount of sample by controlling the sample size (flow rate) in the sonication vessel (Soria & Villamiel, 2010; Vilkhu et al., 2008). Further optimisation studies might be

required during upscaling studies using a continuous ultrasound process to identify the best conditions (i.e. ultrasound power, ultrasound time, flow rate, temperature, solids concentration) (Bhargava et al., 2021; Rastogi, 2011) to modify lupin protein structure with gel quality similar to optimised lab scale process the developed on the current study. Producing modified lupin protein with high gel quality on a commercial scale may significantly affect many community sectors. The agriculture industry will benefit through increase demand for lupin protein as a food ingredient, which may increase lupin crop returns compared with its current status as low-price animal feed. The food manufacturing industry will benefit by introducing lupin as a low-cost abundant new source of protein with high protein functionality. The potential increase in lupin crop market value and establishing/expanding food ingredient manufacturing industry will have a positive economic impact. Positive social impacts include benefits to vegetarian and vegan consumers by diversifying their protein sources at affordable price with higher nutritional value compared with other sources such as soybean. Lupin farmers and their families will benefit through an increase in lupin seed price point and reduced-price fluctuation. Positive environmental impacts include encouraging lupin farmers to regrow lupin, which will increase farming system sustainability and soil health through its nitrogen-fixing ability, which will reduce the use of synthetic nitrogen fertilisers in cereal production.

7.4.Limitations

The studies included in this thesis have several limitations as follows.

7.4.1. Effects of genotype and growing environment on lupin kernels composition and protein functionality

This study was unable to investigate the effects of season (growing years) as we have lupin seeds harvested in 2015. The current study focused on investigating the effect of two growing locations (environments), which have minimal environmental differences (section 3.2.1) (Atnaf, Wegary, Dagne, & Tesfaye, 2018; Clements, Dracup, 2002). The use of lupin seed grown in low rainfall areas such as Agriculture zone 7 (311.6 mm) (Merredin, Western Australia) (BOM, 2018) compare to Wongan Hills (medium rainfall 388.4 mm, may represent better

environmental diversity to investigate their influence on lupin seed composition and protein techno-functional properties.

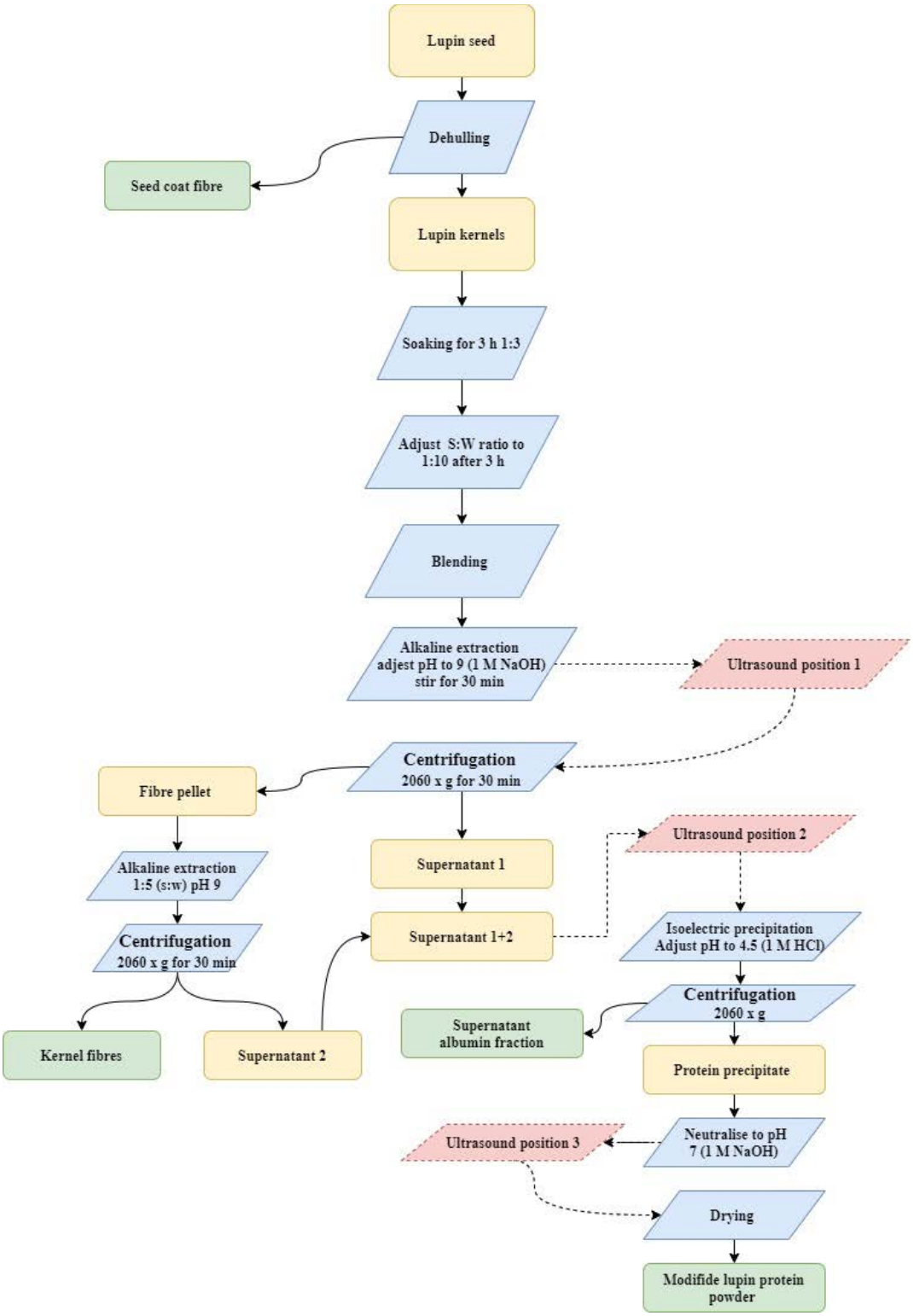
7.4.2. The effects of ultrasound treatment on lupin protein physicochemical properties and gel quality

1. A bench scale, in-line ultrasound treatment would be more appropriate for designing a commercial process to modify lupin protein using ultrasound.
2. The effects of lupin genotype on ultrasound-treated protein gel quality still need to be investigated.

7.5.Future studies

Some possible ideas for future studies are as follows:

1. Upscale the process based on the fundamental knowledge gained through the research in this thesis, especially using in-line ultrasound treatment.
2. Reduce the number of processing steps. The method used here has two drying steps, which may be reduced to only one by changing the location of the ultrasound treatment. The ultrasound treatment can be applied at various points in the process (Figure 7.1). Incorporation of ultrasound treatment during lupin protein extraction may help to improve the protein yield as well as gel quality.
3. Increase protein concentration of the products to produce an isolate (protein content > 90%) by ultrafiltration. The total protein extract (first stage of the process) can be concentrated by ultrafiltration to remove sugars and salts but retain all protein. The concentrated purified extract can then be ultrasound treated and dried (Figure 7.2).
4. Examine the γ-conglutin fraction gelation properties and the influence of sonication on its protein structure and functionality.
5. Developing a lab-scale process to separate the α and β fractions to determine their physiochemical changes and gel quality after ultrasound treatment.
6. Investigate the oxidative load effect of ultrasound treatment on lupin protein structural changes and fat rancidity.
7. Examine the performance of this new material (modified lupin protein) in a range of food systems such as salt-induced gelation, emulsion gels and gluten-free food matrix.



