School of Public Health

Maximising the Nutritional and Sensory Quality of Lupin Bread Made Using Western Australian Bakers Flour

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To the best of my knowledge and belief this thesis 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

Australian sweet lupin (*Lupinus angustifolius*) (ASL) is a legume undervalued and underutilised as a food ingredient despite evidence from human clinical studies of its potential to lower risk factors for chronic diseases attributed to its high protein and dietary fibre content. However, due to these same proteins and dietary fibre, quality problems associated with lupin flour addition to wheat bread can occur. There is a lack of information on the effects of ASL variety and formulation and process parameters on ASL-wheat bread quality. The objective of this thesis was to optimise the formulation and processing of ASL-wheat bread made using Western Australian bakers flour to maximise lupin flour addition (for maximum nutritional and health benefits) whilst maintaining high consumer acceptability.

The nutritional, chemical and physical properties of ASL flours and ASL-wheat breads (with 20% ASL flour) quality were evaluated using six ASL varieties, *Belara*, *Coromup*, *Gungurru*, *Jenabillup*, *Mandelup* and *Tanjil*, and compared to refined wheat flour and breads. There was a significant effect of ASL variety on the following ASL-flour attributes: protein and fat contents; total phenolics and antioxidant activity; carotenoids content; and particle size distribution. ASL-wheat breads significantly differed in dietary fibre and fat contents, total available carbohydrates, total phenolics and antioxidant activity, protein digestibility corrected amino acid score (PDCAAS), moisture loss, crumb specific volume, crumb characteristics and texture properties. Results suggest that ASL varieties *Belara*, *Coromup* and *Tanjil* can be incorporated into wheat flour for bread manufacturing with desirable nutritional, chemical and physical properties. Based on these results, *Coromup* variety was used in the subsequent formulation and process modelling and optimisation study.

For the optimisation of ASL (*Coromup* var.) -wheat bread formulation and process, factorial design for screening for important process parameters, and response surface methodology (RSM) for modelling of the effects of formulation and important process parameters were used. Verification experiments were conducted to test

accuracy of the generated RSM models. In factorial screening, the effects of: sponge proofing time (min); sponge and dough mixing time (min); final proofing time (min); final proofing temperature (^{o}C) and; baking time (min) on ASL -wheat bread physical attributes (i.e. crumb specific volume, crumb characteristics and instrumental texture) were investigated. Factorial models show that sponge and dough mixing and baking times were the two most significant process parameters affecting the bread quality.

The effect of sponge and dough mixing and baking times in combination with the formulation parameters of level of ASL flour incorporation (g/100 g ASL-wheat composite flour); ASL flour volume weighted mean particle size (µm) ; and level of water incorporation (g/100 g ASL-wheat composite flour) on crumb specific volume, instrumental texture attributes and consumer acceptability of the breads. Verification experiments were used to validate the accuracy of the predictive models. Statistical optimisation using the RSM models indicated that ASL-wheat breads containing 21.4 to 27.9 % ASL flour, with particle size of 415 to 687 µm, 59.5 to 71.0 % water, mixed for 4.0 to 5.5 min and baked for 10 to 11 min would have acceptable physical and sensory properties at maximum ASL flour addition. Verification experiments revealed that the generated RSM models were able to accurately predict the responses.

This thesis demonstrated that ASL variety had a significant effect on the nutritional, chemical and physical properties of ASL flour and ASL-wheat breads. *Coromup* results in acceptable bread when substituted for WA bakers flour. These findings are important for the lupin industry to move towards segregation of lupin varieties for specific end use e.g. for bread making to ensure consistent and high quality of lupin-wheat breads and may help direct growers towards planting specific lupin varieties more suited to bread making for higher financial returns on their crop. The use of factorial screening and RSM proved effective in identifying levels of key formulation and process variables to maximise the level of ASL flour addition to wheat bread whilst maintaining good quality. These formulation and process specifications will assist millers and bakers to produce quality lupin-wheat bread that may benefit the health of consumers.

Future research is required on the scale-up of the optimised formulation and process identified in the present study. Optimisation studies are recommended to further increase the level of ASL incorporation and quality of ASL bread for example through gluten addition, and dough sheeting techniques. In addition, research on the development of gluten-free ASL bread is warranted to meet this expanding market.

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PUBLICATIONS

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CONFERENCE PRESENTATIONS

Villarino, C.B.J., Jayasena, V., Coorey, R., Bell, S. and Johnson, S.K. (2012). Nutritional and technological functionality of lupin flour addition to bread - benefits and challenges. 14th International Association of Cereal Science and Technology (ICC) Bread and Cereal Conference. Beijing, China, August 6-9. *Poster presentation.*

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AWARDS/RECOGNITION

Curtin University winner and Trans-Tasman semi-finalist - 2013 Three Minute Thesis Competition "Designing high-fibre, high-protein bread using lupin- a double-edged sword"

Finalist- 2014 Curtin Commercial Innovation Award "An innovative tool to optimise the manufacturing process of lupin-enriched bread with good consumer acceptability"

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ABBREVIATIONS

ANOVA	Analysis of variance

AOAC	Association of Analytical Chemists
ASL	Australian sweet lupin
BCIP/NBT	5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium
BMI	Body mass index
BU	Brabender Unit
CGFI	Centre for Grain Foods Innovation
CHAPS	(3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
CI	Confidence interval
CSIRO	Commonwealth Scientific and Industrial Research Organisation
CSV	Crumb specific volume
CV	Coefficient of variation
CVD	Cardiovascular disease
DAFWA	Department of Agriculture and Food Western Australia
db	Dry basis
DHA	Department of Health and Ageing
DMRT	Duncan's Multiple Range Test
DPPH	2,2-diphenyl-1-picrylhydrazyl
DTNB	5,5-dithio-bis (2-nitrobenzoic acid)
DTT	1, 4-dithiothreitol
EC	Effect contribution
FAO	Food Agricultural Organization
FID	Flame ionization detector
FSANZ	Food Standards Australia and New Zealand
GAE	Gallic acid equivalent
GCO	Gas chromatography-olfactometry
GI	Glycaemic index
GM	Genetically modified
GRDC	Grains Research and Development Corporation
HDL-C	High density lipoprotein cholesterol
HPLC	High performance liquid chromatography
IAUC	Incremental areas under curves

IVPD	In vitro protein digestibility
kDA	Kilodalton
LDL-C	Low density lipoprotein cholesterol
LKF	Lupin kernel fibre
LPI	Lupin protein isolate
MAFF	Ministry of Agriculture, Fisheries and Food
NHFA	National Heart Foundation of Australia
NHMRC	National Health and Medical Research Council
NSP	Non starch polysaccharide
NTSB	2-nitro 5-tho sulfo benzoic acid
PDCAAS	Protein digestibility corrected amino acid score
PI	Prediction interval
PUFA	Polyunsaturated fatty acids
QA	Quinolizidine alkaloids
RDI	Recommended dietary intake
RFO	Raffinose family of oligosaccharide
RH	Relative humidity
SCFA	Short chain fatty acid
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFA	Saturated fatty acid
TBST	Tris-buffered saline and Tween-20
TC	Total cholesterol
TDF	Total dietary fibre
TE	Trolox equivalent
TG	Triglycerides
TPA	Texture profile analysis
TPC	Total phenolic content
UK	United Kingdom
US	United States
USDA	United States Department of Agriculture
UV	Ultraviolet
WA	Western Australia

WBC	Water binding capacity
WHO	World Health Organization

CHAPTER ONE

GENERAL INTRODUCTION

1.1. BACKGROUND

Lupin is a legume of which two-thirds of the global supply is produced in Australia (FAO-STAT, 2014). It is a major rotation crop in Australia and parts of Europe for sustainable farming systems such as wheat and other cereals, due to its nitrogen fixation ability (French et al., 2008; GL-PRO 2005,). However, it still remains underutilized and undervalued as a food source (Jayasena et al., 2010b) despite its unique nutritional (combined high protein and dietary fibre, moderate fat and low total available carbohydrates) and health benefits. Lupin flour and its fractions (i.e. protein isolates, protein concentrates and dietary fibre) have been investigated as substitutes for wheat flour in bakery products, instant noodles, and pasta. These lupin fractions have also been used in meat products, tofu, and Asian fermented foods.

Bread is commonly made from cereals such as wheat and rye along with water, yeast and salt. The use of refined wheat flour in bread is due to its gluten-forming proteins, gliadins and glutenins (Barak et al., 2013), which when mixed with water form a matrix providing the elastic dough structure needed for the development of desirable bread volume and texture. However, the consumption of refined wheat flour bread may have some potential negative nutritional and health implications. The refining process of wheat grain to “white” bread flour reduces its nutritional quality in terms of vitamins, minerals, and dietary fibre (Rosell, 2011). One legume with great potential to increase the nutritional and health benefits of bread is the Australian sweet lupin (ASL) (*Lupinus angustifolius)* or the narrow-leafed lupin (Hall and Johnson 2004).

ASL kernel flour has higher protein content (~40%) and total dietary fibre content (~40%) but lower energy value compared to wheat flour (Hall and Johnson, 2004). ASL kernel flour contains carotenoids, phenolics, vitamins and minerals. ASL has demonstrated the potential to decrease risk factors for chronic disease in human clinical studies of lupin bread consumption through beneficial effects on obesity (Lee

et al., 2006), and risk factors for cardiovascular disease (Belski et al., 2011) and type 2 diabetes mellitus (Hall et al., 2005a).

The use of lupin flour addition to refined wheat flour bread is however, a "double-edged sword" as quality issues arise i.e. poor bread volume and texture, most probably due to the same proteins and dietary fibre that make lupin a nutritious alternative to refined wheat flour. Problems with bread quality i.e. poor bread volume and texture may be attributed to the non-gluten proteins in lupin and the high water binding capacity of lupin dietary fibre (Turnbull et al., 2005) that may weaken the gluten matrix in the dough. The weakening of the matrix results in poor texture and loaf volume of the resulting wheat-lupin composite flour bread (Guemes-Vera et al., 2008). When using refined wheat flour from Western Australia (WA), these quality issues of lupin incorporation are accentuated, due to the lower protein in WA wheat compared to that grown in other wheat-growing regions, for instance North America. Lupin flour has been used to substitute for wheat flour in breads. However, substitution of more than above 10% substitution of refined wheat flour with lupin flour resulted in decrease in dough and bread quality (Doxastakis, et al., 2002; Mubarak, 2001). Therefore, there is a need to examine the effects of bread making process and formulation parameters of ASL breads used in combination with WA wheat flour on bread quality and to optimise these parameters for maximum incorporation of lupin flour whilst maintaining bread quality, e.g. volume, texture and consumer acceptability.

No published study has been reported demonstrating the role of ASL variety on bread nutritional (i.e. protein content and quality, and dietary fibre content), chemical (i.e. carotenoids, total phenolics, antioxidants), and physical (i.e. bread volume, crumb structure and instrumental texture) qualities. Six ASL varieties (*Tanjil, Mandelup, Coromup, Jenabillup, Belara and Gungurru*) are commercially grown in Australia, with the vast majority of production occurring in Western Australia (French et al., 2008). Currently there is a lack of information on the varietal differences in nutritional composition of the seed kernel nor its impact on the physical quality of the dough and nutritional and physical quality and of ASL-refined wheat composite flour breads.

Aside from the lack of information on varietal differences in the protein content and dietary fibre content, published reports on the amino acid profile of ASL flours from different varieties and the protein quality of these varieties as a food ingredient in bread is still scarce. The amino acid profile of lupin complements that of wheat, which is higher in sulphur-containing amino acids (i.e. methionine) but lower in lysine. Therefore, addition of ASL flour into wheat bread has the potential not just to increase the protein content but improve the amino acid balance and protein quality of the final product (Duodu and Minnaar, 2011). Protein quality for human use is measured by calculating the Protein Digestibility-Corrected Amino Acid Score (PDCAAS) using the amino acid profile and protein digestibility of the food product.

Rigorous studies on the effects of formulation and process parameters on ASL-wheat bread quality are scarce in literature. Previous studies on breads containing lupin have mainly focused on the effect of the level of lupin used to substitute for refined wheat flour in bread. However, the effects of other formulation parameters, for example the amount of water incorporation and ASL flour particle size still need to be investigated. The high water binding capacity (WBC) of the dietary fibre in ASL flour may affect the development of gluten, thus the amount of water in bread formulation consequently needs to be adjusted when ASL flour is used to replace wheat flour to allow proper development of the gluten matrix. There should be a proper balance in the amount of water that gluten proteins need for development of the gluten matrix and the amount which will be bound to ASL flour. Inadequate amount of water can lead to dry and crumbly dough or excessive water can result to batter-like dough affecting bread quality. Particle size of lupin flour has potential to impact on bread volume as shown in previous studies of other wheat flour substitutes which reported an increase (de Kock et al., 1999) or decrease (Moder et al., 1984) in bread volume with increase in particle size. Studies on the effects of level of water incorporation and particle size of ASL flour on ASL-WA wheat composite flour bread quality is still lacking.

Aside from formulation parameters, bread making process parameters i.e. mixing, proofing and baking can also affect ASL-wheat bread quality. Two commonly used methods of bread making are the “straight-dough” and the “sponge and dough” processes. The straight-dough process is the simplest method which involves mixing all ingredients, proofing, punching, shaping, final proofing and baking (Atwell, 2001).

The sponge and dough process, involves firstly mixing and fermenting a portion of flour, water and yeast, after which this pre-dough (sponge) is mixed with the rest of the ingredients to form the final dough. The final dough is proofed for a short time before it is divided, rounded, moulded, fermented (final proof) and baked (Collado-Fernández, 2003a).

Most of the reported studies have used the straight-dough method for lupin bread manufacturing. However the sponge and dough process may be a more effective method for incorporating lupin into bread because of its robustness to process fluctuations (i.e. mixing and proofing). It may be useful to establish separate mixing and proofing parameters (i.e. time and/or temperature) for wheat sponge and lupin sponge given the differences in their rheological properties (i.e. proximate composition). This would allow the wheat gluten matrix to develop initially without disruption of the low-elasticity proteins and high water binding of dietary fibres in lupin flour and thus may help reduce the negative effects of lupin flour addition. There are no published reports highlighting the effects of sponge and dough bread making process parameters on lupin bread quality.

As previously mentioned physical qualities (i.e. crumb specific volume, measures of crumb structure and instrumental texture properties) are used in describing bread quality. These characteristics are key quality attributes that determine overall bread quality both for the baker and consumer (Pyler, 1988). Information is lacking on the effects of ASL-wheat composite flour bread formulation and process with regards to the physical properties and consumer acceptability of bread. The information on the relationship of formulation and process with physical properties and consumer acceptability of ASL-wheat composite flour bread can in turn be used for optimisation of bread making parameters to maximise ASL flour addition in wheat bread with acceptable physical and sensory properties.

In-depth optimisation of the formulation and process parameters for high quality ASL bread manufacture has not been reported in the literature. A tool that is commonly used in optimisation studies is a statistical approach known as response surface methodology (RSM). RSM is a collection of mathematical and statistical methods that are effective in the analysis of the effects of several factors (i.e. formulation and

process parameters) on an output (response, i.e. bread physical property and consumer acceptability) of interest based on experiments or situation, and the aim is to optimise the response (Montgomery, 2009). RSM has been used to optimise formulation and process parameters of other "healthy" breads such as wholemeal oat bread, gluten-free breads and legume (i.e. soy beans and chickpea)-wheat flour composite flour breads. There is no published report on the use of RSM in optimizing bread formulation and process to maximise ASL flour substitution for WA wheat flour for maximum nutritional and health benefits from ASL proteins and dietary fibre whilst having acceptable bread properties and consumer acceptability.

1.2. AIMS

The overall objective of this study was to maximise the nutritional and sensory quality of ASL-refined wheat composite flour bread referred to as "ASL-wheat bread" for the remainder of the thesis made using Western Australian refined wheat flour.
Specifically, the study:

a. Examined the effects of ASL varietal differences on nutritional, chemical and physical properties of ASL-wheat bread and selected the variety for bread optimisation with maximum potential for manufacture of high quality bread,
b. Evaluated the effects of formulation and sponge and dough process parameters of ASL-wheat bread making on the physical properties and consumer acceptability of the bread,
c. Optimised formulation and process parameters for ASL-wheat breads using RSM to maximise lupin flour incorporation whilst maintaining acceptable bread volume, instrumental textural properties and consumer acceptability within the range found for 100% wheat flour bread.

1.3. KEY OUTPUT

The key output for this study is new knowledge and information on:

a. The ASL variety most suitable for manufacture of high quality ASL-wheat bread.

b. Predictive models for ASL-wheat bread physical and consumer acceptability using various combinations of ASL-wheat bread formulation and process parameters.
c. An optimised formulation and processing regime for maximising the nutritional quality and palatability of ASL-wheat bread.

CHAPTER TWO

REVIEW OF LITERATURE

Information in this chapter has been submitted for publication as follows:

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2.1 ABSTRACT

Lupin is an undervalued legume for human nutrition despite its high protein and dietary fibre content and potential health benefits. This review focuses on the nutritional value, health benefits and technological effects of incorporating lupin flour into wheat-based bread. Results of clinical studies suggest that consuming lupin compared to bread and other baked products made from wheat reduces chronic disease risk markers; possibly due to increased protein and dietary fibre levels and presence of bioactive compounds. However, lupin protein allergy has also been recorded. Bread textural quality has been improved when 10% lupin flour is substituted for refined wheat flour; possibly due to lupin-wheat protein cross-linking assisting bread volume combined with the high water binding capacity (WBC) of lupin fibre that may delay staling. Above 10% substitution of lupin for refined wheat flour appears to reduce bread textural quality due to the low-elasticity of lupin proteins and the high WBC of its dietary fibre that interrupts gluten network development in the dough. Major gaps in understanding of the role of lupin flour in bread quality include the optimal formulation and processing conditions to maximise lupin incorporation, the effects of protein cross-linking in lupin-gluten matrix, anti-staling properties of lupin flour and effects of processing on levels of γ-conglutin bioactive peptide.

2.2 INTRODUCTION

In this review, the term “baked products” includes breads, cakes, pastries, cookies, crackers and other products which use wheat flour as the primary ingredient, and undergo heat processing. Baked products, in particular bread have been an important

part of the diet for centuries (Smith et al., 2004) and have remained a staple food across the world (IBISWorld, 2011). It is predicted that the global baked products market will reach US$ 410 billion by 2015 (Anon, 2011b). However, the food industry is pressurised to continuously address dynamic consumer preferences for healthy and novel foods incorporating alternative grains and legumes as substitutes for refined wheat.

Wheat flour is the major component of baked products due to the presence of proteins, gliadins and glutenins that form the gluten matrix needed for the viscoelastic characteristics of dough, providing products with the desired volume and texture (Rosell, 2011). However, health and nutritional issues can arise from the use of wheat flour in bread. For example, coeliac disease due to gluten intolerance is a growing health concern (Mandala and Kapsokefalou, 2011). In addition, during refining, the removal of bran and germ leads to reduced nutritional value through significant losses in protein, dietary fibre, vitamins, minerals and phytochemicals (Rosell, 2011). These issues related to over-consumption of refined wheat products have catalysed the search for alternative flour ingredients for baked products including legumes. Legumes (e.g. soybeans, chickpea and faba beans) are good sources of protein, dietary fibre, vitamins and minerals, do not contain gluten and have been added at a rate of 10 to 30% to baked products without reducing quality (Duodu and Minnaar, 2011; Farooq and Boye, 2011).

A legume that can potentially address consumers' desire for healthier baked products is lupin. Lupin grain has a unique combination of high protein and dietary fibre content with very little available carbohydrate (Hall et al., 2005b; Petterson et al., 1997). Moreover, lipid in lupin grain is mainly composed of "healthy" fatty acids, e.g. linoleic, linolenic and oleic acids (Trugo et al., 2003). The grain also contains vitamins and antioxidants including: tocopherols (Boschin and Arnoldi, 2011); carotenoids (Wang et al., 2008); B-vitamins (Erbas et al., 2005) and phenolic compounds (Oomah et al., 2006). In addition, lupin is low in anti-nutritional factors such as trypsin inhibitors and saponins compared to many other legumes (Martínez-Villaluenga et al., 2006a). Studies have demonstrated that lupin flour can be used to formulate acceptable baked products (Hall and Johnson, 2004), as well as other foods such as pasta (Martínez-Villaluenga et al., 2010), meat products (Drakos et al., 2007) and dairy

products (Martínez-Villaluenga et al., 2005b). Human clinical studies have demonstrated the consumption of these lupin-containing products, in particular bread, can lower biomarkers of risk of obesity (Lee et al., 2006), cardiovascular disease (Belski et al., 2011) , type 2 diabetes mellitus (Hall et al., 2005b) and gastrointestinal problems (Johnson et al., 2006).

Despite the potential of lupin as a unique, healthy food ingredient, most of the lupin grain production is utilised as stockfeed. The use of lupin as human food, specifically in baked products has been limited, due mainly to poor sensory quality of resulting products (Paraskevopoulou et al., 2010). The continuing rise in food prices, demand for healthier and non-genetically modified (GM) products, and sustainably produced food, suggest the potential for a rapid increase in utilization of lupin as a food ingredient.

This review paper covers the nutritional, health, and technological functionality of lupin flour in baked products and the role of lupin protein and dietary fibre in these functionalities. In this review, "nutritional functionality" refers to the impact on nutritional composition; "health functionality" refers to the evidence from clinical trials on the effect of lupin food consumption on biomarkers of risk of chronic diseases; and "technological functionality" refers to impacts of lupin addition to baked products on product quality (i.e. instrumental measures and consumer acceptability), and processing efficiency. In this review, "lupin flour" refers to milled dehulled and non-defatted kernels, and other types of lupin flour i.e. wholemeal, defatted) were specified as such. The terms *lupin, wheat flour, bread, proteins, dietary fibre, γ-conglutin, disulphide, dityrosine crosslinking and bread staling* were used to search *Web of Knowledge, ScienceDirect, Scopus, Wiley Online Library and Google Scholar.* For nutritional and health functionality the period was limited to 2000 to present and only articles discussing human clinical studies were included in health functionality. For technological functionality, the search period was extended back to 1980 due to the small number of recent publications.

Information on lupin agronomy, history, production, lupin flour composition and uses as a food ingredient will be presented first. This will be followed by a brief overview of bread and bread making The benefits and challenges of lupin flour addition to bread

and other baked products in terms of the: (a) nutritional and health functionality then (b) technological functionality of the food products will then be reviewed in detail.

2.3 LUPIN

Taxonomy, agronomy and general uses

Lupins (lupine) belong to the genus Lupinus under the Genisteae tribe of Fabaceae (or Leguminosae) family (Uzun et al., 2007) to which soybeans, chickpeas and other types of beans also belong. Lupin seeds from different species and varieties vary greatly in size, shape (i.e. round, oval, and flat), and colour (i.e. white, brown, and grey) (Kurlovich et al., 2002).

Wild species of *Lupinus* found in North and South America, the Mediterranean region and northern Africa were introduced to southern Africa and Australia during the early days of colonization (Cowling et al., 1998). Full domestication of a few lupin species (i.e. *L. angustifolius, L. albus, and L. luteus)* for animal feed and human food use occurred in the latter half of the 20^{th} century in Australia and Europe. Wild lupins have: (a) bitter quinolizidine alkaloids (QA) which renders them unpalatable and potentially toxic, (b) hard seed coats (that do not readily imbibe water) allowing them to survive in the soil for several seasons prior to germination thus delay harvest and use, and (c) shattering pods that scatter seed on the ground at maturity which decreases yield. On the other hand, domesticated lupins have been selectively bred to have: (a) low alkaloid content, making them edible to domestic animals and humans, (b) softer seeds that immediately germinate in moist soil and (c) non-shattering pods which keep the seeds on the plant for efficient harvesting.

Lupin grows well in acidic and sandy soils, as for example those found in Western Australia (French et al., 2008). The lupin plant (foliage) has been used as green manure or forage, as organic material for soil enrichment and stabilization and for soil erosion control (Cowling et al., 1998). Due to its nitrogen fixation ability, lupin is a critical rotation crop for the sustainability of some farming system, such as wheat and other cereals in Western Australia and Europe (e.g. Germany, Poland, Spain) (French et al., 2008; GL-PRO, 2005).

Species

The genus Lupinus consists of hundreds of species, of which only a few have been domesticated (Foley et al., 2011) including *L. albus, L. angustifolius, L. luteus and L. mutabilis* (Table 2.1). *L. albus* is grown mainly in Europe (Harzic et al., 2000) while *L. angustifolius* is largely produced in Western Australia (Cowling and Gladstones, 2000). *L. luteus* is widely distributed in the Mediterranean region (Parra-Gonzalez et al., 2012) while *L. mutabilis* is grown primarily in South America (Erbas, 2010).

Table 2.1.Taxonomic and common names of some commercially grown lupin species.

Species	Common names
Lupinus albus	White lupin, Egyptian lupin, tremoo, altramuz
Lupinus angustifolius	Blue lupin, narrow-leafed lupin, Australian sweet lupin
Lupinus luteus	Yellow lupin, tremosilla
Lupinus mutabilis	Tarwi, tauri, tarhui, chocho, Andean lupin

[1] Trugo et al. (2003)

L. angustifolius, also known as the Australian sweet lupin (ASL), blue lupin or the narrow-leaf lupin, has the highest production of any legume crop grown in Australia (Lawrance, 2007). In recent years, interest in the use of ASL as human food has been increasing in both Australia and Europe due to its potential health benefits (Sirtori et al., 2010). *L. albus* has been used as food since the pre-Roman and Greek times (Cowling et al., 1998). Traditionally it was soaked and boiled to eliminate the bitter alkaloids (Annicchiarico et al., 2010). *L. luteus* typically has yellow flowers (Cowling et al., 1998), thus its common name, the yellow lupin. There is scarcity of reports on human studies or food applications involving yellow lupin despite it being reported to having higher protein and fibre contents than both ASL and *L. albus* (Petterson et al. (1997). *L. mutabilis* has long been utilised for soil enrichment and as food in the Andean region (Gross et al., 1988). The seeds of this species have the highest protein content among the four commercial species (Trugo et al., 2003) at a similar level to soybeans (Cowling et al., 1998).

Agricultural production

According to the Food and Agricultural Organisation of the United Nations (FAO-STAT, 2014), the top five lupin grain producing countries in 2012 were Australia, Poland, Chile, Russian Federation, and Germany. Australia produces 85% of the total global supply of lupin grain, by far the majority of which is ASL. Lupin is lower in cost compared to other legumes: for instance in Australia, lupin is currently sold 33% lower than the price of soybean (igrain.com.au, 2014). However in Australia, lupin grain is still mainly used as animal feed with only around 4% of the total production processed for human consumption (Lawrance, 2007).

2.4 LUPIN FLOUR

Definition and description

Food Standards Australia and New Zealand (FSANZ, 2009), Food Standards Code for Cereals and Cereal Products (Standard 2.1.1), defines flours as "*products of grinding or milling of cereals, legumes or other seeds*". Based on this definition, lupin flour refers to the product from milling lupin seeds. ASL flour has been described as having a pale yellow colour and slight beany flavour (Hall et al., 2005b). According to Australian and UK standards lupin flour should not have more than 200 mg/kg of alkaloids and not more than 0.005 mg/kg of phomopsins (FSANZ, 2011a; MAFF-DOH, 1996).

Lupin flour manufacture

Lupin flour is made from milled dehulled kernels. Seeds are first sorted, graded and then cleaned of any foreign objects using a vibrating screen or metal detector. The cleaned whole seeds are passed through a de-huller to remove the hull from the kernel. Hulls are the separated from the kernels by air classification. Since the hull is rarely incorporated into lupin flour for baked products it will no longer be discussed within this review. The split kernels are then milled and sieved to separate into varying particle size ranges from <150 to 300 microns (Anon, 2011a). The lupin flour milling process is important in producing flour of optimal quality for specific food

applications including bread. Decreasing the particle size of wheat flour substitutes (i.e bran or whole wheat) used in bread making has been reported to either increase (Moder et al.,1984) or decrease (de Kock et al., 1999) loaf volume. However, no study has been reported on the effects of lupin flour particle size on loaf volume.

Composition

Lupin flours are a rich source of nutrients with ASL flour having higher protein (~40 g/100 g db.), higher total dietary fibre (~40 g/100g db.) but lower energy value compared to refined wheat flour (Table 2.2). All lupin flours are also very low in starch, unlike wheat flour in which starch is the major component (Hall and Johnson, 2004). Fat content of lupin flours ranged from 7 g/100 db. to 15 g/100 g db., depending on species (Doxastakis et al., 2002. Hall et al., 2005b). Lupin seeds also contain vitamins such as thiamine, niacin, riboflavin, and tocopherols, as well as minerals including iron, zinc and manganese (Trugo et al., 2003). It was also reported that ASL seeds have high levels of carotenoids compared to those of *L. luteus and L. albus* (Wang et al., 2008). Lupin flour contains antioxidants (Martínez-Villaluenga et al., 2009) in the form of the polyphenolic flavonoids and tannins (Oomah et al., 2006). Compared to soybeans, lupins have lower levels of anti-nutritional components such as phytate and saponins (Trugo et al., 2003).

Table 2.2. Nutritional composition of Australian sweet lupin (ASL) and refined wheat flours[1]

Composition (db.)	**ASL Flour**	**Wheat Flour**
Energy (kJ/100g)	981	1416
Protein (g/100g)	42	12
Fat (g/100g)	7	1
Total dietary fibre (g/100g)	42	3
Soluble dietary fibre (g/100g)	*11*	*1*
Insoluble dietary fibre (g/100g)	*31*	*2*
Available carbohydrate (g/100g)	1	69

[1]Hall et al. (2005b)

Lupin proteins and dietary fibre have the potential to increase the nutritional quality and modify the technological properties of bread and other baked products when lupin flour is used to substitute for wheat flour.

Protein content and nutritional quality

Protein at an adequate intake and of a balanced essential amino acid composition is an essential dietary component (Rolfes et al., 2009). ASL flour has been reported to contain 41.8% protein (Hall et al., 2005b), however protein content was reported to be affected by both genotype (e.g. variety) and environment (e.g. year of harvest) (Cowling and Tarr, 2004). Other lupin species, e.g. *L. albus* and *L. luteus* are not widely utilized as flour on the commercial scale and thus reports on their protein content as flours are limited. Dervas et al. (1999) reported that the protein content of *L. albus* flours ranged from 31-36%. Lupin grains contain two classes of proteins, albumins and globulins (Duranti et al., 2008). The storage proteins in the lupin grains are attributed to the 2S albumins and mainly the globulins. The globulins are classified into four families: α- conglutin (11S globulin), β- conglutin (7S globulin), γ-conglutin (7S basic globulin), and δ-conglutin (2S sulphur-rich albumin) (Foley, et al., 2011). γ-conglutin is a peptide with reported bioactivity (i.e. blood glucose lowering) and thus potential health and pharmaceutical benefits (Duranti et al., 2008).

The main limiting essential amino acids in lupin grain are the sulphur-amino acids (methionine and cysteine), valine and tryptophan (Doxastakis et al., 2002). The amounts of the other essential amino acids i.e. lysine, isoleucine, leucine, phenylalanine and tyrosine in lupin are comparable to the Food and Agricultural Organization (FAO) standards for amino acids of the ideal reference protein appropriate for adults (FAO/WHO/UNU, 1985). The amino acid profile of lupin complements that of wheat, which is higher in sulphur-containing amino acids but lower in lysine. The complementarity of essential amino acids is one of the many advantages of using legume flours, including lupin, in combination with wheat flour in baked products (Duodu and Minnaar, 2011). However, legumes including lupin, lack the gliadins and glutenins which in combination with water develops the gluten matrix required for a viscoelastic dough, leading to good volume and soft and springy texture in bread. This lack of gluten-forming protein in lupin flours limits its incorporation rate in bread and hence its potential to improve the nutritional attributes of baked products (Angioloni and Collar, 2012b). There still remains a lack of information on the effect of genotype x environment on protein quality of lupin flours. Little is also

known on the levels of the bioactive peptide, γ-conglutin in different lupin varieties, nor the stability of γ-conglutin during food processing.

Dietary fibre content and physical properties

Types of dietary fibre in lupin. Increased dietary fibre intake is a general dietary recommendation for a healthy diet across the developed world (DHA-NHMRC, 2005; USDA-CNPP, 2012). In whole lupin seeds, both the hull and the kernel contain high levels of dietary fibre (Pfoertner and Fischer, 2001). However, since lupin flour is generally manufactured only from the dehulled kernels, this review will focus only on dietary fibre in lupin kernels. ASL flour has been reported to contain 41.5 % dietary fibre, 11% of which is soluble and 31.5% is insoluble (Hall et al., 2005b). Dietary fibre in lupin flour, located in the thickened endosperm cell walls, mainly consists of non-starch polysaccharides and raffinose family oligosaccharides such as raffinose, stachyose and verbascose (Evans et al., 1993; Trugo et al., 2003). The non-starch polysaccharides are composed of a rhamnogalacturonan backbone with galactose and arabinose containing side chains (Pfoertner and Fischer, 2001). Both the non-starch polysaccharides and raffinose family of oligosaccharides are involved in the nutritional and technological functionality of lupin flour when used in bread.

Physical properties of lupin dietary fibre. The main physicochemical property of the dietary fibre in lupin flour that may influence its functionality when lupin flour is used in bread is the water binding capacity (WBC). WBC refers to the amount of water a gel system retains within its structure after it is subjected to any form of stress (Tungland and Meyer, 2002); an example being mixing or kneading during bread manufacture. Lupin kernel fibre has a WBC of 8-11 ml water/g dry solids (Pfoertner and Fischer, 2001; Turnbull et al., 2005).

The addition of lupin flour has great potential to elevate the levels of dietary fibre in baked products. However, information is still required on the effect of lupin genotype and production environment on its dietary fibre content and high WBC. This information can then assist in the selection of the optimal lupin variety for incorporation into consumer acceptable baked and other food products with maximum dietary fibre content.

Other lupin fractions as food ingredients

Lupin flour can be fractionated into protein isolates, purified dietary fibre, oil and water-soluble by-products (i.e. whey proteins and oligosaccharides). Protein isolates are prepared either by isoelectric precipitation (Ruiz and Hove, 1976) or ultrafiltration (Chew et al., 2003) of a protein extract of lupin flour. The total protein is conventionally extracted by solubilisation of protein from kernel flour (defatted or non-defatted) at pH 9, centrifugation to remove the insoluble portion (kernel dietary fibre), followed by acid precipitation of the major globulin proteins at pH 4.5 (Sipsas, 2008). The acid-precipitated protein is then separated from the acid-soluble "whey" fraction by centrifugation. Both the acid-precipitated protein and the fibre fractions are then dried to produce the final dry powder ingredients.

Oil can also be obtained from lupin grain (Hill, 2005) by enzyme-assisted aqueous extraction (Jung, 2009) or solvent extraction (Ortiz and Mukherjee, 1982). Lupin oil has similar fatty acid profile to peanut and rapeseed oils (Erbas et al., 2005) and consist mostly of polyunsaturated fatty acids (PUFA) i.e. linoleic, linolenic and oleic with low levels of saturated fatty acids (SFA) (Trugo et al., 2003). Higher PUFA: SFA ratios are of importance for coronary heart disease prevention (Trugo et al., 2003). Lipid in lupin may also have a technological functionality when added to bread. It was shown that lipids in wheat flour positively affected bread quality by forming lipid monolayers at the gas/liquid interphase of the gas cells thus increasing gas retention of the dough (Goesaert et al., 2005) and helping stabilize the gas cells (Gan et al.,1995). The positive effects of wheat flour lipids may also hold true for lipids in lupin flour. ASL flour lipids comprise mainly of the non-polar lipids (i.e. triglycerides) and polar lipids (i.e. phospholipids) (Hansen and Czochanska, 1974). Polar lipids in wheat flour were reported to increase loaf volume while the effects of non-polar lipids were dependent on the presence of other types of lipids (MacRitchie, 1977). There is a need to further investigate the effect of ASL flour lipids on bread quality.

The acid-soluble whey fraction from lupin protein isolate and dietary fibre manufacture, was once considered a waste stream but has demonstrated potential as a source of foaming proteins (Wong et al., 2013) and blood-glucose lowering bioactive

peptides (Sironi et al., 2005). In addition, this whey fraction contains oligosaccharides which may have prebiotic activity similar to those found in soybean (Patel and Goyal, 2012). Prebiotics refer to non-digestible food ingredients that stimulate the growth and/or activity of one or a limited number of beneficial bacteria in the gastrointestinal tract resulting to improved health (Roberfroid, 2007).

Use of lupin flour in wheat-based food

Lupin flour and its fractions have been investigated as a partial substitute for wheat flour in bread, including: white breads (Doxastakis et al., 2002; Guemes-Vera et al., 2008; Mubarak, 2001; Paraskevopoulou et al., 2010); Chilean breads (Ballester et al., 1988) and; sourdough bread (Bartkiene et al., 2011). Lupin has been used in other baked goods such as muffins, cookies and brownies (Clark and Johnson, 2002; Doxastakis et al., 2002; Hall and Johnson, 2004; Nasar-Abbas and Jayasena, 2012), gluten-free cakes (Levent and Bilgiçli, 2011), and biscuits (Jayasena and Nasar-Abbas, 2011) and other wheat-based foods including instant noodles (Jayasena et al., 2010) and pasta (Clark and Johnson, 2002; Martínez -Villaluenga et al., 2010).

A limited number of commercial breads containing lupin flour are available. Bodhi's Bakehouse (Fremantle, Australia) produces *Lupin Loaf*, a gluten-free bread which contains 5.6g/100g of protein and 4.2 g/100 g of dietary fibre, and *Wupper Soft with Lupin* which contains 17.5 g/100g of protein and 10.4g/100g of dietary fibre. *Lupin Loaf* (10% lupin flour) is a pan bread, described as having a dense crumb while *Wupper Soft with Lupin* (40% lupin flour) is a sourdough rye bread. These breads are marketed as niche healthy products and have not reached mainstream consumption possibly due to limited consumer acceptance. Consumers' preference for refined white bread is one of the reasons cited for the relatively low consumption of whole-wheat (Bakke and Vickers, 2007) or high-fibre breads. In the case of lupin, published reports demonstrate that a maximum of only 10% lupin flour can be substituted for refined wheat flour before quality is reduced (Doxastakis et al., 2002). This may be attributed to the low elasticity proteins and high WBC of dietary fibre in lupin flour (Turnbull et al., 2005), that weakens the gluten matrix and thus results in poor texture and loaf volume of the bread (Guemes-Vera et al., 2008). There is therefore, a need for research to identify optimal formulations and processing methods to further increase

the rate of incorporation of lupin into bread whilst maintaining high palatability. This will provide a nutritious bread acceptable to mainstream consumers.

2.5 BREAD

Bread is typically formulated from wheat flour, water, yeast and salt (Popper et al., 2006). Ingredients such as non-wheat flour, shortening, sugar, enzymes, dough conditioners, vitamins and minerals may also be added to improve sensory, textural and nutritional quality (Atwell, 2001; Collado-Fernández, 2003a). There are many types of bread, originating in different parts of the world, including pan breads, rolls or bun, steamed buns, artisan breads and flat breads. These breads are differentiated by their shape, ingredients and equipment and baking processes used for manufacture. For example, pan bread is so named as the dough is fermented and baked in a pan, and flat breads are generally made from dough that has been rolled and thus remains flattened after baking. Breads may also be classified according to the type of heat processing applied in the case of oven-baked and steamed breads. Breads may be leavened by either yeast or lactic acid bacteria (i.e. sourdough breads). Artisan breads are made by hand and produced in small batches, while many commercial breads are made in large volumes on fully automated highly controlled production lines.