S:W, Sample to water ratio.

Figure 7.1. Possible locations of ultrasound treatments during lupin protein extraction process

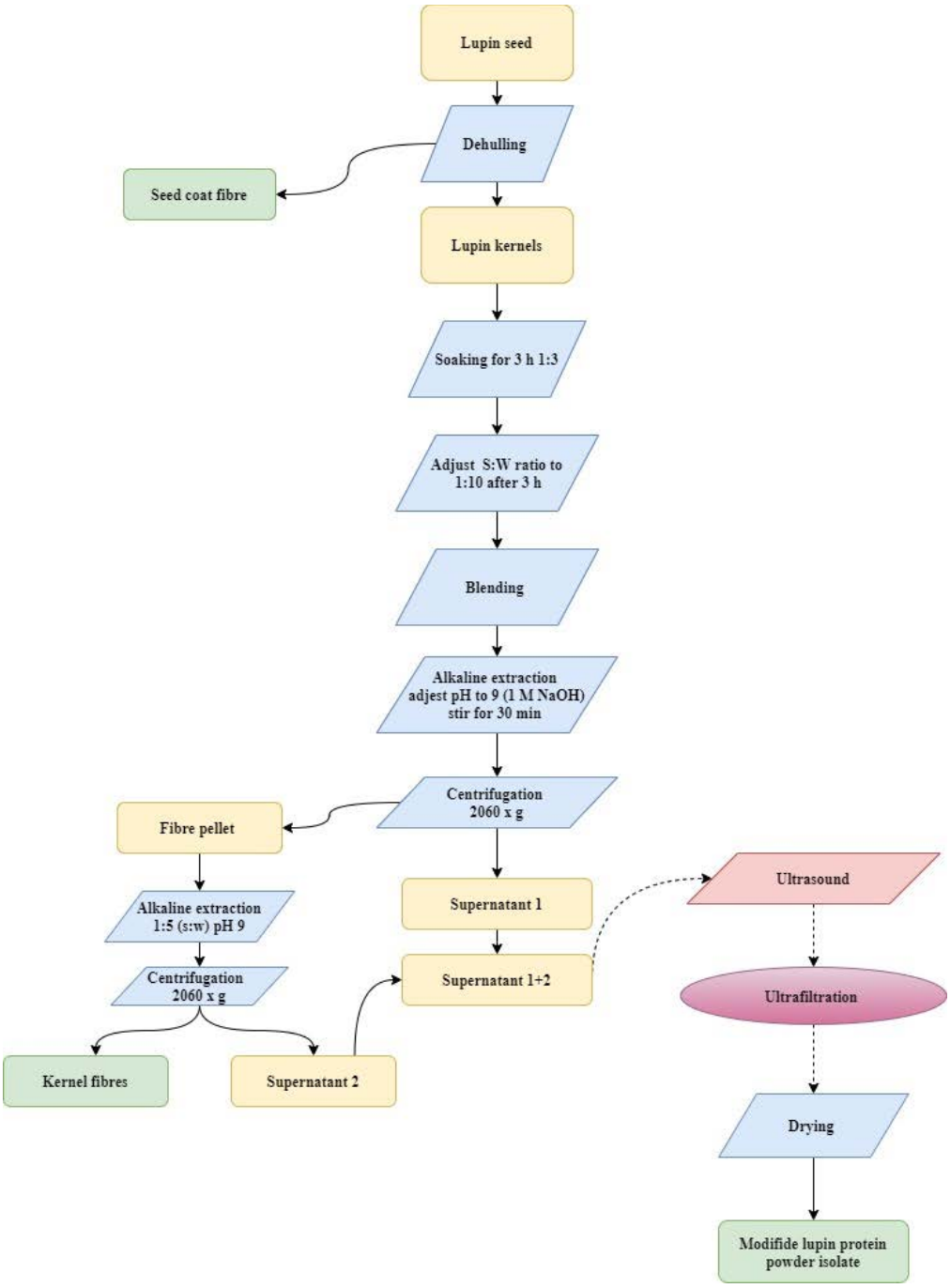


Figure 7.2 Possible lupin protein extraction and modification by including ultrasound and ultrafiltration steps.

7.6 Conclusion

Protein is a food ingredient in high demand because of its essential role in food structure and human nutrition. Plant protein is considered as an important source of protein given the accumulating evidence of its positive metabolic effects on reducing risk factors for some diseases and production sustainability compared with animal sources. However, protein functionality such as its gelling ability limits the use of plant protein in the food industry. Current research suggests that ultrasound-treated LPC can be used as an effective gelling agent in a range of novel food applications. The results of the work in this thesis highlight the impact of ultrasound on lupin physiochemical properties, protein gel quality and gelation mechanism. The research presented here has identified the optimal conditions for producing higher values for Gs, WHC and Gy of lupin protein gels. This new knowledge may provide a promising commercial approach for manufacturing lupin protein and may encourage the food industry to incorporate lupin protein as a gelling agent in a range of food products. This new material may hold great potential as an affordable healthier plant protein source with excellent gelation properties, which can diversify the plant protein food manufacturing market. However, the results presented in the current study focused on limited sets of conditions and possibilities, and further studies are needed to continue the innovation and refinement journey.

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APPENDICES

Appendix A. ANOVA of fractional factorial screening models (Chapter 5) of each response factor and diagnostics; Half-Normal Probability plot, and Plot of residuals versus predicted response values.

Table A.1 ANOVA for selected factorial model for LPC acid-induced gel strength

Source	Sum of Squares	df	Mean Square	F-value	*P* value	
Model	61945.92	5	12389.18	46.32	< 0.0001	Significant
A	21658.03	1	21658.03	80.97	< 0.0001	
B	2862.25	1	2862.25	10.70	0.0084	
C	26950.69	1	26950.69	100.76	< 0.0001	
AC	8494.69	1	8494.69	31.76	0.0002	
BC	1980.25	1	1980.25	7.40	0.0215	
Residual	2674.72	10	267.47			
Cor Total	64620.64	15				

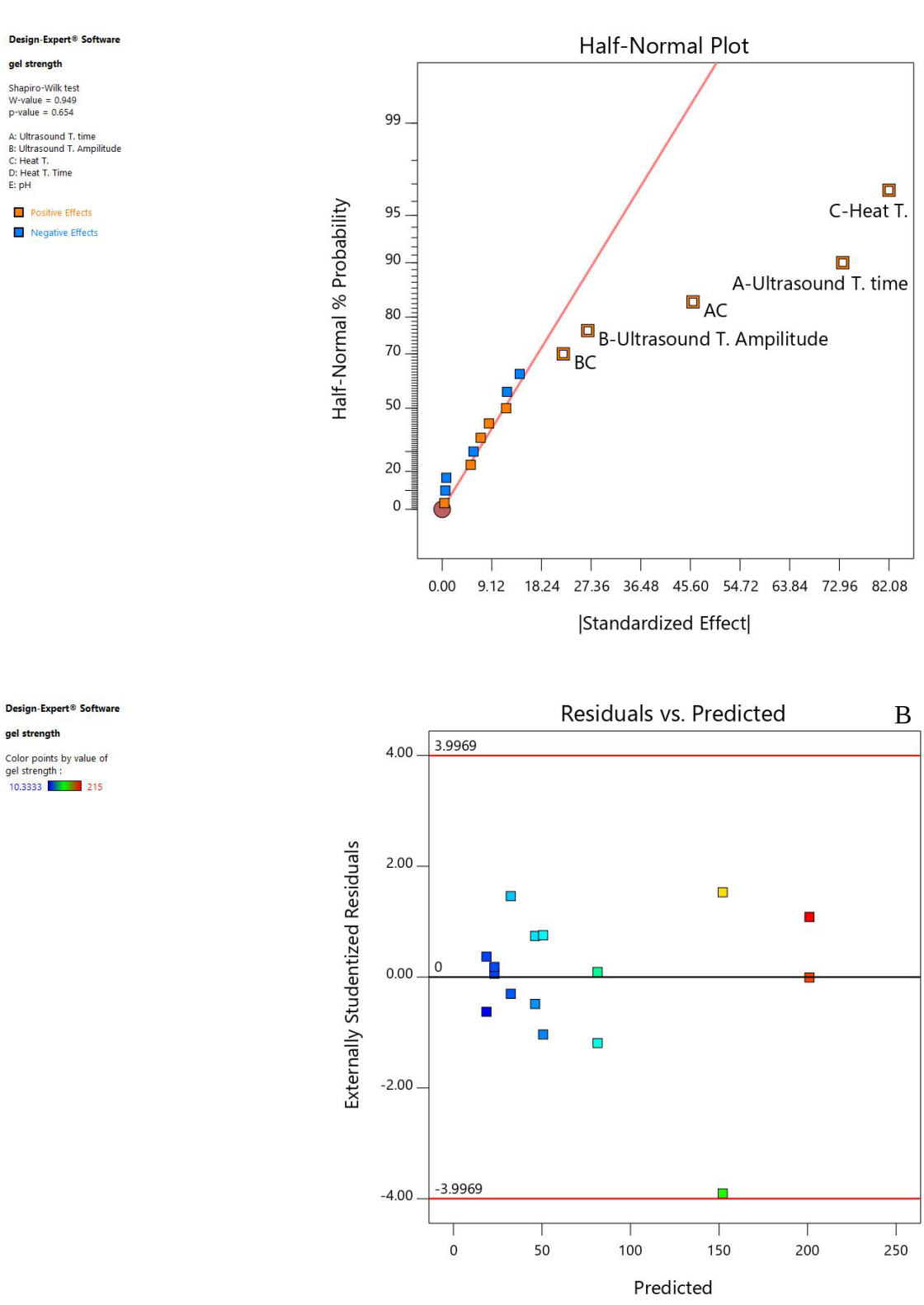


Figure A.1 Diagnostics graphs of the selected model for LPC acid-induced gel strength, (A) Half-Normal Probability plot of effects, and (B) Plot of residuals versus predicted response values

Table A.2 ANOVA for selected factorial model for LPC acid-induced gel WHC

Source	Sum of Squares	df	Mean Square	F-value	*P* value	
Model	2551.99	3	850.66	14.89	0.0002	Significant
A	763.28	1	763.28	13.36	0.0033	
B	386.02	1	386.02	6.76	0.0232	
C	1402.69	1	1402.69	24.56	0.0003	
Residual	685.48	12	57.12			
Cor Total	3237.47	15				

A, Ultrasound treatment time(min); B, Ultrasound treatment power (W/ cm2); C, Heat treatment temperature (℃); WHC, water holding capacity

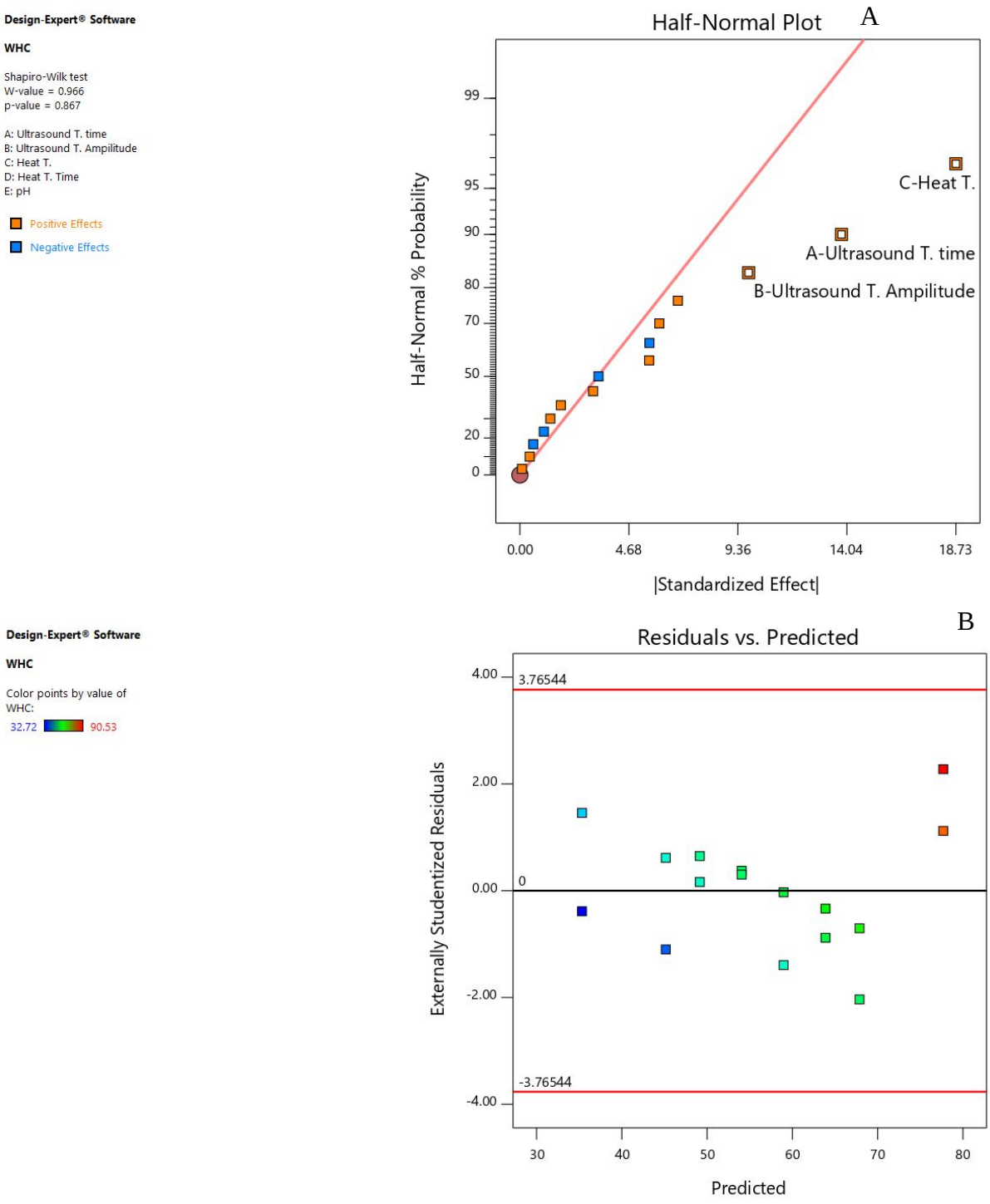


Figure A. 2 Diagnostics graphs of the selected model for LPC acid-induced WHC, (A) Half-Normal Probability plot of effects, and (B) Plot of residuals versus predicted response values