Role of bread in a healthy diet

Bread is one of the most commonly eaten food items, with per capita global consumption ranging from 41 to 303 kg/year (Rosell, 2011) and thus it is a main source of energy and nutrition for humans (Collado-Fernández, 2003a). In America and Australia, typical breads manufactured from refined wheat flour have been reported to contain 9.2 g/100 of protein and 2.7 g/100 g of dietary fibre (USDA, 2012) and 9.7 g/100 g of protein and 2.8 g/100 g of dietary fibre (FSANZ, 2011b) respectively. Bread is also a good source of available carbohydrates, minerals (i.e potassium, calcium, iron) and B vitamins (Southgate, 2003).

It is therefore very important to continue research on developing breads with a healthy micro- and macro-nutrient profile without compromising their consumer acceptability. Substituting lupin flour for refined wheat flour in bread has the potential to increase its

protein, dietary fibre content, improve micronutrient profile and possibly enhance its health functionality due the bioactive compounds such as, γ-conglutin, a blood-glucose lowering peptide (Bertoglio et al., 2011); these topics will be discussed in detail later in this review.

Bread manufacturing

There are different types of bread making processes which vary in their combination of three principal stages: kneading of dough (mixing), fermenting and baking. Two commonly used methods of bread making are the "straight-dough" and the "sponge and dough" processes. The straight-dough process is the simplest method which involves mixing all ingredients, fermenting, punching, shaping, final proofing and baking (Atwell, 2001). The sponge and dough process, involves firstly mixing and fermenting a portion of flour, water and yeast, after which this pre-dough (sponge) is mixed with the rest of the ingredients to form the final dough. The final dough is proofed for a short time before it is divided, rounded, moulded, fermented (final proof) and baked (Collado-Fernández, 2003a). The sponge and dough method is less sensitive to process fluctuations such as over mixing or over proofing than the straight dough method and also enhances loaf volume, texture and shelf-life. The longer mixing allows for more air to be incorporated leading to greater volume and softer texture (Amr and Ajo, 2005). The longer fermentation in the sponge and dough method also results in improved aroma but this method entails more time and effort compared to the straight dough method. The dough may also be less flexible and thus difficult to divide and mould (Collado-Fernández, 2003a).

The technological aspects of bread making have been thoroughly discussed by several authors (Cauvain, 2007; Collado-Fernández, 2003a; Rosell, 2011) and therefore will not be discussed in detail in this review. Table 2.3 shows the main stages of bread making, the mechanisms involved in each stage, the related quality parameters, and quality issues that arise when the processes are performed sub-optimally. Mixing and kneading generate the mechanical energy needed to develop the gluten matrix (Rosell, 2011) and the incorporation of air to form the dough with the required rheological properties (Collado-Fernández, 2003a). Dough properties important to bread making are: water absorption capacity, dough development time, stability, and elasticity which

are conventionally measured by farinographs (Puppo et al., 2005). The quantity of water added to flour is extremely important to the hydration of the dough ingredients and hence the amount of time to develop the gluten network (dough development time) and the quality (stability and elasticity) of the gluten network. During the fermentation stage of bread making, endogenous or added enzymes (e.g. α-amylase, α-glucosidase) convert starch to sugars. The yeasts metabolise the sugars resulting in production of carbon dioxide and ethanol. As carbon dioxide is produced, the dough expands and retains the gas in the resulting bubble structure. The amount of gas retained mainly depends on the quality of the gluten network; the higher the gas retention, the larger the resulting loaf volume. Loaf volume is also influenced by the proofing time and temperature. Lastly, baking results in further expansion of the formed gas bubbles, firming of the dough through coagulation of gluten and gelatinization of starch (Collado-Fernández, 2003a) and development of typical bread aromas (e.g. 1-propanol, acetaldehyde, propanal, butanal, furfural, acetic acid, and ethyl acetate) (Collado-Fernández, 2003b).

The various stages in bread making are sensitive to the substitution of wheat-flour by non-gluten, low-starch lupin flour leading in particular to the disruption of gluten development (Guemes-Vera et al., 2008) and a reduction in carbon dioxide production during fermentation, resulting in bread with poor loaf volume, and hard and crumbly texture (Doxastakis et al., 2002).

Table 2.3. Summary of the main stages of bread making, the mechanisms involved related dough and bread quality parameters and quality issues that arise when processes are performed sub-optimally.

Stage	Mechanisms involved	Related quality parameters	Quality issues	
			Over processing	Under processing
Mixing and kneading	❖ Hydration of wheat proteins and starch ❖ Energy generated to develop gluten matrix through covalent bonds (protein cross-linking) ❖ Incorporation of air bubbles ❖ Enzymatic production of sugars from starch	❖ Water absorption ❖ Dough development time ❖ Viscosity ❖ Stability ❖ Elasticity ❖ Extensibility ❖ Gas retention	❖ Slack and sticky dough ❖ Collapsed bread ❖ Dense bread ❖ Chewy and hard bread	❖ Underdeveloped dough ❖ Dense bread ❖ Chewy and hard bread
Fermentation	❖ Yeasts ferment sugars to produce carbon dioxide ❖ Bubbles surrounded by gluten expand	❖ Loaf volume ❖ Crumb cell structure ❖ Textural properties	❖ Collapsed bread ❖ Low volume ❖ Dense crumb ❖ Chewy and hard bread	❖ Flat bread ❖ Low volume ❖ Dense crumb ❖ Chewy and hard bread
Baking	❖ Rate of fermentation increases in initial stages, expanding dough ❖ Proteins are denatured and gluten matrix becomes rigid ❖ Yeasts and enzymes are heat inactivated ❖ Starch gelatinizes and stabilises structure ❖ Maillard reaction gives browning of the crust ❖ Formation of bread flavours	❖ Loaf volume ❖ Crumb cell structure ❖ Textural properties ❖ Colour and flavour properties	❖ Burnt crust ❖ Burnt flavour	❖ Low volume ❖ Dense crumb ❖ Pale dough ❖ Poor flavour

2.6. NUTRITIONAL AND HEALTH FUNCTIONALITY OF LUPIN FLOUR IN BREAD

Benefits

This review will focus primarily on the impact of lupin protein and dietary fibre on bread quality. Lupin flour addition to wheat flour bread results in increased nutritional quality and potential health benefits by increasing: (a) protein content and protein nutritional quality; (b) dietary fibre content; (c) carotenoid content and; (d) levels of the potentially bioactive peptide γ-conglutin. The importance of bread as a vehicle of nutrients is demonstrated by the fact that Australians obtain 45% of their dietary fibre and 25% of their protein from cereals and cereal products, including bread (NHMRC, 2005). Substitution of 20% refined wheat flour by lupin flour has the potential to add 8 g (~25% of RDI) each of dietary fibre and protein per 100 g of bread (~4 slices).

Beneficial nutritional functionality

The effects of wheat flour substitution by lupin flour in bread and other baked products on protein content, protein nutritional quality and dietary fibre content are presented in Table 2.4. These reports demonstrate that high levels (30-40%) of substitution of wheat flour by lupin flour can increase the protein content ranging from 46 to 352% and dietary fibre content ranging from 106-346%, of wheat bread. Even low levels substitution (e.g. 3%) can increase protein and dietary fibre levels significantly.

In order to maximise the level of lupin flour incorporation into bread, to maximise its nutritional quality, the new product requires systematic optimisation of formulation, processing parameters and their interactions in order to maintain consumer acceptability. However, this systematic optimisation of lupin bread has not been reported in the literature and most studies focused on only a single parameter (e.g. rate of lupin flour incorporation). In addition, some published studies used *L. albus* flour, some used ASL and some did not specify the species.

Table 2.4. Studies on wheat flour substitution by lupin flour in baked products: effects on protein content and dietary fibre content.

Lupin species/variety	Lupin fraction	Lupin incorporation rate (% wheat flour)[1]	Product	Increase (%)	Reference
Not cited	Flour	40	Pan bread	Protein: 110 Dietary fibre: 106	Belski et al. (2011)
		30 (wholemeal wheat flour)	Biscuit	Protein: 352 Dietary fibre : 211	
Not cited	Flour	40 (wheat flour used in white bread)	Pan bread	Protein: 108 Dietary fibre: 346	Hodgson et al. (2010)
Not cited	Flour	40 (wheat flour used in white bread)	Pan bread	Protein: 108 Dietary fibre: 341	Lee et al. (2009)
Not cited	Flour	40 (wheat flour used in white bread)	Pan bread	Protein: 65 Dietary fibre: 252	Lee et al. (2006)
L. angustifolius	Flour	10	Pan bread	Protein: 14 Dietary fibre: 112	Hall and Johnson (2004)
		60	Muffins	Protein: 46 Dietary fibre: 294	
		28 (unbleached bakers flour)	Chocolate chip cookies	Protein: 51 Dietary fibre: 316	
L. albus	Flour, protein concentrate and isolate	3, 6, 9, 12 (not specified)	Bread	Protein:11- 53 Dietary fibre: 7-44	Mubarak (2001)
L. albus cv. *Multolupa*	Flour	3, 6, 9, 12 (wheat flour enriched with vitamins and minerals; with potassium bromate)	Bread	Protein: 20-23	Ballester et al. (1988)
L. angustifolius	Kernel fibre	9 24 (bread flour)	Pan bread Muffins	Protein 132 Dietary fibre: 285	Clark and Johnson (2002)

[1]Type of wheat flour used in parenthesis

However, it has been confirmed (V. Jayasena, personal communication, June 06, 2013), that ASL was used in the studies presented in Tables 4 and 5 that did not cite the species (Lee et al., 2006; Lee et al., 2009; Hodgson et al., 2010; Yang et al., 2010; Belski et al., 2011). No investigations, however, have reported on the effects of ASL variety on the quality of lupin bread.

Beneficial health functionality

This section will focus on the clinical study evidence that consumption of lupin bread and other baked products can modify biomarkers for the risk of chronic diseases (i.e. obesity, cardiovascular diseases, type 2 diabetes mellitus) and other health biomarkers (i.e. bowel health). A summary of relevant studies is presented in Table 2.5. The findings of these studies have revealed that the consumption of lupin bread and other baked products can help reduce risk factors for obesity, type 2 diabetes mellitus, cardiovascular disease and bowel dysfunction.

Obesity biomarkers

Post-prandial self-reported perception of satiety is a valuable tool to rank foods for their potential ability to reduce overall energy intake and hence risk of obesity (ADA, 2005). It has been reported that breakfast meals (energy intake for all meals was matched) with lupin-supplemented bread (40% lupin flour) gave higher satiety than regular white bread when consumed by healthy male and female adults (Lee et al., 2006). This led to a reduction in energy intake at subsequent meals; effects that the authors attributed to the higher protein and dietary fibre contents of the lupin-containing bread. The increased protein from lupin may have increased plasma amino acids,which subsequently stimulated the production of the gastrointestinal hormone cholecystokinin sending signals of fullness to the brain (Paddon-Jones et al., 2008).

Another biomarker for appetite is plasma ghrelin, a gut hormone that stimulates appetite leading to increased food intake and thus its suppression leads to onset of satiety (Benelam, 2009; Kirsz and Zieba, 2011).

Table 2.5. Clinical studies on the effect of lupin baked products on biomarkers of various diseases.

Lupin species/variety	Lupin fraction	Product	Inclusion levels (% by wt. of lupin-wheat composite flour)[1]	Specification of test population	Experimnetal design	Effects of lupin treatment	Reference
A. Obesity biomarkers							
Postprandial							
Not cited	Flour	Bread	40 (wheat flour used in wheat-only bread)	Healthy male and female subjects (n=16) Fasting blood glucose ≤ 5.6 $mmolL^{-1}$. Mean age 58.6±7.2 y	• Randomized controlled crossover trial • Lupin bread vs. wheat-onlybread at breakfast (toast) and lunch (sandwich) • Dose of lupin: 38 g/meal • Energy intake at breakfast controlled (1655KJ) • Outcome measures : self-reported satiety, energy intake, plasma ghrelin	• Increased self-reported satiety • Lowered energy intake • Decreased plasma ghrelin	Lee et al. (2006)
L. angustifolius	Flour	Bread	10 (white wheat flour)	Healthy male and female subjects (n=11), 25-45 y	• Post-meal study • Lupin bread vs. wheat-only bread • Dose of lupin: 7 g/meal • Total available carbohydrate was controlled • Outcome measure: self-reported satiety and post-	• No effect on satiety and food intake	Hall et al. (2005b)

					meal food intake		
Long-term							
Not cited	Flour	Bread	40 (wheat flour used in white bread)	Overweight and obese male and female subjects (n=88), 21-70 y. with fasting blood glucose of ≤ 5.6 $mmolL^{-1}$	• Randomized controlled parallel-design trial for 16 wk • Lupin bread vs. wheat-only bread (to replace 15-20% of daily energy intake) • Dose of lupin : 38 g/day • Total, saturated, monounsaturated and polyunsaturated fat, protein derived from wheat (gluten) and sodium were controlled • Outcome measures: body weight and composition, plasma leptin and plasma adiponectin	• No effect on body weight and composition nor plasma leptin and adiponectin	Hodgson et al. (2010)
Not cited	Flour	Bread Biscuit	40 30 (wholemeal flour)	Overweight and obese male and female subjects (n=131), 21-71 y. with fasting blood glucose of ≤ 6 $mmolL^{-1}$	• Randomized, controlled, double-blind parallel design for 12 mo • Lupin bread and biscuit vs. wholemeal wheat bread and biscuit • Dose of lupin: not cited • Energy, fat and sodium were controlled • Outcome measures; body weight and composition	Did not enhance weight loss or improve maintenance of weight loss	Belski et al. (2011)

B. Type 2 diabetes mellitus biomarkers

Postprandial							
Not cited	Flour	Bread	40 (of flour used in white bread)	Healthy male and female subjects (n=16), fasting blood glucose of ≤ 5.6 mmolL^{-1} Mean age : 58.6±7.2 y	• Randomized controlled crossover trial • Lupin bread vs. white bread at breakfast (toast) and lunch (sandwich) • Dose of lupin : 38 g/meal • Energy intake (1655KJ) at breakfast was controlled • Outcome measures: fasting serum glucose and insulin	Reduced postprandial glucose and insulin levels	Lee et al. (2006)
L. angustifolius	Flour	Bread	10 (white wheat flour)	Healthy male and female subjects (n=11), 25-45 y	• Post-meal randomised cross-over study • Lupin bread breakfast vs. wheat-only bread breakfast • Dose of lupin: 7 g/meal • Total available carbohydrate was controlled • Outcome measures: plasma glucose and serum insulin	Lupin bread had lower glycaemic index but higher insulinaemic index then wheat bread	Hall et al. (2005b)
L. angustifolius	Kernel fibre	Bread	17 (high gluten white wheat flour)	Healthy male and female subjects (n=21) Mean age: 28.9 ± 8.2 y	• Single-blind, randomized cross-over design • Lupin bread breakfast vs. white bread breakfast • Dose of lupin fibre: 9 g/meal • Macronutrient composition except for dietary fibre was controlled • Outcome measures: plasma glucose and insulin	No effect on plasma glucose and insulin levels, nor glycaemic index. Reduced incremental areas under curves (IAUC) for insulin	Johnson et al. (2003)
Long-term							
Not cited	Flour	Bread	40	Overweight and	Randomized controlled	No effect on	Hodgson et al.

			(flour used in white bread)	obese male and female subjects (n=88), 21-70 y. with fasting blood glucose of ≤ 5.6 $mmolL^{-1}$	parallel-design trial for 16 wk • Lupin bread vs. wheat-only bread (to replace 15-20% of daily energy intake) • Dose of lupin: 38 g/day Total, saturated, monounsaturated and polyunsaturated fat, protein derived from wheat (gluten) and sodium were controlled • Outcome measures: serum fasting glucose and serum insulin	glucose and insulin levels	(2010)
Not cited	Flour	Bread Biscuit	40 30 (wholemeal flour)	Overweight and obese male and female subjects (n=131), 21-71 y. with fasting blood glucose of ≤ 6 $mmolL^{-1}$	• Randomized, controlled, double-blind parallel design for 12 mo • Lupin bread and biscuit vs. wholemeal wheat bread and biscuit • Dose of lupin: not cited • Energy, fat and sodium were controlled • Outcome measures: fasting serum glucose and insulin	Lowered fasting insulin levels No effect on fasting glucose levels	Belski et al. (2011)
L. angustifolius	Kernel fibre	Bread Muffin Chocolate brownie	7.5 5.7 7.1 (by weight of product; unbleached bakers flour)	Healthy male subjects (n=38), 24-64 y	• Single-blind, randomized, crossover, dietary intervention design for 28 days • Lupin products vs. wheat-only products • Dose of dietary fibre in lupin kernel fibre diet: 55 g /day (when prescribed energy intake was >9 MJ/day) and 35 g/day (when prescribed energy intake was ≤9 MJ/day)	No effect on fasting plasma glucose or serum insulin levels	Hall, et al. (2005a)

					• Macronutrient composition except for dietary fibre (higher in lupin diet) was controlled • Outcome measures: fasting plasma glucose and serum insulin		

C. Cardiovascular diseases biomarkers

Long-term

Not cited	Flour	Bread	40 (flour used in white bread	Overweight and obese male and female subjects (n=88), 21-70 y. with fasting blood glucose of ≤ 5.6 $mmolL^{-1}$	• Randomized controlled parallel-design trial for 16 wk • Lupin bread vs. wheat-only bread (contributing 15-20% of daily energy intake) • Dose of lupin: 38g/day • Total, saturated, monounsaturated and polyunsaturated fat, protein derived from wheat (gluten) and sodium were controlled • Outcome measures: serum total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and triacylglycerides	No effect on TC, LDL-C and triglycerides Decreased HDL-C	Hodgson et al. (2010)
Not cited	Flour	Bread	40 (flour used in white bread)	Overweight and obese male and female subjects (n=88), 21-70 y with fasting blood glucose of ≤ 5.6 $mmolL^{-1}$	• Randomized controlled parallel-design trial for 16 wk • Lupin bread vs. wheat-only bread (contributing 15-20% of daily energy intake)	Lowered blood pressure	Yang et al. (2010)

					• Dose of lupin: 38g/day • Total, saturated, monounsaturated and polyunsaturated fat, protein derived from wheat (gluten) and sodium were controlled • Outcome measure: blood pressure		
Not cited	Four	Bread	40 (flour used in white bread	Overweight and obese non-smoking male and female subjects (n=88), 20-70 y with fasting blood glucose of ≤ 5.6 $mmolL^{-1}$	• Randomized controlled parallel-design trial for 16 wk • Lupin bread vs. wheat-only bread (to replace 15-20% of daily energy intake) • Dose of lupin : 38g/day • Total fat and saturated, monounsaturated and polyunsaturated fat, protein derived from wheat (gluten) and sodium were controlled • Outcome measures: blood pressure	Lowered blood pressure	Lee et al. (2009)
Not cited	Flour	Bread Biscuit	40 30 (wholemeal flour)	Overweight and obese male and female subjects (n=131), 21-71 y with fasting blood glucose of ≤ 6 $mmolL^{-1}$	• Randomized, controlled, double-blind parallel design for 12 mo • Lupin bread and biscuit vs. wholemeal wheat bread and biscuit • Dose of lupin: not cited • Energy, fat and sodium were controlled • Outcome measures: blood pressure	Lowered blood pressure	Belski et al. (2011)
L. angustifolius	Fibre	Bread	7.5	Healthy male	• Single-blind, randomized,	Decreased TC,	Hall et al.

		Muffin Chocolate brownie	5.7 7.1 (by weight of product; unbleached bakers flour)	subjects (n=38), 24-64 y	crossover, dietary intervention design for 28 days • Lupin products vs. wheat-only • Dose of dietary fibre in lupin kernel fibre diet: 55 g /day (when prescribed energy intake was >9 MJ/day) and 35 g/day (when prescribed energy intake was ≤9 MJ/day) • • Macronutrient composition except for dietary fibre (higher in lupin diet) was controlled • Outcome measures: Fasting TC, HDL-C, LDL-C, HDL-C: LDL-C, and triacylglycerol.	LDL-C, TC: HDL-C and LDL-C:HDL-C No effects on HDL-C and triacylglycerol	(2005a)
D. Colonic health biomarkers **Long-term**							
L. angustifolius	Kernel fibre	Bread Muffin Chocolate brownie	7.5 5.7 7.1 (by weight of product; unbleached bakers flour)	Healthy male subjects (n=38), 24-64 y	• Single-blind, randomized, crossover, dietary intervention design • Lupin products vs. wheat-only bread • Dose of dietary fibre in lupin kernel fibre diet: 55 g /day (when prescribed energy intake was >9 MJ/day) and 35 g/day (when prescribed energy intake was ≤9 MJ/day)	Increased frequency of defecation, faecal output, faecal moisture content, faecal butyrate levels and output; and decreased transit time, faecal pH, and β-glucoronidase	Johnson et al. (2006)

					• Macronutrient composition except for dietary fibre (higher in lupin diet) was controlled • Outcome measures: bowel function self-perception, frequency of defecation, transit time, faecal output, pH and moisture, faecal levels of SCFA and ammonia, faecal bacterial β-glucuronidase activity		
L. angustifolius	Kernel fibre	Bread Muffin Chocolate brownie	7.5 5.7 7.1 (by weight of product; unbleached bakers flour)	Healthy male subjects (n=38), 24-64 y.	• Single-blind, randomized, crossover, dietary intervention design for 28 d • Lupin products vs. wheat only bread • Dose of dietary fibre in lupin kernel fibre diet: 55 g /day (when prescribed energy intake was >9 MJ/day) and 35 g/day (when prescribed energy intake was ≤9 MJ/day) • Macronutrient composition except for dietary fibre (higher in lupin diet) was controlled • Outcome measures: levels of major colonic bacterial groups in faeces (e.g.*Bifidobacteria*,*Clostridium*)	Increased *Bifidobacterium spp.*and decreased clostridia group of *C. ramosum, C. spiroforme* and *C. cocleatum*	Smith et al. (2006)

[1]Type of wheat flour used in parenthesis

Dietary fibre viscosity, and the release of the gut peptide cholecystokinin mediated by protein ingestion can induce delayed gastic emptying that helps regulate ghrelin (Blom et al., 2006; Koliaki et al., 2010). Blom et al. (2006) hypothesized that delayed gastric emptying decreases total ghrelin concentrations through a postgastric feedback mechanism. Consumption of high protein and high dietary fibre lupin bread led to decreased post-meal ghrelin levels, increased satiety and lower short-term energy intake compared to wheat bread in a study by Lee et al. (2006).

The high WBC of lupin dietary fibre (Turnbull et al., 2005), may have also induced satiety by: (a) increasing stomach distension triggering signals of fullness to the brain; (b) delaying gastric emptying, and; (c) prolonging small intestine transit time and absorption rate of nutrients such as glucose from wheat starch digestion (Kristensen and Jensen, 2011). The role of lupin dietary fibre in the satiating effect of lupin bread is supported by the findings of Archer et al. (2004) who found that sausage patties in which purified lupin kernel fibre replaced some of the fat where more satiating than the full fat version.

In contrast, the study by Hall et al. (2005b) showed that consumption of lupin-containing bread did not affect satiety perception and food intake. The authors attributed this lack of effect to the small number of participants, resulting in insufficient statistical power to detect significant differences between the two treatments. In addition, the dose of lupin per meal (7 g) received by the participants in the study by Hall et al. (2005b) was lower than that in the study of Lee et al. (2006) at 38 g per meal.

The current evidence that lupin-wheat bread compared to wheat-only bread can increase post-prandial satiety and reduce short term energy intake suggests that long term replacement of wheat bread by lupin bread in the diet may result in weight loss in overweight or obese people. However, long-term studies have not demonstrated significant effects on lowering body weight or maintenance of weight loss in overweight and obese adults after either a 16-wk (Hodgson et al., 2010) or a 12-mo (Belski et al., 2011) intervention of regular consumption of lupin-wheat bread compared to wheat-only bread. The authors reasoned that the positive effects of

lupin-enriched bread on short-term appetite and energy intake were offset by other dietary, lifestyle and environmental factors that may have influenced energy balance in the long-term.

Type 2 diabetes mellitus biomarkers

Commonly used biomarkers to rank foods for their potential for reducing risk of development of type 2 diabetes mellitus are post-prandial glycaemia (American Diabetes Association, 2001) and glycaemic index (a property of available carbohydrate-containing foods that can predict postprandial glycaemia) (Alfenas and Mattes, 2005) and post-prandial insulinaemia. Fasting blood glucose and insulin levels after long-term dietary intervention are commonly used biomarkers (Anderson, 2005). Studies investigating the effect of consumption of lupin-containing baked foods on these biomarkers are presented in Table 2.5.

The findings of post-prandial studies investigating the effects of lupin bread consumption on glycaemia and insulinaemia are conflicting. Lee et al. (2006) reported lower post-meal plasma glucose and insulin response after consumption of ASL-wheat bread compared to white bread meals that were matched for energy intake. The authors explained that the lower total glycaemic carbohydrate load of the ASL bread breakfast (which was lower in starch compared to the wheat-bread breakfast) was the main reason for the lowering effect of lupin on plasma glucose and insulin response. Hall et al. (2005b) reported that addition of ASL flour to wheat bread lowered its post-meal plasma glucose response but increased insulin response. The authors hypothesised that the lowered glucose response may be due to the: (1) higher protein content of lupin bread; (2) higher dietary fibre content of lupin bread; and (3) presence of phytochemicals (e.g. polyphenols), oligosaccharides, phytic acid, tannins and saponins in lupin bread that could slow down starch digestion and glucose absorption. Hall et al. (2005b), postulated that the increased insulinaemia after consumption of lupin bread might be due to amino acids such as arginine and phenylalanine and to stearic acid present in ASL.

The hypoglycaemic efect of lupin may also be in part due to the peptide, γ-conglutin, which accounts for 4-5% of total proteins in mature lupin seed (Duranti

et al., 2008). A purified lupin protein with 47% γ-conglutin was reported to reduce blood glucose in humans (Bertoglio et al., 2011).

Johnson et al. (2003) reported no differences between the post-prandial plasma glucose and insulin responses of a breakfast containing refined wheat bread with added lupin fibre compared to refined wheat-only bread. This suggests the possibility that the proteins (including γ-conglutin), and perhaps the phytochemicals present in the lupin flour and not the purified lupin fibre, may be responsible for the glycaemia and insulinaemia effects seen in the flour studies (Hall et al., 2005a; Hall et al., 2005b; Lee et al., 2006).

Long-term consumption (i.e 1 and 4 mo) of lupin bread did not affect fasting glucose and insulin levels in healthy or overweight/obese subjects (Belski et al., 2011; Hall et al., 2005a; Hodgson et al., 2010). The authors attributed this lack of effect to the difficulty of observing changes in these biomarkers when the baseline values (e.g. fasting glucose pre-intervention) were all within the normal range. Therefore in future studies on the type 2 diabetes protective effect of lupin foods, it is recommended that participants should be at high risk; such as those with insulin resistance or be type 2 diabetics well controlled through diet. Longer term intervention (12 mo), likewise did not reduce fasting glucose levels but did reduce fasting insulin (Belski et al., 2011). The authors suggested that longer term consumption (> 4 months) of lupin bread may lead to improved insulin sensitivity due to its high-protein and dietary fibre contents.

Cardiovascular disease (CVD) biomarkers

Commonly used biomarkers for risk of CVD monitored in dietary intervention studies are total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), LDL-C:HDL-C ratio, triglycerides and blood pressure (Herder et al., 2011). Decreasing TC, LDL-C, and LDL-C: HDL-C ratio, triglycerides, blood pressure and increasing HDL-C in dietary intervention studies are considered beneficial. In a study by Hall et al. (2005a), consuming lupin kernel fibre-supplemented foods, including baked foods, compared to equivalent wheat-only products for 28 d, resulted in a beneficial decrease in TC, LDL-C,

TC:HDL-C ratio, LDL-C:HDL-C ratio and triglycerides, but did not change HDL-C levels. This beneficial effect of lupin dietary fibre on CVD biomarkers may have been due to its high WBC which may have had a cholesterol-lowering effect by increasing viscosity in the gastrointestinal tract and thus reduced the diffusion rate of bile acids, inhibiting their re-absorption (Zacherl et al., 2011). Hall et al. (2005a), also hypothesised that the residual proteins in the lupin kernel fibre may also have played a role in modifying the CVD biomarkers. A recent review by Cam and de Mejia (2012) highlighted the potential of dietary proteins and peptides, including lupin proteins, to beneficially affect CVD risk biomarkers. The potential of lupin to reduce CVD risk biomarkers is supported by studies that involved consumption of food products (i.e. beverage and dietary bars) containing lupin proteins (Naruszewicz et al., 2007; Sirtori et al., 2012).

No significant effects on TC, LDL-C, triglyceride but decrease in HDL-C were found by Hodgson et al. (2010) after participants consumed lupin flour-supplemented bread compared to white bread for 16 wk. The authors attributed the decrease in HDL-C entirely due to the increase from baseline to 16 wk in the control group with no change in the lupin group. According to the authors, the lack of positive effects of lupin consumption on CVD biomarkers may be due to the: (1) insufficient protein contributed by lupin flour to give a significant effect, and (2) high baseline dietary fibre intake of the subjects. These results conflict with the findings of Hall et al. (2005a) probably due to the: (a) use of different lupin flour fractions, lupin kernel fibre by Hall et al. (2005a) vs lupin flour by Hodgson et al. (2010), and (b) the difference in the amount of total dietary fibre consumed by the subjects in the conflicting studies. Hodgson et al. (2010) argued that lupin fibre used by Hall et al. (2005a) was more effective in improving the CVD biomarkers because: (1) isolation and purification of kernel fibre may have altered its chemical structure and physical properties, and (2) use of purified kernel fibre delivered more total dietary fibre in the test diet compared to lupin flour.

Blood pressure is another important CVD biomarker for which the effect of lupin bread consumption has been measured. Consumption of lupin bread for 16 wk resulted in lowered blood pressure compared to wheat-only bread (Belski et al., 2011b; Lee et al., 2009; Yang et al., 2010). The authors suggested that the beneficial

effects observed were related to the high protein content, specifically the amino acid arginine found at high levels in lupin protein, as well as the polyphenols in lupin.

Colonic health biomarkers

Commonly used biomarkers for evaluating the effect of foods on colonic health include intestinal transit time, frequency of defecation, stool weight and faecal short-chain fatty acid (SCFA) content in particular levels of butyrate (Meyer and Stasse-Wolthuis, 2009). Johnson et al. (2006) mentioned that short transit time, and increased frequency of defecation and stool weight are considered to reduce colon cancer risk by decreasing exposure of colonocytes to potential carcinogens that may be present in the bolus. Two studies have reported the effect of lupin consumption on biomarkers for colonic health (Table 2.5). Four-wk addition of lupin kernel dietary fibre to diets consisting of control and lupin fibre incorporated breads, muffin, chocolate muffin, chocolate drink, toasted muesli, pasta and instant mashed potato, resulted in increased frequency of defecation, faecal output, faecal moisture content, faecal butyrate levels and output and decreased transit time, faecal pH, and β-glucoronidase activity compared to products without lupin kernel fibre. The authors suggested that these positive effects on bowel function were due to fibre fermentation in the colon and high water-binding capacity of the residual unfermented fibre in the faeces (Johnson et al., 2006). In another report using the same diets by Johnson et al. (2006), there was areported an increase in the levels of the potentially beneficial *Bifidobacterium* spp. in the faeces and reduced levels of the potentially pathogenic clostridia group (Smith et al., 2006) in diets containing lupin compared to diets without lupin (. The authors (Smith et al, 2006) consequently classified the lupin kernel fibre as a “prebiotic” based on the positive effects of lupin-incorporated diets on gastro-intestinal flora.

Glycaemic index (GI) lowering potential

GI refers to the incremental area under the blood glucose response curve (**AUC**) within a 2-h period from consuming food (e.g. lupin bread) containing 50 g of available CHO, relative to the AUC produced by 50 g of glucose or white bread (Chiu et al., 2011). GI is mainly used for the purpose of labelling food products to

guide consumers in their food intake. According to Buyken et al. (2010), GI of food in the diet has a positive relationship with risk of type 2-diabetes. Lupin bread has demonstrated lower GI than refined wheat bread (Hall et al., 2005b). GI of lupin flour cannot be measured as it has negligible amount of available carbohydrates. However, its addition to wheat flour will lower its GI. Another concept, glycaemic load may be more appropriate in describing lupin flour *per se*. Glycaemic load is calculated by multiplying the amount of available carbohydrates with the GI of the food and divided by 100 (Henry et al., 2005).

Gluten-free

Coeliac disease is an autoimmune intestinal disorder, caused by permanent intolerance to gluten, affecting ~1% of the general population (Niewinski, 2008). The increasing number of diagnosed cases of celiac disease and perceived gluten intolerance has resulted in an increase in the demand for gluten-free products such as breads. Lupin, like any other grain legume, is gluten-free and studies have investigated the use of lupin flour in formulating gluten-free products such as cakes (Levent and Bilgiçli, 2011), and pasta (Capraro et al., 2008). However, there appears to be no published study reporting the use of lupin flour for gluten-free bread formulation. Lupin could however be a suitable substitute to the genetically-modified, more expensive and high-phytoestrogen soybean flour, in producing gluten-free bread. According to Sirtori et al. (2005) the potential negative effects of phytoestrogens in soybeans may have led to growing interest in research on phytoestrogen free legumes such as lupin.

Perceived health benefit as a non-genetically-modified (GM) food

One major advantage of lupin compared soybean is its non-GM status (Dijkink et al., 2008; Pedersen and Gylling, 2000). Due to the “perceived” health and environmental risks of genetic modification of foods and food ingredients, consumers are now demanding more non GM food products (Bredahl, 2001), and one such product is lupin. The more widespread use of lupin as an ingredient in bread could address the growing consumer desire for non-GM products.

Challenges

The high protein and dietary fibre levels in lupin flour can pose some nutritional and health-related challenges when incorporated into bread. These challenges include: lupin allergenicity; presence of flatulence-inducing oligosaccharides; presence of potentially toxic lupin alkaloids and; contamination with phomopsin fungal toxins.

Lupin allergy

Severe allergenic responses to lupin consumption have been recorded (Hieta et al., 2009; Reis et al., 2007; Sanz et al., 2010). A cross-reactivity study, using blood samples from 34 subjects, indicated that the allergenicity was due mainly to the α-conglutin peptide of lupin (Sirtori et al., 2011). Beta-conglutin and γ- and δ-conglutin peptides, have also been reported in review papers as causes of anaphylactic and other allergenic reactions from foods containing lupin (Jappe and Vieths, 2010; Sanz et al., 2010). Food processing has been used for partial or total reduction of allergens by protein denaturation or hydrolysis (Sathe and Sharma, 2009). Several food processing methods have been investigated to reduce the allergenic effects of lupin, including extrusion, autoclaving, boiling and microwave heating (Alvarez-Alvarez et al., 2005) as well as steam pressure at high temperature and short time (Guillamón et al., 2008). Extrusion, boiling and microwave heating had no significant effect on lupin allergenicity determined by IgE-immunoblotting and CAP inhibition using a serum pool from patients with lupin-specific IgE (Alvarez-Alvarez et al., 2005). Autoclaving of lupin seeds at 138^{o}C for 30 min and controlled pressure drop at 6 bar for 3 min, destroyed their allergenic potency (quantified by the CAP-fluorescent enzyme immunoassay system from a serum pool of patients with lupin-specific IgE), without affecting acceptance of the lupin bread as judged by an expert panel (Guillamon et al., 2010). However, the acceptance of lupin bread incorporating heat-treated lupin still needs to be validated as the use of an expert panel in consumer acceptability of foods is deemed inappropriate (Lawless and Hayman, 1999). There is a need to further investigate the effects of other food processing methods (i.e. fermentation, high-pressure treatment) on lupin allergenicity and its effects on acceptability of foods into which the processed lupin is incorporated.

Flatulence

A potential drawback of the use of lupin flour in foods is the presence of high levels of raffinose family of oligosaccharides (RFOs), raffinose, stachyose, and verbascose, which can cause flatulence (Martínez-Villaluenga et al., 2006b). It was reported that raw intact lupin seeds contain 7-15% of RFOs, the highest level amongst all types of grain legumes (Martínez -Villaluenga, et al., 2005a). Oligosaccharides cause flatulence since they are not hydrolysed nor absorbed in the small intestine but instead enter the colon where they are rapidly fermented by colonic microflora (Price et al., 1988). Soaking of legumes, including lupin, has been used as a pre-treatment to reduce their levels of oligosaccharides and hence their flatulence potential (Fernandes et al., 2010). However, soaking of lupin as a pre-treatment may also lead to protein losses as reported by (Wong et al., 2013).

Quinolizidine alkaloids (QA)

A potential food safety issue of lupin consumption is the presence of bitter quinolizidine alkaloids (QAs) (Resta et al., 2008), which can result in moderate acute teratogenic (congenital malformations) toxicity (Erbas, 2010). According to Resta et al. (2008), QA intoxication from feed fed to domestic mammals results in trembling, shaking, excitation and convulsion, and moderate oral toxicity can lead to loss of motor coordination and control. Breeding of varieties of lupin low in QAs has resulted in the current commercial "sweet" varieties of *L. angustifolius*. This breeding program has decreased the QA to safe levels for human consumption (Pilegaard and Gry, 2008). Australian (FSANZ, 2011a) and British (MAFF-DOH, 1996) standards state that the QA content of lupin and lupin products (i.e. flour) should not exceed 200 mg/kg. Sujak et al. (2006) reported that lupin seeds of different species may contain 118-650 mg/kg alkaloids, however processing of the seeds can significantly decrease their levels. Traditionally, lupins were soaked and boiled to eliminate QAs (Annicchiarico et al., 2010). Defatting and drying of lupin seeds (El-Adawy et al., 2001) and dilution by incorporation into food products (Resta et al., 2008) have been reported to decrease the amount of alkaloids in the final product. Evaluation of lupin food products available in the Swiss market,

showed that all samples tested had alkaloid contents below the maximum levels legislated in Australia and Great Britain (Reinhard et al., 2006).

Phomopsins

Lupins, similar to other grains and grain legumes, may be contaminated with phomopsin, the mycotoxins produced by the fungus *Diaporthe toxica* (known formerly as *Phomopsis leptostromiformis*) (European Food Safety Authority, 2012). Phomopsin causes the liver disease *lupinosis* in sheep which can cause death (Prieto-Simón et al., 2007), and these compounds may pose potential health risks to humans. Australia and Great Britain have set the limit for phomopsin content in lupin foods at 0.005 mg/kg (FSANZ, 2011a; MAFF-DOH, 1996). Control of phomopsin relies on breeding resistant varieties (Kurlovich et al., 2002), which has translated to phomopsin-free lupin food products in the Swiss market (Reinhard et al., 2006).

2.7 TECHNOLOGICAL FUNCTIONALITY OF LUPIN IN BREAD MANUFACTURING

Benefits

The protein and fibre components of lupin flour have potential to profoundly influence the technological aspects of bread manufacture, including bread process efficiency and dough and bread sensory qualities. Published reports have demonstrated that a substitution rate of ~10%, lupin can provide the following beneficial effects during bread making (Table 2.6): increased dough stability, mixing tolerance, loaf volume and weight; decreased mixing time; improved tolerance to mixing and handling during fermentation and; delayed staling and bread firmness after 24 h storage (Paraskevopoulou et al., 2010; Guemes-Vera et al., 2008; Doxastakis et al., 2002; Pollard et al., 2002; Dervas et al., 1999; Ballester et al.,1988). Sensory properties of bread were also not affected at a substitution rate of 9% *L. albus* flour to refined wheat flour (Mubarak, 2001).

Table 2.6. Studies investigating the effects of lupin incorporation into bread on dough and loaf quality.