Table A.3 ANOVA for selected factorial model for LPC acid-induced gel yield

Source	Sum of Squares	df	Mean Square	F-value	*P* value	
Model	256.50	7	36.64	24.53	< 0.0001	significant
A	40.10	1	40.10	26.85	0.0008	
BC	21.17	1	21.17	14.18	0.0055	
BD	46.62	1	46.62	31.21	0.0005	
BE	15.52	1	15.52	10.39	0.0122	
CD	45.24	1	45.24	30.29	0.0006	
CE	57.87	1	57.87	38.75	0.0003	
DE	29.99	1	29.99	20.08	0.0021	
Residual	11.95	8	1.49			
Cor Total	268.45	15				

A, Ultrasound treatment time(min); B, Ultrasound treatment power (W/ cm2); C, Heat treatment temperature (ºC**).**

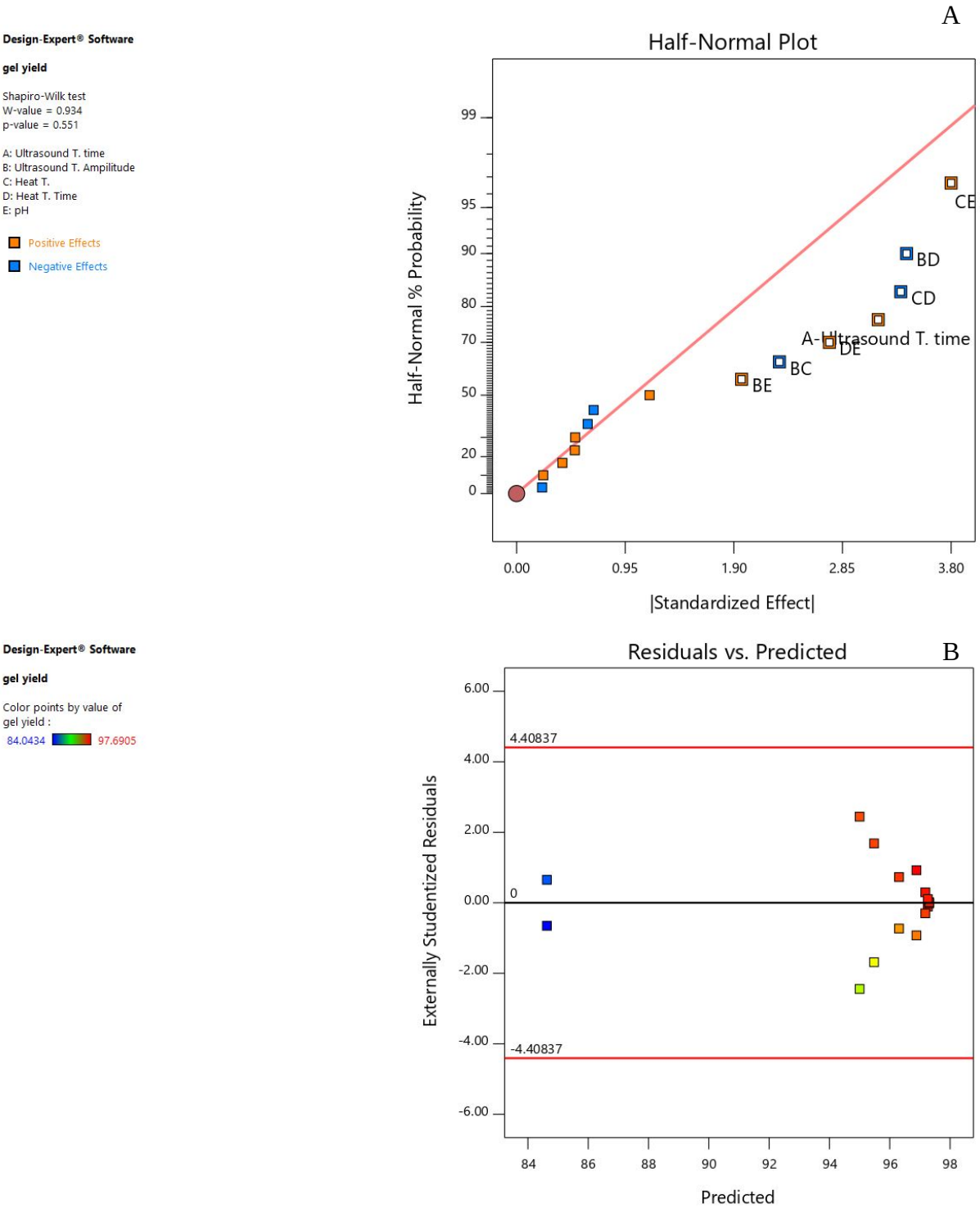
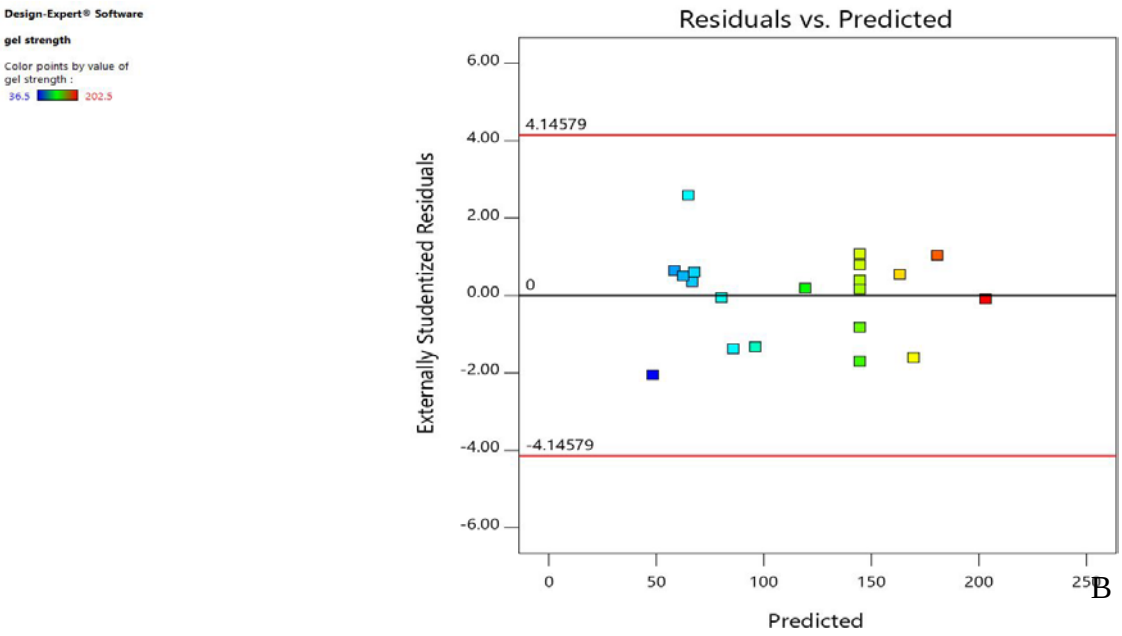