Lupin species/variety	**Lupin fraction**	**Product**	**Lupin Incorporation rate (% of lupin-wheat composite flour)[1]**	**Water incorporation rate (% of total formulation)**	**Bread making process**	**Positive effects of lupin incorporation**	**Negative effects of lupin incorporation**	**Reference**
L. luteus *L. angustifolius*	Wholegrain flours	Sourdough dome bread	10 (not cited)	Based on reference moisture content of raw materials, water absorption and required humidity of the end product	Straight dough	Fermenting the wholegrain flour with *Pediococcus acidilacti* lessened negative effects on quality	Decrease loaf specific volume and porosity Increased crumb hardness	Bartkiene et al. (2011)
L. albus cv. Multolupa	Flour (raw and heat treated)	Pan bread	10 (not cited)	31.4	Straight dough	Panel favoured bread made using heat-treated lupin flour compared to non-heated flour due to texture and flavour	Decreased volume and increased density	Guillamon (2010)
L. albus ssp. Graecus	Protein isolates (albumin and globulin)	Pan bread	5, 10 (not cited)	Farinograph value 500 BU	Straight dough	Increased dough stability Good handling behaviour and tolerance during fermentation stage Delayed staling	Increased development time Decreased dough elasticity and bread volume Increased	Paraskevopoulou et al. (2010)

							hardness, gumminess and chewiness	
L. mutabilis	Flour Protein concentrate Protein isolate	Pan bread	5, 10, 15, 20 2.5, 5.0, 7.5, 10 0.5, 1.0, 3.0, 4.0 (not cited)	Not cited	Straight dough	Decreased firmness compared to wheat-only bread after 24 h Increased specific volume compared to wheat-only bread Texture were rated as good in general	Increased firmness after baking	Guemes-Vera et al. (2008)
L. mutabilis	Flour Protein concentrate Protein isolate	Dough	5, 10, 15, 20 2.5, 5.0, 7.5, 10 0.5, 1.0, 3.0, 4.0 (not cited)	Not cited	Straight dough		Gluten matrix was less interconnected on microscopic examination due to the presence of lupin proteins	Guemes-Vera et al. (2004)
Lupinus albus ssp. Graecus	Flour	Pan bread	5,10 (not cited)	Based on Farinograph value of 500 BU	Straight dough	Increased stability and tolerance of dough at 5 and 10%	Lowered volume as % lupin flour increased	Doxastakis et al. (2002)
L. albus *L. angustifolius*	Flour	Pan bread	2, 5, 10, 15, 20 (bakers flour)	35.7	Straight dough	*L. albus* addition decreased mixing time of dough *L. angustifolius* addition allowed for greater tolerance to over mixing of dough	Decreased dough strength and loaf height Increased darkness of crust and crumb	Pollard et al. (2002)
L. albus	Flour Protein	Pan bread	3, 6, 9 and 12 (not cited)	Based on Farinograph	Straight dough	Up to 6% flour or protein concentrate	Increased dough	Mubarak (2001)

	concentrate Protein isolate			value		could be added and 9% protein isolate without detrimental effects on sensory properties	development time and dough weakening Decreased dough stability and loaf volume	
Lupinus albus ssp. Graecus	Flour (full-fat, concentrated, defatted and concentrated)	Pan bread	5, 10, 15 (commercial wheat flour of medium strength)	Based on Farinograph value of 500 BU	Straight dough	Increased stability and tolerance of dough at 5% addition	Decreased dough strength at 15%additon Lowered volume as % lupin flour increased	Dervas et al. (1999)
L. albus cv. Multolupa	Full-fat flour	Rolled bread	0, 3, 6, 9, 12 (wheat flour enriched with vitamins and minerals; with potassium bromate)	Not cited	Straight dough	Increased loaf volume at all addition levels		Ballester et al. (1988)

[1]Type of wheat flour used in parenthesis

Studies on other baked products such as biscuits, gluten-free cakes and muffins have reported that lupin flour incorporation rates of 20-30% can be achieved without reducing sensory quality and acceptability (Jayasena and Nasar-Abbas, 2011; Levent and Bilgiçli, 2011; Nasar-Abbas and Jayasena, 2012).

Protein crosslinking

The crucial step of gluten matrix formation during bread dough mixing can be explained in part by protein crosslinking, which is the formation of covalent or non-covalent bonds between amino acid side chains in polypeptides, either within a protein or between proteins (Feeney and Whitaker, 1988). Two types of protein crosslinks have been identified during gluten development in bread: disulphide and dityrosine (Gerrard et al., 2005). Disulphide crosslinks are produced from two cysteine residues that are adjacent within a food protein matrix (Lindsay and Skerritt, 1999), while dityrosine crosslinks are formed between two or three tyrosine residues (Tilley et al., 2001).

Lupin does not contain gluten but contains globulins and albumins that do have cysteine and tyrosine residues. Cysteine and tyrosine levels for ASL have been reported as 1.6 and 4.2 g/100 g protein (Petterson et al.1997) while those for wheat have been reported as 2.2 and 1.4 g/100 g protein (Shoup et al., 1966). It is a possibility that the availability of cysteine and tyrosine residues in lupin proteins may result in crosslinks between lupin and gluten proteins and thus can help form the desirable structure for dough and bread. An example of a legume protein that can form a highly viscous and elastic dough, characteristic needed for good bread volume and texture is marama bean protein (Amonsou et al., 2012). It was reported that high levels of tyrosine in marama bean protein led to dityrosine crosslinks in the dough leading to its desirable dough properties (Amonsou et al., 2012). These findings may as well apply to lupin which contains the amino acid tyrosine.

Bread making processes such as mixing, proofing and baking lead to formation of dityrosine (Rodriguez Mateos et al., 2006) and disulphide bonds (Gerrard et al., 2005). The amount of disulphide bonds and the strength of these bonds influence the rheological properties of dough (Shewry and Tatham, 1997) and an optimal level of

disulphide crosslinking during dough mixing is important in bread making (Buchert et al., 2010). Some studies show that increased levels of disulphide bonds can either negatively affect (Manu and Prasada Rao, 2008) or have no effect (Poulsen, 1998) on dough and bread quality. The effects of dityrosine bond concentration on dough and bread quality is not well understood. Tilley et al. (2003) reported that dityrosine bonds form during mixing and baking contributing to formation of gluten network, however the authors did not investigate the levels of tyrosine bonds formed and how their level relates to quality. In contrast, other investigators have reported that dityrosine levels do not influence gluten formation (Pena et al., 2006; Rodriguez Mateos et al., 2006) and consequent dough and bread quality. The beneficial effect of up to 10% lupin flour incorporation to wheat bread may possibly be due to crosslinking between wheat and lupin proteins. There is however still a need to further investigate the effects of the levels of disulphide and dityrosine bonds on lupin-wheat dough and bread quality.

Most of the reported studies for lupin bread manufacturing (Table 2.6) have used the straight-dough method. However the sponge and dough process may be a more effective method for incorporating lupin into bread because of its robustness to process fluctuations (i.e. mixing and proofing). It may be useful to establish separate mixing and proofing parameters (i.e. time and/or temperature) for the wheat sponge and the lupin sponge given the differences in their physical and chemical properties (Hall et al., 2005b) which may contribute to differences in their rheological properties. Separate mixing and proofing for wheat and lupin sponges would allow the wheat gluten matrix to develop by hydration of proteins and starch from wheat, without disruption by the high water binding of the dietary fibre and low-elasticity proteins in lupin flour and thus may help reduce the negative effects of lupin flour addition. There is now a need to explore the use of sponge and dough method in making lupin breads.

Anti-staling properties

Ronda and Roos (2011) defined staling in bread as hardening of the crumb, mainly caused by starch retrogradation, in which water distribution plays a critical role. When starch retrogradation occurs, water molecules are incorporated into crystallites

(as moisture is redistributed from gluten to starch) causing dehydration of gluten which results to crumb hardening (Gray and Bemiller, 2003). Moisture migration from crumb to crust can also lead to crumb staling and can increase firming rate (Baik and Chinachoti, 2000). According to Hug-Iten et al. (2003), another important determinant of crumb firmness is the extent of gluten network plasticisation (fluidity), and water is the most important plasticiser in food; highlighting that high water absorption during mixing, proofing and baking of bread can delay staling. The high WBC of lupin (Turnbull et al., 2005), has potential to lead to staling-inhibition by providing the extra moisture to prevent gluten dehydration and slowing down of firming rate (by retaining more moisture in the crumb); and by providing plasticising function. According to Gray and Bemiller (2003), proteins also can delay bread staling by diluting and interacting with starch leading to reduced extent of starch retrogradation and by serving as a moisture reserve to reduce firming rate. In support of this hypothesis, substitution of wheat flour with 10% lupin protein isolates has been reported to delay bread firming (Paraskevopoulou et al., 2010). There is however only limited information on the effects of lupin flour addition to bread on staling.

Challenges

The main quality problem arising from lupin incorporation into wheat bread is low loaf volume and hard and chewy texture, most likely due to the low-elasticity of lupin proteins and the high water binding capacity of lupin dietary fibre. In addition, microscopic examination of wheat and lupin flour doughs has revealed that the gluten matrix was less interconnected in the presence of lupin proteins (Güemes-Vera et al., 2004).

There are published studies on approaches to improve the quality of bread supplemented with gluten-free flours which may be applicable to lupin flour. Angioloni and Collar (2012a) reported that high-pressure treatment of non-wheat flours (i.e. oats, millet and sorghum) resulted in more acceptable breads than using untreated flours at substantial (40-60%) rate of wheat flour substitution. The authors postulated that high-pressure treatment may have altered the folding/unfolding and the aggregation/disaggregation of the flour proteins improving their functionality in

bread. This may have been due to the pressure-induced denaturation of proteins leading to increased reactivity of sulphydryl bonds and higher disulphide crosslinking (Galazka et al., 2000). No study however has used high-pressure treatment with the aim of improving the quality of lupin-supplemented bread.

The use of bread improvers has been widely used to improve quality of breads supplemented with non-wheat flours. Joye et al. (2009) summarized the various bread improvers which are either chemicals (e.g. potassium bromate, iodate, chlorine dioxide azocarbonamide, ascorbic acid and peroxides) or enzymes (e.g. transglutaminase, glucose oxidase, hexose oxidase), which promote the formation of covalent bonds between gluten matrix proteins during bread making. Joye et al. (2009) also presented the mechanisms of action of these additives. In general, the chemical agents act as oxidants of the cysteine (SH) residues and tyrosine (OH) residues to form crosslinks whereas the enzymes act as catalysts for the oxidation of these same residues to produce disulphide and dityrosine crosslinks, or in the case of transglutaminase, the crosslinking of lysine and glutamine residues.

The use of chemical agents has been a major safety concern for consumers, manufacturers and regulatory agencies and thus enzymes are considered as safer alternatives in bread making. The effects of enzymes in non-wheat flour supplemented- or gluten-free breads (which may be applicable to lupin bread), were explored by several investigators (Alaunyte et al., 2012; Gujral and Rosell, 2004; Renzetti and Arendt, 2009; Renzetti et al., 2010; Ribotta et al., 2010; Roccia et al., 2012). There is now a need to investigate the effects of enzymes for protein-crosslinking in lupin-wheat bread making and the association between crosslinking level and dough and bread quality of lupin-wheat composite flour breads.

The use of carbohydrate-degrading enzymes and sourdough fermentation have been widely applied to improve the quality of high-fibre baked products and therefore such approaches may be applicable to lupin bread. An example is the enzyme xylanase that degrades and thus reduces the water binding properties of non-starch polysaccharides (NSP) (Courtin et al., 2001). The action of xylanase leads to a redistribution of water from the NSP to the gluten matrix (Shah et al., 2006), This in turn prevents the undesirable effects of the high water binding capacity of NSP on

dough quality and consequently bread quality. Sourdough fermentation or use of lactic acid bacteria to leaven the bread can enhance quality of lupin-wheat bread. A study by Bartkiene et al. (2011) showed that the sourdough fermentation by *Pediococcus acidilacti* of lupin flour (at 10% substitution of wheat flour), resulted in better bread quality compared to breads produced using unfermented flours. According to Ktenioudaki and Gallagher (2012), sourdough fermentation alters dough components through acidification, proteolysis of gluten and starch hydrolysis leading to improved quality of high-fibre breads. Sourdough fermentation of breads using wheat with coarse durum wheat bran, (Rizzello et al., 2012) and composite non-wheat flours (i.e. buckwheat, amaranth, chickpea, and quinoa flours) (Coda et al., 2010) resulted in improved textural, sensory and nutritional properties compared to breads that did not undergo sourdough fermentation. To date, however there are very few studies investigating the potential of sourdough fermentation to produce high quality lupin-wheat bread.

The optimisation of water incorporation rate is a critical step to maximise the quality of dough and bread. Most studies on lupin bread formulation have however not focused on this aspect (Table 2.6). In some studies, the amount of water used for the control breads (wheat bread) were the same for the lupin-wheat breads (Guillamon et al., 2010; Pollard et al., 2002). However, as previously explained lupin dietary fibre disrupts gluten matrix due to its high WBC which necessitates adjustment of water added to the dough. The amount of water added to lupin-wheat composite flour needs to be increased to account for the water tightly bound by the lupin dietary fibre and the free water needed to form the gluten matrix in the dough. Likewise, most published studies reported the effects of discrete levels for water and lupin incorporation rates without examining the interactive effects of these two parameters on the quality of lupin-wheat dough and bread. In addition, no in depth process optimisation studies aimed at simultaneously optimising multiple processing parameters have been reported for lupin-wheat bread.

A useful methodological and statistical approach that could be applied to optimisation of bread formulation and processing is response surface methodology (RSM). RSM is a collection of mathematical and statistical methods that are efficient in the modelling and analysis of experiments or situations in which an output or

response of interest is dependent on several factors, and the aim is to optimise the response (Montgomery, 2009). RSM had been used to optimise formulation and process parameters of other "healthy" breads such as wholemeal oat bread (Flander et al., 2007) and gluten-free breads (McCarthy et al., 2005; Sanchez et al., 2004). Likewise, studies have been reported optimizing both the formulation and processing method of breads made from blends of wheat and legume flours using statistical designs including RSM (Angioloni and Collar, 2012b; Jideani and Onwubali, 2009; Yamsaengsung et al., 2010). Similar optimisation studies are however still required to optimise both the formulation and processing variables in lupin-wheat bread manufacture to maximise lupin addition whilst maintaining acceptable sensory quality and consequently maximising the nutritional and health potential of the bread.

Another challenge for the incorporation of lupin into wheat bread is the potential for undesirable aftertaste. Sensory evaluation of baked products with lupin flour showed that consumers detected aftertaste or unusual taste (Hall and Johnson, 2004). Incorporation of more than 30% lupin flour in muffins and 20% in biscuits lowered flavour acceptance of the products, which was attributed to a beany flavour imparted by lupin (Jayasena and Nasar-Abbas, 2011; Nasar-Abbas and Jayasena, 2012). Lupin flour from *L. angustifolius cv. Boregine* has been described as having a grassy, metallic, fatty, hay-like, meat-like and cheese-like odour characteristics (Bader et al., 2009). Volatile compounds (e.g. pyrazines, aldehydes, alcohols, ketones) were also detected when lupin protein isolate (LPI) was added to bread (Paraskevopoulou et al., 2012).

Studies have been conducted to resolve this issue of off-odours/ flavours in lupin-based foods. It has been reported that roasting lupin seeds may help remove its "beany" flavour (Yañez et al., 1986). A patented method for *L. albus* flour suggests an optional step of heating seeds to inhibit lipoxygenase activity extends the shelf-life of the flour (Auger and Corre, 1993) and possibly products incorporated with the heated lupin flour. Inactivation of lipoxygenase prevents hydrolysis of fatty acids in lupin that causes rancidity. De-oiling of lupin flakes with ethanol and 2-propanol resulted in protein isolates with less "legume-like" flavour and improved consumer acceptance without affecting technological functionality of the lupin flakes (Bader et

al., 2011). However, the use of a trained panel by the authors (Bader et al., 2011) for consumer acceptance of the de-oiled lupin flakes may not be appropriate (Lawless and Hayman, 1999), since there is a requirement to validate the results of the consumer acceptance using untrained panel. Sourdough fermentation may also have the potential to reduce undesirable flavours imparted by lupin when used in bread. Schindler et al. (2011) found that volatile compounds (determined by gas chromatography-olfactometry (GCO) using 2 trained panellists) produced from sourdough fermentation of lupin protein extracts (and potentially of lupin dough), with sweet, solvent, fungal, musty, earthy, burnt, dusty or cereal-like characteristics , may have masked the undesirable odorants in lupin. This was evident in the higher overall and flavour acceptance scores of breads with sourdough fermented lupin flours compared to unfermented breads (Bartkiene et al., 2011).

OTHER LUPIN FOOD USES

Aside from wheat-based food products, lupin has been used in a wide range of other foods. Lupin fibre and protein isolates have been used as fat replacers and vegetable protein extenders in meat products such as sausages and frankfurters (Alamanou et al., 1996; Archer et al., 2004). Lupin also has the potential to be used as dairy substitute in ice cream (Yap, 2006), and fermented milk (Martínez-Villaluenga and Gómez, 2007). Lupin may also be used as a substitute for egg yolks in brioche due to its yellow colour and emulsifying properties (Kohajdova et al., 2011). Jayasena et al. (2010) developed a lupin-based tofu analogue. Other Asian fermented foods that have successfully incorporated lupin include analogues of tempe (a traditional Indonesian food), miso (Japanese condiment and soup base) and soy sauce (Sipsas, 2008). The nutritional and technological functionality of lupin fractions in food should be further explored using other food products (e.g. snacks, cereal bars, confectionery) to maximise the potential of lupin as a healthy alternative to traditional ingredients.

2.8 CONCLUSION

This review presented a comprehensive and critical analysis of published scientific work on the nutritional, health and technological functionality of lupin flour addition to bread and other baked products. Scientific evidence shows that incorporation of lupin flour into baked products is accompanied by both benefits and challenges. The high protein and dietary fibre contents of lupin provide a "double-edged sword" effect when lupin flour is used to substitute for wheat flour in bread. Evidence has been presented that supplementing baked products with lupin flour improves their nutritional profile mainly through increased protein and dietary fibre. There is mounting evidence that these lupin products when included in the diet can reduce biomarkers of risk of obesity, cardiovascular diseases, type 2 diabetes mellitus and bowel dysfunction. In addition, lupin is gluten-free, low in anti-nutritional factors compared to other legumes, and has antioxidant activities. Lupin may also be a more suitable alternative to soybeans as it is not genetically modified, has lower levels of phytoestrogens and is lower in cost. On the other hand, lupin protein allergens, dietary fibre-induced flatulence, and to a minor extent, risk of toxic effects of alkaloids and phomopsins, pose some health-related barriers for the widespread use of lupin flour in baked products.

Investigations on lupin flour incorporation into baked products have demonstrated that a 10% rate substitution of wheat flour resulted in equal or better quality compared to wheat-only bread. Technological drawbacks such as lowered volume, denser pore structure and firmer crumb in the final product are common when lupin substitution was beyond 10%. These negative effects may be attributed to the low elasticity of lupin proteins, and high water binding of its dietary fibre; both of which interrupt the development of the desired wheat gluten network. This review has highlighted the lack of evidence on species/varietal differences in the effects of lupin flour incorporation into bread. Likewise, there is a need to investigate the effects of bread making on the potentially anti-diabetic peptide γ-conglutin. The information on the role of protein crosslinking in lupin-wheat dough and bread structure and how this crosslinking can be manipulated to optimise bread quality needs to be explored. Lastly, systematic optimisation of the formulation and processing parameters of

lupin-wheat bread, to maximise lupin incorporation rate and nutritional benefits whilst maintaining bread quality is lacking.

CHAPTER THREE-Experimental

Varietal differences in composition of lupin (*Lupinus angustifolius)* flour and lupin-wheat flour composite breads

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3.1. ABSTRACT

Flours of six Australian sweet lupin (*Lupinus angustifolius*; ASL) varieties: *Belara*, *Coromup*, *Gungurru*, *Jenabillup*, *Mandelup* and *Tanjil* and their ASL-refined wheat composite flour breads (at 20% ASL flour incorporation) were evaluated for their nutritional (including protein quality of bread) and chemical composition and compared to refined wheat flour and 100% refined wheat flour bread. Protein, dietary fibre, total phenolic and total carotenoid content and antioxidant activity were significantly ($p<0.05$) higher and available carbohydrates significantly lower ($p<0.05$) in all ASL flours compared to the wheat flour and in all ASL-wheat breads compared to wheat-only bread. Likewise, protein quality (i.e. protein digestibility corrected amino acid score, PDCAAS) of ASL-wheat breads were significantly ($p<0.05$) higher compared to wheat-only bread. There was a significant effect ($p<0.05$) of ASL variety on the protein, fat and total phenolic content and antioxidant activity of the ASL flours and on the dietary fibre, fat, available carbohydrate, PDCAAS and total polyphenolic content and antioxidant capacity of the ASL-wheat bread. These results will aid in choosing which ASL variety can be used to most improve nutritional and health functionality of wheat bread.

3.2 INTRODUCTION

Bread, being one of the most consumed food items in the world, is a main source of nutrition for humans (Collado-Fernández, 2003). It is forecasted that the global

market for bakery products, including bread, will amount to US$ 410 billion by 2015 (Anon, 2011). Along with this growth in demand for bakery products is the increasing shift of consumer preferences towards healthier baked goods with high dietary fibre, protein, vitamins and minerals. Bread is traditionally produced using wheat flour due to its proteins, gliadins and glutenins (Goesaert et al., 2005) that help provide the desired bread texture and volume. However, nutritional and health issues arise from the over-consumption of baked products made with refined wheat flour. It was reported that refining of wheat leads to significant losses in protein, dietary fibre, vitamins, minerals and phytochemicals (Rosell, 2011). Non-wheat flours from cereals and grain legumes have been added to refined wheat flour in bread to improve nutritional value and meet consumer demands. Australian sweet lupin (ASL) is a grain legume which can potentially enhance the nutritional profile of refined wheat flour. ASL is so named due to its very low level of bitter alkaloids that render it suitable for human consumption.

ASL kernel flour has been described as having a pale yellow colour and slight beany flavour (Hall et al., 2005). ASL flour is a rich source of nutrients with higher protein (~40%) and dietary fibre (~40%) but lower energy value compared to wheat flour (Hall and Johnson, 2004). ASL seeds also contain other potentially health beneficial components including carotenoids (Wang et al., 2008), phenolics (Oomah et al., 2006), and a range of vitamins and minerals (Petterson et al., 1997). ASL flour has been used in various food products such as muffins, cookies and brownies (Hall and Johnson, 2004; Nasar-Abbas and Jayasena, 2012), biscuits (Jayasena and Nasar-Abbas, 2011), and noodles (Jayasena et al., 2010). In addition, lupin is a global major rotation crop for sustainable farming systems such as wheat and other cereals, due to its nitrogen fixation ability (French, et al., 2008; GL-PRO, 2005).

Studies show that substitution of wheat by lupin flour and its fractions (i.e. protein isolates and concentrates) can significantly improve nutritional profile of wheat bread (Belski et al., 2011; Hall and Johnson, 2004; Mubarak, 2001). In addition the amino acid profile of lupin complements that of wheat, which is higher in sulphur-containing amino acids (i.e. methionine) but lower in lysine. Therefore, addition of ASL flour into wheat bread has the potential not just to increase the protein content but improve the amino acid balance and protein quality of the final product (Duodu

and Minnaar, 2011). Addition of ASL flour to refined wheat bread has also been reported to decrease its glycaemic index (Hall et al., 2005). Aside from its valuable nutritional profile, ASL foods have demonstrated potential through clinical trials to decrease risk factors for obesity (Lee et al., 2006), cardiovascular disease (Belski et al., 2011) and gastrointestinal problems (Johnson et al., 2006) which were attributed to lupin protein and dietary fibre content. Substitution of refined wheat flour with lupin flour in bread may further enhance its health functionality due to its bioactive compound, γ-conglutin, a blood-glucose lowering peptide (Bertoglio et al., 2011).

Examples of ASL varieties commercially grown in Australia include *Belara, Coromup, Gungurru, Jenabillup, Mandelup and Tanjil* (French et al., 2008). However, there is a lack of information on the differences in nutritional and phytochemical composition of these commercially-produced ASL varieties when incorporated into ASL-wheat composite flour bread.

This study aimed to compare the nutritional and chemical characteristics of ASL flour and ASL-wheat composite flour bread made from different varieties of ASL.

3.3. MATERIALS AND METHODS

Materials

Six varieties of ASL seeds namely *Belara, Coromup, Gungurru, Jenabillup, Mandelup* and *Tanjil* were used in the study. These varieties were chosen as they are varieties commercially grown in Western Australia. Five kg of each variety were obtained from the Department of Agriculture and Food Western Australia (DAFWA). Seed samples were harvested in 2010 from Geraldton, Western Australia except *Mandelup* which was grown in Wongan Hills, Western Australia. Both Geraldton and Wongan Hills belong to Northern Agricultural Region and are in the same climatic zone (Zone 4) (ABCB, 2012). The seed samples were vacuum-packed in plastic bags and stored at ~10^{o}C until use. The kernel was separated from the seed coat by using an LH 5095 dehuller (Codema Inc., MN, USA) followed by air-induced separation and manual sorting. The kernels were milled (Retsch ZM200, Retsch GmbH, and Haan, Germany) to pass 100% through a 250 µm sieve.

Duplicate 1 kg flour samples for each variety was vacuum-packed in plastic bags and stored at ~ 10^{o}C until use.

Western Australian produced refined wheat flour ("bakers flour") was purchased from Miller's Food (Byford, WA, Australia). Bread making ingredients i.e. dry yeast (Tandaco, Cerebos Export, Seven Hills, NSW, Australia), bread improver (Healthy Baker, Manildra Group, Gladesville, NSW, Australia), sugar (Coles Brand, Tooronga, VIC, Australia), salt (Coles Brand, Tooronga, VIC, Australia), and vegetable oil (Crisco, NSW, Australia) were purchased from Coles Supermarket (Perth, WA, Australia). The bread improver contained stearoyl lactylate, soy flour, calcium sulphate, ascorbic acid, L-cysteine monohydrochloride, wheat flour, maltflour and amylase. It has been reported that the use of oil in bread does not increase bread volume as solids fat do (Watanabe et al., 2002). However, there is an increasing trend in the use of oil instead of the solid fat in the baking industry for reasons of health and nutritional considerations, availability, bulk handling and storage and reduced usage level (Kamel, 1992). Kamel (1992) reported that disadvantages of using vegetable oil in bread include slow proof time, poor oven spring, open grain, dull crumb, weak side walls and low loaf volume which can however be countered with the use of surfactants. Surfactants or surface active agents are typically used as ingredients in bread improvers such as the one used in this study which contained stearoyl lactylate. The use of bread improver in this study allowed the use of vegetable oil without compromising the quality of the bread. Chemical reagents for analyses were all of analytical grade and were supplied by Thermo Fisher Scientific Pty Ltd. (Scoresby, VIC, AUS) and Sigma-Aldrich (St. Louis, MO, USA)

Experimental design

The study composed of duplicates of: six ASL flour varieties and six breads from ASL-wheat composite flour; one refined wheat flour and one 100% refined wheat flour bread. ASL-wheat bread samples were prepared in a randomised order over a total of 5 d. Three to four samples were prepared each day, which included a dummy control (wheat bread), internal control (wheat bread), and 2 to 3 ASL-wheat bread samples. The dummy control was baked at the start of the day to condition bread

making equipment (i.e. mixer, proofer and oven) and was discarded after baking. The order of internal control samples was randomised within each baking session. A total of 9 bread samples were produced for each replicate sample. Three samples from each treatment were chosen randomly for analyses.

Bread making

The sponge and dough method (Figure 3.1) at 20% ASL flour substitution for wheat flour was used in this study. Preliminary studies (unpublished) at the Centre for Grain Foods Innovation (CGFI, Kensington, WA, Australia) indicated that the use of this sponge and dough method for lupin-wheat composite flour bread resulted in better bread quality characteristics compared to the straight dough process. Separate mixing and proofing for wheat sponge and lupin sponge may allow for the initial development of the wheat gluten matrix without its disruption by the high water binding capacity of lupin dietary fibre (Turnbull et al., 2005). In this study, we fermented the sponges and dough for a total of 1 h and 35 min instead of the typical 2 to 3 h for the sponge and dough method. During the preliminary studies (unpublished) fermentation of the lupin sponge and dough for more than 2 h, led to intense off odour and flavour (beany like) being perceived in the resulting bread which may have been due to lipoxygenase activity.

Five hundred gram doughs were prepared using a composite flour (58.7% of the total ingredients) comprising of 234.8 g wheat flour and 58.7 g ASL flour. Water (183.5 g) was used at 36.7% of total ingredients. The remaining ingredients (4.6% of total ingredients) comprised of 7.5 g yeast, 5.5 g vegetable fat, 4 g bread improver, 3 g salt and 3 g sugar.

Wheat and ASL flour sponges were prepared separately. Refined wheat flour (88.05 g) was combined with 55 g water, 4 g bread improver, and 6 g yeast, and mixed for 2.5 min using a Hobart N50 mixer (Hobart, Troy, OH, USA) set at low (No. 1) speed. ASL flour (58.7 g) was combined with 101.22 g water and 1.5 g yeast. Refined wheat and ASL flour sponges were proofed separately at 35^{o} C and 80% RH for 1 h. The two sponges were then combined and mechanically mixed for 6.5 min

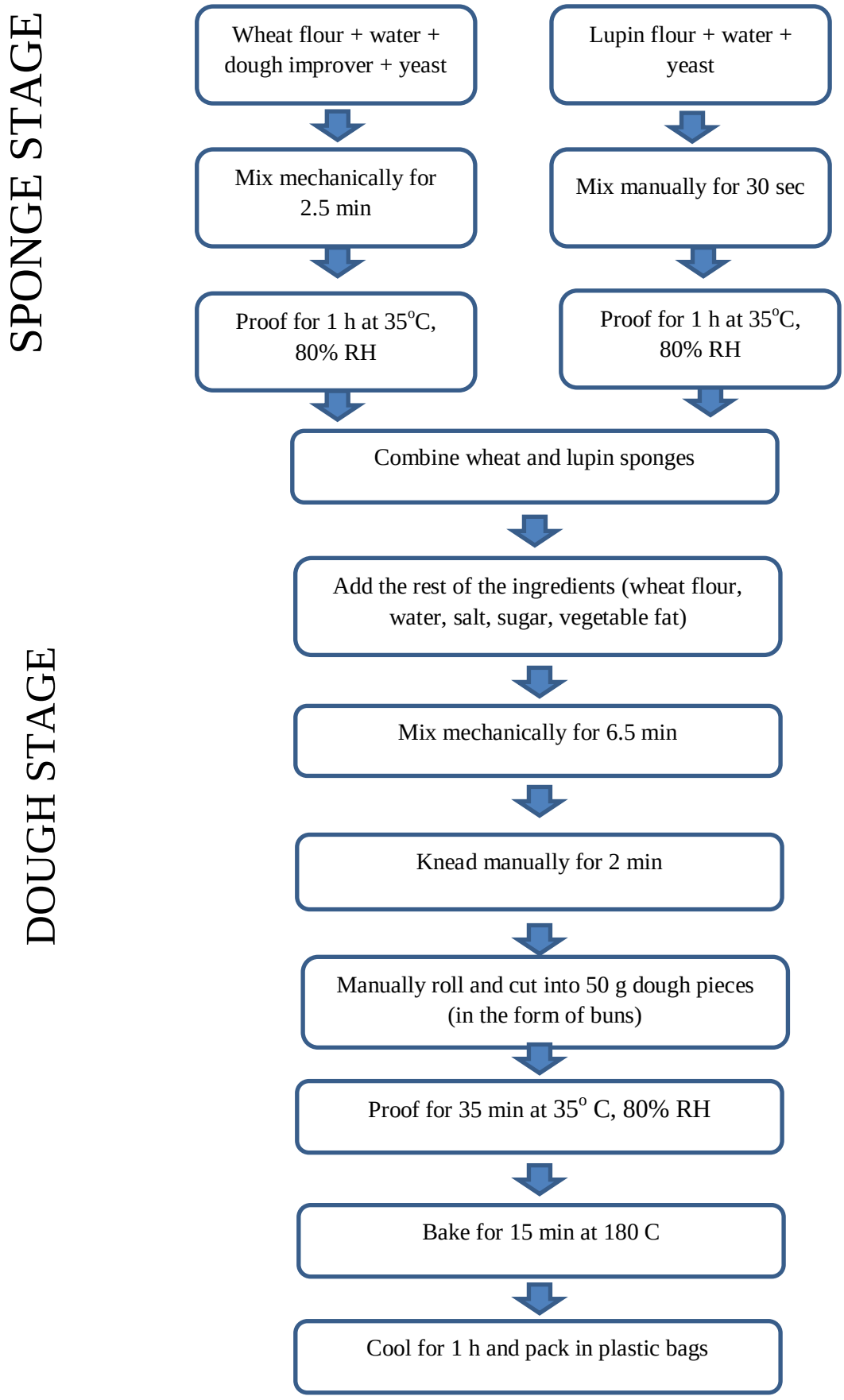


Figure 3.1. Sponge and dough method used in making ASL-wheat and wheat breads

with the remaining ingredients (146.75 g wheat flour, 27.27 g water, 3 g salt, 3 g sugar and 5.5 g vegetable fat) using a Hobart N50 mixer (Hobart, Troy, OH, USA) with a dough hook, set and maintained at low (No. 1) speed. The Hobart mixer was used as this is a typical mixer used in commercial mechanical mixing of dough. The resulting dough was kneaded for 2 min, hand-rolled, cut and formed (by the author of this thesis to ensure consistent dough handling across treatments; kneading by hand was done using constant forward and backward rolling with the aim of using the same force throughout the entire baking day) into 50 g dough pieces (as buns) and proofed at 80% RH for 35 min. The average final dough temperature was 31^{o}C.

After proofing, the dough pieces were baked at 180^{o}C for 15 min in a convection oven (LG Oven LF 96105SS, LG Electronics Inc., Eastern Creek, NSW, Australia). The bread bun samples were cooled at room temperature for 1 h on a baking rack before packing and sealing in plastic bags. For chemical analysis, 3 randomly selected breads were freeze-dried (Alpha 1-2 LD Plus, Christ, Osterode, Germany) for 48 h. The freeze-dried samples were ground (100% though <500 µm sieve) using a Retsch Grindomix GM 200 (Retsch GmbH, Haan, Germany), and stored in moisture proof containers at -20^{o}C until analysed.

Analytical methods

Proximate composition of flours

All analyses of flour and bread samples were conducted in at least duplicate measurements. Proximate composition was determined using AOAC Methods (AOAC, 2008). Moisture content was determined using the AOAC oven drying method, 925.10. Crude protein (Kjeldahl digestion and distillation method, N × 5.7 for refined wheat flour and bread; N × 5.4 for ASL flour and N × 5.66 for ASL-wheat bread) was measured according to AOAC Method 920.87. Total dietary fibre (TDF) content was determined using the Megazyme TDF Kit KTDFR (Bray, Co.Wicklow, Ireland) based on AOAC enzymatic gravimetric method. Crude fat was determined by petroleum ether extraction (Buchi E-816, Flawil, Switzerland) using AOAC Method 945.16, and ash was determined by dry-ashing at 550^{o}C according to

the AOAC Method 923.03. Total available carbohydrates were calculated by difference, i.e. 100 − (% moisture +% protein + % fat + % ash + % TDF). All values were expressed as g/100 g dry sample.

Protein quality

Amino acid content of bread samples

Amino acids profile of the breads was analysed by the ChemCentre (Bentley, WA, Australia) using high-performance liquid chromatography HPLC with UV detection. The bread samples were hydrolysed with 6N HCl for 24 h. After hydrolysis, the solution was diluted, filtered and neutralised with sodium hydroxide (Llames and Fontaine, 1994). All individual amino acids were determined, except for cysteine and cystine which were degraded to cysteic acid and were reported as a combined outcome based on AOAC Method 994.12 (AOAC, 2008). Tryptophan was not measured due to the limitation of the available method for quantifying this amino acid. Since amino acids have low absorptivity in the UV/Vis range, the analysis used pre-column derivatisation to form adjunct compounds which have high absorption in the UV and therefore could be determined by HPLC with greater sensitivity.

The amino acid content (mg/g protein) was calculated by dividing the amino acid content of the sample (mg/100g) (Appendix 1) with the protein content of the sample (g/100 g) (Table 3.1) as shown in Eq. 3.1.

$$\text{Amino acid content} = \frac{\text{amino acid of the sample } (\frac{\text{mg}}{100\text{g}})}{\text{protein content of the sample } (\frac{\text{g}}{100\text{g}})} \quad \text{Eq. 3.1.}$$

Amino acid scoring

Amino acid scores were calculated according to the FAO/WHO (1991) computation of amino acid scores. Amino acid score was calculated by dividing the amino acid content of the bread samples (mg/g protein) by the suggested reference pattern of amino acid requirements for pre-school children (2-5 y.o.) for 9 essential amino

acids plus tyrosine and cysteine. The suggested reference patterns (mg/g protein) are as follows: Histidine, 19; isoleucine, 28; leucine, 66; lysine, 58; methionine + cysteine, 25; phenylalanine + tyrosine, 63; threonine, 34; valine, 35 (FAO/WHO, 1991)

In-vitro protein digestibility (IVPD) and protein digestibility corrected amino acid score (PDCAAS)

IVPD of the breads was determined following the modified pepsin pancreatin digestion method of Faki et al. (1984) and Akeson and Stahmann (1964). Bread samples equivalent to approximately 50 mg protein were incubated at 37^{o}C with 0.75 mg pepsin (in 7.5 ml of 0.1 N HCl, pH 2; 2500 units/mg activity, Chem-Supply, Gillman, SA, Australia) for 3 h. The solution was neutralized with 3.75 ml of 0.2 N NaOH. The 2 mg pancreatin (in 3.75 ml of pH 8.0 phosphate buffer; Chem-Supply, Gillman, SA, Australia) was added and the sample incubated for 24 h at 37^{o}C. The undigesteded protein in 5 ml of digesta was then precipitated by addition of 25 ml of 10% TCA and centrifuged for 30 min at 1000 x *g*. Nitrogen in the supernatant was determined using the Kjeldahl digestion and distillation method following the method used for bread samples (i.e. proximate composition of flours) in this Chapter. IVPD was calculated by expressing the difference between total nitrogen and residual nitrogen as a percentage of total nitrogen in the sample (Eq. 3.2). PDCAAS was determined by multiplying the IVPD with the limiting amino acid score for each sample (Eq. 3.3). The limiting amino acid score is the score of the amino acid with the lowest amino acid score.

$$\text{IVPD}=\left(\frac{\text{Total nitrogen-nitrogen in the supernatant}}{\text{Total nitrogen}}\right) \text{x } 100 \qquad \text{Eq. 3.2}$$

$$\text{PDCAAS}=\text{IVPD x limiting amino acid score} \qquad \text{Eq. 3.3}$$

Electrophoresis and Western blotting

Extraction of proteins. Extractions of ASL and wheat flour proteins, and ASL-wheat and wheat bread proteins were performed according to the method of Capraro et al.