Figure A. 3 Diagnostics graphs of the selected model for LPC acid-induced gel yield,(A) Half-Normal Probability plot of effects, and (B) Plot of residuals versus predicted response values

Appendix B. Central composite rotatable design (CCRD) (Chapter 6) and diagnostics of each response: Half-Normal Probability plot of effects and Plot of residuals versus predicted response values)

A



B

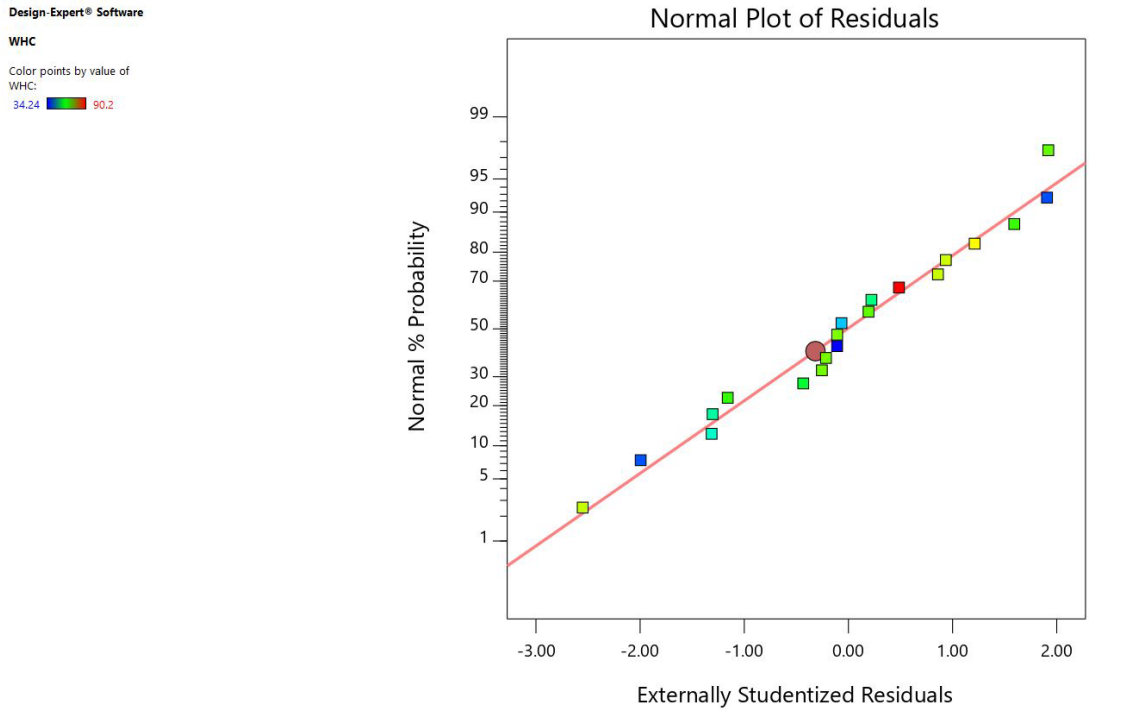


Figure B.1 Diagnostics graphs of the selected model for LPC acid-induced gel strength: (A) normal probability plot and (B) residuals vs. predicted plot

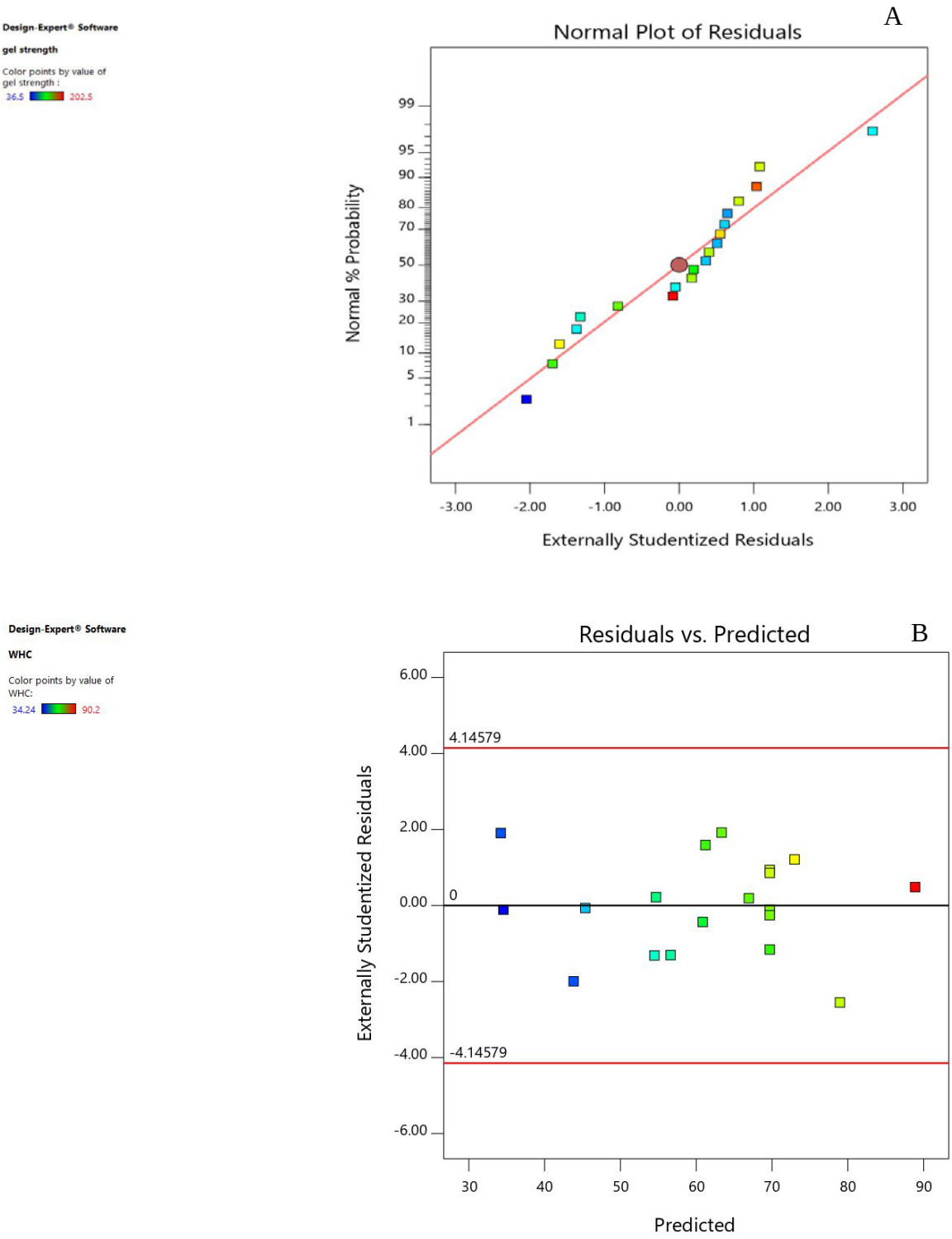


Figure B.2 Diagnostics graphs of the selected model for LPC acid-induced gel WHC: (A) normal probability plot and (B) residuals vs. predicted plot