(2008). Five mg of bread or 2 mg of flour were weighed into 1.5 mL Eppendorf tubes. The samples were suspended in 100 µL of extraction solution comprising of 8 $molL^{-1}$ urea, 20 mg/ml CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate and 65 $mmolL^{-1}$ 1,4-dithiothreitol (DTT). Extraction was carried out under shaking at room temperature for 2 h. The slurry was centrifuged at 10,000 x *g* for 30 min at room temperature, and the supernatant containing the dissolve protein was separated and were kept frozen at 80^{o}C until use.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE of the flour and bread proteins under reducing conditions was performed using a method as reported by Wong et al. (2013). NuPAGE Novex 10% Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) were used for the electrophoresis. Protein samples were diluted with NuPAGE sample buffer (Invitrogen) to give 5µg of protein in the 10µL of final solution that was loaded onto the gel. Electrophoresis was performed with MES SDS running buffer (Invitrogen) at 200 V for 1 h until the electrophoretic front was approximately 1 cm from the bottom of the gel. Proteins were fixed and stained using 50 mL Bio Safe Coomassie G-250 stain (Bio-Rad Laboratories, Hercules, CA, USA). Destaining was performed by washing and soaking the stained gels 5 to 10 times with deionized water. The molecular weights of the major peptide bands were estimated by comparison with bands of molecular weight markers (Prestained SDS-PAGE standards, broad range, Bio-Rad). The lupin protein subunits (α, β, γ and δ conglutins) were then tentatively identified by comparing their estimated molecular weights with literature values and subjectively quantified by visual assessment of band staining intensity.

Western blotting. Verification of the identity of γ-conglutin peptides in the flour and bread samples was performed using Western blotting based on the method of Foley and Singh (2002). Unstained SDS-PAGE gels (prepared as described above) with molecular weight markers were placed in transfer chambers filled with 1 L transfer buffer (100 mL of tris-base glycine solution [24 g tris-base and 112 g glycine per 1L distilled water]), 2.5% SDS, 200 mL of methanol and 700 mL of distilled water). Separated proteins on the SDS-PAGE gels were transferred by electroblotting onto nitrocellulose filters (Amersham Hybond-C, GE Healthcare Australia Pty. Ltd., Rydalmere NSW, Australia) overnight at 25 volts and 4^{o}C with stirring. The

nitrocellulose filter was then incubated with ~ 30 mL of Tris-buffered saline and Tween-20 (TBST) solution for 30 min at room temperature with gentle shaking. The TBST solution was prepared by mixing 50 $mmolL^{-1}$ Tris (pH 7.5), 200 $mmolL^{-1}$ NaCl and solution and 0.05% (v/v) Tween-20. After incubation with TBST, the nitrocellulose filter was then incubated in 20 mL of the TBST blocking solution containing 20 µL primary antibody (Rhonda Foley, CSIRO, Floreat, WA, Australia) overnight at room temperature with gentle shaking. The TBST blocking solution was prepared by adding 10% (w/v) skim milk powder (Diploma, Fonterra, Auckland, New Zealand), to the TBST solution. The primary antibody was a rabbit serum containing polyclonal antibodies raised against the ~30 kDA and ~50 kDA γ-conglutin subunits. After this incubation, the filter was washed (3x, 10 min each wash) with TBST solution. The washed filter was incubated in 20 mL TBST solution with 0.4 µL secondary antibody (monoclonal anti rabbit IgG alkaline phosphatise conjugated; Sigma-Aldrich (St. Louis, MO, USA) for 30 min at room temperature with gentle shaking and then washed with TBST solution (3x, 10 min each wash). The washed nitrocellulose filter was then incubated with 1-2 mL of 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT; Sigma-Aldrich, St. Louis, MO, USA) until intense bluish purple bands of γ-conglutin developed (~ 1-2 min). The nitrocellulose filter was then dried at room temperature.

Phytochemical analysis

Alkaloids

Quinolizidine alkaloid (QA) content of ASL and wheat flour samples was analysed by the ChemCentre (Bentley, WA, Australia) based on the method by Harris and Wilson (1988). Alkaloids were extracted with a polar solvent, derivatised and analysed using an Agilent 6890 Gas Chromatograph (Agilent Technologies Australia Pty Ltd, VIC, Australia) using a flame ionisation detector (FID). QA content of the samples was expressed as g/100 g dry sample.

Oligosaccharides

The amount of raffinose family of oligossacharides (RFOs) of ASL flour samples was determined by the ChemCentre (Bentley, WA, Australia). Defatted flour samples were extracted with 70% v/v ethanol at 65°C for 30 min after which the solvent was removed under reduced pressure. The RFOs in the residue were extracted with water, and then separated and quantified by High-Performance Liquid Chromatograph (HPLC) with refractometer detection (Smith et al.,1986). RFO content of the samples were expressed as g/100 g dry sample.

Carotenoids

The levels of carotenoids in the ASL and wheat flours, and ASL-wheat and wheat-only breads were determined by Dr. Kent Fanning (Department of Agriculture, Fisheries and Forestry Health and Food Sciences Precinct, Coopers Plains Qld, Australia).

Extraction. Carotenoid extracts were prepared and analysed following the procedure of Fanning et al. (2010). Samples (0.4g) were mixed with 5 ml of acetone and vortexed. Ten ml of hexane and 5 ml of 10% NaCl (aq) was then added and re-vortexed. These samples were then centrifuged at 5000 x *g* or 4 min at 4^{o}C. The top layer of hexane containing the extracted carotenoids was transferred to clean tubes. A further 10 ml of hexane was added, and the extraction repeated. Further aliquots of hexane were used for extraction until the hexane layer was colourless indicating full extraction of the carotenoids. The combined hexane extracts were dried in a centrifugal evaporator prior to reconstitution in 2 ml of 50/50 (v/v) methanol/dichloromethane.

Analysis, identification, and quantification. The sample extract was analysed using high-performance liquid chromatography (HPLC) as described by Fanning et al. (2010) using a diode YMC C30 Carotenoid Column, 3 µm, 4.6 × 250 mm (Waters, Milford, MA, USA) and a SPD-M10 A VP diode array detector (Shimadzu, Kyoto, Japan). Individual carotenoids were identified by comparison with retention times

and absorption spectra of carotenoid standards. Standard curves were constructed for each carotenoid using concentrations ranging from 0.03 to10 µg mL^{-1}. Carotenoid concentrations were expressed as µg g^{-1} dry weight of flour and bread samples. Total carotenoids were calculated by summing the amount of individual carotenoids in the sample.

Phenolics and antioxidant capacity

The levels of phenolics and antioxidant capacity in the ASL and wheat flours, and ASL-wheat and wheat-only breads was determined by Ms. Jiayue Chu (School of Public Health, Curtin University, Bentley, WA, Australia).

Extraction. The extraction of samples for total phenolic compounds and antioxidant activity was performed as described by Martinez-Villaluenga et al. (2009). One g of sample was added to 10 mL of 80% methanol in 50mL centrifuge tube and shaken for 2 h at 37°C. The mixture was centrifuged at 4000 x *g* for 10min and the supernatant was collected, filtered and stored at -20°C in the dark until analysed.

Total phenolic content. The determination of total phenolic content (TPC) was performed as previously described by Adom and Liu (2002). Fifty µL of sample extract was diluted with 650 µL ultrapure water then 50 µL Folin-Ciocalteu reagent was added and the sample neutralized with 500 µL of 7% sodium carbonate. The absorbance of the resulting blue complex was measured at 750 nm after 90 min against a blank of 80% methanol. A calibration curve using gallic acid (dissolved in 80% methanol) with concentrations ranging from 0 – 250 µg/ml gallic acid was constructed. The TPC of the samples was expressed as gallic acid equivalents (mg of GAE /g dry sample).

Total antioxidant capacity. Total antioxidant capacity was determined using a method modified from that of Martinez-Villaluenga et al. (2009). Freshly prepared 0.6 mM 2, 2-diphenyl-1-picrylhydrazyl (DPPH) in 80% methanol solution was stored at 4°C (in the dark) prior to use. The sample extract (100 µL) was mixed with 250 µL of the methanolic DPPH solution and 2ml of 80% methanol then shaken for

40 min at room temperature in the dark. A standard curve using Trolox (dissolved in 80% methanol) with concentrations ranging from 0-150 µg/L Trolox, was constructed. Absorbance was then measured at 517nm against a blank comprised of: 100 µL Trolox, 250 µL methanolic DPPH solution, and 2 mL 80% methanol. Antioxidant capacity of the samples was expressed as Trolox equivalents (TE) (µmol TE/g dry sample).

Statistical analysis

Data are presented as mean values with standard deviations. Normality of data was evaluated by Kolmogorov-Smirnov test. One-way ANOVA was used to compare means followed by Duncan's Multiple Range Test to separate the means when F was significant. Additionally, Dunnet's Test was used as a post-hoc operation to compare mean values of wheat flour and wheat-only bread against mean values of individual ASL flour and ASL-wheat breads. ANOVA and post-hoc tests were performed using IBM SPSS Statistics V.21 (IBM Corp., NY, USA).

3.4 RESULTS AND DISCUSSION

Nutritional profile of flour and bread samples

Proximate composition and dietary fibre

Table 3.1 gives the proximate and total dietary fibre composition of ASL and wheat flours, and the ASL-wheat and wheat-only bread samples. Protein content of the ASL flours ranged from 39.6 to 42.2 g/100 g dry basis (db.). The values are comparable to that previously reported for ASL flour of 41.8 g/100 g db. (Hall et al., 2005). There was a significant effect of variety ($p<0.05$) on ASL protein content. Of the ASL flours *Coromup* had the highest ($p<0.05$) protein contents with Belara having the lowest ($p<0.05$). However, the varietal effect on the protein content of the ASL flours was not evident in the ASL-wheat breads; possibly due to the relatively low level (20%) of ASL flour incorporation. All of the ASL flour varieties had significant higher ($p<0.05$) protein content than the wheat flour and all of the

ASL-wheat breads had a significant higher ($p<0.05$) protein content than the wheat-only bread. On average the ASL-wheat breads had a ~42% higher protein content than the wheat-only bread.

The total dietary fibre content of the ASL flours ranged from 37.5 to 40.2 g/100 g db. (Table 3.1) which are slightly lower than a previously reported value of 41.5 g/100g db. (Hall, et al., 2005). There was no varietal effect ($p>0.05$) on the total dietary fibre content of the ASL flours. However, an unexplained small but statistically significant varietal effect ($p<0.05$) on the total dietary fibre content of the ASL wheat-bread was observed. All of the ASL flour varieties had significant higher ($p<0.05$) total dietary fibre content than the wheat flour, and consequently all of the ASL-wheat breads had a significant higher ($p<0.05$) total dietary fibre content than the wheat-only bread. On average the ASL-wheat breads had a ~75% higher dietary fibre content than the wheat-only bread.

The fat content of ASL flours ranged from 7.8-8.8 g/100 g db. which were higher than a previously reported value of 6.9 g/100g db. (Hall, et al., 2005). There was a significant varietal effect ($p<0.05$) on the fat content of the ASL flours with *Belara*, *Tanjil* and *Gungurru* having significantly ($p<0.05$) higher fat contents than the other varieties and *Mandelup* having the lowest ($p<0.05$) fat content. This varietal effect in the ASL flour translated to a similar significant effect ($p<0.05$) in the ASL-wheat breads of which that incorporating *Belara* had the highest ($p<0.05$) and that incorporating *Mandelup* the lowest ($P<0.05$) fat content. All of the ASL flour varieties had significant higher ($p<0.05$) fat content than the wheat flour but only ASL-wheat breads incorporating *Belara* and *Coromup* had slightly but significantly greater ($p<0.05$) fat content than the wheat-only bread.

Table 3.1. Proximate composition of ASL and wheat flours and ASL-wheat and wheat-only breads[1]

Sample	Protein[2] (g/100g)		Total dietary fibre[2] (g/100g)		Fat[2] (g/100g)		Ash[2] (g/100g)		Total available carbohydrates[2] (g/100g)	
	Flour	Bread	Flour	Bread	Flour	Bread	Flour	Bread	Flour	Bread
Belara	39.6±0.4^{a*}	18.7±0.6^{a*}	38.9±1.9^{a*}	15.8±0.5^{bc*}	8.8±0.4^{c*}	5.7±0.6^{d*}	3.28±0.01^{a*}	2.54±0.35^{a}	9.4±3.0^{a*}	57.2±0.3^{a*}
Coromup	42.4±0.3^{e*}	19.3±0.3^{a*}	39.3±0.9^{a*}	16.2±0.6^{c*}	7.8±0.8^{b*}	4.4±0.2^{c*}	2.87±0.88^{a*}	2.26±0.30^{a}	7.4±1.7^{a*}	57.9±0.5^{a*}
Gungurru	41.4±0.4^{c*}	19.2±0.8^{a*}	40.0±1.3^{a*}	14.8±0.6^{ab*}	8.5±0.0^{c*}	3.2±0.2^{ab}	2.75±0.21^{a*}	2.66±0.35^{a}	7.4±0.9^{a*}	60.1±0.4^{b*}
Jenabillup	41.9±0.4^{d*}	19.1±0.2^{a*}	39.2±1.9^{a*}	14.6±0.5^{a*}	7.8±0.5^{b*}	3.5±0.5^{b}	3.82±0.09^{a*}	2.50±0.31^{a}	7.4±1.6^{a*}	60.3±0.5^{b*}
Mandelup	40.8±0.5^{b*}	18.9±1.4^{a*}	37.5±0.4^{a*}	15.1±0.3^{ab*}	7.1±0.3^{a*}	2.6±0.4^{a}	3.41±0.04^{a*}	2.09±0.38^{a}	11.1±0.1^{a*}	61.3±1.1^{b*}
Tanjil	40.5±0.4^{b*}	19.0±0.7^{a*}	40.2±0.4^{a*}	14.6±0.2^{a*}	8.8±0.2^{c*}	3.9±0.6^{bc}	3.18±0.09^{a*}	2.62±0.37^{a}	7.3±1.3^{a*}	59.5±0.7^{g*}
Wheat	*12.3±0.1*	*13.4±0.4*	*6.4±0.0*	*9.2±1.8*	*1.8±0.1*	*3.4±0.5*	*0.62±0.04*	*2.04±0.28*	*78.9±0.0*	*71.9±0.2*

[1]Means ± standard deviation (expressed as dry basis); Moisture contents (g/100g): *Belara* and *Coromup*-6.9;*Gungurru*- 8.6; *Jenabillup*-7.9; *Mandelup*-7.0; *Tanjil*-6.8; Wheat- 11.7; as reported by Villarino et al. (2015a)

[2]Values within the ASL flour and bread columns with different superscript letter denote significant difference ($p<0.05$) using Duncan's Test

[*] Denotes significant difference ($p<0.05$) from wheat flour or wheat-only bread using Dunnett's Test

The ash contents of the ASL flours ranged from 2.75 to 3.82 % with a significant varietal effect ($p<0.05$); however no varietal effect ($p<0.05$) was observed amongst the ASL-wheat breads. The ash content of all ASL flours was significantly higher ($p<0.05$) than that of the wheat flour, but no differences were observed ($p>0.05$) between the ASL-wheat breads and the wheat only bread in ash content.

There was no effect of variety ($p>0.05$) on ASL flour available carbohydrate content. However, an unexplained small but statistically significant varietal effect ($p<0.05$) on the ASL wheat-bread available carbohydrate content was observed. All of the ASL flour varieties had substantially and significantly less ($p<0.05$) available carbohydrate content than the wheat flour and consequently all of the ASL-wheat breads had a significant lower ($p<0.05$) levels than the wheat-only bread. The ASL-wheat breads had ~17% lower available carbohydrates than the wheat-bread control.

Protein quality of bread samples

Amino acid profile and amino acid scoring of bread samples

Table 3.2 presents essential amino acid content of ASL-wheat and wheat-only breads. Histidine content of the ASL-wheat breads ranged from 21 to 26 mg/g protein. Isoleucine content ranged from 42-44 mg/g protein. Leucine content of the ASL-wheat breads ranged from 77 to 80 mg/g protein. Lysine content of the ASL-wheat breads ranged from 22 to 28 mg/g protein. Methionine+cysteine content of the ASL-wheat breads ranged from 28 to 33 mg/g protein. Phenylanine+tyrosine content of the ASL-wheat breads ranged from 91 to 97 mg/g protein. Valine content of the ASL-wheat breads ranged from 42 to 46 mg/g protein. This is the first report on the essential amino acid content of ASL-wheat breads.

It can be observed from Table 3.2 that ASL variety had a significant effect ($p<0.05$) on leucine, methionine+cysteine, threonine and valine content of the ASL-wheat breads. *Belara*-wheat bread had the significantly ($p<0.05$) higher amounts of leucine, threonine and valine compared to the other varieties, On the other hand *Coromup*

Table 3.2. Essential amino acid content of ASL-wheat and wheat-only breads[1,2]

Amino acid	Bread						
	Belara-wheat	Coromup-wheat	Gungurru-wheat	Jenabillup-wheat	Mandelup-wheat	Tanjil-wheat	Wheat-only
Histidine	25.4±1.1	21.2±1.1	24.0±0.7	25.7±1.5	24.9±2.2	24.5±1.1	25.7±0.5
Isoleucine	43.8±0.0	42.5±0.5*	43.3±0.0	43.0±0.0*	43.1±0.4	42.3±0.4*	44.4±0.5
Leucine	81.2±1.1^{e}	78.5±0.4^{bc}*	78.2±0.0^{b}	79.5±0.4^{cd}*	80.2±0.4^{de}	76.8±0.7^{a}*	84.3±0.0
Lysine	28.0±1.9*	26.9±0.7*	23.4±0.0*	26.8±0.0*	28.1±0.7*	22.4±2.6*	17.2±0.0
Methionine + cysteine	32.6±0.8^{b}*	30.0±1.5^{a}*	28.1±0.7^{a}*	31.2±0.4^{b}*	31.5±0.4^{b}*	32.4±1.9^{b}*	38.4±0.5
Phenylalanine + tyrosine	97.0±1.9*	91.4±2.6*	93.8±1.5*	94.4±2.2*	93.7±0.7*	91.5±1.5*	102.9±1.1
Threonine	44.6±0.4^{e}	41.9±0.0^{b}*	40.6±0.7^{a}*	43.3±0.4^{cd}*	43.7±0.4^{de}*	42.3±0.4^{bc}*	45.5±0.0
Valine	46.2±0.4^{e}*	44.3±0.4^{c}*	43.3±0.0^{b}*	43.3±0.4^{b}*	45.0±0.0^{d}*	42.1±0.0^{a}*	48.1±0.5
Tryptophan[4]							

[1]g/100 g sample dry basis
[2]Means ± S.D.
^{abcde} Values within a row with different superscript denotes significant difference ($p<0.05$) using Duncan's Test
* Denotes significant difference ($p<0.05$) with wheat flour using Dunnett's Test

and Gungurru-wheat breads had significantly ($p<0.05$) lower methionine+cysteine compared to the bread samples produced from other varieties. All ASL wheat breads had levels of methionine+cysteine, phenylalanine+tyrosine, threonine (except *Belara)* and valine which were significantly lower ($p<0.05$) compared to those in wheat bread. The levels of isoleucine and leucine in *Coromup*-, *Jenabillup*- and *Tanjil*-wheat breads were significantly ($p<0.05$) lower compared to those in wheat bread. On the other hand the levels of lysine in all ASL-wheat breads were significantly ($p<0.05$) higher compared to those in wheat bread. Histidine levels in all ASL-wheat breads were not significantly ($p>0.05$) different from that found in wheat bread.

Table 3.3 presents the essential amino acid scores of the ASL-wheat and wheat-only breads. There was no ($p>0.05$) varietal effect on the essential amino acid scores of ASL-wheat breads. Tryptophan content of the samples was not measured, however reported values for ASL whole grain was 7 mg/g protein (Petterson et al., 1997) and that for wheat gluten, 10.94 mg/g protein (Woychik et al., 1961). Using these literature values of tryptophan content of ASL and wheat the approximate amount of the tryptophan in the ASL-wheat breads containing 20 g per 100g of composite flour is 10.2 mg/ g protein which is equivalent to an amino acid score of 0.92. The amounts of all essential amino acids except for lysine and possibly tryptophan, in all ASL-wheat breads were higher (with amino acid scores >1.0) compared to the Food and Agricultural Organization (FAO) standards for amino acids of ideal reference protein appropriate for children ages 2 to 5 (which also covers the range appropriate for human adults) (FAO/WHO/UNU, 1985). Given the results of the essential amino acid scores, the limiting amino acid is lysine for all ASL-wheat breads. Based on this finding, the scores of lysine were then used in the computation of the PDCAAS of the ASL-wheat breads.

Table 3.3. Amino acid scores of the essential amino acids[1,2] of ASL-wheat and wheat-only breads.

Amino acid	Bread samples						
	Belara-wheat	Coromup-wheat	Gungurru-wheat	Jenabillup-wheat	Mandelup-wheat	Tanjil-wheat	Wheat-only
Histidine	1.3±0.1	1.1±0.2	1.3±0.0	1.4±0.1	1.3±0.1	1.3±0.1	1.4±0.0
Isoleucine	1.6±0.0	1.5±0.0	1.5±0.0	1.5±0.0	1.5±0.0	1.5±0.0	1.6±0.0
Leucine	1.3±0.8	1.2±0.0	1.2±0.0	1.2±0.0	1.2±0.0	1.2±0.0	1.3±0.0
Lysine	0.5±0.0	0.5±0.0	0.4±0.0	0.5±0.0	0.5±0.0	0.4±0.0	0.3±0.0
Methionine + cysteine	1.3±0.0	1.2±0.1	1.1±0.0	1.2±0.0	1.5±0.0	1.3±0.1	1.5±0.0
Phenylalanine + tyrosine	1.5±0.0	1.5±0.0	1.5±0.0	1.5±0.0	1.5±0.0	1.5±0.0	1.6±0.0
Threonine	1.3±0.0	1.2±0.0	1.2±0.0	1.3±0.0	1.3±0.0	1.2±0.0	1.3±0.0
Valine	1.3±0.1	1.3±0.0	1.3±0.0	1.3±0.0	1.3±0.0	1.2±0.0	1.4±0.0

[1]Mean± S.D. (n=2)
[2]Based on standard FAO/WHO 2-5 year old reference pattern (mg/g protein): Histidine-19; Isoleucine-28; Leucine-66; Lysine-58; Methionine+Cysteine-25; Phenylalanine+Tyrosine- 63; Threonine- 34; Valine-3

The PDCAAS (Table 3.4) of the ASL-wheat bread samples ranged from 0.31 to 0.40 with *Tanjil*-wheat bread having lower ($p<0.05$) values compared to other ASL-wheat breads. ASL variety significantly ($p<0.05$) affected the PDCAAS of the ASL-wheat breads which may be attributed to differences in the levels of limiting amino acid lysine in the ASL varieties. Results imply that substitution of wheat flour with 20 g ASL flour /100 g of composite flour can potentially increase the PDCAAS of wheat bread by ~50%.

Table 3.4. Effects of ASL variety on in- vitro protein digestibility and PDCAAS of ASL-wheat and wheat breads[1].

Bread	In vitro protein digestibility (%)	PDCAAS
Belara-wheat	$82.1\pm1.7^{a*}$	$0.40\pm0.03^{c*}$
Coromup-wheat	$82.3\pm4.2^{a*}$	$0.38\pm0.01^{bc*}$
Gungurru-wheat	$81.0\pm0.7^{a*}$	$0.33\pm0.03^{ab*}$
Jenabillup-wheat	$83.6\pm0.3^{c*}$	$0.38\pm0.00^{bc*}$
Mandelup-wheat	$82.3\pm0.6^{b*}$	$0.40\pm0.01^{c*}$
Tanjil-wheat	80.0 ± 0.0^{a}	$0.31\pm0.01^{a*}$
Wheat-only	78.0±0.0	0.23±0.00

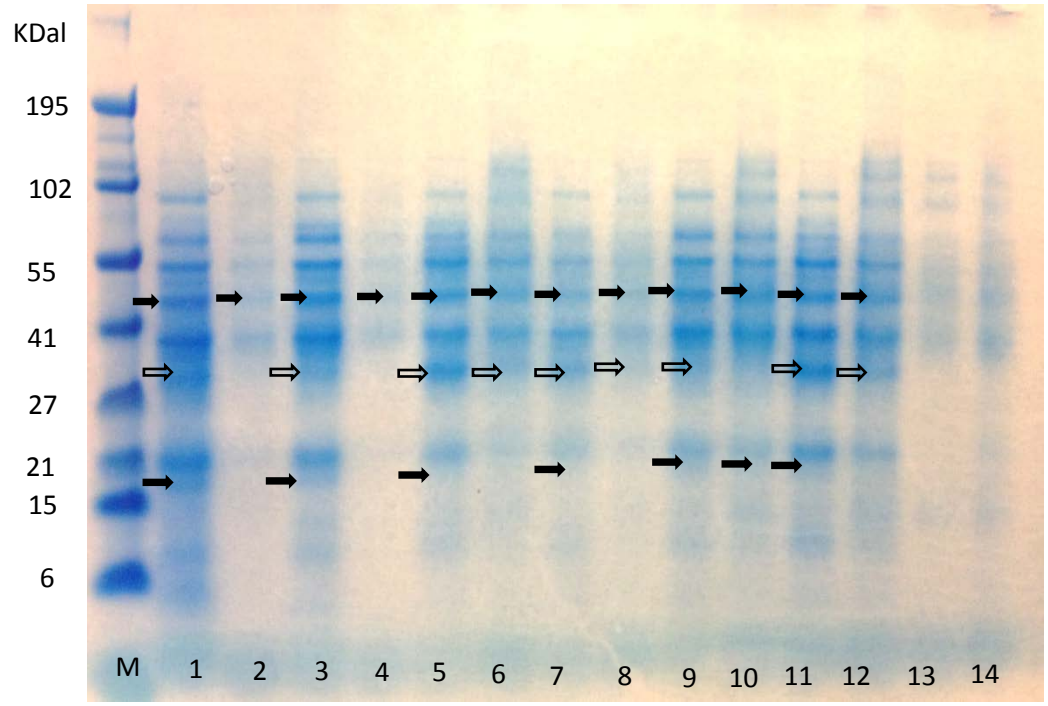
[1]Means ± S.D.
[ab] Values within a column with different superscript denotes significant difference ($p<0.05$) using Duncan's Test
[*] Denotes significant difference ($p<0.05$) with wheat flour using Dunnett's Test

Electrophoresis and Western blotting

Figure 3.2 presents a photographic image of the SDS-PAGE gel and a Western blot of ASL and wheat flour and ASL-wheat breads and wheat-only bread. The SDS-PAGE image (Figure 3.2 (a)) shows intense bands in the ASL- flour and ASL-wheat bread sample that correspond in molecular weight to the subunits of α- (50 and 80 kDa), β-(20 and 60 kDa) and γ-(17 and 30 kDa) conglutins (Capraro et al., 2008). These bands are not visible in the wheat flour and wheat-only bread sample

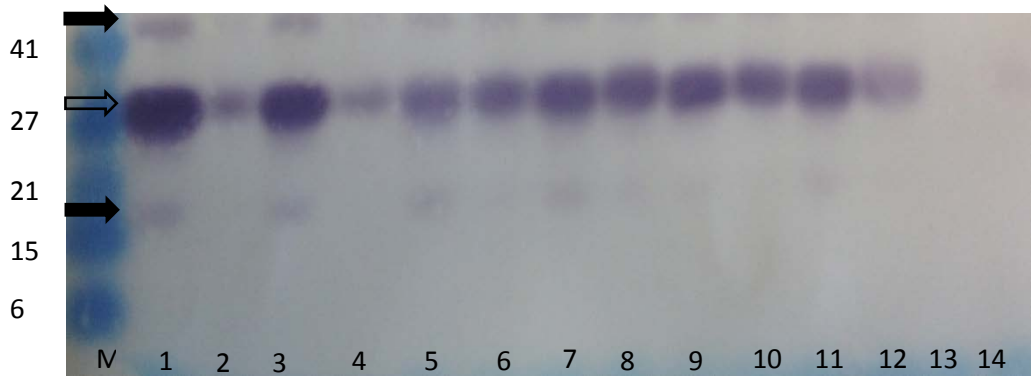
In terms of the potentially bioactive peptide γ-conglutin, the SPS-PAGE gel (Figure 3.2 (a)) shows that all ASL flour lanes have a band of molecular weight corresponding to 30 kDa γ-conglutin subunit. On the other hand, bands of molecular weight corresponding to 30 kDa γ-conglutin subunit in the ASL-wheat breads lanes were not as intense as the bands in the ASL flour lanes. Western blots (Figure 3.2 (b)) confirms that all ASL flour and ASL-wheat breads have intense bands of reactivity with the γ-conglutin antibody at 30 kDa. The similar intensities of the bands in the Western blots of the ASL flour and the corresponding ASL-wheat breads for each ASL flour variety (except *Belara* and *Coromup)* indicates that the bread making process did not greatly affect the integrity of the 30 kDa γ-conglutin subunit. However, further studies quantifying the 30 kDa γ-conglutin subunit are needed.

The absence of the 17 kDa γ-conglutin bands in the ASL-wheat bread lanes (2, 4, 6, 8, 10 and 12) of the SDS-PAGE gel may indicate that the bread-making process may have led to the breakdown of this peptide or rendered it non-extractable. According to Islam et al. (2011), lupin proteins may interact with wheat proteins via crosslinking during baking reducing their extractability. Although the Western blots showed bands at ~17 kDa molecular weight, this result needs to be confirmed as a γ-conglutin subunit as the primary antibody used was designed to react with the~30 kDA and~50 kDA molecular weights only. Dr. Rhonda Foley (CSIRO, Floreat, WA, Australia) (Personal communication, 17 June 2014), who was one of the lead designers of the primary antibody, advised that the Western blot reactivity at ~17 kDa is difficult to interpret since no reactivity to the antibody used is expected at this molecular weight. However, both the SDS-PAGE gels and Western blots display bands with a molecular weight of ~50 kDa that may represent the un-reduced γ-conglutin dimer. The SDS-PAGE and Western blotting were both run under reducing conditions, however the presence of Western blot reactivity at ~50 kDa suggests that the γ-conglutin dimer was not fully reduced during the analytical procedures.



(a)

Figure 3.2. (a) SDS-PAGE (b) Western blots of flour and bread proteins: lane M, molecular weight standards; lane 1, Belara flour; lane 2, Belara-wheat bread; lane 3, Coromup flour; lane 4, Coromup-wheat bread; lane 5, Gungurru flour; lane 6, Gungurru-wheat bread; lane 7, Jenabillup flour; lane 8, Jenabillup-wheat bread; lane 9, Mandelup flour; lane 10, Mandelup-wheat bread; lane 11, Tanjil flour; lane 12, Tanjil-wheat bread; lane 13, wheat flour; lane 14, wheat bread; ➡ bands corresponding to molecular weights of ~17 and ~50 kDa; and ⇨ corresponding to molecular weights of ~30 kDa.



Reports on the effects of the bread maki (b) ss on γ-conglutin are lacking and the mechanism by which the bread making process affect γ-conglutin still needs to be understood as any changes to the γ-conglutin may reduce its potential for health-related bioactivity.

Phytochemical composition of flour and bread samples

Alkaloid and oligosaccharides of flour samples

No varietal effect ($p > 0.05$) was observed for the alkaloids contents of the ASL flours for which the mean values ranged from 0.007 to 0.016 g/100g db. (full data not presented). These values are below the maximum level permitted for lupins for human food use of 0.020g/100g as defined by the Australian (FSANZ, 2011) and Great Britain (MAFF-DOH, 1996) national food standards.

No varietal effect ($p > 0.05$) was observed for the total oligosaccharide contents of the ASL flours for which the mean values ranged from 4.2 to 6.6 g/100 g db. (full data not presented). These levels are below those previously reported of 7.4 to 8.0 g/100 g as is basis by Evans et al. (1993).

Carotenoid contents of flour and bread samples

Table 3.5 presents the carotenoid contents of the flours and breads. Significant varietal effect ($p<0.05$) were observed for the contents of individual and the total carotenoids amongst the ASL flours. These effect of variety were translated into significant varietal effect ($p<0.05$) in the ASL-breads, except for alpha-carotene for which no significant ($p>0.05$) varietal effect was observed. All ASL flours had significantly higher ($p<0.05$) levels of lutein, zeaxanthin, alpha-carotene, beta-carotene and total carotenoids than the wheat flour, however this was only translated into higher ($p<0.05$) levels of these carotenoids in the Belara-wheat compared to the wheat-only bread. Of the ASL varieties, Mandelup has the significantly highest ($p<0.05$) total carotenoid level, whereas there were no significant differences ($P>0.05$) in the levels of total carotenoids amongst the ASL-wheat breads.

Values for carotenoid content in ASL reported in the literature are conflicting. Wang et al. (2008) reported that ASL seeds have 229 µg/g total carotenoids with 24.1 µg/g lutein and 134.4 µg/g zeaxanthin. In stark contrast, Fryirs et al. (2008) reported that lupin flour has 44 µg/g of combined lutein and zeaxanthin. These values are higher

than those reported in the present study however the reason for this remains unclear. The recovery of carotenoids in ASL-wheat breads compared to that calculated for the raw composite flours were: lutein ~24%; zeaxanthin ~15%; alpha-carotene ~71%; beta-carotene48%; total carotenoids ~38%. According to Hidalgo et al. (2010), mixing and baking led to significant decreases in the carotenoid contents of refined einkorn and bread flours. During mixing, in the presence of water and oxygen, lipoxygenase (LOX) oxidises polyunsaturated fatty acids which in turn causes oxidation of carotenoids (Leenhardt et al., 2006). ASL has been shown to have high LOX activity (Yoshie-Stark and Wäsche, 2004) and this may have resulted in the low recovery rates of carotenoids in the present study. In addition, the thermal process of baking can decrease carotenoid content (Namitha and Negi, 2010). ASL flour appears to be a good source of carotenoids however further research is required to develop processing approaches to reduce their losses during bread making.

Total phenolics and total antioxidant capacities of flours and breads

Table 3.6 presents the content of total phenolics and the total antioxidant capacities of the flours and breads. There was a significant effect of ASL variety ($p<0.05$) on the total polyphenolic content and the antioxidant capacity amongst both the ASL flours and the ASL-wheat breads. Of particular note was the higher ($p<0.05$) antioxidant capacity of the Tanjil flour compared to the other ASL-flours. In addition, all ASL flours had significantly higher ($p<0.05$) total phenolic levels and antioxidant capacity than the wheat flour. The total phenolic levels of the ASL samples in this study are lower than those reported for milled whole ASL seeds (including hulls) that ranging from 2.6 (Siger et al., 2012) to 5.8 (Wang and Clements, 2008) mg GAE/g dry matter. This difference may be due to the presence of hull material in the samples used in other studies which may have had higher concentrations of polyphenolics than the kernel.

Table 3.5. Carotenoid contents of ASL and wheat flours, and ASL-wheat and wheat-only breads[1,2].

	Lutein (µg/g)		Zeaxanthin (µg/g)		Alpha-Carotene (µg/g)		Beta-carotene (µg/g)		Total Carotenoids (µg/g)	
Sample	Flour	Bread	Flour	Bread	Flour	Bread	Flour	Bread	Flour	Bread
Belara	7.5±0.4^{c*}	0.6±0.1^{b*}	4.1±0.2^{b*}	0.2±0.1^{b*}	2.2±0.3^{ab*}	0.7±0.0^{a*}	4.7±0.5^{b}	1.1±0..3^{c*}	18.4±1.3^{bc}	2.5±0.5^{a}
Coromup	7.3±0.3^{c*}	0.5±0.1^{ab}	3.9±0.2^{b*}	0.1±0.1^{ab}	1.8±0.2^{a*}	0.6±0.0^{a}	4.6±0.3^{b}	0.7±0.1^{ab}	17.6±0.8^{bc}	2.0±0.3^{a}
Gungurru	4.9±0.2^{b*}	0.3±0.0^{a}	4.0±0.2^{b*}	0.1±0.0^{a}	3.3±0.5^{c*}	0.6±0.2^{a}	6.9±0.6^{c}	0.6±0.0^{ab}	19.1±1.4^{bc}	1.5±0.1^{a}
Jenabillup	3.4±0.1^{a*}	0.3±0.0^{a}	4.0±0.0^{b*}	0.1±0.0^{ab}	1.8±0.0^{a*}	0.5±0.2^{a}	2.6±0.6^{a}	0.6±0.2^{b}	11.9±0.6^{a}	1.5±0.4^{a}
Mandelup	7.6±0.3^{c*}	0.4±0.2^{ab}	4.4±0.1^{b*}	0.1±0.0^{ab}	2.6±0.3^{bc*}	0.5±0.1^{a}	5.5±1.1^{bc}	0.7±0.1^{ab}	20.1±1.8^{c}	1.8±0.4^{a}
Tanjil	5.4±0.5^{b*}	0.3±0.1^{a}	3.4±0.3^{a*}	0.1±0.0^{a}	2.7±0.2^{bc*}	0.5±0.1^{a}	6.6±5.9^{b}	0.6±0.0^{b}	16.1±0.3^{b}	1.4±0.1^{a}
Wheat	*0.6±0.0*	*0.2±0.0*	*0.02±0.0*	*0.025±0.0*	*0.4±0.0*	*0.3±0.0*	*5.0±0.8*	*0.4±0.1*	*1.6±0.0*	*0.9±0.1*

[1]Means ± standard deviation (expressed as dry basis)
[2]Data supplied by Dr. Kent Fanning (Department of Agriculture, Fisheries and Forestry Health and Food Sciences Precinct, Coopers Plains Qld, Australia)
[abc]Values within each column relating to ASL varieties with different superscript denotes significant difference ($p<0.05$) using Duncan's Test
[*] Denotes significant difference ($p<0.05$) with wheat flour or wheat–only bread using Dunnett's Test

Table 3.6. Total phenolic content and total antioxidant capacity of ASL flour and ASL-wheat breads[1,2].

Sample	Total Phenolics (mgGAE/g db)		Total Antioxidant capacity (µmolTE/g db)	
	Flour	Bread	Flour	Bread
Belara	1.8±0.1^{cd*}	0.7±0.0^{b*}	3.0±0.0^{bc*}	1.2±0.1^{ab}
Coromup	1.6±0.1^{a*}	0.7±0.0^{bc*}	2.8±0.1^{ab*}	1.6±0.1^{c*}
Gungurru	1.6±0.1^{a*}	0.6±0.0^{a*}	2.6±0.2^{a*}	1.1±0.1^{a}
Jenabillup	1.9±0.1^{d*}	0.7±0.0^{bc*}	3.0±0.1^{bc*}	1.3±0.1^{b*}
Mandelup	1.6±0.1^{ab*}	0.7±0.1^{ab*}	3.3±0.2^{c*}	1.3±0.0^{b*}
Tanjil	1.7±0.0^{bc*}	0.8±0.0^{c*}	5.4±0.5^{d*}	1.5±0.1^{c*}
Wheat	*0.4±0.0*	*0.5±0.0*	*0.9±0.0*	*1.0±0.0*

[1]Means ± standard deviation (expressed as dry basis)
[2]Data supplied by Ms. Jiayue Chu (School of Public Health, Curtin University, Bentley, WA, Australia)
[abcd]Values within the each column relating to ASL varieties with different superscript denotes significant difference ($p<0.05$) using Duncan's Test
[*] Denotes significant difference ($p<0.05$) to wheat flour or wheat-only bread within a column using Dunnett's Test

The total antioxidant capacity of the ASL flours in this study are within the range of the reported for milled ASL seeds (including hulls) ranging from 3.0 (Martínez-Villaluenga et al., 2009) to 7.5 µmolTE/g (Siger, et al., 2012). Again the fact that the values in the present study are at the lower range of those previously reported may be due to the presence of hulls in other studies.

Recovery rates of total phenolics and total antioxidant capacities of lupin flour after baking were at least 85% (full data not presented).The ASL-wheat breads had almost double the total phenolics content compared to the wheat-only bread, while the total antioxidant capacity was as much as 50% higher. These results indicate that the

bread making process did not dramatically diminish the total phenolics content and antioxidant capacity of the ASL-wheat composite flours and imply that substitution of refined wheat flour for ASL flour can increase the polyphenolic content and antioxidant capacity of bread.

3.5. CONCLUSION

This study identified varietal effects on the nutritional and chemical properties of ASL flours and ASL-wheat composite flour breads. The results indicate that *Belara, Coromup* and *Tanjil* flours may be good choices from the ASL varieties investigated to increase the nutritional and health attributes of wheat bread.

CHAPTER FOUR-Experimental

The effects of Australian sweet lupin (ASL) variety on physical properties of flours and breads

Information contained in this chapter has been published as follows:

Villarino, C.B.J., Jayasena, V., Coorey, R., Bell, S. and Johnson, S.K. (2015).The effects of Australian sweet lupin (ASL) variety on physical properties of flours and breads. *LWT-Food Science and Technology* 60, 435-443.

4.1. ABSTRACT

Physical characteristics of Australian sweet lupin (ASL) flours and breads made using ASL (20 g/100 g):refined wheat (80 g/100 g) composite flours of ASL varieties *Belara, Coromup, Gungurru, Jenabillup, Mandelup* and *Tanjil* were evaluated and compared to wheat-only flour and bread. There was a significant ($p<0.05$) effect of ASL variety on flour particle size distribution and surface area. Moisture loss, bread specific volume, crumb characteristics and texture properties of ASL-wheat breads were also significantly ($p<0.05$) affected by ASL variety. Of the ASL varieties, *Mandelup*-wheat bread had the lowest ($p<0.05$) moisture loss, bread volume, and height; most dense pore appearance and higher number of smaller cells; hardest, chewiest and least springy instrumental texture. *Tanjil*-wheat bread had the highest bread volume and was comparable with other ASL-wheat breads in terms of moisture loss, crumb cell and texture characteristics. Results suggest that ASL varieties *Belara, Coromup, Gungurru, Jenabillup* and *Tanjil* can be incorporated into wheat flour for bread manufacturing with desirable bread volume, crumb cell and texture attributes.

4.2 INTRODUCTION

The addition of ASL flour to wheat flour bread can result in low bread volume and hard crumb texture, due to the disruption of the gluten matrix by the non-elastic lupin proteins and high water absorbance of ASL dietary fibre (Turnbull et al., 2005). However the influence of ASL variety on bread quality has not been previously reported. Varietal differences in the proximate composition of ASL flour may, based on findings for other legume flours (Sosulski and Youngs, 1979), influence particle size distribution of the flour. Any differences in ASL flours particle size may in turn affect bread volume; since decreasing particle size of refined wheat flour substitutes (bran or whole wheat) either increased (Moder et al., 1984) or decreased (de Kock et al.,1999) loaf volume. ASL variety may impact on the key bread quality attributes of crumb specific volume, cell structure and instrumental texture since it has been reported that subtle differences in the proximate composition of legume flours can affect dough rheology and bread quality (Farooq and Boye, 2011; Angioloni and Collar, 2012). This study therefore assessed the effects of ASL variety on the physical characteristics of ASL flours and ASL-refined wheat composite flour breads.

4.3. MATERIALS AND METHODS

Materials

Materials described in Chapter **3.3** were used. The proximate and dietary fibre composition (g/100g as is) of ASL and refined wheat flours measured in Chapter **3** was used in this Chapter for the purpose of correlation with the physical characteristics of the ASL flours and ASL-wheat breads presented in section **4.3**. It was reported in Chapter **3** that there was a significant varietal effect ($p<0.05$) on moisture, protein and fat was observed between the ASL flours and may help explain any differences in the physical properties of the ASL flours and ASL-wheat breads. The refined wheat flour was significantly ($p<0.05$) different to all ASL flours in proximate composition and dietary fibre level.

Experimental design

The experimental design described in section **3.3** was used.

Bread making

The bread making procedure described in section **3.3** was followed.

Analytical methods

Physical tests were performed on 3 randomly chosen breads from each treatment after storing at room temperature for up to 24 h after baking. For disulphide bonds density determination, the same samples in Chapter **3** were analysed.

Physical characteristics of flours

The particle size distribution of the flours was analysed in triplicate following the method of Licata et al. (2014) by laser light scattering using a Mastersizer 2000 (Malvern instruments Ltd, Malvern, UK). Five g of sample was dry-dispersed into the apparatus using a Scirocco 2000 dry powder dispersion unit (Malvern instruments Ltd, Malvern, UK). Data was calculated by the instrument software as d (0.1) (µm), d (0.5) (µm) and d (0.9) (µm) representing the maximum diameter of 10%, 50% and 90% of the particles, respectively. Particle size distribution curves were also software generated using volume (%) data of particle size at various ranges (4- 1000 µm). The volume weighted mean particle size, D[4,3] (µm) and surface area (m^2/g) were also calculated by the software. Detection limit of the instrument is 20 nm while accuracy is ± 3% of d (0.5) and ± 5% of d (0.1) and d (0.9). Specifications on sensitivity are not available as the instrument cannot measure individual particles and is not a particle counter (thus cannot measure concentration).

Moisture loss

Moisture loss (%) during baking was determined as conducted by Paraskevopoulou et al. (2010) and calculated as:

$$\text{Moisture loss} = \frac{\text{weight of dough (before baking)} - \text{weight of baked bread}}{\text{weight of dough (before baking)}} \times 100 \qquad \text{Eq. 3.1}$$

Crumb specific volume (CSV)

Specific volume (cm^3/g) of crumb was determined based on the method of Miñarro et al. (2012) by cutting cube from the centre of the bun (one cube per bun) using an electric knife (KN400, Kenwood, Delonghi Australia Pty Limited, Casula, NSW, Australia), after which the volume in cm^3 (length (cm) x width (cm) x height (cm)) of the cube was measured and divided by the weight (g) of the cube.

Crumb cell characteristics

Crumb cell characteristics were measured using C-Cell (Calibre Control International Ltd, Warrington, UK) as reported by Alvarez-Jubete et al. (2010). Breads were sliced parallel to their base with a thickness of 1 cm using an electric slicer and the two middle slices were used for evaluation. The imaging system captured photos of the bun slices and measured crumb cell properties: slice area (cm^2), number of cells per cm^2, cell wall thickness (mm), and cell diameter (mm). Likewise, bun slice height was measured using a slice cut perpendicular to the base of the bun. Detection limit of the instrument is 1 pixel or $0.14mm^2$ while precision is approximately 5% coefficient of variation. Sensitivity of the digital imaging system was not available.

Instrumental textural properties

Instrumental textural properties of hardness, springiness, cohesiveness and chewiness were determined with TA.XT^{plus} Texture Analyser (Stable Microsystems Ltd., Surrey, UK) with 5 kg load cell based on the method of Angioloni and Collar (2012). Texture profile analyses (TPA) were performed using a 36 mm cylindrical

aluminium probe. Each slice (slice cut parallel to the base of the bun used in C-Cell crumb characterisation) was subjected to a double cycle of compression with crosshead speed of 1 mm/s and maximum deformation of 50%. One measurement was taken on the mid portion of the slice surface from which force-time curves were generated, providing values to determine texture characteristics. Hardness (g) was the peak force at first compression. Springiness was the ratio of the length (L2) under the curve to the point of peak force at second compression and the length (L1) under the curve to the point of peak force at first compression. Cohesiveness was the ratio of the area of work during second compression and the area of work during first compression. Chewiness (g) was calculated as hardness (g) × cohesiveness × springiness. Information on the detection limit, accuracy and sensitivity of the texture analyser were not available.

Disulphide bond density

The disulphide bond density in the bread samples were quantified based on the direct colorimetric method of Chan and Wasserman (1993). The method involved two separate extractions and colorimetric measurements to determine both the amount of free thiols, and the amount of total sulphydryls. Disulphide bond density (nmoles/mg sample db.) was calculated as the difference between total sulphydryls (nmoles/mg sample db.) and free thiols (nmoles/mg sample db.). Lysozyme (40,000 units/mg protein, Sigma Aldrich, New South Wales, AU) was used as an internal control. Tests were run in duplicate and against a sample blank.

Free thiols content was determined by suspending 30 mg of bread sample in 1.0 ml of reaction buffer (RB1) consisting of 8M urea,10 mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), 3 mM ethylene-diaminetetraacetic acid (EDTA), 0.2M Tris-HCl, pH 8.0 and 1% sodium dodecyl sulphate (SDS) for 20 min under N_2 and in the dark. The suspension was centrifuged at 13,600 x *g* in a microcentrifuge for 10 min at room temperature and 0.1 ml of the supernatant was diluted with 0.9 ml of RB1 without DTNB. The solution was centrifuged at 13,600 x *g* for 10 min at room temperature and absorbance was read at 412 nm against a sample blank which underwent the same treatment as above.

Prior to determination of total sulphydryl content, 2-nitro 5-thio sulfo benzoic acid ($NTSB^{2-}$) was synthesized from DTNB in the presence of sodium sulphite and O_2 for 20 min in the dark at room temperature as described in Thannhauser et al. (1987). Total sulphydryl content was then determined by suspending 30 mg of bread sample in 1.0 ml of reaction buffer (RB2) consisting of 8M urea,0.1M sodium sulphite, 3 mM EDTA, 0.2M Tris-HCl, pH 9.5, 1% SDS and 10 mM $NTSB^{2-}$ at room temperature and in the dark. The sample suspension was immediately centrifuged at 13,600 x *g* in a microcentrifuge for 10 min at room temperature and 0.1 ml of the resulting supernatant was diluted with 0.9 ml of RB2 without $NTSB^{2-}$. The solution was then centrifuged at 13,600 x *g* for 10 min at room temperature and its absorbance was read at 412 against a sample blank which underwent the same treatment as above.

Statistical analysis

Data are presented as mean values with standard deviations. Normality of data was evaluated by Kolmogorov-Smirnov test. One-way ANOVA was used to compare means followed by Duncan's Multiple Range Test to separate the means when F was significant. Additionally, Dunnet's Test was used as a post-hoc operation to compare mean values of wheat-only bread against mean values of individual ASL-wheat breads. Linear correlation of all flour and bread physical characteristics were analysed using Pearson's correlation test while non-linear models of these relationships were evaluated using the scatter plot option in Microsoft Excel 2013 (Microsoft Corp., Redmond, WA, USA). Only significant Pearson's correlations and non-linear models which had $R^2 > 0.50$ are presented. ANOVA, post-hoc tests and Pearson's correlation were performed using IBM SPSS Statistics V.21 (IBM Corp., NY, USA).

4.4. RESULTS AND DISCUSSION

Particle size distribution

Particle size distribution curves of ASL flours are shown in Figure 4.1. The particle size of ASL flours had trimodal distributions, except for those of *Gungurru* and *Tanjil* which had bimodal distributions. This appears to be the first report on the particle size distribution of ASL flours, however those reported for other legume flours had bimodal distributions (Kerr et al., 2000; Petitot et al., 2010). Wheat flour had a singular mode distribution (Figure 4.2), however previous reports are conflicting; some showing singular mode of distribution (Sabanis and Tzia, 2009) others bimodal (Hareland, 1994; Wang and Flores, 2000). According to Hareland (1994), particle size distribution of wheat flour depends on wheat hardness and class, type of grinder and grinding time which may explain the different reported distributions. In the present study, the differences in mode of distribution between wheat flour and the ASL-flours and the varietal effect amongst the ASL-flours study may be linked to differences in proximate and dietary fibre composition (Chapter 3, **Table 3.1**).

The ASL flour particle size can be divided into two modes: <100 microns (fine) and >100 microns (coarse). This partition may be attributed to protein and dietary fibre rich fractions with different particle size distribution. According to Sosulski and Youngs (1979), ASL flour "fine" particles comprised mainly of protein (86 g/100 g db.), however the "coarse" (larger-sized) particles may be enriched in dietary fibre. The dietary fibre component of ASL kernels consists mainly of non-starch polysaccharides within thickened endosperm cell walls (Evans et al., 1993; Trugo et al., 2003). These cell wall structures may have been more resistant to the milling process, thus contributing to the larger-particle size fraction. However, protein and dietary fibre composition of the fine and coarse particles in ASL flours still requires investigation.

The volume weighted mean particle sizes of ASL flour samples ranged from 124-144 µm (Table 4.1). *Gungurru* flour had the significantly ($p<0.05$) largest mean particle size amongst ASL flours. ASL flour volume weighted mean particle size had a positive and quadratic association with moisture content (g/100 g as is; volume weighted mean= $250.7-(38.1\times\text{Moisture}) + (2.93\times \text{Moisture}^2)$, $R^2 =0.63$) suggesting moisture content may play a major role in determining particle size distribution. It

was previously reported that there is a direct relationship between moisture content and particle size of wheat flour (Gaines and Windham, 1998). The authors explained that at higher moisture content wheat kernels become more pliable to cracking leading to larger sized flour particles. Pearson correlation test demonstrated that the surface area of the ASL flours was negatively and linearly associated with moisture content (Pearson's correlation, $r=-0.81$, $p<0.001$). Particle size characteristics of lupin flour has potential to impact on bread volume based on previous studies of other non-wheat substitutes (de Kock et al., 1999; Moder et al., 1984).

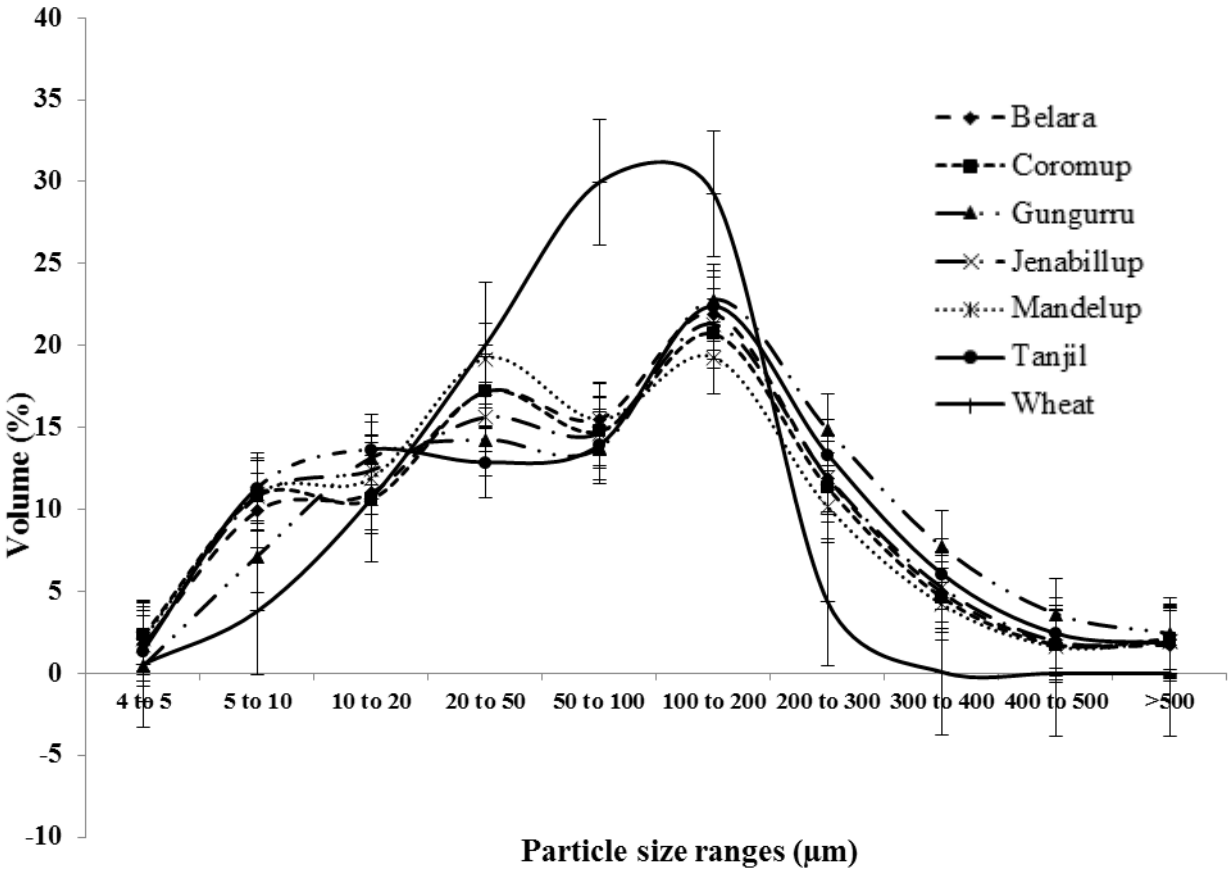


Figure 4.1. Particle size distribution curves of Australian sweet lupin and wheat flours

Note: Error bars denote standard deviation

Table 4.1. Particle size characteristic of ASL and wheat flours[1].

Flour	d[0.1][2] (µm)	d[0.5][2] (µm)	d[0.9][2] (µm)	D[4,3][3] (µm)	Surface area (m^2/g))
Belara	8.1±0.1^{c*}	74.1±0.8^{a}	288.1±7.0^{a*}	123.7±4.1^{a*}	0.2±0^{bc}
Coromup	7.1±0.1^{a*}	68.2±0.7^{a}	294.1±4.8^{ab*}	129.9±4.0^{a*}	0.3±0^{e}
Gungurru	11.4±0.3^{e*}	105.4±7.4^{c*}	339.3±4.3^{c*}	144.0±4.1^{b*}	0.2±0^{a}
Jenabillup	8.0±0.1^{c*}	74.1±2.7^{a}	299.1±12.5^{ab*}	129.7±9.7^{a*}	0.3±0^{cd}
Mandelup	7.7±0.4^{b*}	65.8±11.8^{a}	289.2±11.7^{a*}	126.8±10.2^{a*}	0.3±0de
Tanjil	8.5±0.1^{d*}	85.2±2.6^{b*}	304.7±7.6^{b*}	128.4±5.5^{a*}	0.3±0^{b}
Wheat	*14.2±0.3*	*72.3±1.9*	*164.6±1.6*	*81.6±1.4*	0.3±0

[1]Mean± standard deviation (n=6)
[2] d[0.1], d[0.5] , d[0.9] represents the maximum diameter of 10%, 50% and 90% of the particles, respectively.
[3]D[4,3] represent the volume weighted mean particle size
[abcde]Values within column with different superscript denotes significant difference ($p<0.05$) using Duncan's Test
* Denotes significant difference ($p<0.05$) with wheat flour using Dunnett's Test

Bread moisture loss

Figure 4.2 presents the moisture loss during baking of ASL-wheat and wheat-only breads. Amongst the ASL-wheat breads, *Mandelup* had the significantly lowest ($p<0.05$) loss of moisture during baking while *Jenabillup* had the highest ($p<0.05$). Moisture loss (baking loss) is an important parameter influencing bread texture and staling. According to Kotoki and Deka (2010) too much water lost during baking may produce a dry crust and may lead to early staling.

Moisture loss may be influenced by the water binding capacity (WBC) of the dough ingredients. For instance, non-wheat ingredients such as potato flour (Kotoki and Deka, 2010) and potato fibre (Kaack et al., 2006) were found to decrease moisture loss when used to substitute wheat flour in bread due most likely to their high WBC. The high WBC ASL kernel dietary fibre (Turnbull et al., 2005), may affect the moisture loss in ASL-wheat bread, however, there were no significant ($p>0.05$) differences in the dietary fibre content of the ASL flours (**Table 3.1**). Moisture loss

was however significantly (Pearson's correlation, r=0.40, p=0.02) associated with the moisture content of the ASL flour. The high WBC of the ASL dietary fibre may explain why all ASL-wheat bread samples had lower ($p<0.05$) moisture loss compared to the wheat-only bread.

Additon of lupin protein isolate has been reported to delay bread firming (Paraskevopoulou et al., 2010) while dietary fibre addition to bread has been reported to delay staling (Angioloni and Collar 2009). In light of this, further exploration of the potential anti-staling effects of ASL flour incorporation into wheat bread is warranted.

Figure 4.2. Moisture loss during baking (%) of ASL-wheat and wheat-only breads.

Note: Dark-coloured columns with different letters denote significant difference ($p<0.05$) using Duncan's Test
*Denotes significant difference ($p<0.05$) with wheat flour sample using Dunnett's Test
Error bars denote standard deviation

Crumb specific volume (CSV)

Amongst the ASL-wheat breads, *Tanjil* exhibited the significantly highest ($p<0.05$) crumb specific volume (CSV) while *Mandelup* had the lowest ($p<0.05$) (Figure 4.3). Specific volume of the ASL-wheat breads had a positive and quadratic relationship ($CSV= 24.4 + (7.0\times Fat) – (0.4 \times Fat^2)$; $R^2=0.61$) with the fat content of ASL flour implying that increased fat content of ASL flours may beneficially influence bread volume. This hypothesis is supported by findings of Pollard et al. (2002) in which use of defatted ASL flour resulted in lower loaf volume than full-fat. ASL flour lipids, like those in wheat flour may positively influence loaf volume in by forming lipid monolayers at the gas/liquid interphase of the gas cells thus increasing gas retention of the dough (Goesaert et al., 2005) and help stabilize the gas cells (Gan et al., 1995).

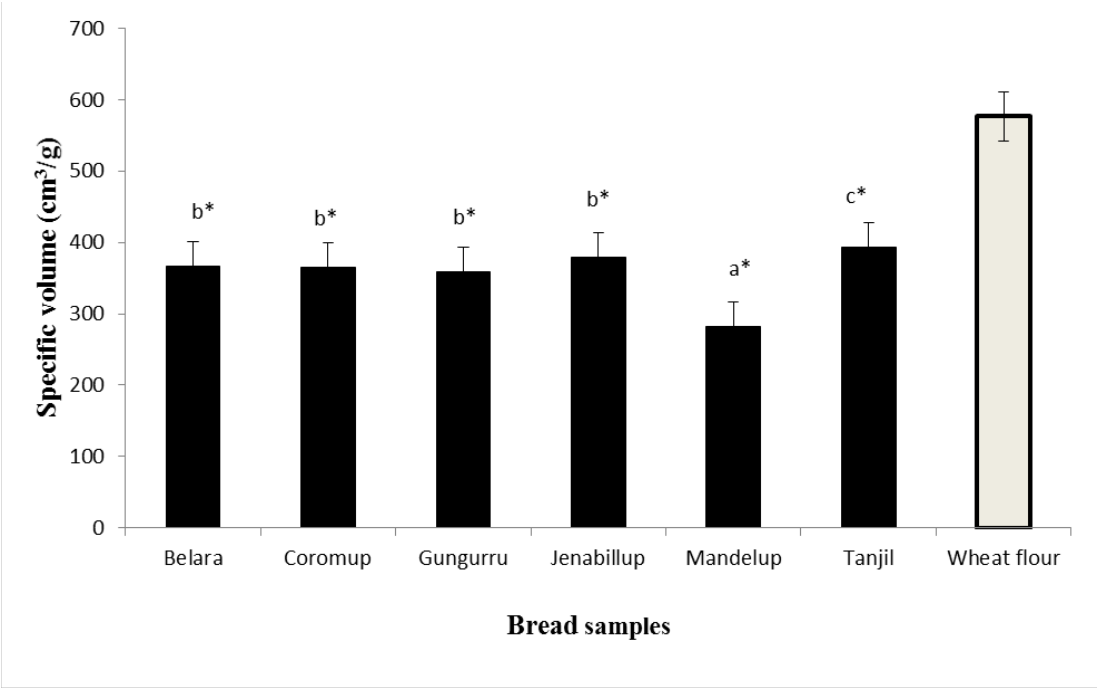


Figure 4.3. Specific volume of ASL-wheat and wheat-only bread samples.

Note: [abc]Dark- coloured columns with different letters denote significant difference ($p<0.05$) using Duncan's Test
*Denotes significant difference ($p<0.05$) with wheat flour sample using Dunnett's Test
Error bars denote standard deviation

CSV of the ASL-wheat breads was positively and linearly correlated (Pearson's correlation: r=0.43, p=0.04) with volume % of coarse particles in ASL flours. de Kock et al. (1999) suggested that the large flaky shapes of coarse bran may have encapsulated air during the bread making process leading to the more open structure and higher loaf volume they reported. The smaller surface area to volume ratio of coarse particles in ASL flours (Table 4.1) may have lowered water absorption by the ASL flour allowing for more water for gluten matrix development and consequently higher CSV.

The CSV of all ASL-wheat breads were significantly ($p<0.05$) lower than that of the wheat-only bread. This is consistent with published reports on substitution of refined wheat flour for lupin flours and fractions (i.e. protein isolates and concentrates) in bread (Doxastakis et al., 2002; Paraskevopoulou et al., 2010). However, all of the ASL-wheat breads except for *Mandelup* were within the CSV range of 3.4 to 5.6 g/cm^3 found for commercially available breads in Western Australia (Centre for Grain Foods Innovation, unpublished).. The maximum amount of ASL flour that can replace wheat flour and still provide bread with an acceptable volume now requires investigation.

Crumb cell characteristics

Table 4.2 shows the crumb cell characteristics of ASL-wheat and wheat-only bread samples as determined by C-Cell image analysis. Figure 4.4 presents bread and crumb photographic images. Crumb of *Mandelup*-wheat bread had significantly smaller ($p<0.05$) slice area, height, cell wall thickness, and cell diameter but had greater number of cells per cm^2 compared to other ASL-wheat breads (Table 4.2). Except for crumb height, most of the crumb characteristics of the other ASL-wheat breads did not significantly ($p>0.05$) differ with that of the wheat-only bread.

Protein content of the ASL flours was linearly and positively associated (Pearson's correlation, r=0.34, p=0.04) with crumb area of ASL-wheat breads. This finding suggests that the protein in lupin may have some useful technological functionality

in bread making, perhaps through protein crosslinking. This finding appears contrary to that of Paraskevopoulou et al. (2010) who reported that addition of lupin protein isolates to wheat bread decreased crumb area

Table 4.2. Crumb cell characteristics of ASL-wheat and wheat-only breads[1].

Bread	Slice area[2] (cm^2)	Bun height[2] (cm)	Number of cells[2] (per $cm^{2)}$	Cell wall Thickness[2] (mm)	Cell diameter[2] (mm)
Belara	40.6.±1.8^{ab*}	4.3±0.3^{b*}	83.1±7.9^{ab*}	0.42±0.01^{b}	1.7±0.2^{b}
Coromup	44.8.±2.6^{c}	4.5±0.3^{bc*}	76.5±6.3^{ab}	0.43±0.02^{b}	1.8±0.2^{b}
Gungurru	42.6±2.6^{bc}	4.6±0.2^{c*}	80.2±4.8^{ab}	0.42±0.01^{b}	1.8±0.1^{b}
Jenabillup	43.7±2.7^{c}	4.3±0.2^{b*}	80.8±5.0^{ab}	0.42±0.01^{b}	1.7±0.1^{b}
Mandelup	38.9±3.4^{a*}	3.8±0.2^{a*}	102.3.±8.6^{c*}	0.39±0.01^{a*}	1.3±0.1^{a*}
Tanjil	43.8±2.6^{c}	4.7±0.2^{c*}	74.8±3.1^{a}	0.43±0.01^{b*}	1.9±0.1^{b}
Wheat	*43.7±1.7*	*5.8±0.2*	*75.6±6.9*	*0.42±0.01*	*1.8*±0.2

[1]Means ± standard deviation (n=6)
[abc]Values within the lupin flour column with different superscript denotes significant difference ($p<0.05$) using Duncan's Test
* Denotes significant difference from wheat bread using Dunnett's Test

Fat content of the ASL flours was positively and linearly associated (Pearson's correlation) with crumb height ($r=0.72$, $p=0.01$), cell wall thickness ($r=0.64$, $p=0.02$), cell diameter ($r=0.70$, $p=0.01$) but negatively associated with the number of cells per cm^2 ($r= -0.79$, $p=0.01$). These associations may have been a result of increased ASL flour lipids assisting gas retention and gas cell stability in the dough.

Amongst ASL-wheat breads there was a positive linear correlation (Pearson's correlation) between CSV and both slice area ($r=0.50$, $p<0.001$) and bun height ($r=0.40$, $p=0.01$). Likewise, bread volume was positively associated with cell wall thickness ($r=0.63$, $p<0.001$), cell diameter ($r=0.62$, $p<0.001$) but negatively associated with the number of cells per cm^2 ($r=-0.62$, $p<0.001$). These results are in agreement with the findings of Paraskevopoulou et al. (2010).

The thinner cell wall and smaller cell diameter of *Mandelup*-wheat bread implies that the gluten matrix was not able to retain the gas bubbles (which may in part be due to the low fat content of *Mandelup* flour) formed during mixing and proofing which resulted in a higher number of smaller cells compared to the other ASL-wheat bread

samples. Alternatively, the production of more stable gas cells in *Mandelup*-wheat dough may have resulted in the lack of their coalescence during baking giving the greater number of smaller gas cells (Paraskevopoulou et al., 2010). The higher number of smaller gas cells in *Mandelup*-wheat bread created a denser pore appearance compared to other ASL-wheat breads (Figure 4.4).

Instrumental textural properties

Mandelup-wheat bread was significantly ($p<0.05$) the chewiest compared to the other ASL-wheat breads samples (Table 4.3**).** This appears to be the first report of an effect of ASL variety on the texture profile of bread.

There were negative linear correlations (Pearson's correlation) between the fat content of the ASL flour and the hardness ($r=-0.79$, $p=0.01$) and chewiness ($r=-0.51$, $p=0.04$) of ASL-wheat breads. Increased fat content of ASL flour may have assisted in increased gas retention and more stable gas cells resulting in a more open cell structure and softer and less chewy crumb. The lower fat content of *Mandelup* flour compared to the other ASL flours may have accounted for the stark difference in the texture profile between *Mandelup*-wheat breads and the other ASL-wheat breads.

Table 4.3 Texture profile of ASL-wheat and wheat-only breads[1].

Bread	Hardness[2] (g)	Springiness[2]	Cohesiveness[2]	Chewiness[2] (g)
Belara	381.4±15.0^{a*}	0.925±0.035^{ab}	0.7±0.1^{a}	261.9±32.0^{a}
Coromup	353.4±27.1^{a}	0.953±0.008^{bc}	0.7±0.0^{a}	250.8±30.7^{a}
Gungurru	323.6±28.9^{a}	0.943±0.006^{abc}	0.8±0.0^{a}	255.2±33.8^{a}
Jenabillup	332.2±35.6^{a}	0.957±0.00^{bc}	0.7±0.0^{a}	246.0±53.1^{a}
Mandelup	636.9±57.0^{b*}	0.918±0.023^{a}	0.7±0.0^{a}	464.6±35.4^{b*}
Tanjil	327.0±103.4^{a}	0.963±0.004^{c}	0.7±0.1^{a}	247.4±88.0^{a}
Wheat	*293.8±130.5*	*0.999±0.00*	*0.7±0.06*	*221.06±109.4*

[1]Means ± standard deviation (n=6)
[abc]Values for lupin bread within a column with different superscript denotes significant difference ($p<0.05$) using Duncan's Test
* Denotes significant difference from wheat bread using Dunnett's Test

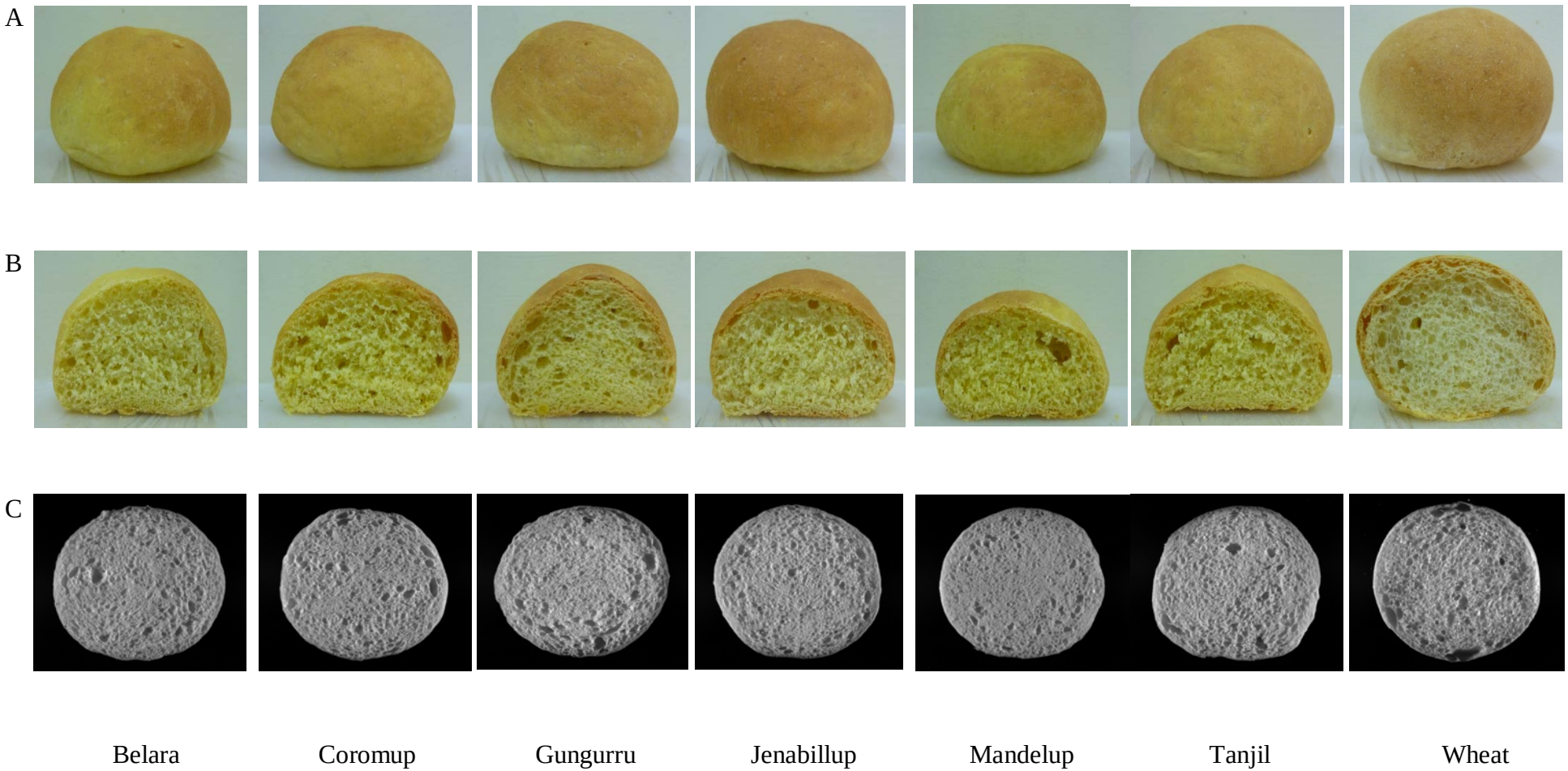


Figure 4.4. Photographic images of the ASL-wheat and wheat-only breads (A) whole bread (B) longitudinal cut (C) cross-sectional cut (C-Cell image)

The volume of coarse particles (>100 µm) in ASL flour samples showed a linear negative correlation (Pearson's correlation) with hardness ($r=-0.66$, $p<0.00$) and chewiness ($r=-0.58$, $p=0.02$) but positively correlated with springiness ($r=0.54$, $p=0.03$) of ASL-wheat breads. This indicates that ASL flours with more coarse particles produced softer, springier and less chewy bread, supporting the possible role of particle size of ASL flour in ASL-wheat bread quality.

ASL-wheat bread hardness demonstrated linear negative correlations (Pearson's correlation) with CSV ($r=-0.64$, $p=0.01$), cell wall thickness ($r=-0.77$, $p < 0.01$) and cell diameter ($r=-0.82$, $p=0.01$) but positively correlated with number of cells per cm^2 ($r=0.84$, $p<0.01$). Springiness of ASL-wheat breads showed linear and positive correlations with bread specific volume ($r=0.64$, $p=0.01$), bun slice area ($r=-0.54$, $p=0.02$), cell wall thickness ($r=0.67$, $p<0.01$) and cell diameter ($r=0.62$, $p=0.01$) but negative correlation with number of cells per cm^2 ($r=-0.70$, $p<0.01$). Chewiness of ASL-wheat breads showed negative linear correlations with bread specific volume ($r=-0.79$, $p<0.01$), cell wall thickness ($r=-0.72$, $p<0.01$), cell diameter ($r=-0.78$, $p<0.01$) but was positively correlated with number of cells per cm^2 ($r=0.79$, $p<0.01$). These results imply that ASL-wheat breads with higher CSV, cell diameter and cell wall thickness and lower number of smaller cells were softer, springier and less chewy. Findings on the interrelationships of these bread parameters reported by Scanlon and Zghal (2001) are consistent with the present study.

Except for the hardness ($p<0.05$) of *Belara* - and *Mandelup*-wheat breads, the instrumental textural properties of the ASL-wheat breads, unexpectedly did not significantly ($p>0.05$) differ with those of the wheat-only bread. This is in contrast to previous reports using ASL flour, and flour from other lupin species (*L. albus and. L. mutabilis*) (Bartkiene et al., 2011; Guemes-Vera et al., 2008; Paraskevopoulou et al., 2010). The results of the present study indicate that the lipid and protein components of the ASL flour may have assisted the ASL-wheat breads to attain some of the texture profile properties of the wheat-only bread.

Disulphide bond density

Table 4.4 presents the levels of free thiols, total sulphydryls and disulphide bonds in ASL-wheat and wheat-only breads. Free thiols levels of the ASL-wheat breads did not significantly ($p>0.05$) differ while *Gungurru* -wheat bread had the lowest ($p<0.05$) levels of total sulphydryls. Of the composite flour breads, *Belara*- and *Mandelup*-wheat breads had the significantly highest ($p<0.05$) level of disulphide bonds while *Gungurru*-wheat bread had the significantly lowest ($p<0.05$) level. The levels of disulphide bonds in the present study are lower than published reports however this appears to be the first report of the disulphide bond levels in any legume-wheat composite flour bread.

Table 4.4. Free thiols, total sulphydryl and disulphide bonds in ASL-wheat and wheat-only breads[1].

Bread	Free thiols (nmoles/mg sample)	Total SH (nmoles/mg sample)	Di-sulfide (nmoles/mg sample)
Belara	6.3±1.2^{a}	23.4±1.3^{b*}	17.0±2.0^{c*}
Coromup	5.2±3.3^{a}	18.6±3.4^{ab}	13.4±0.7^{b}
Gungurru	5.6±1.5^{a}	16.0±2.8^{a}	10.4±2.3^{a}
Jenabillup	6.6±2.1^{a}	20.7±3.6^{b*}	14.1±3.0^{b}
Mandelup	5.3±2.4^{a}	22.2±1.2^{b*}	16.9±1.3^{c*}
Tanjil	5.6±3.5^{a}	21.1±4.2^{b*}	15.4±0.8^{bc*}
Wheat	*3.0±2.6*	*14.5±3.3*	*11.4±0.9*

[1]Means ± standard deviation (n=4)
^{abc}Values for lupin breads within a column with different superscript denotes significant difference ($p<0.05$) using Duncan's Test
* Denotes significant difference with wheat flour using Dunnett's Test

The levels of free thiols in ASL-wheat breads did not significantly ($p>0.05$) differ from that of the wheat-only bread. Levels of total sulphydryls of ASL-wheat bread except for *Coromup*- and *Gungurru*-wheat breads were significantly ($p<0.05$) higher compared to that of wheat-only bread. *Belara*- , *Mandelup*- and *Tanjil*-wheat breads had significantly ($p<0.05$) higher levels of disulphide bonds than the wheat-only bread. These differences may be due to the different protein types in ASL and wheat flour. Lupin proteins are comprised mainly of globulins (Foley et al., 2011), whereas

wheat proteins include albumins, globulins, gliadins and glutenins. Disulphide crosslinks involving gliadins and glutenins (Gerrard et al., 2005) form during mixing of wheat flour and water produce the viscoelastic protein network required for bread making (Lindsay and Skerritt, 1999). Addition of ASL flour to wheat flour may have altered the disulphide crosslinks formed in the dough resulting in the observed differences in their levels in the ASL-wheat compared to the wheat-only breads.