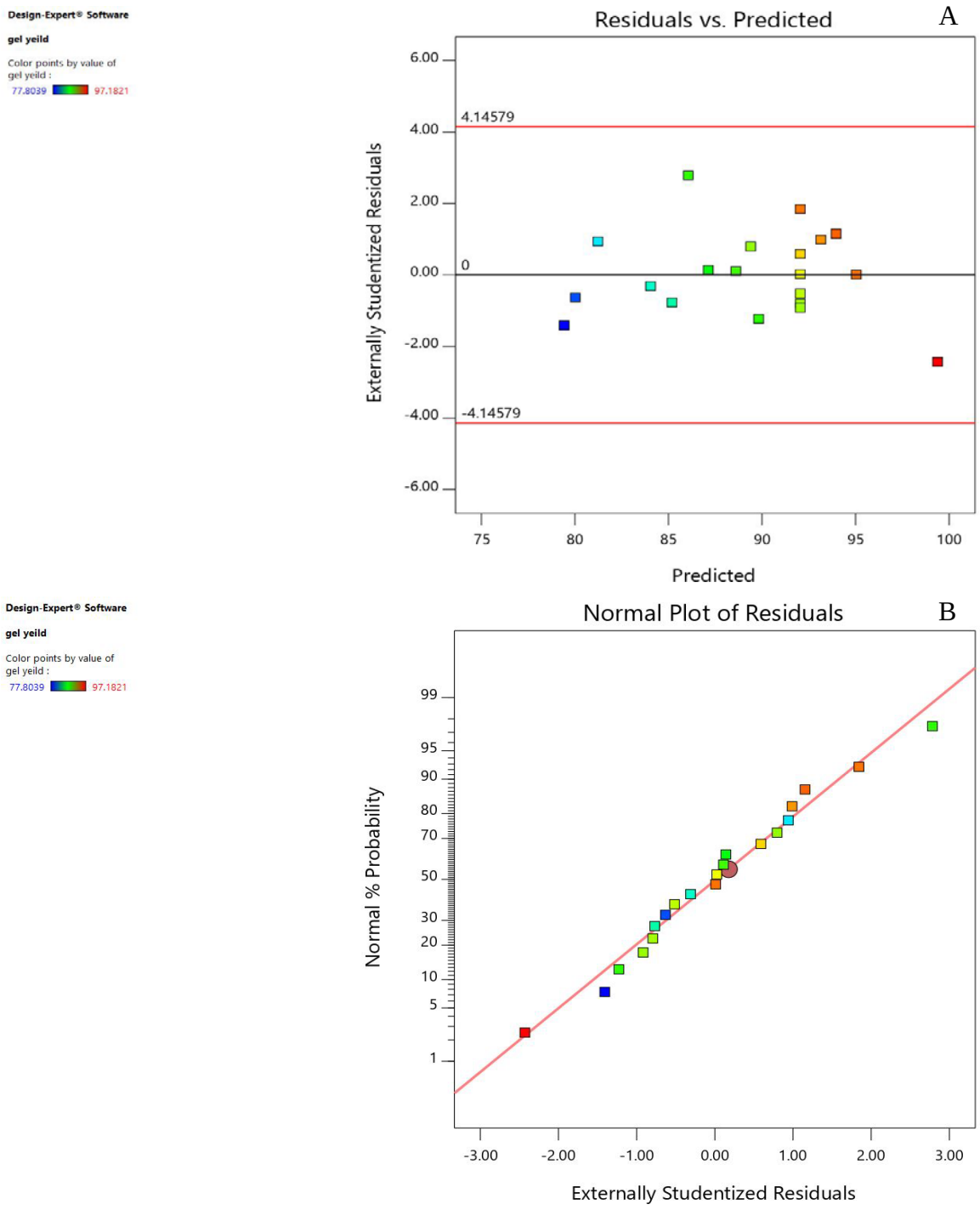


Figure B.3 Diagnostics graphs of the selected model for LPC acid-induced gel yield: (A) normal probability plot and (B) residuals vs. predicted plot