The levels of disulphide bonds in wheat flour directly influence the rheological properties of dough (Shewry and Tatham, 1997) and according to Buchert (2010) optimal disulphide crosslinking during dough mixing is important in bread making. The level of disulphide bonds have been reported to either negatively affect (Manu and Prasada Rao, 2008) or have no effect (Poulsen, 1998) on dough and bread quality.

Disulphide bond levels in ASL-wheat bread were not significantly associated (Pearson's correlation, $p>0.05$) with bread quality attributes however the role of disulphide bonds or other protein cross-links such as di-tyrosine in ASL-wheat breads quality requires further investigation**.**

4.5. CONCLUSION

This study demonstrated significant effects of ASL variety on some physical properties of ASL-wheat bread. Moisture content may have influenced the particle size characteristics of ASL-wheat flour that in turn impacted on bread quality. Though fat and protein content of the ASL flour had a significant effect on ASL-wheat bread quality these relationships require further validation given that the Pearson's correlation r and non-linear model R^2 were generally < 0.80. The results indicate that all of the ASL varieties, except for *Mandelup*, may be suitable to replace wheat flour in bread to give desirable volume, crumb cell characteristics and instrumental textural properties. The findings in this Chapter highlight the importance of choosing the most suitable variety of ASL flour that can be substituted

for wheat flour in bread that would result in acceptable volume, crumb properties and instrumental texture. Given the results presented in this Chapter and Chapter 3, the *Coromup* variety was selected for use in the succeeding Chapters (5 and 6) due its good nutritional, chemical and physical properties when added to bread. *Coromup* is also the second largest produced ASL variety in Western Australia (CBH, personal communication). In addition, studies on shelf life of the ASL-wheat bread and the economics of its manufacture are needed to support commercialisation. Given the varietal effects identified in this study, investigations into how environment and its interaction with genotype affect ASL flour and bread quality are warranted. The varietal effects presented here may also assist lupin breeders to identify and optimise genetic traits in ASL to enhance its functionality for bread making.

CHAPTER FIVE-Experimental

The effects of bread-making process factors on Australian sweet lupin-wheat bread quality characteristics

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5.1. ABSTRACT

Factorial experimental design was used to investigate the effects of: sponge proofing time (min); sponge and dough mixing time (min); final proofing time (min); final proofing temperature (^{o}C) and; baking time (min) on Australian sweet lupin-wheat bread physical attributes. Factorial models show that crumb specific volume was positively associated with sponge and dough mixing time ($p=0.01$) and baking time ($p=0.02$). Crumb area was positively associated ($p=0.01$) with sponge and dough mixing time. Final proofing time positively influenced cell wall thickness ($p<0.01$), cell diameter ($p<0.01$) but negatively affected number of cells ($p<0.01$). Cell wall diameter also positively associated with baking time ($p=0.04$) while number of cells was negatively influenced by sponge and dough mixing time ($p=0.01$) Instrumental springiness was positively associated with sponge and dough mixing time ($p=0.02$). Sponge and dough mixing and baking times were the two most significant process parameters affecting the bread physical quality and hence should be optimised.

5.2. INTRODUCTION

The use of ASL flour in wheat bread can lower bread quality. This may be attributed to the low elasticity of lupin proteins and the high water binding capacity of lupin dietary fibre (Turnbull et al., 2005) that may weaken the gluten matrix and thus

result in poor loaf texture and volume (Guemes-Vera et al., 2008). Published results show that more than 10% substitution of wheat flour by lupin in bread led to poor volume and texture in bread (Doxastakis et al., 2002; Paraskevopoulou et al., 2010). There is however a lack of rigorous studies, such as by factorial screening, to determine effects of processing parameters on lupin-wheat bread quality. These studies are required to inform the development of optimised processes for high quality lupin bread.

The effects of bread making process parameters i.e. kneading of dough (or mixing); fermentation (or proofing); and baking on bread quality have been thoroughly discussed and reviewed by several authors (Cuvain and Young, 2006, Rosell, 2011). The control of these parameters is critical to obtain bread with good quality characteristics such as high crumb specific volume, desirable crumb cell structure and instrumental textural properties. The present study assessed for the first time the effects of important bread making process factors on the physical quality attributes of ASL-wheat breads.

5.3. MATERIAL AND METHODS

Materials

ASL variety *Coromup* was used in this study based on its good performance in our varietal screening study of physical, nutritional and chemical properties of ASL-wheat breads (data not presented). Thirty kg of *Coromup* whole grain harvested from east of Geraldton, Western Australia in 2012, was vacuum packed in plastic bags, and stored at ~10^{o}C until use. The kernel was separated from the seed coat using an LH 5095 dehuller (Codema Inc., Maple Grove, MN, USA) followed by air-induced separation (Kimseed Vacuum Separator, Kimseed International Pty Ltd, Osborne Park, WA, Australia) and manual sorting. The kernels were milled (Retsch SR 300, Retsch GmbH, Haan, Germany) to pass 100% through a 250µm screen. The resulting flour was vacuum-packed in plastic bags, placed in a sealed plastic box,

and stored in a dry and cool area (~ 10^{o}C) until use. Other bread making ingredients (WA wheat flour, yeast, bread improver, salt, sugar and vegetable oil) previously described in section **3.2** were used in this study.

Experimental design

Identifying limits of processing parameters

The processing variables for evaluation in the factorial screening (Table 5.1) were selected for their potential to influence bread physical characteristics (Collado-Fernández, 2003, Flander et al., 2007). Their lower and upper limits encompassed were based on those of Flander et al. (2007) and on preliminary processing experiments performed by the authors (data not presented).

Factorial experimental design

A two-level incomplete factorial experimental design 2^{5-1} (resolution V) was used to investigate the effects of the following process parameters (Table 5.1): sponge proofing time (min); sponge and dough mixing time (min); final proofing time (min); final proofing temperature (^{o}C) and baking time (min) on the physical bread attributes of: crumb specific volume (cm^3/g); crumb area (cm^2); number of cells per cm^2; cell wall diameter (mm); cell wall thickness (mm); hardness (g); springiness and chewiness (g) (Table 5.2) using Design-Expert Version 8 software (Stat-Ease Inc. Minneapolis, MN, USA). Factorial experimental design was used to screen multiple independent variables and establish the few significant ones affecting the dependent variables (responses) of interest. The incomplete design is considered as robust as the full design but requiring less number of experimental runs (Stat Ease Inc., 2011).

Four samples were prepared each day, which included a dummy control (wheat bread), internal control (wheat bread), and 2 ASL-wheat bread samples. The dummy control was baked at the start of the day to condition bread making equipment (i.e.

mixer, proofer and oven) and was discarded after baking. The order of internal control samples was randomised within each run. A total of 9 bread samples (buns) were produced for each run. Three samples from each treatment were chosen randomly for analyses.

Table 5.1. Factorial independent variables with actual and coded values.

Independent variable	Units	Actual values		Coded Values	
		Minimum	Maximum	Minimum	Maximum
Sponges proofing time	min	45	75	-1	1
Sponge and dough mixing time	min	4	12	-1	1
Final proofing time	min	20	50	-1	1
Final proofing temperature	^{o}C	30	40	-1	1
Baking time	min	10	25	-1	1

Bread making

A modified sponge and dough method at 20% ASL flour substitution for wheat flour and separate mixing and proofing for wheat sponge and lupin sponge previously described in section **3.3** was used in this study. Modification of the method in this study was done by combining the mechanical mixing of the sponges and dough and kneading of the dough (**Figure 3.1)** to minimize errors coming from manual handling of the samples. Specifications of the process parameters evaluated in this study i.e. sponges proofing time, sponges and dough mixing time, final proofing time and temperature, and baking time are presented in Table 5.1.

Analytical methods

Flour particle size distribution

The particle size distribution of the *Coromup* flour and wheat flour samples was measured using the method previously described in section **4.3.**

Proximate and dietary fibre analyses

The proximate composition and dietary fibre content of the *Coromup* flour and wheat flour samples were measured using the methods previously described in section **3.3.**

Crumb specific volume (CSV)

Specific volume (cm^3/g) of crumb of the breads was evaluated using the method previously described in section **4.3.**

Crumb cell characteristics

Crumb cell characteristics of the breads were measured following the method previously described in section **4.3.**

Instrumental textural properties

Instrumental textural properties of hardness, springiness, cohesiveness and chewiness of the breads were determined using the methods previously described in section **4.3.**

Table 5.2.Factorial experimental design in uncoded form of processing (independent) variables and response (dependent) variables[1].

Run	Uncoded formulation and processing variables					Responses							
	Sponges proofing time (min)	Sponges and dough mixing time (min)	Final proofing time (min)	Final proofing temp (^{o}C)	Baking time (min)	Crumb specific volume (cm^3/g)	Crumb area (cm^2)	Cell wall thickness (mm)	Cell diameter (mm)	No. of cells per cm^2	Hardness (g)	Springiness	Chewiness (g)
1	45	12	20	40	25	3.6±0.4*	31.8±10.7*	0.39±0.00	1.4±0.0	93.8±2.6*	422±30	0.88±0.02*	226±4
2	45	12	50	40	10	3.3±0.3	31.5±22.4*	0.43±0.01*	1.7±0.1*	77.5±4.8*	227±6*	0.90±0.02*	128±12*
3	75	4	20	30	10	3.1±0.2	28.7±15.2	0.40±0.00*	1.4±00	96.7±1.1*	363±20	0.85±0.01	184±11*
4	75	12	50	40	25	4.1±0.1*	30.0±10.8	0.43±0.00	1.8±0.1*	77.8±7.8	346±52	0.90±0.01*	179±37*
5	45	12	20	30	10	3.4±0.1	29.6±8.8	0.38±0.00	1.2±0.0	102.9±2.3*	331±21*	0.95±0.05*	206±19*
6	45	4	20	30	25	2.9±0.1	27.5±15.9	0.40±0.01*	1.5±0.2*	91.8±6.3*	432±41	0.86±0.06	183±25*
7	45	12	50	30	25	4.3±0.3*	33.2±10.2*	0.40±0.01*	1.6±0. 1*	86.9±4.9*	283±60*	0.94±0.02*	189±4*
8	75	4	50	30	25	3.4±0.3	29.2±3.4	0.43±0.00*	1.7±0.0*	79.8±0.7	379±19	0.90±0.04*	154±1*
9	75	4	50	40	10	2.9±0.1	22.8±10.6	0.43±0.02*	1.7±0.3*	84.0±9.8	431±30	0.87±0.00	216±14
10	45	4	50	30	10	2.6±0.2	25.4±10.6	0.41±0.00*	1.5±0.1*	91.3±2.2	388±5	0.86±0.01	209±19*
11	75	12	20	40	10	3.4±0.3	31.2±14.2*	0.38±0.01	1.2±0.1	104.9±6.4	315±30*	0.92±0.02*	200±17*
12	45	4	50	40	25	2.7±0.3	21.3±2.3	0.43±0.01	1.5±0.2*	87.9±8.3	587±34	0.85±0.05	220±10
13	75	12	50	30	10	3.0±0.2	28.3±13.8	0.40±0.00*	1.4±0.0	91.4±3.9*	339±22*	0.87±0.02	194±20*
14	75	4	20	40	25	3.6±0.2*	29.7±16.6	0.41±0.02*	1.5±0.1*	85.2±7.6*	359±40	0.90±0.01*	204±29*
15	75	12	20	30	25	3.7±0.0*	30.1±9.3	0.40±0.02*	1.5±0.2*	89.3±7.6*	419±27	0.92±0.00*	254±15
16	45	4	20	40	10	2.8±0.2	28.6±4.7	0.39±0.01	1.3±0.1	103.8±6.1*	361±39	0.89±0.02*	210±30*
Control	*60*	*8*	*35*	*35*	*25*	*4.3±0.6*	*34.0±2.3*	*0.41±0.01*	*1.7±0.1*	*80.9±4.9*	*279±52*	*0.92±0.02*	*166±30*

[1]Mean± standard deviation

*Denotes NO significant difference ($p>0.05$) from the control sample using Dunnet's Test

Statistical analysis

Design Expert V8 software (Stat-Ease, Inc. Minneapolis MN, USA) was used to generate the factorial screening study sample run sequence and to generate a regression analysis on the responses in order to determine the association of the formulation and processing variables with the response variables. Particle size and composition of ASL and wheat flours were compared using one-way ANOVA followed by Duncan's Multiple Range Test (DMRT) to separate the means when F was significant. The properties of the ASL-wheat bread samples were compared with the control sample (wheat-only bread) using one-way ANOVA followed by Dunnet's Test. Pearson's Correlation test was used for correlation of bread physical characteristics. Descriptive and correlation analyses were performed using IBM SPSS Statistics V.21 (IBM Corp., NY, USA).

5.4 RESULTS AND DISCUSSION

Figure 5.1 shows photographic images of the ASL-wheat and wheat-only control breads.

Flour particle size distribution

Particle size characteristics of ASL and wheat flours are presented in Table 5.3. The particle size of ASL flour (volume weighted mean) was significantly ($p<0.05$) larger than that of the refined wheat flour. Published reports indicate that decreasing particle size of wheat flour substitutes (i.e. bran or whole wheat) used in bread making can either increase (Moder et al., 1984) or decrease (de Kock et al., 1999) loaf volume. Given these contradictory reports in the literature, further investigations are still needed to determine the effects of particle size of ASL flour on ASL-wheat bread quality.

Table 5.3. Particle size characteristics of ASL and wheat flours[1].

Sample	d[0.1][2] (µm)	d[0.5][2] (µm)	d[0.9][2] (µm)	D[4,3][3] (µm)	Surface area (m^2/g)
ASL	9.0±0.1[a]	82.4±1.2[b]	301.4±4.4[b]	126.2±2.7[b]	0.22±0.00[b]
Wheat	15.1±0.1[b]	77.7±0.3[a]	169.1±0.5[a]	85.8±0.3[a]	0.17±0.00[a]

[1]Mean± standard deviation
[2] d[0.1], d[0.5], d[0.9] represents the maximum diameter of 10%, 50% and 90% of the particles, respectively.
[3]D[4,3] represent the volume weighted mean particle size
[ab]Values within column with different superscript denotes significant difference ($p<0.05$) using Duncan's Test

Proximate and dietary fibre composition

This ASL flour sample had significantly ($p<0.05$) higher levels of protein, fat, ash and total dietary fibre but significantly ($p<0.05$) lower levels total available carbohydrates than the wheat flour sample (Table 5.4). These results are comparable to the findings of Hall et al. (2005) and similar to the results presented in Chapter **3**. Based on this flour composition data the 20% substitution of wheat flour by ASL flour used in this study and Chapter **3** can increase the protein content and dietary fibre content of wheat breads by 42% and 75%, respectively and hence improve the bread's nutritional profile. However, the increase in non-gluten protein and high water-binding dietary fibre from ASL flour may reduce bread quality through interfering with gluten matrix development.

Table 5.4. Proximate and total dietary fibre content of ASL and wheat flours[1].

Component (g/100 g dry basis)	ASL	Wheat
Protein	40.4 ± 0.5[b]	12.6±0.3[a]
Total dietary fibre	41.7±0.3[b]	6.9±0.1[a]
Fat	7.6±0.2[b]	0.2±0.0[a]
Ash	2.9±0.2[b]	0.7±0.0[a]
Total available carbohydrates	7.0±0.9[a]	79.5.±1.0[b]

[1]Mean± standard deviation
[ab]Values within the same row with different superscript denotes significant difference ($p<0.05$) using independent sample T-test

Influence of process parameters on crumb specific volume

The CSV of the ASL-wheat bread samples (Table 5.2) ranged from 2.6-4.3 cm^3/g with some samples (i.e. Runs 1, 4, 7, 14 and 15) not significantly different ($p>0.05$) from the wheat-only control. Factorial analysis demonstrated that CSV of ASL-wheat bread samples was positively associated with sponges and dough mixing time ($p = 0.01$) and baking time ($p= 0.02$) (Table 5.5) which accounted for 65% of the total effect contribution.

The positive relationship between CSV and sponges and dough mixing time supports previous reports of an increase in dough development time when lupin flour or lupin flour fractions (i.e. protein isolates) are added to wheat flour (Paraskevopoulou et al., 2010, Mubarak, 2001). These authors explained that the increase in time required for dough development could have been due to the addition of non-gluten proteins and dietary fibre from ASL flour resulting in disruption or delay in the development of the gluten matrix. In the present study, those lupin-wheat breads with similar CSV to that of the wheat-only control had a longer mixing time of 12 min, compared to the 8 min used for the control. This increased mixing time will have allowed for (a) increase opportunity for development of the gluten matrix; (b) better assimilation of the lupin proteins and dietary fibre into the matrix; and (c) incorporation of more air cells into the dough, which in turn could have positively influenced the CSV of the ASL-wheat breads. Amr and Ajo (2005) also reported that increased mixing in the sponge and dough method had a positive effect on the specific volume of flat breads. The authors reasoned that the increased mixing time led to more subdivision of the air bubbles developed during proofing giving dough with a larger number of smaller sized bubbles and producing breads with a desirable porous, spongy crumb, and fine grain.

The positive association of baking time with CSV implies that increased baking time allowed more time for the air bubbles produced during mixing and proofing to expand during baking. Therdthai et al. (2002) explained that dough changes during the whole duration of baking, reporting that the baking process can be divided into 3

stages: (1) firstly there is increase of dough volume, loss of surface skin elasticity and thickening and browning of the crust; (2) then moisture from the crumb evaporates, starch gelatinises and proteins coagulate, and; (3) finally there is volatilisation of organic substances. Increased baking time of ASL-wheat bread would therefore allow for all of these stages to be realised fully resulting in increased CSV.

In future studies, the extended mixing and baking times required in the present study for increased CSV, need to be optimised in conjunction with other process and formulation parameters to maximise ASL-bread quality.

Influence of process parameters on crumb cell characteristics

Crumb area

The crumb area of the ASL-wheat breads ranged from 21.3-33.2 cm^2 with some samples (i.e. Runs 1, 2, 7 and 11) not significantly different ($p>0.05$) to the wheat-only control (Figure 5.1). Factorial analysis demonstrated that crumb area was positively associated with sponges and dough mixing time ($p = 0.01$) which represented 43.6% of the total effect contribution (Table 5.5). This result is related to the positive effect of mixing time on CSV since CSV and crumb area were highly correlated (Pearson correlation: r=0.75, p=0.001). The increased air cell incorporation as a result of prolonged mixing may have led to increased crumb area of the bread.

Cell wall thickness

The cell wall thickness of the ASL-wheat flour breads ranged from 0.38-0.43 mm with some samples (i.e.Runs 2, 3, 6, 7, 8, 9, 10, 13, 14, 15 and 16) not significantly different ($p>0.05$) from the wheat-only control. Cell wall thickness was positively associated with final proofing time ($p <0.001$) accounting for 57.7% of the total effect contribution (Table 5.5).

Table 5.5. Associations between processing variables and ASL-wheat bread responses in factorial screening.

Processing variables	Responses											
	Bread specific volume (cm^3/g)		Crumb area (cm^2)		Cell wall thickness (mm)		Cell diameter (mm)		No. of cells per cm^2		Springiness	
	EC[a]	*P* value	EC[a]	*P* value	EC[a]	*P* value	EC[a]	*P* value	EC[a]	*P* value	EC[a]	*P* value
Sponges proofing time (min)	3.5	0.32	0.06	0.91	4.95	0.11	2.73	0.35	0.96	0.54	0.12	0.88
Sponges and dough mixing time (min)	38.49	**0.01**	43.63	**0.01**	7.09	0.06	0.17	0.81	24.44	**0.01**	41.69	**0.02**
Final proofing time (min)	0.06	0.89	10.11	0.16	57.68	**<0.01**	49.36	**<0.01**	45.63	**<0.01**	2.61	0.51
Final proofing temp (^oC)	0.01	0.96	1.14	0.62	6.96	0.06	4.38	0.24	1.94	0.38	0.3	0.82
Baking time (min)	26.15	**0.02**	1.84	0.53	7.59	0.05	15.45	**0.04**	3.74	0.23	0.22	0.84

[a]% Effect Contribution, calculated by dividing each factors sum of squares by the total of all the term sum of squares and multiplying by 100, ranking the magnitude of each factor's effect

This finding is consistent with those of Zghal et al. (1999, 2001) on wheat breads who suggested that a greater degree of gas cell coalescence occurred with increased proofing time producing more stable and thicker cell walls in the bread. However, thin-walled, finer and uniformly-sized cells result in a softer and more elastic textured bread compared to thick-walled, coarse and open structures and hence an increase in cell wall thickness may negatively influence consumer acceptability (Pyler (1988). The results of the present study indicate that proofing time needs to be optimised in future studies in order to minimise any undesirable effect of thick cell walls on consumer acceptability of ASL-wheat bread.

Cell diameter

Cell diameter of the ASL-wheat flour breads ranged from 1.2-1.8 mm into which range the control bread also fell (Table 5.2). Cell diameter was positively associated with final proofing time ($p < 0.01$) and baking time ($p = 0.04$), together accounting for 64.8% of the total effect contribution (Table 5.5). The positive relationship of cell diameter with final proofing time is similar to the findings of Zghal et al. (1999 and 2001). Increased proofing time may have resulted in a greater degree of gas coalescence and thus producing larger cells. The positive relationship between cell diameter and baking time similar to the findings of Hayman et al. (1998), who reported that the important mechanism of gas cell coalescence occurs between 12 to 18 mins of baking. Therefore ASL-wheat breads baked for 25 min will have had far greater opportunity for gas cell coalescence resulting to its larger cell diameter compared to those baked for only 10 min.

Number of cells per cm^2

The number of cells per cm^2 of the ASL-wheat flour breads ranged from 77.5-104.9 into which range the wheat-only control fell (Table 5.2). The number of cells per cm^2 was negatively influenced by final proofing time ($p < 0.01$) and baking time ($p = 0.02$) accounting for 66.7% of the total effect contribution (Table 5.5).

Figure 5.1 (page1)

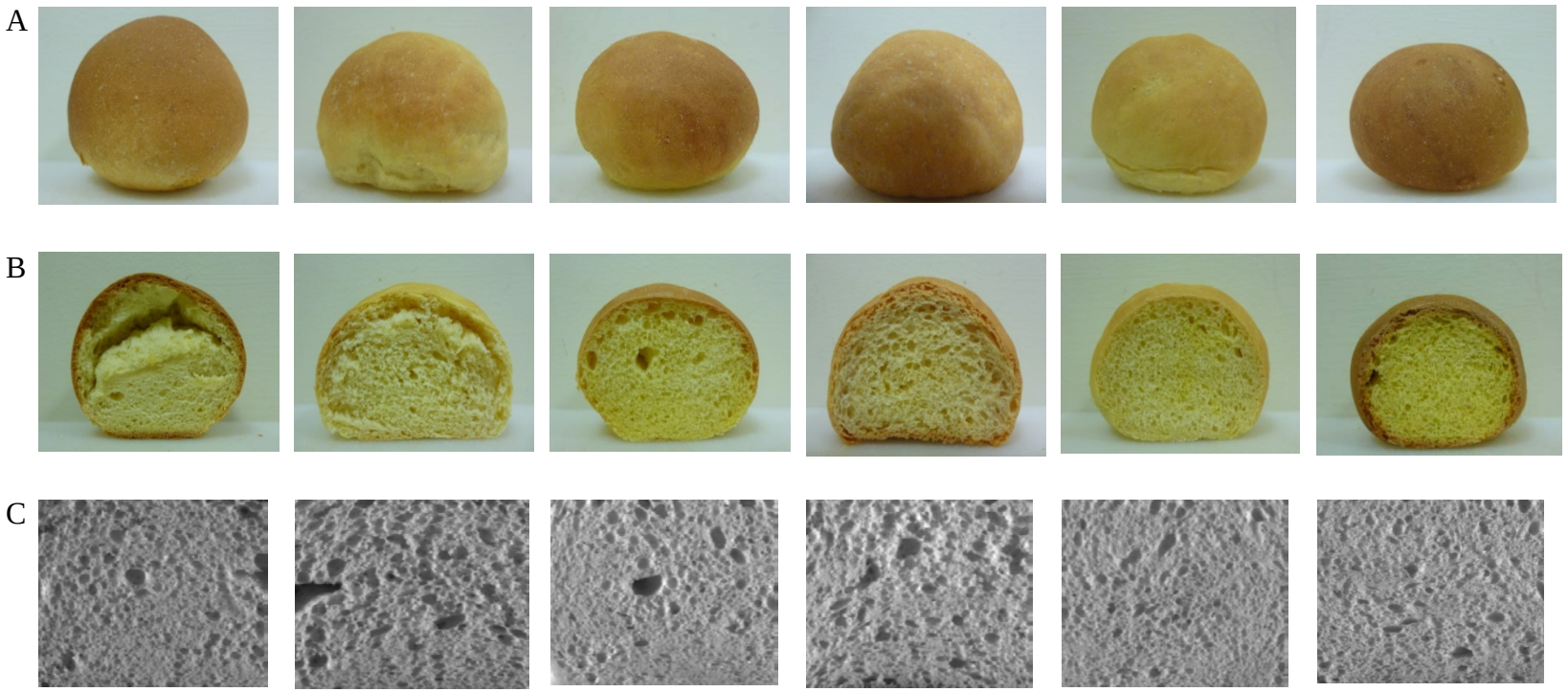


Figure 5.1 (page 2)

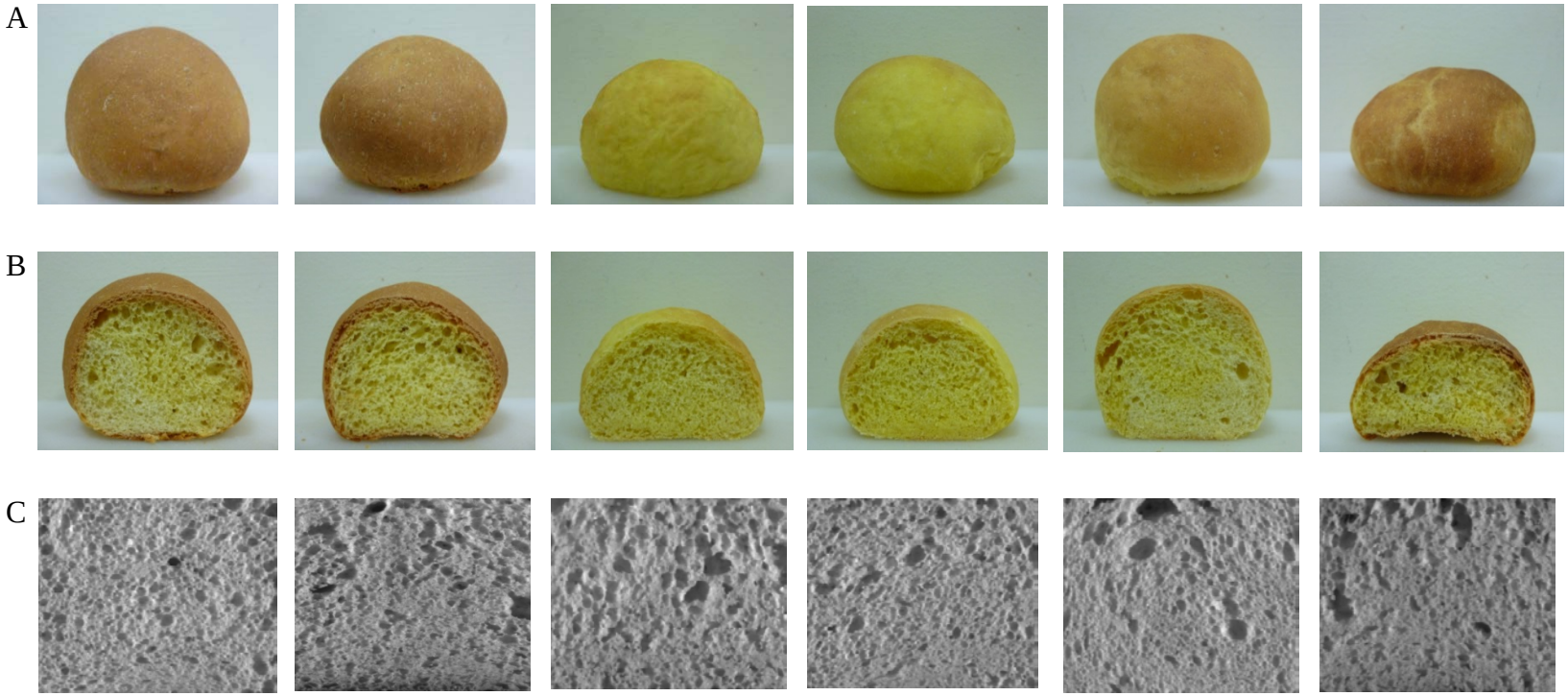


Figure 5.1 (page 3)

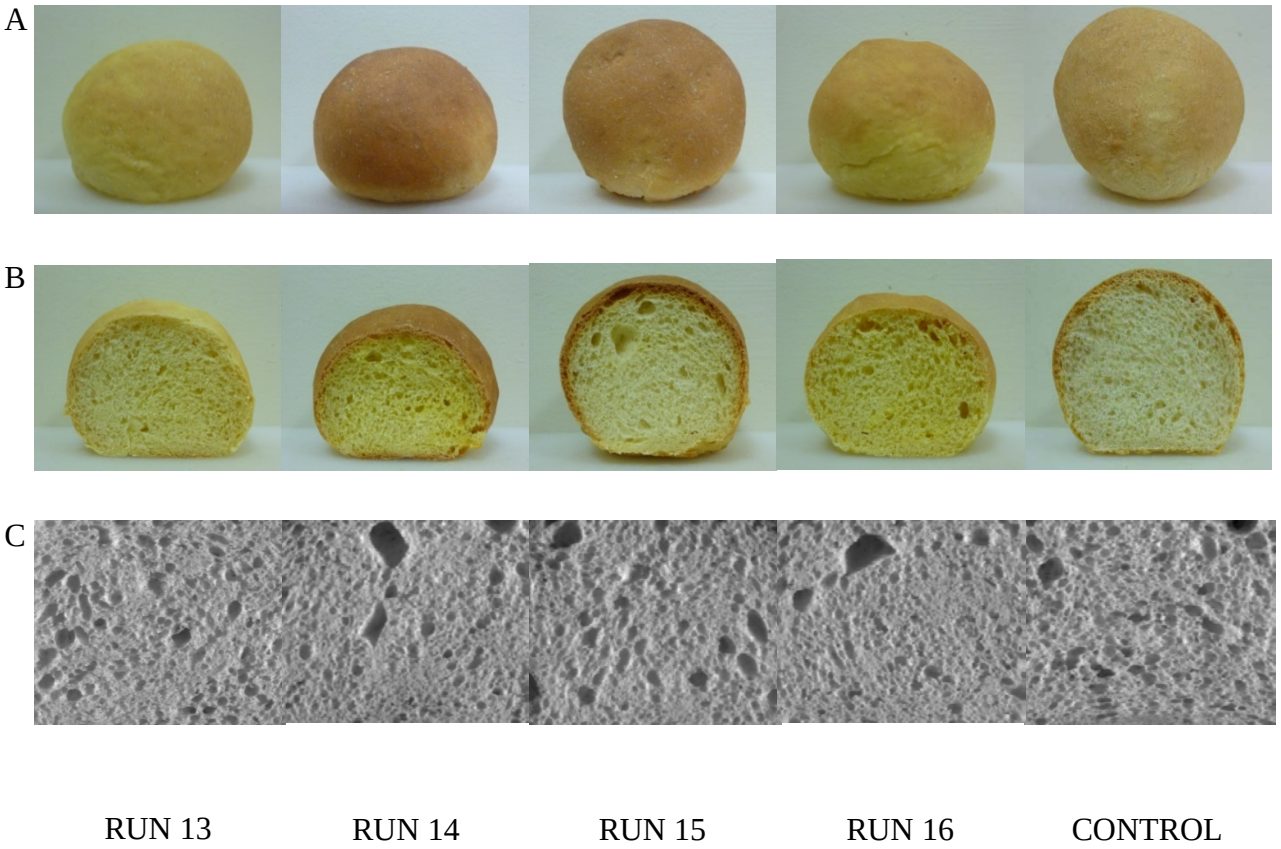


Figure 5.1. Photographic images of ASL-wheat (runs 1- 16) and wheat-only (control) breads (A) whole bread bun (B) longitudinal cut (C) cross-sectional cut (C-Cell image).

The results imply that increased proofing and baking decreased the cell density of the crumb, perhaps due to bubble coalescence leading to fewer larger sized air bubbles. This is also supported by the positive relationship of proofing and baking times with cell diameter.

Influence of process parameters on instrumental texture characteristics

The instrumental texture characteristics of the breads are given in Table 5.2. Hardness of the ASL-wheat flour breads ranged from 227-587 g, springiness from 0.85 to 0.95, and chewiness from 129- 254 g. The values of all of these parameters for the ASL-wheat breads encompassed those of the wheat-only control bread. These results indicate that substitution of 20% ASL-flour into the wheat flour bread has the potential to result in comparable textural properties to that of wheat-only bread. There is however still a need to further explore the maximum amount of ASL flour (to maximise nutritional and health benefits) that can be added without sacrificing textural quality.

Factorial analysis showed that amongst the textural properties measured, only springiness was significantly affected by any of the processing parameters, being positively affected by sponge and dough mixing time ($p = 0.02$) accounting for 42% of the total effect contribution (Table 5.5). This finding may be related to the positive effect of mixing time on CSV since CSV and springiness were highly correlated (Pearson's correlation: r=0.67, p=0.005), indicating that the higher-volume ASL-wheat breads had softer and springier texture. This is supported by previous studies that reported an inverse relationship of bread volume with hardness (Every et al.,1998) and of hardness with springiness (Carson and Sun, 2001; Gambaro et al., 2002). In addition, the incorporation of more air cells with increased mixing time as described earlier, may have led to increase in ASL-wheat bread volume and thus producing springier bread.

5.5. CONCLUSION

Factorial screening was an effective tool for identifying that mixing time of sponges and dough (min) and baking time (min) are the two most significant process

parameters influencing the ASL-wheat bread physical parameters, CSV (cm^3/g), crumb area (cm^2) and springiness using the sponge and dough bread making method. Interestingly, there were some ASL-wheat bread samples which had similar properties to the control sample. The results presented in this Chapter emphasizes the importance of understanding the effects of bread making process parameters on ASL-wheat bread quality to maximise addition of ASL flour into wheat bread with acceptable physical characteristics. The two most significant process parameters (i.e. mixing time of sponges and dough and baking time) that affected ASL-wheat bread physical properties can be used in optimisation experiments along with formulation parameters (i.e. amount of ASL flour, amount of water and ASL flour particle size) using a more robust experimental design such as response surface methodology (RSM). Optimisation of the aforementioned process and formulation parameters (as presented in Chapter 6) to determine the conditions that maximised the physical, sensory and nutritional quality of ASL bread at maximum ASL incorporation rate to design a product with potential consumer health benefits will be presented in Chapter 6.

CHAPTER SIX-Experimental

Optimisation of formulation and process of Australian sweet lupin (ASL)-wheat bread

Information in this chapter has been published/submitted as follows:

Villarino, C.B.J., Jayasena, V., Coorey, R., Bell, S. and Johnson, S.K. (2015).Optimisation of formulation and process of Australian sweet lupin (ASL) bread. *LWT-Food Science and Technology*, 61, 359-367.

Johnson, S. K., Villarino, C. B., Jayasena, V., Coorey, R. & Chakrabarti-Bell, S. 2014. A lupin flour based foodstuff and a method of manufacturing a lupin flour based foodstuff, Australian Provisional Patent Application No. 2014903932. Curtin University.

6.1 ABSTRACT

This study aimed to optimise formulation and process factors of Australian sweet lupin (ASL)-refined wheat bread bun to maximise the ASL level whilst maintaining bread quality using response surface methodology (RSM) with a central composite face-centered design. Statistical models were generated that predicted the effects of level of ASL flour incorporation (g/100 g of ASL-wheat composite flour), ASL-flour volume weighted mean particle size (µm), water incorporation level (g/100 g ASL-wheat composite flour), mixing time of sponge and dough (min) and baking time (min) on crumb specific volume, instrumental texture attributes and consumer acceptability of the breads. Verification experiments were used to validate the accuracy of the predictive models. Optimisation of the formulation and process parameters using the models predicted that formulations containing ASL flour at 21.4 - 27.9 g/100 g of ASL-wheat composite flour with volume weighted mean particle size of 415 - 687 µm, incorporating water at 59.5 - 71.0 g/100 g ASL-wheat composite flour, with sponges and dough mixed for 4.0 - 5.5 min and bread baked for 10 - 11 min would be within the desirable range of CSV, instrumental hardness and overall consumer acceptability. Verification experiments confirmed that the statistical models accurately predicted the responses.

6.2. INTRODUCTION

Lupin incorporation above 10% results in poor dough and bread quality (Doxastakis, et al., 2002; Mubarak, 2001) but higher levels are desirable to obtain nutritional and health benefits from the lupin-containing bread. There is however a lack of investigations on the effects of formulation and processing parameters and their interaction on lupin-wheat composite flour bread quality and the optimisation of the levels of these parameters to maximise the level of lupin incorporation whilst maintaining acceptable bread quality.

Flour particle size and the amount of added water are important formulation parameters that affect bread quality. Previous studies of non-wheat flour substitutes have reported that increased particle size either increased (de Kock et al., 1999) or decreased (Moder et al., 1984) bread volume. The amount of water added to ASL-wheat bread formulations needs to be carefully adjusted to compensate for the water absorbed by the ASL flour. It has been demonstrated in Chapter 5 that mixing time and baking times were positively associated with bread volume, crumb area and springiness, therefore these factors should also be considered in any optimisation studies.

The mathematical and statistical approach of response surface methodology (RSM) has been used to optimise formulation and process parameters for the manufacture of "healthy" breads such as wholemeal oat bread (Flander et al., 2007), gluten-free breads (McCarthy et al., 2005) and wheat-legume flour composite breads (Angioloni and Collar, 2012; Jideani and Onwubali, 2009). There is however no published study using RSM to optimise the formulation and process parameters to deliver high quality lupin-wheat composite flour bread with maximum lupin incorporation.

The aim of this study was to use RSM to assess the effects of formulation and process parameters on the physical and sensory qualities of ASL-wheat composite flour bread and to optimize the levels of these parameters to produce acceptable quality bread with maximum level of ASL flour incorporation.

6.3 MATERIALS AND METHODS

Materials

The same ASL (var. *Coromup*) seeds previously described in section **5.3** were used in this study. Ten kg of seeds harvested in 2012 at a site 70 km east of Geraldton, Western Australia were vacuum packed in moisture-proof plastic bags, and stored at ~10^{o}C until use. The seeds were de-coated and milled as previously discussed in section **3.3** into flours of three differing target particle sizes (1) 120 µm screen to give 27 µm volume weighted mean particle size; (2) 750 µm screen to give 357 µm volume weighted mean particle size; and (3) 2000 µm screen to give 687 µm volume weighted mean particle size. Screen sizes were determined by preliminary milling experiments. Particle size was determined by laser light scattering using a Mastersizer 2000 (Malvern Instruments Ltd, Malvern, UK) as previously discussed in **3.3.** Flour samples were vacuum-packed in plastic bags and stored in moisture-tight boxes ~ 10^{o}C until use.

Details of the Western Australian refined wheat flour ("bakers flour") and the other bread ingredients (yeast, bread improver, sugar, salt and vegetable oil) are as previously discussed in section **3.3.**

Experimental design and statistical analyses

Identifying limits of formulation and processing parameters

The formulation and processing variables evaluated in this study (Table 6.1) were selected for their potential to influence ASL-wheat bread quality based on findings of previous studies (Flander et al., 2007; Gularte et al., 2012) and the results previously presented in Chapter **5**. Their lower and upper limits were chosen as extreme levels at which a bread product could still be manufactured based on preliminary experiments by the authors (data not presented).

Table 6.1. Central composite experimental design showing independent variables with actual and coded values.

Factor	Independent variable	Units	Actual values		Coded values	
			Minimum	Maximum	Minimum	Maximum
X1	ASL flour volume weighted mean particle size	µm	27	687	-1	1
X2	Level of ASL flour incorporation	g/100 g composite flour	5	40	-1	1
X3	Level of water incorporation	g/100 g composite flour	40	80	-1	1
X4	Sponge and dough mixing time	min	4	12	-1	1
X5	Baking time	min	10	25	-1	1

ASL, Australian sweet lupin

Modelling of responses

A central composite face-centered response surface methodology (RSM) design (1/2 fraction) with 5 independent variables and six replicates at the centre point for a total of 32 experimental samples (Table 6.2) was generated and analysed using Design-Expert Version 8 software (Stat-Ease Inc. Minneapolis, MN, USA). Central composite design is the most common RSM method and is used to estimate coefficients of quadratic models (Stat-Ease Inc., 2011) that can be used for accurate optimisation. The formulation and processing independent variables investigated were: X_1, ASL flour volume weighted particle size (µm); X_2, level of ASL flour incorporation (g/100 g of ASL-wheat composite flour); X_3, level of water incorporation (g/ 100 g composite flour), X_4, mixing time of sponges and dough (min); and X_5, baking time (min). Centre points were replicated to measure reproducibility of the method.

Table 6.2. Actual values of formulation and process parameters of the 32 samples used in central composite experimental design.

Run	X_1, ASL flour volume weighted mean particle size (µm)	X_2, Level of ASL flour incorporation (g/100 g composite flour)	X_3, Level of water incorporation (g/100 g composite flour)	X_4, Sponge and dough mixing time (min)	X_5, Baking time (min)	Run	X_1, ASL flour volume weighted mean particle size (µm)	X_2, Level of ASL flour incorporation (g/100 g composite flour)	X_3, Level of water incorporation (g/100 g composite flour)	X_4, Sponge and dough mixing time (min)	X_5, Baking time (min)
1	27	40	40	4	10	**17**	687	40	40	12	10
2	27	5	80	4	10	**18**	27	22.5	60	8	17.5
3	687	22.5	60	8	17.5	**19**	27	40	40	12	25
4	357	22.5	40	8	17.5	**20**	687	5	40	12	25
5	687	40	80	12	25	**21**	27	40	80	4	25
6	27	5	80	12	25	**22**	357	22.5	60	8	17.5
7	357	40	60	8	17.5	**23**	687	40	40	4	25
8	357	22.5	60	8	17.5	**24**	357	22.5	60	8	25
9	357	22.5	60	12	17.5	**25**	27	5	40	12	10
10	357	22.5	60	8	17.5	**26**	27	5	40	4	25
11	357	22.5	60	8	17.5	**27**	357	22.5	60	4	17.5
12	687	5	80	4	25	**28**	687	5	40	4	10
13	687	40	40	12	10	**29**	357	22.5	60	8	17.5
14	27	22.5	60	8	17.5	**30**	27	40	80	12	10
15	27	40	40	12	25	**31**	357	5	60	8	17.5
16	687	5	40	12	25	**32**	357	22.5	60	8	10

ASL, Australian sweet lupin

Multiple linear regression analysis was applied to fit data for each response variable to linear and quadratic models. Experimental data were transformed when required based on Box-Cox tests and the most accurate model was chosen through sequential F-tests, lack-of fit tests and other adequacy measures (i.e. R^2, adj R^2, PRESS, DFFITS, DFBETAS, Cook's D). The generalized quadratic equation used for each response variable is given in Eq. 6.1:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_0 X_i + \sum_{i=1}^{n} \beta_{ii} X_i + \sum \sum_{i<j=1}^{n} \beta_{iJ} X_i X_j \qquad \text{Eq. 6.1}$$

where Y is the predicted response; β_0, βi, βii, and βij are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively, and Xi, and Xj corresponds to the independent variables. Two dimensional contour plots were generated for each response variable, showing the relationship between two independent variables with the three other independent variables fixed at centre levels. Design-Expert Version 8 software (Stat-Ease Inc. Minneapolis, MN, USA) was used for model generation, tests of model adequacy, and contour plot generation. Pearson's Correlation test was used for correlation of bread physical characteristics and were performed using IBM SPSS Statistics V.21 (IBM Corp., NY, USA).

Optimisation

Optimisation was primarily based on generating a solution with the maximum level of ASL flour incorporation to give maximum CSV, minimum instrumental hardness and minimal consumer overall acceptability of 6 ("like slightly"). The secondary optimisation objectives were maximum ASL flour particle size and minimum mixing and baking times based on cost minimisation for commercial bread production. Optimisation of the formulation and process variables were performed using a multiple response method, "desirability". Desirability is a measure of success when optimising multiple responses and ranges in value from 0 to 1 (least to most desirable, respectively) (Dhinda et al., 2012). This approach combined desires and priorities for each of the response and independent variables identified above as the basis of optimisation. The desirability scores were generated by the Design-Expert Version 8 software (Stat-Ease Inc. Minneapolis, MN, USA) by specifying the criteria: i.e. goal ("maximise", "minimise", "target", "in range", "equal to"); limits,

weights and importance for CSV, instrumental hardness and overall acceptability, ASL flour incorporation, ASL flour particle size, mixing times and baking times (Table 6.3). Level of ASL flour incorporation was set at maximum as a proxy variable for maximum protein and dietary fibre content of the bread. ASL flour particle size was also specified at maximum level while mixing and baking times were specified at minimum levels. CSV was set at maximum and instrumental hardness at maximum. The target level of overall acceptability by consumer evaluation panel was fixed to a score of 6 ("like slightly") in a 9 point-hedonic scale rating. The limits for CSV and instrumental hardness were based on the upper and lower values determined for wheat-only bread (data not shown). "Weights" for all variables were set at 1. "Importance" for both the ASL flour incorporation and overall acceptability were set at maximum (+++++), since the main objective of the optimisation was to maximize ASL incorporation rate whilst maintaining high sensory acceptability of the bread. The software generated the "desirability" scores of different combinations of formulation and process parameters and only scores with >0.70 were considered in the reported optimum range for each variable.

Table 6.3. Specifications of criteria for the optimisation of independent and response variables used in optimisation.

Factors	Optimisation criteria			
	Goal	Limits	Weights	Importance
Independent variables				
ASL flour incorporation (g/100 g composite flour)	Maximise	5-40	1	+++++
Volume weighted mean particle size µm)	Maximise	27-687	1	+
Mixing time (min)	Minimise	4-12	1	+
Baking time (min)	Minimise	10-25	1	+
Dependent variables				
Crumb specific volume(cm^3/g)	Maximise	3.0-5.6	1	+
Instrumental hardness (g)	Minimise	110-222	1	+
Overall acceptability	Target=6	5.5-9.0	1	+++++

.

Verification experiments were performed to estimate the predictive capacity of the RSM models. Two bread samples were produced and analysed: one "optimal" and the other "sub-optimal". Experimental data for each response variable were compared to the predicted value of the response using confidence and prediction intervals at $\alpha = 0.95$. Experimental values of the responses within the confidence and/or prediction interval signify that the model can accurately predict responses.

Bread making

The sponge and dough method presented in section **3.3** which was modified in section **4.3** was used for making breads in this study. Each baking run comprised of 5 samples namely, a dummy control (wheat bread), internal control (wheat bread), and 3 ASL-wheat bread samples. Formulation and processing conditions at various levels evaluated in the present study are shown in Tables 6.1 and 6.2. Other processing and formulations were as detailed in section **3.3.** Doughs were prepared using a total of 550 g of composite ASL- refined wheat flour with water added at various combinations specified in Tables 6.1 and 6.2. The remaining ingredients comprised of 14.3 g yeast, 7.7 g bread improver, 5.5 g salt, 5.5 g sugar and 10.4 g vegetable oil. Physical tests were performed on 3 randomly chosen breads from each treatment after storing at room temperature for up 24 h after baking.

Analytical methods

Crumb specific volume (CSV)

Specific volume (cm^3/g) of bread crumb was evaluated using the method previously described in section **4.3.**

Instrumental textural properties

Instrumental textural properties of hardness, springiness, cohesiveness and chewiness of the bread samples were determined using the method previously described in section **4.3.**

Consumer evaluation

Two consumer panel groups were used in the study: Group 1 for modelling of the effects of formulation and process parameters and; Group 2 for verification of the models. Group 1 consisted of 74 panellists (14 male and 60 female) and Group 2, 50 panellists (13 male and 37 female). The participants were 18 to 55 years of age, regular bread consumers, not allergic to any food, and not pregnant or lactating. Ethics approval was obtained from the Human Ethics Committee of Curtin University.

During the evaluation of the modelling samples, each panellist (Group 1) received a random selection of nine samples from the total of thirty seven (32 experimental and 5 control samples), served in two sessions, with a 5 min break between each session. Sample presentation was based on a replicated incomplete balanced block design, Plan 13.15 of Cochran and Cox (1957). During the evaluation of the verification samples, each panellist (Group 2) evaluated all 3 samples consisting of the optimal, non-optimal and control (wheat-only) using a randomized complete block design.

The panellists received 10 g of each sample coded with 3-digit random numbers along and were instructed to evaluate the samples from left to right and to cleanse their palate with water between samples. Panellists rated their acceptability of colour, appearance, flavour/aroma, texture and overall acceptability of the samples using a questionnaire with 9-point hedonic scales (1=dislike extremely; 2=dislike very much; 3=dislike moderately; 4= dislike slightly; 5=neither like nor dislike; 6= like slightly; 7= like moderately; 8= like very much; and 9= like extremely). Evaluations were performed in individual booths illuminated with artificial daylight.

Proximate and dietary fibre analyses of optimal bread sample

The proximate composition and dietary fibre content of the optimal bread formulation was measured using the methods previously described in section **3.3.**

6.4. RESULTS AND DISCUSSION

Effects of formulation and process parameters on CSV

The CSV of the ASL-wheat breads ranged from 1.0 to 4.0 cm^3/g. Table 6.4 shows the effects of formulation and process parameters on CSV expressed as their corresponding regression coefficients in the quadratic models. Tests for reliability of the models (Table 6.4) indicate that the equations can adequately predict the CSV as a function of the formulation and process factors.

The generated model showed that all formulation and process parameters except for ASL flour particle size had significant ($p<0.05$) effects on CSV. Figure 6.1(A) presents the contour plot of the effects of level of ASL flour vs level of water incorporation on CSV. This plot illustrates how at a constant level of water incorporation, increasing the level of ASL flour reduces ($p<0.05$) CSV. In addition, at a constant level of ASL flour incorporation, increasing the level of water gives increasing CSV to a maximum, after which further addition of water results in CSV lowering again. This illustrates the quadratic effect ($p<0.05$) of level of water incorporation on CSV.

Published reports have previously demonstrated that above 10% substitution of refined wheat flour by lupin flour decreases bread volume (Dervas et al., 1999; Mubarak, 2001). However, most studies on lupin bread have not considered the effects of other formulation and process parameters and their interaction on bread volume. For instance, in some previous studies, the amount of water used for the lupin-wheat breads and control wheat bread were the same (Guillamon et al., 2010). However, the quadratic effect of water on CSV observed in the present study and the high water binding capacity of lupin highlight the importance of adding an optimal amount of water to attain desirable ASL-wheat bread volume.

Table 6.4. Effects of formulation and process factors on crumb specific volume (CSV) and instrumental texture of ASL-wheat bread expressed as their corresponding coefficients in the quadratic predictive models

Factor[a]	Crumb specific volume (cm^3/g)	Instrumental texture		
		Hardness (g)	Springiness	Chewiness (g)
Constant	2.267	13.385	0.595	-0.07
PS	-	-0.002*	0.000*	-
LF	0.004*	0.022*	0.006*	0.000*
W	-0.059*	-0.354*	0.002*	0.007*
MT	0.022	0.230	-0.022	-
BT	0.006	0.354*	0.016	-0.011*
PS × *LF*	-	-	-	-
PS × *W*	-	-	-	-
PS × *MT*	-	-	-	-
PS × *BT*	-	0.000*	-	0.000*
LF × *W*	-	-0.000*	-	-
LF × *MT*	-	-	-	-
LF × *BT*	-	-0.002*	-	0.000*
W × *MT*	-	0.055	0.000*	Ns
W × *BT*	-	-	-	Ns
MT × *BT*	-0.001*	-	0.000	
PS^2	-	-	0.000	-
LF^2	-	0.002*	-0.000*	-0.000*
W^2	0.000*	0.003*	-	-0.000*
MT^2	-	-	-	Ns
BT^2	-	-0.008*	-	0.000*
R^2	0.90	0.95*	0.92	0.83
R^2_{adj}	0.88	0.91*	0.88	0.76
CV (%)	7.35	3.72*	3.56	3.41
Lack of fit	0.22	0.10	0.04*	0.22
Transformation	$1/\sqrt{Y}$	*ln(Y)*	*None*	$1/\sqrt{Y}$

*Coefficients significant (95% confidence level)

[a]*PS*, volume weighted mean particle size (µm); *LF*, level of ASL flour incorporation (g/100 g composite flour); *W*, level of water incorporation (g/100 g composite flour); *MT*, mixing time (min); *BT*, baking time; (min)

R^2, R^2_{adj}, *CV (%)* and *Lack of fit are* measures of fit of the model

Transformation is data transformation used to improve fit of models

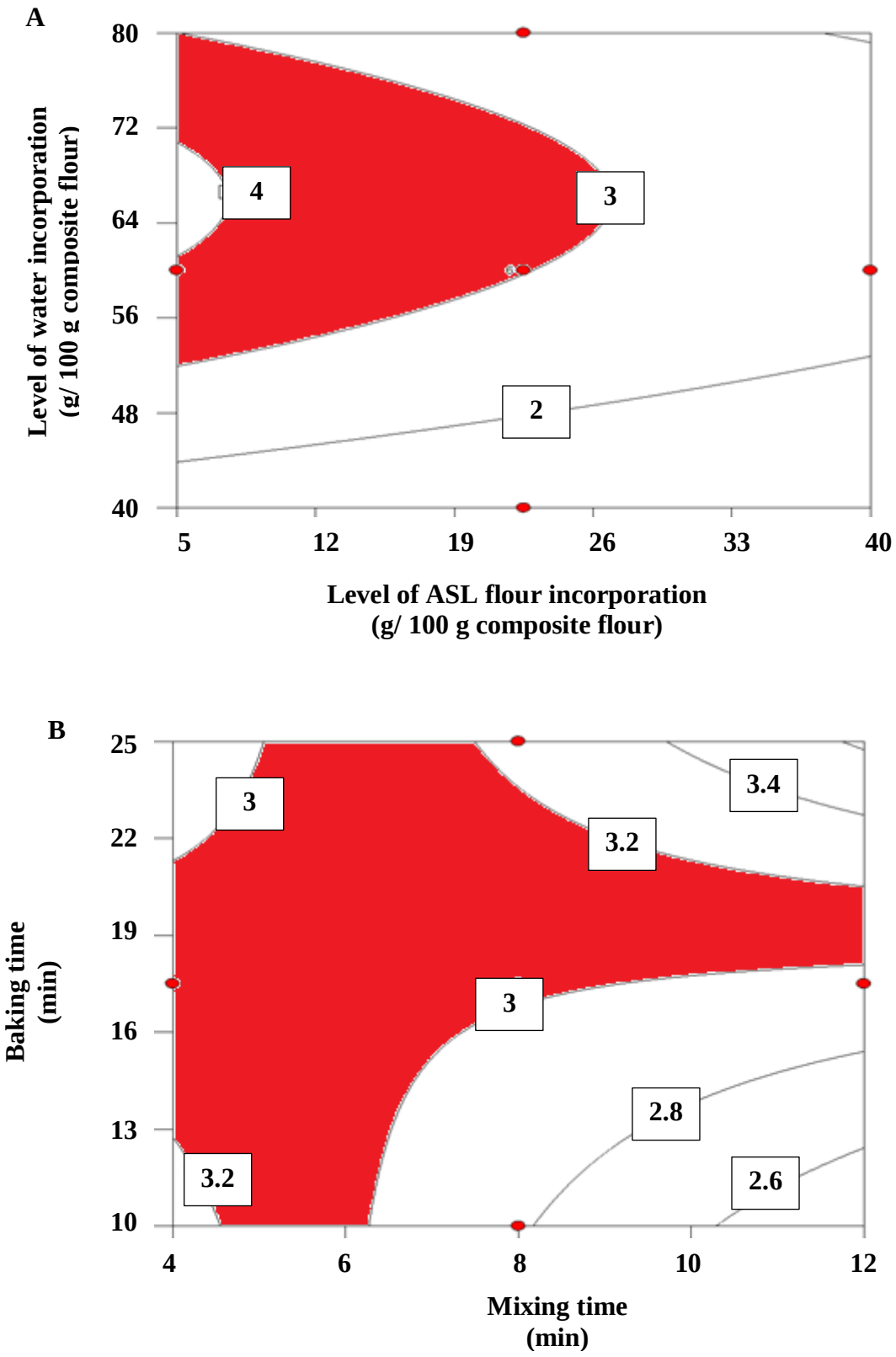


Figure 6.1.Contour plots showing effects on crumb specific volume (cm^3/g) of: (**A**) level of ASL flour and level of water incorporation and (**B**) mixing time and baking time.

CSV was not significantly associated ($p>0.05$) with either mixing or baking time (Table 6.4), however the interaction between mixing and baking times (*MT* x *BT*; Table 6.4) was significant ($p<0.05$), hence the coefficients for the individual factors are included in the model (Table 4) due to the hierarchical conditions of regression models. Figure 6.1 (B) presents the response surface contour plot of the effect of mixing time vs baking time on CSV. This plot illustrates that mixing time of 4.0-6.4 min with baking time of 10-21 min or mixing time of 5-12 min with baking time of 17.5-25.0 min, give CSV values above the target of 3 cm^3/g. The results indicate that the required gas cell expansion to reach target CSV values of 3 cm^3 /g occurred even at short mixing and baking times.

Given the wide range of possible combinations of mixing and baking times to attain target CSV, it should be possible to minimise these process times to reduce overall bread manufacturing time without comprising the bread quality.

Effects of formulation and process parameters on instrumental texture

The effects of formulation and process parameters on measures of instrumental texture expressed as their corresponding regression coefficients in the quadratic models are given in Table 6.4. Tests for reliability of the models (Table 6.4) generally indicated that the equations can adequately predict the responses as a function of the formulation and process factors. The springiness acceptability model however had a significant ($p<0.05$) lack of fit suggesting it may not be highly accurate. Pearson correlation tests showed significant association between hardness and springiness ($r=-0.79$, $p<0.05$) and hardness and chewiness ($r=0.82$, $p<0.05$). Due to these correlations and that hardness is the most common textural characteristic measured for bread, the following discussion will focus on hardness.

Instrumental hardness of ASL-wheat breads ranged from 256-4834 g and the generated model showed linear, interactive and quadratic associations with formulation and process parameters (Table 6.4). Figure 6.2(A) presents the contour plot of the effects of the level of ASL flour vs water incorporation level.

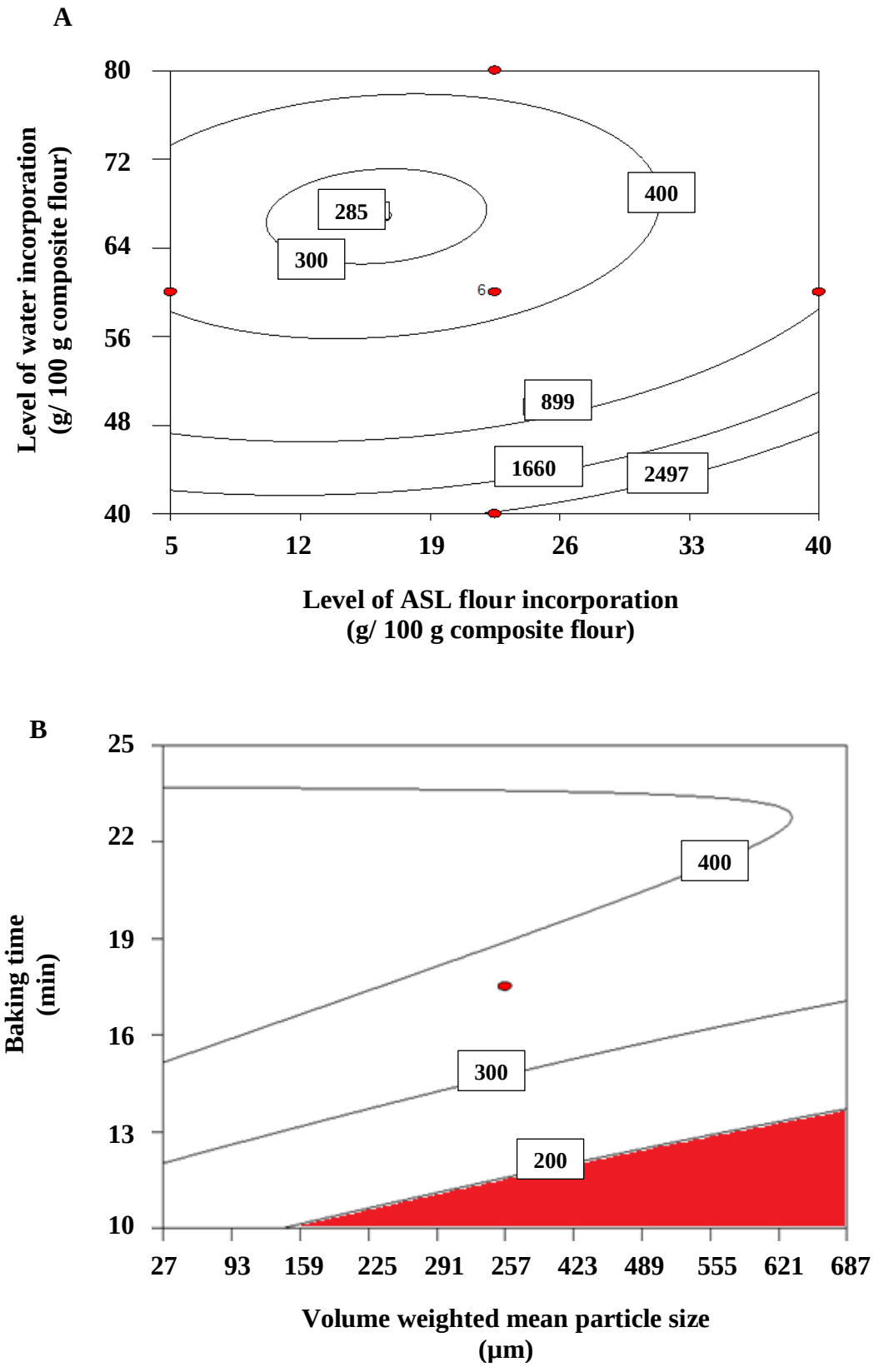


Figure 6.2. Contour plots showing effects on instrumental hardness (g) of: (**A**) level of ASL flour and level of water incorporation and (**B**) volume weighted mean particle size and baking time.

This plot demonstrates that there is a limited and specific combination of the amount of ASL flour (~ 16 g /100 g of composite flour) and water (~64 g /100 g of composite flour) that is predicted to produce ASL-wheat breads with the target level of hardness (below 222 g). This limited and specific combination is due to the quadratic effects of both the level of ASL flour and water incorporation and their interaction. The results demonstrate the importance of adding the optimal amount of water to attain desirable ASL-wheat bread texture.

Baking time alone had a quadratic effect on instrumental hardness and particle size of ASL flour had an interactive effect with baking time (Table 6.4). Figure 6.2 (B) shows the contour plot of the effects of ASL flour volume weighted mean particle size vs baking time, demonstrating that a minimum ASL flour volume weighted mean particle size of ~192 µm combined with 10 min baking time would produce ASL-wheat breads with the target hardness of < 222 g. The negative linear effect of volume weighted mean particle size on hardness implies that the use of larger ASL flour particle size in ASL-wheat bread results in softer crumb. Larger ASL flour particle size may have resulted in less water absorption (due to their smaller surface area to volume ratio) leading to decreased ability of the ASL flour to compete with the gluten-forming proteins of the wheat flour and improved development of the gluten matrix.

According to de Kock et al. (1999) the large flaky shapes of the coarse bran can encapsulate air during the bread making process leading to the more open structure, higher loaf volume and softer and springier crumb. Larger particle size in ASL flour may also have had this type of effect. The interactive effect of ASL flour particle size and baking time might be explained by larger particle size ASL flour giving maximum gas cell expansion during early stages of baking resulting in less time needed for baking to produce softer bread. Likewise, less baking time intuitively would lead to less moisture loss resulting in softer bread.

Based on these findings it appears possible to maximise ASL particle size and minimise baking time to help reduce bread manufacturing costs whilst not compromising the bread quality.

Effects of formulation and process parameters on ASL-wheat bread consumer acceptability

The effects of formulation and process parameters on consumer acceptability of colour, appearance, flavour, texture and overall acceptability of the breads expressed as their corresponding regression coefficients in the quadratic models are shown in Table 6.5. Tests for reliability (Table 6.5) indicate that generally the equations can adequately predict these responses as a function of the formulation and process factors. The appearance acceptability model had a significant ($p<0.05$) lack of fit suggesting it may not be highly accurate. Pearson correlation tests show that acceptability of colour, appearance, flavour and texture are all highly correlated ($p<0.05$) with overall acceptability and therefore this discussion will focus on overall acceptability.

Overall acceptability scores of the ASL-wheat breads ranged from 2 ("dislike very much") to 7 ("like moderately') and was significantly ($p<0.05$) associated with formulation and process parameters (Table 6. 5). Figure 6.3 (A) shows the contour plot of the effect of level of ASL flour vs water incorporation which indicates that to give the target overall acceptability score of 6, a maximum ASL flour incorporation of ~30 g/100 g composite flour combined with ~68 g water/100 g composite flour is needed. As the level of ASL flour incorporation increases from 5 to 30 g/100 g composite flour there is a corresponding decrease in the range of the amount of water that can be added owing to the quadratic effect of water and its interactive effect with ASL flour incorporation. It can also be observed that the contour plots of the effects of ASL flour vs water incorporation on CSV (Figure 1A) and overall acceptability (Figure 6.3 A) are almost identical. This is reflected in a high Pearson's correlation ($r=0.88$, $p<0.05$) between CSV and overall acceptability, demonstrating how bread volume is strongly and positively associated with consumer acceptability.

The contour plot of the effect of level of ASL flour incorporation vs mixing time on overall acceptability (Figure 6.3(B)), demonstrates that a maximum level of ASL flour incorporation of ~28 g/100 g composite flour, mixed for 4 to 12 min, would produce breads with the target minimum overall acceptability score of 6.

Table 6.5. Effects of formulation and process factors on consumer acceptability scores of ASL-wheat bread expressed as their corresponding coefficients in the quadratic predictive models

Factor[b]	Consumer acceptability				
	Colour	**Appearance**	**Flavour**	**Texture**	**Overall**
Constant	1.044	1.051	-5.620	1.045	1.109
PS	-0.000*	-0.000*	-	-0.000*	0.000
LF	0.004*	0.006*	-0.079*	0.010*	0.008*
W	-0.020*	-0.027*	0.359*	-0.026*	-0.021*
MT	0.006	0.010	-0.115*	0.009	0.007*
BT	0.002*	-0.004*	0.225*	0.006	-0.013
PS × *LF*	0.000	-	-	0.000*	0.000
PS × *W*	0.000*	0.000*	-	0.000*	0.000*
PS × *MT*	0.000*	0.000*	-	-	-
PS × *BT*	0.000*	0.000*	-	0.000*	0.000*
LF × *W*	0.000*	0.000*	-	0.000*	0.000*
LF × *MT*	-0.000*	-0.000*	0.003*	-0.000*	-0.000*
LF × *BT*	-	0.000*	0.001	-0.000*	-0.000*
W × *MT*	-0.000*	-0.000*	-	-	-
W × *BT*	-	-	-	0.000*	-
MT × *BT*	0.000*	-	-	-	-
PS^2	-	-	-	-	-
LF^2	-	-	-	-	-
W^2	0.000*	-	-0.003*	0.000*	0.000*
MT^2	-	-	-	Ns	-
BT^2	-	0.000*	-0.006*	ns	0.000*
R^2	0.99	0.99	0.90	0.96	0.96*
R^2_{adj}	0.98	0.98	0.87	0.94	0.94*
CV (%)	1.78	4.31	6.61	3.87	3.35*
Lack of fit	0.26	0.02*	0.16	0.21	0.30
Transformation	$1/\sqrt{Y}$	*1/Y*	$(Y)^1$	$1/\sqrt{Y}$	$1/\sqrt{Y}$

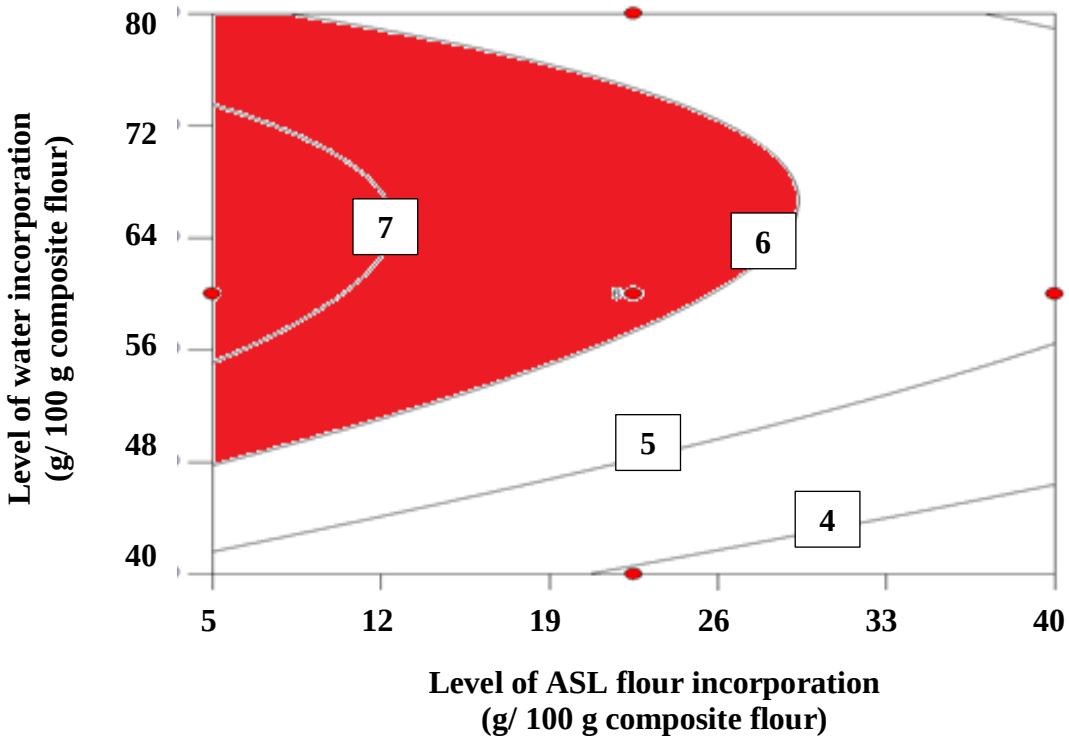
*Coefficients significant (95% confidence level)

[a]*PS*, volume weighted mean particle size (µm); *LF*, level of ASL flour incorporation (g/100 g composite flour); *W*, level of water incorporation (g/100 g composite flour); *MT*, mixing time (min); *BT*, baking time; (min)

R^2, R^2_{adj}, *CV (%)* and *Lack of fit are* measures of fit of the model

Transformation is data transformation used to improve fit of models

A



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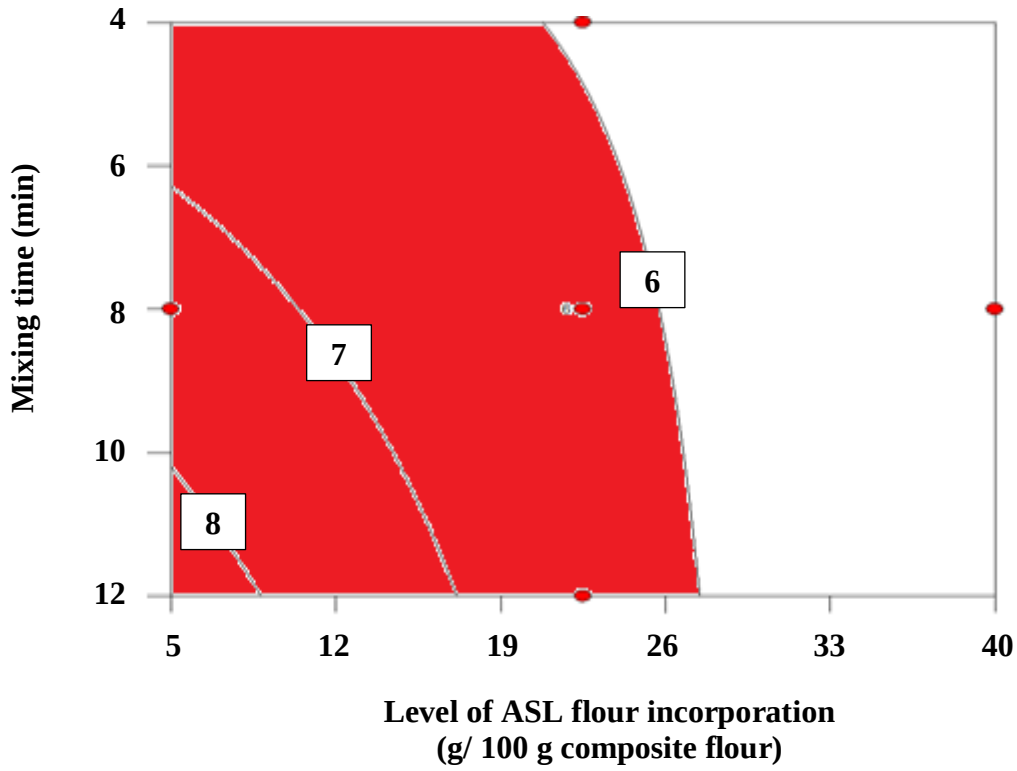

Figure 6.3 (page 1)

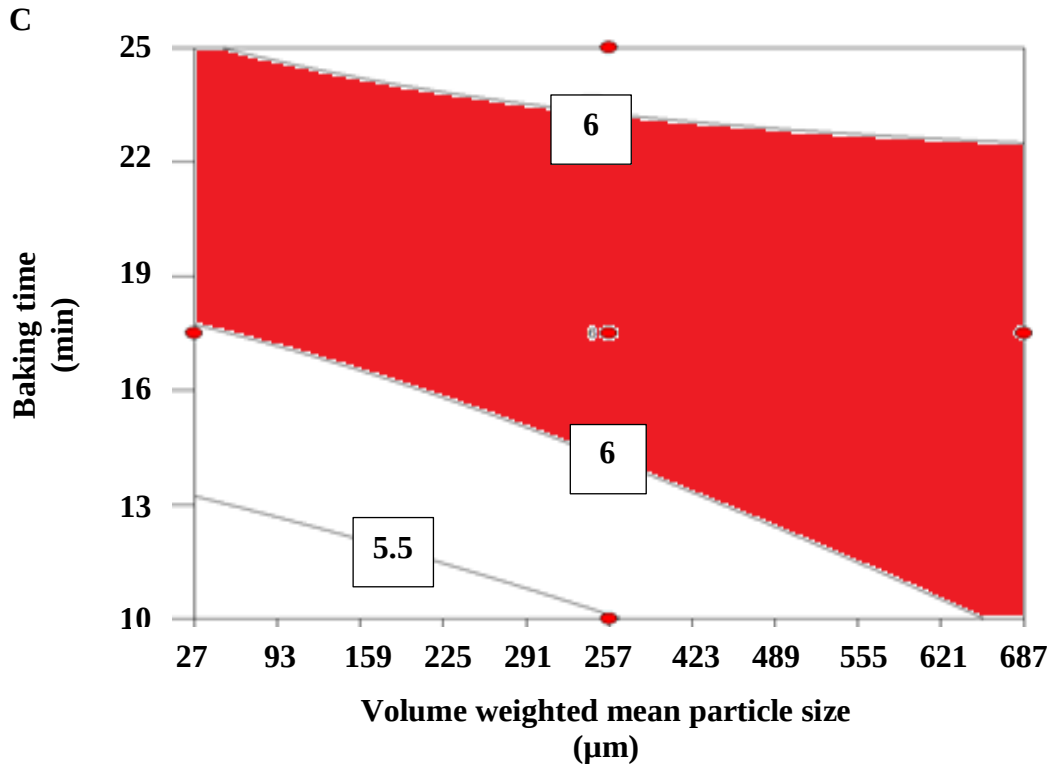

Figure 6.3. Contour plots showing effects on overall acceptability score of: (**A**) level of ASL flour and level of water incorporation, (**B**) volume weighted mean particle size and baking time, and (**C)** level of ASL flour and mixing time

Decreasing the amount of ASL flour by ~40% (to 17 g/100 g composite flour) combined with a mixing time of 4 to 9.5 min would result in an increase in overall acceptability score to 7 ("like moderately"). These results indicate that short mixing times are possible, which may assist with the cost-effectiveness of ASL-wheat bread production.

The contour plot of the effect of volume weighted mean particle size of ASL flour vs baking time (Figure 6.3 (C)) demonstrates that a particle size of > 654 µm combined with a baking time of 10.0 - 23.5 min would produce ASL-wheat breads meeting the target overall acceptability score of 6. Decreasing the particle size below 654 µm reduced the range of baking time that gave breads with overall acceptability score of 6 due to a quadratic effect of baking time and its interactive effect with particle size. The effects of particle size of ASL flour and baking time on overall acceptability

may be related to their effects on instrumental texture illustrated by the high negative correlation ($r=-0.83$, $p<0.05$) between overall acceptability and instrumental hardness. Based on these findings in may be possible to maximise ASL particle size and minimise baking time to reduce costs of ASL-wheat bread manufacturing.

Optimisation and verification of models

The following ranges of optimized formulation and process parameters satisfied the optimisation criteria (Table 6.3) and had a "desirability" of >0.70: (a) ASL flour volume weighted mean particle size 415 to 687 µm; (b) level of ASL flour incorporation 21.4 to 27.9 g/100 g composite flour; (c) level of water incorporation 59.5 to 71.0 g/100 g composite flour; (d) mixing time 4.0 to 5.5 min; and (e) baking time 10 to 11 min. This is the first report of using RSM to optimise formulation and process parameters of ASL-containing bread to maximise addition level of ASL whilst maintaining acceptable physical and sensory properties.

An "optimal" sample was produced with: ASL flour volume weighted particle size 687 µm; ASL flour incorporation 26.8 g/100 g composite flour; water incorporation 66g/100 g composite flour; mixing time 4 min; baking time 10 min. A "non-optimal" sample was produced with: ASL flour volume weighted particle size 122 µm; ASL flour incorporation 26.8 g/100 g composite flour; water incorporation 48 g/100 g composite flour; mixing time of 8 min; baking time 20 min. Photographic images of the "optimal" and "non-optimal" breads are given in Figure 6.4.

Verification experiments using the "optimal" and "non-optimal" samples demonstrated that that in general, the generated models were able to predict CSV, instrumental hardness and overall acceptability responses (Table 6.6). Actual values of the sample responses were within the confidence and prediction intervals of the predicted values except for the instrumental hardness of the "optimal" sample.

Table 6.6. Predicted and actual values of crumb specific volume (CSV), instrumental hardness and overall acceptability scores of "optimal" and "non-optimal" ASL-wheat bread.

Response	"Optimal" bread[1]		"Non-optimal" bread[2]	
	Predicted value	Actual value	Predicted value	Actual value
Crumb specific volume (cm/g^3)	3.2±0.0	3.0±0.0	2.0±0.0	2.1±0.0
Hardness (g)	105.1±0.3	198.4±17.5*	1110±0.3	1106.3±145.3
Overall acceptability	6.0±0.0	5.8±2.2	4.6±0.0	5.1±2.2

[1]Conditions: ASL flour volume weighted mean particle size, 687µm; level of ASL flour incorporation, 26.8 g/100 g composite flour; level of water incorporation 66g/100 g composite flour; mixing time of sponge and dough, 4 min; baking time, 10 min

[2]Conditions: ASL flour volume weighted particle size, 122 µm; level of ASL flour incorporation, 26.8 g/100 g composite flour; level of water incorporation, 48 g/100 g composite flour; mixing time of sponge and dough, 8 min; baking time, 20 min

*Denotes significant difference ($p<0.05$) between predicted and actual values using prediction intervals

Proximate and dietary fibre composition of "optimal" bread sample

The proximate and dietary fibre composition (as is basis) of the "optimal" ASL-wheat bread sample were as follows: protein 19 g/100 g; fat 5 g/100 g; total dietary fibre 19 g/100 g; ash 2 g/100 g; total available carbohydrate 55 g/100 g. The protein and dietary fibre content of the optimal ASL-wheat bread are 62% and 126% respectively higher compared to that of the wheat-only control bread (data not shown), allowing "increased protein" and "good source of dietary fibre" nutrient content claims according to Australia and New Zealand regulations (FSANZ, 2013).

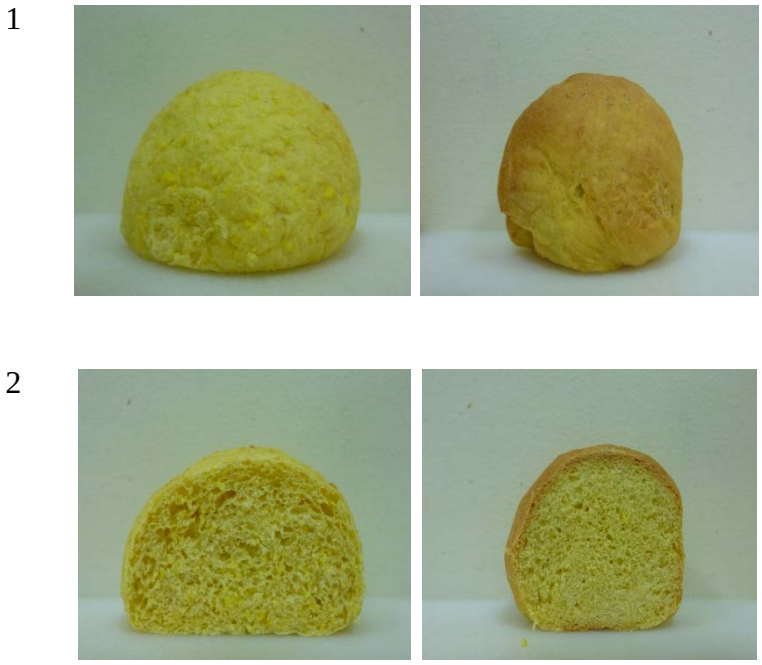

Figure 6.4. Photographic images of ASL-wheat bread (optimal and non-optimal) (1) whole bun, and (2) longitudinal cut. (A) level of ASL flour incorporation (g/100 g composite flour), (B) crumb specific volume (cm^3/g), (C) instrumental hardness (g) and (D) overall acceptability score.

6.5. CONCLUSION

This study successfully used RSM to model the effects of formulation and process parameters on CSV, instrumental hardness and overall acceptability of ASL-wheat composite flour breads. The statistical models were verified and then used for optimising of the formulation and process parameters to maximise addition of ASL flour in bread for maximum nutritional benefits whilst maintaining acceptable bread quality. This is the first report to present an in-depth investigation of how lupin bread

making formulation and process parameters affect bread qualities. Furthermore, this is the first study which has used RSM to optimise formulation and process parameters to maximise addition of ASL flour in wheat bread for maximum nutritional benefits (and potential health benefits) whilst maintaining acceptable physical and sensory properties. Our findings have increased the understanding of the effects of formulation and process parameters on ASL-wheat bread quality. This information will assist the grains industry in providing ASL flour of appropriate specifications for quality bread manufacture to their customers and assist bread manufacturers to develop high quality breads with maximum lupin addition that may assist in consumer nutrition and health. Future research is now required to better understand on one-hand the impact of gluten addition on ASL-wheat bread quality and on the other hand the process and formulation conditions required to manufacture gluten-free ASL based breads to meet this expanding market.

CHAPTER SEVEN

GENERAL CONCLUSION

The findings presented in the Chapters 3-6 of this thesis demonstrate that the aims of the thesis were achieved. The first aim was to examine the effects of ASL variety on nutritional, phytochemical and physical properties of ASL-wheat bread and select an ASL variety with good potential for manufacture of high quality bread for optimisation studies was achieved in Chapters 3 and 4. The results of Chapter 3 and 4 demonstrate that ASL variety had significant effects on the nutritional, chemical and physical properties of ASL flour and ASL-WA wheat bread. ASL varieties *Belara*, *Coromup* and *Tanjil* flour were identified as good choices for use in ASL-wheat bread considering the nutritional, phytochemical and physical properties of the end product. These results highlight the importance of specifying the ASL variety when supplying flour to bread manufacturers to ensure a high and consistent bread quality.

Chapter 3 showed that similar to previous studies (presented in Table 2.4), addition of lupin flour (regardless of species) to wheat bread increased its protein and dietary fibre content. Chapter 4 on the other hand revealed that addition of lupin flour to wheat bread decreased bread volume and increased hardness (similar to findings in previous studies; Table 2.6) due most likely to the non-gluten proteins in lupin and high WBC of the lupin dietary fibre.

Chapters 3 and 4 presented a comprehensive description of ASL and the commercially available varieties not just as flour but as applied to bread. This information advanced the state of knowledge of ASL specifications as raw material and its effect when added to food (i.e. bread) compared to what is available in literature (Tables 2.4 and 2.6). There is one study (Hall and Johnson, 2004) which presented the nutritional properties of ASL (var *Meritt*) as flour and its effects on the nutritional properties when added to bread. In Chapter 3, not only were the nutritional properties of six ASL varieties reported but also their effects on the nutritional and bioactive properties (i.e. total phenolics, antioxidants, gamma-

conglutin and protein quality) of wheat bread. No other study has reported such wide-ranging data for lupin flour or lupin bread. Chapter 4 is the first report on the effects of ASL varieties on bread physical properties and the use of the sponge and dough method in lupin bread making (all studies presented in Table 2.6 used the straight dough method). From our preliminary experiments (data not presented), the use of the sponge and dough method resulted in better volume and texture than using the straight dough method, although it is suggested that further studies are conducted to compare these two common bread making methods. In addition, the results presented in Chapters 3 and 4 can serve as basis for future investigations involving the use of other lupin species and varieties for high quality bread manufacture.

The findings of Chapters 3 and 4 are however limited due to the use of ASL from only one geographical region and one year of harvest of the ASL. In addition, only one type of wheat flour (Western Australian bakers flour) was investigated. It is therefore recommended that effects of environmental factors such as growing location and year of harvest be investigated to further understand how such factors can affect ASL flour and consequently ASL-wheat bread qualities. In addition, it would valuable to determine how other types of wheat flour may influence the qualities of the resulting ASL-wheat bread. The wheat flour used in this study has lower protein content (10.8% as is) compared to flours produced from North American wheat. The use of such flour with higher protein contents ranging from 13 to 14% (which may translate to better bread quality), may be more robust to ASL flour addition allowing high lupin breads with good acceptability to be developed. It has been reported that protein content of wheat flour positively influences bread quality (Zhu et al., 2001; Lukow et al., 1990).

The second aim of this thesis to evaluate the effects of formulation and process parameters for ASL-wheat bread production on its physical properties and consumer acceptability and the third aim to optimise formulation and process parameters for ASL-wheat bread using RSM to maximise lupin flour incorporation whilst maintaining acceptable bread volume, instrumental textural properties and consumer acceptability, were achieved in Chapters 5 and 6. Findings presented in Chapters 5 and 6 demonstrated that statistical tools such as factorial design and RSM were useful in optimising ASL-wheat bread formulation and process parameters for

maximum nutritional (and potential health benefits) and sensory quality. Optimisation of formulation and process parameters predicted that formulations containing ASL flour at 21.4 - 27.9 g/ 100 g of ASL-wheat composite flour with volume weighted mean particle size of 415 - 687 µm, incorporating water at 59.5 - 71.0 g/100 g ASL-wheat composite flour, with sponges and dough mixed for 4.0 - 5.5 min and bread baked for 10 - 11 min would be within the desirable level of CSV, instrumental hardness and overall acceptability.

The use of more robust statistical designs in Chapter 5 and 6 advanced the state of knowledge of how various formulation and processing parameters affect lupin-wheat bread quality. Existing studies (Paraskevopolou et al., 2010; Doxastakis et al., 2002) on lupin-wheat bread merely looked at the effects on bread quality of lupin incorporation levels in wheat bread without consideration of the interactions between other formulation and process parameters. Likewise, unlike most reports on lupin-wheat breads (Table 2.6) which used the traditional "one at a time" experimental design (i.e. study of individual factor effects), the statistical tools used in this thesis permitted examination of individual, interactive and more complex effects of the important formulation and process parameters on lupin-wheat bread quality. This information aided in formulation and process optimisation for maximum lupin flour addition to wheat bread with acceptable physical and sensory properties. The use of more robust designs led to a more than "doubling" of the amount of lupin flour (compared to the maximum amount of ~10% as found in studies in Table 2.6) which can be substituted for wheat flour in bread whilst maintaining acceptable bread volume, texture and sensory properties. This increase in lupin incorporation rate was possible due to the use of more appropriate combinations of levels of formulation and process parameters that allowed for maximum addition of ASL flour to wheat bread without compromising its quality. The optimized formulation and processing parameters in this study can serve as baseline information from which studies on the incorporation of lupin of other species into bread can be maximised.

The limitations of these findings are that the optimisation study was performed on a laboratory scale and may not accurately predict ASL-wheat bread quality in scaled-up commercial production. For future studies, it is suggested that the optimum

formulation and process be evaluated and if necessary adjusted in large scale production scenarios.

Other future recommended studies include shelf-life determination of the ASL-wheat bread optimised in this thesis. There is some, but limited published evidence of delayed staling when lupin flour is incorporated into bread, possibly related to its high water retention properties. Another area of interest is the use of heat-treated lupin flour to reduce the off-flavour/aroma (described as having a beany and bitter characteristic) that has been reported for lupin bread. Reduction of any undesirable flavour/aroma has good potential to increase the overall acceptability of lupin bread and may allow increased level of ASL flour incorporation. The effects of manipulating levels of protein crosslinking (i.e. disulphide and dityrosine) on ASL-wheat bread quality is worthy of investigation as establishing optimal level of these cross-links may help maximise ASL-wheat bread volume and improve its texture. Previous studies have used gluten to try and minimise the disruption by lupin proteins and dietary fibre on the gluten matrix of lupin-wheat breads, therefore further studies investigating the optimal use of added gluten are warranted. The use of bread improvers such as the enzyme xylanase, that could partially hydrolyse the non-starch polysaccharides in ASL flour to reduce its water binding capacity and thus reduce its negative effect on the development of gluten matrix is worthy of investigation. The use of alternative bread making processes will be of value to try and further improve the quality of ASL-wheat breads. For instance, sourdough fermentation of lupin-wheat dough has been reported to reduce the off-flavour imparted by lupin in bread as well as improve the bread volume and texture. In addition, there were accounts that sheeting of dough increased volume in bread made using composite flours compared to other bread making methods such as that used (i.e. sponge and dough method) in this thesis. Lastly, the development of gluten-free bread using ASL flour in combination with other non-gluten flours and ingredients to meet the rapidly expanding consumer demand for this class of food products is warranted.

In conclusion, the findings presented in this thesis have a range of potential beneficial outcomes as follows: **Farmers** may benefit through increased demand and financial returns for lupin and Western Australian wheat grain to supply the

emerging ASL-wheat bread market. As communicated by David Feinberg of Lupin Foods Australia (LFA, a subsidiary of CBH Group), the availability of functional food products with lupin can stabilise and increase market share for lupin growers (GRDC, 2014). According to Mr. Feinberg, with the increased demand of lupin foods globally, LFA can offer growers a fixed-price contract for up to two years that would secure a stable supply and to distribute lupins into a high-quality global market. The author is currently working with LFA for possible adoption of the provisional patent application covering the results of the study. **Lupin breeders and seed suppliers** have new information that may assist in the breeding and supply to food industry of the ASL variety most suited to bread manufacture. The breeders can now consider enhancing the traits of varieties that were identified as suitable for bread making (i.e. *Belara, Coromup and Tanjil)*. Traits that may be enhanced by breeders are the amount of fat (which may assist in improved bread quality) and increased PDCAAS and gamma-conglutin (for increased health potential).**The food industry** may benefit through availability of new formulation and process specifications for the manufacture of high quality, high-value and healthy ASL-wheat bread. (d) **Consumer** health may benefit through availability of a new, palatable ASL-wheat bread product with high protein and fibre levels and improved protein quality and; **Health professionals** may benefit through availability of a new healthier bread option to recommend to clients.

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APPENDIX 1

AMINO ACID PROFILE OF ASL-WHEAT AND WHEAT BREADS

Amino acid	Bread samples[1]						
	Belara	Coromup	Gungurru	Jenabillup	Mandelup	Tanjil	Wheat
Alanine	0.76±0.00^{d*}	0.75±0.01^{cd*}	0.72±0.00^{b*}	0.74±0.01^{c*}	0.75±0.01^{cd*}	0.69±0.01^{a*}	0.52±0.00
Arginine	1.56±0.00^{a*}	1.62±0.08^{ab*}	1.68±0.03^{b*}	1.58±0.03^{a*}	1.58±0.01^{a*}	1.54±0.01^{a*}	0.85±0.00
Aspartic	1.64±0.01^{a*}	1.62±0.07^{a*}	1.67±0.07^{a*}	1.74±0.03^{a*}	1.71±0.02^{a*}	1.63±0.11^{a*}	1.03±0.01
Cysteine	0.28±0.03^{ab}	0.27±0.02^{a}	0.25±0.01^{a}	0.30±0.00^{ab}	0.30±0.01^{ab}	0.33±0.03^{b*}	0.24±0.01
Glutamic	5.38±0.12^{a*}	5.43±0.14^{a*}	5.58±0.02^{a*}	5.52±0.01^{a*}	5.44±0.06^{a*}	5.52±0.15^{a*}	4.63±0.01
Glycine	0.88±0.01^{b*}	0.88±0.01^{b*}	0.83±0.01^{a*}	0.87±0.01^{b*}	0.88±0.00^{b*}	0.82±0.01^{a*}	0.62±0.00
Histidine	0.48±0.02^{a*}	0.41±0.06^{a}	0.46±0.01^{a*}	0.49±0.03^{a*}	0.47±0.04^{a*}	0.47±0.02^{a*}	0.34±0.01
Isoleucine	0.82±0.00^{ab*}	0.82±0.01^{ab*}	0.83±0.00^{b*}	0.82±0.00^{ab*}	0.82±0.01^{ab*}	0.81±0.01^{a*}	0.59±0.01
Leucine	1.52±0.01^{b*}	1.52±0.01^{b*}	1.50±0.00^{b*}	1.52±0.01^{b*}	1.52±0.01^{b*}	1.46±0.01^{a*}	1.13±0.00
Lysine	0.53±0.04^{b*}	0.52±0.01^{b*}	0.45±0.04^{ab*}	0.51±0.04^{b*}	0.53±0.01^{b*}	0.43±0.05^{a*}	0.23±0.04
Methionine	0.33±0.04^{a*}	0.32±0.01^{a}	0.29±0.00^{a}	0.30±0.01^{a}	0.30±0.00^{a}	0.29±0.01^{a}	0.27±0.00
Phenylalanine	1.00±0.00^{bc*}	0.98±0.01^{ab*}	1.00±0.00^{bc*}	1.01±0.01^{c*}	0.99±0.01^{bc*}	0.97±0.01^{a*}	0.80±0.01
Proline	1.79±0.06^{a*}	1.82±0.01^{a*}	2.14±0.39^{a*}	1.76±0.01^{a*}	1.81±0.00^{a*}	1.75±0.06^{a*}	1.73±0.01
Serine	1.17±0.00^{b*}	1.15±0.01^{a*}	1.17±0.01^{b*}	1.20±0.00^{c*}	1.17±0.01^{ab*}	1.17±0.01^{b*}	0.88±0.01
Taurine	0.08±0.01^{c*}	0.06±0.01^{ab*}	0.04±0.00^{a*}	0.05±0.00^{ab*}	0.06±0.00^{b*}	0.04±0.01^{a*}	0.02±0.00
Threonine	0.84±0.01^{c*}	0.81±0.00^{b*}	0.78±0.01^{a*}	0.83±0.01^{bc*}	0.83±0.01^{bc*}	0.81±0.01^{b*}	0.61±0.00
Tyrosine	0.82±0.04^{a*}	0.79±0.04^{a*}	0.80±0.03^{a*}	0.80±0.04^{a*}	0.78±0.03^{a*}	0.78±0.04^{a*}	0.58±0.01
Valine	0.87±0.01^{d*}	0.86±0.01^{cd*}	0.83±0.00^{b*}	0.83±0.01^{b*}	0.85±0.00^{c*}	0.80±0.00^{a*}	0.64±0.01

[1]g/100 g sample dry basis

[2]Means ± S.D.

[ab] Values within a row with different superscript denotes significant difference ($p<0.05$) using Duncan's Test

[*] Denotes significant difference ($p<0.05$) with wheat flour using Dunnett's Test

APPENDIX 2

INCOMPLETE BLOCK DESIGN USED IN SAMPLE PRESENTATION DURING THE CONSUMER ACCEPTABILITY FOR THE MODELLING EXPERIMENTS IN CHAPTER SIX-BASED ON PLAN 13.15 (COCHRAN AND COX, 1957)

Panelist	SAMPLE								
	SESSION 1					SESSION 2			
	1	2	3	4	5	6	7	8	9
1	1	7	9	10	12	16	26	33	34
2	2	8	10	11	13	17	27	34	35
3	3	9	11	12	14	18	28	35	36
4	4	10	12	13	15	19	29	36	37
5	5	11	13	14	16	20	30	37	1
6	6	12	14	15	17	21	31	1	2
7	7	13	15	16	18	22	32	2	3
8	8	14	16	17	19	23	33	3	4
9	9	15	17	18	20	24	34	4	5
10	10	16	18	19	21	25	35	5	6
11	11	17	19	20	22	26	36	6	7
12	12	18	20	21	23	27	37	7	8
13	13	19	21	22	24	28	1	8	9
14	14	20	22	23	25	29	2	9	10
15	15	21	23	24	26	30	3	10	11
16	16	22	24	25	27	31	4	11	12
17	17	23	25	26	28	32	5	12	13
18	18	24	26	27	29	33	6	13	14
19	19	25	27	28	30	34	7	14	15
20	20	26	28	29	31	35	8	15	16
21	21	27	29	30	32	36	9	16	17
22	22	28	30	31	33	37	10	17	18
23	23	29	31	32	34	1	11	18	19
24	24	30	32	33	35	2	12	19	20
25	25	31	33	34	36	3	13	20	21
26	26	32	34	35	37	4	14	21	22
27	27	33	35	36	1	5	15	22	23
28	28	34	36	37	2	6	16	23	24
29	29	35	37	1	3	7	17	24	25
30	30	36	1	2	4	8	18	25	26
31	31	37	2	3	5	9	19	26	27
32	32	1	3	4	6	10	20	27	28
33	33	2	4	5	7	11	21	28	29
34	34	3	5	6	8	12	22	29	30
35	35	4	6	7	9	13	23	30	31
36	36	5	7	8	10	14	24	31	32
37	37	6	8	9	11	15	25	32	33

APPENDIX 3

CONSUMER ACCEPTABILITY QUESTIONNAIRE USED IN CHAPTER SIX

CONSUMER ACCEPTABILITY QUESTIONNAIRE

Block reference number: **Date:**
Sample code:

Samples will be presented from left to right. Please evaluate the samples in the order presented. Please consume all of your sample in order to evaluate all attributes. Please answer the following questions by ticking the box that best reflects your feelings about this sample. Rinse mouth before and after evaluation of the sample with the water provided. Expectorate rinse water to avoid being full during the session.

1. How much do you like the **COLOUR** of this sample?

❑	❑	❑	❑	❑	❑	❑	❑	❑
Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely

2. How much do you like the **APPEARANCE** of this sample?

❑	❑	❑	❑	❑	❑	❑	❑	❑
Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely

3. How much do you like the **FLAVOUR/AROMA** of this sample?

❑	❑	❑	❑	❑	❑	❑	❑	❑
Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely

4. How much do you like the **MOUTHFEEL/TEXTURE** of this sample?

❑	❑	❑	❑	❑	❑	❑	❑	❑
Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely

OVERALL, how much do you like this sample?

❑	❑	❑	❑	❑	❑	❑	❑	❑
Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely

Comments:_____
_____
_____

APPENDIX 4

FACTORIAL SCREENING ANOVA TABLES GENERATED IN CHAPTER FIVE

A. CRUMB SPECIFIC VOLUME

Parameters	Sum of squares	df	Mean Square	F value	p-value
Model	2.38	5.00	0.48	4.29	0.02
Sponge proof time	0.12	1.00	0.12	1.10	0.32
Mixing time of sponge dough	1.34	1.00	1.34	12.11	0.01
Final proof time	0.00	1.00	0.00	0.02	0.89
Final proof temp	0.00	1.00	0.00	0.00	0.96
Baking time	0.91	1.00	0.91	8.23	0.02
Residual	1.11	10.00	0.11		
Cor Total	3.49	15.00			

B. CRUMB AREA

Parameters	Sum of squares	df	Mean Square	F value	p-value
Model	86.72	5.00	17.34	2.63	0.09
Sponge proof time	0.10	1.00	0.10	0.02	0.91
Mixing time of sponge dough	66.63	1.00	66.63	10.10	0.01
Final proof time	15.46	1.00	15.46	2.35	0.16
Final proof temp	1.74	1.00	1.74	0.26	0.62
Baking time	2.80	1.00	2.80	0.42	0.53
Residual	65.94	10.00	6.59		
Cor Total	152.66	15.00			

C. CELL WALL THICKNESS

Parameters	Sum of squares	df	Mean Square	F value	p-value
Model	0.00	5.00	0.00	10.71	0.00
Sponge proof time	0.00	1.00	0.00	3.14	0.11
Mixing time of sponge dough	0.00	1.00	0.00	4.50	0.06
Final proof time	0.00	1.00	0.00	36.65	0.00
Final proof temp	0.00	1.00	0.00	4.43	0.06
Baking time	0.00	1.00	0.00	4.82	0.05
Residual	0.00	10.00	0.00		
Cor Total	0.01	15.00			

D. CELL DIAMETER

Parameters	Sum of squares	df	Mean Square	F value	p-value
Model	0.01	1.00	0.01	0.98	0.35
Sponge proof time	0.00	1.00	0.00	0.06	0.81
Mixing time of sponge dough	0.23	1.00	0.23	17.70	0.00
Final proof time	0.02	1.00	0.02	1.57	0.24
Final proof temp	0.07	1.00	0.07	5.54	0.04
Baking time	0.13	10.00	0.01		
Residual	0.47	15.00			
Cor Total	0.01	1.00	0.01		

E. NO. OF CELLS PER CM^2

Parameters	Sum of squares	df	Mean Square	F value	p-value
Model	813.15	5.00	162.63	5.17	0.01
Sponge proof time	45.43	1.00	45.43	1.45	0.26
Mixing time of sponge dough	1.05	1.00	1.05	0.03	0.86
Final proof time	526.93	1.00	526.93	16.76	0.00
Final proof temp	14.44	1.00	14.44	0.46	0.51
Baking time	225.30	1.00	225.30	7.17	0.02
Residual	314.34	10.00	31.43		
Cor Total	1127.49	15.00			

F.HARDNESS

Parameters	Sum of squares	df	Mean Square	F value	p-value
Model	38902.30	5.00	7780.46	1.41	0.30
Sponge proof time	405.53	1.00	405.53	0.07	0.79
Mixing time of sponge dough	23808.38	1.00	23808.38	4.30	0.06
Final proof time	28.48	1.00	28.48	0.01	0.94
Final proof temp	814.54	1.00	814.54	0.15	0.71
Baking time	13845.37	1.00	13845.37	2.50	0.14
Residual	55337.10	10.00	5533.71		
Cor Total	94239.40	15.00			

G. SPRINGINESS

Parameters	Sum of squares	df	Mean Square	F value	p-value
Model	0.01	5.00	0.00	1.63	0.24
Sponge proof time	0.00	1.00	0.00	0.02	0.88
Mixing time of sponge dough	0.01	1.00	0.01	7.57	0.02
Final proof time	0.00	1.00	0.00	0.47	0.51
Final proof temp	0.00	1.00	0.00	0.05	0.82
Baking time	0.00	1.00	0.00	0.04	0.84
Residual	0.01	10.00	0.00		
Cor Total	0.02	15.00			

H. CHEWINESS

Parameters	Sum of squares	df	Mean Square	F value	p-value
Model	2242.20	5.00	448.44	0.42	0.82
Sponge proof time	16.03	1.00	16.03	0.02	0.90
Mixing time of sponge dough	0.19	1.00	0.19	0.00	0.99
Final proof time	1987.97	1.00	1987.97	1.87	0.20
Final proof temp	4.94	1.00	4.94	0.00	0.95
Baking time	233.06	1.00	233.06	0.22	0.65
Residual	10627.40	10.00	1062.74		
Cor Total	12869.60	15.00			

APPENDIX 5

RESPONSE SURFACE METHODOLY (RSM) ANOVA TABLES GENERATED IN CHAPTER SIX

A. CRUMB SPECIFIC VOLUME

Parameters	Sum of squares	df	Mean Square	F value	p-value
Model	0.57	6.00	0.10	38.50	< 0.0001
Level of ASL flour incorporation (LF)	0.09	1.00	0.09	35.77	< 0.0001
Level of water incorporation (W)	0.20	1.00	0.20	82.41	< 0.0001
Mixing time (MT)	0.00	1.00	0.00	0.29	0.60
Baking time (BT)	0.01	1.00	0.01	3.01	0.10
MT× BT	0.02	1.00	0.02	8.59	0.01
W^2	0.25	1.00	0.25	100.92	< 0.0001
Residual	0.06	25.00	0.00		
Lack of Fit	0.06	20.00	0.00	2.06	0.22
Pure Error	0.01	5.00	0.00		
Cor Total	0.63	31.00			

B. INSTRUMENTAL HARDNESS

Parameters	Sum of squares	df	Mean Square	F value	p-value
Model	31.28	13.00	2.41	25.65	< 0.0001
Volume weighted particle size (PS)	0.59	1.00	0.59	6.29	0.02
Level of ASL flour incorporation (LF)	2.83	1.00	2.83	30.16	< 0.0001
Level of water incorporation (W)	12.91	1.00	12.91	137.60	< 0.0001
Mixing time (MT)	0.17	1.00	0.17	1.82	0.19
Baking time (BT)	0.67	1.00	0.67	7.14	0.02
PS×BT	0.74	1.00	0.74	7.90	0.01
LF×W	0.60	1.00	0.60	6.41	0.02
LF×BT	1.00	1.00	1.00	10.61	0.00
W×MT	0.39	1.00	0.39	4.20	0.06
MT×BT	0.36	1.00	0.36	3.86	0.07
LF^2	0.58	1.00	0.58	6.20	0.02
W^2	3.67	1.00	3.67	39.09	< 0.0001
BT^2	0.56	1.00	0.56	5.99	0.02
Residual	1.69	18.00	0.09		
Lack of Fit	1.51	13.00	0.12	3.32	0.10
Pure Error	0.18	5.00	0.04		
Cor Total	32.97	31.00			

C. INSTRUMENTAL SPRINGINESS

Parameters	Sum of squares	df	Mean Square	F value	p-value
Model	31.28	13.00	2.41	25.65	< 0.0001
Volume weighted particle size (PS)	0.59	1.00	0.59	6.29	0.02
Level of ASL flour incorporation (LF)	2.83	1.00	2.83	30.16	< 0.0001
Level of water incorporation (W)	12.91	1.00	12.91	137.60	< 0.0001
Mixing time (MT)	0.17	1.00	0.17	1.82	0.19
Baking time (BT)	0.67	1.00	0.67	7.14	0.02
PS×BT	0.74	1.00	0.74	7.90	0.01
LF×W	0.60	1.00	0.60	6.41	0.02
LF×BT	1.00	1.00	1.00	10.61	0.00
W×MT	0.39	1.00	0.39	4.20	0.06
MT×BT	0.36	1.00	0.36	3.86	0.07
LF^2	0.58	1.00	0.58	6.20	0.02
W^2	3.67	1.00	3.67	39.09	< 0.0001
BT^2	0.56	1.00	0.56	5.99	0.02
Residual	1.69	18.00	0.09		
Lack of Fit	1.51	13.00	0.12	3.32	0.10
Pure Error	0.18	5.00	0.04		
Cor Total	32.97	31.00			

D. INSTRUMENTAL CHEWINESS

Parameters	Sum of squares	df	Mean Square	F value	p-value
Model	0.01	9.00	0.00	12.02	< 0.0001
Volume weighted particle size (PS)	0.00	1.00	0.00	4.25	0.05
Level of ASL flour incorporation (LF)	0.00	1.00	0.00	8.20	0.01
Level of water incorporation (W)	0.00	1.00	0.00	34.92	< 0.0001
Baking time (BT)	0.00	1.00	0.00	5.08	0.03
PS×BT	0.00	1.00	0.00	5.55	0.03
LF×BT	0.00	1.00	0.00	7.64	0.01
LF^2	0.00	1.00	0.00	5.17	0.03
W^2	0.00	1.00	0.00	15.90	0.00
BT^2	0.00	1.00	0.00	9.31	0.01
Residual	0.00	22.00	0.00		
Lack of Fit	0.00	17.00	0.00	2.02	0.22
Pure Error	0.00	5.00	0.00		
Cor Total	0.01	31.00			

E. COLOUR ACCEPTABILITY

Parameters	Sum of squares	df	Mean Square	F value	p-value
Model	0.08	13.00	0.01	77.94	< 0.0001
Volume weighted particle size (PS)	0.00	1.00	0.00	38.70	< 0.0001
Level of ASL flour incorporation (LF)	0.00	1.00	0.00	40.41	< 0.0001
Level of water incorporation (W)	0.02	1.00	0.02	275.01	< 0.0001
Mixing time (MT)	0.00	1.00	0.00	3.74	0.07
Baking time (BT)	0.00	1.00	0.00	61.35	< 0.0001
PS×BT	0.00	1.00	0.00	3.41	0.08
PS×W	0.00	1.00	0.00	32.54	< 0.0001
PS×MT	0.00	1.00	0.00	9.05	0.01
PS×BT	0.00	1.00	0.00	6.59	0.02
LF×W	0.00	1.00	0.00	30.29	< 0.0001
LF×BT	0.00	1.00	0.00	31.60	< 0.0001
W×MT	0.00	1.00	0.00	9.69	0.01
W^2	0.04	1.00	0.04	470.89	< 0.0001
Residual	0.00	18.00	0.00		
Lack of Fit	0.00	13.00	0.00	2.43	0.17
Pure Error	0.00	5.00	0.00		
Cor Total	0.08	31.00			

F. APPEARANCE ACCEPTABILITY

Parameters	Sum of squares	df	Mean Square	F value	p-value
Model	0.00	1.00	0.00	59.51	< 0.0001
Volume weighted particle size (PS)	0.00	1.00	0.00	38.39	< 0.0001
Level of ASL flour incorporation (LF)	0.04	1.00	0.04	566.90	< 0.0001
Level of water incorporation (W)	0.00	1.00	0.00	2.15	0.16
Mixing time (MT)	0.01	1.00	0.01	71.20	< 0.0001
Baking time (BT)	0.01	1.00	0.01	78.34	< 0.0001
PS×W	0.00	1.00	0.00	13.32	0.00
PS×MT	0.00	1.00	0.00	64.60	< 0.0001
PS×BT	0.00	1.00	0.00	39.50	< 0.0001
LF×W	0.01	1.00	0.01	109.67	< 0.0001
LF×MT	0.00	1.00	0.00	7.36	0.01
LF×BT	0.00	1.00	0.00	5.74	0.03
W×MT	0.06	1.00	0.06	757.27	< 0.0001
W^2	0.00	18.00	0.00		
Residual	0.00	13.00	0.00	6.83	0.02
Lack of Fit	0.00	5.00	0.00		
Pure Error	0.13	31.00			
Cor Total	0.00	1.00	0.00	59.51	< 0.0001

G. FLAVOUR/AROMA ACCEPTABILITY

Parameters	Sum of squares	df	Mean Square	F value	p-value
Model	26.65	7.00	3.81	27.26	< 0.0001
Level of ASL flour incorporation (LF)	4.81	1.00	4.81	34.44	< 0.0001
Level of water incorporation (W)	3.15	1.00	3.15	22.55	< 0.0001
Mixing time (MT)	0.61	1.00	0.61	4.35	0.05
Baking time (BT)	1.63	1.00	1.63	11.68	0.00
LF×MT	0.73	1.00	0.73	5.23	0.03
LF×BT	0.57	1.00	0.57	4.10	0.05
W^2	15.15	1.00	15.15	108.49	< 0.0001
Residual	3.35	24.00	0.14		
Lack of Fit	3.06	19.00	0.16	2.73	0.13
Pure Error	0.29	5.00	0.06		
Cor Total	30.00	31.00			

H. TEXTURE ACCEPTABILITY

Parameters	Sum of squares	df	Mean Square	F value	p-value
Model	0.15	13.00	0.01	38.26	< 0.0001
Volume weighted particle size (PS)	0.00	1.00	0.00	5.87	0.03
Level of ASL flour incorporation (LF)	0.03	1.00	0.03	81.65	< 0.0001
Level of water incorporation (W)	0.04	1.00	0.04	133.72	< 0.0001
Mixing time (MT)	0.00	1.00	0.00	0.76	0.40
Baking time (BT)	0.00	1.00	0.00	1.21	0.29
PS×LF	0.00	1.00	0.00	8.47	0.01
PS×W	0.00	1.00	0.00	6.11	0.02
PS×BT	0.00	1.00	0.00	12.40	0.00
LF×W	0.00	1.00	0.00	13.91	0.00
LF×MT	0.01	1.00	0.01	31.53	< 0.0001
LF×BT	0.00	1.00	0.00	8.00	0.01
W×BT	0.00	1.00	0.00	10.34	0.00
W^2	0.06	1.00	0.06	183.35	< 0.0001
Residual	0.01	18.00	0.00		
Lack of Fit	0.00	13.00	0.00	2.10	0.21
Pure Error	0.00	5.00	0.00		
Cor Total	0.16	31.00			

J. OVERALL ACCEPTABILITY

Parameters	Sum of squares	df	Mean Square	F value	p-value
Model	0.15	13.00	0.01	38.26	< 0.0001
Volume weighted particle size (PS)	0.00	1.00	0.00	5.87	0.03
Level of ASL flour incorporation (LF)	0.03	1.00	0.03	81.65	< 0.0001
Level of water incorporation (W)	0.04	1.00	0.04	133.72	< 0.0001
Mixing time (MT)	0.00	1.00	0.00	0.76	0.40
Baking time (BT)	0.00	1.00	0.00	1.21	0.29
PS×LF	0.00	1.00	0.00	8.47	0.01
PS×W	0.00	1.00	0.00	6.11	0.02
PS×BT	0.00	1.00	0.00	12.40	0.00
LF×W	0.00	1.00	0.00	13.91	0.00
LF×MT	0.01	1.00	0.01	31.53	< 0.0001
LF×BT	0.00	1.00	0.00	8.00	0.01
W^2	0.00	1.00	0.00	10.34	0.00
BT^2	0.06	1.00	0.06	183.35	< 0.0001
Residual	0.01	18.00	0.00		
Lack of Fit	0.00	13.00	0.00	2.10	0.21
Pure Error	0.00	5.00	0.00		
Cor Total	0.16	31.00			

APPENDIX 6

MEAN VALUES OF CSV AND MEASURES OF INSTRUMENTAL TEXTURE OF ASL-WHEAT BREADS PRODUCED IN 32 RUNS DURING THE MODELLING EXPERIMENTS IN CHAPTER SIX

		INSTRUMENTAL TEXTURE		
RUN	**CSV**	**HARDNESS**	**SPRINGINESS**	**CHEWINESS**
1	1.7	1964	0.80	749
2	2.3	364	0.80	176
3	1.1	4834	0.67	1274
4	1.1	3654	0.71	699
5	2.9	307	0.90	184
6	2.8	875	0.88	438
7	2.0	629	0.83	281
8	2.0	400	0.86	203
9	1.3	1828	0.73	582
10	1.6	2957	0.77	1167
11	1.3	4516	0.67	1043
12	1.1	4176	0.64	984
13	3.9	410	0.93	247
14	2.6	252	0.93	170
15	1.6	858	0.77	347
16	2.6	499	0.93	315
17	2.2	736	0.87	387
18	3.8	208	0.94	142
19	2.7	333	0.90	312
20	2.2	431	0.86	726
21	1.2	3978	0.89	2406
22	2.4	411	0.94	270
23	2.8	411	0.90	250
24	2.9	449	0.92	292
25	3.3	288	0.93	231
26	3.6	273	0.92	173
27	3.6	325	0.90	191
28	2.7	344	0.92	215
29	3.6	245	0.93	166
30	3.3	288	0.93	231
31	3.0	346	0.91	222
32	3.9	222	0.94	151

APPENDIX 7

MEAN VALUES OF MEASURES OF CONSUMER ACCEPTABILITY SCORES OF ASL-WHEAT BREADS PRODUCED IN 32 RUNS DURING THE MODELLING EXPERIMENTS IN CHAPTER SIX

RUN	COLOUR	APPEARANCE	FLAVOUR/ AROMA	TEXTURE	OVERALL
1	5	4	5	4	5
2	5	5	6	6	6
3	3	2	3	2	2
4	4	4	4	3	3
5	5	5	6	7	6
6	6	6	6	6	6
7	6	6	5	5	5
8	5	5	4	4	4
9	4	3	4	4	4
10	5	4	4	3	4
11	4	4	4	3	3
12	4	4	4	4	3
13	6	6	6	5	6
14	5	5	5	5	5
15	6	5	4	4	4
16	6	6	5	5	5
17	5	6	6	5	5
18	7	7	7	7	7
19	7	7	7	6	7
20	6	6	6	5	6
21	4	3	4	4	4
22	6	5	6	5	6
23	7	7	6	7	6
24	7	7	6	6	6
25	7	7	6	6	6
26	7	7	7	6	6
27	6	7	6	6	6
28	6	7	6	6	6
29	7	7	6	6	6
30	7	7	6	6	6
31	7	7	6	6	6
32	7	7	7	7	7