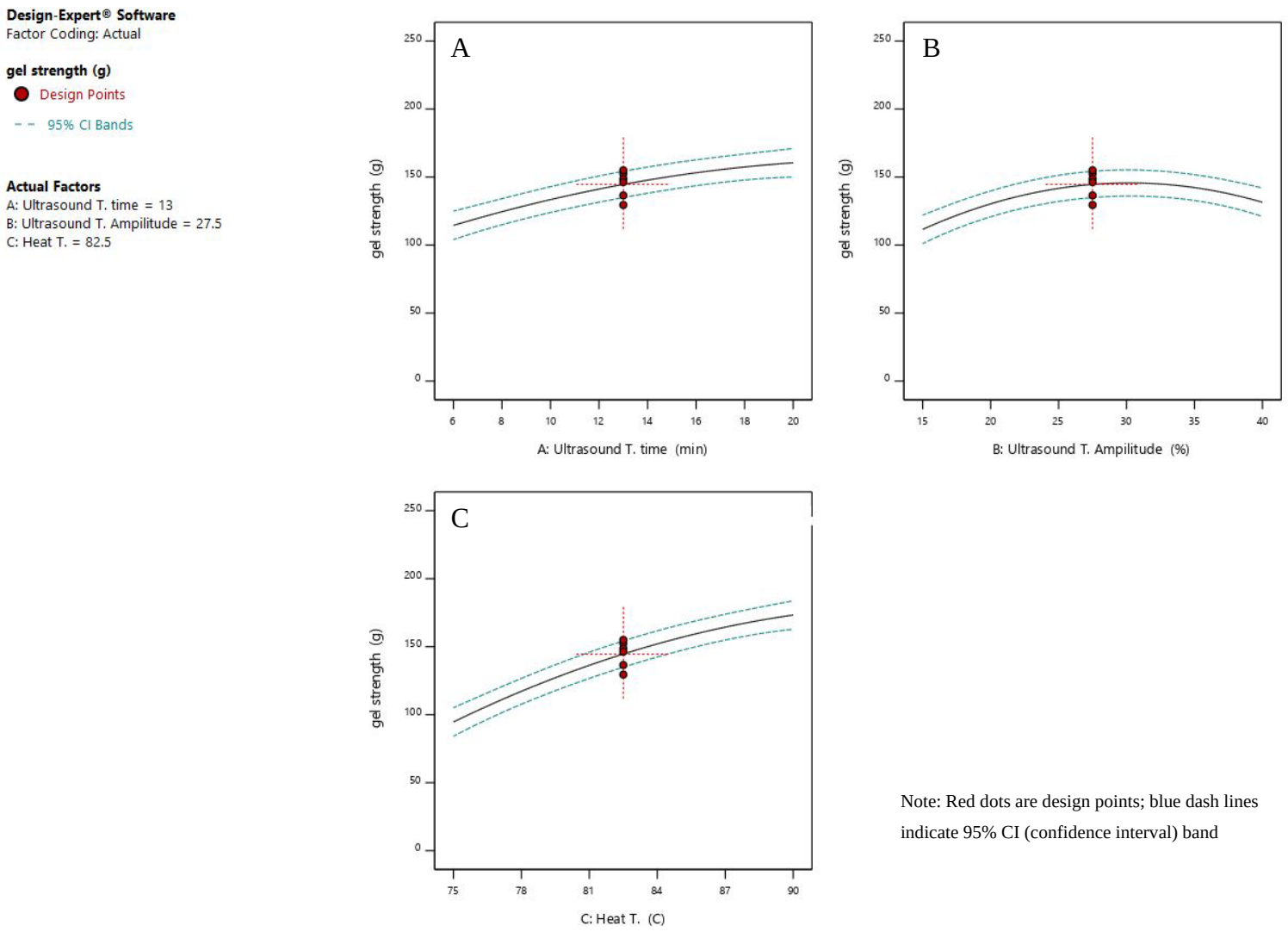
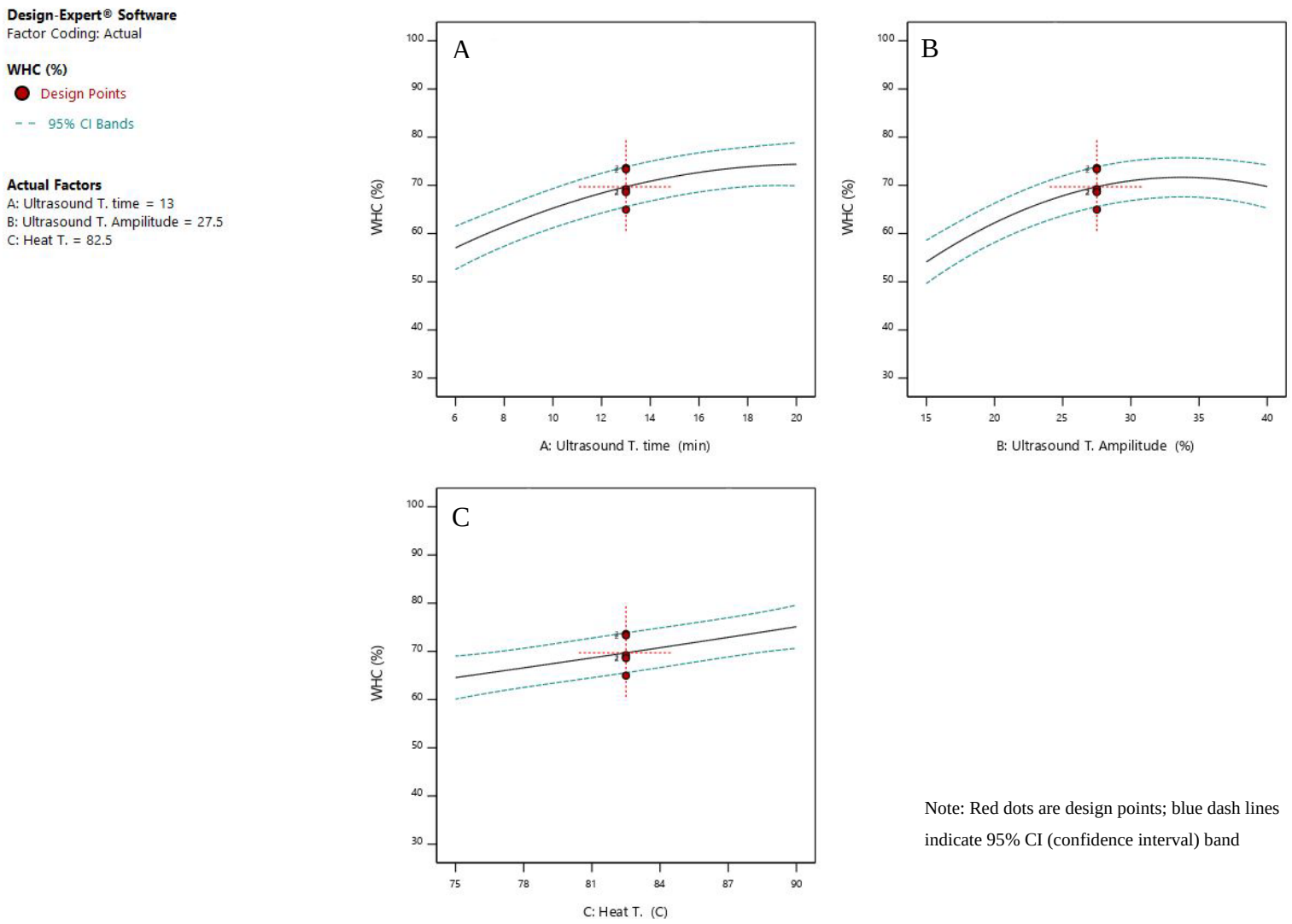


Figure B.4 One-factor plots of LPC acid-induced gel strength as a function of (A) ultrasound treatment time (min), (B) ultrasound treatment power (W/ cm^2) and (C) Heat treatment temperature (°C)



Note: Red dots are design points; blue dash lines indicate 95% CI (confidence interval) band

Figure B.5 One-factor plots of LPC acid-induced gel WHC as a function of (A) ultrasound treatment time (min), (B) ultrasound treatment power (W/ cm^2) and (C) Heat treatment temperature (°C)

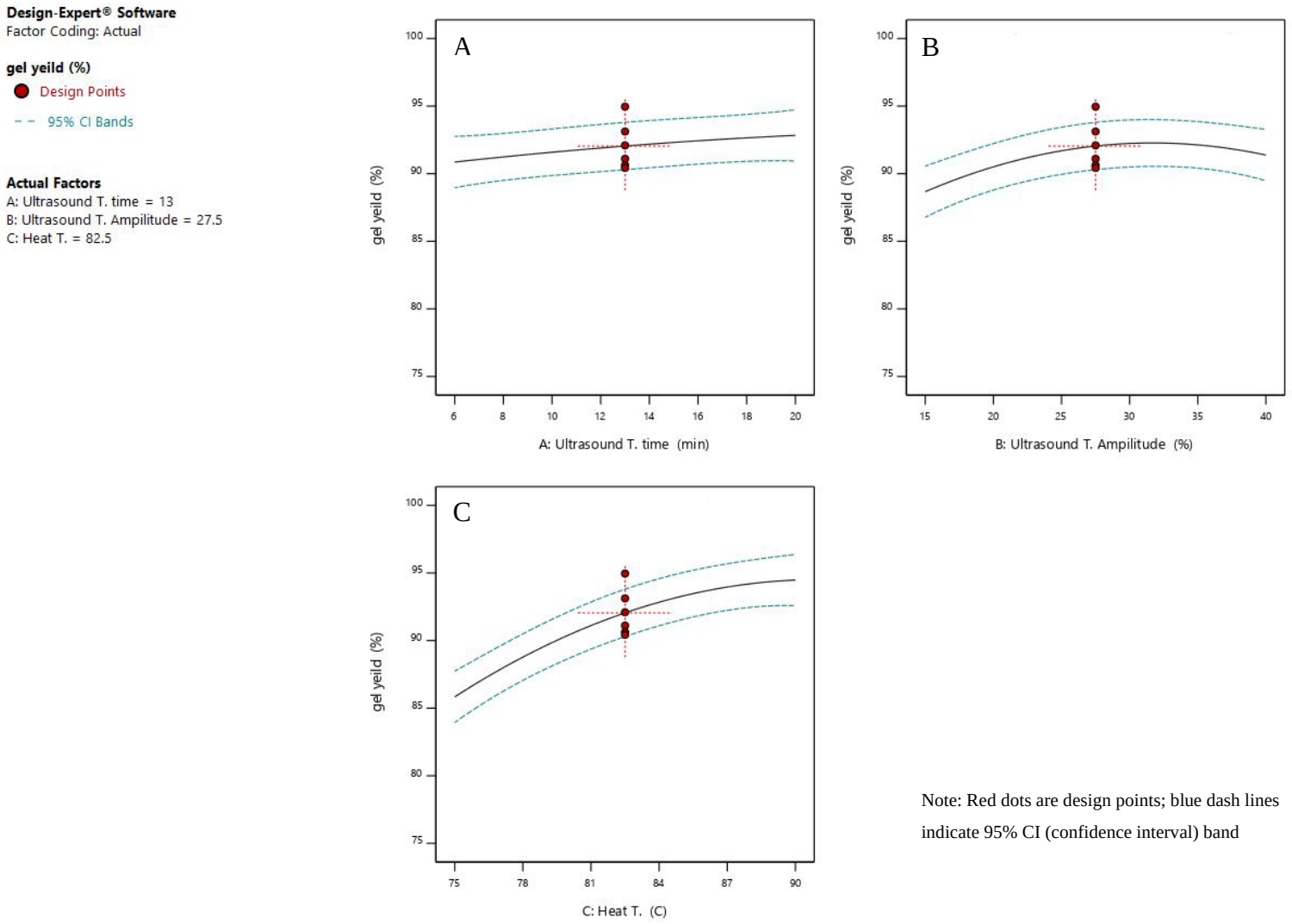
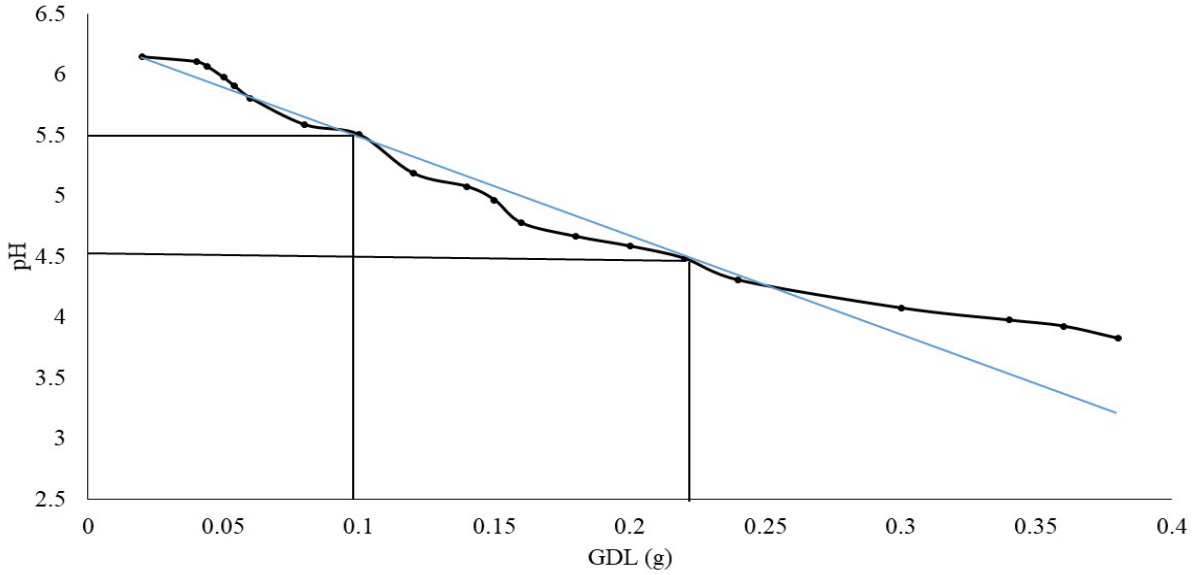


Figure B.6 One-factor plots of LPC acid-induced gel yield as a function of(A) ultrasound treatment time (min), (B) ultrasound treatment power (W/ cm2) and (C) Heat treatment temperature (°C)

Appendix C.



GDL, Glucono-δ-lactone.

Figure C.1 Identifying pH values after adding GDL to acidifying LPC solution to trigger cold-set gelation

Appendix D.

Statement of Contribution of Others

To Whom It May Concern，

I, Hayder A. Al-Ali, made the major contribution to the study design, literature search, data acquisition and interpretation, discussion, writing and editing of the manuscript "Technological strategies to improve gelation properties of legume proteins with the focus on lupin, published at *Innovative Food Science and Emerging Technologies* journal.

Hayder A. Al-Ali Signed:

I, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

Stuart K. Johnson	Signed:
Mark Hackett	Signed:
Mohammad Gulzar	Signed:
Emmanuel Karakyriakos	Signed:
Umar Shah	Signed:

To Whom It May Concern，

I, Hayder A. Al-Ali, made the major contribution to the study design, sample preparation, data acquisition and interpretation, discussion, writing and editing of the manuscript: "Effect of Variety and Growing Location on the Composition of Australian Sweet Lupin Kernels and the Composition and Techno-functionality of Protein Concentrates" that will be submitted to a journal for potential publication.

Hayder A. Al-Ali Signed:

I, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

Stuart K. Johnson	Signed:
Mark Hackett	Signed:
Mohammad Gulzar	Signed:
Emmanuel Karakyriakos	Signed:

To Whom It May Concern，

I, Hayder A. Al-Ali, made the major contribution to the study design, sample preparation, data acquisition and interpretation, discussion, writing and editing of the manuscript, "Effect of high intensity ultrasound on the physiochemical and gelation properties of acid-induced gelation of lupin protein concentrate" that will be submitted to a journal for potential publication.

Hayder A. Al-Ali Signed:

I, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

Stuart K. Johnson	Signed:
Mark Hackett	Signed:
Mohammad Gulzar	Signed:
Emmanuel Karakyriakos	Signed:

To Whom It May Concern，

I, Hayder A. Al-Ali, contributed the study design, sample preparation, data acquisition and

interpretation, discussion, writing and editing of the manuscript, “Identification of significant factors controlling lupin protein concentrate acid-induced gel quality” that will be submitted to a journal for potential publication

Hayder A. Al-Ali Signed:

I, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

Stuart K. Johnson	Signed:
Mark Hackett	Signed:
Mohammad Gulzar	Signed:
Emmanuel Karakyriakos	Signed:

To Whom It May Concern，

I, Hayder A. Al-Ali, made the major contribution to the study design, sample preparation, data acquisition and interpretation, discussion, writing & editing of the manuscript, “Optimisation of ultrasound treated lupin protein concentrate acid-induced gel quality using response surface methodology” that will be submitted to a journal for potential publication.

Hayder A. Al-Ali Signed:

I, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

Stuart K. Johnson	Signed:
Mark Hackett	Signed:
Mohammad Gulzar	Signed:
Emmanuel Karakyriakos	Signed: