School of Public Health

Exploring and Improving Functional Properties of Underutilised Lupin Seed Coat

Liezhou Zhong

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DECLARATION

To the best of my knowledge and belief this thesis titled “EXPLORING AND IMPROVING FUNCTIONAL PROPERTIES OF UNDERUTILISED LUPIN SEED COAT” contains no material previously published by any other person except where due acknowledgement has been made.

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

Signature:

Date: 20 February 2020

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ABSTRACT

Seeds of legume lupin (*Lupinus spp.*) are a sustainable, high-quality protein source for both livestock and humans, with multiple economic and environmental benefits. Lupin is attracting worldwide attention as a potential future staple food, primarily as lupin kernel flour based food products. However, the lupin flour production generates high milling loss, with the removal of the lupin seed coat. This accounts for 24% of the whole seed on average, being the main by-product. In this study, the aim is to value-add to lupin seed coat as a novel human food ingredient. To achieve this, the chemical (e.g. proximate, dietary fibre, polyphenols and minerals compositions) and physicochemical properties (e.g. water solubility, water/oil binding capacity, swelling capacity) of seed coats from a range of recent commercially grown Australian sweet lupin (ASL, *Lupinus angustifolius*) cultivars grown at two sites were investigated. Moreover, a high temperature, high pressure, high mechanical shear twin-screw extrusion cooking process was developed and optimised to improve desirable ASL seed coat compositional and physicochemical functionalities.

In chapters 3 & 4, six recent Australian sweet lupin cultivars grown at two Western Australian lupin production zones were sampled to investigate their properties using a genotype × environment study. Results showed that ASL seed coats had water-binding capacities and water solubilities of 3.39-3.74 g/g water on a dry basis (db) and <8% respectively, which were similar to or slightly lower than those of field pea, lentil and mung bean seed coats. Dietary fibre (DF) is the major component of ASL seed coat, while insoluble dietary fibre (IDF) took up over 95% of total dietary fibre (TDF, 80-87 g/100 g db). The seed coats also contained 2.81-3.01 g/100 g db of ash, 1.62 - 2.42 g/100 g db of fat and 6.34 - 8.59 g/100g db (N×5.40) of protein. Calcium was the main mineral of the seed coats (up to 0.64 g/ 100 g db), followed by potassium (0. 31 g/ 100 g db) and magnesium (0.15 g/ 100 g db). The range of total free polyphenol content in ASL seed coats varied from 57.24 to 93.52 mg gallic acid equivalents (GAE)/ 100 g db. Total bound polyphenol content ranged from 16.71 to 34.78 mg GAE/100 g, accounting for up to 27.15% of the total polyphenol content in the lupin seed coats. Three flavones (apigenin-7-*O*-β-apiofuranosyl-6,8-di-*C*-β-glucopyranoside, vicenin 2, and apigenin-7-*O*-β-glucopyranoside), one isoflavone (genistein), one

dihydroflavonol derivative (aromadendrin-6-*C*-β-D-glucopyranosyl-7-*O*-[β-D-apiofuranosyl-(1→2)]-*O*-β-D-glucopyranoside), and several hydroxybenzoic and hydroxycinnamic acid derivatives were identified and semi-quantified using a high-performance liquid chromatography with diode array detector which coupled with a triple quadrupole mass spectrometer and electrospray ionization source (HPLC-DAD-ESI-MS/MS). Considerable variations in levels of individual polyphenols were found among the seed coats but apigenin-7-*O*-β-apiofuranosyl-6,8-di-*C*-β-glucopyranoside was the predominant polyphenol in all samples accounting for 73.08 - 82.89 % of the total free polyphenols. In contrast, the majority of individual phenolics in bound fraction were phenolic acid derivatives. Significant effects of genotype, environment and their interaction on ASL seed coat chemical and physicochemical properties were observed. These results suggest that ASL seed coat could be a valuable dietary source of dietary fibre and polyphenols.

In chapters 5 & 6, a co-rotating twin-screw extrusion cooking processing was developed and optimised to maximise the SDF content and retention of total free polyphenol content (TFPC) and total free individual polyphenols content (TFIPC) simultaneously. In chapters 5, a fractional factorial design was used to characterise the interactive and individual effects of extrusion cooling processing parameters (independent factors) and screen the most important few factors that can exert significant impacts on extrusion cooked lupin seed coat properties. Water solubility increased (4.02- 4.47 % vs 5.37-9.64 % db) whereas water-binding capacity slightly decreased (3.84-3.88 g/g vs 3.15-3.73 g/g db) after extrusion cooking. Extrusion cooking significantly increased SDF content of lupin seed coat, from 2.90 g/100 g db in un-extruded seed coat to a maximum of 9.03 g/100 g db, while decreasing insoluble dietary fibre content from 89.89 to 82.89 g/ 100 g db. The screw speed (rpm), the total moisture content in barrel (%), and barrel temperature (°C) were identified as the most important processing parameters. In chapters 6, a central composite rotatable design together with the desirability function were used to optimise further the three extrusion cooking processing parameters and to maximise the levels of SDF, TFPC and TFIPC. Optimal solution (barrel temperature at 138.7°C, total moisture in the barrel at 25 per cent and a screw speed of 450 rpm) which generated by the software (Design-Expert) were obtained. Under the optimal condition, levels of seed coat SDF, TFPC and TFIPC were 11.37 g/ 100 g db of, 59.93 mg GAE /100 g db and 354.56 μg/g db respectively.

In chapter 7, effects of optimised extrusion cooking processing on bioaccessibility, bioavailability and dialysability of selected minerals and polyphenols of the extrusion cooked lupin seed coats under optimal conditions were investigated using a standardised *in vitro* digestion method. Iron content of the extrusion cooked lupin seed coat was doubled compared to the untreated seed coat, while other mineral contents (Ca, K, Na, Mg, Zn and Cu) were constant. Extrusion cooking showed no or slight effects on bioaccessibility and the bioavailability of all the selected minerals and the apigenin-7-*O*-β-apiofuranosyl-6,8-di-*C*-β-glucopyranoside. These findings suggest that extrusion cooking could be a practical technology to increase the soluble dietary fibre content in lupin seed coat and thus expand its application in human food as a high fibre ingredient.

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PUBLICATIONS

1. **Zhong, L.**, Fang, Z., Wahlqvist, M. L., Wu, G., Hodgson, J. M., & Johnson, S. K. (2018). Seed coats of pulses as a food ingredient: characterization, processing, and applications. *Trends in Food Science & Technology, 80*, 35-42.
2. **Zhong, L.**, Wu, G., Fang, Z., Wahlqvist, M. L., Hodgson, J. M., Clarke, M. W., Junaldi, E., & Johnson, S. K. (2019). Characterization of polyphenols in Australian sweet lupin (*Lupinus angustifolius*) seed coat by HPLC-DAD-ESI-MS/MS. *Food Research International, 116*, 1153-1162.
3. **Zhong, L.**, Fang, Z., Wahlqvist, M. L., Hodgson, J. M., & Johnson, S. K. (2019). Extrusion cooking increases soluble dietary fibre of lupin seed coat. *LWT-Food Science and Technology*, 99, 547-554.
4. **Zhong, L.**, Al-Ali, H. Fang, Z., Wahlqvist, M. L., Hodgson, J. M., & Johnson, S. K. (2020). Lupin seed coat as a promising food ingredient: physicochemical, nutritional, antioxidant properties, and effect of genotype and environment. *International Journal of Food Science and Technology*, 55(4), 1816-1824.

The first pages of these articles are attached in the appendices with the expressed permission from Elsevier B.V. for article [1-3] and from John Wiley and Sons for article [4].

CONFERENCE PRESENTATIONS

Zhong, L., Fang, Z., Wahlqvist, M. L., Wu, G., Hodgson, J. M., & Johnson, S. K. (2019). Exploring and improving functional properties of underutilized lupin seed coat. Innovative Horticulture for Healthy Foods Conference 2019 (IHHF 2019), Bunker Bay, Western Australia, Australia, 8-10 December.

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STATEMENT OF CONTRIBUTION

To Whom It May Concern，

I, Liezhou Zhong, am the major contributor to this thesis titled "Exploring and Improving Functional Properties of Underutilised Lupin Seed Coat". The thesis and all publications and presentations listed in "PUBLICATIONS" section and "CONFERENCE PRESENTATIONS" section, and a submitted manuscript entitled: "*Multi-response surface optimisation of extrusion cooking to modify functional properties of lupin seed coat*" were primarily executed, interpreted, discussed, written and edited by Liezhou Zhong under the main supervision and guidance of A/Prof Stuart K. Johnson and co-supervision of Dr Zhongxiang Fang, Prof Mark L. Wahlqvist and Prof Jonathon M. Hodgson.

Liezhou Zhong

A/Prof Stuart K. Johnson (Lead supervisor)

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AWARDS

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LIST OF ABBREVIATIONS

ABTS	2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
ANFs	Anti-nutritional factors
(F/B/T) AOXs	(Free/Bound/Total) Antioxidant capacity assays
Api	Apigenin
ASL	Australian sweet lupin
CA	Caffeic acid
CCRD	Central composite rotatable design
CE	Catechin equivalent
DAD	Diode array detector
db	Dry basis
DF	Dietary fibre
DPPH	2-2-diphenyl-1-picrylhydrazy
FTIR	Fourier Transform infrared spectroscopy
FFD	Fractional factorial design
ER	Eradu
ESIMS	Electrospray ionization mass spectrometry
GAE	Gallic acid equivalent
G × E	Genotype by environment
HPLC	High performance liquid chromatography
IDF	Insoluble dietary fibre
Mw	Molecular weight
NSP	Non-starch polysaccharides
(F/B) ORAC	(Free/ Bound) Oxygen radical absorbance capacity
RSM	Response surface methodology
SD	Standard deviation
SDF	Soluble dietary fibre
SEM	Scanning electron microscopy
SME	Specific mechanical energy
SOL	Solubility in water
TA	Taxifolin

TE	Trolox equivalent
T (F/B) PC	Total (Free/Bound) phenolic content
T (F/B) IPC	Total (Free/Bound) individual phenolic content
(F/B) TPC	(Free/Bound) Total polyphenol content
UV	Ultraviolet
VE	Vitexin equivalent
WA	Western Australia
WBC	Water-binding capcity
WH	Wongan Hills
WHO	World Health Organization

CHAPTER 1
General introduction

1.1. BACKGROUND

Lupin (*Lupinus spp.*) is a non-starchy legume seed which belongs to the Fabaceae family (Gladstones, Atkins, & Hamblin, 1998). Globally, four lupin species, *L. angustifolius*, *L. luteus*, *L. albus* and *L. mutabilis*, are commercially grown. Western Australia (WA) is the world's largest producer of lupin, with estimated 600,000 tonnes in 2018-19 season comprising over 86% of Australian production and 64% of world production (ABARES, 2019). Varieties of *L. angustifolius*, better known as narrow-leafed lupin and Australian sweet lupin (ASL), with low potentially toxic alkaloids are the dominant lupin crop in WA. Lupin has low agronomic input requirements and high tolerance under drought and poor soils, thus greatly contributing to WA's cropping systems and improving soil conditions with its ability to fix atmospheric nitrogen. Lupin is gluten-free, non-genetically modified and has lower levels of some conventional anti-nutritional factors (ANFs) (e.g. phytates, saponins, lectins, and trypsin inhibitors) and phytoestrogenic compounds than those of soybean, in contrast, high contents of carotenoids and polyphenols (including condensed tannins) which shown multiple health benefits have been found in lupin seed (Villarino, Coorey, Bell, & Johnson, 2016). As such, lupin is attracting international attention as an emerging staple food (Johnson, Clements, Villarino, & Coorey, 2017).

Dehulling of lupin is an essential process to produce lupin kernels that are subsequently processed into high protein, high fibre flour. However, lupin seed coat comprises ~24% of the total seed weight which is much higher than in soybean (5-8%) and pea (*Pisum sativum* L.) (9-14%), resulting in a high proportion of commercial milling loss during lupin kernel manufactory (Sipsas, 2008). Mainly due to its impermeable matrix structure and high concentration of insoluble non-starch polysaccharides (NSP), the primary market lupin seed coat is low-value animal feed with only very limited use in foods such as high fibre bread, and it represents a tough disposal problem for the industry (Sipsas, 2008). Australian lupin milling sector

produces over 10,000 tonnes of lupin seed coats annually, while less than 2% of them are used in human food products (Tucek, 2009). Nevertheless, lupin seed coat contains considerable levels of dietary fibre (DF), minerals, and phytochemicals (bioactive secondary metabolites in plant, e.g. polyphenolic antioxidants) indicating its potential as a functional food ingredient.

Up to date, no studies have reported the bioactive nutrients (e.g. phytochemicals) nor physicochemical functionalities (e.g. solubility, and water binding capacity) of the seed coat of the recent and agriculturally dominant ASL varieties. Most of the DF in lupin seed coat is insoluble fractions (i.e. insoluble dietary fibre, IDF) which has less physiological and technological functionalities. Moreover, DF have been postulated to lower bioavailability of nutrients and phytochemicals (Baye, Guyot, & Mouquet-Rivier, 2017; O'Grady, O'Connor, & Shanahan, 2019). Hence, technologies are required to modify the physicochemical properties of lupin seed coat to raise the content of the more physiological functional soluble dietary fibre (SDF) and increase the bioavailability of its minerals and phytochemicals.

Twin-screw extrusion cooking combines moisture, heat, pressure and mechanical shear contributing to cell wall disruption and chemical transformation (Singh, Gamlath, & Wakeling, 2007; Wolf, 2010). It was reported that twin-screw extrusion could increase the functionalities of high-fibre by-products such as soybean seed coat and wheat bran mainly through increasing soluble dietary fibre level, water-binding capacity of the DF and bioavailability of their nutrients (Duque, Manzanares, & Ballesteros, 2017; Offiah, Kontogiorgos, & Falade, 2019; Sharma, Bansal, Mangal, Dixit, Gupta, & Mangal, 2016). However, there are no literature reports on the process development and optimisation of extrusion cooking to modify the functionalities of lupin seed coat beneficially.

1.2. AIM

Therefore, the hypotheses of this study are (a) compositional and physicochemical properties of ASL seed coat can vary across the genotypes and growing environments; (b) ASL have considerable levels of polyphenols thus showing high antioxidant capacities; (c) extrusion cooking technology can be optimised to improve the physicochemical and compositional properties of the seed coat, and (d) extrusion

cooking will improve bioaccessibility and bioavailability of selected polyphenols and minerals. Accordingly, this study aims to understand the genotypic and environmental impacts on the biochemical and physicochemical functionalities of ASL seed coat; develop and optimise an extrusion cooking process to increase the desirable compositional and physicochemical functionalities of the seed coat. This study will benefit lupin growers, food manufacturers and consumers by generating new knowledge of a novel functional fibre food ingredient. This may contribute to consumer health, and add value to lupin waste, thus increasing the financial returns on the lupin crop.

CHAPTER 2

Review of literature

Information contained in this chapter has been published as follows:

Zhong, L., Fang, Z., Wahlqvist, M. L., Wu, G., Hodgson, J. M., & Johnson, S. K. (2018). Seed coats of pulses as a food ingredient: characterization, processing, and applications. *Trends in Food Science & Technology, 80*, 35-42.

ABSTRACT

In recognition of their multiple benefits on the environment, food security, and human health, pulses are attracting worldwide attention. The seed coat is a major by-product of pulse processing, and its only markets are as low-value ruminant feed and very limited use in high fibre foods. Recently, accumulating studies have suggested that this underutilised by-product has greater potential as a novel natural "nutritious dietary fibre" which can be used as a functional food ingredient. This review discusses biochemical and physicochemical functionalities of seed coats of six globally important pulses: chickpea, field pea, faba/broad bean, lentil and mung bean with a special emphasis on the emerging food pulse lupin. Food process modification and recent human food applications of the seed coats are summarised. Bioavailability of the seed coat compounds, and phomopsins contaminated lupin seed coats as a typical example of safety issue are discussed. High levels of dietary fibre, minerals and potential health-promoting phytochemicals in the seed coats indicate their great potential to be used as a natural "nutritious dietary fibre". However, further in-depth studies are required to improve their desirable nutritional, physiological and techno-functional properties whilst minimizing any undesirable ones.

2.1. Introduction

"Pulses" refers to those low-fat content leguminous seeds which are harvested for dry grain (FAO, 1994). So, oilseeds (e.g. soybean and peanut), leguminous green

vegetables (e.g. green peas and green beans) and leguminous fodder plants (e.g. clover and alfalfa) are traditionally excluded. Pulses are historically important in both the human diet and cropping systems as crop rotations, due to their rich-protein and biological nitrogen fixation ability. Although most pulses are not traditionally typical Western-style foodstuffs, international events like "International Year of Pulses 2016" and "Global Pulses Day" suggest that they are being promoted to be important human food world widely (Foyer et al., 2016).

As shown in Table 2.1, six of the 11 pulses which are covered in the FAO list, chickpea (*Cicer arietinum*), lupin (*Lupinus*), field pea (*Pisum sativum*), faba/broad bean (*Vicia fabae*), lentil (*Lens culinaris*) and mung bean (*Vigna radiate*), are the most important pulses globally, totally accounting for 79.89% of the world pulse production (81.8 million tonne) in 2016 (FAOSTAT, 2018). India is the largest pulse producer globally, followed by Canada, Myanmar and China. However, Australia is the largest lupin producer in the world, contributing an average of 58.22% of the world production in 2012-2016 (ABARES, 2018). Australian sweet lupin (ASL, *L. angustifolius*), which is also named "narrow-leafed lupin", is the most important lupin specie, constituting 93% of Australian lupin production and 52% of the world production (Pulse Australia, 2016). However, chickpea has overtaken lupin as Australia's largest pulse crop since 2011-12, with a production estimated at over 2 million tonnes in 2016-17 (ABARES, 2018). As a leading pulse exporter, Australia exports over 90% of its chickpeas, faba beans, lentils and mung beans, and 60% of field peas were exported, being the largest exporter of *desi* chickpea and faba bean in the world. Notably, although Australia exported only 50% of its lupin, this accounted for 90% of world lupin export in 2013.

Pulse seed has three distinctive parts, namely the seed coat, embryonic axe and cotyledon, which generally accounts for 8-16%, 1-3% and 80-90% of the whole seed respectively (Dueñas, Hernandez, & Estrella, 2006). However, the proportions of seed coat show great genetic and environmental variability both between and within species (Table 2.2). For example, lupin uniquely contains a much higher percentage of seed coat than others, with up to 24% in Australian sweet lupin and around 18% in white lupin (Clements et al., 2014). Removal of pulse seed coat (dehulling) is a primary process to produce dehulled splits, ground flours and other fractionated pulse ingredients like pulse protein and fibre. In practice, by-product generated from the

dehulling process is a mixture of seed coats, embryonic axes and brokens of cotyledons (Oomah, Caspar, Malcolmson, & Bellido, 2011; Sherasia, Garg, & Bhanderi, 2017). As a consequence, the dehulling loss which is the main waste stream of pulse processing represents as much as 31% for sweet lupin in Australia (Sipsas, 2008), and up to 28% for lentil and chickpea in India (Tiwari & Cummins, 2011). Currently, the primary markets for pulse seed coats are low-value animal feed and only very limited use in human foods such as that added to make high fibre bread and meat products (like sausage and nuggets). This by-product not only leads to a tough disposal problem for the millers, but also wastes a potential source of novel, nutritious and health-promoting food ingredient (Sherasia et al., 2017).

Growing evidence suggests that pulse seed coats have considerable amount of dietary fibre which is associated with diverse types of minerals and phytochemicals (bioactive secondary metabolites in plants, e.g. polyphenolic antioxidants). Therefore, besides the well-documented physiological benefits of dietary fibre, seed coats provide the potential for various physiological benefits, such as those related to antioxidant and anti-inflammatory activities. Available studies on pulse seed coats mainly focus on proximate compositions and anatomical structures, with little attention paid to their phytochemical properties and physiological functionalities. The present review brings together the current research on the characterization, processing and applications of seed coats from six selected pulses, i.e., chickpea, lupin, field pea, faba/broad bean, lentil and mung bean. This information should encourage strategies which might enable the more extended use of pulses and their seed coats in human foods.

2.2. Seed coat morphology and physical properties

The pulse seed coat (often referred to as hull or testa) is a protective outer layer of the pulse seed. Structures of pulse seed coats have been overviewed by Moïse, Han, Gudynaitę-Savitch, Johnson, and Miki (2005) and Smykal, Vernoud, Blair, Soukup, and Thompson (2014). Anatomical structures of seed coats of field pea (Van Dongen, 2003), faba/broad bean (Youssef & Bushuk, 1984), chickpea (Wood, Knights, & Choct, 2011), lentil (Hughes & Swanson, 1986), lupin (Clements, Dracup, Buirchell, & Smith, 2005; Clements et al., 2014) and mung bean (Joseph & Swanson, 1993) have been extensively examined and show great similarities. Largely, there are three specialized cross-sectional layers in typical pulse seed coats: palisade cells (macrosclereids) layer,

thick-walled hourglass cells (osteosclereids) layer, and a few layers of parenchyma (Figure 2.1) (Clements et al., 2014; Moïse et al., 2005; Tiwari et al., 2011).

The seed coats significantly affect chemical exchange (e.g. water and gas), biochemistry, mechanical properties (e.g. permeability, hardness and porosity) and physiological activities (e.g. germination and metabolism) of the pulse seeds (Moïse et al., 2005). In addition, their chemical and physical characteristics, including composition, shape, mass, smooth or rough surface, thickness, colour, density and thermal properties (e.g. thermal conductivity and thermal diffusivity) strongly affect the whole seed properties (such as density, dehulling efficiency, and cooking quality), further determine post-harvest processing, end application and market price of the seeds (Souza & Marcos-Filho, 2001). In this review, the most essential physical properties of the seed coats (i.e. colour, thickness and permeability) are discussed.

2.2.1. Colour

As a key quality indicator, the colour of the pulse seeds is controlled by several genes and crucial to consumer acceptance (Gulisano, Alves, Martins, & Trindade, 2019; Moïse et al., 2005; Oomah, Tiger, Olson, & Balasubramanian, 2005). Seed coat colour varies significantly across different varieties (Table 2.2). In Australia for example, over 90% of field pea is *dun* coloured type, and principally light brown *desi* chickpea, beige or light brown faba bean, white with/without spots lupin, red lentil and green mung bean. It has been revealed that the pigmentations of seed coats are mainly attributed to chlorophyll and polyphenols (mainly flavonoids) (Hossain, Panozzo, Pittock, & Ford, 2011), which mainly are located in the external palisade layer (Wood et al., 2011). The colours are associated with levels of those compounds, and thus their physiological properties like antioxidant capacity. For example, dark coloured pulses are reported to contain higher levels of polyphenols, mainly anthocyanins and condensed tannins, and correspondingly higher antioxidant activities than those of pale ones (Xu, Yuan, & Chang, 2007).

Colours of pulse seed coats are unstable during post-harvest processing and strongly affected by processing conditions. For example, the extremely high temperature (>40 ºC) may accelerate undesirable colour darkening process in faba bean seed coat. This is accompanied by a significant loss, ranging up to 86% of total polyphenols, which

may be explained by the polymerization of polyphenols into insoluble, un-extractable high molecular weight polymers (Nasar-Abbas et al., 2009). Similar browning was found in lentils (Nozzolillo & Debezada, 1984) and chickpea (Reyes-Moreno, Okamura-Esparza, Armienta-Rodelo, Gomez-Garza, & Milan-Carrillo, 2000) when stored at high temperature. Moreover, unpigmented varieties are supposed to be more vulnerable to seed deterioration during storage (Souza et al., 2001). However, the exact principles which are responsible for the darkening of pulse seed coats are still unclear.

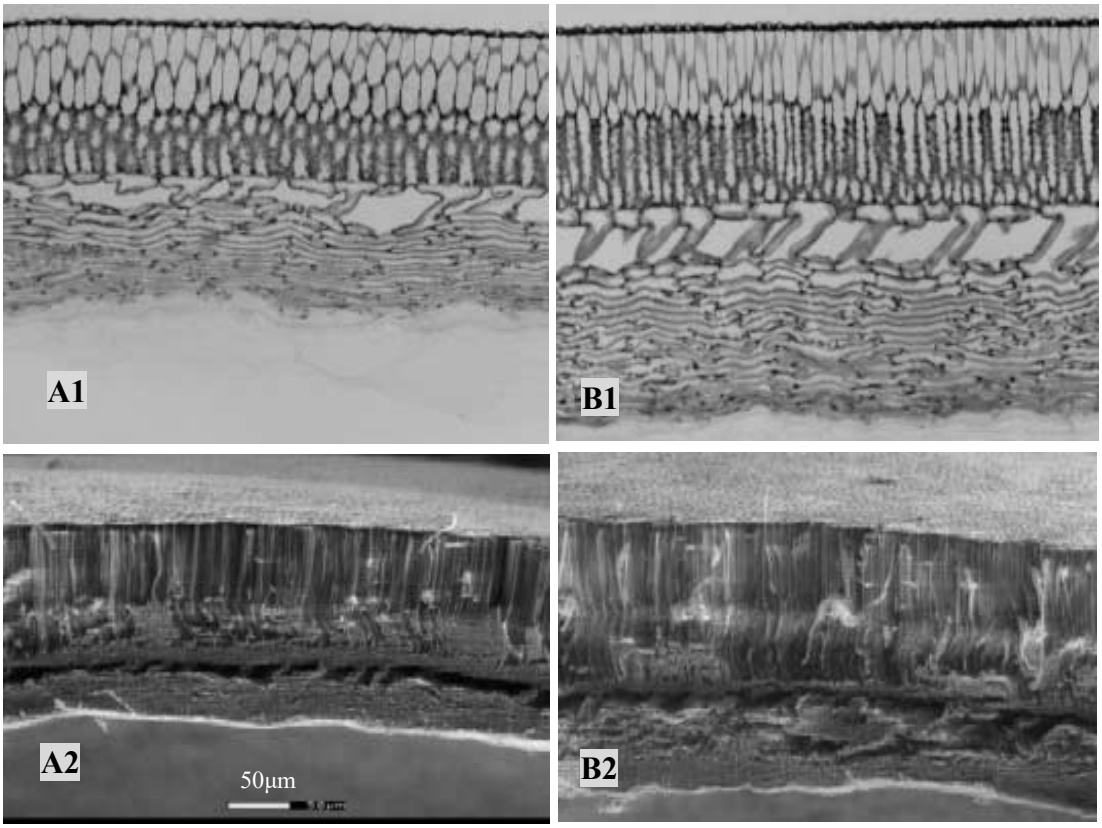

Figure 2.1 Light microscopy (A1, B1) and environmental scanning electron microscopy (A2, B2) of cross-sectional images of *Lupinus angustifolius* mutant (A) and parent (B) genotypes. pa, palisade layer; hg, hour-glass cell layer; par, parenchymatous layer. Adapted from Clements, Zvyagin, Silva, Wanner, Sampson, and Cowling (2004).

Table 2.1 Names and major types of the selected six pulses.

Botanical name	FAO commodity class	Common/Alternative names	2017 World production (million tonne)	Main market types in Australia (• most common)
Pisum sativum	Peas, dry	Field pea; Protein pea; Austrian winter peas (black peas); Canadian field peas (spring peas)	16.21	• Dun (including Kaspa type) White/blue Green
Vicia fabae	Broad beans, dry	Faba bean /Tickbean Broad bean	4.84	• *V. faba* var. minor (faba bean) *V. faba* var. major (broad bean)
Cicer arietinum	Chickpeas	Garbanzo beans (US); Bengal gram (India)	14.78	• Desi Kabuli
Lens culinaris	Lentils	Masurdahl, adas	7.59	• Red Green
Lupinus spp.	Lupins	Blue lupin; narrow-leaved lupin European white lupin	1.61	• ASL (*L. angustifolius*) Albus (*L. albus*)
Vigna radiate	Beans, dry	Mung bean (Australia) Green/golden gram (India)	~3.0	• *Vigna radiata* (green) *Vigna mungo* (black)

Ref: FAO (1994); FAOSTAT (2019); Sherasia, et al. (2017); Tiwari and Singh (2011) (2011); Pulse Australia (2016).

Table 2.2 Morphological and physical properties of the selected six pulse seed coats.

Pulses	Colour	Seed weight (g/100 seeds)	Seed coat percentage (%)*	Seed coat thickness (μm)
Lupin				
L. angustifolius	speckled	3.1-23.8	19.4-38.8	257.0-335.0
L. albus	white	12.0-86.9	12.2-27.5	nd
Field pea	green/yellow	18.7-25.6	7.2-14.0	55.9-72.0
Vicia fabae				
Faba bean	beige	40.0-95.0	11.0-15.4	141.0-248.0
Broad bean	beige	110.0-145.0	nd	
Chickpea				
desi	dark/brown	12.0-27.0	10.1-22.0	343.0-423.0
kabuli	beige/yellow	20.0-65.0	4.5-9.5	251.0
Lentil	red/green	4.5-7.5	7.0-11.0	25.0-65.0
Mung bean	green	2.5-4.7	8.6-23.5	30.0-330.0

* Data are on a dry basis; nd: no data were found;

Ref.: Hung, et al. (1983); Miao, et al. (2001); Clements, et al. (2005, 2014); Van Dongen (2003); Youssef & Bushuk, 1984; Wood, et al., 2011; Tajoddin, et al., 2013.

2.2.2. Thickness and permeability

In general, the palisade cell layer mainly decides the thickness of the seed coat. Domesticated pulse varieties have thinner, softer, more permeable seed coats than wild counterparts mainly due to decreases in thickness of the palisade layer. Moreover, the proportion and thickness of the seed coat are negatively correlated with seed size. The seed coat characteristics should be carefully considered during food processing (especially dehulling) and application. For example, *kabuli* chickpea has a larger seed size and thinner seed coat than the *desi* type (Table 2). As a result, cultivars of *kabuli* are normally used as whole seeds without dehulling for paste, salads, roasted or fried to make snacks (Wood, Knights, Campbell, & Choct, 2014). By contrast, cultivars of the *desi* type are often dehulled to *dahl* (split) which are directly cooked or milled to flour. Another example to show the associations between processing properties and seed coat thickness is that lentils have thinner seed coats and thus shorter cooking

times than do other pulse seeds. Additionally, thicker seed coats result in longer cooking-time in field peas (Wang, Daun, & Malcolmson, 2003) and faba beans than those which have thin seed coats (Youssef et al., 1984).

The permeability of pulse seed coats changes as the seed matures and are related to their structure and chemistry (Ma, Cholewa, Mohamed, Peterson, & Gijzen, 2004). Although impermeability of pulse seed coats is important to seed vitality, it is undesirable during food processing. The impermeability will contribute to lower whole-seed cookability ("hard-to-cook" phenomenon) and customer acceptability. For example, during soaking, the thick and impermeable seed coat will slow water imbibition by the seed, restrict its expansion and thus decrease the wet dehulling efficiency. The hydrophobic waxy cuticle and condensed palisade cells layer of the seed coat are major contributors to seed impermeability (Ma et al., 2004).

2.3. Pulse seed coat composition

The nutritional composition of whole pulse seeds has been reviewed in the FAO/INFOODS global food composition database for pulses (uPulses 1.0) (FAO, 2017). The composition of seed coats of the selected six pulses are summarised (Table 3). Generally, pulse seed coats have about 8-10% moisture, 3% ash, 1-3% lipids and 2-8% protein, with a major carbohydrate components (60-90%), mainly insoluble non-starch polysaccharides (NSPs) (Tiwari et al., 2011). Of the macronutrients, we focus on carbohydrates and minerals since they make up the majority and provide a basis for the usage of the seed coat as a food ingredient.

In general, pulse seed coats have a neutral to slightly nutty flavour, although their volatile profiles are largely unknown (Pfoertner & Fischer, 2001). Pulse seed coats are the major contributors to the phytochemical content of the whole seeds (Dueñas et al., 2006; Luo, Cai, Wu, & Xu, 2016). Some of the phytochemicals are, historically, referred to as "anti-nutritional factors (ANFs)", as with polyphenols, phytic acid and alkaloids. However, numerous epidemiological studies now indicate their potential benefits for human health (Rochfort & Panozzo, 2007). Investigation of the micronutrients (vitamins and minerals) and other bioactive compounds in the six pulse seed coats is embryonic.

Table 2.3 Main carbohydrates and dietary fibre of three selected pulses seed coats.

Pulses	ASL	Field pea	Chickpea
Starch (g/100g)	0.4-0.9	0.16-1.8	0.2-0.5
Oligosaccharides (g/100g)	0.4	nd	nd
NSP (g/100g)			
Total	79.8-89.1	68.0	45.9-72.4*
Soluble	5.0	3.0-4.0	1.9-2.5
Insoluble	80.6-84.1	64.0-65.0	49.1-52.9*
Cellulose (g/100g)	44.5-51.7	62.3	18.2-29.0
Hemicellulose (g/100g)	12.7-14.4	8.2	30.4
Pectins (g/100g)	15.6-27.7	nd	0.1
Lignin (g/100g)	0.3-2.1	3.5	1.4-4.1
Dietary fibre (g/100g)			
Total	88-90.5	81.0-91.5	74.9-84.2
Soluble	3.1-3.8	4.1-11.0	nd
Insoluble	84.2-87.4	70.0-87.4	nd

Data are on a dry basis.

nd: no data were found

ASL: Australian sweet lupin.

NSP: Non-starch polysaccharides; nd: no data were found.

*: A remaining 15% was not hydrolysed by the NSP analyses which was supposed to be “highly bound ligno-cellulosic compounds” (Wood, et al. 2014).

Ref.: Miao, et al. (2001); Evans, et al. (1993); Brillouet, et al. (1983); Guillon & Champ (2002); Hashemi, et al. (2015); Bailey, et al. (1974); Sosulski and Wu (1988); Dalgetty & Baik (2003); Ralet, et al. (1993a); Wood, et al. (2014).

2.3.1. Carbohydrates

As mentioned above, pulse seed coats have negligible amounts of starch and oligosaccharides. Instead, they are predominantly composed of structural polysaccharides (non-starch polysaccharides, NSPs), which are mainly cellulose, hemicellulose, pectin (Table 2.3). As such, over 50 per cent of the monosaccharides in seed coat are glucose from the cellulose. The other principal sugars vary between

species. For example, the high concentrations of xylose (21.6%), uronic acids (10.0%) and arabinose (8.4%) in lupin seed coat indicate relatively high contents of arabinoxylan hemicellulose and pectin (Evans, Cheung, & Cheetham, 1993). On the contrary, uronic acids (22.3%), xylose (10.8%) and arabinose (5.2%) are the main sugars in field pea seed coat cell walls (except glucose), indicating a high content of pectin (Guillon & Champ, 2002). It is worthwhile to note that there are also significant differences between NSPs in cotyledons and seed coats. For instance, the major constituent NSPs of lupin seed coat are cellulose (from 45 to 56 g/100g dry basis (db), arabinoxylan hemicelluloses (~13 g/100g db) and pectins, whereas pectic substances and hemicellulose are the predominant parts in cotyledon (Brillouet & Riochet, 1983).

Non-starch polysaccharides are classified as the principal components of the plant dietary fibre (DF) (Lovegrove et al., 2017). In principle, seed coat contributes a significant proportion of the DF level of the pulse because of their high content of NSPs, ranging from 75 to 91 g/100g db (Table 2.3). In addition, most of the DF in pulse seed coats are insoluble dietary fibre (IDF), only 3.5% of total dietary fibre (TDF) in lupin seed coat is soluble for example (Evans et al., 1993). IDF levels of dehulled lentils, peas and chickpeas decreased by 64%, 53% and 35% respectively compared to raw seeds, but no significant reduction in SDF was found (Dalgetty & Baik, 2003). However, regarding the newly proposed DF definition and analytic method (i.e. AOAC 2011.25), contemporary information on DF (including oligosaccharides) for the six pulse seed coats is scarce.

2.3.2. Minerals and trace elements

Pulses provide substantial amounts of minerals. Pulse seed coats are rich in several minerals, e.g. Ca, Mg, Mn, Cu, Zn, B, Al and Na etc. (Tiwari et al., 2011). Notably, 67.5% of total Ca, and 41.3% of total Al of the whole lupin seed were reported to concentrate in its seed coat (Hung, Handson, Amenta, Kyle, & Yu, 1988). Likewise, over 70% of Ca and 50% of iron in mung bean (Singh, Singh, & Sikka, 1968), lentil (Tiwari, et al., 2012) and chickpea (Jambunathan & Singh, 1981) are found in their seed coats. Besides the inter-species variations, minerals in seed coats vary inner species widely. For instance, contents of most of the minerals, especially Ca, Zn, Cu, and Mn, in *kabuli* chickpea seed coat are higher than *desi* type (Jambunathan et al.,

1981). Consequently, differences in the seed coats (like thickness and proportion) between pulses are used to explain the variations in mineral levels of the whole seeds.

2.3.3. Phytochemicals

The major phytochemicals in different pulses vary significantly. For instance, chickpea was found to be one of the major sources of dietary saponins (Oakenfull, 1981), but alkaloids are characteristically present in lupin. Although, carotenoids (a group of lipid-soluble natural plant pigments) contents of field pea (Marles, Warkentin, & Bett, 2013) and chickpea (Ashokkumar, Tar'an, Diapari, Arganosa, & Warkentin, 2014) are suggested to be associated with seed coat colours, pulse seed coats are generally known as a poor source of carotenoids since they have low level of fat. In some cases, phytochemicals may cause toxic effects (e.g. favism caused by vicine and convicine in faba beans) (Klupšaitė & Juodeikienė, 2015). However, this review will discuss polyphenols and phytic acid in the six pulse seed coats, and alkaloids in lupins since they are more relevant to the potential positive physiological properties of the seed coats.

2.3.3.1.Polyphenols

Polyphenols are a wide range of secondary plant metabolites, which typically have one or more aromatic rings bearing several hydroxyl groups. The major polyphenols in whole pulse seeds are phenolic acids (e.g., benzoic/cinnamic acids and their derivatives), flavonoids (e.g., flavone and flavonol glycosides) and condensed tannins (Oomah, Patras, Rawson, Singh, & Compos-Vega, 2011). Recently, a few studies have investigated polyphenols in legume seed coats, including a systematic evaluation in 28 pigmented edible bean coats (Gan et al., 2016), chickpea (Sreerama, Neelam, Sashikala, & Pratape, 2010), faba bean (Boudjou, Oomah, Zaidi, & Hosseinian, 2013), field pea (Guo et al., 2019; Marles et al., 2013), lentil (Dueñas et al., 2006; Oomah, Caspar, et al., 2011), and mung bean (Luo et al., 2016; Muhammed, Manohar, & Junna, 2010).

In general, these studies confirmed that the seed coats have a high total polyphenol level, ranging 68.34-5798 mg gallic acid equivalents /g db (mg GAE/ 100 g db). Of particular, polyphenols of whole pulses seeds are essentially concentrated in the seed coats, and hence they are the predominant *in vitro* antioxidant capacity contributors.

For example, 80.3-84.2% of the total polyphenol and over 83.9% of total flavonoid content of whole mung bean seed were reported to be present in the seed coat (Luo et al., 2016; Muhammed et al., 2010). The proportions of total polyphenol and total flavonoid content in faba bean seed coat are up to 80.0% and 89.3% of the whole seed, respectively (Boudjou et al., 2013). Similarly, total polyphenol content of chickpea seed coat (75.94 mg GAE /g db) was relatively higher than that of the cotyledon (15.24 mg GAE /g db) (Sreerama, Neelam, et al., 2010). Condensed tannins in faba bean (Boudjou et al., 2013), mung bean (Xu et al., 2007), and lentil (Dueñas, Hernández, & Estrella, 2002) seed coats were reported to represent over 75%, 50% and 54% respectively of the total tannins in the whole seeds. Notably, Xu et al. (2007) found that polyphenols levels and *in vitro* antioxidant activities of dark coloured (like red, bronze, and black) lentil and chickpea seeds were significantly higher than those of light coloured (like white, yellow, and green) varieties. Total free phenolic acids and condensed tannins in coloured pea seed coat reached to 78.53 g/g db and 1560 mg catechin equivalents /g db (mg CE/g db) comparing to 17.17 g/g db and not detected for those in the white seed coat (Troszyńska & Ciska, 2002).

In the case of lupins, total polyphenol content in seed coats of *L. mutabilis*, *L. albus*, and *L. angustifolius* which grown in Brazilia were reported to be 1.15-4.49 mg CE/g db which were much lower than cotyledons (7.38-12.42 mg CE/g db) (Ranilla, Genovese, & Lajolo, 2009). The results accord with findings from Lampart-Szczapa, Siger, Trojanowska, Nogala-Kalucka, Malecka, and Pacholek (2003), who found that polyphenols in seed coats of *L. luteus*, *L. albus* and *L. angustifolius* grew in Poland (ranging from 0.16 to 0.42 mg caffeic acid equivalents/g db), were 1.30-6.52 times lower than those in cotyledons (0.32 to 1.88 mg caffeic acid equivalents/g db). Additionally, these authors revealed that free phenolic acids, primarily procatechuic acid and *p*-hydroxybenzoic acid, were mainly present in the seed coats. Likewise, they found that concentrations of tannins in the cotyledons were 4.33-31.00 times higher than that in the seed coat. On contrast, Petterson (1998) reported that most tannins (include proanthocyanidins) of lupin occurred in the seed coat, however, the initial data are unavailable.

These different and sometimes conflicting results of studies on polyphenols in pulse seed coat are difficult to interpret and compare since the lack of consensus on

extraction methods and reporting (i.e., equivalents). Nonetheless, most of the previous published studies have only extracted polyphenols with organic solvents in which case appreciable amounts of "bound" polyphenols in the seed coat matrix may remain un-extracted and thus the total polyphenol levels and antioxidant capacity may be underestimated (Saura-Calixto, 2012).

2.3.3.2.Phytic acid

Phytic acid (PA), its lower substituted homologues and its salts are referred to as phytates which are commonly present in pulse seeds. Phytic acid has been implicated in the "hard-to-cook" phenomenon in pulse seeds. In addition, they are considered as the main anti-nutritional factor because of their capacity to chelate cations (in particular calcium, iron and zinc) to form insoluble complexes and therefore reduce their bioavailability (Sanchez-Chino, Jimenez-Martinez, Davila-Ortiz, Alvarez-Gonzalez, & Madrigal-Bujaidar, 2015). The content of phytic acid can be affected significantly by genetic and environmental factors, alone and in combination. However, phytic acid in mung bean (1.8-5.8 mg/g db), pea (3.1-7.1 mg/g db), lentil (2.5-12.2 mg/g db), chickpea (2.8-13.6 mg/g db), lupin (6.0-8.9 mg/g db) and faba bean (5.9-15.0 mg/g db) are generally lower than soybean (4.8-20.1 mg/g db) (Campos-Vega, Loarca-Piña, & Oomah, 2010). Moreover, the majority of phytic acid is demonstrated to present in "the proteins bodies" in legume cotyledons, what is supported by Campos-Vega et al. (2010). For example, phytic acid of chickpea is presented in a low level (0.79 mg/g db) in seed coat but high in cotyledon (9.82 mg/g db) (Sreerama, Neelam, et al., 2010). Beal and Mehta (1985) indicated that little (1 mg/g) or no phytic acid was found in pea seed coats. Dehulling significantly increased the phytic acid levels in lupins (Embaby, 2010) and other pulses (Ghavidel & Prakash, 2007), reflecting that phytic acid is mainly located in the cotyledons.

2.3.3.3.Alkaloids

Alkaloids are mainly present in lupins. Quinolizidine alkaloids, mainly lupanine, 13-hydroxylupanine and angustifoline, are major contributors to the bitter taste of some varieties of lupin seeds and are potentially toxic (Petterson, 1998). Bitter lupin varieties have alkaloid contents ranging between 0.5-6%, in contrast, the sweet varieties have less than 0.02% (Resta, Boschin, D'Agostina, & Arnoldi, 2008). Moreover, alkaloids

can be removed by washing with water. A maximum legal limit on alkaloid concentration in lupin flours and lupin products has been set at 0.02% by authorities of France, UK, Australia and New Zealand (Resta et al., 2008). Little is known on distributions of alkaloids in lupin seeds, though Sipsas (2008) reported that no alkaloids were found in Australian sweet lupin seed coat, but no detailed data was found.

2.4. Mycotoxins contamination

Pulses are vulnerable to be contaminated by fungus and the resulting mycotoxins (e.g., alfatoxins, ochratoxins and phomopsins) during pre- or post-harvest (CAST, 2003). A further increase in human exposure of them by consuming products containing contaminated pulses may occur. However, recent systematic surveys on mycotoxins in pulses based human food are lacking. Here, phomopsins in contaminated lupin seeds, a highly representative example of mycotoxins contamination of pulses, will be discussed as a detailed case study.

Phomopsins are toxins produced by the fungus *Diaporthe toxic* (EFSA, 2012). The fungus mainly infects lupin stems but also the seeds under high humidity storage conditions (>13%). Lupin seed coat, being the outermost layer of the seed, is the most vulnerable part of seed to be invaded by the fungus and thus may contain the highest level of phomopsins (EFSA, 2012). Phomopsins are suspected as the cause of lupinosis in grazing animals. A maximum legal limit (5 μg/kg) of phomopsins in lupin seeds and lupin foods has been established in Australia, New Zealand, UK and FAO (Schloss, Koch, Rohn, & Maul, 2015). As other mycotoxins, phomopsins are stable to food processing including soaking, cooking, and fermentation. However, seeds contaminated by phomopsins can be easily removed by seeds grading and screening since phomopsins is “almost entirely limited to dis-coloured seeds” (EFSA, 2012). In addition, resistant varieties have been developed. Extrusion which combines high pressure, high temperature and severe shear has shown the capacity to reduce other mycotoxins (e.g. alfatoxins and zearalenone) (Bullerman & Bianchini, 2007), but no studies on detoxifying phomopsins in lupin by extrusion cooking have been reported.

2.5. Bioavailability of nutrients in pulse seed coats

Bioavailability refers to the extent that nutrients can be released from food matrix into digestive fluid, and thereby available for intestinal transport, biotransformation, absorption and metabolism (Versantvoort, Van de Kamp, & Rompelberg, 2004). There is strong evidence that the structure and composition of a food matrix will govern the bioavailability of many nutrients in the gastrointestinal tract (Wahlqvist, 2016).

A few published clinical studies have suggested that pea seed coat consumption may benefit cardiovascular and gastrointestinal biomarkers in humans, that may be due to multiple mechanisms caused by the high dietary fibre in the seed coat (Dahl, Whiting, Healey, Zello, & Hildebrandt, 2003; Flogan & Dahl, 2010; Mollard, Luhovyy, Smith, & Anderson, 2014). However, dietary fibre has been shown to significantly reduce the bioavailability of several nutrients. For example, lupin seed coat in the diet decreased protein digestibility in rats (Bailey, Mills, & Hove, 1974). In contrast, removal of lentil seed coat significantly improved lentil iron bioavailability (DellaValle, Vandenberg, & Glahn, 2013). The compact inert insoluble fibre matrix of the seed coat may be a physical barrier to block the release of nutrients, give increased viscosity of digesta and therefore impair absorption. Besides, dietary fibre, polyphenols and alkaloids can also inhibit enzymes, and chemically bind some nutrients thus lowering their bioavailability (Khattab & Arntfield, 2009).

Cellulose and hemicellulose in the colon can be degraded by some specialized series of gut bacteria and their fermentability of was surprisingly reported to be high, up to 70% and 72% respectively (Flint, Scott, Duncan, Louis, & Forano, 2012; O'Grady et al., 2019; Williams, Mikkelsen, Flanagan, & Gidley, 2019). It suggests that “trapped” compounds (e.g., minerals and polyphenols) in pulse seed coats could be released in colon. In this context, dietary fibre could also modulate pH of the human gastrointestinal tract, especially lower pH level in the colon, to enhance the release and absorption of minerals (Baye et al., 2017). A large proportion of polyphenols are reported to be not bioavailable in the upper part of the human gastrointestinal tract. Instead, they will reach the colon and be metabolized at a large extent by gut microbiota (Saura-Calixto, 2012). However, more studies are needed to investigate digestibility of pulse seed coats in human, as well as their physiological effects on human health (including impacts on colon and gut bacteria).

Table 2.4 Selected physicochemical properties of pulse seed coats.

Physical properties	ASL	Field pea	Chickpea	Lentil	Mung bean
Direct Density (g/mL)	nd	0.6	0.4	0.7	nd
Bulk Density (g/mL)	nd	0.8	0.7	0.8	0.45-0.64
Swelling capacity(mL/g)	nd	1.9-6.0	3.6	2.4	5.51-9.20
Water binding (mL/g)	7.0-8.0	4.0-7.1	6.2	3.6	3.13-4.44
Oil binding (mL/g)	1.6-1.7	1.5-2.0	1.8	1.6	1.49-1.83

nd: no data were found.

ASL: Australian sweet lupin.

Ref.: Guillon & Champ (2002); Dalgetty and Baker (2003); Pfoertner and Fischer (2001); Turnbull, Baxter, and Johnson (2005); Ralet, et al. (1993b); Huang, et al. (2009).

2.6. Effect of processing on pulse seed coats

Generally, pulses are dried in the field to achieve the target moisture of 9-20% for threshing (i.e. removal of pods), then cleaned, graded and further dried to approximate 13% for storage. Storage conditions (e.g. seed moisture, relative humidity, duration and temperature) significantly affect the seed coat characteristics. For example, the seed coat colour of faba bean has been observed to darken from beige to dark brown depending upon the storage conditions (Nasar-Abbas et al., 2009). Although pulses can be consumed either whole or dehulled splits, they require processing before consumption to (1) reduce or eliminate anti-nutritional factors, (2) improve consumer acceptability (e.g. texture, flavour), and (3) enhance nutritional properties like nutrient bioavailability. There are several conventional whole seeds processing methods, including soaking, dehulling, milling, cooking, puffing, germination (or sprouting) and fermentation (Patterson, Curran, & Der, 2017). But only few studies are found to treat isolated pulse seed coat using milling, boiling, and more recently extrusion cooking. All have shown to affect composition, and physicochemical and nutritional properties of the seed coats.

2.6.1. Conventional processing

Seed coat bulk density (weight of seed coat per unit volume) is low such that further processing (like grinding) is required to increase their density to reduce its storage and

transport fees after dehulling (Table 2.4). Grinding was reported to increase solubility of pea seed coat, from 4.1% to 8.6%, accompanied by a reduction in water-binding capacity (WBC) and swelling capacity by 35.2% and 21.7% respectively (Ralet, Della Valle, & Thibault, 1993a). Similarly, water solubility of mung bean seed coat was 0.97% with particle size of <50 mesh (<300 μm), whereas a much lower water solubility (0.79%) was found with particle size of >35 mesh (>500 μm) (Huang, Lia, Cheng, Chan, Hwang, & Hwang, 2009). The authors also found that mung bean seed coat with smaller particle size had a significantly higher swelling capacity, WBC, and oil binding capacity but lower bulk density compared with those with bigger particle size.

Soaking followed by cooking of whole pulse seeds is the traditional domestic operation to produce edible pulse products. During soaking, pulses imbibe water to expand the seed coats, and activate endogenous enzymes (cell wall polysaccharidases which can disrupt the cell wall, and phytase which can reduce phytic acid content, for example) (Wang et al., 2003). Moreover, water-soluble compounds like minerals, soluble tannins, phytic acid, alkaloids and polyphenols may leach into soaking, cooking and canning water (Tajoddin, Manohar, & Lalitha, 2013). As the outer layer, the seed coat plays a crucial role in controlling these exchanges during soaking and cooking. Additionally, Güzel (2012) found that atmospheric pressure cooking (APC) and high-pressure cooking (HPC) caused darkening of chickpea and faba bean seed coats, with greater effect for HPC. The colour changes may be the results of pigment degradation. Hashemi, Yang, Yang, Jin, Ozga, and Chan (2015) found that starch in pea seed coat increased from 0.16% to 0.59% on a dry basis after boiling for 30 min, what may be due to the increase of starch bioavailability and losses of soluble compounds during boiling.

Mung bean (Tajoddin et al., 2013), lentil and field pea (López-Amorós, Hernández, & Estrella, 2006), chickpea (Ghavidel et al., 2007), and lupins (Dueñas, Hernandez, Estrella, & Fernandez, 2009) have been used to germinate sprouts. Most of these studies confirmed that germination will increase polyphenols (prominently flavonoids) and vitamins, whereas decrease anti-nutritional factors (e.g. α-galactosides, trypsin inhibitors and phytic acid). As a result, germination can increase antioxidant capacity and bioavailability of the nutrients. Seed coat impermeability is the main regulator for pulse germination. Moreover, the structure and composition of the seed coat will

change significantly just before and during germination, possibly by enzymes (Finch-Savage & Leubner-Metzger, 2006). Although no study on the effect of germination on pulse separated seed coat has been found so far, it can be hypothesised that changes in composition and physicochemical properties of pulse seed coat may occur.

2.6.2. Extrusion cooking

Extrusion cooking is a high-temperature short-time unit operation in which food will be cooked in a sealed cylinder by high pressure, high temperature and high mechanical shear, then passed through a die (Alam, Kaur, Khaira, & Gupta, 2016). Depending on extrusion conditions (such as material particle size, feed rate, moisture, screw speed and configuration, barrel temperature and die geometry), the process results in disruption of cell wall structures, chemical reactions (such as polysaccharides depolymerization, Maillard reaction and starch gelatinization), and physical changes (e.g. solubility, morphological and rheological properties) (Singh et al., 2007; Wolf, 2010). Moreover, extrusion has been used to incorporate seed coats of field pea (Schmidt, 1987), lupin (Tucek, 2009) into breakfast, pasta and snacks to increase their dietary fibre levels. But they are beyond the scope of this review.

Extrusion cooking, mainly twin-screw extrusion, is the most used technology to modify the functional properties of high fibre materials (Rashid, Rakha, Anjum, Ahmed, & Sohail, 2015; Wolf, 2010; Yan, Ye, & Chen, 2015). The water solubility of pea seed coat was reported to increase by 3.6-15.3% after extruded using twin-screw extruder, accompanied by a dramatic increase (up to 220%) in soluble dietary fibre (Ralet, Della Valle, & Thibault, 1993b). Similarly, single screw extruder increased soluble dietary fibre in pea seed coat from 5.3% to 6.7% (Arrigoni, Caprez, Amadò, & Neukom, 1986). Correspondingly, technical properties of pea seed coat, like water binding capacity and swelling capacity, were increased by extrusion cooking. On the contrary, extrusion has shown no or slightly increased effects on the technical properties of yellow pea seed coats (Arrigoni et al., 1986). Except the conflicting results mentioned above, data from extruded wheat bran (Rashid et al., 2015; Yan et al., 2015), sugar beet pulp (Rouilly, Jorda, & Rigal, 2006), onion waste (Ng, Lecain, Parker, Smith, & Waldron, 1999) support the increase in the solubility of dietary fibre, as well as the improvements on their physicochemical characteristics.

Extrusion cooking has been revealed to reduce the levels of heat sensitive extractable polyphenols, which can be extracted by aqueous/organic solvents (Singh et al., 2007). However, it can release non-extractable polyphenols, which remain in the resulting residues of the aqueous/organic extraction, from food matrix. Depolymerization of high molecular weight polyphenols (such as condensed tannins) was also reported (Awika, Dykes, Gu, Rooney, & Prior, 2003). Additionally, extrusion cooking can increase the bioavailability of minerals, mainly by reducing the chelating properties of dietary fibre and the contents of other chelating compounds such as phytic acid and condensed tannins (Singh et al., 2007). Taken together, extrusion cooking could be an applicable technology to improve the properties of pulse seed coats. However, more comprehensive studies are required to investigate its effects on compositional and physicochemical properties of the pulse seed coats.

2.7. Application of pulse seed coats in human food

Pulses have been historically important sources of energy, protein and dietary fibre in the human diet. Currently, pulse seed coats have only limited use in human food such as in high fibre bread and meat products. For example, the Australian lupin milling sector produces about 10,000 tonnes of lupin seed coats annually, while less than 2% of them are used in human food products (Tucek, 2009). However, the high content of dietary fibre in pulse seed coats, along with considerable amounts of minerals, phytochemicals (e.g. polyphenols) suggests they could be more widely utilised as novel functional dietary fibre ingredients (Oomah, Caspar, et al., 2011). There are several commercial dietary fibre ingredients manufactured from pea seed coat and lupin seed coat, both of them have been classified as GRAS (Generally Recognized as Safe). However, lupin has been listed as a food allergen what requires mandatory labelling in Europe since 2007, and most recently in Australia and New Zealand (March 2017) (FSANZ, 2017). Moreover, there are several specific regulations on contaminants and natural toxins levels of the six pulses and their derived food products, phomopsins and lupin alkaloids in lupin seed coat for example.

Like other dietary fibre ingredients, pulse seed coats have been incorporated into baked goods, in which they have shown to change physical, nutritional, and sensory properties of the products. Dalgetty and Baik (2006) found that incorporations of pea, lentil, and chickpea seed coats significantly increased dough mixing time, water

absorption, and loaf weight but decreased loaf volume. The observations are in accordance with the results of Sosulski and Wu (1988) who added up to 7.7% of pea seed coat into dough. The authors of these studies concluded that bread with 5% pulse seed coat addition was comparable to whole wheat bread in sensory quality but had desirable higher dietary fibre content. Likewise, incorporation of 2-6% of lupin seed coat flour into bread dough increased water absorption but decreased stability time of the dough. Moreover, the number and size of the bread pores, and thus the specific volume of the bread were significantly lowered due to lupin seed coat flour addition, though the bread showed good consumer acceptability (Wandersleben et al., 2018).

In terms of adding pulse seed coats into meat products, Verma, Banerjee, and Sharma (2012, 2015) used pea hull flour (PHF) and chickpea hull flour (CHF) as dietary fibre sources to improve qualities of chicken nuggets. The studies found that incorporation of the two hull flours significantly increased product yield and dietary fibre content. However, both reduced emulsion stability of the product, and lowered its hardness, gumminess and chewiness dramatically. Product colour was also affected by initial colours of the two hull flours and formulation differences. The sensory evaluation suggested that an 8% PHF addition in low salt (40% reduction) chicken nuggets were acceptable to consumers.

2.8. Conclusions

To date, pulse seed coats are little utilised in human food. However, there is potential for the seed coat to be used as a natural "nutritious dietary fibre" which could (1) fill the "fibre intake gap", (2) provide considerable levels of minerals and antioxidants, and (3) achieve greater safe and sustainable utilization of pulses by exploiting value-added applications of their by-products (Saura-Calixto, 2012; Sharma et al., 2016). However, in-depth studies on biochemical, and nutritional properties of pulse seed coats are still lacking. In addition, physicochemical properties (e.g. solubility, swelling capacity, water and oil binding capacities) of pulse seed coats will significantly associate with physiological functionalities (Wahlqvist, 2016). However, the impacts of processing on physicochemical properties of pulse seed coats are still unclear. Moreover, to minimise the negative effects of dietary fibre and other "anti-nutritional factors", while improving their desirable physiological properties, further work is needed to optimise the processing. Finally, parallel to the study of pulse seed coats

incorporation into food products, more nutritional and safety studies on the products are needed. These will add to what are likely to be favourable cost and sustainability profiles.

To address the mentioned issues, the following chapters will use as Australian sweet lupin (ASL) a typical example to (1) screen chemical and physicochemical functionalities of seed coats of six dominated ASL varieties, (2) evaluate the feasibility of extrusion cooking on lupin seed coat properties enhancement and its effects on bioavailabilities of lupin seed coat minerals and phytochemicals.

CHAPTER 3

Effect of genotype and environment on the physicochemical and nutritional properties of lupin seed coats

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ABSTRACT

Genotype, environment and their interaction have been found to show significant effects on lupin seed properties. In this chapter, the physicochemical, nutritional properties and antioxidant capacities of seed coats of six Australian sweet lupin (ASL) cultivars grown at two locations in Western Australia were evaluated to explore their use for food purposes. The genotypic and environmental effects on the properties were investigated. Results indicated that genotype, environment and their interaction were significant for seed weight, seed coat percentage, proximate composition, dietary fibre content, polyphenols and antioxidant capacities. Strong correlations between seed coat lightness (L*) value, polyphenol content and antioxidant capacities were found. A comparison using multivariate analysis of the seed coat properties exhibited clear separation based on growing sites. This study supports the great potential of ASL seed coat as an "antioxidant dietary fibre". Given the significant effects of genotype, environment and their interaction on ASL seed coat physicochemical and compositional properties, the results also could provide a new breeding strategy to produce lupin varieties with desirable levels of nutrients and phytochemicals.

3.1. Introduction

Legume lupin is a sustainable, high-quality protein source for both livestock and humans, with multiple economic and environmental benefits to combating climate change (Foyer et al., 2016; Johnson et al., 2017). Take Australia as an example, lupins play an important role in its farming systems through wheat: lupin rotation. Since the release of the initial domesticated cultivars of Australian sweet lupin (ASL, *Lupinus angustifolius*) in the late 1960s and early 1970s, ASL cultivars have been subsequently bred to further improve ASL performance, such as yield, seed qualities, adaptation, harvestability, disease resistant and herbicide tolerance (DPIRD, 2018). Recently, the ASL varieties that have dominated Australian production are Mandelup (released in 2004), Coromup (2006), Jenabillup (2007), PBA Gunyidi (2010), PBA Barlock (2013), PBA Jurien (2015) and PBA Leeman (2017) (Pulse Australia, 2015).

In spite of lupin's positive characteristics thus that its expanding use as human food, the high milling loss during flour production, with lupin seed coat being the main by-product, complicates its use as a food ingredient. However, the by-product could be an economically available source of nutraceutical components, including natural dietary fibres and polyphenols (Chapter 2). Accordingly, increasing studies have been bringing new options to utilise lupin seed coat into human foods (Tucek, 2009). For example, the seed coat of albus lupin has been commercially developed as an insoluble fibre ingredient (Vitafiber®, Avelup Ltd., Chile). More recently, lupin seed coat flour was incorporated into bread as a source of insoluble dietary fibre (IDF) (Wandersleben et al., 2018), and used to produce cellulose nanofiber aerogel (Ciftci, 2017). But only a few studies have investigated the nutritional and functional properties of seed coats of several now redundant lupin varieties and the results showed great variations (Bailey et al., 1974; Brillouet et al., 1983; Evans et al., 1993; Eleonora Lampart-Szczapa, Korczak, Nogala-Kalucka, & Zawirska-Wojtasiak, 2003; Lush & Evans, 1980; Michalczyk, Piotrowicz-Cieslak, Sawicka-Sienkiewicz, Galek, Bochenek, & Glowacka, 2006; Mohamed & Rayas-Duarte, 1995). In this context, a systematic study was needed to investigate the properties of seed coats of the recent and agriculturally dominant ASL varieties.

Clements, Dracup, and Galwey (2002) reported that genotype, environment and their interaction showed significant effects on ASL seed coat percentage while Winnicki et

al. (2019) identified effects of irrigation level on ASL seed characteristics. In light of this, the purposes of the study in this chapter are to (i) provide a new characterisation of nutritional and functional properties of seed coats of the current ASL varieties under production in Australia, (ii) investigate the genotypic and environmental effects on the properties of the selected ASL seed coats. To this end, six recent ASL cultivars grown in 2015 at two locations in Western Australia (WA) were analysed for their seed coat instrumental colour, physiochemical properties (water-binding capacity, oil binding capacity, swelling capacities), proximate and dietary fibre composition, as well as total polyphenols and antioxidant capacities. Univariate and multivariate analyses were performed to evaluate the significance of genotype, environment and their interaction on these properties.

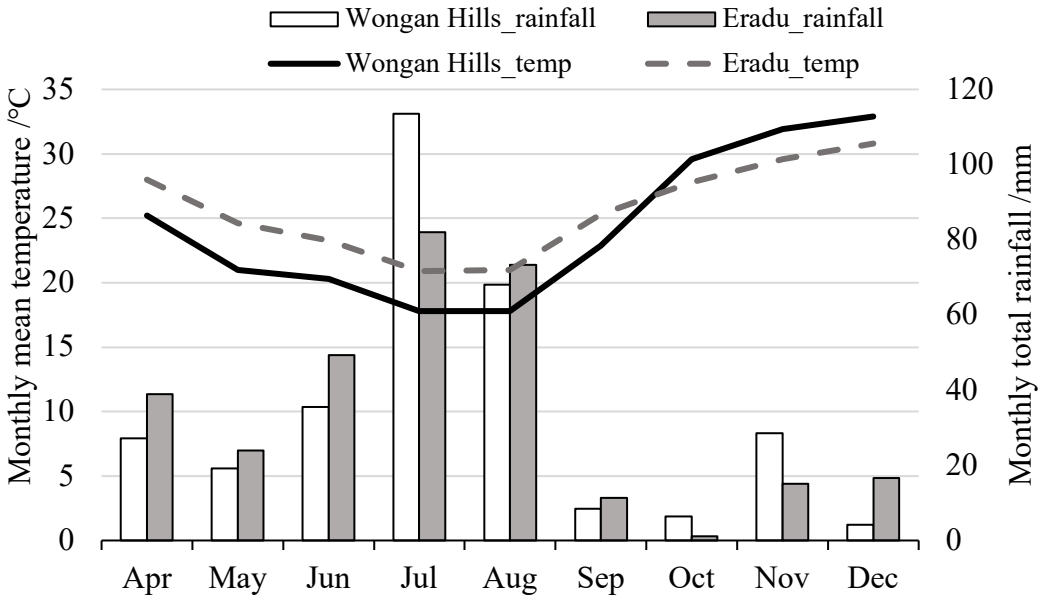

Figure 3.1 Monthly mean temperature and monthly total rainfall at two different growing sites (Wongan Hills, WH; Eradu, ER) (manipulated basing on BOM climate data, http://www.bom.gov.au/climate/data/index.shtml).

3.2. Materials and methods

3.2.1. Lupin seed coat samples

Whole seeds (ca 4 kg) of six ASL genotypes (Coromup, Mandelup, Jenabillup, PBA Barlock, PBA Gunyidi and PBA Jurien) harvested from two locations within 2015 growing season were obtained from the with the Department of Primary Industries and

Regional Development - Agriculture and Food (DPIRD, Kensington, WA, Australia). The lupins were grown in Wongan Hills (WH; 30.54°S, 116.43°E) and Eradu (ER; 28.70°S, 115.05°E) Western Australia (WA). In 2015 lupin growing season (April-December), the two locations had similar solar radiation exposure with annual mean daily global exposure being 19.5 MJ m^{-2} (BOM, 2018). Other climatic characteristics (monthly mean temperature and rainfall) of the two growing locations are presented in Figure 3.1 (BOM, 2018). The two locations belong to lupin Agzone 5 and Agzone 2, respectively based on their rainfalls (White, French, McLarty, & Grains Research and Development Corporation, 2008).

The seeds as shown in Figure 3.2 were dehulled by a laboratory-scale AMAR dehuller (NSIC.SSI, India). Coarse seed coats were separated by a vacuum separator (Kimseed Pty Ltd., WA, Australia) with manual removal of any remaining broken cotyledons. The seed coat percentage of whole lupin seed was calculated. Western Australia has a typical Mediterranean climate. The seed coats were pre-dried at 50 °C overnight prior to being milled into flours using a ZM 200 Retch Mill (Retsch Gmbh & Co, Haan, Germany) then passed through (100%) a 500 μm screen. The resulting flours were vacuum-packaged into two bags which stored separately at -20°C (only for polyphenol and antioxidant capacity analyses) and 4°C.

3.2.2. Reagents and equipment

Hydrochloric acid, sodium hydroxide and ethyl acetate were purchased from Thermo Fisher Scientific (Scoresby, Vic, Australia). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), gallic acid, and catechin were purchased from Aldrich (Sigma-Aldrich St. Louis, MO, USA). Integrated total dietary fibre assay kit (K-TDFR) and total starch test kit were from Megazyme (Megazyme International Ireland Ltd, Bray Ireland). Fluorescein (sodium salt, FL) and 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) were obtained from Cayman Chemical (Ann Arbor, Michigan, USA). Folin-Ciocalteu's reagent, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS•+) and most of the other solvents, as well as 96-well black-walled, clear-bottom polypropylene microplates (Nunc) were purchased from Thermo Fisher Scientific Australia (Scoresby Vic, Australia). UV-1800 spectrophotometer (Shimadzu, Canby, OR, USA) and a multi-detection micro-plate reader (BioTek Instruments, Inc., Winooski, VT)

were used. Ultrapure water was prepared by Arium ® pro Ultrapure Water Systems (Sartorius Stedim Biotech, Göttingen, Germany) and used in all experiments.

3.2.3. Determination of functional properties of lupin seed coat

Instrumental colour of whole seeds and the seed coat flour were determined by a HunterLab spectropolarimeter with the CIE L* (100 representing white; 0 representing black), a* (+ is red; - is green) and b* (+ is yellow; - is blue) colour space. Colour measurement was measured on average of six replicate samples. The colour difference (ΔE) before and after milling was calculated as the following equation,

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where ΔL*, Δa*and Δb* were the differences of L*, a* and b* value respectively.

Water-binding capacity (WBC) and oil-binding capacity (OBC) of the seed coat were evaluated in triplicate as described by Robertson, de Monredon, Dysseler, Guillon, Amado, and Thibault (2000). Briefly, 3 g of dried lupin seed coat flour and 30 mL ultrapure water or olive oil (density is around 0.915 g/cm^3) was added into a tared centrifuge tube and equilibrated at ambient temperature for 18 h. After that, the tube was centrifuged at 3,000 ×*g*, 25 °C for 20 min. The supernatant was discarded carefully, and the pellet was inverted and drained for 15 min in the tube before being weighed as fresh pellet weight. WBC/OBC was expressed as g water/ oil retained in the pellet per g of dry sample. The resulting pellet from WBC analysis was moved to a tared aluminium dish and dried to constant weight at 105 °C. Solubility in water (SOL) was expressed as a percentage of losing weight during soaking (Fuentes-Alventosa et al., 2009).

For determination of swelling capacity (SC), approximately 200 mg dry seed coat accurately weighed was hydrated in 10 mL ultrapure water in a calibrated cylinder (1.5 cm diameter) at ambient temperature (Robertson et al., 2000). The seed coat was dispersed with gentle stirring, and wrapped in foil, then equilibrated without stirring for 18 h. The bed volume (settled volume occupied by seed coat) was recorded. SC was expressed as volume/g original substrate dry weight.

Figure 3.2 Six modern genotypes of Australian sweet lupin (ASL) seeds grown at two locations and harvested in 2015 (Eradu, ER; Wongan Hills, WH)

3.2.4. Determination of proximate composition

Proximate composition analyses of the seed coat were carried out following standard methods (AOAC, 2011) in at least duplicate and reported as g/100 g dry basis (db). Briefly, moisture level was determined by 105 °C oven drying method to constant weight. Protein content was measured by the Kjeldahl digestion distillation method

using 5.40 as the conversion factor (Mosse, 1990). Lipid content was determined by the Soxhlet extraction distillation method using an E-816 extraction unit (Buchi Labortechnik AG, Postfach, Switzerland). Ash content was determined by furnace combustion at 550 °C over-night.

3.2.5. Determination of dietary fibre

Insoluble dietary fibre (IDF), dietary fibre soluble in water but precipitated in 95% aqueous ethanol (SDF) were determined principally based on an integrated enzymatic-gravimetric analysis procedure (AOAC 2009.01 & 2011.25) using Megazyme K-TDFR Kit (Megazyme International Ireland Ltd, Bray Ireland). Duplicate 1.0 g samples were accurately weighed and then digested by 40 mL of pancreatic α-amylase/amyloglucosidase (AMG) mixture (50 units/mL and 3.4 units/mL respectively in 50 mM, pH 6.0 sodium maleate buffer which contained 2 mmol/L $CaCl_2$) in a 37 °C water shaking bath for exactly 16 h. After that, pH of the solutions was immediately adjusted to approximately 8.2 by adding 3.0 mL of 0.75 M Tris base solution to terminate the reaction, followed by incubation in boiling water for 20 min. After cooled to 60 °C, the mixture was reacted with 0.1 mL of protease solution (350 tyrosine units/mL) at 60 °C for exactly 30 min. pH of enzymatically digested mixture was subsequently adjusted to approximately 4.3 by adding 4.0 mL of 2 mol/L acetic acid. The resulting solutions were filtered through tared Gooch crucibles which contained Celite to obtain IDF. The filtrate was precipitated for 60 min by adding 280 mL of 95% (v/v) ethanol which was pre-heated to 60 °C, followed by filtration to get SDF. All the residues from filtrations were washed by 15 portions of 78% (v/v) ethanol, 95% (v/v) ethanol and acetone subsequently, followed by drying to constant weight at 105 °C oven overnight. After weighted, the duplicates were used to correct protein and ash, respectively.

3.2.6. Extraction of free and bound phenolic compounds

Free polyphenols extraction: Polyphenols were extracted as described by our research team (Wu, Johnson, Bornman, Bennett, Clarke, et al., 2016). Duplicate 2 g samples of each ground lupin seed coat was mixed with 10 mL chilled 80% methanol (methanol/water, v/v) in a 15 mL tube with polyvinyl lined cap. After vortexed for 10 s, the tube was covered by aluminium foil and mixed by rotational-shaking for 2 h in

the dark followed by centrifugation at 3220 ×*g*, 4 °C for 10 min. The residue was extracted twice more with 7 mL of 80% methanol. The supernatants were then collected, pooled and made up to 25 mL with 80% methanol. Extracts were stored at -80 °C before free phenolics analysis (FP fraction).

Bound polyphenols extraction: The seed coat residue from free fraction extraction was transferred to 50 mL tube and mixed with 15 mL 2 mol/L NaOH. The mixture was vortexed for 10 s; then the tube was flushed with N_2, capped and sealed. After covered by aluminium foil and rotational-shaken for 2 h at ambient temperature, the mixture was acidified to pH 2 with 12 mol/L HCl. Alkaline hydrolysed previously-matrix bound phenolics (BP fraction) was extracted with 15 mL ethyl acetate, intermittently shaken for 10 min then centrifuged at 3220 ×*g*, 4 °C for 5 min. The organic layer was collected, and the extraction was repeated for 5 times. The combined extract was evaporated at 30 °C under N_2 to dryness. The extract was re-suspended with 10 mL of 100% methanol and stored at -80 °C before use.

3.2.7. Determination of total polyphenol content

For both free and bound fractions, total polyphenol content (TPC) was determined using Folin-Ciocalteu assay (Zhong et al., 2018). Briefly, 100 µL of each extract, gallic acid standards (10-100 mg/L) or 80% (vol/vol) methanol blank was mixed with 200 µL 0.2 N Folin-Ciocalteu phenol reagent and shaken vigorously. After stood for 5 min in the dark, 800 µL of Na_2CO_3 (5%) was then added followed mixed thoroughly and stood in the dark for another 90 min. 200 µL of the mixture was transferred into triplicate wells of a clear 96-well microplate. The absorbance of each well was read at 765 nm using a Synergy HT Multi-Detection Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA) (Ainsworth & Gillespie, 2007). The results were expressed as mg gallic acid equivalent (mg GAE/100 g db) per 100 g dry sample.

3.2.8. Total antioxidant capacity assays

Three assays were performed to measure the antioxidant capacities of both free and bound extracts, namely DPPH and ABTS• + free radical scavenging activity assay and oxygen radical absorbance capacity (ORAC) assay, as described previously (Zhong et al., 2018). Results were expressed as mg of Trolox equivalents (TE)/ 100 g db.

3.2.8.1.DPPH free radical scavenging activity

DPPH stock solution (1 mmol/L, 24 mg DPPH in 100 mL methanol) was prepared and stored at -20 °C in the dark until use (Wu, Johnson, Bornman, Bennett, Singh, & Fang, 2016). 5 mL of stock solution was freshly diluted with 45 mL methanol to obtain the DPPH working solution which has an absorbance of 1.1 ± 0.02 units at 517 nm (50 µmol/L, 0.02 mg/mL). 50 µL of each extract (in triplicate), Trolox standards (10 - 128 µmol/L) or 80% (vol/vol) methanol blank were reacted with 200 µL of 0.02 mg/mL DPPH solution, followed by maximum shaking for 10 s in the Microplate Reader. The plate with lid was kept in the reader for 60 min at 25 °C prior to the measurement of absorbance at 517 nm using a Synergy HT Multi-Detection Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA).

3.2.8.2.ABTS free radical scavenging activity

ABTS+ (7.4 mM) and potassium persulphate (2.6 mM) were mixed (1:1, vol/vol) and reacted for 12 h in the dark to prepare the fresh stock solution (Wu, Johnson, Bornman, Bennett, Singh, & Fang, 2016). The ABTS+ working solution was freshly prepared by diluting 2 mL of the stock solution with around 120 mL methanol to get an absorbance of 1.1 ± 0.02 units at 734 nm. 150 µL of the extracts, Trolox standards (10-200 mg/L) or 80% (vol/vol) methanol blank were mixed with 1.4 mL of the ABTS+ working solution in 3 mL centrifuge tubes and stood in the dark for 2 h. 200 µL of the mixture was immediately transferred into triplicate wells of a clear 96-well microplate. The absorbance was then determined at 515 nm on the microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

3.2.8.3.Oxygen radical absorbance capacity (ORAC)

The ORAC assay was performed in a 96-well black-walled, clear-bottom polypropylene microplate essentially as described by Huang, Ou, Hampsch-Woodill, Flanagan, and Prior (2002). The exterior wells on all sides were abandoned as there was much more variation from them than from the interior wells. 25 µL each of the two fractions, in triplicate, was mixed with 150 µL fluorescein (0.084 µmol/L) and incubated at 37 °C for 30 min. 25 µL of 153 mmol/L AAPH was added quickly to initiate the reaction followed by shaking at maximum intensity for 10 s. Fluorescence intensity at 37°C was monitored kinetically at 485 nm excitation and 528 nm emission

every minute in total 120 min using the microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The areas under the average fluorescence-reaction time kinetic curve (AUC) for both blank and samples were integrated.

3.2.9. Statistical analysis

All the results were reported on a dry basis and expressed as mean ± standard deviation ($n \geq 2$ unless otherwise specified). Two-way ANOVA was performed on IBM SPSS V25.0 (SPSS Inc., Chicago, IL, USA) to investigate the main effects of genotype (G), environment (E) and their interaction (G×E). Independent samples t-test was used to compare across different locations. $p < 0.05$ indicated significant difference. All analyses were performed on SPSS Statistics V22 (SPSS Inc., Chicago, Illinois, US). RStudio (Version 1.1.463) and R (Version 3.5.2, R Core Team, 2019) was employed to perform Pearson's correlation and principal component analyses (PCA) using "FactoMineR" and "ggplot2" packages. Means of dependent variables were used for PCA and correlation analyses.

3.3. Results and discussion

3.3.1. Lupin seed coat colour, seed weight and seed coat percentage

3.3.1.1. Lupin seed coat colour

Seed coat colour is genetically and environmentally determined and a commercially important indicator of maturity and qualities of the lupin seeds (Moïse et al., 2005). As shown in Figure 3.2, all the six varieties had similar speckled beige seed coats, but the colour values varied greatly among individuals within and across the two sites. Although location exhibited no significant effects on the L* values, genotypic effects and its interactive effects with the environment were significant (Table 3.1). In contrast, both site and genotype showed significant effects on the redness a* values, but the interaction was not significant. However, neither genotypic nor environmental factors showed significant effects on the yellowness of b* (Table 3.2).

The speckling of ASL seed coat develops soon after the seed reaches physiological maturity (largely early November in WA) (Dracup & Kirby, 1996). Black legume seed coat colour is controlled by several genes in the flavonoid pathway and affected by a

range of biotic and abiotic factors, being a response to diseases and insects for instance (Gulisano et al., 2019; Moïse et al., 2005; Oomah et al., 2006). Therefore, the darker seeds from WH could be explained by the higher temperature in November and December 2015 (Figure 3.1). Lignin and flavonoids (including anthocyanins, proanthocyanidins, and condensed tannins) were reported to contribute to pulse seed coat colour (Moïse et al., 2005). However, in contrast, ASL seed coats were reported to be low in both lignin (<2%) and condensed tannins (0.03 g/ 100 g, as catechin) (Brillouet et al., 1983; Eleonora Lampart-Szczapa et al., 2003).

In the present study, milling increased L* values significantly, and no significant difference was found among L* values of all milled seed coats. The spotted colour fragments of chickpea seed coat were reported to be mainly located at external palisade region of seed coats, and other parts of seed coat are less stained but have homogeneous light yellow (Wood et al., 2011). On this basis, the increases in the lightness of the milled lupin seed coats may be attributed to the increased proportion of un-pigmented or light colour particles from internal parts of the coats.

3.3.1.2. Lupin seed weight and seed coat percentage

All of the six ASL varieties investigated are high yielding across most lupin growing areas of Western Australia (WA), with PBA Jurien being the highest. However, Coromup is the most popular variety due to its highest protein content. As shown in Figure 3.2, the average seed size of the varieties was Coromup> Jenabillup> PBA Jurien> Mandelup> PBA Barlock> PBA Gunyidi. This was further supported by the seed weight ranking within both two sites (Table 3.3). Both genotype and environment, as well as their interaction, showed significant effects on seed weight (Table 3.2). Across the two grown locations, seed weights of all the six ASL varieties from ER were significantly higher than those from WH. The lower temperature and rainfall during sowing (middle of April to middle of May), and lower temperature during flowering (July) but consistently higher temperature (>30 °C) during seed development (October) at WH may have reduced the accumulation of biomass and thus weight of individual seeds (Dracup et al., 1996).

Lupins are characterised by their higher percentage of seed coat as compared to other pulses, with about 24% dry basis in ASL (Section 2.1). In this work, the seed coats of

the six ASL varieties comprised 19.74 to 23.93% of total seed dry mass (Table 3.3), which is in the range of the existing data (19.4-38.8%) (Table 2.2). Seed coat percentage in 944 ASL varieties that were grown in WA was reported to range from 19.4 to 38.8 % (Clements et al., 2005 & 2002). The authors also revealed a negative relationship between seed weight and seed coat proportion, suggesting that large seeds have reduced seed coat thickness and seed coat percentage (Clements et al., 2005). Similarly, a significant albeit weak negative correlation (r = -0.38, p = 0.022) between seed weight and seed coat percentage was found in the current study. According to the univariate analyses, genotype, environment and their interaction showed significant effects on seed coat percentage (Table 3.2). The results are comparable with those reported by Clements et al. (2002), who also observed that seed coat thickness was positively correlated to grow-season rainfall. However due to the differences dehulling efficiency of lab-scale and commercial equipment, commercial ASL millers were indicated to produce a higher level of milling loss at around 31% (Sipsas, 2008).

3.3.2. Lupin seed coat physicochemical characteristics

Results of water solubility (SOL), water binding capacity (WBC), oil binding capacity (OBC) and swelling capacity (SC) are presented in Table 3.3. The univariate analyses revealed that genotype was the determining factor for SOL, with the effects of the environment being nonsignificant (Table 3.2). In contrast, genotypic, environmental effect and their interaction were significant on WBC. In fact, values of WBC may be affected by the lost soluble fractions that were discarded after centrifugation (Robertson et al., 2000). Regarding OBC and SC, effects of all the investigated factors were not significant.

Moreover, both OBC and SC were relatively constant independent of varieties and locations. However, WBC levels of ASL seed coats were lower than those reported by Pfoertner et al. (2001) (7-8 g/g). The current WBC of lupin seed coat was similar to those of lentil and mung bean seed coat but lower than field pea (4.0-7.1 g/g) and chickpea (6.2 g/g) (Table 2.4). The variability in reported WBC may be a result of differences in seed coat composition, particle size, and milling process (Robertson et al., 2000). The range of the OBC values was less than other legume seed coats (1.4-1.9 g/g) (Table 2.4). As anticipated, the values of SC were low, ranging from 4.47 to

5.07 mL/g db, but were consistent with the existing data of other legume seed coats (Table 2.4).

3.3.3. Proximate composition

The moisture content of ASL seed decreases sharply from around 60% at physiological maturity to 12-14% of harvest maturity (Dracup et al., 1996). However, to compare their properties, all seed coats were pre-dried to achieve similar moisture of around 5% before milling. The proximate composition of seed coats of some earlier ASL cultivars were found that the seed coats contain 9-11% moisture, 3-6.6% protein, around 1.5% lipid, 2-3% ash and 80-90% non-starch polysaccharides (NSP) (Bailey et al., 1974; Evans et al., 1993; Lampart-Szczapa et al., 2003). In the present study, the proximate composition of the six modern cultivars was relatively consistent (Table 3.4). Ash content varied from 2.81 to 3.01 g/100g db, in agreement with the reported results above. However, the fat and protein content in this study, ranging from 1.62 to 2.42 g/100g db and 6.34 to 8.59 g/100g db (N×5.40) respectively. The protein results here were slightly higher as compared to those reported (4.6 to 6.7% after adjusting N conversion factor from 6.25 to 5.40) (Evans et al., 1993; Lampart-Szczapa et al., 2003). As noted previously, the lab-based dehulling procedure used in this study produced small cotyledon broken and embryonic axes, both of which could not be removed manually and may have contributed to the high fat and protein values. As summarised in Table 3.2, genotypic, environmental and their interactive effects on ash, fat and protein content in lupin seed coat were significant, with PBA Gunyidi and Mandelup showing higher ash, fat and protein levels. Besides, significant correlations ($p < 0.01$) among ash, fat and protein levels were observed and all Pearson's correlation coefficients were higher than 0.527.

Table 3.1 Lupin seed coat colour

			PBA Jurien	Coromup	PBA Gunyidi	Mandelup	PBA Barlock	Jenabillup
Intact seed	L*	ER	66.12±0.58^{bA}	66.26±1.88^{bA}	57.89±0.08^{aA}	60.72±0.66^{aA}	59.05±0.37^{aA}	67.19±0.94^{bA}
		WH	69.83±0.63^{bA}	66.04±0.44^{bA}	60.80±0.89^{aB}	60.61±0.73^{aA}	59.70±0.51^{aA}	64.34±1.56^{bB}
	a*	ER	4.23±0.37^{aA}	5.19±0.50^{abcA}	5.85±0.61^{cA}	5.33±0.60^{bcA}	5.82±0.31^{cA}	4.58±0.54^{abA}
		WH	4.67±0.43^{aB}	5.67±0.31^{aA}	5.83±0.30^{aA}	5.34±0.78^{aA}	5.77±0.18^{aA}	5.49±0.39^{aA}
	b*	ER	12.67±0.55^{aA}	13.69±0.28^{aA}	12.68±0.56^{aA}	12.48±0.86^{aA}	13.32±0.47^{aA}	12.73±0.55^{aA}
		WH	13.01±0.57^{aA}	13.44±0.41^{aA}	13.26±0.75^{aA}	12.45±1.43^{aA}	13.48±0.47^{aA}	13.44±0.43^{aA}
Seed coat flour	L*	ER	81.56±0.89^{aA}	80.66±0.28^{aA}	79.85±0.64^{aA}	79.80±0.53^{aA}	80.63±0.51^{aA}	82.35±1.71^{aA}
		WH	81.40±1.44^{aA}	80.83±1.31^{aA}	80.47±1.15^{aA}	80.68±1.32^{aA}	80.38±1.74^{aA}	81.99±0.99^{aA}
	a*	ER	2.41±0.26^{aA}	3.03±0.07^{bcA}	2.93±0.19^{bcA}	2.86±0.05^{abcA}	2.57±0.06^{abA}	3.26±0.20^{cA}
		WH	2.53±0.17^{aA}	2.78±0.39^{aA}	2.60±0.24^{aA}	2.20±0.15^{aB}	2.18±0.13^{aB}	2.50±0.35^{aB}
	b*	ER	16.45±0.44^{aA}	17.31±0.14^{abA}	17.98±0.56^{bcA}	18.57±0.50^{cA}	18.24±0.27^{bcA}	19.09±0.46^{cA}
		WH	18.30±0.44^{aB}	18.66±0.56^{aB}	17.71±0.68^{aA}	17.68±0.34^{aA}	17.56±0.26^{aA}	17.92±0.20^{aB}
ΔE		ER	16.00	15.00	22.78	20.18	22.36	16.49
		WH	12.90	15.94	20.42	20.98	21.38	18.45

Means ± standard deviation (n = 6).

ER, Eradu; WH, Wongan Hills.

Means assigned with different small letters in the same row, and capital letters in the same column within each dependent variable indicate significant differences ($p < 0$ .05).

Table 3.2 Effects of genotype (G), environment (E) and their interaction (G×E) presented as p-values from analysis of variance (ANOVA)

Sees coat properties	*p*-value											
Physicochemical properties												
	Seed weight	% Hull	L*	a*	b*	SOL	WBC	OBC	SC			
Genotype	<0.0001	<0.0001	<0.0001	<0.001	0.339	<0.0001	0.025	0.999	0.182			
Environment	<0.0001	<0.0001	0.443	0.010	0.173	0.825	0.006	0.798	0.407			
G×E	0.011	<0.0001	<0.0001	0.235	0.963	0.038	<0.0001	0.820	0.070			
Proximate composition and dietary fibre												
	Ash	Fat	Protein	IDF	SDF	TDF						
Genotype	<0.0001	<0.0001	<0.0001	<0.0001	<0.01	<0.0001						
Environment	<0.001	<0.01	0.011	<0.0001	<0.01	<0.0001						
G×E	<0.01	<0.0001	<0.0001	<0.001	0.311	<0.01						
TPC and antioxidant capacity												
	FTPC†	BTPC‡	TTPC§	FDPPH†	BDPPH‡	TDPPH§	FABTS†	BABTS‡	TABTS§	FORAC†	BORAC‡	TORAC§
Genotype	<0.0001	<0.0001	<0.0001	<0.01	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Environment	<0.0001	<0.0001	<0.0001	<0.0001	<0.01	<0.0001	<0.0001	<0.0001	<0.0001	0.010	<0.01	<0.01
G×E	<0.001	0.023	<0.0001	0.031	0.473	0.013	<0.01	0.015	<0.01	0.341	0.450	0.278

SOL, water solubility; WBC, water binding capacity; OBC, oil binding capacity; SC, Swelling capacity; IDF, insoluble dietary fibre; SDF, soluble dietary fibre; TDF, total dietary fibre.

† total polyphenol content (TPC) and antioxidant capacities (DPPH, ABTS, ORAC) in free fractions; ‡ TPC and antioxidant capacities in bound fractions; § total TPC and antioxidant capacities.

Table 3.3 Seed weight (mg, db), seed coat percentage (%, db) and functional properties of lupin seed coats

		PBA Jurien	Coromup	PBA Gunyidi	Mandelup	PBA Barlock	Jenabillup
Seed weight	ER	136.38±0.27^{cA}	144.88±1.18^{dA}	115.44±2.34^{aA}	130.48±3.17^{bA}	130.28±2.51^{bA}	142.53±1.72^{dA}
	WH	117.08±1.61^{bB}	120.78±1.74^{bB}	103.95±2.21^{aB}	112.33±4.51^{abB}	106.38±1.65^{aB}	119.41±2.43^{bB}
Seed coat	ER	22.54±0.17^{aA}	23.04±0.22^{bA}	22.92±0.38^{eA}	23.92±0.01^{dA}	23.93±0.11^{dA}	23.27±0.14^{cA}
	WH	22.62±0.13^{aA}	19.74±0.02^{aB}	22.19±0.50^{dA}	22.45±0.26^{bcB}	22.41±0.14^{cdA}	21.94±0.35^{bA}
SOL	ER	6.06±0.01^{aA}	7.67±0.27^{bA}	6.92±0.03^{bA}	7.23±0.32^{bA}	7.28±0.12^{bA}	7.42±0.30^{bA}
	WH	5.62±0.06^{aB}	7.34±0.05^{bA}	7.40±0.18^{bA}	8.06±0.54^{bA}	7.25±0.01^{bA}	7.08±0.57^{bA}
WBC	ER	3.40±0.02^{aA}	3.74±0.04^{bdA}	3.63±0.04^{cdA}	3.59±0.00^{cA}	3.80±0.03^{bA}	3.74±0.05^{bdA}
	WH	3.72±0.05^{aB}	3.48±0.02^{bcB}	3.64±0.02^{acA}	3.71±0.11^{acA}	3.56±0.05^{abcB}	3.39±0.06^{bB}
OBC	ER	0.73±0.09^{aA}	0.74±0.06^{aA}	0.67±0.01^{aA}	0.68±0.00^{aA}	0.71±0.07^{aA}	0.69±0.01^{aA}
	WH	0.68±0.01^{aA}	0.68±0.03^{aA}	0.71±0.00^{aA}	0.70±0.16^{aA}	0.69±0.02^{aA}	0.71±0.01^{aA}
SC	ER	4.80±0.02^{aA}	4.92±0.10^{aA}	4.79±0.02^{aA}	4.81±0.23^{aA}	5.06±0.12^{aA}	5.05±0.13^{aA}
	WH	5.07±0.04^{aB}	4.88±0.01^{aA}	4.84±0.00^{aA}	4.75±0.03^{aA}	5.07±0.11^{aA}	4.47±0.49^{aA}

ER, Eradu; WH, Wongan Hills.

SOL, water solubility (% db); WBC, water binding capacity (g/g db); OBC, oil binding capacity (g/g db); SC, Swelling capacity (mL/g db).

Means assigned with different small letters in the same row, and capital letters in the same column within each dependent variable indicate significant differences ($p < 0.05$).

3.3.4. Dietary fibre

Dietary fibre (DF) is the major component of ASL seed coat (Section 2.3). DF of lupin seed coat mainly consists of cellulose (representing from 57% to 87%), arabinoxylan hemicelluloses (~13%) and pectins (Brillouet et al., 1983; Evans et al., 1993; Miao, Fortune, & Gallagher, 2001). As shown in Table 3.4, all the lupin seed coats had a high content of total dietary fibre (TDF), accounting for 79.84 to 86.59 g/100 g db which primarily consisted of IDF. Similarly, it was reported that only 3.5% of TDF is soluble in the lupin seed coat (KohaJdoVá, Karovicova, & Schmidt, 2011). The results were also in accordance with the previous studies, which SDF comprised of less than 3.5% of TDF (Section 2.3.1). However, a higher IDF were reported by Evans et al. (1993), 88.4-90.9 g/ 100g db, and consequently higher TDF, which could be explained by the different dehulling methods as discussed earlier.

From the results of univariate analyses, both genotypic and environmental factors, together with their interaction, contributed to the observed differences in the levels of IDF and TDF (Table 3.2). With respect to SDF, despite the non-significant G × E interaction, genotype and environment were significant determinates of SDF (Table 3.2). However, except PBA Jurien and PBA Gunyidi, IDF, SDF and TDF of ASL seed coats from the two sites were similar ($p > 0.05$). In contrast, the high content of fibre was found in the seed coat of wild lupins and lupins grown in dry conditions (Miao et al., 2001).

3.3.5. Total polyphenol content and antioxidant capacity

3.3.5.1. Total polyphenol content

As presented in Table 3.5, the range of total polyphenol content in free fraction (FTPC) varied from 57.24 mg gallic acid equivalents (GAE)/ 100 g db in PBA Jurien to 93.52 mg GAE/ 100 g db in PBA Barlock, both of which were from WH. Moreover, according to the combined analysis of variance analysis, significant effects of environment and genotype and their interaction on FTPC values were detected (Table 3.2). Seed coat FTPC values of seeds from WH were higher than those from ER. In addition, among the six varieties, PBA Jurien was consistently and significantly the lowest in FTPC across the two locations (Table 3.5).

Regarding bound polyphenols, the ASL varieties showed a range in total bound polyphenols (BTPC) from 16.71 mg GAE/ 100 g db in PBA Jurien (ER) to 34.78 mg GAE/100g db in PBA Barlock (WH) (Table 3.5). BTPC accounting for up to 27.15% of the total phenolics in the lupin seed coats. By examining the results of the analysis of variance for BTPC, the main effects of genotype and site dominated the variation, but the genotype × environment was also a significant albeit weak source (Table 3.2). Additionally, ANOVA analyses revealed that BTPC in seed coats of the seeds grown at ER was significantly lower than those from WH. Similar to the pattern in FTPC, the highest BTPC were found in PBA Gunyidi within both two locations while the values in PBA Jurien were the lowest (Table 3.2). Collectively, highly significant effects of the genotype, site and their interaction (G×E) were detected in explaining the variations among TPC (FTPC+BTPC) levels (Table 3.2). Besides, PBA Barlock (WH) contained the highest TPC among the varieties over the two sites, followed by PBA Gunyidi and Mandelup, with PBA Jurien being the lowest (Table 3.5).

Total phenolic content in the seed coats of ASL cultivars was reported to be 18.4-28.8 mg caffeic acid equivalents /100 g (Lampart-Szczapa et al., 2003) and 106 mg catechin equivalents/100 g fresh weight (Ranilla et al., 2009). Since the authors did not investigate bound polyphenols, the results largely equate to free TPC (FTPC) of the current study. Of particular interest, FTPC and BTPC were significantly and negatively correlated with L* values, suggesting that darker seeds contained higher FTPC, BTPC and thus TPC (Table 3.6; Section 2.2.1 and Section 2.3.3). Polyphenols, principally flavonoids, play a key role as protectants adopted by plants to respond to various environmental stresses, serving as UV-B attenuators in photoprotection, inhibiting oxidation and improving osmotic under exposure to thermal stress (Agati, Azzarello, Pollastri, & Tattini, 2012). As discussed earlier, genes in the flavonoid pathway along with environmental factors significantly affect the biosynthesis and accumulation of polyphenols in the epidermal layer of the seed coat (Moïse et al., 2005). Against this, the higher temperature during seed physiological maturity period (November and December 2015) in WH may benefit to the build-up of phenolics in the lupin seed coats, reflecting as higher values in FTPC, BTPC and TPC.

Table 3.4 Proximate compositions and the dietary fibre content of lupin seed coats (g/100 g db)

		PBA Jurien	Coromup	PBA Gunyidi	Mandelup	PBA Barlock	Jenabillup
Ash	ER	2.92± 0.02^{aA}	2.93± 0.02^{abA}	3.01± 0.01^{bA}	2.89± 0.03^{aA}	2.93± 0.02^{abA}	2.88± 0.02^{aA}
	WH	2.81± 0.01^{aB}	2.96± 0.03^{dA}	2.95± 0.01^{dB}	2.86± 0.01^{bcA}	2.90± 0.00^{cA}	2.85± 0.02^{abA}
Fat	ER	1.83±0.01^{aA}	2.13±0.04^{cA}	2.28±0.06^{dA}	1.85±0.00^{abA}	1.96±0.01^{bA}	1.94±0.01^{abA}
	WH	1.67±0.04^{aB}	2.41±0.08^{dB}	1.77±0.00^{abB}	2.15±0.02^{cB}	1.97±0.09^{bcA}	1.62±0.03^{aA}
Protein	ER	8.09± 0.12^{aA}	8.31± 0.07^{bA}	8.29± 0.00^{bA}	8.23± 0.04^{bA}	7.75± 0.06^{cA}	7.65± 0.05^{cA}
	WH	6.34± 0.01^{aB}	8.59± 0.01^{bB}	8.35± 0.08^{cA}	8.44± 0.18^{bcA}	8.06± 0.03^{bcB}	8.02± 0.01^{cB}
IDF	ER	80.36±0.59^{aA}	78.51±0.04^{abA}	78.64±0.13^{abA}	76.95±0.15^{bA}	79.32±0.75^{abA}	79.79±0.04^{abA}
	WH	83.30±0.01^{aB}	78.91±0.01^{bA}	81.13±0.02^{cB}	77.46±0.14^{dA}	79.72±0.19^{bcA}	80.68±0.44^{abA}
SDF	ER	2.97±0.31^{aA}	2.74±0.17^{aA}	2.79±0.23^{aA}	3.31±0.18^{aA}	3.17±0.29^{aA}	2.86±0.05^{aA}
	WH	3.22±0.24^{aA}	3.42±0.28^{aA}	2.96±0.04^{aA}	3.76±0.09^{aA}	3.25±0.19^{aA}	3.00±0.04^{aA}
TDF	ER	83.33±0.90^{aA}	81.24±0.21^{abA}	81.43±0.10^{abA}	80.26±0.33^{bA}	82.49±1.04^{abA}	82.65±0.01^{abA}
	WH	86.52±0.23^{aB}	82.33±0.27^{bcA}	84.09±0.02^{cB}	81.22±0.05^{bB}	82.97±0.37^{bcA}	83.67±0.47^{cA}

ER, Eradu; WH, Wongan Hills.

IDF, insoluble dietary fibre (g/100 g db); SDF, soluble dietary fibre (g/100 g db); TDF, total dietary fibre (g/100 g db).

Means assigned with different small letters in the same row and capital letters in the same column within each dependent variable indicate significant differences ($p < 0$ .05).

Table 3.5 Total phenolics content (mg GAE/100 g db) and antioxidant capacities (mg TE/100 g db)

			PBA Jurien	Coromup	PBA Gunyidi	Mandelup	PBA Barlock	Jenabillup
TPC	Free	ER	60.10±1.13^{aA}	82.02±4.22^{bA}	82.17±2.17^{bA}	77.72±0.25^{bA}	74.23±3.40^{bA}	84.11±3.13^{bA}
		WH	57.24±0.20^{aA}	86.07±3.61^{bA}	93.24±1.98^{bB}	91.82±1.29^{bB}	93.52±0.79^{bB}	89.94±1.36^{bA}
	Bound	ER	8.23±1.54^{aA}	10.36±1.43^{abA}	21.76±1.08^{dA}	14.25±2.01^{abcA}	20.46±0.89^{cdA}	16.01±2.60^{bcdA}
		WH	16.71±2.23^{aB}	20.54±0.92^{aB}	34.75±0.46^{bB}	30.23±0.18^{bB}	34.78±1.50^{bB}	31.08±0.68^{bB}
	Total	ER	68.34±0.41^{aA}	92.38±2.79^{bA}	103.93±1.09^{bA}	91.97±2.26^{bA}	94.68±4.29^{bA}	100.13±5.73^{bA}
		WH	73.94±2.43^{aA}	106.61±2.69^{bB}	127.99±2.44^{cB}	122.04±1.11^{cB}	128.31±0.71^{cB}	121.02±0.67^{cB}
DPPH	Free	ER	23.96±0.35^{aA}	25.97±1.39^{abA}	27.29±0.02^{abA}	29.82±1.89^{bA}	27.16±0.92^{abA}	24.87±1.01^{aA}
		WH	25.04±0.79^{aA}	31.78±2.70^{abA}	36.59±2.33^{bB}	32.26±0.25^{abA}	34.58±1.03^{bB}	33.35±4.00^{abA}
	Bound	ER	9.01±0.21^{aA}	10.88±1.58^{abA}	13.71±0.82^{bA}	10.23±0.15^{aA}	11.08±0.24^{abA}	9.22±0.84^{aA}
		WH	9.06±0.40^{aA}	10.98±0.81^{abA}	14.50±0.84^{dA}	12.03±0.04^{bcB}	14.03±0.18^{cdB}	11.65±0.49^{bA}
	Total	ER	33.22±0.32^{aA}	36.85±0.19^{abA}	41.00±0.80^{cA}	40.05±1.74^{bcA}	38.24±1.17^{bcA}	34.09±0.16^{aA}
		WH	34.09±0.39^{aA}	42.76±3.52^{abA}	51.09±1.49^{bB}	44.29±0.29^{bA}	48.61±1.21^{bB}	45.00±4.49^{bA}
ABTS	Free	ER	28.14±1.19^{aA}	35.73±0.10^{bA}	37.65±3.07^{bA}	28.91±0.38^{aA}	37.48±1.24^{bA}	32.00±0.49^{abA}
		WH	33.98±0.85^{aB}	39.53±0.94^{abB}	52.13±0.49^{cB}	46.12±0.07^{bcB}	51.16±0.33^{cB}	37.60±3.11^{abA}
	Bound	ER	17.73±1.06^{abA}	15.69±1.71^{aA}	26.44±1.48^{dA}	20.57±1.53^{bcA}	24.29±0.68^{cdA}	21.07±0.52^{bcA}
		WH	18.64±0.67^{aA}	23.24±0.69^{bB}	28.58±1.52^{cA}	23.05±0.60^{bA}	25.80±1.32^{bcA}	22.42±1.00^{abA}
	Total	ER	45.37±2.07^{aA}	51.42±1.82^{aA}	64.10±4.56^{cA}	49.48±1.91^{aA}	61.78±0.55^{bcA}	53.06±1.01^{abA}
		WH	52.62±0.18^{aB}	62.78±0.24^{bcB}	80.71±2.00^{eB}	69.17±0.67^{cdB}	76.97±0.99^{deB}	60.03±4.67^{abA}

			PBA Jurien	Coromup	PBA Gunyidi	Mandelup	PBA Barlock	Jenabillup
ORAC	Free	ER	354.48±13.33^{aA}	589.03±5.89^{bcA}	554.25±36.20^{bcA}	548.69±7.35^{bcA}	632.00±42.93^{cA}	508.16±26.87^{bA}
		WH	429.79±39.48^{aA}	600.42±36.11^{bcA}	586.44±15.43^{bcA}	549.14±21.43^{abA}	688.65±9.69^{cA}	577.67±46.57^{bcA}
	Bound	ER	114.73±2.63^{aA}	158.81±8.12^{abA}	225.69±4.56^{cA}	193.67±10.53^{bcA}	216.43±10.85^{bcA}	161.54±31.48^{abA}
		WH	131.83±3.42^{aB}	178.08±7.36^{abA}	224.29±15.43^{cdA}	211.83±3.98^{cdA}	262.15±21.72^{dA}	180.28±11.01^{abA}
	Total	ER	469.21±10.70^{aA}	747.84±2.23^{bcA}	779.94±40.76^{cdA}	742.36±17.88^{bcA}	848.43±32.08^{dA}	669.70±4.61^{bA}
		WH	561.62±36.06^{aA}	778.49±43.48^{bcA}	788.25±57.98^{bcA}	760.98±25.41^{bA}	950.79±31.41^{cA}	757.95±57.58^{bA}

ER, Eradu; WH, Wongan Hills.

Means assigned with different small letters in the same row and capital letters in the same column within each dependent variable indicate significant differences ($p < 0$ .05).

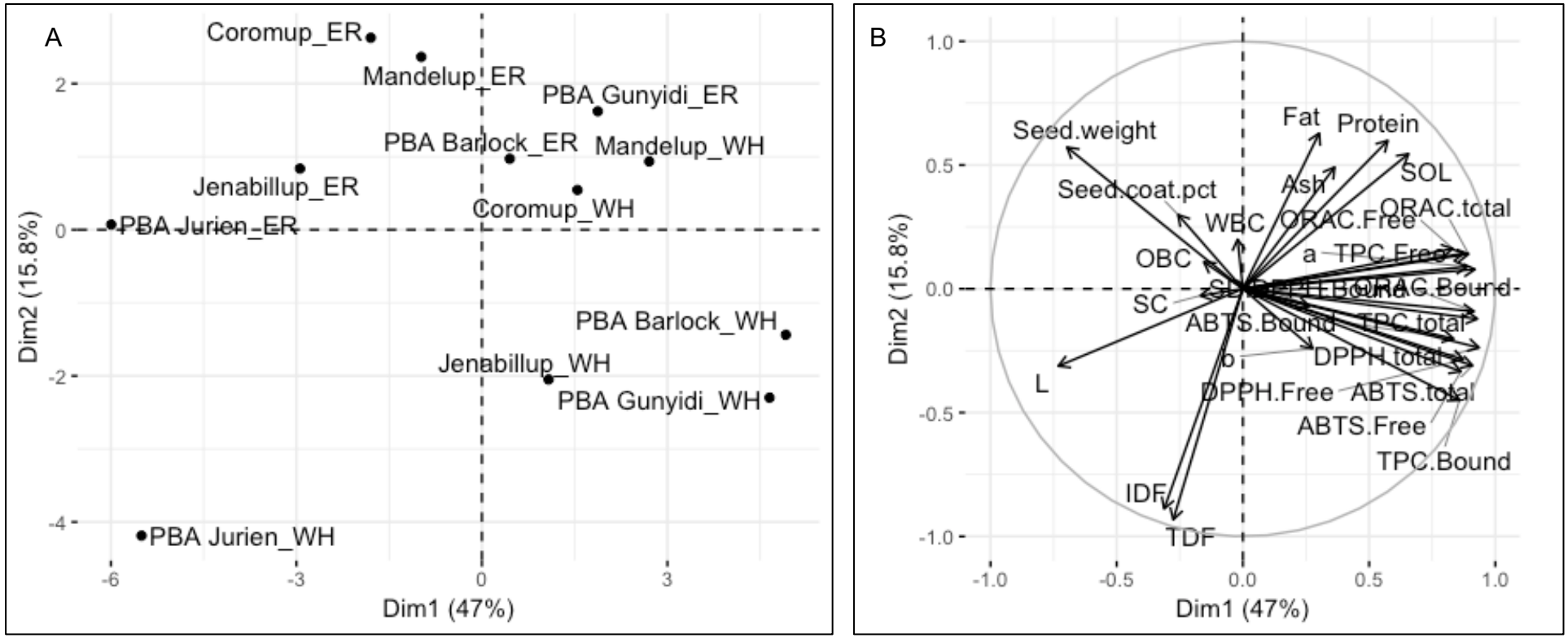

Figure 3.3 Scores (A) and loadings (B) of principal component analysis of the physicochemical and compositional properties for six lupin cultivars grown at two locations.

3.3.5.2.Antioxidant capacity assays (AOXs)

The antioxidant activities of the lupin seed coats were investigated using three assays, DPPH, ABTS and ORAC. Both DPPH and ABTS are electron transfer based assays which are similar to TPC assay, while ORAC is a hydrogen atom transfer-based assay which uses kinetic quantitation (Niki, 2010). As presented in Table 3.5, the values of free DPPH varied from 23.96 to 36.59 mg TE/ 100 g db. Previously, the antioxidant capacity of seed coat of a Brazilian ASL cultivar was demonstrated to be 13 mg TE/100 g fresh weight using DPPH assay, which equivalent to free DPPH in the current study (Ranilla et al., 2009). Besides, the results of DPPH and ABTS assays were significantly affected by genotype, environment and G×E interaction, whereas G×E interaction was nonsignificant on ORAC levels despite the significant effects of genotype and site (Table 3.2). Similar to the trend of TPC, seed coat of the lupin seeds from WH had significantly higher in both free and bound AOXs than ER.

3.3.5.3.Pearson correlation among L, TPCs and AOXs*

Pearson's correlations were conducted to determine relationships among L* values, TPC and AOXs. As shown in Table 3.6, the AOXs of both free and bound fractions were significantly and positively correlated with the corresponding TPCs (FTPC, BTPC and TPC) values ($p < 0.01$) highlighting that phenolics are the major contributors to the antioxidant activity (Jacobo-Velazquez & Cisneros-Zevallos, 2009). Of note, both TPCs and AOXs, showed high negative correlations with L* values of the seeds, implying the darker seeds generally had higher levels of polyphenols (bound polyphenols of particular) and therefore higher AOXs.

3.3.6. Principal component analysis

In this study, 44 responses of 12 samples were analysed, consequently generated a large amount of data (528 data points). To reduce the dimensionality of the data and visualize potential differences attributed to site, principal component analysis (PCA) was conducted. Figure 3.3 shows PCA scores plot and factor loadings pilot. The first two principal components (PC1, PC2) were detected to explain 46.96% and 15.83% of total variation in the data set respectively. PC1 differentiates the seed coat samples based on contents of TPC (free, bound and total), levels of all the three antioxidant

capacity assays (free, bound and total), a*, L*, SOL and seed weight (factor loadings > 0.60) (Granato, Santos, Escher, Ferreira, & Maggio, 2018). In contrast, PC2 separates the samples according to contents of IDF, TDF, fat and protein. Taken together, samples harvested from ER were clearly separated from those originating from WH, implying the environmental effects as discussed earlier (Section 3.3.1-3.3.5). Notably, PBA Jurien from both sites were distanced from others and located at left of the plot which was determined by the low TPC and AOXs values but high IDF and TDF content in PBA Jurien.

Table 3.6 Pearson's correlation coefficients (*r*) among L*, antioxidant activities and total phenolics values of free and bound phenolics fractions

Trait	TPC	DPPH	ABTS	ORAC
Free fractions				
DPPH	0.745**			
ABTS	0.696**	0.788**		
ORAC	0.718**	0.522**	0.557 **	
L* value†	-0.423*	-0.473*	-0.462*	-0.472*
Bound fractions				
DPPH	0.783**			
ABTS	0.794**	0.781**		
ORAC	0.741**	0.818**	0.822**	
L* value†	-0.504*	-0.691**	-0.713**	-0.798**

† lightness of intact seeds;

*, ** Significant at 0.05 and 0.01, respectively.

3.4. Conclusions

This chapter examines physicochemical, nutritional properties and antioxidant capacities of seed coats of six new Australian sweet lupin (ASL) cultivars grown in two locations in Western Australia. Although great genotypic and environmental variations are found in those investigated properties, contents of IDF, TDF, fat and protein ect., the results support that ASL seed coats can be used as a natural "antioxidant dietary fibre" in human food products. Besides, given the genotypic and environmental effects on ASL seed coat properties, this study suggests that variety and growing location-based utilisation must be of interest. For example, it appears feasible to select specific genotypes and production environments to further improve the

nutritional and functional properties of ASL seed coats including increased levels of polyphenols and higher antioxidant capacities.

However, the methods used in this chapter to determine polyphenol content is not specific. Many non-phenolic reducing compounds (like vitamins, monosaccharides, oligosaccharides, amino acids and proteins) from fibre-based by-products can interfere the Folin-Ciocalteu assay (Everette, Bryant, Green, Abbey, Wangila, & Walker, 2010; Max, Salgado, Cortes, & Dominguez, 2010; Torre, Aliakbarian, Rivas, Domingue, & Converti, 2008). Therefore, the following chapter will employ HPLC-DAD-ESI-MS/MS, more advanced and accurate method, to identify and quantify individual polyphenols in the seed coats.

CHAPTER 4

Characterization of polyphenols in Australian sweet lupin (*Lupinus angustifolius*) seed coat by HPLC-DAD-ESI-MS/MS

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Zhong, L., Wu, G., Fang, Z., Wahlqvist, M. L., Hodgson, J. M., Clarke, M. W., Junaldi, E., & Johnson, S. K. (2019). Characterization of polyphenols in Australian sweet lupin (*Lupinus angustifolius*) seed coat by HPLC-DAD-ESI-MS/MS. *Food Research International*, 116, 1153-1162.

ABSTRACT

In this chapter, seed coats of six ASL commercial varieties from two growing sites were sampled for identification and quantification of polyphenols using a high-performance liquid chromatography (HPLC) with diode array detector (DAD) and coupled with a triple quadrupole mass spectrometer which equipped with electrospray ionization source (ESI-MS/MS). Three flavones (apigenin-7-*O*-β-apiofuranosyl-6,8-di-*C*-β-glucopyranoside, vicenin 2, and apigenin-7-*O*-β-glucopyranoside), one isoflavone (genistein) and one dihydroflavonol derivative (aromadendrin-6-*C*-β-D-glucopyranosyl-7-*O*-[β-D-apiofuranosyl-(1→2)]-*O*-β-D-glucopyranoside), and several hydroxybenzoic and hydroxycinnamic acid derivatives were identified. Considerable variations in levels of individual polyphenols were found but apigenin-7-*O*-β-apiofuranosyl-6,8-di-*C*-β-glucopyranoside was the predominant polyphenol in all samples accounting for 73.08 - 82.89 % of the total free polyphenols. These results suggest that ASL seed coat could be a valuable dietary source of polyphenols.

4.1. Introduction

Lupin seeds are increasingly recognised for their high protein (around 40%), and uniquely high dietary fibre (about 30%) but low fat and negligible starch (Johnson et

al., 2017). As aforementioned, modern lupins are also welcomed for their low several "anti-nutritional factors" (e.g. alkaloids, phytic acid, saponins, lectins, and trypsin inhibitors) (Section 2.3). Recently, however, many secondary metabolites have shown multiple health benefits, with polyphenols being of particular interest because of their well demonstrated physiological benefits and thus have been more widely investigated (Rochfort et al., 2007). In this respect, numerous studies have revealed high levels of polyphenols in whole lupin seeds, and suggested that polyphenols are the main primarily contributor to their antioxidant capacity (Johnson et al., 2017; Khan, Karnpanit, Nasar-Abbas, Zill-e-Huma, & Jayasena, 2015). As summarised by Khan et al. (2015), the main groups of lupin polyphenols are phenolic acids and flavonoid derivatives, which are mostly occur as phenolic compounds conjugate with mono-, di- and oligosaccharides. Particularly, few new flavone *C*-glucosides are uniquely found in lupins (Kamel, 2003; Ranilla et al., 2009; Siger, Czubinski, Kachlicki, Dwiecki, Lampart-Szczapa, & Nogala-Kalucka, 2012). The results can provide new strategies to value add to lupin seed coat.

The seed coat of pulses, including chickpea (Sreerama, Neelam, et al., 2010), faba bean (Boudjou et al., 2013), field pea (Marles et al., 2013), lentil (Oomah, Caspar, et al., 2011), and mung bean (Luo et al., 2016), is the predominant contributor of polyphenols to the whole seeds, and showed distinct phenolic composition compared to the kernels (Section 2.3.3.1). Additionally, significantly higher polyphenol levels are found in dark coloured (black and red) chickpea and lentil seeds than those in lighter coloured (white and beige) counterparts, indicating the genotypic effects on seed coat polyphenols (Xu et al., 2007). In contrast, the total polyphenol content, which based on Folin-Ciocalteu assay, in seed coats of *L. mutabilis*, *L. albus*, and *L. angustifolius* grown in Brazil is reported to be 1.15 - 4.49 mg catechin equivalents (CE)/g dry basis which is much lower than in cotyledons (7.38 - 12.42 mg CE/g db) (Ranilla et al., 2009). As mentioned in the preceding chapter, high level of total polyphenol content (68.34 - 121.02 mg gallic acid equivalents /100g db) was found in seed coats of all the six ASL varieties. However, the non-specific properties of Folin-Ciocalteu assay on polyphenol quantification and the differences in data reporting require a more accurate method to investigate phenolic composition in the seed coat (Section 3.4).

To the best of our knowledge, no work on identification and quantification of individual polyphenols in lupin seed coats have been reported. Moreover, the effects of genotype and environment on the individual polyphenols in the ASL seed coat has not been investigated. To this end, in this chapter, individual polyphenols in ASL seed coat were identified and semi-quantified using HPLC-DAD-ESI-MS/MS. Six commercial varieties of ASL grown in two locations in WA were used to evaluate the effects of genotype, environment, and their interaction (G × E) on contents of the individual polyphenolics.

4.2. Materials and methods

4.2.1. Materials

Milled seed coat of six currently produced ASL cultivars (Coromup, Mandelup, Jenabillup, PBA Barlock, PBA Gunyidi and PBA Jurienp) grown at Wongan Hills (WH) and Eradu (ER) WA were prepared as previously described (Section 3.2.1).

4.2.2. Reagents and polyphenols standards

LC-MS grade acetonitrile, formic acid and methanol were purchased from Thermo Fisher Scientific (Scoresby, Vic, Australia). Authentic standards, including caffeic acid, *trans*-cinnamic acid and ferulic acid were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Vitexin (apigenin-8-*C*-glucoside), *p*-coumaric acid, protocatechuic acid and genistein were purchased from Cayman Chemical (Redfern, NSW, Australia). Other reagents were purchased from commercial suppliers as detailed in Section 3.2.2.

4.2.3. Extraction of free and bound phenolic compounds

Free and bound polyphenol extracts of the seed coats were obtained as described in Section 3.2.6.

4.2.4. HPLC-DAD-ESI-MS/MS analysis

Individual polyphenols were identified and quantified according to the procedure developed by our research group (Wu, Johnson, Bornman, Bennett, Clarke, et al., 2016) with minor modifications. An Agilent 1200 auto-sampler HPLC system was coupled

to diode array detector (DAD) and an MS/MS system (Agilent 6460 LC-QQQ, Agilent Technologies, Palo Alto, CA, USA). The DAD was set to monitor signals at 190-600 nm with a resolution of 2 nm. After filtering through a millipore membrane (0.22 μm), samples (20 μL) were injected into the Kinetex XB-C 18 reversed phase-HPLC column (5 μm, 250 × 4.6 mm, Phenomenex, Torrance, CA, USA) at 25 °C. Solvent A consisted of 0.05% formic acid in water; solvent B was acetonitrile. The flow rate was set at 0.5 mL/min to facilitate the coupling to mass spectrometer. A linear gradient elution was conducted: linear to 8% B from 0% B in 18 min, and changed to 18% B in 2 min, to 20% B in 15 min, then linear to 80% B in 10 min, and finally to 100% B in 2 min. This was followed by extra 8 min for column washing (100% B) and re-equilibration (100% A) respectively.

Two-stage MS/MS spectra were acquired in the electrospray ionisation (ESI) negative ion mode with full scan ranging from *m/z* 50 to 1300. 5 L/min of N_2 was employed as the nebulising gas at 45 psi, 300°C. Capillary voltage and nozzle voltage were set at 3.5 kV and -500 V respectively. Sheath gas (N_2) was maintained at 11 L/min and 250 °C. Collision energy was selected as 25 eV based on the abundance of the daughter ions. Data acquisition and analyses were performed on the Agilent MassHunter workstation. The identification confidence level of each compound was presented in Table 4.1 following La Barbera et al. (2017) described.

For quantification of individual phenolic compounds, only those peaks with signal/noise (S/N) >10 were selected. Due to the lack of available standards, individual phenolic compound (except protocatechuic acid, ferulic acid and genistein) were semi-quantified using compounds with similar chemical structures and UV absorption. Apigenin-7-*O*-β-apiofuranosyl-6,8-di-*C*-β-D-glucopyranoside (**F3**), vicenin 2 (**F4**) and apigenin-7-*O*-β-glucopyranoside (**F5**/**B5**) were quantified using vitexin as standard (Siger et al., 2012). Phenolic acid hexosides, including cinnamic acid glucoside (**F1**), *p*-coumaric acid glucoside (**B3**) and ferulic acid glucoside (**B4**) were quantified using their corresponding phenolic acids. Dicaffeoylquinic acid (**F6**) content was calculated using caffeic acid standard curve. Aromadendrin-6-*C*-β-D-glucopyranosyl-7-*O*-[β-D-apiofuranosyl-(1→2)]-*O*-β-D-glucopyranoside (**F2**) was quantified using dihydroquercetin (or taxifolin). **B1** and **F7**/**B7** were not quantified since the peaks were not confidently identified. All results for the compounds above

were expressed as μg standards equivalent per g of dry sample (μg vitexin equivalent per g of dry sample for **F3** and **F4**, for example).

Linearity of the selected seven standards and their spike recovery which was performed using lupin (Coromup, ER) seed coat and detected in free fraction are presented in Table 4.2. The limit of detection (LOD) and quantification (LOQ) were calculated at S/N ratios of 3 and 10 respectively. Six replicates of a mixed standards solution at the same concentration were freshly prepared and analysed in a single day and six separate days to evaluate the intraday and inter-day precision respectively. The intra- and inter-day variations were calculated as relative standard deviation (RSD) of the peak area.

4.2.5. Statistical analysis

Fragmentations of flavonoid aglycones and glycosides were designated using nomenclature systems proposed by Ma, Li, VandenHeuvel, and Claeys (1997) and Domon and Costello (1988) respectively. Proposed mass spectrum fragmentation pathways were drawn using ChemDraw Prime software (V 16.0, PerkinElmer, VIC, Australia). The results were presented and analysed as described in Section 3.2.9, except that Unscrambler X (Camo Software AS., Norway) was employed to perform principal component analyses (PCA) using the mean values of free and bound individual polyphenol contents.

4.3. Results and discussion

4.3.1. Identifications of individual polyphenols

The HPLC-DAD chromatograms of free polyphenol extract (FP) and bound polyphenol extract (BP) of PBA Barlock are shown in Figure 4.1. Peaks were labelled as **F1-8** (FP) and **B1-8** (BP) respectively. HPLC retention time (Rt), UV-Vis absorption maximum wavelength (λ_{max}), deprotonated molecular ions and fragment ions of the 13 individual polyphenols are listed in Table 4.1.

4.3.1.1.Flavones in free faction

F3 was the dominant individual polyphenol in FP fraction. The UV-Vis absorbance at 275 nm and 340 nm of **F3** (Figure 4.2) showed the characteristic UV absorption bands

of flavones (Wu, Johnson, Bornman, Bennett, Clarke, et al., 2016). The absorption properties were largely the same as that of the authentic apigenin-8-*C*-β-D-glucopyranoside (vitexin) standard (270 nm, 332 nm). The mass spectra of the **F3** revealed the $[M-H]^-$ ion at *m/z* 725. The yield of two-fold neutral 120 amu fragments (*m/z* 725 to *m/z* 605, $^{0,2}X_{1,1}$; and *m/z* 455 to *m/z* 335, $^{0,2}X_{1,2}$), as well as ions at *m/z* 383 (apigenin + 113) and *m/z* 353 (apigenin + 83) are characteristics of apigenin-di-*C*-glycosyl flavone fragmentations (Figure 4.2 & 4.3) (Ferreres, Silva, Andrade, Seabra, & Ferreira, 2003). In addition, fragments at *m/z* 593 ($[(M-H)-132]^-$, Y_0), *m/z* 575 ($[(M-H)-150]^-$, $Z_{0,1}$) and *m/z* 455 ($[(M-H)-120-150]^-$, $^{0,2}X_{1,1}Z_{0,2}$) suggested the presence of *O*-pentose which was supported by diagnostic ion pairs at *m/z* 575 and at *m/z* 455 (Cuyckens & Claeys, 2004; Vukics & Guttman, 2010). The pentose can be attached either to the flavone aglycone giving an -*O*-glycoside or one of the two glucoses forming a *C*,*O*-glycoside, which is difficult to distinguish by MS/MS (Cavaliere, Foglia, Pastorini, Samperi, & Lagana, 2005). However, this compound was tentatively identified as apigenin-7-*O*-β-apiofuranosyl-6,8-di-*C*-β-D-glucopyranoside (Api-7-*O*-Api*f*-6,8-di-*C*-Glc*p*), mainly because it was previously isolated and identified using MS and nuclear magnetic resonance (NMR) spectra in the whole seeds of *L. hartwegii* by Kamel (2003) and *L. termis* by Elbandy and Rho (2014). The identification is further supported by the results of Siger et al. (2012) who identified the compound in *L. angustifolius*, *L. luteus* and *L. albus* seeds grown in Poland, and reported a highly similar mass spectra.

The UV-Vis absorption properties and fragment performances of **F4** were very similar with those of **F3**, with typical UV absorption at 273 nm (Band II) indicating the introduction of di-*C*-glycosides substituents at C6 or C8 positions (Santos-Buelga, García-Viguera, & Tomás-Barberán, 2003). Likewise, the same two successive losses of 120 amu neutral portions (*m/z* 593 to *m/z* 473, $^{0,2}X_1$; and *m/z* 473 to *m/z* 353, $^{0,2}X_2$), indicated that it was flavone di-*C*-glycosides either. Moreover, the $[M-H]^-$ of **F4** (*m/z* 593) was 132 amu smaller than that of **F3** (*m/z* 725) but accompanied by the same fragment ions at *m/z* 383 ($[(M-H)-120-90]^-$) and *m/z* 353 ($[(M-H)-120-120]^-$), indicating it was apigenin di-*C*-glycosides but was not conjugated by the pentose, i.e., apiose. Ions at *m/z* 297 $[(M-H)-120-120-56]^-$ corresponded to the further losses of 2×CO. As such, **F4** was tentatively identified as apigenin-6,8-di-*C*-β-D-glucopyranoside (vicenin 2). This was consistent with the mass spectra of the standard

(Cao, Yin, Qin, Cheng, & Chen, 2014), and the compound was previously reported in seeds of lupins (Siger et al., 2012).

Both **F5** and **B5** had λ_{max} at 278 nm and 320 nm. The deprotonated molecular ion $[M-H]^-$ of the compound was *m/z* 431 but this study failed to obtain its ion fragments (MS^2). However, based on the UV absorption and deprotonated molecular ion, as well as the slightly longer elution time (33.94 min) then the authentic standard vitexin (33.75 min), it could be tentatively identified as apigenin-7-*O*-β-glucopyranoside (Api-7-*O*-Glc*p*) (Santos-Buelga et al., 2003). The longer retention time of **F4** (di-*C*-glycosides) than that of **F3** (*O*-pentosyl-di-*C*-glycosides) in our study could be explained by the induction of a third sugar moiety in **F3** which can increase polarity and thus shorter retention time (Santos-Buelga et al., 2003). A longer retention time of vitexin (mono-*C*-glycosides) than **F4** supported the elution order. In contrast, Siger et al. (2012) reported a reverse elution order of the two compounds (**F3** and **F4**). This inconsistency could be attributed to the different HPLC conditions and column used. In the case of the longer retention time of **F5**/**B5** (mono-*O*-glycosides) than vitexin (mono-*C*-glycosides), Santos-Buelga et al. (2003) revealed that flavone *O*-glycosides generally elute after the corresponding *C*-glycosides.

Besides the compound **F3**, **F4** and **F5**/**B5** found in our ASL seed coat samples, much more complex but similar flavone-glycosides were also reported, e.g., apigenin-7-*O*-β-apiofuranosyl-6-*C*-β-glucopyranosyl-8-*C*- (6’’’’-*O*-*E*-feruloyl)-β-glucopyranoside (molecular weight (Mw) = 902) in *L. hartwegii* (Kamel, 2003) ; apigenin-6-*C*-β-D-glucopyranosyl-8-*C*-[α-L-rhamnopyranosyl-(1→2)]-β-glucopyranoside (Mw = 740) and apigenin-6-*C*-β-D-glucopyranosyl-8-*C*-[β-D-apiofuranosyl-(1→2)]-β-glucopyranoside (Mw = 726) in *L. termis* (Elbandy et al., 2014); apigenin-7-neohesperidoside (Mw = 578), apigenin-7-apioglucoside (Mw = 564) and several unidentified apigenin derivatives in raw and germinated *L. angustifolius* seeds (Dueñas et al., 2009). Apart from apigenin as the aglycone, luteolin derivatives and diosmetin derivatives are also found in *L. angustifolius* seeds. All of these studies suggest the high complexity of flavones in lupins.

4.3.1.2.Isoflavones in free (FP) and bound polyphenol (BP) faction

F8/B8 had a deprotonated ion at *m/z* 269, and showed a characteristic ion at *m/z* 133 which was the same as that of the authentic genistein standard but different from those of the apigenin standard (*m/z* 151 and *m/z* 117) (Vukics et al., 2010). It was therefore identified as genistein and this identification was also supported by its UV spectra (λ_{max} = 267 nm). Some studies reported that no isoflavones were detected in *L. angustifolius* species, whereas high level of isoflavones (e.g., mutabilein, mutabilin) were found in *L. mutabilis* (Dini, Schettino, & Dini, 1998; Ranilla et al., 2009). Other researchers, however, found genistein and its derivatives in raw and/or germinated *L. angustifolius* seeds. Examples include genistein apiofuranosyl diglycoside (Mw = 726), genistein diglucoside (Mw = 594), genistein-7-*O*-β-glucopyranoside (Mw = 432), genistein diglycoside (Mw = 564, attaching pentose-hexose moiety) and several types of genistein acetyl glycosides (Dueñas et al., 2009). The first three compounds which had the same deprotonated molecular ions as **F3**, **F4** and **F5**/**B5** in the current study, however, were identified as flavones, with apigenin being the corresponding aglycone instead of genistein, due to their characteristic UV absorptions.

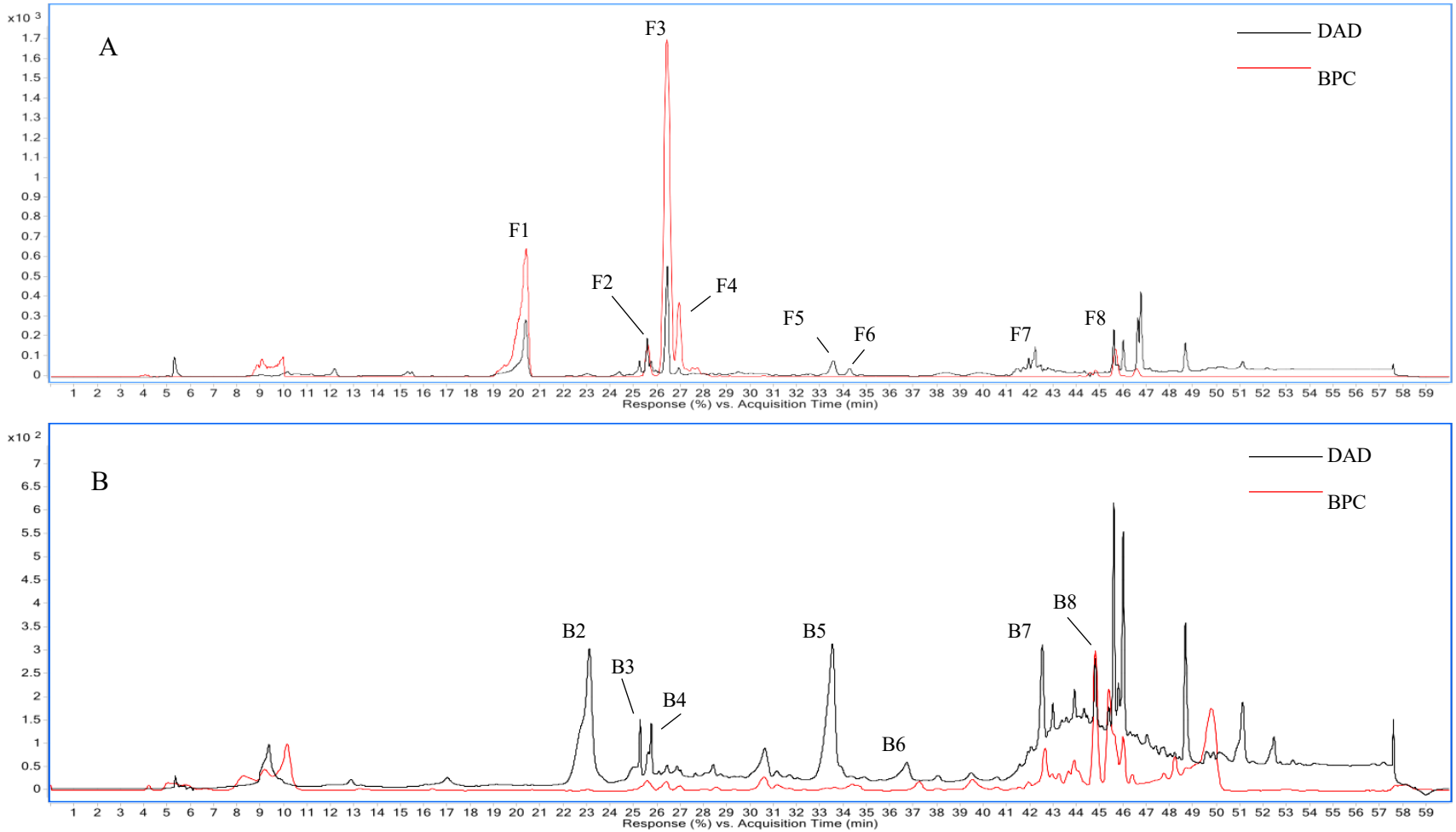


Figure 4.1 UV chromatogram at 280 nm (DAD) and base peak chromatogram (BPC, all) of the free (A) and bound fraction (B) of PBA Barlock (ER) seed coat.

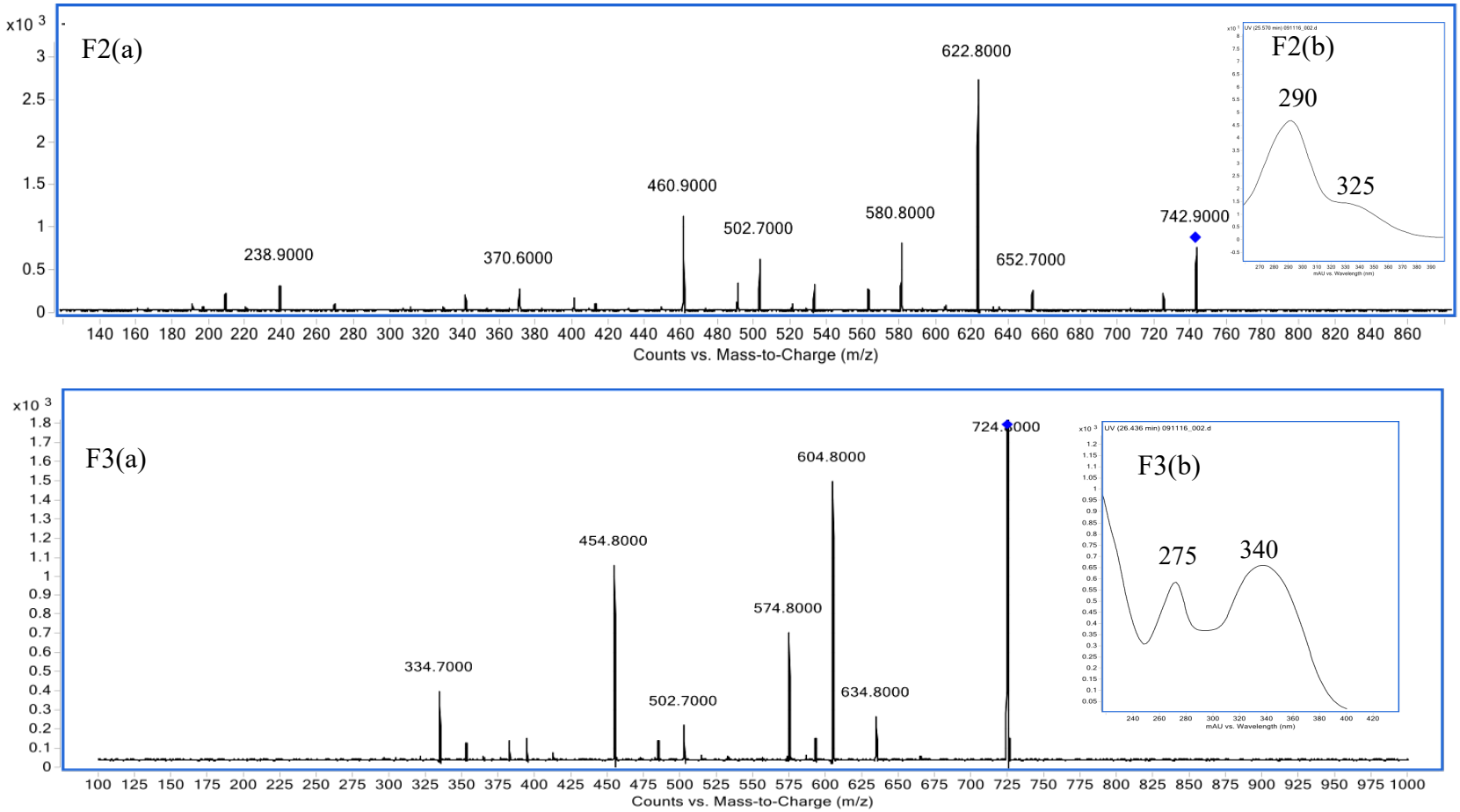


Figure 4.2 ESI-MS/MS spectra (a) and UV-Vis absorption profile (b) of aromadendrin-6-*C*-β-D-glucopyranosyl-7-*O*-[β-D-apiofuranosyl-(1→2)]-*O*-β-D-glucopyranoside (**F2**) and apigenin-7-*O*-β-apiofuranosyl-6,8-di-*C*-β-D-glucopyranoside (**F3**).

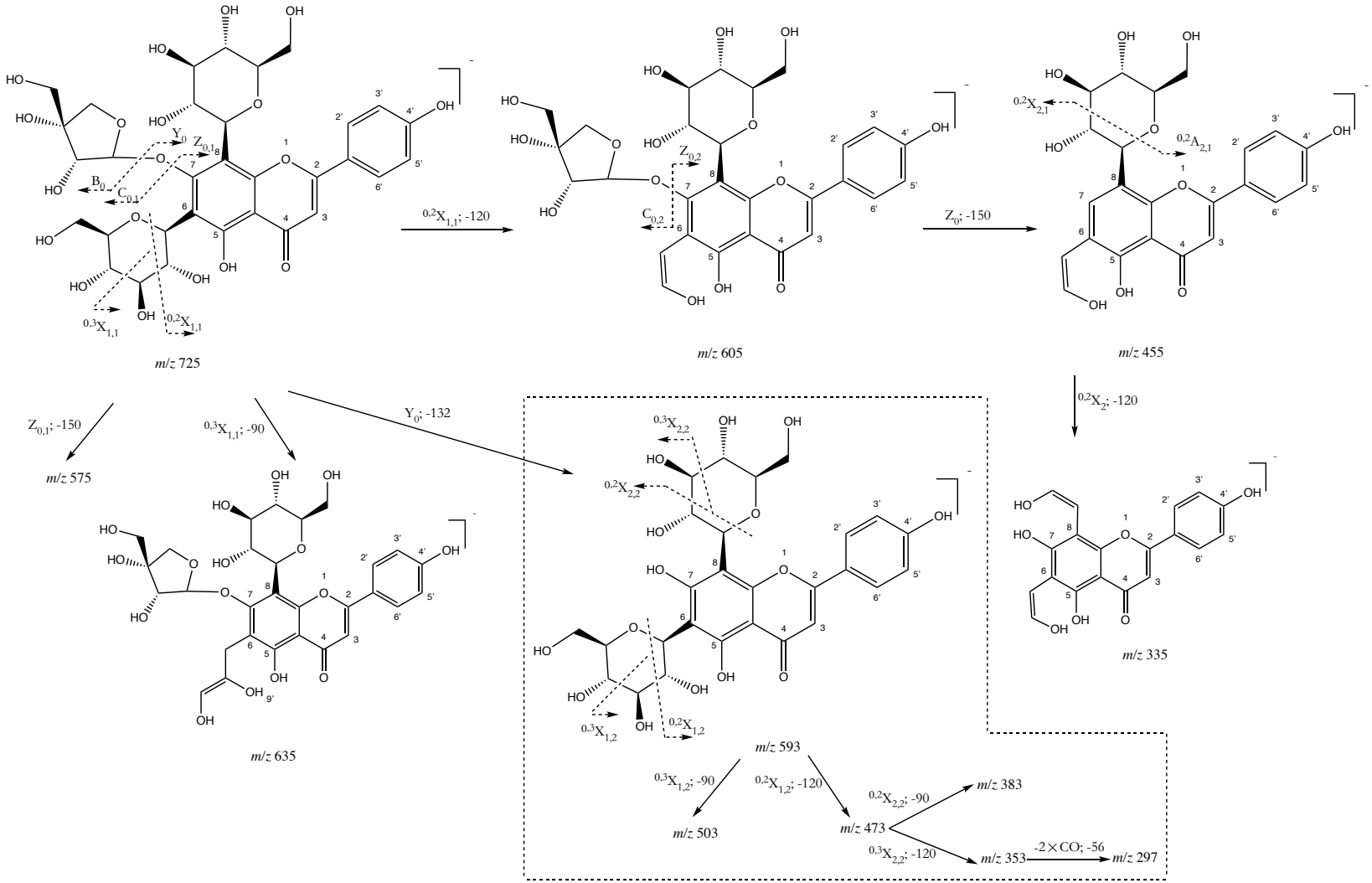


Figure 4.3 Proposed mass spectrum fragmentation pathway for apigenin-7-*O*-β-apiofuranosyl-6,8-di-*C*-β-D-glucopyranoside (F3) and apigenin-6,8-di-*C*-β-D-glucopyranoside (F4, dashed box).

Table 4.1 Polyphenols identified by HPLC-ESI-MS/MS in samples.

Peak No.	t_R (min)	λ_{max}	$[M-H]^-$ *m/z*	*m/z* MS/MS (Abundance %)	Assigned identity	Identification confidence level	Reference
F1	20.63	275	309a	291(64); 247 (3); 180(30); 128(99)	Cinnamic acid glucoside	2a	Gruz, Novák, & Strnad, 2008
B1	9.89	202	405a	191(44);111(100)	Quinic acid derivative		Clifford, Knight, & Kuhnert, 2005
B2	23.08	275	153b	135(40); 109(80)	Protocatechuic acid	1	Gruz, Novák, & Strnad, 2008
B3	25.25	226; 310	325b	163 (87); 119(64)	*p*-coumaric acid glucoside	2a	Gruz, Novák, & Strnad, 2008
F2	25.57	290; 325sh	743b	653(17); 623(100); 581(32); 563 (13); 533(31); 503 (22); 461(54); 371(15)	Aromadendrin-6-*C*-β-D-glucopyranosyl-7-*O*-[β-D-apiofuranosyl-(1→2)]-*O*-β-D-glucopyranoside	2b	Dueñas et al., 2009
B4	25.73	290	355b	193(34);178(30);134(100)	Ferulic acid glucoside	2a	Gruz, Novák, & Strnad, 2008

Peak No.	t_R (min)	λ_{max}	$[M-H]^-$ *m/z*	*m/z* MS/MS (Abundance %)	Assigned identity	Identification confidence level	Reference
F3	26.43	275; 340	725a	635(2); 605(10); 593(5); 575(7); 503(1); 455(7); 383(1); 353(1); 335 (3)	Apigenin-7-*O*-β-apiofuranosyl-6,8-di-*C*-β-glucopyranoside	2a	Siger et al., 2012
F4	26.92	273; 340	593b	503 (9); 473 (16); 383(11); 353(22); 297(3)	Vicenin 2	2a	Cao, Yin, Qin, Cheng, & Chen, 2014
F5/B5	33.94	278; 320	431c	n.d.	Apigenin-7-*O*-β-glucopyranoside	2a	Santos-Buelga et al., 2003
F6	34.58	327	515c	249(5); 179(2); 135(6)	Dicaffeoylquinic acid	2a	Clifford, Knight, & Kuhnert, 2005
B6	36.94	300sh; 330	n.d.	n.d.	Ferulic acid	1	Gruz, Novák, & Strnad, 2008
F7/B7	42.93	300sh; 335	361c	n.d.	Hydroxycinnamic acid derivative		Gruz, Novák, & Strnad, 2008
F8&B8	44.97	267; 330sh	269a	269(100); 195(25); 133 (52)	Genistein	1	Vukics & Guttman, 2010

t_R: Retention time; sh: Shoulder; n.d.: no data.

Abundances of ions in the MS spectra: (a) abundance over 1×10^5; (b) abundance in the range 1×10^4-1×10^5; (c) abundance below 1×10^4; n.d.: not detected.

Identification confidence level: Level 1, confirmed structures where a reference standard is available; level 2a, evidence by spectrum matching with a spectrum from the literature; level 2b, diagnostic evidence where no other structure fits the experimental MS^2 information (La Barbera et al., 2017)

Table 4.2 Method validation parameters of the seven selected standards and their recovery in lupin (Coromup, ER) seed coat using the HPLC-DAD.

Compound	Linear range (mg/L)	Regression equation	R^2	LOQ (μg/L)	LOD (μg/L)	Recovery (%)	RSD (%) of recovery	RSD (%) of intra-day (n=6)	RSD (%) of inter-day (n=6)
Protocatechuic acid	0.32-8.00	y=58.989x-3.4128	0.9994	35	119	97.94	1.80	0.37	0.52
Caffeic acid	0.29-7.28	y=122.62x-57.419	0.9989	9	30	100.76	4.43	0.10	1.06
Vitexin	0.80-20.00	y=47.05x-0.4737	1	30	100	97.72	4.25	0.37	0.46
Ferulic acid	0.26-6.40	y=146.81x+2.1822	1	11	36	97.61	0.75	0.43	0.56
Taxifolin	0.17-4.20	y=78.267x-23.037	0.9983	19	65	104.38	0.84	0.48	2.74
trans-Cinnamic acid	0.19-4.80	y=358.73+2.3522	1	1	4	99.32	2.30	0.11	0.34
Genistein	0.12-3.00	y=105.96x+1.5452	0.9997	4	13	97.89	4.42	0.23	0.37

Table 4.3 Individual polyphenol profile (μg/g dry basis) in free fraction of Australian sweet lupin seed coats.

			PBA Jurien	Coromup	PBA Gunyidi	Mandelup	PBA Barlock	Jenabillup
Hydroxycinnamics								
F1	CIA[2]	ER	19.51±1.69^{aA}	31.14±0.54^{dA}	28.15±0.52^{cdA}	27.12±0.38^{bcA}	24.32±0.82^{bA}	31.40±0.09^{dA}
		WH	17.10±0.11^{aA}	27.83±0.77^{bcB}	26.88±0.01^{bcA}	26.49±1.44^{bA}	25.63±0.51^{bA}	29.68±0.35^{cB}
F6	diCQA[3]	ER	67.88±2.60^{abA}	89.03±15.01^{bA}	71.50±2.69^{abA}	57.35±0.48^{aA}	63.18±0.51^{aA}	65.25±0.08^{abA}
		WH	49.08±0.20^{bB}	55.52±0.35^{cA}	42.31±0.02^{aB}	43.62±1.63^{aB}	46.56±2.66^{abB}	46.90±0.02^{abB}
$\boldsymbol{\Sigma}$ Subgroup		ER	87.40±0.91^{aA}	120.18±15.55^{bA}	99.65±3.21^{abA}	84.47±0.85^{aA}	87.50±1.32^{aA}	96.65±0.01^{abA}
		WH	66.18±0.09^{aB}	83.35±1.13^{cA}	69.19±0.03^{abB}	70.11±3.07^{abB}	72.19±3.17^{abB}	76.58±0.32^{bcB}
Flavones								
F3	Api-Api*f*-di-Glc*p*[4]	ER	717.28±2.22^{aA}	960.32±6.33^{cA}	764.60±3.47^{aA}	958.7±14.76^{cA}	883.18±19.52^{bA}	900.52±16.15^{bcA}
		WH	697.85±10.96^{aA}	954.85±7.87^{cdA}	804.18±6.21^{bB}	977.00±9.73^{deA}	1011.82±8.25^{eB}	928.95±18.85^{cA}
F4	Vicenin 2	ER	36.02±1.81^{bA}	25.70±0.06^{aA}	26.38±0.14^{aA}	33.68±0.92^{bA}	44.02±1.17^{dA}	28.48±0.05^{aA}
		WH	34.51±0.53^{bA}	24.65±0.20^{aB}	25.78±0.28^{aA}	33.84±0.06^{bA}	59.53±1.05^{cB}	32.62±2.35^{bA}
F5	Api-*O*-Glc*p*[5]	ER	80.21±8.62^{abcA}	74.80±7.51^{abcA}	81.96±17.59^{bcA}	46.50±1.20^{aA}	91.67±0.61^{cA}	57.21±0.59^{abA}
		WH	40.76±2.27^{aB}	32.54±11.15^{aB}	22.42±0.03^{aB}	23.65±2.31^{aB}	48.02±11.77^{aB}	36.36±0.21^{aB}
$\boldsymbol{\Sigma}$ Subgroup		ER	833.51±9.04^{aA}	1060.82±13.9^{cA}	872.94±21.21^{aA}	1038.88±16.88^{bcA}	1018.86±21.3^{bcA}	986.21±15.51^{bA}
		WH	773.12±13.76^{aB}	1012.04±3.08^{cB}	852.38±6.46^{bA}	1034.49±12.11^{cA}	1119.37±2.47^{dB}	997.93±20.99^{cA}
Isoflavones								
F8	Genistein	ER	24.89±0.39^{abA}	25.01±0.72^{bA}	30.79±0.25^{cA}	22.30±1.35^{aA}	34.01±0.17^{dA}	25.82±0.36^{bA}
		WH	41.79±4.22^{abB}	47.92±6.03^{abcB}	32.53±0.45^{aB}	33.13±2.19^{aB}	51.00±5.59^{bcA}	62.60±5.19^{cB}

			PBA Jurien	Coromup	PBA Gunyidi	Mandelup	PBA Barlock	Jenabillup
Dihydroflavonols								
F2	Aro-Glc*p*-Api*f*Glc*p*[6]	ER	35.83±1.08^{aA}	56.57±0.16^{dA}	42.88±0.01^{bA}	40.92±0.54^{bA}	50.87±1.05^{cA}	61.03±0.74^{eA}
		WH	37.52±0.75^{aA}	38.83±2.91^{aB}	34.20±0.13^{aB}	40.93±3.65^{aA}	48.29±0.14^{aA}	39.01±13.8^{aA}
Total		ER	986.63±15.55^{aA}	1262.58±28.89^{cA}	1046.26±24.66^{aA}	1186.58±16.93^{bcA}	1191.25±23.85^{bcA}	1169.71±15.89^{bA}
		WH	918.6±10.38^{aB}	1182.14±5.08^{cA}	988.3±6.76^{bA}	1178.66±3.19^{cA}	1290.84±6.44^{dB}	1176.12±1.68^{cA}

[1] Means ± standard deviation (n=2).

[2] cinnamic acid glucoside (as *trans*-cinnamic acid equivalent); [3] dicaffeoylquinic acid (as caffeic acid equivalent); [4] apigenin-7-*O*-β-apiofuranosyl-6,8-di-*C*-glucopyranoside (as vitexin equivalent); [5] apigenin-7-*O*-β-glucopyranoside (as vitexin equivalent); [6] aromadendrin-6-*C*-β-D-glucopyranosyl-7-*O*-[β-D-apiofuranosyl-(1→2)]-*O*-β-D-glucopyranoside glucopyranoside (as taxifolin equivalent).

ER, Eradu; WH, Wongan Hills.

Means assigned with different small letters in the same row, and capital letters in the same column within each dependent variable indicate significant differences ($p < 0.05$).

Table 4.4 Individual polyphenol profile (μg/g dry basis) in bound fraction of Australian sweet lupin seed coats.

			PBA Jurien	Coromup	PBA Gunyidi	Mandelup	PBA Barlock	Jenabillup
B2	Protocatechuic acid	ER	6.46±1.02^{aA}	8.12±0.27^{aA}	29.13±3.28^{bA}	34.72±0.22^{bA}	51.45±4.96^{cA}	40.03±4.03^{bcA}
		WH	5.68±0.42^{aA}	13.08±2.37^{aA}	58.21±1.87^{bcB}	60.03±1.42^{bcB}	63.38±2.03^{cA}	44.96±0.50^{bA}
B3	*p*-coumaric acid glucoside[2]	ER	5.31±0.84^{abA}	4.88±0.97^{abA}	6.19±0.33^{abA}	5.39±0.11^{abA}	7.09±0.47^{bA}	4.00±0.48^{aA}
		WH	3.80±0.59^{aA}	3.13±0.23^{aA}	6.40±0.18^{bA}	5.67±0.02^{bA}	6.02±0.27^{bA}	3.91±0.18^{aA}
B4	Ferulic acid glucoside[3]	ER	7.36±1.10^{aA}	9.41±0.55^{aA}	8.32±0.11^{aA}	8.26±0.14^{aA}	8.24±0.31^{aA}	7.30±0.54^{aA}
		WH	6.30±0.63^{aA}	7.53±0.25^{abA}	8.75±0.51^{bA}	8.45±0.03^{bA}	7.91±0.26^{bA}	7.42±0.22^{abA}
B6	Ferulic acid	ER	5.83±0.22^{aA}	6.53±0.15^{abA}	7.51±0.26^{bA}	5.57±0.01^{aA}	6.20±0.11^{aA}	5.38±0.60^{aA}
		WH	5.63±0.21^{aA}	6.40±0.20^{aA}	9.15±0.48^{bA}	5.93±0.04^{aB}	6.32±0.03^{aA}	6.01±0.07^{aA}
Σ Hydroxycinnamics		ER	24.96±3.18^{aA}	28.94±1.94^{abA}	51.15±3.98^{cA}	49.21±7.14^{bcA}	72.97±5.85^{dA}	56.70±5.65^{cdA}
		WH	21.41±1.84^{aA}	30.14±1.68^{aA}	82.51±2.08^{cB}	80.07±1.47^{cB}	83.64±1.54^{cA}	62.30±0.18^{bA}
B5	Apigenin-7-*O*-glucoside[4]	ER	27.12±5.94^{aA}	41.41±9.42^{aA}	68.33±5.45^{bA}	40.03±2.87^{aA}	51.6±1.27^{abA}	49.32±5.51^{abA}
		WH	24.17±2.76^{aA}	47.42±7.23^{bA}	83.14±4.11^{cA}	40.11±0.25^{bA}	50.57±2.14^{bA}	54.51±2.79^{bA}
B8	Genistein	ER	4.16±0.06^{aA}	4.67±0.31^{aA}	5.85±0.11^{aA}	4.42±0.25^{aA}	5.19±0.17^{aA}	4.78±0.94^{aA}
		WH	4.02±0.19^{aA}	4.88±0.21^{abA}	6.00±0.57^{bA}	4.07±0.28^{aA}	4.75±0.06^{aA}	4.89±0.01^{abA}
Total		ER	56.24±9.07^{aA}	75.03±11.05^{abA}	125.33±9.32^{cA}	98.40±3.08^{abcA}	129.77±7.28^{cA}	116.04±8.69^{bcA}
		WH	49.60±4.79^{aA}	82.43±5.34^{bA}	171.65±2.59^{dB}	124.26±0.95^{cB}	138.96±3.74^{cA}	121.48±2.96^{cA}

[1] Means ± SD (n=2); [2] *p*-coumaric acid equivalents; [3] as ferulic acid equivalent; [4] apigenin-7-*O*-β-glucopyranoside (as vitexin equivalent).

ER, Eradu; WH, Wongan Hills. Means assigned with different small letters in the same row, and capital letters in the same column within each dependent variable indicate significant differences ($p < 0.05$).

4.3.1.3. Dihydroflavonols in free faction

The strong UV-Vis absorption peak at 295 nm and the small peak of lower intensity (shoulder) at 325 nm implied that the **F2** could be flavanone or dihydroflavonol (Figure 4.2) (Mabry, Markham, & Thomas, 1970). However, flavanones almost only occur in *Citrus* genus, but dihydroflavonols are ubiquitously distributed in legumes (Santos-Buelga et al., 2003). Dueñas et al. (2009) have found 4 dihydroflavonols, namely 3 dihydroquercetin (taxifolin) derivatives and dihydrokaempferol (aromadendrin) acetylglycoside in *L. angustifolius* seeds.

The 162 amu (*m/z* 743 to *m/z* 581, Y_1) and 180 amu (*m/z* 743 to *m/z* 563, Z_1) losses from deprotonated molecular ion of **F2** were typical pattern of *O*-glycoside fragmentations, suggesting the presence of hexose then giving an *O*- or *C*, *O*-diglycosyl structure (Gattuso, Barreca, Gargiulli, Leuzzi, & Caristi, 2007). Moreover, a series of ions, $[M-H-18]^-$ (*m/z* 725, E_1^-), $[M-H-90]^-$ (*m/z* 653, $^{0,3}X_{2,1}$), $[M-H-120]^-$ (*m/z* 623, $^{0,2}X_{2,1}$), $[M-H-120-90]^-$ (*m/z* 533, $^{0,2}X_{2,1}{}^{0,3}X_{2,2}$), $[M-H-120-120]^-$ (*m/z* 503, $^{0,2}X_{2,1}{}^{0,2}X_{2,2}$) were observed as the characteristic fragments of two glucoside moieties. The further subsequent losses from *m/z* 461 ($[M-H-162-120]^-$, $Y_1{}^{0,2}X_{1,1}$) to *m/z* 401 (60 amu, $Y_1{}^{0,2}X_{1,1}{}^{0,3}X_0$), *m/z* 371 (90 amu, $Y_1{}^{0,2}X_{2,1}{}^{0,2}X_0$) and *m/z* 341 (120 amu, $Y_1{}^{0,2}X_{2,1}{}^{0,1}X_0$) were *C*-bound cleavages of pentose. Together, although further aglycone analyses are needed, the compound was proposed to be aromadendrin-6-*C*-β-D-glucopyranosyl-7-*O*-[β-D-apiofuranosyl-(1→2)]-*O*-β-D-glucopyranoside (Aro-6-*C*-Glc*p*-7-*O*-Api*f* Glc*p*).

4.3.1.4. Phenolic acid derivatives in free (FP) and bound polyphenol (BP) faction

F1 showed deprotonated molecular ion $[M-H]^-$ at *m/z* 309, with ion at *m/z* 291 being its dehydrated fragment (Demarque, Crotti, Vessecchi, Lopes, & Lopes, 2016). Decarboxylated ion (*m/z* 247), glycoside fragment (*m/z* 180) indicated that **F1** could be cinnamic acid glucoside (Mw = 310). **F6** had a λ_{max} at 327 nm, and parent ion at *m/z* 515 accompanied by distinguished fragment ion at *m/z* 179 and then lost a carboxyl group to *m/z* 135, indicating the caffeic acid moiety. It was thus tentatively identified as dicaffeoylquinic acid (diCQA) (Clifford, Knight, & Kuhnert, 2005). Czubinski, Siger, and Lampart-Szczapa (2016) also reported two nearby peaks at the similar retention times of **F5**/**B5** (Api-7-*O*-Glc*p*) and **F6**. Instead, the authors postulated the

compounds to be apigenin and cinnamic acid derivative respectively due to the lack of MS data in their study.

In terms of phenolic acid hexosides in bound fraction, *p*-coumaric acid glucoside (**B3**) and ferulic acid glucoside (**B4**) showed diagnostic 162 amu losses ($[M-H-Glu]^-$) with resulting in the corresponding ions at deprotonated phenolic acids. Deprotonated protocatechuic acid (**B2**, $[M-H]^-$ at *m/z* 153) and *p*-coumaric acid moiety ($[M-H-162]^-$ at *m/z* 163) of **B3** showed characteristic 44 amu carboxyl group losses and then giving signals at $[M-H-COO]^-$ and $[M-H-162-COO]^-$, namely ions at *m/z* 109 and *m/z* 119 respectively (Gruz, Novák, & Strnad, 2008; Wu, Johnson, Bornman, Bennett, Clarke, et al., 2016). Moreover, fragment at *m/z* 178 of **B4** was due to a methyl radical loss ($[M-H-162-CH_3]^-$, 15 amu) of the ferulic acid moiety and then a further 44 amu loss to *m/z* 134 (Wu, Johnson, Bornman, Bennett, Clarke, et al., 2016). We failed to get MS data for **B6**, but it had same retention time and UV-Vis absorption properties with the authentic ferulic acid standard. **B1** showed deprotonated ion at *m/z* 405 but produced fragments at *m/z* 191 and *m/z* 111 which are characteristic for quinic acid (Clifford et al., 2005). Therefore, the compound was denoted as quinic acid derivative. Due to the lack of MS^2 data for either, **F7** and **B7** were designated as cinnamic acid derivative mainly basing on their characteristic maximum UV absorption at 340 nm. Protocatechuic acid (**B2**), ferulic acid glycoside (**B4**), ferulic acid (**B6**), *p*-coumaric acid and other hydroxybenzoic and hydroxycinnamic compounds previously found in *L. angustifolius* seeds (Dueñas et al., 2009).

4.3.2. Quantification of individual polyphenols.

Results for HPLC-DAD method validation are shown in Table 4.2. Briefly, the R^2 of the 7 analysed standards were all greater than 0.99, indicating good linearities within the ranges used. The intra- and inter-day variations of all the standards were lower than 0.48% and 2.74% respectively. Moreover, the percentage of recovery of these standards which spiked in lupin (Coromup, ER) seed coat ranged from 97.61% to 104.38% with acceptable precision. The results suggested that the HPLC-DAD method is adequate for quantifying the selected phenolics.

4.3.2.1.Quantifications of individual polyphenols in free fraction.

Of all individual polyphenols of the 12 lupin seed coat samples (6 genotypes by 2 locations), apigenin-7-*O*-β-apiofuranosyl-6,8-di-*C*-β-D-glucopyranoside (Api-7-*O*-Api*f*-6,8-di-*C*-Glc*p,* **F3**) was the dominant compound in the free fraction, ranging from 697.85 μg/g db to 1011.82 μg/g db (as vitexin equivalent), which accounted for 73.08 - 82.89 % of the total polyphenols in free fraction (Table 4.3). High contents of this compound have also been found in Polish grown *L. angustifolius*, *L. luteus* and *L. albus* whole seeds, 409.6 - 428.8 μg/g db, 709.8-876.9 μg/g db and 257.5 - 259.5μg/g db respectively (Siger et al., 2012). The authors also reported high levels of vicenin 2 (**F4** in the present study) in the whole seeds of the three-lupin species, being 277.8 - 302.5 μg/g db, 536.3 - 631.4 μg/g db and 119.0 - 143.0 μg/g db respectively. In contrast, much lower contents of **F4** were found in our seed coats, from 24.65 μg/g db up to 59.53 μg/g db. The differences might partly be related to the genetic and environmental differences, because considerable variability was also apparent in our collection as will be discussed below. In addition, the differences in distributions of the two compounds in the seed coat and cotyledon may have also contributed. Luo et al. (2016) reported provocatively high vitexin (apigenin-8-*C*-glucoside) and isovitexin (apigenin-6-*C*-glucoside) contents (37,430 μg/g db and 47,180 μg/g db, respectively) in mung bean seed coat, while the two compounds were not detected in mung bean cotyledons. In the current study the high percentage of api-7-*O*-Api*f*-6,8-di-*C*-Glc*p* (**F3**) suggested that lupin seed coat could be a good plant source of the compound. For example, raw celery which is regularly the main dietary source of apigenin usually contains between 28.5 and 240.2 μg/g, but up to 786.5 μg/g in the seeds (Haytowitz, 2018). Total flavone intakes have been associated with lower risk for all-cause mortality (Mink, 2007). Followed by purification and investigations of the potential bioactivities of **F3**, the compound also could be used to produce other apigenin derivatives (e.g., vitexin and isovitexin) by removing particular glycosides (e.g., by using acid hydrolysis) resulting in compounds that may have multiple pharmacological effects (He, Min, Kong, He, Li, & Peng, 2016).

Phenolic acids (in free or esterified form) of lupins seeds, including *p*-hydroxybenzoic acid, procatechuic acid, chlorogenic acid, vanillin acid, *p*-coumaric acid and ferulic acid, have previously been indicated to mainly occur in the seed coat of *L. luteus*, *L. albus*, and *L. angustifolius* rather than the cotyledon (Lampart-Szczapa et al., 2003). The HPLC conditions in our study were carefully developed to maximise separation

of gallic acid, protocacheuic acid, catechin, caffeic acid, *p*-coumaric acid, ferulic acid and *trans*-cinnamic acid standards (not shown). In this context, contrary to expectations, this study found only three hydroxycinnamic derivatives (**F1**, **F6** and **F7**) in free fraction. But total hydroxycinnamics level was up to 99.65 μg/g db (as standards equivalents), that was much higher than published data of 6.06 μg/g db in *L. albus* seed coat, and around 2.50 μg/g db in *L. angustifolius* seed coat (Lampart-Szczapa et al., 2003). Oppositely, Frank Sosulski (1984) revealed that only *trans*-*p*-coumaric acid, *trans*-ferulic acid and *p*-hydroxybenzoic acid occurred in *L. albus* seed coat but totally up to 44 μg/g db. In fact, the existing data on content of phenolic acids in whole *L. angustifolius* seeds show a large variability, from 4.9 μg/g db to 58.14 μg/g db as reported by Dueñas et al. (2009) and Siger et al. (2012) respectively.

It was also somewhat surprising that high levels of isoflavones (genistein) were found in the free fraction in our study at 22.30 - 62.60 μg/g db, which differed from some previous studies that reported very low levels of isoflavones (0.9 μg/g db) in whole *L. angustifolius* seeds and did not detect them in its seed coat (Dueñas et al., 2009; Ranilla et al., 2009). However, compared to the results of the present study, much higher levels of genistein and its derivative were previously reported in *L. mutabilis* seed coats, 98.09-870 μg/g fresh weight (Ranilla et al., 2009). Not unexpectedly, no anthocyanins were found in the lupin seed coats by the HPLC-MS/MS, which could be supported by the results at 520 nm of the DAD. Oomah et al. (2006) reported that only negligible anthocyanins were found in Australian sweet lupin seed using UV-Vis method. However, positive ion mode is generally used to identify the anthocyanins using mass spectrometry (Motilva, Serra, & Macia, 2013). Therefore, further investigations should be performed. Nevertheless, the results of this study indicated that the polyphenols in lupin seed coats were primary flavonoids (flavones plus isoflavone), 90.64 % to 94.41 % of total free polyphenol content, a finding that in agreement with that of the lentil seed coat polyphenol profile (Dueñas et al., 2002), and that of whole seeds of *L. angustifolius*, *L. luteus* and *L. albus* (Siger et al., 2012).

As mentioned, the quantity of individual phenolic compounds varied across the six genotypes and the two locations, whereas the profile was found to be constantly stable. Statistical analysis revealed that the levels of free individuals and total free polyphenol content of ASL seed coats were significantly affected by genotype, location and their

interaction, with all of the effects being significant at $p < 0.01$. In contrast, location exhibited no effects on total flavones ($p = 0.189$). Among the 6 genotypes, the lowest **F3** level, and accordingly, the lowest total flavone content and total polyphenol content were measured in PBA Jurien ($p < 0.01$), which followed by PBA Gunyidi ($p < 0.01$) and consistently so for both ER and WH. Comparisons across the two locations, seeds of all genotypes harvested from WH accumulated a lower level of total hydroxycinnamics than that of the counterparts from ER ($p < 0.001$). The higher temperature and UV radiation, but lower rainfall at WH tended to decrease free phenolic acid derivatives levels in the lupin seed coats. A reverse trend, however, was observed in genistein content. Collectively, location exerted effects on the levels of phenolic individuals but was in genotype-dependent manner; genotype was the determining contributor of the observed variations.

4.3.2.2. Quantification of individual polyphenols in bound fraction.

It was the first time to evaluate bound polyphenols in lupin seed coat. Bound polyphenols could reach colon then largely be metabolized by gut bacteria and show physiological benefits (Saura-Calixto, 2012). Although acid hydrolysis was used to hydrolyse the polyphenols from seed coats of chickpea (Sreerama, Sashikala, & Pratape, 2010) and lentil (Dueñas, Sun, Hernández, Estrella, & Spranger, 2003), alkaline hydrolysis was used in our study since alkaline hydrolysis was reported to be a better procedure to release polyphenols from polysaccharides than acid hydrolysis because it (1) can reduce polyphenols (especially flavonoids and phenolic acids) losses; (2) is an effective method of cleavage of ester bonds which bind polyphenols to the cell wall (Acosta-Estrada, Gutierrez-Uribe, & Serna-Saldivar, 2014). This approach has been employed to release polyphenols of 10 legumes seed coats (Frank Sosulski, 1984), and more recently lentil seed coat (Dueñas et al., 2002). As shown in Table 4.4, the majority of phenolic individual types in bound fraction were phenolic acid derivatives. No api-7-*O*-Api*f*-6,8-di-*C*-Glc*p* (**F3**) and vicenin 2 (**F4**) were detected in the bound fraction. Ferulic acid derivatives widely occur in plant cell walls, and contribute to cell wall rigidity by crosslinking polysaccharides and lignin (Agati et al., 2012; Rosazza, Huang, Dostal, Volm, & Rousseau, 1995). In addition, they also esterify with various compounds (e.g., flavonoids, sterols and hydroxycarboxylic acids), that can be cleaved by alkaline hydrolysis.

Following the trends in the results of polyphenol contents in the free fraction, the total polyphenol contents varied among the genotypes and locations, but the results in bound fraction showed much larger standard deviations. Generally, the effects of genotype, location and their interaction on bound polyphenol quantity were found in great similarities with free fraction, but with location and genotype × location showing no influences on levels of both api-7-*O*-Glc*p* (**B5**, $p = 0.117$ and 0.269 respectively) and genistein (**B8**, $p = 0.613$ and 0.717 respectively). The hydroxycinnamics and api-7-*O*-Glc*p* were the dominant bound phenolic individuals, totally accounted up to 96.58 % of total bound phenolic compounds. In addition, individual and total bound polyphenol content of PBA Jurien of the two locations were the lowest among the genotypes ($p < 0.001$). Conversely, PBA Barlock of the two locations had the highest total bound polyphenol content which was mainly contributed by the highest levels of total hydroxycinnamics. Particularly, the protocatechuic acid levels in PBA Jurien, 5.07 ± 2.99 µg/g db (ER) and 5.68 ± 0.42 µg/g db (WH) respectively, were much lower than PBA Barlock (51.45 ± 4.96 µg/g db and 63.38 ± 2.03 µg/g db respectively). In contrast to free fraction with respect to total bound polyphenol content, only PBA Gunyidi and Mandelup seeds from WH showed statistically significantly higher than those of ER, but the remaining did not.

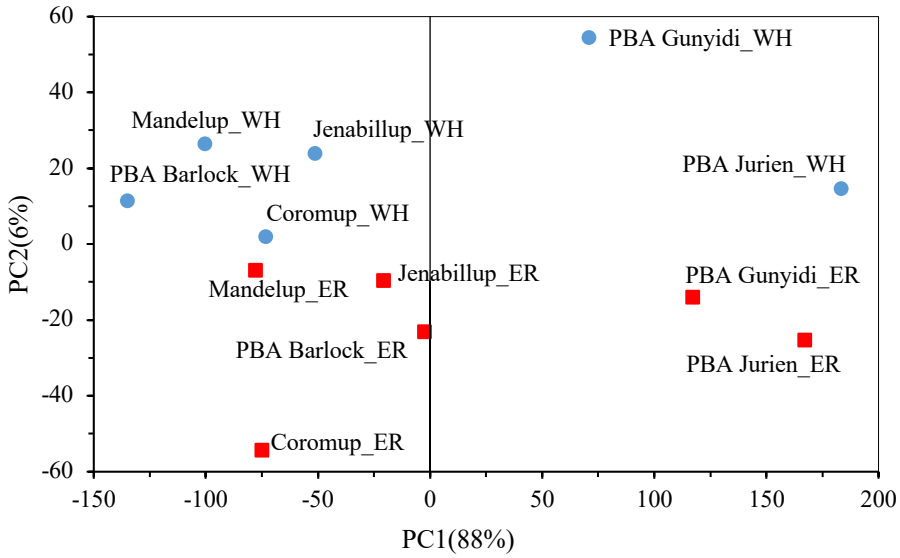


Figure 4.4 Scores plot of principle component analysis (PCA) on free and bound phenolic profiles of six ASL genotypes from two sites (ER, Eradu; WH, Wongan Hills).

4.3.3. Principle component analysis (PCA)

The mean values of free and bound polyphenol contents were used for PCA to evaluate any genetic and environmental effects. As illustrated in Figure 4.4, two main components (namely, PC1 and PC2) were detected to explain 88% and 6% variations of the data, respectively. PC1 clearly distanced PBA Gunyidi and PBA Jurien from both locations from other varieties. By examining the corresponding loadings data, the level of **F3** (Api-7-*O*-Api*f*-6,8-di-*C*-Glc*p*) in free fractions which showed a negative loading was the main contributor of PC1, suggesting that PBA Gunyidi and PBA Jurien from both locations contained lower **F3** than others. Moreover, PC2 evidently separated the examined samples basing on their grown locations with **F5** (Api-7-*O*-Glc*p*) being the major contributor.

4.4. Conclusions

Up to the present time, lupin seed coat is a low-value animal feed and a waste disposal issue for lupin flour millers. In this study, three flavones (apigenin-7-*O*-β-apiofuranosyl-6,8-di-*C*-β-glucopyranoside (**F3**), vicenin 2 and apigenin-7-*O*-β-glucopyranoside), one isoflavone (genistein) and one dihydroflavonol derivative (aromadendrin-6-*C*-β-D-glucopyranosyl-7-*O*-[β-D-apiofuranosyl-(1→2)]-*O*-β-D-glucopyranoside), together with several hydroxybenzoic and hydroxycinnamic acid derivatives were, for the first time, identified in ASL seed coats using HPLC-ESI-MS/MS. Mass spectrum fragmentation pathways **F3** and vicenin 2 were also proposed. The very high concentration of **F3** in the free polyphenol extracts highlighted the potential that lupin seed coat could be a good source for the compound. This study has also examined the impacts of genotype, environment and their interaction on ASL seed coat polyphenols. The results demonstrated that both free and bound polyphenols in ASL seed coat were significantly affected by all of the genotypic and environmental factors. However, the observed variation was largely attributed to genotype.

Taken together, these results of this study support and promote the idea that ASL seed coat could be further value-added by exploring the potential for it as a fibre-polyphenol bioactive ingredient, manufacturing flavonoid-fortified high fibre foods and flavonoid-based nutraceuticals, for example (Cory, Passarelli, Szeto, Tamez, & Mattei, 2018). However, future studies are required to further optimize the phenolic extraction

method and MS/MS conditions (like using positive ion mode and higher collision energy); and isolate and standardize **F3** to confirm structures (especially its glycosylation patterns) using higher resolution MS spectrometry and NMR; investigate the influences of processing, including harvest, storage, dehulling, milling and food development technologies (e.g., baking, boiling, extrusion) on polyphenol composition and bioavailability in ASL seed coat.

It is also worth to note that weaknesses of studies in Chapter 3 and 4 are that they do not include seasonal factors (different years). Moreover, due to challenges in sample collection, only two locations were selected, and their environmental conditions were similar. Further screenings, using a broader range of environmental conditions, are important to obtain more comprehensive insights on this matter. Notwithstanding, the effects of food processing on the ASL properties, as well as the interactive effects among ASL seed coats and other food components are yet unclear and merit further investigation.

The results of investigated ASL seed coat chemical and physicochemical properties, the very low level soluble dietary fibre in particular, imply that processing is required to improve the unfavourable properties of ASL seed coat, whilst maximising its polyphenol retention to enhance its feasibility in human food. In following chapters, the possibility of extrusion cooking in ASL seed coat improvement will be investigated.

CHAPTER 5

Identification of significant extrusion cooking parameters on lupin seed coat soluble dietary fibre using a fractional factorial design

Information contained in this chapter has been published as follows:

Zhong, L. Z., Fang, Z. X., Wahlqvist, M. L., Hodgson, J. M., & Johnson, S. K. (2019). Extrusion cooking increases soluble dietary fibre of lupin seed coat. *LWT-Food Science and Technology*, 99, 547-554.

ABSTRACT

Extrusion cooking is widely used to improve the functional properties of dietary fibre of food processing by-products. This chapter investigated the capacity of extrusion cooking to increase the proportion of dietary fibre in the Australian sweet lupin seed coat, which is soluble, and modify its physicochemical properties (water binding capacity and water solubility). A fractional factorial design was used to identify which of the extrusion cooking factors most affected seed coat dietary fibre composition and physicochemical properties. Extrusion cooking significantly increased soluble dietary fibre content of lupin seed coat, from 2.90 g/100 g dry basis to a maximum of 9.03 g/100 g dry basis, while decreasing insoluble dietary fibre content from 89.89 g/100 g dry basis to 82.89 g/100 g dry basis. Water solubility increased (4.02- 4.47 % vs. 5.37-9.64 % dry basis) whereas water-binding capacity slightly decreased (3.84-3.88 g/g vs. 3.15-3.73 g/g dry basis) after extrusion cooking. The screw speed, total moisture content in barrel and barrel temperature were identified as the most important processing factors. These findings suggest that extrusion cooking could be a practical technology to increase the soluble dietary fibre content in lupin seed coat and enhance health benefits of the seed coat.

5.1. Introduction

Due to multiple health benefits of dietary fibre (DF), efforts have been made to incorporate high fibre food processing by-products into human diets (Elleuch, Bedigian, Roiseux, Besbes, Blecker, & Attia, 2011). These may be derived from vegetables (e.g. onion and carrot wastes), fruits (e.g. residues after juice or wine productions), cereals (e.g. wheat bran and rice bran), oil cake, pulses (e.g. seed coat), etc. (Chapter 2). The seed-coat, at ~25% of the whole *Lupinus angustifolius* (Australian sweet lupin, ASL) seed, is the main by-product of lupin kernel flour production and has great potential to be a source of DF and phytochemicals such as phenolic antioxidants (Chapter 2-4). It is well established that physiological benefits of different DF fractions are determined by their chemical structure (Wahlqvist, 2016). For example, it has been generally assumed that insoluble dietary fibre (IDF, except resistant starch which is not in lupin seed coat) shows no or slight effect on digesta viscosity and is poorly fermentable in colon whereas soluble dietary fibre (SDF) can significantly increase digesta viscosity and be well-fermented to produce short-chain fatty acids (Kumar, Sinha, Makkar, Boeck, & Becker, 2012; O'Grady et al., 2019; Williams et al., 2019). Given that over 95 per cent of DF fraction in lupin seed coat is IDF, optimised technologies are required to increase SDF level to enhance the health benefits of lupin seed coat.

Extrusion cooking is the most widely used technology to increase SDF content in high IDF food material (Rashid et al., 2015; Wolf, 2010). During extrusion cooking, food materials are cooked in a sealed barrel under high pressure, high temperature and high mechanical shear in a short time. The process results in various chemical reactions, disruption of cell wall structures and rheological changes (Singh et al., 2007). Extrusion cooking increased SDF content of a range of high IDF sources such as pea seed coat and sugar-beet pulps , soybean residue (Jing & Chi, 2013), carrot residue (Gao, Yan, Xu, Ye, & Chen, 2015), orange pomace (Huang & Ma, 2016), wheat bran (Rashid et al., 2015), rice and rye bran (Andersson, Andersson, Jonsall, Andersson, & Fredriksson, 2017), along with beneficially modified physicochemical properties (e.g., viscosity, solubility and water binding capacity). Moreover, extrusion cooking was also indicated to increase the nutritional properties of those by-products. For example, addition of extrusion cooked soybean residue in the high fat diet lowered mice weight

gain and increased the level of serum high-density lipoprotein cholesterol, while levels of low-density lipoprotein cholesterol, total cholesterol and triglyceride in mice were decreased (Chen, Ye, Yin, & Zhang, 2014). Overall, the extrusion cooked soybean residue showed more health benefit than un-treated residue (Chen et al., 2014). Similarly, extrusion cooked potato peels bound more bile acids *in vitro* than un-extruded peels (Camire, Zhao, & Violette, 1993).

Several processing factors of extrusion cooking, including feed-rate, moisture level in barrel, barrel temperature, screw-speed, are of great impacts on the qualities of the end products. As a consequence, "factor screening" is essential when operating extrusion cooking to obtain the most desirable products (Montgomery, 2012). Screening experiments using fractional factorial designs should allow all relevant processing factors to be identified, understood and, potentially, to predict the effects and interactions of the factors. Subsequently, the factors identified can be used for optimisation (Licata et al., 2014; Montgomery, 2012).

No published work appears to have evaluated the effect of extrusion cooking on the compositional and physicochemical properties of the lupin seed coat. It is not known which of the main independent process factors might influence lupin seed coat properties. In this chapter, the fractional factorial design was used to investigate the effects of extrusion cooking factors (i.e., the seed coat particle size, moisture in barrel, barrel temperature profile, feed rate, screw speed) on lupin seed coat properties (i.e., DF compositions, water solubility, water binding capacity). Fourier transform infrared spectroscopy (FTIR) was used to evaluate any structural changes of the lupin seed coat caused by the extrusion cooking.

5.2. Materials and methods

5.2.1. Materials

Fifty kilograms (kg) of clean and dried ASLseed coats (Coromup) were kindly provided by Coorow Seeds Company (Coorow, Western Australia, Australia). The seeds were grown at Coorow (116.02°E, 29.88°S) and harvested in 2016. The seed coat was sealed in plastic barrels and stored at room temperature. The seed coats (20 kg) were further dried in a 40 °C oven overnight. Around half of the dried seed coats were then milled using a ZM 200 Retch Mill (Retsch Gmbh & Co, Haan, Germany)

and passed through (> 97 per cent) a 500 μm sieve. The remaining half were milled separately and passed through (> 97 per cent) a 1000 μm sieve. Their average particle size ($PSD_{0.5}$) were 382.03 μm and 682.58 μm respectively, as determined by a Mastersizer 2000 (Malvern Instruments Ltd, Malvern, UK).

5.2.2. Experimental design and extrusion cooking

5.2.2.1. Fractional factorial design

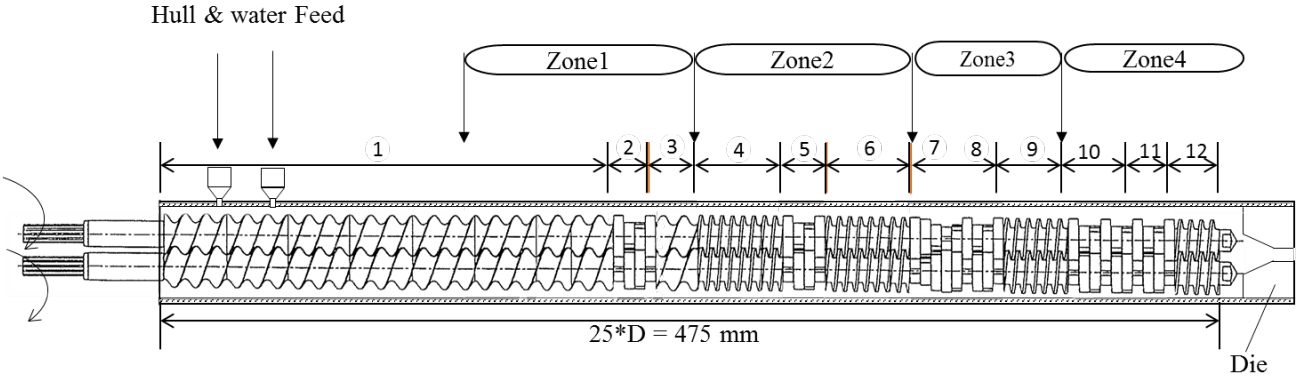
Five operation factors (independent variables) were evaluated, namely seed coat particle size (μm), barrel temperature profile (60/90/100/100 °C, 60/90/140/125 °C), total moisture in barrel (%), screw speed (rpm) and feed rate (kg/h). Design-Expert software (V11, Stat-Ease, Inc. Minneapolis, MN, USA) was used to generate the fractional factorial design runs (2_V^{5-1}, Table 5.2). Maximum (+1) and minimum (-1) extrusion levels of each factor were identified through preliminary experiments to make sure the extruder would operate properly (stable, no over-torque, over-pressure and blockage). The 3rd run and 12th run were selected to be repeated as validation runs.

5.2.2.2. Twin-screw extrusion operation

A co-rotating intermeshing twin-screw extruder (MPF 19:25, APV Baker Inc., Peterborough, England) equipped with 3 mm circular die and a twin-screw volumetric feeder (K-MV-KT20, K-Tron, New Jersey, USA) was used to perform extrusion cooking (Figure S1). The motor power and maximum screw speed of the extruder was 2200 W and 500 rpm respectively. The ratio of barrel length to screw diameter (L/D) was 25 with screw diameter (D) of 19.0 mm. The segmented screw consisted of feed screws (D), single lead screws (D) and kneading paddles (0.25D). The barrel was divided into four temperature zones (Z1/Z2/Z3/Z4) which can be set at different temperatures independently (Figure 5.1 & Table 5.1). This screw configuration has been standardised and reported to generate a great deal of pressure and shear (Licata et al., 2014). A melt pressure transducer (2076, Terwin, Bottesford, UK) was located at die block. A peristaltic pump (504 U/RL, Watson Marlow, Falmouth, UK) was used to inject room temperature distilled water through a port adjacent to the barrel (at 4D) to obtain target total moisture in barrel (corrected for moisture content in lupin seed

coat flour). A control panel was used to set and continuously monitor feed-rate, motor torque, barrel and die temperatures, and die pressure.

Figure 5.1 Schematic diagram of screws of the twin-screw extruder used in study



(manipulated basing on instructions (MPF 19:25, APV Baker Inc., Peterborough, England) of the equipment. Figure S1 is the photo of the equipment)

Table 5.1 The screw configuration of the twin-screw extruder

Order	Quantity	Length	Components
1	11	D*	Feed screws
2	4	¼ D	60° Forwarding paddles
3	1	D	Feed screw
4	2	D	Single lead screws
5	4	¼ D	60° Forwarding paddles
6	2	D	Single lead screws
7	5	¼ D	30° Forwarding paddles
8	4	¼ D	30° Reversing paddles
9	1	D	Single lead screws
10	6	¼ D	60° Forwarding paddles
11	5	¼ D	60° Reversing paddles
12	1	D	Single lead screws

*D is the screw diameter (19 mm)

About 400 g of extrudates were collected after the extruder was under steady-state to achieve stable die pressure and torque readings (after about 2 min running). Real-time motor torque, die pressure, barrel and die temperature were recorded manually at an interval time around 40 seconds during the sample collection period (about 2 min in total). Average values of torque and screw speed were used to calculate the specific mechanical energy (SME, kW·h/kg) of each run as Licata et al. (2014) described. Motor power and the maximum screw speed of the twin-screw extruder are 2.2 kW and 500 rpm respectively. The extrudates were then cooled at ambient temperature then dried at 40 °C for 24 h to achieve moisture content at 3.5-4.5%. The sample was then milled to match the particle size of raw lupin seed coat (100 per cent passed through the screens). Immediately, around 300 g was vacuum-packed in polyethylene bags and stored at 4 °C in the dark until dietary fibre analyses. Dietary fibre composition (i.e. total dietary fibre, soluble and insoluble dietary fibre), water binding capacity, and water solubility of extruded lupin seed coat were used as dependent variables of the design.

Table 5.2 Factorial independent variables with actual and coded values.

Factors	Independent variables	Units	Actual values		Coded values	
			Min	Max	Min	Max
A	Particle size[1]	μm	500	1000	-1	+1
B	Highest barrel temperature[2]	°C	100	140	-1	+1
C	Screw speed	rpm	300	400	-1	+1
D	Total moisture in barrel	%	35	46	-1	+1
E	Feeding rate	kg/h	3.5	6	-1	+1

[1] The screen sizes which material was passed through, the actual particle size distributions are shown in Section 5.2.1.

[2] The highest barrel temperature set at zone 3, temperature profiles are 60/90/100/100 ℃, 60/90/140/125 ℃ respectively.

5.2.3. Determination of dietary fibre

Insoluble dietary fibre (IDF), dietary fibre soluble in water but precipitated in 78% aqueous ethanol (SDF) of the extrusion cooked lupin seed coats (n=16) and the initial

seed coat (n=2) were determined, in duplicated, using Megazyme K-TDFR kit (Megazyme International Ireland Ltd, Bray, Ireland) (Section 3.2.5).

5.2.4. Free and bound phenolic compounds extraction and quantification of individual polyphenols using HPLC-DAD

Free and bound polyphenols were extracted using 80% methanol and alkaloid hydrolysis method as described in Section 3.2.6. Individual phenolics were quantified using validated HPLC-DAD methods as described in Section 4.2.4.

5.2.5. Determination of water-binding capacity and water solubility of lupin seed coat

The water-binding capacity (WBC) and water solubility (SOL) of lupin seed coat flours before and after extrusion cooking were determined as described in Section 3.2.3.

5.2.6. Fourier transform infrared spectroscopy (FTIR)

To investigate the structure changes in lupin seed coat fibres induced by extrusion cooking, IDF and SDF fractions of initial seed coat (500 μm) and extruded seed coat of the 12th run (due to the highest SDF among all runs) were obtained independently using the same protocols as the Section 3.2.5, except that SDF fraction was collected using centrifugation (5000 ×*g*, 25 °C for 25 min) instead filtration. Then, the intact initial seed coat (500 μm) and extruded samples of the 12th run, along with their fractionated IDF (around 2 g) and SDF (around 0.5 g) fractions were frozen dried, vacuum packaged and analysed by FTIR. The FTIR spectra were recorded by a Thermo Scientific Nicolet iS50 FTIR spectrometer, which was coupled to a Smart iTR Attenuated Total Reflectance (ATR) sampling accessory (Thermo Scientific, Madison, WI, USA) (Ciftci, Ubeyitogullari, Huerta, Ciftci, Flores, & Saldaña, 2017). FTIR spectra were recorded across the range 4000-400 cm^{-1} at a spectral resolution of 4 cm^{-1} with the co-addition of 64 scans. A background spectrum was recorded from the clean diamond ATR crystal before each sample, with the co-addition of 64 scans. Post-processing was performed using OPUS v7.0 (V7.0, Bruker, Ettlingen, Germany), and the FTIR spectra were background corrected and vector normalised across the range 1100-1000 cm^{-1}, similar to methods described by others (Chylinska, Szymanska-Chargot, Kruk, & Zdunek, 2016).

5.2.7. Statistical analysis

All the results were calculated on a dry basis (db) and expressed as mean ± standard deviation (n≥2). Design-Expert software (V11, Anderson & Whitecomb, 2018) was used to generate the model and analyse the results. Incremental changes of independent variables caused by extrusion cooking were inputted as responses of the design. The most significant three factors on each response (i.e. SDF, IDF, TDF, SOL and WBC) were selected using Daniel half-normal plot, along with ANOVA analyses generated by the Design-Expert. The adequacies of the selected best-fitting models were diagnosed using residue analyses (Montgomery, 2012), which are presented in Appendix (Appendices Section II). Pearson-correlations among dependent variables and one-way ANOVA using Tukey test among results of each independent variable were analysed using SPSS Statistics (V25, SPSS Inc., Chicago, Illinois, US).

To further process and analyse the FTIR spectra, second-derivative spectra were calculated from normalized raw data (2300-1800 cm^{-1}) using a 9-smoothing points Savitzky-Golay algorithm by using OPUS (V7.0, Bruker, Ettlingen, Germany). The Unscrambler X (Camo Software AS., Norway) was employed to perform principal component analyses (PCA) of the second-derivative spectra at 1800-650 cm^{-1} (Chylinska et al., 2016). Non-derivative and second-derivative spectrum were presented using ORIGIN (v2018 PRO, OriginLab Corporation, MA, USA).

5.3. Results and discussion

5.3.1. Twin-screw extruder operation

Raw material properties and operation parameters such as screw speed, feed rate, barrel temperature and moisture content in the barrel are the most important independent variables to affect the extrusion performance (Duque et al., 2017). During extrusion cooking in this study, the lupin seed coat lost moisture quickly to generate very dry material and large amount of steam, what likely owing to the low water-binding capacity of the seed-coat (Table 5.3). As a result, the processing could lead to blasting output and fluctuations in torque and die pressure in some cases (Chen et al., 2014). In current study, levels of the parameters as given Table 5.2 were selected carefully to avoid inconsistent output, blockage and over-torque.

After extrusion, lupin seed coat showed pale brown from the light beige of the raw one (Figure 5.2). Similar browning on citrus fibre after extrusion was reported by (Redgwell, Curti, Robin, Donato, & Pineau, 2011). The initial colour of lupin seed coat, Maillard reaction, caramelization, subsequent oxidation, degradations and polymerizations of polyphenols (especially flavonoids), as well as the potential of partial charring of the fibre were supposed to contribute the browning formation in extruded lupin seed coat (Guy & Campden, 2001). As shown in Table 5.3, the selected extrusion conditions generated a range of specific mechanical energy (SME) between 0.09 and 0.36 kW·h/ kg, which had a strong correlation with moisture content in barrel (r = -0.777, p < 0.001). The pronounced effect of moisture on SME is relatively consistent with the results previously found in citrus fibre extrusion (Redgwell et al., 2011).

5.3.2. Effects of extrusion cooking on dietary fibre composition

As presented in Table 5.3, the majority of the raw lupin seed coat is dietary fibre. Total dietary fibre (TDF) accounts for over 92% of seed coat weight, indicating it could be a suitable dietary fibre source. As expected, however, the soluble dietary fibre (SDF) level was low (< 3%). The results are slightly lower than those of reported previously (3.1-3.8%) (Table 2.3 and Table 3.4). With regard to the SDF difference due to the particle size of the seed coat, reducing particle size was suggested to increase SDF as discussed in Section 2.6.

Extrusion cooking significantly increased SDF content in the lupin seed coat, while concomitantly decreased insoluble dietary fibre (IDF) level, and collectively showed no effects on TDF content (p = 0.484) (Table 5.3). The highest increases of both 500 μm and 1000 μm seed coats were observed under the conditions of high screw speed (400 rpm), or high temperature (140 °C) but low moisture content in barrel (35%). Likewise, Wang, Klopfenstein, and Ponte (1993) reported that SDF content of extruded wheat bran increased with increasing temperature and screw speed, with a highest SDF increase being found using the same temperature in barrel and screw speed as the present study. Similarly, the highest “fibre extractability” (ratio of SDF to TDF) of extruded wheat bran and rye bran was also found at high screw speed (400 rpm), high temperature (130 °C), and low moisture in barrel (24% for wheat and 30% for rye) (Andersson et al., 2017).

Table 5.3 Experimental levels used for each factor of fractional factorial design compared to the raw lupin seed coat and experimental results for responses.

Std	Run	Factors[1]					SME[2]	Responses				
		A	B	C	D	E		SDF[3]	IDF[3]	TDF[3]	WBC[4]	SOL[4]
		µm	°C	rpm	%	kg/h	kWh/kg	g/100 g db	g/100 g db	g/100 g db	g/g db	% db
Raw lupin seed coat¶												
	C500	500	-	-	-	-	-	2.90±0.49^{a}	89.89±0.16^{e}	92.79±0.65^{b}	3.84±0.00^{i}	4.47±0.37^{a}
	C1000	1000	-	-	-	-	-	2.20±0.07^{a}	90.72±0.38^{e}	92.94±0.45^{b}	3.88±0.08^{i}	4.02±0.14^{a}
Extrusion experiments												
5	1	500	100	400	35	3.5	0.34	6.43±0.43^{cd}	85.99±0.15^{bc}	92.42±0.58^{b}	3.45±0.06^{cdefg}	7.47±0.37^{efg}
9	2	500	100	300	46	3.5	0.22	5.44±0.72^{bc}	87.30±0.72^{cd}	92.74±0.00^{b}	3.19±0.15^{ab}	6.28±0.04^{cdfe}
3	3	500	140	300	35	3.5	0.36	8.01±0.14^{df}	82.30±0.21^{a}	90.32±0.34^{a}	3.41±0.00^{bcdefg}	9.15±0.39^{ij}
4	4	1000	140	300	35	6	0.14	6.20±0.21^{bcd}	86.08±0.06^{bc}	92.28±0.15^{b}	3.49±0.09^{defgh}	7.86±0.27^{ghi}
15	5	500	140	400	46	3.5	0.19	6.63±0.28^{cde}	85.30±0.45^{b}	91.92±0.18^{ab}	3.15±0.06^{a}	6.98±0.69^{defgh}
16	6	1000	140	400	46	6	0.09	5.56±0.40^{bc}	86.47±0.14^{bc}	92.03±0.54^{ab}	3.23±0.02^{abc}	6.44±0.05^{cdefg}
8	7	1000	140	400	35	3.5	0.25	7.27±0.12^{de}	85.43±0.21^{b}	92.71±0.09^{b}	3.64±0.00^{gh}	8.40±0.34^{hij}
12	8	1000	140	300	46	3.5	0.16	4.90±0.56^{b}	87.94±0.16^{d}	92.84±0.40^{b}	3.24±0.06^{abcd}	5.93±0.41^{bcdf}
1	9	500	100	300	35	6	0.15	5.89±0.25^{bcd}	86.15±0.51^{bc}	92.03±0.27^{ab}	3.73±0.05^{hi}	6.45±0.35^{cdefg}

Std	Run	Factors[1]					SME[2]	Responses				
		A	B	C	D	E		SDF[3]	IDF[3]	TDF[3]	WBC[4]	SOL[4]
		µm	°C	rpm	%	kg/h	kWh/kg	g/100 g db	g/100 g db	g/100 g db	g/g db	% db
13	10	500	100	400	46	6	0.17	5.59±0.21^{bc}	86.34±0.12^{bc}	91.93±0.09^{ab}	3.28±0.04^{abcde}	5.37±0.45^{bc}
10	11	1000	100	300	46	6	0.15	5.26±0.12^{bc}	87.26±0.62^{cd}	92.52±0.50^{b}	3.51±0.10^{efgh}	5.52±0.86^{bcd}
7	12	500	140	400	35	6	0.22	9.03±0.47^{f}	82.89±0.20^{a}	91.91±0.27^{ab}	3.40±0.01^{abcdefg}	9.64±0.08^{j}
14	13	1000	100	400	46	3.5	0.2	4.88±0.28^{b}	87.92±0.42^{d}	92.80±0.70^{b}	3.33±0.10^{abcde}	6.91±0.05^{defgh}
2	14	1000	100	300	35	3.5	0.25	6.49±0.51^{cd}	85.83±0.40^{b}	92.32±0.91^{b}	3.55±0.00^{ghi}	7.23±0.27^{efgh}
6	15	1000	100	400	35	6	0.2	6.65±0.14^{cde}	86.63±0.07^{bcd}	93.28±0.21^{b}	3.52±0.03^{efgh}	7.21±0.06^{efgh}
11	16	500	140	300	46	6	0.13	6.06±0.47^{bcd}	86.34 ±0.32^{bc}	92.40±0.79^{b}	3.24±0.02^{abcd}	5.82±0.19^{bcdf}

¶Raw lupin seed coat was was not part of the design.

[1] See Table 1 for the code for each letter.

[2] SME, specific mechanical energy, was calculated as Licata et al. (2014) described.

[3] SDF, soluble dietary fibre; IDF, insoluble dietary fibre; TDF, total dietary fibre;

[4] WBC, water binding capacity; SOL, water solubility; Data are represented as mean ± standard deviation (n = 3).

Results are expressed as mean ± standard deviation (n = 2).

Means assigned with different letters in the same column indicate significant differences ($p < 0.05$).

The ANOVA of the factorial analysis revealed that the model for incremental changes of SDF was significant and had an adequate fit (Table 5.3 & 5.4, Table S 1). To further test the precision of the model, extrusion cooking processes of the 3rd and 12th run were duplicated, and their dietary fibre compositions were analysed and listed in Table S 5. Results showed that their SDF, IDF and TDF were respectively not significantly different ($p > 0.05$, using one-sample t-tests) from the values presented in Table 5.3.

According to the model, the moisture in barrel showed the most significant effects on SDF, followed by barrel temperature and screw speed (Figure S 2A). On the contrary, only temperature and moisture in barrel displayed significant effects on IDF, while no significant effects of any of the extrusion factors were observed on TDF. The results are consistent with those of Andersson et al. (2017) and Rashid et al. (2015) who specified the same three significant factors for extruded wheat bran and rye bran. According to the main effect plots, screw speed presented positive effect on SDF increase. Conversely, moisture content in barrel showed a negative effect. Extrusion cooking has been suggested to disrupt the macro- and micro-structure of fibre matrix, break down bonds in polysaccharides, resulting IDF degradation (Berrios, 2011; Wolf, 2010). Increasing screw speed can shorten the residence time but generate higher shear stress and friction which may (1) facilitate breakage of cell wall and fibre matrix; (2) accelerate mixing operation and heat transfer, enable to build up high mechanical energy and temperature (Duque et al., 2017). A high moisture content in barrel may moderate shearing forces and consume more energy to develop steam (Section 3.1) which thereby reduce the energy exerted on the material (Alam et al., 2016). This was supported by the observed significant and positive correlation ($r = 0.525$, $p = 0.037$) between SDF and the SME. The SME is the indicator of the total mechanical energy input on per unit of extrudate (Duque et al., 2017).

Extrusion cooking has been commonly reported to increase SDF level of high fibre food by-products (Section 2.6.2). For example, SDF in pea seed coat was doubled by extrusion cooking using a twin-screw extruder (Ralet et al., 1993b). Likewise, an increase of peas seed coat SDF using a single-screw extruder, from 5.3% to 6.7%, was also reported by Arrigoni et al. (1986). In current study, strong and negative correlations between SDF and IDF ($r = - 0.963$, $p < 0.0001$) and their incremental changes ($r = - 0.832$, $p < 0.001$) were found, which implied that extrusion cooking

transformed IDF to SDF. This conversion was previously found in extruded soybean seed coat (Dust, Gajda, Flickinger, Burkhalter, Merchen, & George C. Fahey, 2004) and wheat bran, rice bran and soybean residues (Chen et al., 2014; Yan et al., 2015), and the conversion rate varied due to processing conditions and material properties.

Table 5.4 Analysis of variance (ANOVA) for the fractional factorial model for incremental changes of each response.

Response		Model	A-PSD	B-Final barrel temperature	C-Screw speed	D-Total moisture	E-Feed rate
SDF	F	14.5850		15.5948	4.4757	42.5145	
	p	<0.0001		0.0006	0.0353	<0.0001	
IDF	F	10.8163	9.3229	8.8360		14.2901	
	p	0.0010	0.0100	0.0116		0.0026	
WBC	F	22.7057				22.7057	
	p	0.0003				0.0003	
SOL	F	15.4093		7.1225		23.6960	
	p	0.0004		0.0193		0.0003	

SDF, soluble dietary fibre; IDF, insoluble dietary fibre; WBC, water binding capacity; SOL, water solubility.

See supplemental information Section II for complete results of the ANOVA analyses.

5.3.3. Effects of extrusion cooking on water-binding capacity and water solubility

Extrusion cooking reduced water-binding capacity (WBC) of lupin seed coat by 3.1 g/g db to 18.1 g/g db (Table 5.3). According to the ANOVA of the factorial analysis, only moisture content in barrel showed significant effects on WBC that was positive (Table 5.4; Table S 3). These decreases in after extrusion cooking are consistent with those found in pea seed coat and sugar-beet pulps (Ralet et al., 1993a), as well as citrus fibre (Redgwell et al., 2011), but contrast with those of orange pomace (increased up to 16.03%) (Huang et al., 2016) and yellow pea seed coat (no significant changes) (Arrigoni et al., 1986). Extrusion cooking can significantly change morphology of fibres like reducing particle size, increasing specific surface area and specific porosity, what affect the WBC (Duque et al., 2017). The breakdown of fibre matrix and losses

of SDF which were removed along with the soaking water may have contributed to the WBC decreases. Fibres with different chemical compositions show differences in resistances to extrusion cooking (Ralet et al., 1993a).

The water solubility (SOL) of extruded lupin seed coat ranged from 5.37% to 9.64%, which are significantly higher than those of un-extruded controls (4.02% - 4.47%) (Table 5.3). Both barrel temperature and moisture content in barrel were the significant factors for changes of SOL (Table 5.4; Table S 4). Moreover, SOL increased with increasing of barrel temperature, whereas moisture content in barrel showed an inverse relationship. A strong and positive correlation between SOL and SDF ($r = 0.908$, $p < 0.001$) was found and implied that the increases in SOL may be attributed to the increases in SDF (Huang et al., 2016). SOL which can be determined using easy and fast procedures thus could be considered as an indicator of SDF. Furthermore, SOL was also well correlated to the SME ($r = 0.603$, $p = 0.013$). The same correlations were previously found in extruded wheat bran (Ralet, Thibault, & Della Valle, 1990) and citrus fibre (Redgwell et al., 2011).

*: passed through 500 µm and 1000 µm sieve respectively.

Figure 5.2 Lupin seed coats before and after extrusion cooking.

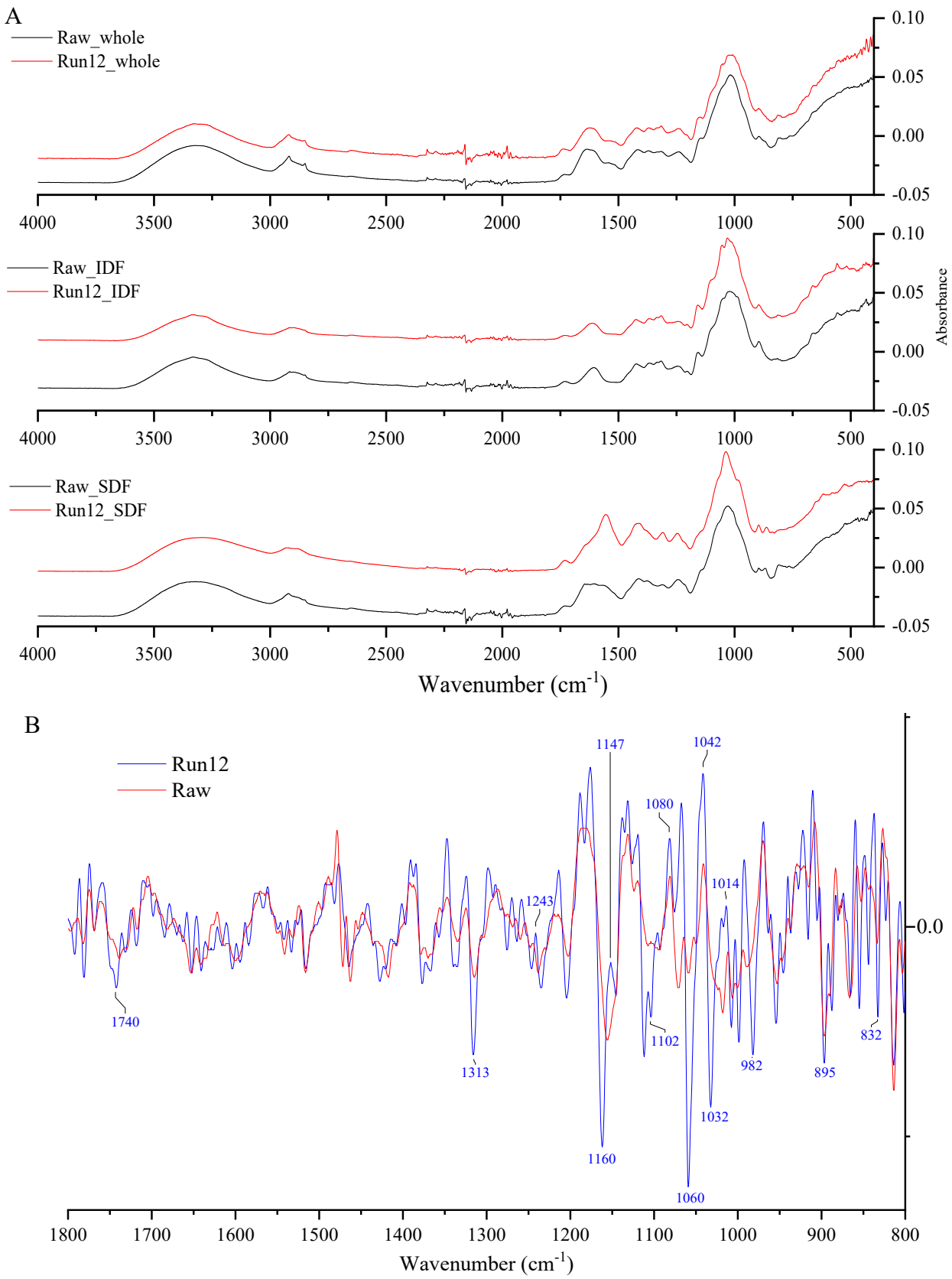


Figure 5.3 Non-derivative (A) FTIR spectrum of raw lupin seed coat and extruded seed coat of the 12th run and second-derivative spectrum (B) of raw and the 12th run seed coat in the range of 1800-800 cm^{-1}.

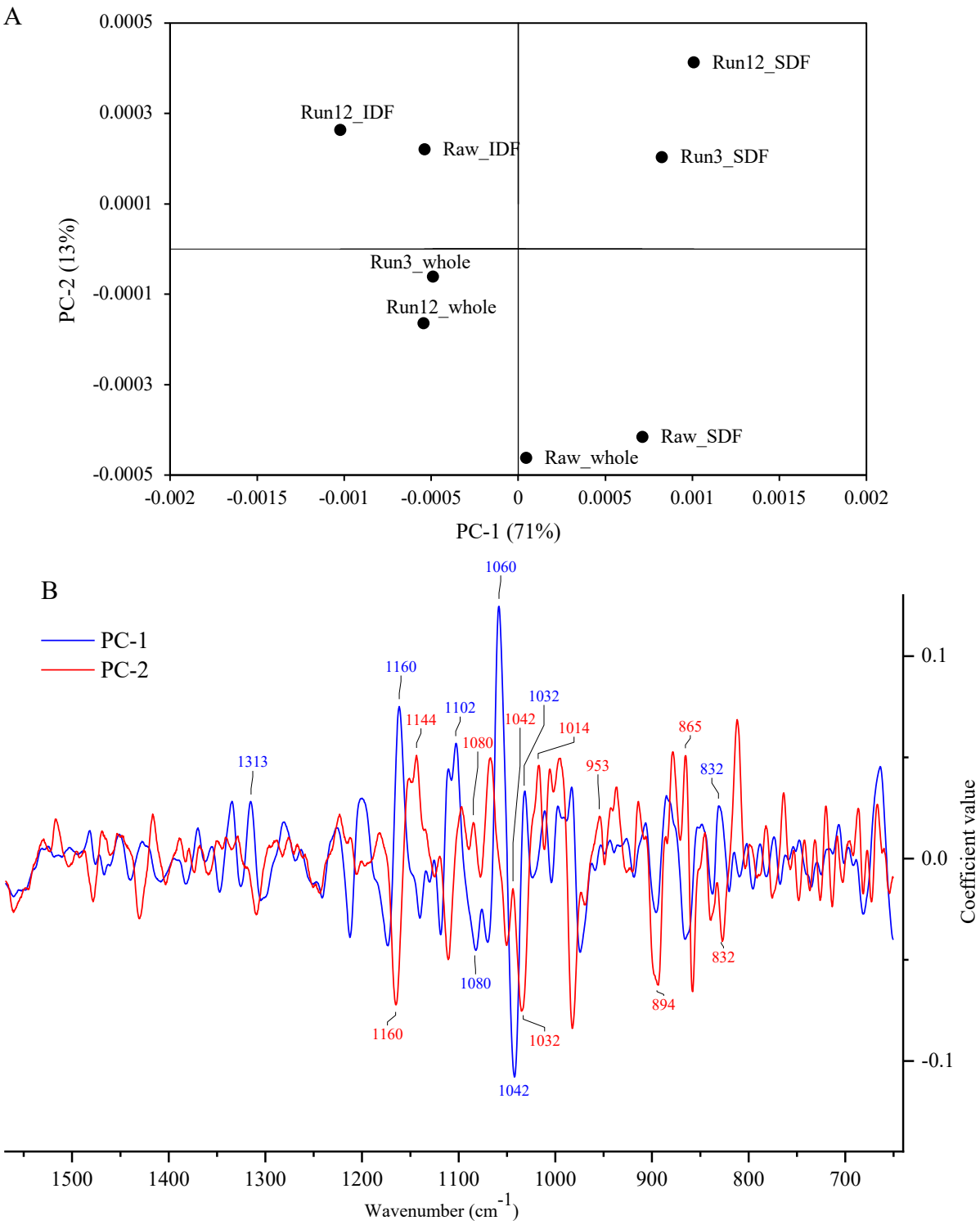


Figure 5.4 PCA scores plot (A) and loadings plot (B) of the soluble dietary fibre (SDF), insoluble dietary fibre (IDF) and whole seed coats based on the second-derivative FTIR spectra at region of 1800-650 cm^{-1}.

5.3.4. Fourier transform infrared (FTIR) spectroscopy

As seen in Figure 5.3A, FTIR spectra of lupin seed coats exhibited typical carbohydrates profiles (Chylinska et al., 2016), and were in close agreement with those reported by Ciftci (2017). A broad band region at 3600 cm^{-1} to 2800 cm^{-1} was found due to the overlapping stretching vibrations of OH and CH groups (Alemdar & Sain, 2008). Within the fingerprint region (1850 - 800 cm^{-1}), the band at 895 cm^{-1}, can be assigned to the characteristic wavenumber of cellulose (C-H bending, β-linkages between the sugars) (Chylinska et al., 2016; Ciftci, 2017). Intriguingly, after extrusion cooking, the big band at 1014 cm^{-1} (C-O and C-C stretching of pectin) observed in the raw lupin seed coat was overlapped by two new bands, 1032 cm^{-1} and 1008 cm^{-1}, which are C-O and C-C stretching of cellulose (Figure 5.3) (Kacurakova, Capek, Sasinkova, Wellner, & Ebringerova, 2000; Szymanska-Chargot & Zdunek, 2013). After extrusion cooking, increased intensities of the band at 1042 cm^{-1} and 1740 cm^{-1} were found, which because of the C-O and C-C stretching of xyloglucan, and the C = O stretching vibration of alkyl esters of pectin respectively (Szymanska-Chargot et al., 2013). Additionally, the observed bands at 1370 cm^{-1} and 1147 cm^{-1} may also originate from xyloglucan (Szymanska-Chargot et al., 2013). Xylan is the backbone of the hemicellulose in the lupin seed coat (Evans et al., 1993).

FTIR spectra of IDF from extruded lupin seed coats, especially in the 800-1400 cm^{-1} region, were in accord with that of cellulose purified from lupin seed coat (Ciftci, 2017), and of commercial cellulose (Chylinska et al., 2016), with absorbance maxima located at 1102 cm^{-1}, 1059 cm^{-1}, 1032 cm^{-1}, and 1006 cm^{-1} (Figure 5.3A). In contrast, SDF fraction from extruded lupin seed coat showed absorbance bands that could be assigned to low-methylated pectin: 1726; 1411; 1313 and 1247 cm^{-1} (Szymanska-Chargot et al., 2013). The band centred at 832 cm^{-1} was only found in SDF fractions, and a likely assignment is the ring vibration of pectin. The absorbance bands in SDF factions support the results that SDF after extrusion cooking mainly consists of pectins and hemicelluloses (Ralet et al., 1993a; Redgwell et al., 2011).

Similar distinctions among the dietary fibre fractions are also found using PCA analysis (Figure 5.4). At the region of 1800-650 cm^{-1} of the second-derivative spectra, two components which explained 71% and 13% of variability respectively are identified (Figure 5.4A). The loadings plot (Figure 5.4B) indicates the main

contributors to group the fractions. Whole seed coats before and after extrusion cooking are separated by PC1 (Figure 5.4A). The strong positive loading value at 1160 cm^{-1} and 1060 cm^{-1} of PC1 could be due to the cellulose and hemicellulose respectively (Chylinska et al., 2016). In contrast, PC2 of the PCA scores plot clearly distinguishes SDF fractions of extrusion cooked seed coats from the counterpart of raw seed coat (Figure 3A). The separation mainly based on the positive peaks at 1144 cm^{-1} (C-C stretching), 1080 cm^{-1} (C-C stretching) and 1004 cm^{-1} (C-O stretching), all are attributed to pectin. Besides, negative peaks at 1032 cm^{-1} (C-O valence) and 894 cm^{-1} (C-H bending) could be assigned to hemicellulose and pectin respectively (Cao, Yang, Han, Jiang, & Ji, 2015). Nevertheless, IDF fractions of all seed coats are grouped in the same zone. The separations and the loading plots collectively reflect the chemical changes in lupin seed dietary fibre caused by the processing, particularly in SDF fractions.

5.4. Conclusions

The soluble dietary fibre content of the lupin seed coat is increased by extrusion cooking, by up to 3-fold compared to the raw material, along with a significant decrease in insoluble dietary fibre. Water solubility is improved, but water-binding capacity is lowered by the extrusion cooking processing. Screw speed, moisture content in barrel and barrel temperature are identified as the most important processing factors affecting the seed coat fibre composition and physicochemical properties. These results demonstrate that extrusion cooking could be a feasible option to manufacture lupin seed coat into a food ingredient with increased SDF level. To maximise soluble dietary fibre level of ASL seed coat and simultaneously high polyphenol retention, next chapter will use a more robust design to optimise the identified significant processing factors.

CHAPTER 6

Multi-response surface optimisation of extrusion cooking on modification of functional properties of lupin seed coat

ABSTRACT

In chapter 5, twin-screw extrusion cooking has been indicated to increase desirable soluble dietary fibre (SDF) in Australian sweet lupin seed coat. In this chapter, we optimised the co-rotating twin-screw extrusion cooking processing using a central composite rotatable design to increase SDF in lupin seed coat from 4.42 g/ 100 g up to 11.37 g/ 100 g dry basis. The high levels of polyphenols in the seed coat were retained while the extruded seed coat had enhanced antioxidant capacities simultaneously based on desirability function. The optimal twin-screw extrusion conditions were validated. The results demonstrate multiple applications of twin-screw extrusion technology in processing high fibre by-products.

6.1. Introduction

Soluble dietary fibre (SDF) generally has more desirable technological functionalities and health benefits as discussed in Chapter 5 (Elleuch et al., 2011). In this context, however, over 80 per cent of the lupin seed coat is insoluble dietary fibre (IDF) such that an extrusion cooking technology was employed to elevate SDF level in the lupin seed coat. In chapter 5, a fractional factorial design (FFD) as a first-order study of response surface methodology (RSM) was conducted to identify significant processing parameters of the extrusion cooking process that affect SDF (Duque et al., 2017). However, given "the sequential nature of RSM", two stages of studies are required, namely a first-order screening study to identify those most important processing factors and a second-order study (central composite rotatable design (CCRD) for example) to obtain a more robust model to optimise the processing (Montgomery, 2012; Myers, Montgomery, & Anderson-Cook, 2016). The first-order screening study has demonstrated that twin-screw extrusion cooking could be a feasible approach to increase SDF content in seed coat of Australian sweet lupin (Chapter 4). In addition,

the study identified three extrusion parameters, i.e., extrusion temperature, total moisture in barrel and screw speed, were the main factors of the SDF increases.

To further investigate the capacity of extrusion cooking on increasing SDF level in the lupin seed coat, a second order, CCRD, was used to optimise the three identified significant factors from the previous study. Dietary fibre composition, total and individual phenolics, as well as antioxidant capacities, were used as the dependent variables (also referred to responses). Simultaneous optimisation using the desirability function was conducted to optimise the process.

6.2. Materials and methods

6.2.1. Materials

Around twenty kilograms of *Lupinus angustifolius* seed coat (Coromup) provided by Coorow Seeds Company (Coorow, Western Australia, Australia) were dried, milled and passed through 500 μm sieve (>98 per cent) as previously described (Section 5.2.1). The particle size of the material was selected according to the results obtained from screening experiment (Chapter 5).

6.2.2. Reagents

Reagents were purchased as described in Section 3.2.2

6.2.3. Experimental design

6.2.3.1. Response surface methodology (RSM)

The central composite rotatable design (CCRD) was generated and analysed using the Design-Expert software (V11, Stat-Ease Inc. Minneapolis, MN, USA). Three independent variables (processing parameters) identified in the first-order model, namely extrusion temperature, total moisture in barrel and screw speed were investigated (Chapter 5). As shown in the design matrix, the CCRD consists of a total of 20 experimental runs (Table 6.2). The analytical ranges were set according to the preliminary factorial design (Chapter 5). The experiments were separated into two blocks (two halves) such that the experimental runs can be conducted on two consecutive days.

Total dietary fibre (TDF), soluble dietary fibre (SDF), insoluble dietary fibre (IDF), total polyphenols content (TPC), individual polyphenols and antioxidant capacities (DPPH, ABTS, ORAC) of both free and bound polyphenol extracts were analysed as the dependent variables (responses). A polynomial quadratic regression equation was used to describe the effects of the three identified independent variables on the selected dependent variables:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j$$

Where Y represents the dependent variable; X_i and X_j are selected independent variables (vary from 1 to 3); β_0 denotes the intercept coefficient, while β_i, β_{ii}, and β_{ij} are the first order, quadratic and interaction coefficients, respectively. Each dependent variable was analysed separately, and their most-fitting models were established independently based on the following criteria as suggested by Myers et al. (2016): (1) model is significant with *p* being lower than 0.05; (2) lack-of -fit is insignificant with $p > 0.10$; (3) the adequate precision is greater than 4; (4) acceptable residual tests by examining diagnostic plots (normal plot, Cook's Distance) generated by the software. All the mentioned information of each model is summarised in Table 6.3, while the diagnostic plots are presented as Appendices (Section III).

Table 6.1 Central composite rotatable design (CCRD)

Factors	Independent variables	Units	Actual values		Coded values	
			Low	High	-α	+α
A	Highest barrel temperature*	°C	120	150	109.77	160.23
B	Screw speed	rpm	300	400	265.91	434.09
C	Total moisture in barrel	%	30	40	26.59	43.41

* The highest barrel temperature set at zone 3, temperature profiles are 70/100/120/120 °C, 70/100/150/125 °C, 70/100/110/110 °C, 70/100/160/125 °C, respectively.

6.2.3.2. Simultaneous optimisation using desirability function

The global desirability function (*D*) with relative "importance" degrees of the variables was performed on the Design-Expert software to simultaneously optimise the selected

dependent variables (Myers et al., 2016). The "importance" degree (r_i) varies from 1 for the least importance to 5 for the most importance. The desirability function is a combination of each desirability of all dependent variables, which accompanies with "its own error" and "compromises between important responses" (Candioti, De Zan, Camara, & Goicoechea, 2014; Myers et al., 2016). Given the current study initially focuses on SDF level, only SDF, total free phenolic content (TFPC) which determined using Folin-Ciocalteu assay and total free individual phenolic content (TFIPC) quantified by HPLC-DAD were selected as the main responses to get the optimum output while reducing possible errors and compromises on SDF. We selected TFPC and TFIPC as the key dependent variables for two reasons: Firstly, significant correlations were observed among polyphenol content and antioxidant assays as will be detailed later. Secondly, free polyphenols were the main contributors of both the total polyphenol content and total individual polyphenol content, hence dominate the model analyses as will be highlighted later either. Consequently, the objective function (D) is shown below:

$$D = (d_1^{r_1} \cdot d_2^{r_2} \cdot \ldots \cdot d_n^{r_n})^{\frac{1}{\sum r_i}} = (\prod_{i=1}^{n} d_i^{r1})^{\frac{1}{\sum r_i}}$$

where d_1, ..., d_n represent the individual desirability function for each dependent variables; n denotes the number of dependent variables (n =3 in this study); The "importance" degree (r_i) varies from 1 for the least importance to 5 for the most importance in the Design-Expert software (Stat-Ease Inc., 2018).

6.2.3.3. Model verification

To obtain the maximum responses, we extrapolated the limits of independent variables and responses (namely dependent variables or "goals"). Optimal solutions (Opti), which suggested the levels of independent variables to achieve the highest levels of responses, were generated by the design (Table 6.7) (Stat-Ease Inc., 2018). Meanwhile, a second optimal solutions (Sub-opti) based on the un-extrapolated design space were separately generated (Table 6.8). Both were duplicated to validate the model. Actual SDF, total free polyphenol content (TFPC) and total free individual polyphenol content (TFIPC) were compared to the predicted outcomes.

6.2.4. Twin-screw extrusion operation

The extrusion cooking and extrudates collection were performed as described in Section 5.2.2.2, except that the extrudates from each experimental run were split into two bags and stored in 4 °C for fibre analyses and -20 °C for polyphenol characterisations respectively. The values of the three selected independent variables from the screening experiment are presented in Table 6.2. Feed rate was fixed at 4 kg per hour (feeder configuration was conducted).

6.2.5. Extrudate analyses

6.2.5.1. Determination of dietary fibre composition

A Megazyme K-TDFR kit was used to quantify the dietary fibre composition of the extrusion cooked lupin seed coats (n = 20) and the initial seed coat. Insoluble dietary fibre (IDF), soluble dietary fibre (SDF) and total dietary fibre (TDF, i.e. IDF+ SDF) were evaluated as detailed in Section 3.2.5.

6.2.5.2. Determination of total free and bound polyphenols

Free and bound polyphenols were extracted as previously described in Section 3.2.6. Individual polyphenols in extracts were identified and quantified following a validated HPLC-DAD method using an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA) (Section 4.2.4). Total polyphenols content (TPC) in free and bound extracts were evaluated using Folin-Ciocalteu assay (Section 3.2.7).

6.2.5.3. Determination of antioxidant capacities of free and bound polyphenols

DPPH, ABTS and ORAC assays were performed as Section 3.2.8 described.

Table 6.2 Experimental levels of barrel temperature (℃), screw speed (rpm) and moisture in barrel (%) used for the CCRD compared to the raw lupin seed SME (kW·h/kg) coat on dietary fibre composition (g/ 100 g db) and total polyphenol content (using Folin-Ciocalteau assay, mg GAE/100 g db).

	Factor 1	Factor 2	Factor 3		Dietary fibre			Total polyphenol content		
Run	Barrel Temperature†	Screw Speed	Moisture in Barrel	SME	SDF	IDF	TDF	Free	Bound	Total
Control¶	-	-	-	-	*4.42±0.06*	*91.00±0.45*	*95.42±0.40*	*36.11±0.31*	*18.14±1.00*	*54.26±0.69*
1	135	350	43.409§	0.14	6.73±0.24	87.25±0.98	93.98±1.22	32.02±0.54	14.63±0.02	46.65±0.56
2	120	400	40	0.17	7.04±0.34	87.41±1.26	94.45±1.60	32.30±1.71	15.25±0.36	47.55±1.35
3	150	300	30	0.27	9.05±0.23	83.91±1.00	92.95±1.23	41.21±0.69	13.72±0.07	54.93±0.76
4	150	400	30	0.29	9.04±0.49	84.12±0.48	93.16±0.01	41.84±1.13	15.17±0.93	57.01±2.06
5	109.773‡	350	35	0.27	7.86±0.47	85.78±1.40	93.64±1.87	34.30±1.13	16.74±0.67	51.04±1.80
6	135	265.91‡	35	0.22	8.99±0.28	85.39±1.22	94.38±1.50	36.99±0.30	13.29±0.53	50.29±0.83
7	135	350	35	0.23	8.89±0.16	85.86±0.67	94.75±0.83	37.93±0.36	13.93±0.08	51.86±0.28
8	150	300	40	0.17	8.20±0.19	86.83±0.66	95.04±0.85	32.27±1.31	16.76±0.11	49.03±1.41
9	135	350	35	0.23	8.72±0.12	85.62±0.52	94.33±0.64	36.78±0.55	15.44±0.70	52.21±1.25
10	120	300	40	0.18	7.75±0.01	87.69±1.15	95.43±1.16	30.59±0.92	15.61±0.21	46.20±0.71
11	135	434.09‡	35	0.27	8.96±0.10	86.07±1.06	95.02±1.16	40.51±0.59	12.12±0.31	52.63±0.29
12	135	350	26.591§	0.34	10.38±0.40	83.33±0.19	93.70±0.21	45.70±0.50	12.96±1.11	58.66±0.60

Run	Factor 1	Factor 2	Factor 3	SME	Dietary fibre			Total polyphenol content		
	Barrel Temperature†	Screw Speed	Moisture in Barrel		SDF	IDF	TDF	Free	Bound	Total
13	150	400	40	0.19	7.55±0.02	85.49±0.11	93.04±0.10	33.67±1.31	16.65±0.18	50.32±1.49
14	135	350	35	0.25	8.98±0.21	84.69±1.06	93.67±1.26	36.39±1.51	13.13±0.77	49.51±0.73
15	120	300	30	0.23	8.50±0.00	84.79±0.20	93.29±0.20	36.92±0.67	13.91±0.95	50.84±1.62
16	135	350	35	0.25	9.04±0.13	84.63±0.66	93.66±0.53	37.22±1.99	13.80±0.99	51.02±1.00
17	120	400	30	0.34	9.56±0.06	83.09±0.15	92.66±0.09	43.48±1.25	13.96±1.86	57.44±3.12
18	160.227‡	350	35	0.25	8.55±0.24	85.23±0.37	93.78±0.60	36.93±0.93	12.90±0.93	49.84±1.86
19	135	350	35	0.25	9.28±0.18	84.07±0.14	93.35±0.32	39.22±0.44	12.81±1.43	52.03±1.88
20	135	350	35	0.25	9.12±0.25	84.84±1.21	93.96±0.95	37.41±2.56	13.69±0.01	51.11±2.57

† The highest barrel temperature set at zone 3 (Figure 5.1), temperature profiles are 70/100/120/120 °C, 70/100/150/125 °C, 70/100/110/110 °C, 70/100/160/125 °C, respectively.

‡ Rounded to integer in actual operation.

§ Rounded to one decimal in actual operation

¶Control was un-extrusion cooking lupin seed coat, and it was not part of the design.

6.2.6. Statistical analysis

All the results were calculated on a dry basis (db) and expressed as mean ± standard deviation (n ≥ 2). One-way ANOVA using Tukey test among results of each independent variable (n = 2), as well as Pearson-correlations (n = 20) among specific mechanical energy (SEM) and means of each independent variable were analysed using SPSS Statistics (V23, SPSS Inc., Chicago, Illinois, US). ORIGIN (V2018 Pro, OriginLab Corporation, MA, USA), GraphPad Prism 8 (GraphPad software, USA) and RStudio (using the "Hmisc" package and "corrplot" package) (http://www.R-project.org/) were used for data visualisation.

6.3. Results and discussion

6.3.1. Effect of extrusion cooking on lupin seed coat dietary fibre composition

6.3.1.1. Soluble dietary fibre (SDF)

Extrusion cooking processing significantly increased SDF content in the lupin seed coat, ranging 6.73 to 10.38 g/100 g db after extrusion cooking versus 4.42 g/100 g db in the un-extruded sample (Table 6.2). The increases were in accordance with the screening study (Chapter 5). A quadratic model which best fit the criteria discussed in Section 6.2.3 was "suggested" by the Design-Expert software to unfold the collective impacts of the three independent variables on SDF. As anticipated, total moisture in the barrel had the most significant effect on SDF ($p < 0.0001$), followed by the barrel temperature ($p = 0.0053$) (Table 6.3 & 6.4), what are comparable with the results of Chapter 5. The underlying mechanisms were discussed in Section 5.3.2. In the present study, barrel speed showed insignificant effects on SDF, but significant interactive effects of the screw speed and total moisture in the barrel were identified. The established mathematical predictive equation for SDS are presented in Table 6.4. By examining the 3D surface plots and contour plots, moisture in barrel exhibited strong negative effects on SDF (Figure 6.1A & Figure 6.2A). In contrast, a reverse U-shaped association between barrel temperature and SDF was found: as barrel temperature increased, so did SDF but after the peak at around 138 -140 °C) the SDF decrease with a further increase in temperature. Besides, higher SDF levels were obtained at high

screw speed and low moisture content in the barrel when the barrel temperature was kept constant at 135 °C (Figure 6.1C& Figure 6.2C).

High extrusion cooking temperature is supposed to disintegrate the macro-structure of the food material, resulting in fibre degradation and increase water solubility (Alam et al., 2016). Given the strong and positive correlation between SDF and SME ($r = 0.848$, $p < 0.0001$), the downward trend of SDF under very high barrel temperature could be explained by the decreased SME under high temperature. The biomass can be softened under high temperature and therefore the material rheology undergoes significant changes, decrease in viscosity for instance, in the extruder barrel. The high temperature lowers the shear load, reflecting as reduced torque as found in the current study (Alam et al., 2016; Duque et al., 2017). A similar, albeit weaker, correlation between SDF and SME was observed in the previous screening study (Section 5.3.2). In terms of the negative effect of moisture content in barrel, high moisture in the barrel could moderate thermal energy exerted on the material and reduce shearing forces. The result was also supported by the strong negative correlation between moisture in the barrel and SME ($r = -0.895$, $p < 0.0001$) in this study and the screening study (Section 5.3.2). In fact, the torque increases as the moisture decreases (Duque et al., 2017). Nonetheless, in contrast to the screening experiment, screw speed did not show significant effects on SDF in the current study (Table 6.3). As the screw speed was increased, mechanical energy exerted on the material was increased meanwhile the retention time was decreased, resulting in complex net-effects on SDF of the seed coat.

6.3.1.2. Insoluble (IDF) and total (TDF) dietary fibre

Along with the increases of SDF after extrusion, IDF witnessed significantly decreases such that TDF being constant (Table 6.2). The same pattern, which indicates the redistribution from IDF to SDF, was widely found in extruded cereal brans, pea and soybean seed coats, as well as by-products of fruits and vegetable processing (Section 2.6.2). Multiple mechanisms were established to explain the transformation, like particle size reduction, degradation of fibres and increased enzymatic digestibility (Offiah et al., 2019). Furthermore, in agreement with the screening study (Chapter 5), only IDF data generated a significant model with the total moisture content in barrel showing significant effects (Table 6.3 & 6.4).

Table 6.3 Analysis of variance (ANOVA) of the selected models for each dependent variable

Source	SDF	IDF	TFIPC	TBIPC	TFPC	TPC	Api-Api*f*-di-Glc*p*	Api-Glc*p*	Genistein	FDPPH	FABTS	FORAC
Type of model	Quadratic	Linear	Quadratic	Linear	Linear	Linear	Quadratic	Linear	Quadratic	Quadratic	Quadratic	Linear
p-value												
Model	< 0.0001	< 0.0001	0.0033	0.0059	< 0.0001	< 0.0001	0.0137	< 0.001	0.0070	0.0106	< 0.0001	0.0026
A-Barrel Temperature	0.0053	0.1875	0.0471	0.9796	0.1020	0.2456	0.0429	0.5793	0.2918	0.0701	0.0024	0.3220
B-Screw Speed	0.1915	0.9605	0.0012	0.5473	0.0099	0.0627	0.0065	0.2309	0.0040	0.0608	0.0022	0.8674
C-Moisture in Barrel	< 0.0001	< 0.0001	0.0037	0.0008	< 0.0001	< 0.0001	0.8680	< 0.0001	0.0206	0.0097	< 0.0001	0.0003
AB	0.1097	-	0.6627	-	-	-	0.2211	-	0.0484	0.4599	0.2000	-
AC	0.3057	-	0.1880	-	-	-	0.1158	-	0.1357	0.1062	0.3728	-
BC	0.0007	-	0.6127	-	-	-	0.9720	-	0.0168	0.2347	0.7890	-
A^2	0.0006	-	0.2090	-	-	-	0.7999	-	0.0981	0.2394	0.0045	-
B^2	0.9374	-	0.0036	-	-	-	0.0029	-	0.7037	0.8871	0.5411	-
C^2	0.0306	-	0.0343	-	-	-	0.0684	-	0.0404	0.0043	0.0002	-
Lack of Fit	0.3066	0.0666	0.8857	0.2139	0.5391	0.2020	0.7640	0.2324	0.2022	0.0238	0.1118	0.4989

R-squared												
Adjusted	0.9289	0.7199	0.7612	0.4650	0.8413	0.7662	0.6592	0.6288	0.7115	0.6802	0.9353	0.5237
Predicated	0.7741	0.5664	0.5406	0.1552	0.7406	0.6081	0.2502	0.3895	0.0029	-0.2638	0.7616	0.2832
Adeq Precision	19.1400	15.6236	9.7193	8.6288	18.4315	15.0126	8.2272	10.6940	8.2844	7.8602	20.4691	8.5142

Table 6.4 Predictive equations for each response using their corresponding actual coefficients acquired in the models

Source	SDF	IDF	TFIPC	TBIPC	TFPC	TPC	Api-Api*f*-di-Glc*p*	Api-Glc*p*	Genistein	FABTS	FORAC
	g/ 100g db		µg/g db		mg GAE/100g db			µg/g db		mg TE/100 g db	
Intercept	-54.5366	79.0570	788.3022	-35.6094	51.2046	55.9636	809.4574	117.1403	61.9074	30.9219	401.8983
A	0.4942‡	-0.0150	0.6692‡	0.0035	0.0481	0.0474	-2.0881‡	0.0457	-0.2867	3.0278‡	0.4901
B	0.0819	-0.0002	-1.5635‡	0.0264	0.0260	0.0251	-1.7200‡	0.0321	-0.0676‡	0.0952‡	0.0259
C	1.0228‡	0.2382‡	-17.1317‡	1.7897‡	-0.8483‡	-0.9654‡	-13.5840	-1.4759‡	-0.9751‡	-8.3814‡	-7.1192‡
AB	-0.0002	-	0.0013	-	-	-	0.0030	-	0.0002‡	-0.0012	-
AC	-0.0014	-	0.0476	-	-	-	0.0469	-	0.0021	-0.0095	-
BC	-0.0016‡	-	-0.0044	-	-	-	-0.0002	-	0.0009‡	0.0007	-
A^2	-0.0014‡	-	-0.0094	-	-	-	-0.0015	-	0.0005	-0.0080‡	-
B^2	-	-	0.0024‡	-	-	-	0.0020	-	-	0.0001	-
C^2	-0.0061‡	-	0.1557‡	-	-	-	0.1042	-	0.0057‡	0.1155‡	-

A-Barrel Temperature (°C), B-Screw Speed (rpm), C-Moisture in Barrel (%); ‡ Significant coefficients ($p < 0.05$); The predictive equation for FDPPH is not presented becase of the significant *Lack of Fit* of the model (Table 6.2).

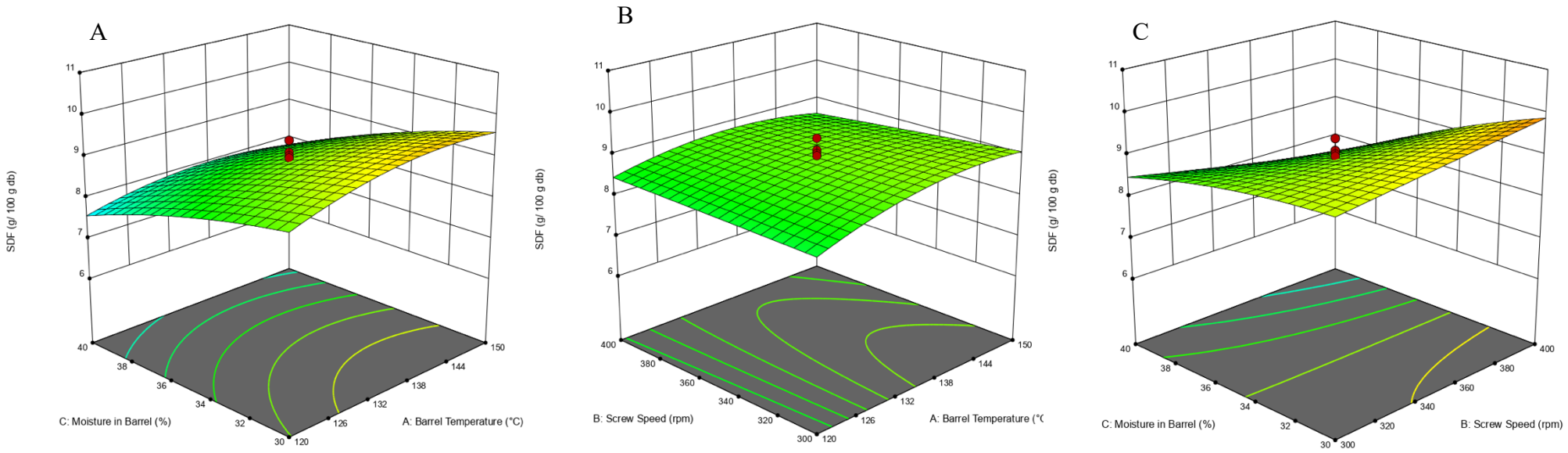


Figure 6.1 3D surface plot demonstrating the effects (A) barrel temperature and total moisture in barrel at a constant screw speed of 350 rpm; (B) barrel temperature and screw speed at moisture of 35%; (C) screw speed and total moisture in barrel at barrel temperature of 135 °C on SDF of lupin seed coat

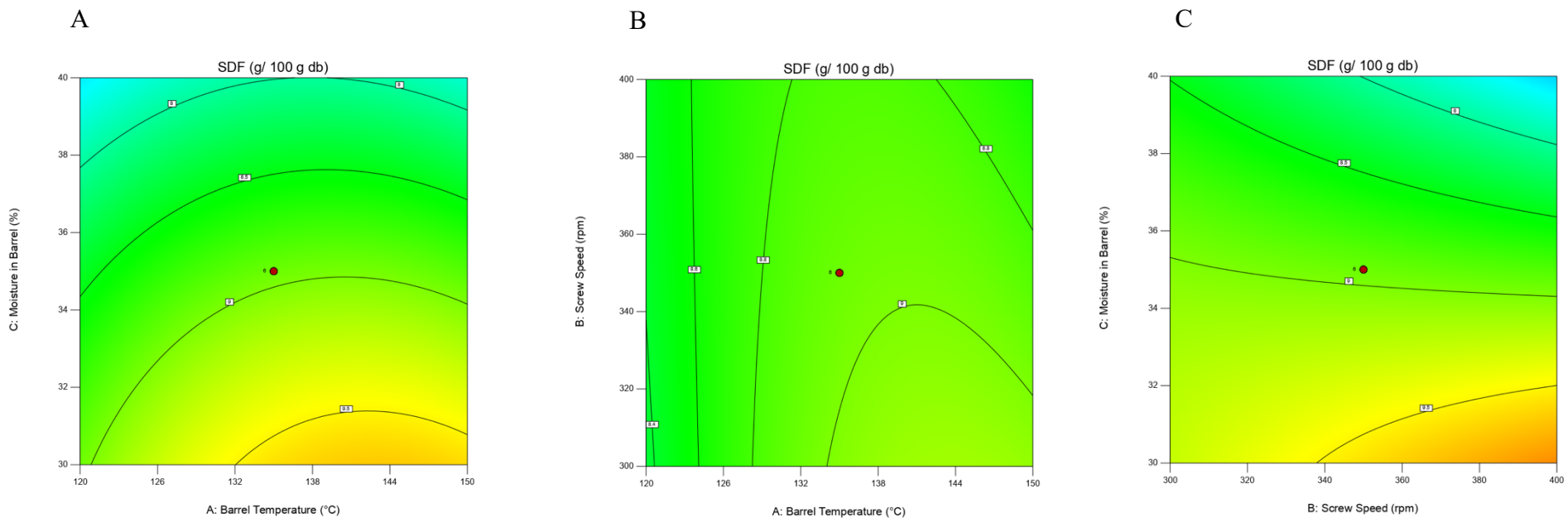


Figure 6.2 Contour plots demonstrating the effects (A) barrel temperature and total moisture in barrel at a constant screw speed of 350 rpm; (B) barrel temperature and screw speed at moisture of 35%; (C) screw speed and total moisture in barrel at barrel temperature of 135 °C on SDF of lupin seed coat

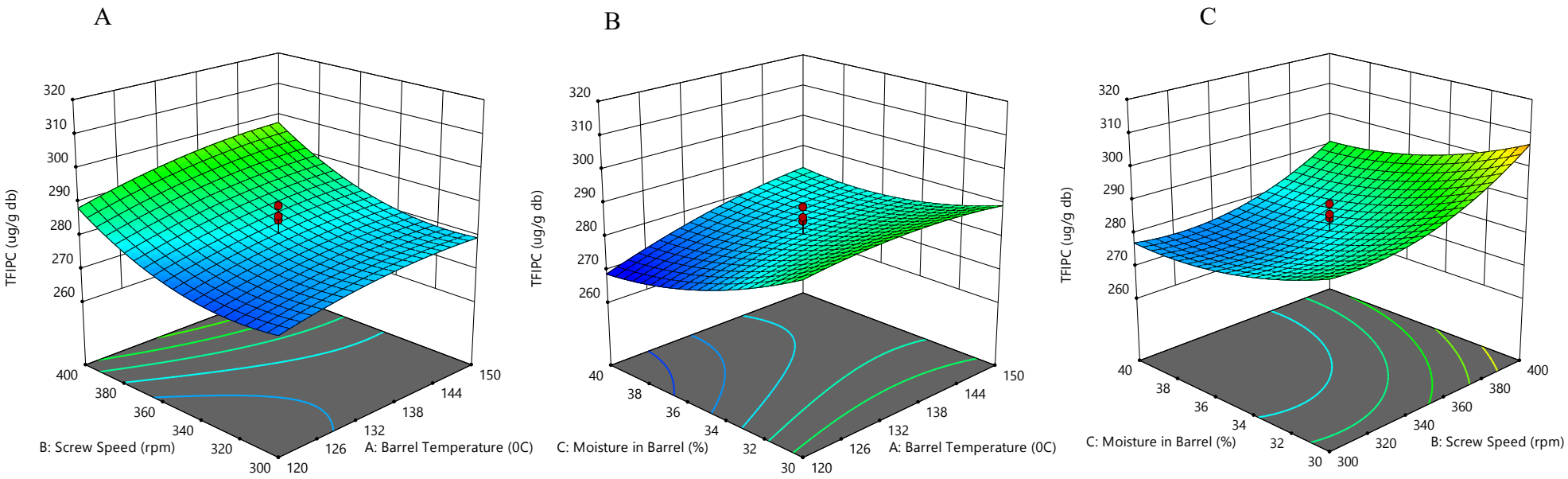


Figure 6.3 3D surface plot of TFIPC (μg/g db) demonstrating the effects (A) barrel temperature (°C) and screw speed (rpm) at moisture of 35%; (B) barrel temperature (°C) and total moisture in barrel (%) at a constant screw speed of 350 rpm; (C) screw speed (rpm) and total moisture in barrel (%) at barrel temperature of 135 °C

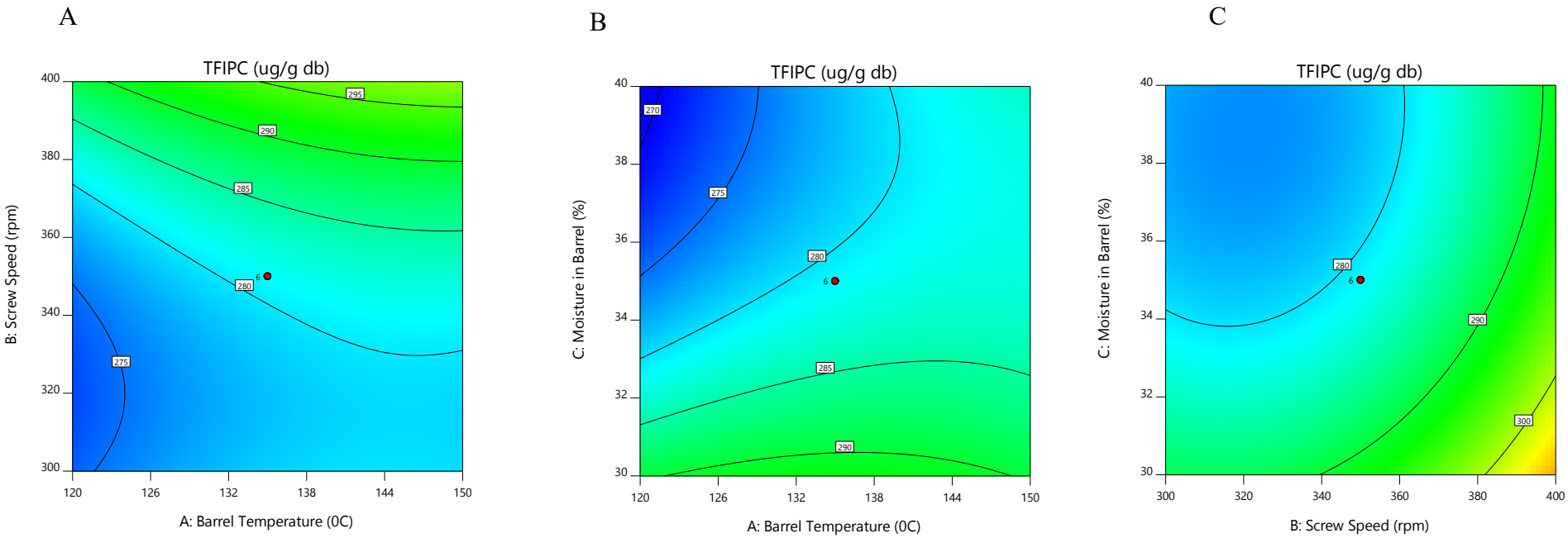


Figure 6.4 Contour plot of TFIPC (µg/g db) demonstrating the effects (A) barrel temperature (°C) and screw speed (rpm) at moisture of 35%; (B) barrel temperature (°C) and total moisture in barrel (%) at a constant screw speed of 350 rpm; (C) screw speed (rpm) and total moisture in barrel (%) at barrel temperature of 135 °C

6.3.2. Effect of extrusion cooking on total polyphenol content (TPC)

6.3.2.1. Total free polyphenol content (TFPC)

Total free polyphenol content (TFPC) of lupin seed coat was 36.11 GAE mg/100 g db, as can be seen in Table 6.2. Extrusion cooking increased TFPC levels under some circumstances (e.g., run 3, 13 and 17), while significant decreases were also observed (e.g., run 1, 2 and 10) (Table 6.2). A similar pattern in TFPC of extrusion cooked wheat bran was found by Ramos-Enriquez, Ramirez-Wong, Robles-Sanchez, Robles-Zepeda, Gonzalez-Aguilar, and Gutierrez-Dorado (2018). According to the ANOVA analysis of the selected linear model, the moisture content in barrel exerted the most significant effects on TFPC, followed by barrel temperature and screw speed (Table 6.3 & 6.4). Furthermore, temperature in the barrel and screw speed showed a slight positive effect on TFPC, whilst moisture showed a strong negative effect. It is worthy to note that TFPC in lupin seed coat before and after extrusion cooking was significantly and positively correlated with SDF ($r = 0.844$, $p < 0.0001$) and SME ($r = 0.909$, $p < 0.0001$).

6.3.2.2. Total bound polyphenol content (TBPC)

Extrusion cooking reduced TBPC, maximally by 33.2 per cent (Table 6.2). According to the ANOVA analysis, no fitting model was found for TBPC. Negative correlations between TBPC and SME (r = -0.504, p = 0.024), SDF (r = -0.614, p = 0.004), and TFPC (r = -0.600, p = 0.005) implied that extrusion cooking may liberate bound polyphenols to be free forms, reflecting as the decreased TBPC and simultaneously increased TFPC. Conversely, Ramos-Enriquez et al. (2018) reported increases in TBPC of extruded wheat bran, 248.09-384.24 mg GAE/100 g db comparing to 238.01 mg GAE/100 g db of non-extruded counterpart. The differences could be inferred from the variations in retention rates of different individual polyphenols under extrusion cooking. Moreover, lupin polysaccharides have been revealed to have radical scavenging capacities and reducing power (Thambiraj, Phillips, Koyyalamudi, & Reddy, 2018). Consequently, the Folin-Ciocalteu assay may be strongly interfered by the reducing sugars and oligosaccharides liberated by extrusion cooking and reducing compounds from Maillard reaction during the processing (Acosta-Estrada et al., 2014;

Sanchez-Rangel, Benavides, Heredia, Cisneros-Zevallos, & Jacobo-Velazquez, 2013; Wang, Hu, Nie, Yu, & Xie, 2016).

6.3.2.3.Total polyphenol content (TPC)

Total polyphenol content (TPC), e.g., TFPC plus TBPC, in extruded and initial lupin seed coats are shown in Table 6.2. Ciftci (2017) reported that TPC of Canadian grown lupin seed coat was up to 72 mg GAE/100 g db, agreeing with the current study (54.26 mg GAE/100 g db), though the authors used subcritical water technology to hydrolyse the seed coat. Not surprisingly, TFPC took up over 65 per cent of TPC in all samples such that extrusion cooking factors showed similar effects on TPC as those on TFPC. Likewise, the TFPC dominated the ANOVA analysis of TPC, giving a very similar model as the one of TFPC (Table 6.4). The 12th run which has the lowest moisture content in barrel showed the highest levels of TFPC and TPC. The phenols and tannins were indicated to be polymerised under high temperature and high moisture content extrusion, resulting in lowered extractability and antioxidant activity (Brennan, Brennan, Derbyshire, & Tiwari, 2011). Moreover, the low water content in barrel showed higher shearing force which could enhance the liberation of bound polyphenols. It is also supported by the significant and positive correlation between TPC and SME ($r = 0.906$, $p < 0.0001$), and SDF ($r = 0.794$, $p < 0.0001$).

Extrusion cooking was found to reduce TPC of brans from oat, wheat, barley and rice, up to by 73.38 per cent (Kaur, Sharma, Singh, & Dar, 2015). Conversely, the increases were also widely reported in extruded food material, like wheat bran (Ramos-Enriquez et al., 2018) and sorghum bran (Salazar Lopez et al., 2016). T. Wang, He, and Chen (2014) and Brennan et al. (2011) summarised some contrasting results on this aspect and suggested that it is likely related to extrusion processing conditions and raw material variations. Generally, the decreases may be caused by thermal degradation, and decarboxylation and polymerisation of polyphenols during extrusion (Altan, McCarthy, & Maskan, 2009). In contrast, the increases are largely explained by the disruption of cell wall matrix and thus release of polyphenols from the cell wall matrix (i.e., increased polyphenol extractability), depolymerisation of high molecular weight polyphenols (like condensed tannins) and Maillard reaction (Brennan et al., 2011; Wang et al., 2014). Taken together, the overall effects of extrusion cooking processing on TFPC and TPC were complex because of the transient phenomenon and varied

based on material properties, stabilities of individual polyphenols and operation conditions. Consequently, apart from the significant correlation between TPC and SDF, extrusion cooking did not consistently increase TPC level as it did on SDF.

6.3.3. Effect of extrusion cooking on individual polyphenols

The influences of extrusion cooking on TPC of different food material have been extensively studied but less so with respect to individual polyphenols (Brennan et al., 2011). The results of TPC, as well as antioxidant capacity assays, are strongly linked to the polyphenol profiles (Jacobo-Velazquez et al., 2009). Table 6.5 and Table 6.6 illustrate the individual free and bound phenolic compounds of both extruded lupin seed coats and un-extruded control. The results are comparable with those in Chapter 4. Briefly, apigenin- glucopyranoside (Api-Glc*p*) and genistein were found in both free and bound fraction. In contrast, cinnamic acid glucoside (CIA), aromadendrin-6-*C*-β-D-glucopyranosyl-7-*O*-[β-D-apiofuranosyl-(1→2)]-*O*-β-D-glucopyranoside (Aro-Glc*p*-Api*f*Glc*p*), and apigenin-7-*O*-β-apiofuranosyl-6,8-di-*C*-β-glucopyranoside (Api-Api*f*-di-Glc*p*) were only detected in the free faction. On average, total free phenolic individuals (TFIPC) accounted for around 90 per cent of total individual phenolic individuals (TIPC). Moreover, no significant fitting model for TIPC was generated. In this regard, TIPC will not be discussed in detail.

6.3.3.1. Free individual polyphenols (FIPC)

As illustrated in Table 6.5, the effects of extrusion cooking on FIPC were not observed uniformly across all individual polyphenols. For example, extrusion cooking sharply reduced the content of cinnamic acid glucoside, Aro-Glc*p*-Api*f*Glc*p* and Api-Api*f*-di-Glc*p*, up by 54.7 %, 31.9 % and 45.0 % respectively. Conversely, the processing reproducibly increased the levels of Api-Glc*p* in free fraction, up by 60 per cent, while slightly increased genistein content in most of the runs. Overall, the total free phenolic individuals (TFIPC) witnessed considerable decreases after extrusion cooking, owing to the overwhelming losses of Api-Api*f*-di-Glc*p*, which accounted for more than 70 per cent of the total polyphenols. Extrusion cooking has been reported to decrease the content of heat-sensitive extractable phenolic acids (Altan et al., 2009) and flavonoids (Khanal, Howard, & Prior, 2009). Nonetheless, extrusion cooking also has been found to increase some phenolic individuals by releasing bound ones to be more extractable

(Wang et al., 2014), or depolymerising tannins into lower molecular weight, more extractable compounds (Awika et al., 2003; Khanal et al., 2009).

Among the individual polyphenols in free fraction, only Api-Api*f*-di-Glc*p*, Api-Glc*p*, and genistein generated fitted models by Design-Expert software (Table 6.3 & 6.4). However, extrusion cooking just slightly changed the genistein level (15.09-16.55 μg/g db versus 15.60 μg/g db in raw seed coat). In terms of Api-Glc*p*, the moisture content in barrel showed the most significant negative effects. In terms of Api-Api*f*-di-Glc*p*, barrel temperature and screw speed had significant effects on them. From examinations of Table 6.3 and the contour plots (Figure S 7 & Figure S 8), we found that the retention of Api-Api*f*-di-Glc*p* was more susceptible to the elevated screw speed than increased barrel temperature. Moreover, Api-Api*f*-di-Glc*p* had higher retention under higher screw speed and high temperature, which may attribute to the shorter residence time and enhanced fibre abruption (Kazemzadeh, 2011). In contrast, great Api-Api*f*-di-Glc*p* losses were found under high moisture content in the barrel but relatively low temperature and low screw speed, where Api-Api*f*-di-Glc*p* may be solubilised into the water and then more vulnerable to oxidation during a longer residence time.

Table 6.5 Free individual polyphenol content (FIPC) (μg/g db) and total free individual polyphenol content (TFIPC) (μg/g db) of the extrusion cooked and raw lupin seed coats

Std	Run[1]	F1	F2	F3	F4	F5	F8	TFIPC
		CIA[2]	Aro-Glc*p*-Api*f*Glc*p*[3]	Api-Api*f*-di-Glc*p*[4]	Vicenin 2	Api-*O*-Glc*p*[5]	Genistein	
Control¶		*3.16±0.33*	*22.86±1.46*	*271.50±10.98*	*7.70±0.52*	*60.24±0.23*	*15.60±0.51*	*381.07±14.03*
14	1	2.01±0.14	17.57±0.67	169.47±2.67	4.31±0.23	73.38±1.69	16.38±0.09	283.12±1.65
7	2	1.62±0.22	15.78±0.99	163.47±6.18	4.58±0.62	67.73±8.69	16.23±0.49	264.42±17.18
2	3	1.80±0.02	16.53±0.12	164.02±1.04	3.29±0.24	89.17±13.68	15.61±0.10	290.41±15.19
4	4	1.97±0.08	17.35±0.38	172.97±4.18	4.73±1.77	92.32±5.69	15.82±0.09	305.17±11.28
9	5	1.59±0.06	15.58±0.39	154.02±2.31	4.21±0.05	85.30±8.18	15.96±0.06	276.67±5.31
11	6	1.83±0.07	16.73±0.34	163.03±3.57	4.11±0.03	82.97±6.60	15.10±0.26	283.77±3.15
20	7	1.73±0.11	16.30±0.54	162.17±0.94	3.56±0.22	86.22±9.38	16.02±0.00	286.01±8.01
6	8	1.71±0.14	16.13±0.52	164.94±0.09	3.82±0.26	78.63±13.36	15.95±0.04	281.19±12.91
17	9	1.43±0.36	14.90±1.69	158.30±2.00	3.57±0.18	81.53±1.03	15.65±0.21	275.38±5.11
5	10	1.61±0.15	15.86±0.65	159.33±4.40	4.21±0.07	73.21±2.84	15.80±0.21	270.03±8.33
12	11	1.94±0.06	17.15±0.16	184.81±0.76	4.62±0.35	89.14±8.90	16.07±0.09	313.73±8.99
13	12	2.01±0.00	17.63±0.07	164.31±0.05	5.98±1.28	96.48±0.10	15.47±0.07	301.88±0.99
8	13	1.92±0.13	17.30±0.58	180.10±2.94	4.88±0.38	77.40±1.90	16.55±0.16	298.15±6.10

Std	Run[1]	F1	F2	F3	F4	F5	F8	TFIPC
		CIA[2]	Aro-Glc*p*-Api*f*Glc*p*[3]	Api-Api*f*-di-Glc*p*[4]	Vicenin 2	Api-*O*-Glc*p*[5]	Genistein	
19	14	1.78±0.12	16.47±0.55	149.41±2.97	3.69±0.08	87.12±6.10	15.09±0.15	273.57±2.23
1	15	1.94±0.10	17.27±0.38	165.72±2.19	3.91±0.09	80.17±0.93	15.73±0.12	284.74±0.82
16	16	1.69±0.06	16.09±0.21	162.07±2.97	4.51±0.42	85.22±1.58	15.27±0.25	284.85±2.32
3	17	2.03±0.18	17.57±0.83	172.33±2.35	3.81±0.05	92.54±8.97	15.11±0.16	303.40±5.41
10	18	1.85±0.06	16.82±0.26	163.17±0.48	4.31±0.31	76.27±1.65	15.70±0.02	278.11±1.15
18	19	1.94±0.07	17.19±0.28	165.97±1.87	4.26±0.22	84.44±1.58	15.36±0.26	289.16±3.85
15	20	1.80±0.15	16.63±0.69	158.48±0.90	4.19±0.19	79.55±5.40	15.27±0.07	275.91±5.22

Means ± SD (n=2).

¶Control was un-extrusion cooking lupin seed coat, and it was not part of the design.

F1-F7 are individual polyphenols in free fraction as noted in Table 4.1.

[1] Experimental levels of barrel temperature (℃), screw speed (rpm) and moisture in barrel (%) used for the CCRD as in Table 6.2; [2] cinnamic acid glucoside (as *trans*-cinnamic acid equivalent); [3] aromadendrin-6-*C*-β-D-glucopyranosyl-7-*O*-[β-D-apiofuranosyl-(1→2)]-*O*-β-D-glucopyranoside glucopyranoside (as taxifolin equivalent); [4] apigenin-7-*O*-β-apiofuranosyl-6,8-di-*C*-glucopyranoside (as vitexin equivalent); [5] apigenin-7-*O*-β-glucopyranoside (as vitexin equivalent).

Table 6.6 Bound individual polyphenol content (BIPC) (μg/g db) and total free individual polyphenol content (TBIPC) (μg/g db), and total individual polyphenol content (TFIPC+TBIPC) (μg/g db) of the extrusion cooked and raw lupin seed coats

Std	Run[1]	B2	B3	B4	B5	B6	B8	TBIPC	TIPC
		Protocatechuic acid	*p*-coumaric acid hexoside[2]	Ferulic acid hexoside[3]	Api-*O*-Glc*p*[4]	Feruic acid	Genistein		
Control¶		*5.63±0.65*	*0.87±0.10*	*1.31±0.12*	*23.55±0.82*	*1.82±0.01*	*4.32±0.08*	*37.49±1.63*	*418.56±15.66*
14	1	16.09±1.78	2.44±0.23	2.70±0.26	22.72±2.44	1.81±0.24	2.55±0.05	48.31±4.52	331.43±2.86
7	2	14.51±0.46	2.92±0.23	3.10±0.24	14.06±0.31	2.03±0.08	2.89±0.02	39.51±0.22	303.92±16.96
2	3	10.64±0.61	2.66±0.03	2.85±0.03	9.61±0.19	1.90±0.13	1.73±0.00	29.39±0.62	319.81±14.57
4	4	13.53±1.71	2.57±0.01	2.80±0.02	9.66±2.55	1.81±0.14	1.63±0.00	31.99±4.16	337.15±15.43
9	5	18.61±1.86	2.81±0.16	3.05±0.17	22.08±2.48	1.94±0.14	2.25±0.14	50.72±4.68	327.39±9.99
11	6	13.29±0.25	2.62±0.06	2.78±0.18	6.23±1.25	2.27±0.13	1.77±0.20	28.97±1.81	312.74±4.96
20	7	12.59±0.13	2.69±0.12	2.93±0.14	8.30±2.15	1.62±0.38	1.92±0.10	30.05±1.74	316.05±9.75
6	8	19.61±0.02	3.33±0.04	3.59±0.06	18.52±2.17	2.03±0.09	2.34±0.17	49.43±2.33	330.61±15.23
17	9	14.95±1.67	3.15±0.0	3.40±0.02	14.11±1.03	1.94±0.08	1.96±0.08	39.52±2.69	314.90±2.43
5	10	15.67±2.89	3.61±0.04	3.84±0.02	22.56±2.74	2.08±0.26	2.51±0.08	50.27±5.34	320.30±13.67
12	11	12.61±0.21	2.59±0.07	2.77±0.24	14.08±1.20	1.57±0.03	1.85±0.16	35.46±0.95	349.19±8.03
13	12	5.88±0.72	2.18±0.19	2.14±0.29	7.60±0.06	1.66±0.04	1.44±0.05	20.90±1.22	322.79±2.21
8	13	17.45±1.78	3.69±0.10	3.97±0.05	32.49±1.94	1.66±0.31	2.43±0.03	61.68±0.03	359.83±6.07

Std	Run[1]	B2	B3	B4	B5	B6	B8	TBIPC	TIPC
		Protocatechuic acid	*p*-coumaric acid hexoside[2]	Ferulic acid hexoside[3]	Api-*O*-Glc*p*[4]	Feruic acid	Genistein		
19	14	8.12±1.78	3.02±0.07	3.23±0.16	9.23±0.11	1.78±0.11	1.79±0.03	27.17±2.20	300.73±0.03
1	15	9.57±1.95	3.22±0.19	3.42±0.24	10.63±2.08	1.48±0.33	1.87±0.00	30.18±4.13	314.91±4.96
16	16	7.80±1.95	3.29±0.03	3.48±0.07	9.19±0.32	1.83±0.16	1.86±0.11	27.45±2.32	312.30±0.00
3	17	7.76±1.33	3.19±0.14	3.38±0.23	10.07±2.94	1.79±0.15	1.57±0.11	27.77±4.90	331.17±0.52
10	18	6.95±0.80	3.21±0.31	3.22±0.52	18.48±0.25	1.77±0.03	1.97±0.09	35.60±1.75	313.71±0.60
18	19	9.34±1.55	2.79±0.47	2.93±0.57	16.28±0.72	1.49±0.29	1.74±0.06	34.55±3.08	323.70±0.77
15	20	9.58±0.86	3.41±0.02	3.64±0.00	16.07±3.31	1.80±0.06	1.74±0.07	36.25±4.01	312.17±1.21

Means ± SD (n=2).

¶Control was un-extrusion cooking lupin seed coat, and it was not part of the design.

B1-B8 are individual polyphenols in bound fraction as noted in Table 4.1.

[1] Experimental levels of barrel temperature (℃), screw speed (rpm) and moisture in barrel (%) used for the CCRD as in Table 6.2; [2] *p*-coumaric acid equivalent; [3] as ferulic acid equivalent; [4] apigenin-7-*O*-β-glucopyranoside (as vitexin equivalent).

Table 6.7 Numerical optimisation (Opti, extrapolated design space) criteria using desirability analysis of the multi-responses

Factors	Goal	Goal limits	Importance level (r_i)	Weight	Optimal solution	Actual value	95% PI range
Independent factors							
Barrel temperature (°C)	In range	120-150	-	-	138.7	139	
Total moisture in barrel (%)	In range	25† -40	-	-	25	25	
Screw speed (rpm)	In range	300-450†	-	-	450	450	
Responses							
SDF (g/100 g db)	Maximise	6.73-11.5†	+++++ (5)	1	11.33	11.17±0.06	10.23-12.42
TFPC (mg GAE/100 g db)	Maximise	30.6-60†	+++ (3)	1	48.18	46.05±2.01	44.48-51.88
TIFPC (μg/g db)	Maximise	264-360†	++++ (4)	1	355.05	350.04±1.89	326.26-383.83
Desirability					0.944		

† Outside of the design space to obtain maximum responses.

Table 6.8 Numerical optimisation (Sub-opti, un-extrapolated design space) criteria of desirability analysis

Factors	Goal	Goal limits	Importance level (*r*)	Weight	Optimal solution	Actual value	95% PI range
Independent factors							
Barrel temperature (°C)	In range	120-150	-	-	141.1	141	
Total moisture in barrel (%)	In range	30-40	-	-	30	30	
Screw speed (rpm)	In range	300-400	-	-	400	400	
Responses							
SDF (g/100 g db)	Maximize	6.73-10.38	+++++ (5)	1	9.91	10.15±0.25	9.43-10.39
TFPC (mg GAE/100 g db)	Maximize	30.6-50.8	+++ (3)	1	42.92	42.90±0.30	40.13-45.74
TIFPC (μg/g db)	Maximize	264-313.3	++++ (4)	1	307.01	309.08±1.89	294.44-319.59
Desirability					0.849		

In the case of TFIPC, extrusion cooking decreased TFIPC values regardless of the extrusion conditions. Moreover, all three independent variables showed significant effects on TFIPC levels based on the results of the ANOVA analysis. According to the 3D surface and contour plots, high TFIPC values were observed under high screw speed and barrel temperature but low moisture content in barrel (Figure 6.3 & Figure 6.4). On the one hand, high screw speed shortens the residence time, which further reduces the losses of heat-sensitive polyphenols of the seed coat over extrusion cooking (Kazemzadeh, 2011). More dominantly, high screw speed can induce high shear damage on fibre, resulting in the release of bound polyphenols (Nayak, Liu, & Tang, 2015). Conversely, the moisture content in the barrel had a negative effect on TFIPC. Similar to SDF, TFIPC gradually raised at first as barrel temperature increased, followed by a decreasing trend afterwards. The underlying mechanisms could be the same as those of TPC as stated in Section 6.3.2. Intriguingly, a significant and positive correlation between TFPC and TFIPC ($r = 0.722$, $p = 0.0003$) was detected, implying that polyphenols in free fractions are the main contributors to the TFPC. Among the individuals, TFPC were observed to be strongly related with contents of Api-O-Glcp ($r = 0.890$, $p < 0.0001$), suggesting that it may dominantly contribute to the TFPC values. The results of Folin-Ciocalteu assay are related to the chemical structures of individual polyphenols (Jacobo-Velazquez et al., 2009).

6.3.3.2. Bound individual polyphenols (BIPC)

Supporting the results in Chapter 4, the majority of the phenolic acids found in bound extracts are hydroxycinnamic acid derivatives. Phenolic acids, hydroxycinnamic acids, in particular, engage in the formation of legume cell wall by connecting polysaccharides via C-C, ether and ester bonds (Acosta-Estrada et al., 2014). As noted in TPC, extrusion cooking could liberate bound phenolics from cell wall but also degrade the compounds. Levels of bound individual polyphenols before and after the extrusion cooking were illustrated in Table 6.6. Despite Api-Glc*p* and genistein in bound extracts were reduced after extrusion cooking, the levels of protocatechuic acid, *p*-coumaric acid hexoside, ferulic acid hexoside were surprisingly increased. The results obtained in this study support that the effects of extrusion cooking on bound phenolics are type-dependent (Brennan et al., 2011). The decreases in Api-Glc*p* and genistein were probably because of the degradation and/or chemical changes upon

extrusion, or they were transferred into free forms as discussed in Section 6.3.2.1 and 6.3.3.1. Compared to the flavonoids, the disruption of cell wall matrix caused by extrusion cooking could further enhance the extraction of bound phenolic acids which may offset their losses.

According to the ANOVA analysis, a linear model was generated, and only moisture content in the barrel had significant positive effects on TBIPC (Table 6.3). On one hand, high moisture in the barrel can moderate thermal energy thus mitigate reductions in Api-Glc*p*, and genistein, which collectively constitutes over 74 per cent of TBIPC. On the other hand, high moisture in the barrel could reduce the viscosity of the seed coat in the barrel, which could be supported by the strong negative correlation between moisture content in the barrel and SME (r = -0.895, $p < 0.0001$). Low shearing force reduces the losses of Api-Glc*p* and genistein too. Similar to free fractions, a significant and positive correlation between TBPC and TBIPC (r = 0.774, $p < 0.0001$) was observed. Nevertheless, TBPC and TBIPC were negatively correlated with TPC and TIPC respectively such that no correlations between TPC and TIPC were found.

6.3.4. Effect of extrusion cooking on antioxidant capacities

Three antioxidant capacity assays (AOXs) based on radical scavenging capacity were conducted to evaluate the antioxidant capacities of both free and bound polyphenol extracts. Generally, the AOXs are dependent not only on the content of antioxidant compounds but also the structures of the individual compounds (Rice-Evans, Miller, & Paganga, 1996). Besides the mutual correlations, the AOXs of free fractions (FAOXs) results were observed to significantly and positively correlate with TFPC and TFIPC, whereas similar correlations among AOXs of bound fractions (BAOXs) were not observed. As a result, the correlations between TAOXs and TIPC were not significant. The alkaline hydrolysis used in this study has been reported to solubilise undesirable non-phenolic compounds (like monosaccharides and oligosaccharides) from fibre-based by-products, which can interfere the results of TPC and BAOXs as stated earlier (Max et al., 2010; Torre et al., 2008). Moreover, as presented in Figure 6.5, FAOXs are the main contributors to the total AOXs (TAOXs, namely sums of FAOXs and BAOXs). To this end, only FAOXs, i.e., FDPPH, FABTS and FORAC,

will be analysed by the design. However, it is worth to note that all BAOXs were significantly lowered by the extrusion cooking processing (Figure 6.5).

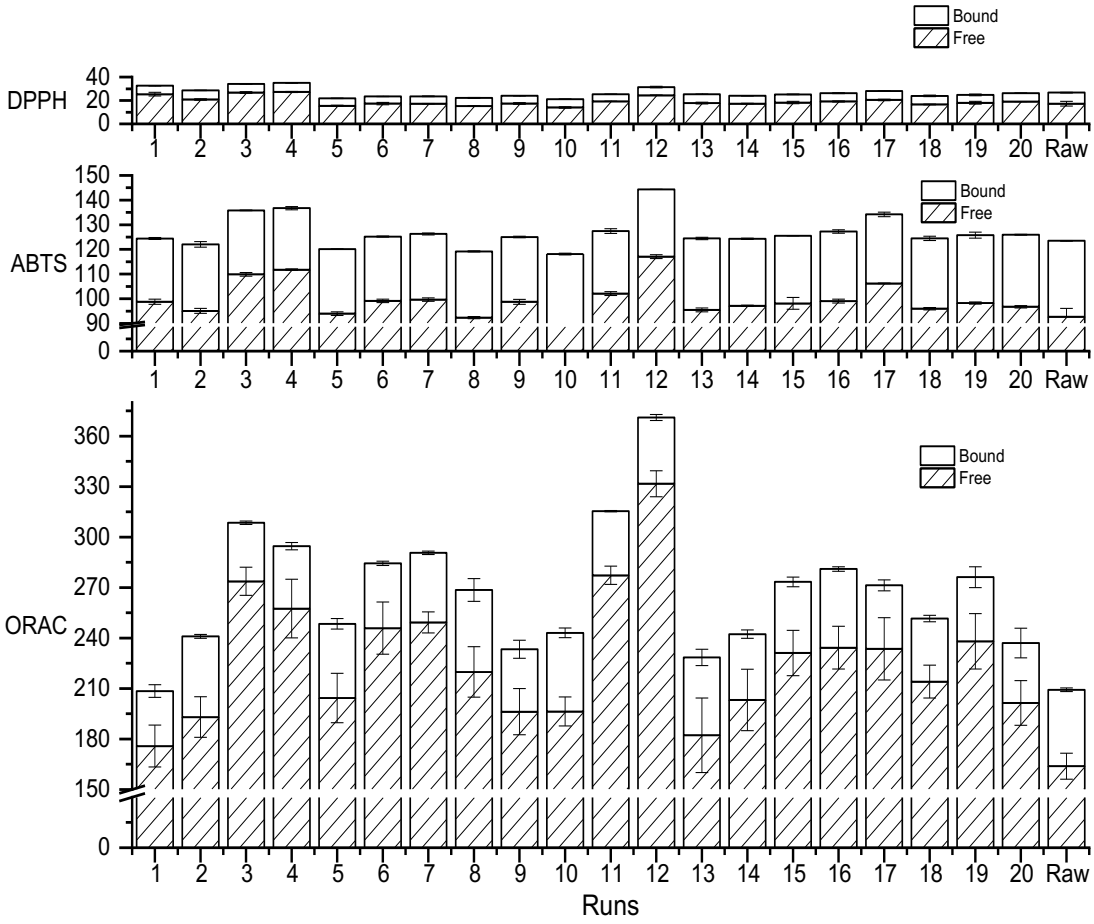


Figure 6.5 Results of antioxidant capacities (mg TE/100 g db)

As illustrated in Figure 6.5, FDPPH results of extrusion cooked lupin seed coat varied from 14.3 mg TE/100 g db to 27.30 mg TE/100 g db versus 17.19 mg TE/100 g db in the un-extruded lupin seed coat. Consistency in the trend was found in FABTS (88.94-117.08 mg TE/100 g vs. 92.68 mg TE/100 g). Similar decreases and increases in FABTS values after extrusion cooking were reported in wheat bran (Ramos-Enriquez et al., 2018). The increases may be induced by the formation of reducing compounds (like melanoidins) originated from Maillard reaction (Brennan et al., 2011). It has been suggested that low moisture and high-temperature extrusion cooking process can promote the Maillard reaction (Singh et al., 2007). With respect to FORAC, the FORAC levels of extrusion cooked lupin seed coats ranged from 182.24 mg TE/100 g db to 331.69 mg TE/100 g db, showing significant increases relative to the untreated lupin seed coat (163.84 mg TE/100 g db). As presented in Table 6.3, results of FDPPH generated significant second-order model but failed to achieve adequate fit ($p < 0.10$),

suggesting that the model is not strong enough to be used for predictions (Stat-Ease Inc., 2018). In contrast, data of FABTS obtained a well fitted quadratic model. According to the ANOVA analysis, only moisture in barrel showed significant effects on FABTS (Table 6.3). The results indicate that high screw speed, high temperature and low moisture content extrusion cooking could expect a higher FABTS. By comparison, a significant well-fitting linear model of FORAC was generated by the Design-Expert software (Table 6.3). As the same as found in FABTS, among the three selected independent factors, only moisture in barrel showed significant and negative effects on FORAC, with *p*-value being 0.0001.

6.3.5. Optimisation and validation of the extrusion cooking processing

Table 6.7 shows the selected numerical optimisation criteria using desirability analysis of the multi-responses. As discussed previously, three responses, i.e., SDF, TFPC and TFIPC, were selected to optimise the extrusion cooking processing. Moreover, this study tried to slightly stretch the design, i.e., extend goal limits of independent factors (nearby the ±α of the design and within the capacities of the twin-screw extruder, however) and responses, to obtain "better than observed" prediction regions of the responses (Stat-Ease Inc., 2018; Vera-Candioti, Garcia, Galera, & Goicoechea, 2008). Hence, the lower limit of total moisture in the barrel and the upper limit of screw speed are extrapolated to be outside the design space as shown in Table 6.7. Under the mentioned optimisation criteria, optimal solution generated by the software (1 of 84 solutions) which showed the maximum in the desirability function (D = 0.944) were barrel temperature at 138.7℃, total moisture in the barrel at 25 per cent and a screw speed of 450 rpm. The predicted SDF, TFPC and TFIPC were obtained, namely 11.37 g/100 g db, 59.93 mg GAE /100 g db and 354.56 μg/g db respectively. An extra optimisation (Sub-Opti.) was obtained following criteria which allow all factors and responses in the range of the original CCRD design Table 6.8. By comparing the two optimisations, the first criteria (Table 6.7) outputted much higher predicted responses than those "all in-range" criteria (Table 6.8).

As shown in Table 6.7, the actual values of the responses at the optimal settings were comparable to the predicted values determined from the expended model, with all being within the 95% PI (prediction interval) ranges. According to the one-sample t-test between actual and predicted values, all actual values was not significantly

different ($p > 0.05$) from those predicted counterparts. By contrast, the actual SDF and FTPC showed well agreement with the ones predicted. In general, these findings confirm that the selected RSM models are capable of predicting the three responses after extrusion cooking.

6.4. Conclusions

Dietary fibre (DF) is an essential component of a healthy diet. The microbiota-accessible and viscous soluble dietary fibre (SDF) are suggested to be more relevant to the potential health benefits of DF. In this chapter, extrusion cooking is optimised to promote SDF content, total free phenolic content (TFPC) and total free individual phenolic content (TFIPC) of lupin seed coat simultaneously using a central composite rotatable design and global desirability function. Under the optimal extrusion conditions, namely barrel temperature at 138.7℃, total moisture in barrel at 25 percent and screw speed of 450 rpm, lupin seed coat SDF content is increased from 4.42 g/100 g db up to 11.17 g/100 g db, while TFPC and TFIPC are found at 46.05 mg GAE /100 g db and 350.04 μg/g db respectively. This study supports that extrusion cooking could be a promising technology to explore the applications of the lupin seed coat, which is the major by-product of lupin kernel production, in human foods, for example as a natural antioxidant DF source.

However, many challenges remain: any higher SDF level is still desired. Combinations of extrusion cooking and other pre- and post-treatments to further increase SDF content merit further investigation. Moreover, the dark/grey colour produced from the extrusion cooking may show negative impacts on the end products. Dietary fibre composition and structure are reported to determine its physiological properties; therefore, next chapter will investigate the impacts of extrusion cooking on the bioaccessibility, bioavailability and dialysability of selected minerals and polyphenols of ASL seed coat.

CHAPTER 7

Extrusion cooking of lupin seed coat: Effects on polyphenol and mineral bioaccessibility, bioavailability, and dialysability

ABSTRACT

Dietary fibre has shown multiple effects on bioaccessibility and bioavailability of other food compounds, whereas the impacts varied greatly depending on the type of dietary fibre. In chapter 5 and 6, lupin seed coat dietary fibre composition, along with the physicochemical properties, were modified by the extrusion cooking. In this chapter, effects of optimised extrusion cooking processing on bioaccessibility, bioavailability and dialysability of selected minerals and polyphenols of the treated lupin seed coats were investigated using a standardised *in vitro* digestion method. Potential changes in the physical structure of food matrix caused by the processing were confirmed by scanning electron microscopy. Results show that iron content in the extrusion cooked lupin seed coat was doubled which may due to contamination from wearing of the screws. The contents of other minerals (Ca, K, Na, Mg, Zn and Cu) remained constant. The extrusion cooking showed no significant effects on the bioaccessibility of Cu, Fe, Mg, Zn and the bioavailability of all the selected minerals. However, the impacts of the extrusion cooking on the mineral dialysability depended on parameters of the processing. The apigenin-7-*O*-β-apiofuranosyl-6,8-di-*C*-β-glucopyranoside level was reduced by the extrusion cooking from 271.50 μg vitexin equivalent (VE)/g db to 165.54 μg/g db and 141.20 μg VE/g db, while bioaccessibility of the phenolic compound was improved. However, both bioavailability and dialysability of the compound were unchanged after extrusion. The results suggest that the extrusion cooking shows both positive and negative effects on the bioaccessibility, bioavailability and dialysability of the selected minerals and phenolic compound such that the global effects are highly variable and dependent on the processing parameters.

7.1. Introduction

Previous chapters demonstrated that lupin seed coat could be an important and affordable dietary fibre and polyphenol source for human. At the same time, optimised extrusion cooking was established to enhance its functionalities as a food ingredient. In addition, Hung et al. (1988) revealed that lupin seed coat has considerable levels of minerals, with 67.5% of total calcium, and 41.3% of total aluminium of the whole lupin seed being concentrated in the seed coat. Before being absorbed to the circulatory system (bioavailable) for human digestion, nutrients and other food substances must be liberated from the ingested food matrix into the gut lumen thus becoming bioaccessible (Ribas-Agusti, Martin-Belloso, Soliva-Fortuny, & Elez-Martinez, 2018). Therefore, the total quantity of a nutrient does not necessarily reflect its available amount for human absorption. Czubinski, Wroblewska, Czyzniejewski, Gornas, Kachlicki, and Siger (2019) indicated that the *in vitro* digestion procedure used in the current study could release up to 92% of the initial Api-Api*f*-di-Glc*p* from raw whole ASL seed flour by comparing the levels of compound in solid pellet before and after digestion. Karnpanit, Coorey, Clements, Benjapong, and Jayasena (2017) found that only 6% of calcium, 17% of iron, and 9% of zinc in whole ASL seed coat were bioaccessible, whilst the mineral bioaccessibilities of the dehulled ASL seed were 11%, 21%, and 12%, respectively.

Structure of the food matrix and interactions among food compounds were indicated to play important roles in the bioaccessibility and bioavailability of most food components (Palafox-Carlos, Ayala-Zavala, & González-Aguilar, 2011; Wahlqvist, 2016). For example, dietary fibre can induce positive or negative effects on bioaccessibility and bioavailability of minerals. On one hand, the binding and physical entrapment properties of dietary fibre can lower their absorption in the small intestine. On the other hand, dietary fibre fermentation in the colon can reduce pH and liberate bound minerals thus enhance colonic absorption and offset the negative impact (Baye et al., 2017). Maillard reaction products can reduce bioavailability of many minerals, calcium in particular (Gharibzahedi & Jafari, 2017). In this respect, extrusion cooking can lead to multiple chemical and structural changes, as demonstrated in Chapter 5 and 6. Minerals are expected to be unchanged before and after extrusion cooking. However, extrusion cooking disrupted ASL seed coat matrix, substantially decreased

polyphenols that inhibit minerals absorption, modified dietary fibre structures and converted insoluble dietary fibre to soluble dietary fibre, as well as induce Maillard reaction, what can show conflicting impacts on mineral absorption (Singh et al., 2007). In terms of polyphenols, although their levels were decreased by extrusion cooking, some positive structural and compositional changes could enhance their absorption in human digest tract. Extrusion was indicated to depolymerise proanthocyanidins in sorghum into more bioavailable low molecular weight compounds and improve the bioavailability of catechins (Gu, House, Rooney, & Prior, 2008). Likewise, extrusion released bound polyphenols of barley and oat, and correspondingly increased their bioavailability in pigs (Hole et al., 2013).

In light of the potential effects of food matrix structure, dietary fibre and polyphenols on the bioaccessibility and bioavailability of food compounds, it is essential to evaluate the bioaccessibility and bioavailability of minerals and polyphenols in this lignocellulosic and compact ASL seed coat. Consequently, the main aim of the research reported in this chapter was to assess the mineral and polyphenol bioaccessibility and bioavailability of both raw and optimally extrusion cooked ASL seed coats. In addition, this chapter evaluated the potential effects of extrusion cooking processing on physical and structural characteristics of the seed coats, and visible changes in the seed coat structures induced by extrusion cooking were investigated using scanning electron microscopy (SEM).

Table 7.1 Dietary fibre composition of raw and extrusion cooked lupin seed coats

	Raw	Optimal	Sub-optimal
SDF (g/100 g db)	4.42±0.06 [a]	11.17±0.06 [b]	10.15±0.25 [c]
IDF (g/100 g db)	91.00±0.45 [a]	81.50±0.06 [b]	82.53±0.21 [b]
TDF (g/100 g db)	95.42±0.40 [a]	92.67±0.11 [b]	92.68±0.46 [b]

Raw, raw ASL seed coat.

Optimal, extrusion cooked ASL seed coats using optimal conditions obtained from a stretched CCRD model.

Sub-optimal, extrusion cooked ASL seed coats using sub-optimal conditions obtained from an un-stretched CCRD model.

Results are expressed as mean ± standard deviation (n = 2).

Means assigned different letters in the same raw indicate significant differences ($p < 0.05$).

7.2. Materials and methods

7.2.1. Lupin seed coat samples

Raw ASL seed coat, extrusion cooked ASL seed coats from optimal conditions obtained from a stretched central composite rotatable design (CCRD) model and sub-optimal conditions obtained from an un-stretched CCRD model were prepared as described in Section 6.3.5. Dietary fibre composition of the lupin seed coats is illustrated in Table 7.1. Duplicate blank control was included.

7.2.2. Enzymes and reagents

Enzyme α-amylase from *Aspergillus oryzae* (10065, 33.8U/mg) was purchased from Sigma (Sigma-Aldrich, Sydney, Australia). Pepsin (PL082, 2500 U/mg) and pancreatin from porcine pancreas (PL378) were obtained from Chem-Supply (Chem-Supply, SA, Australia).

7.2.3. *In vitro* digestion and dialysis

7.2.3.1. In vitro oro-gastro-intestinal digestion

A static *in vitro* stimulated digestion method that simulated the human gastrointestinal digestion conditions was used (Minekus et al., 2014). Three physiological digestion phases include the mouth, stomach, and small intestine phases. Stock solutions of simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF), as well as other stock solutions were prepared as Minekus et al. (2014) described. All of the working solutions were prepared daily and pre-heated to 37 °C before use. Ultrapure water (dH_2O) was prepared by Arium® pro Ultrapure Water Systems (Sartorius Stedim Biotech, Göttingen, Germany) and used in all experiments.

Simulation of mouth conditions: Five-gram lupin seed coat samples and 3.5 mL of SSF electrolyte stock solution was added into a 50 mL tared polypropylene centrifuge tube and preconditioned for 10 min at 37 °C. 25 μL of 0.3 mol/L $CaCl_2$, 0.5 mL of amylase in SSF (1500 U/mL, approx. 750 units in total) and 975 μL of dH_2O was

added and thoroughly mixed in a vortex-stirrer for 10 s. The mixture was subsequently incubated for 2 min at 50 rpm in a 37 °C shaking bath.

Simulation of stomach conditions: SGF stock solution (7.5 mL) was added into the sample bolus resulting from oral phase and vortexed for 10 s, followed by 1.6 mL porcine pepsin solution and 5 µL of 0.3 mol/L $CaCl_2$. The pH of the suspension was acidified to 3 with 1 mol/L HCl (around 0.2 mL) then dH_2O was added to make up the volume to 20 mL. The tube was capped with N_2, incubated in a 37 °C shaking water bath (50 rpm) for 120 min.

Simulation of the duodenum and upper small intestine conditions: SIF stock solution (11 mL), 40 µL of 0.3 mol/L $CaCl_2$, 5 mL of pancreatin and 2.5 mL bile extract solution was subsequently added in the tubes and vortexed for 10 s. The pH was adjusted to pH 6.9 with 1 mol/L NaOH (around 150 µL). 1.31 mL of dH_2O was subsequently added to get a total sample volume of 40 mL. The tubes were capped with N_2 and incubated at 37 °C, 50 rpm water shaking water bath (OLS 200, Grant, UK) for two hours.

7.2.3.2. Mineral and polyphenol dialysability

$NaHCO_3$ (5.5 mL, 0.5 mol/L) and NaCl (5.5 mL, 0.9 g/100g) were added into a dialysis bag with a molecular mass cut off at 10 kDa (diameter 22 mm, 15 cm lengths, SnakeSkin, Thermo Fisher Australian). After 1.5 h of incubation of the gastric phase, the dialysis bag was put into the gastric chime, and the incubation was continued for 30 min more to get 2h in total as described by Shumoy, Lauwens, Gabaza, Vandevelde, Vanhaecke, and Raes (2017). The following intestinal phase was performed as described above. The dialysis method has been successfully used to determine mineral dialysability in sorghum (Wu, Johnson, Bornman, Bennett, Singh, Simic, et al., 2016) and lupin (Karnpanit et al., 2017) in our research group. According to our previous study, the molecular weight of all the identified polyphenols is lower than 1000, which is over 10 times smaller than the molecular weight cut-off of the tubing (Table 4.1).

7.2.4. Sample collections

The resulting digestion suspension was centrifuged at 2750 × *g* for 5 min, yielding supernatant and pellet, which were stimulated chyme and residues after digestion respectively (Versantvoort et al., 2004). Hence, the soluble but non-dialysable (SND)

minerals and polyphenols were located in the supernatant, while the pellets contained insoluble (In) compounds (Shumoy et al., 2017).

For minerals dialysability, the dialysis tubing was collected, rinsed by dH_2O, dried on an absorbent paper and placed into a tared glass falcon tube (20 mL) and oven-dried to constant weight. The supernatants from centrifuging were collected into tared glass tubes and freeze-dried to constant weight along with tubes containing the pellets. The resulting residues were used to analyse mineral contents by ICP-OES (Section 7.2.5). In terms of polyphenol dialysability, the digestion mixture was acidified to pH 2 after the intestinal digestion phase to stabilise the phenolic compounds and terminate reactions (Pineda-Vadillo et al., 2016). The dialysis tubing, supernatants and pellets collected from centrifuging as did for minerals were frozen under -70°C prior to being freeze-dried and stored until further analyses.

7.2.5. Quantitation of minerals

The Vista Pro inductively coupled plasma optical emission spectrometer (ICP-OES) (Varian, Palo Alto, USA) at the National Measurement Institute (Perth, Western Australia) was used to determine the content of minerals (Wu, Johnson, Bornman, Bennett, Singh, Simic, et al., 2016). Levels of potassium (K), sodium (Na), calcium (Ca), magnesium (Mg), iron (Fe), zinc (Zn) and copper (Cu) in the seed coat were investigated. For fractions from digestion procedure, all of the residues resulting from digestion, along with the dialysis tubing, were digested using the strong acids. Mineral contents were corrected against blanks.

7.2.6. Quantitation of polyphenols

Polyphenols of the seed coats, freeze-dried digested fractions (i.e. dialysis tubing content along with tube, supernatant residue and 2 g of pellet from centrifuge) were extracted and quantified as previously described (Section 4.2.4). Briefly, the samples were mixed with 15 mL of 80% methanol, caped with N_2, and mixed using a head-over-heels rotator for 2 hours. After centrifuged at 3220 × g 4 °C for 15 min, the supernatant was collected, and the pellet was re-extracted with 6 mL of 80% methanol twice more. Then, the supernatants were combined, made up to 30 mL and stored at -20°C until analyses. Individual polyphenols were analysed using a validated HPLC-DAD method as previously described, and the content of apigenin-7-*O*-β-

apiofuranosyl-6,8-di-*C*-β-glucopyranoside (Api-Api*f*-di-Glc*p*) was expressed as μg Vitexin equivalent (VE) /g dry sample (Section 4.3.2).

7.2.7. Calculations

Bioavailability (%) and bioaccessibility (%) were defined and calculated as described by Versantvoort et al. (2004). For the current study, bioaccessibility is defined as the percentage of the polyphenol/minerals which were released from the matrix and transferred into digestive fluid after ingestion. By contrast, bioavailability is the fraction of the compounds which is absorbed to the blood comparing to the total amount in the raw sample (serum-available). The ratio of a dialysable (bioavailable) fraction compared to the bioaccessible fraction is defined as dialysability (Shumoy et al., 2017). Therefore, the bioaccessibility (%), bioavailability (%) and dialysability (%) of minerals and polyphenols were calculated as follows:

$$Bioaccessibility\ (\%) = \frac{Qty(D) + Qty(Sup)}{Qty(total)} \times 100$$

$$Bioavailability\ (\%) = \frac{Qty(D)}{Qty(total)} \times 100$$

$$Dialysability\ (\%) = \frac{Bioavailability}{Bioaccessibility} \times 100$$

Where Qty (D), Qty (Sup) and Qty (total) were the quantities of the compounds in dialysis tubing, supernatant (digestion slurry) and the initial raw samples, respectively. Duplicate blank control were included during *in vitro* digestion.

7.2.8. Scanning electron microscopy (SEM)

IDF and SDF fractions of the raw seed coat and extruded ASL seed coats (Optimal and Sub-optimal) were prepared as prepared described in Section 5.2.6. The surface morphology of raw ASL seed coats, as well as their IDF and SDF fractions, were observed using SEM on a dual-beam field emission scanning electron microscope (Zeiss Neon 40EsB FIBSEM, Oberkochen, Germany) at John de Laeter Centre Curtin University. All samples were freeze-dried, then attached on aluminium stubs using carbon tape and coated with a platinum sputter coater (208HR, Cressington). Electron

beam was set at 3 kV. A step sizes of 10 μm (magnification at 2820 ×) and 2 μm (magnification at 8520 ×) images were acquired.

7.2.9. Statistical analysis

As described in Section 3.2.9.

7.3. Results and discussion

7.3.1. Minerals content in the raw and extruded lupin seed coat

In this study, the contents of Ca, K, Na, Mg, Zn, Fe and Cu of lupin seed coat before and after extrusion were investigated. As presented in Table 7.2, Ca was the main mineral of the raw lupin seed coat (6.40 g/kg db), followed by K (3.10 g/kg db) and Mg (1.50 g/kg db). In contrast, the level of Na, Fe, Zn and Cu were relatively low. The results in the current study are in line with the ranges reported in seed coats of several old ASL cultivars grown in New South Wales Australia, except that a much higher Cu were reported previously (3 - 4 mg/kg) (Hung et al., 1988). In particular, 67.5% of Ca in the ASL seed was indicated to locate in the seed coat (Hung et al., 1988). Karnpanit et al. (2017) determined the Ca, Fe and Zn contents of whole seeds of Coromup, Jenabillup, Mandelup, PBA Barlock and PBA Gunyidi that ranged from 2.01 to 2.8 g/kg db, 21.4 to 31.0 mg/kg db and 14.0 to 49.8 mg/kg db respectively. Therefore, the result was supported by the much lower Ca levels found in whole seeds (Karnpanit et al., 2017). Ca can chelate with pectins in the cell wall such that play a critical role in cell wall growth and stabilisation of cell wall structures (Moïse et al., 2005).

The extrusion cooking did not change mineral contents except that Fe content was doubled in both extruded seed coats (Optimal and Sub-optimal) (Table 7.2). Extrusion cooking was reported to increase contents of Fe and Cu in both whole and dehulled ASL seeds (Suliburska, Krejpcio, Lampart-Szczapa, & Wojciak, 2009). However, no significant changes in the level of Cu were found in the present study (Table 7.2). Similar Fe increases were observed in extruded pea and kidney bean seed meals (Alonso, Rubio, Muzquiz, & Marzo, 2001), legume flours (Lombardi-Boccia, Lullo, & Carnovale, 1991), bovine lung (Pinto, Colli, & Areas, 1997) and maize-based snack food (Hazell & Jolinson, 1989). The increases were explained by contaminations during extrusion cooking processing, mainly from wearing of screws.

Table 7.2 Minerals content in raw and extruded lupin seed coats

	Ca	K	Mg	Na	Fe	Zn	Cu
	g/kg	g/kg	g/kg	mg/kg	mg/kg	mg/kg	mg/kg
Raw	6.40±0.00[a]	3.10±0.00[a]	1.50±0.00[a]	213.33±5.77[a]	47.00±2.00[a]	19.00±0.00[a]	0.80±0.10[a]
Optimal	5.80±0.49[a]	2.80±0.30[a]	1.25±0.15[a]	190.00±14.14[a]	90.33±2.52[b]	17.00±2.00[a]	0.75±0.10[a]
Sup-optimal	6.47±0.06[a]	3.17±0.06[a]	1.50±0.00[a]	220.00±0.00[a]	106.67±5.77[b]	19.00±0.00[a]	1.00±0.00[a]

Raw, raw ASL seed coat.

Optimal, extrusion cooked ASL seed coats using optimal conditions obtained from a stretched CCRD model.

Sub-optimal, extrusion cooked ASL seed coats using sub-optimal conditions obtained from an un-stretched CCRD model.

Results are expressed as mean ± standard deviation (n = 2).

Means assigned different letters in the same column indicate significant differences ($p < 0.05$).

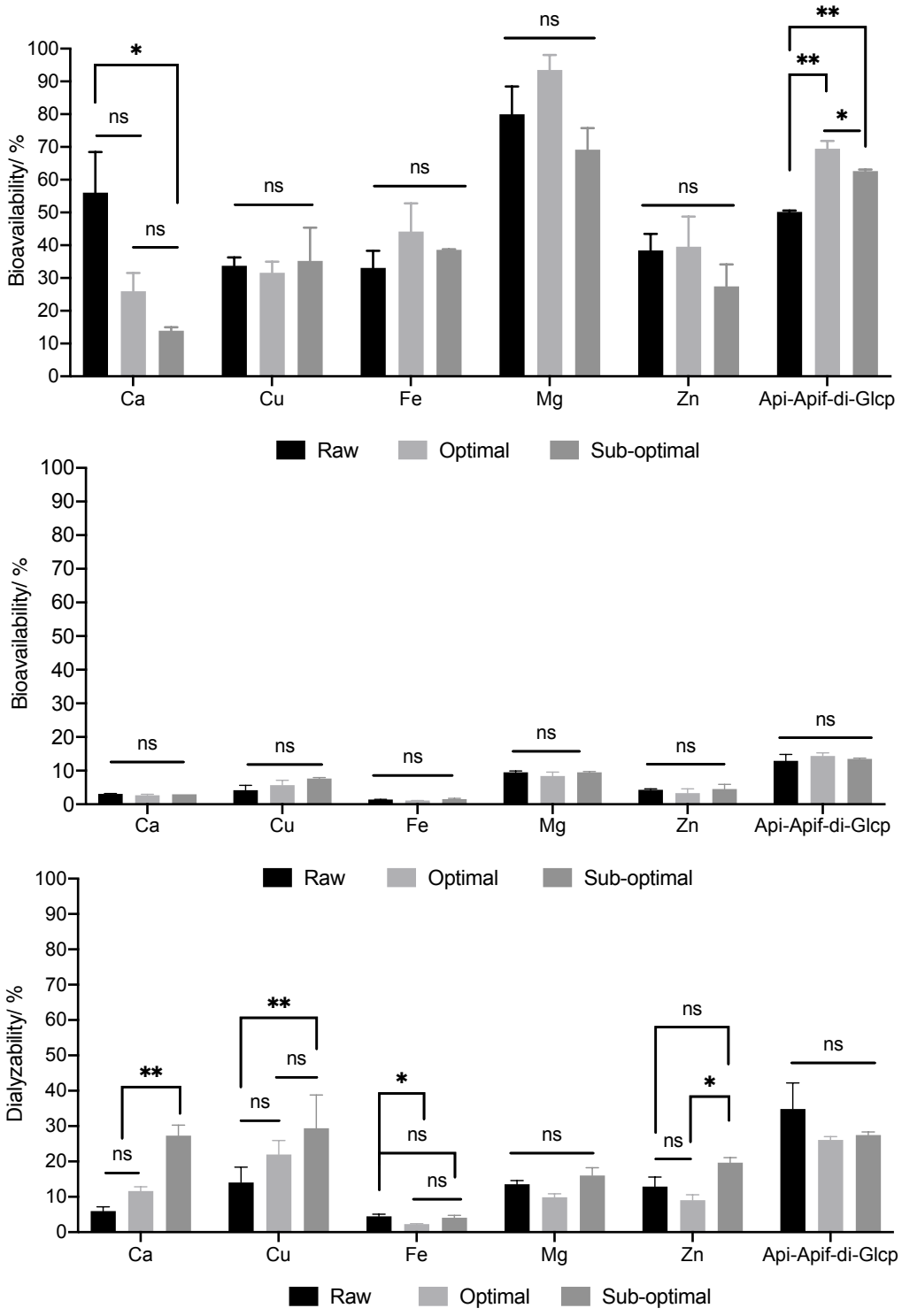


Figure 7.1 Bioaccessibility, bioavailability and dialysability of selected minerals and apigenin-7-*O*-β- apiofuranosyl-6,8-di-*C*-β-glucopyranoside (Api-Api*f*-di-Glc*p*) in raw and extruded lupin seed coats in Optimal and Sub-optimal conditions (ns, $p > 0.05$; *, $p \leq 0.05$; **, $p \leq 0.01$)

7.3.2. Bioaccessibility, bioavailability and dialysability of selected minerals in raw and extruded lupin seed coat

As shown in Section 7.2.3.1, the digestive fluids contained a large amount of K, Na. K, Na that added into the digestion system significantly interfered the results. Therefore, both K and Na were excluded for bioaccessibility, bioavailability and dialysability analyses. As presented in Figure 7.1, extrusion showed no significant effects on bioaccessibility of Cu, Fe, Mg and Zn except that the treatments significantly lowered Ca bioaccessibility. In terms of Fe bioavailability, however, given the occurrence of exogenous Fe after extrusion cooking, the non-significant difference between before and after the processing indicated that Fe from contamination could not solubilise during digestion (Lombardi-Boccia et al., 1991). Likewise, the effects of extrusion cooking on the bioavailability of all the selected minerals were unexpectedly not significant, implying that higher mineral bioaccessibility did not necessarily mean a higher bioavailability. The dialysability of the selected minerals was highly variable and dependent on bioaccessibility and bioavailability. However, the dialysabilities of Ca, Cu and Zn of Sub-optimal seed coat were found to be significantly higher than those of raw sample. In contrast, the values of Optimal sample that had the highest SDF content were affected by extrusion cooking. Taken together, it seems that the effects of extrusion cooking on mineral dialysability depended on the parameters of the processing.

Extrusion cooking was extensively documented to improve mineral absorption directly (disruption of food matrix and enhanced enzyme digestibility for example) or indirectly (reduction of mineral chelating compounds like phytates, and condensed tannins that may hamper mineral absorption) (Alam et al., 2016; Nikmaram et al., 2017; Singh et al., 2007). However, no significant changes in mineral absorption induced by extrusion cooking were also reported (Drago, Velasco-Gonzalez, Torres, Gonzalez, & Valencia, 2007; Watzke, 1998). The conflicting results may be explained by the differences in material and extrusion cooking processing conditions such as the screw profile, feed rate, barrel temperature and moisture and screw speed. In this study, the SDF of Optimal seed coat, and Sub-optimal sample were 11.17 and 10.15 g/ 100 g db respectively comparing to 4.42 g/100 g db in the untreated raw sample. The extrusion cooking changed the composition of lupin seed coat dietary fibre, a redistribution of

IDF to SDF for example, along with the structural properties (Section 5.3.2 and Section 6.3.1). Both IDF and SDF showed mineral binding/ physical trapping capacity (Baye et al., 2017). Moreover, SDF can serve as a thickening agent that affects digesta viscosity such that show negative influences on nutrient infusibility. In this regard, the positive effects on mineral bioaccessibility, bioavailability and dialysability caused by extrusion cooking may be compensated, partly at least, by the potential negative effects, resulting in not-significant overall effects (Figure 7.1). However, given that extrusion cooking changed the seed coat structure, disruption of the cell wall matrix for example, colon fermentability of the extruded seed coat could be improved that may further enhance the release and absorption of minerals.

7.3.3. Bioaccessibility, bioavailability and dialysability of Api-Api*f*-di-Glc*p* in raw and extruded lupin seed coat

The extrusion cooking considerably decreased all individual polyphenols (Section 6.3.3). In line with the previous study, the results in the current study showed that the extrusion cooking significantly decreased the levels of the phenolic compounds in the lupin seed coats. For example, the apigenin-7-*O*-β-apiofuranosyl-6,8-di-*C*-β-glucopyranoside (Api-Api*f*-di-Glc*p*) content was reduced from 271.50 μg vitexin equivalent (VE)/g db to 165.54 μg/g db in Opti sample and 141.20 μg VE/g db in Sub-Optimal sample respectively. Czubinski et al. (2019) indicated that the *in vitro* digestion procedure used in the current study could release up to 92% of the initial Api-Api*f*-di-Glc*p* from raw whole ASL seed flour by comparing the levels of compound in solid pellet before and after digestion. However, the authors did not quantify the compound amount in the digest fluid. In this study, only around 50 per cent of the compound in the raw seed coat were found the digest fluid.

Except for Api-Api*f*-di-Glc*p*, only trace of other individual polyphenols was detected in digestive mixture and dialysate. Therefore, only the dominant phenolic compound of the seed coats, i.e., Api-Api*f*-di-Glc*p*, was monitored in this study. As shown in Figure 7.1, significantly higher levels of bioaccessible Api-Api*f*-di-Glc*p* were found in extrusion cooked samples compared to the respective raw seed coat. Moreover, the two extrusion-cooked samples exhibited no significant differences. In addition, Api-Api*f*-di-Glc*p* bioavailability of raw, Optimal and Sub-optimal samples were similar (12.91%, 14.38% and 13.50% respectively; $p > 0.05$). Likewise, extrusion cooking

showed no significant influences on Api-Api*f*-di-Glc*p* dialysability either. In contrast, extrusion was reported to improve the bioavailability of sorghum catechins (Gu et al., 2008) and phenolic acids of barley and oat in pigs (Hole et al., 2013). Dietary fibre in the lupin seed coat can interact with Api-Api*f*-di-Glc*p* chemically and physically. Moreover, increase SDF level may affect the viscosity of digestion fluids, hampering diffusion and further bioavailability of Api-Api*f*-di-Glc*p* as discussed above (Palafox-Carlos et al., 2011; Ribas-Agusti et al., 2018).

It is well recognized that the *in vivo* bioavailability of flavonoids is very low, with a majority of flavonoid glycosides being generally assumed to reach in the colon directly where they can be deglycosylated and degraded by gut microbiota (Yang, Liu, Yang, Gupta, & Jiang, 2018). However, it is generally assumed that the glucose conjugated flavonoids are more bioaccessible and bioavailable than their corresponding aglycones (Ribas-Agusti et al., 2018). For Api-Api*f*-di-Glc*p*, its pentose (-*O*-glycoside) residue could be hydrolysed by lactase phlorizin hydrolase (LPH) which locates in the brush border of the small intestine epithelial cells (Williamson, Kay, & Crozier, 2018), and the resulting apigenin glucosides possibly could be transported into the epithelial cells through glucose transporter (SGLT1) then be hydrolysed to apigenin by cytosolic *β*-glucosidase (CBG).

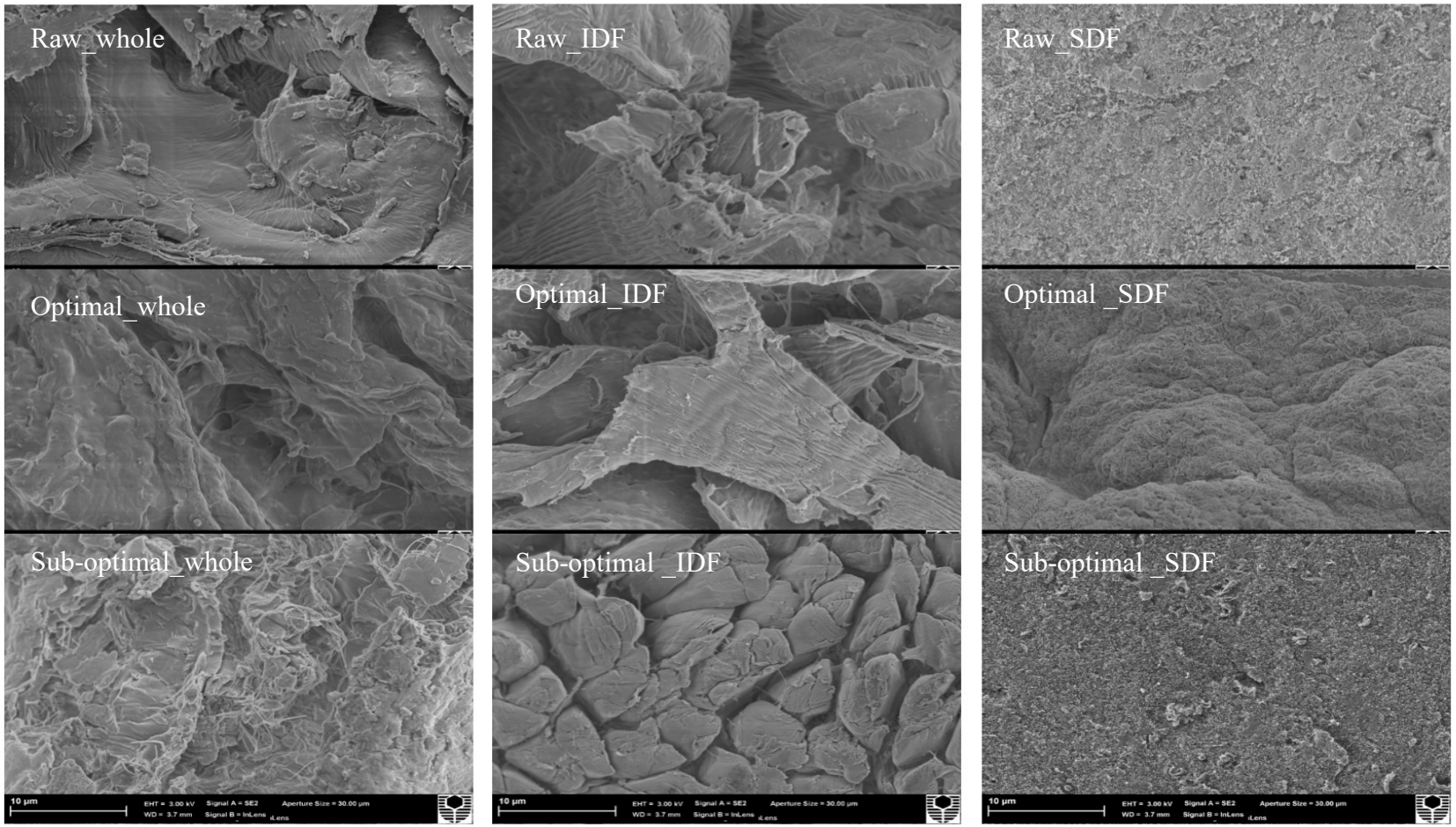


Figure 7.2 SEM images of the surface of raw, Optimal and Sub-optimal ASL seed coat powder, and their corresponding SDF, IDF factions

7.3.4. SEM analysis

The bioaccessibility and bioavailability of minerals and polyphenols from fibrous matrix were reported to be affected by the physical structure of food matrix (Grundy, Edwards, Mackie, Gidley, Butterworth, & Ellis, 2016). To visualise the anatomical structures of lupin seed coat, SEM was previously used to investigate its surface and cross-sectional structures (Clements et al., 2014). In this study, SEM images of the raw and extruded seed coats (Optimal and Sub-optimal), as well as their SDF and IDF fractions, were obtained to analyse their surface morphology and to visualise changes induced by extrusion cooking. As shown in Figure 7.2, the compact surface was found in untreated seed coat powder, with visible "stretching" and "tearing" damages caused by the milling process. The broken cellulose fibrils found in "Raw_IDF" sample also suggested the damages from the milling process. Moreover, in agreement with figures reported by Clements et al. (2014), a preventive un-smooth seed surface was found from IDF of raw samples. In contrast, both Optimal and Sub-optimal seed coats displayed collapsed surface with "paste" and broken cellulose fibrils attached. The "paste" could be SDF, while the cellulose fibres and their debris indicated disruption cell wall structure of ASL seed coat and the defibrillation of fibre caused by the extrusion cooking (Ciftci, 2017). However, a limitation is that the microstructure of seed coats after the simulated digestion were not examined.

7.4. Conclusions

The extrusion cooking processing is hypothesised to affect bioaccessibility and bioavailability of many food compounds, such as minerals and polyphenols. However, using an *in vitro* digestion model, this study reveals that the processing generally showed no or slight effects on bioaccessibility, bioavailability and dialysability of selected lupin seed coats minerals and polyphenols. Extrusion cooking does not change mineral content except that iron is doubled, which may be attributable to equipment contaminations from the process. The extrusion cooking decreases the level of the major phenolic individual, apigenin-7-O-β- apiofuranosyl-6,8-di-C-β-glucopyranoside (Api-Api*f*-di-Glc*p*, whereas its bioaccessibility is improved. However, both bioavailability and dialysability of Api-Api*f*-di-Glc*p* are unchanged.

This chapter, though, evaluates the mineral and polyphenol bioaccessibility and bioavailability of the extruded lupin seed coats only, but not the end food products in which might be incorporated the extruded seed coats. Given the interactions among food ingredients and the effects of subsequent food processes, more studies on the food products are required. Moreover, the *in vitro* digestion study used in this work does not include the colon fermentation, which might further affect the bioavailability of minerals and polyphenols and thus merits further investigation.

CHAPTER 8

General discussion, conclusions and future research

8.1. Introduction

The seed coat of Australian sweet lupin (ASL, *Lupinus angustifolius*), being 24% of the seed weight in average, accounts for a relatively higher percentage of the seed compared to other pulses (Chapter 2). As a result, the ASL milling sector produces a large amount of milling loss during ASL kernel productions. The seed coat is generally used as a stock feed or discarded as a waste. However, to achieve a sustainable and low-carbon food system, increased attention has been paid to valorise the biomass (Garcia-Amezquita, Tejada-Ortigoza, Serna-Saldivar, & Welti-Chanes, 2018). For example, the seed coat of albus lupin has been commercially developed as an insoluble fibre ingredient (Vitafiber®, Avelup Ltd., Chile). More recently, lupin seed coat flour was incorporated into bread as a source of insoluble dietary fibre (IDF) (Wandersleben et al., 2018), and used to produce cellulose nanofiber aerogel (Ciftci, 2017).

We aimed to value-add to ASL seed coat as a natural dietary fibre and source of polyphenols for human food products. Therefore, we hypothesised that extrusion cooking would be a feasible technology to enhance the desirable physicochemical and compositional properties of the seed coat to facilitate its applications in human foods. To test the hypothesises, we firstly screened the chemical and physicochemical properties of seed coats from six recent and agriculturally dominant ASL cultivars (Chapter 3 and 4). Secondly, significant processing parameters of twin-screw extrusion cooking were identified using a fractional factorial design (Chapter 5). Subsequently, the extrusion cooking was optimised using a central composite rotatable design (Chapter 6). Finally, to evaluate nutritional effects of the extrusion cooking on the biomass, bioaccessibility, bioavailability and dialysability of selected minerals and individual polyphenol of extrusion cooked ASL seed coats were investigated using a static *in vitro* stimulated digestion method (Chapter 7).

8.2. Conclusions and discussion

The main purpose of the current study was to examine the feasibility of extrusion cooking to improve functionalities of ASL seed coat, to increase SDF but keep high levels of polyphenols in specific, and to optimise processing conditions. Firstly, this study

8.2.1. The pros, cons of lupin seed coat as a food ingredient

With the numerous advantages in the sustainable food system and human nutrition, lupins are attracting international attention as an emerging staple food (Johnson et al., 2017). This study demonstrated that lupin seed coat, a major processing by-product during lupin kernel flour products, can be a promising food ingredient.

8.2.1.1. The physicochemical properties of ASL seed coat

As a high dietary fibre food material, the key physicochemical properties of ASL, include water solubility (SOL), water- (WBC) and oil- (OBC) binding capacity, and swelling capacity (SC). These properties will determine its applicability in food processing and thus further diversify its processing technology. They could also affect sensory and physiological qualities of the end food products (Elleuch et al., 2011; Stephen et al., 2017). As discussed in Section 2.7, food processing and other food ingredients in return will affect the physicochemical properties of the material (Garcia-Amezquita et al., 2018).

The reported physicochemical properties of seed coats of field pea, chickpea, lentil and mung bean are summarised in Table 2.4. In Chapter 3, we investigate ASL seed coat physicochemical properties from six recent cultivars. Although ASL seed colour varies based on cultivars, the seed coat flours after milling are identical. Their water-binding capacities, being 3.39-3.74 g/g water db, are similar to or slightly lower than those of field pea, lentil and mung bean. Generally, ASL seed coats have a fairy low SOL, which is lower than 8%. However, the SOL is expected to be lower when more efficient dehulling and separation facility is used. In addition, anatomical structures of ASL seed coat is obtained using scanning electron microscopy (SEM). ASL seed coat exhibits a compact surface, with visible structure disruption caused by the milling process (Section 7.3.4). The results indicate that all investigated physicochemical

properties of ASL seed coat are greatly affected by processing technologies and the processing conditions. Both pea seed coat and lupin seed coat have been classified as GRAS (Generally Recognized as Safe). However, processed pea seed coat has been relatively well developed as a commercial food ingredient (Section 2.7). Nonetheless, the comparable physicochemical properties of ASL seed coat to those of other legume seed coats opens the possibility of using ASL seed coat as a food ingredient.

8.2.1.2. The chemical properties of ASL seed coat

Dietary fibre (DF) is the major component of ASL seed coat, with total dietary fibre (TDF) accounting for 80-87 g/100 g db. Among the TDF, insoluble dietary fibre (IDF) took up over 95 per cent of TDF (Table 3.4). The results are supported by the Fourier transform infrared spectroscopy (FTIR) analyses which indicate typical carbohydrates spectra. Moreover, the FTIR spectra suggest that the majority of ASL seed coat IDF are cellulose while SDF fraction mainly composes of pectins and hemicelluloses (Section 5.2.6). Apart from dietary fibre composition, the proximate composition (Table 3.4), plus total polyphenol content and antioxidant capacities (DPPH, ABTS, ORAC) (Table 3.5), as well as levels of seven minerals (Ca, K, Na, Mg, Zn, Fe and Cu) (Table 7.1) are evaluated. Results show that the ASL seed coat contains 2.81-3.01 g/100 g db of ash, 1.62 - 2.42 g/100g db of fat and 6.34 - 8.59 g/100g db (N×5.40) of protein (Table 3.4). Calcium is the main mineral of the raw lupin seed coat (6.40 g/kg db), followed by potassium (3.10 g/kg db) and magnesium (1.50 g/kg db) (Table 7.1). In general, proximate composition and mineral profile of ASL seed coat are comparable with other pulse seed coats (Section 2.3).

Phenolic compounds are identified and quantified using HPLC-DAD-ESI-MS/MS in Chapter 4. Of particular interests, three flavones (apigenin-7-*O*-β-apiofuranosyl-6,8-di-*C*-β-glucopyranoside, vicenin 2, and apigenin-7-*O*-β-glucopyranoside), one isoflavone (genistein) and one dihydroflavonol derivative (aromadendrin-6-*C*-β-D-glucopyranosyl-7-*O*-[β-D-apiofuranosyl-(1→2)]-*O*-β-D-glucopyranoside), and several hydroxybenzoic and hydroxycinnamic acid derivatives are identified in the ASL seed coat (Table 4.1). Considerable variations in levels of individual polyphenols dependent on biotic and abiotic factors are found but apigenin-7-*O*-β-apiofuranosyl-6,8-di-*C*-β-D-glucopyranoside (Api-7-*O*-Api*f*-6,8-di-*C*-Glc*p*) is the dominant compound in the free polyphenol fraction, ranging from 697.85 μg vitexin equivalent

(VE)/g db to 1011.82 µg VE/g db, which accounts for 73.08 - 82.89 % of the total free polyphenols (Table 4.3). In contrast, the majority of phenolic compounds in the bound fraction are phenolic acid derivatives. No api-7-*O*-Api*f*-6,8-di-*C*-Glc*p* and vicenin 2 are detected in the bound fraction (Table 4.4). The results indicate that ASL seed coat also can be used to extract polyphenols, Api-7-*O*-Api*f*-6,8-di-*C*-Glc*p* for instance, which can be further used in human food, pharmaceuticals and cosmetics, thus providing a new strategy to value add to the seed coat (Cory et al., 2018; Lai et al., 2017). Moreover, the bound polyphenols could reach the colon and be released during the fermentation of dietary fibre (O'Grady et al., 2019). These bound polyphenols therefore may alter gut microbiota profile, implying the physiological benefits of the seed coat consumption (Zmora, Suez, & Elinav, 2019).

This thorough compositional characterisation of the seed coat of six the major ASL cultivars provides the latest data on compositional properties of ASL seed coat (Chapter 3 & 4). Of particular, it is the first time that both free and bound individual polyphenols of the seed coat have been identified and quantified. The seed coat, being the outer layer, is a modulator of the seed-environment relationships, governing nutrient supply to the embryo and protecting the seed against pests and disease (Moïse et al., 2005). This study observes that the majority of chemical properties of ASL seed coat are significantly affected by genotype, environment and their interaction. In summary, this work demonstrates that ASL seed coat could be further value-added by exploring the potential for its fibre and bioactive components, while more desired nutritional and functional properties could be obtained by manipulating the genotypic and environmental factors of the lupin production (Clements et al., 2002).

8.2.1.3.Lupin seed coat as a food ingredient: cons vs pros

In summary, despite the genotypic and environmental variations in physicochemical, compositional properties and antioxidant capacities of ASL, the results support the idea that ASL seed coats can be used as a natural and economic “antioxidant dietary fibre” in human food products. However, given the high proportion of DF, IDF in particular, ASL seed coat use for human food purpose is debatable in many aspects.

Pros:

To fulfil current trends in sustainable food systems and circular economy, considerable attention has been paid to value-add by-products and waste streams from the agriculture sector and the food industry. In this respect, the valorisation of ASL seed coat by expanding its use as functional human food ingredient can benefit to lupin growers, millers, manufacture and traders (Lai et al., 2017; Sharma et al., 2016).

It is well established that the consumption of high DF and polyphenols foods can benefit to a range of health outcomes (Cory et al., 2018; Stephen et al., 2017; Veronese et al., 2018). ASL seed coat can be a low-cost new source of both DF and polyphenols that can increase the availability of high fibre and polyphenols food products for consumers, as aforementioned (Garcia-Amezquita et al., 2018).

Besides the potential physiological effects of DF and polyphenols, ASL seed coat showed advantages in their technological properties, i.e. physicochemical properties as discussed in Section 8.2.1.1. Therefore, ASL seed coat can have diverse functions in food processing and formulations, as bulking agents that can replace fats, sugars and other ingredients to produce low-caloric meat and bakery products as discussed in Section 2.7. Additionally, its high antioxidant capacity could stabilise the end products and increase their shelf-life (Elleuch et al., 2011; Garcia-Amezquita et al., 2018).

Cons:

ASL seed coat has a compact structure with low porosity. Moreover, the majority of ASL seed coat dietary fibre is the insoluble dietary fibre, which is mainly cellulose and is poorly fermented in the human colon thus will be excreted intact in the faeces (O'Grady et al., 2019; Williams et al., 2019). Although the water-binding capacity of the IDF will contribute to its benefits in increasing faecal weight and decreasing transit time by stimulating peristalsis, SDF is generally more desirable for its both technological and nutritional properties (Elleuch et al., 2011; Stephen et al., 2017; Williams, Grant, Gidley, & Mikkelsen, 2017).

DF also has been implicated to show some side effects in nutritional perspective. For example, they could reduce the absorption efficacy of other food components like minerals, protein and vitamins through binding capacity. As detailed in Section 2.7, the percentage of ASL seed coat incorporation in food products may be finite to avoid

undesirable changes to qualities of the end products (e.g. consumer acceptability, colour, odour and texture) (Elleuch et al., 2011).

8.2.2. Extrusion cooking: an applicable technology to enhance qualities of lupin seed coat for food application

Extrusion cooking is widely used to improve the functional properties of high fibre by-products from the agricultural section and food processing. It is a low cost, high throughput and its versatility permits effective process optimisation (Alam et al., 2016). In this study, a twin-screw extruder was used to maximise SDF content in lupin seed coat. Processing parameters were successfully optimised.

8.2.2.1.The twin-screw extrusion cooking operation and experimental design

Many variables affect the twin-screw extrusion cooking operation and the properties of the extruded product. Besides the type (co-rotating or counter-rotating) and design (motor size, length to diameter (L/D) ratio) of the twin-screw extruder, there are several operation parameters such as material properties (e.g. composition, particle size, density, viscosity and flow properties, water-binding capacity), feed rate, the moisture content in barrel, screw speed and screw configuration (screw elements number and design), barrel temperature, die geometry (Figure 8.1) (Duque et al., 2017; Kazemzadeh, 2011; Yacu, 2011). These operation parameters (independent factors) collaboratively can further determine shear stress distribution, motor torque, residence time, mechanical energy input, heat transfer, and complex physicochemical transformations during the processing (Yacu, 2011). In addition, there are strong interactions among the operation parameters and their overlapping effects on the processing (Figure 8.1). Consequently, it is a challenge to control the extrusion cooking process precisely.

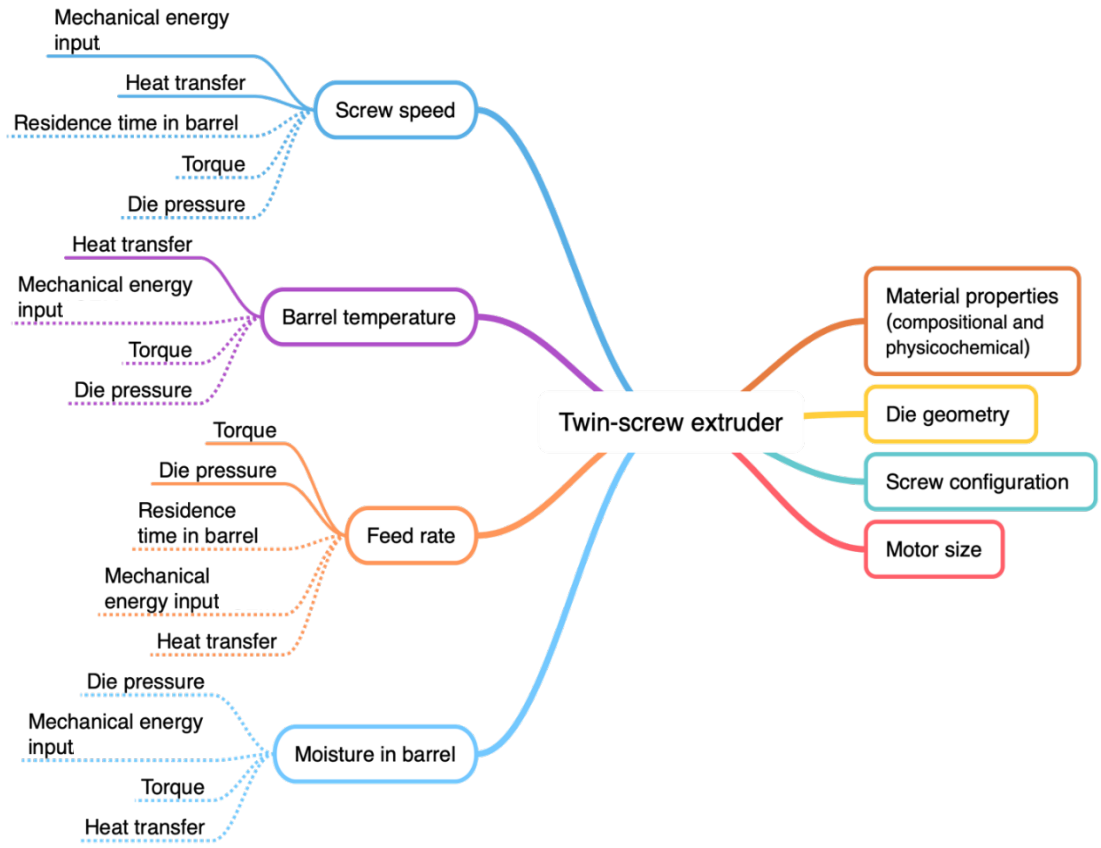


Figure 8.1 The overlapping effects of extrusion cooking independent factors on the properties of lupin seed coat (solid line indicates positive correlations; dashed line indicates negative correlations)

In this study, a co-rotating intermeshing twin-screw extruder (MPF 19:25, APV Baker Inc., Peterborough, England) with motor power and a maximum screw speed of the extruder being 2200 W and 500 rpm is used. Die geometry and screw configuration are fixed to reduce the number of operation parameters (Section 5.2.2). As described in Chapter 5, five primary operation parameters (Table 5.2) are selected for the fractional factorial design to identify the most important independent variables. According to the first-order model, screw speed, barrel temperature and moisture in barrel show significant effects on SDF. Subsequently, the selected independent factors are optimised using the central composite rotatable design (CCRD) (Chapter 6). The real-time torque, die pressure and die temperature are monitored and specific mechanical energy (SME) is calculated to monitor the process indirectly.

Figure 8.2 The dried seed coat during extrusion cooking blocked die and built-up extremely high die temperature and pressure, resulting in over-torque.

As discussed in Chapter 5, the lupin seed coat is a high fibre material with low density, low water-binding capacity. As a result, the material cannot form an expanded porous structure but will lose moisture quickly to generate dehydrated material and a large amount of steam. Subsequently, the processing could lead to blasting output, along with steam, and showed high fluctuations in torque and die pressure (Section 5.3.1). In some cases, the dehydrated seed coat would block the die and increase die pressure dramatically, and finally lead to over-torque and terminate the processing (Figure 8.2). Therefore, the extrusion cooking conditions are carefully identified according to preliminary experiments based on (1) extended maximum and minimum ranges of the models, (2) the extruder worked properly (stable and consistent output, and no over-torque, over-pressure and blockage).

8.2.2.2. The capacity of twin-screw extrusion cooking in lupin seed coat functionalities modifications

The results in Chapter 5-6 demonstrate that the extrusion cooking could purposely increase soluble dietary fibre (SDF) in the lupin seed coat, from 4.42 g/100 g db up to 11.37 g/100 g db depending on the processing parameters, in contrast, insoluble dietary fibre (IDF) content is reduced. Moreover, the changes in the SDF and IDF ratio are attributed to the redistribution of SDF to IDF, which are supported by the results of FTIR (Section 5.3.4). Moreover, collapse in the physical structure of the seed coat matrix and abruptions of cell wall integrity caused by the extrusion cooking are observed by the scanning electron microscopy (SEM) (Section 7.3.4).

Apart from the effects on dietary fibre composition, extrusion cooking shows both positive and negative impacts on total free polyphenol content (TFPC), based on extrusion processing conditions (Section 6.3.2.1). On the one hand, extrusion cooking

can reduce heat-sensitive free polyphenols. On the other hand, the process could liberate bound polyphenols to free forms, reflecting as the simultaneously decreased total bound polyphenol content (TBPC). The results are further supported by the changes in individual polyphenol profiles after extrusion cooking (Section 6.3.3). Besides, extrusion cooking can depolymerise high molecular weight polyphenols (like condensed tannins) and lead to Maillard reaction, all of which can contribute to the increases in TPC (Brennan et al., 2011; Wang et al., 2014). Likewise, the results of antioxidant capacity assays (i.e. DPPH, ABTS, ORAC) (AOXs) are strongly correlated with polyphenol content such that the extrusion cooking showed similar effects on AOXs as polyphenol content (Section 6.3.4).

As a result of the structural and chemical changes in lupin seed coat mentioned above, physicochemical properties like water solubility (SOL) and water-binding capacity (WBC) are also modified. SOL show slight increases while reductions in WBC are found (Section 5.3.3). However, both SOL and WBC are not included for optimisation in this study, mainly due to the strong correlation between SOL and SDF, and the nonspecific determination of WBC. Moreover, the colour of extrusion cooked lupin seed coat turns to pale brown from the initial light beige, which complicates its use in human foods as described in Section 5.3.1. In terms of the effects on mineral and polyphenol bioaccessibility, bioavailability and dialysability, it is unexpectedly found that the processing largely has no effects on bioaccessibility, bioavailability and dialysability of selected lupin seed coats minerals and polyphenols (Section 7.3.2 and Section 7.3.2). Moreover, iron contaminations in extrusion cooked lupin seed coats are found, which may originate from screw and barrel wear, necessitating to monitor heavy metal contamination in the processing.

8.3. Potential impact

There are very few studies on compositional and physicochemical properties of lupin seed coat, while the existing data are from seed coats of several now redundant lupin varieties. In addition, only very limited research has been reported on developing lupin seed coat-based ingredients for human food or development of processing technologies to improve its functionalities to add value to what is currently a major "waste" stream from lupin flour manufacture. Therefore, this study can provide: (a) fundamental new knowledge on lupin seed coat nutrient and phytochemical

composition and their bioavailabilities, and physicochemical properties; (b) an optimised extrusion processing technology to effectively modify lupin seed coat to obtain a highly functional dietary fibre ingredient; a process that may in the future be applied to other underutilised "waste" fibre streams; (c) a new nutritionally functional dietary fibre ingredient to help fill dietary fibre intake gap of the general public.

These outputs may lead to the following outcomes: (a) consumers may benefit through the increased availability of high fibre food products made using the modified lupin seed coat fibre; (b) lupin farmers may benefit through increased demand and financial returns on lupin varieties most suited for functional seed coat ingredient manufacture; (c) millers and seed merchants may benefit through value-addition to the current low value of seed coat by-product stream, and reduced seed coat waste disposal costs; (d) the food industry may benefit through an optimised process to manufacture value-added modified lupin seed coat ingredients.

Nonetheless, there are several limitations in this study: (a) it is unfortunate that the G × E study (Chapter 3 and 4) did not include seasonal factors (different years). Moreover, only two locations were selected but their environmental conditions are not contrasting enough; (b) the individual polyphenols were identified using HPLC-DAD-ESI-MS/MS only. More advanced technologies (e.g. triple quadrupole mass spectrometry and quadrupole-orbitrap mass spectrometry, nuclear magnetic resonance imaging) could be used to improve the identification confidence level (Kamel, 2003); (c) the extrusion conditions were constrained by the capacity of the extruder, more desirable products may be achieved using more a powerful extruder (Chapter 5 &6); (d) polysaccharides (cellulose, hemicellulose, pectin) and oligosaccharides profile, as well as the sugar composition in raw, extrusion cooked lupin seed coats and the dietary fibre fractions were not determined (Evans, 1994; Górecka, Lampart-Szczapa, Janitz, & Sokolowska, 2000); (e) the *in vitro* digestion model rather than animal or human trials was used to evaluate mineral and polyphenol bioaccessibility and bioavailability (Chapter 7). The results may not reflect the actual values in humans (Guerra et al., 2012). Moreover, fermentation in the colon was not included (Gullon, Gullon, Tavaria, Vasconcelos, & Gomes, 2015; Williams et al., 2017).

8.4. Future research

- Quantifying cellulose, hemicellulose, pectin content and the sugar compositions in raw and extrusion cooked lupin seed coats, as well as in SDF and IDF fractions, would be helpful to understand mechanism under the dietary fibre composition changes induced by extrusion cooking.
- To obtain more detail on lupin seed coat individual polyphenol identification, more advanced mass spectrometry could be used.
- Apigenin-7-*O*-β-apiofuranosyl-6,8-di-*C*-β-D-glucopyranoside is the predominant polyphenol which accounts for around 80% of lupin seed coat individual polyphenols content. The compound could be concentrated to produce novel functional food ingredients or be included as a component of nutraceuticals or cosmetics (He et al., 2016).
- Whole-genome sequencing has been performed for Australian sweet lupin (ASL) (Yang et al., 2015), and other nine legumes, such as soybean, chickpea and common bean (Foyer et al., 2016). By comparing the genomics across legume species, candidate genes of ASL, which are related to their seed and seed coat development and some key metabolic pathways (protein, polyphenols accumulations), could be identified to develop new genomics-assisted breeding strategies for ASL quality improvement.
- Combination of extrusion cooking and other treatments, simultaneously or sequentially, to further increase SDF content. For example, raw seed coat could be pre-conditioned with steam/water, cell wall degrading enzymes (e.g. xylanase, cellulase) or other chemicals (e.g. sodium hydroxide, sulfuric acid) (Duque et al., 2017). Reactive extrusion cooking (extrusion cooking along with enzymes or other chemicals) could be used. However, post-extrusion operations (e.g. enzymatic treatments, microwave, second step extrusion cooking) are recommended for industrial application (Dang & Vasanthan, 2019).
- Scale-up studies are required for commercial use purpose.
- Development of new food products that include the extrusion cooked seed coat. Qualities of the end products, sensory properties of particular, should be analysed. In turn, the results could be used to adjust the extrusion cooking process to meet the specific requirements in food product development.

- The *in vitro* fermentation studies, pre-clinical studies in animals and clinical trials in humans could be conducted to investigate the seed coat colon fermentability, as well as its impacts on human gut microbiome and the production of metabolites such as short-chain fatty acids (Gentile & Weir, 2018; Williams et al., 2017).

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APPENDICES

Appendix A Supplemental Materials

Section I.

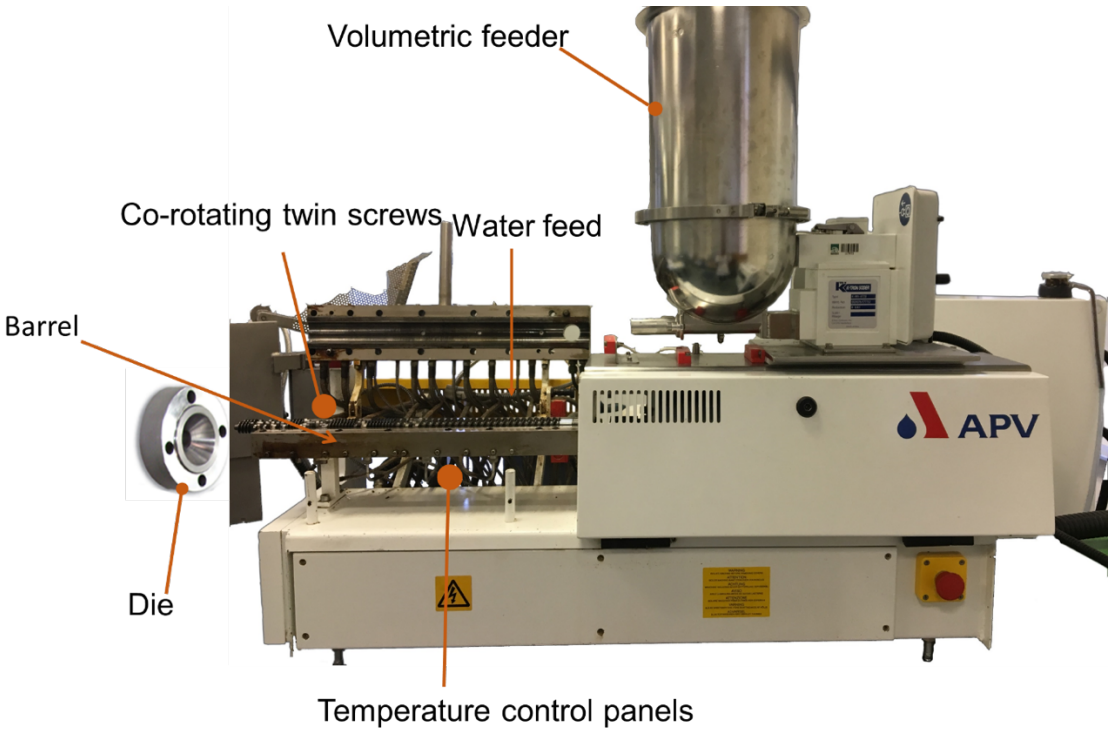


Figure S 1 The co-rotating intermeshing twin-screw extruder (MPF 19:25, APV Baker Inc., Peterborough, England) equipped with 3 mm circular die and a twin-screw volumetric feeder (K-MV-KT20, K-Tron, New Jersey, USA)

Section II. Fractional factorial screening models and diagnostics of each response (tabulated ANOVA (i.e., residue analysis); Half-Normal Probability plot of effects; Plot of residuals versus predicted response values)

Table S 1 ANOVA for selected factorial model for soluble dietary fibre (SDF)

Source	Sum of Squares	df	Mean Square	F-value	*p*-value	
Transform: Inverse						
Model	0.0007	5	0.0001	23.64	3.064E-05	significant
A-PSD	1.253E-06	1	1.253E-06	0.2036	0.6614	
B-Final barrel temperature	0.0001	1	0.0001	24.01	0.0006	
C-Screw speed	0.0000	1	0.0000	5.92	0.0353	
D-Total moisture	0.0004	1	0.0004	71.94	7.031E-06	
AB	0.0001	1	0.0001	16.12	0.0025	
Residual	0.0001	10	6.156E-06			
Cor Total	0.0008	15				

Table S 2 ANOVA for selected factorial model for insoluble dietary fibre (IDF)

Source	Sum of Squares	df	Mean Square	F-value	*p*-value	
Model	2614.67	3	871.56	10.82	0.0010	significant
A-PSD	751.22	1	751.22	9.32	0.0100	
B-Final barrel temperature	711.99	1	711.99	8.84	0.0116	
D-Total moisture	1151.46	1	1151.46	14.29	0.0026	
Residual	966.94	12	80.58			
Cor Total	3581.61	15				

Table S 3 ANOVA for selected factorial model for water binding capacity (WBC)

Source	Sum of Squares	df	Mean Square	F-value	*p*-value	
Model	0.2509	1	0.2509	22.71	0.0003	significant
D-Total moisture	0.2509	1	0.2509	22.71	0.0003	
Residual	0.1547	14	0.0111			
Cor Total	0.4057	15				

Table S 4 ANOVA for selected factorial model for solubility (SOL)

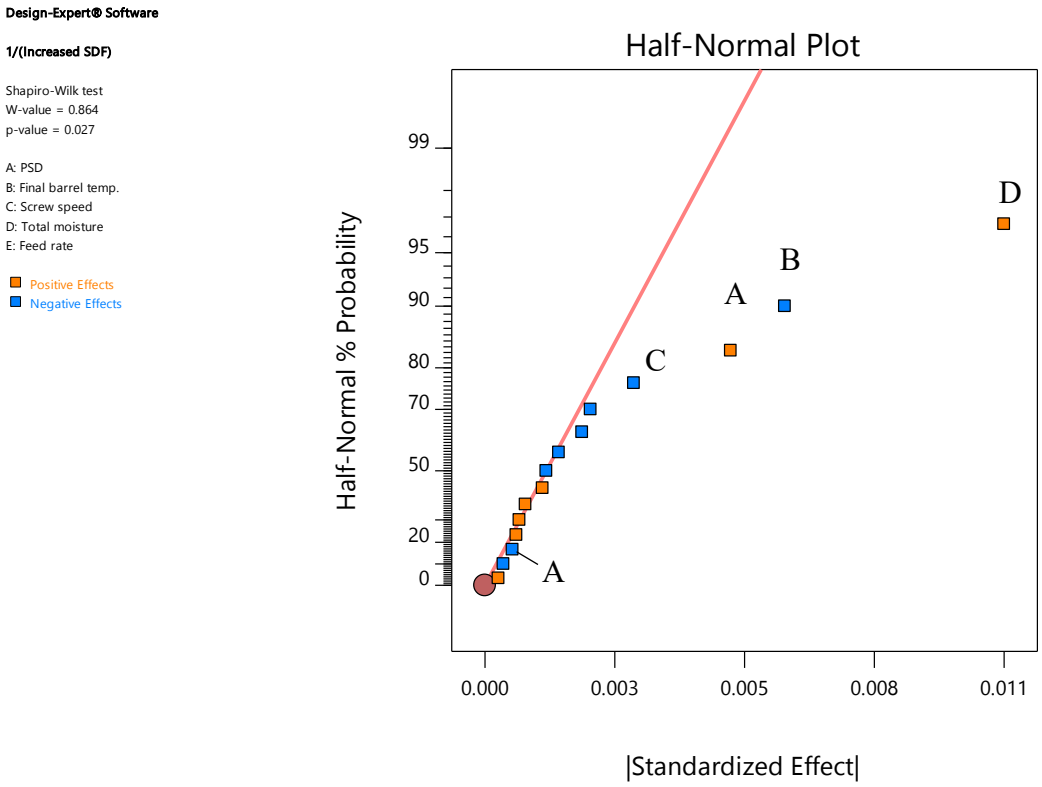
Source	Sum of Squares	df	Mean Square	F-value	*p*-value	
Model	16.30	2	8.15	15.41	0.0004	significant
B-Final barrel temperature	3.77	1	3.77	7.12	0.0193	
D-Total moisture	12.53	1	12.53	23.70	0.0003	
Residual	6.87	13	0.5288			
Cor Total	23.17	15				

Table S 5 Dietary fibre composition of the duplicated the 3rd and 12th run

	IDF (g/100 g db)	SDF (g/100 g db)	TDF (g/100 g db)
Run3	82.99±0.47	8.53±0.45	91.53±0.03
Run12	83.46±0.43	9.32±0.08	92.78±0.51

No significant differences ($p > 0.05$) was observed comparing with the corresponding values in Table 5.3.

A



B

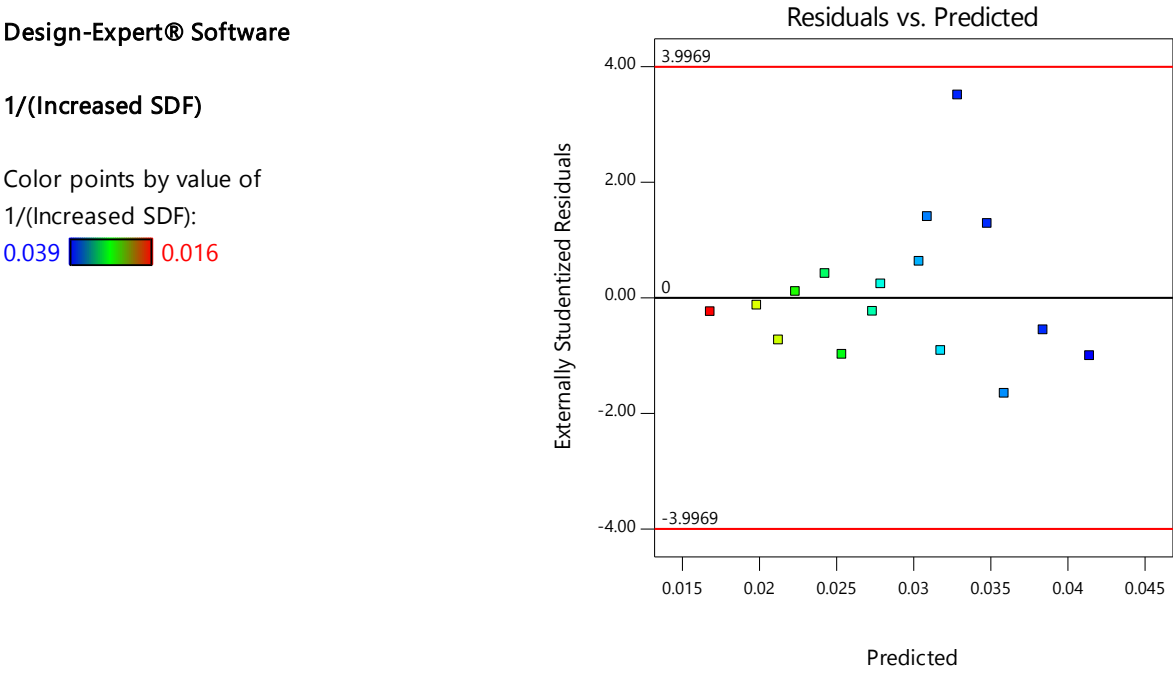
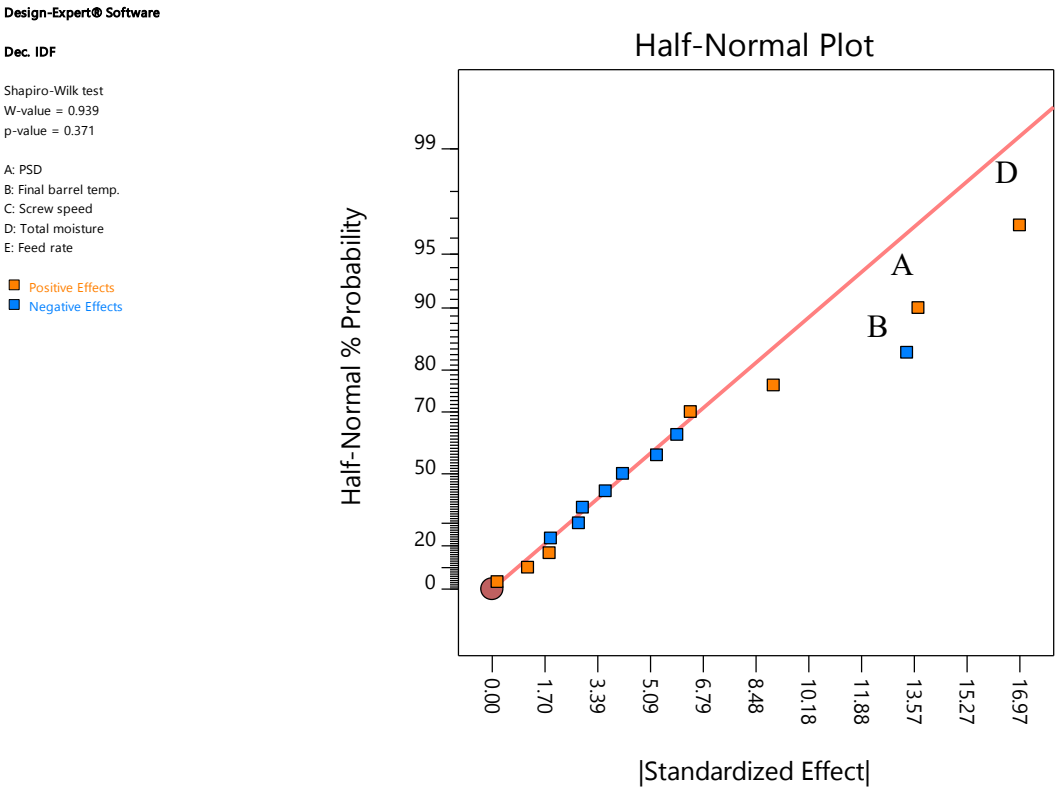


Figure S 2 Diagnostics graphs of the selected model for soluble dietary fibre (SDF), (A) Half-Normal Probability plot of effects, and (B) Plot of residuals versus predicted response values

A



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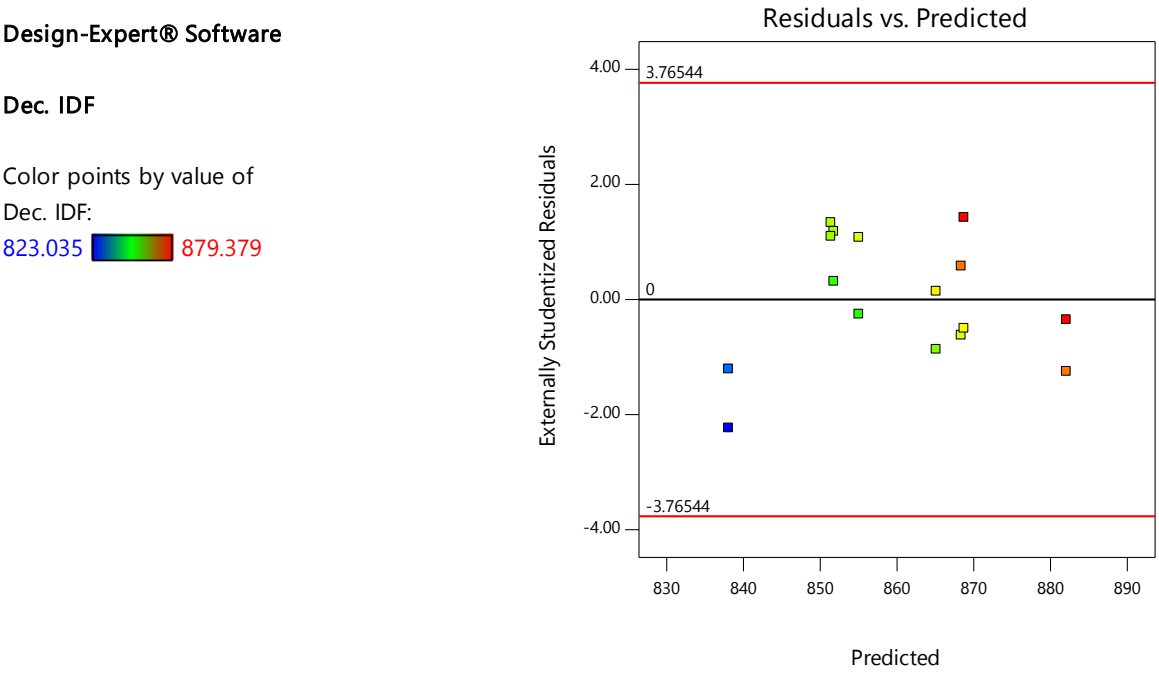
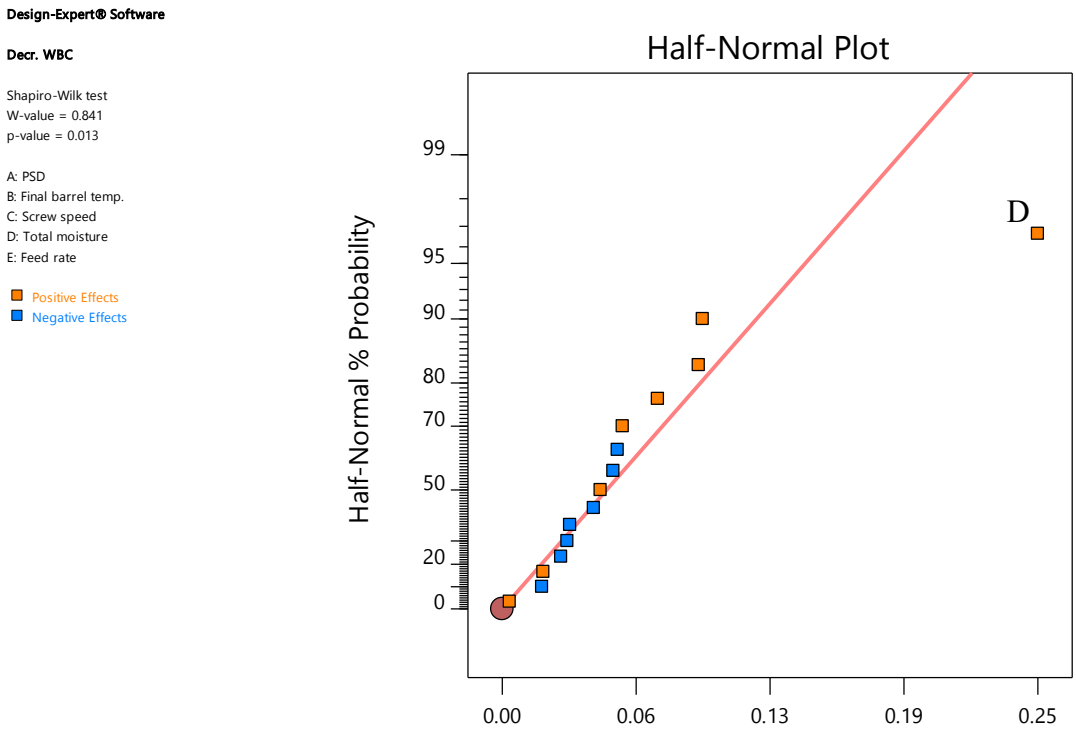


Figure S 3 Diagnostics graphs of the selected model for insoluble dietary fibre (IDF), (A) Half-Normal Probability plot of effects, and (B) Plot of residuals versus predicted response values

A



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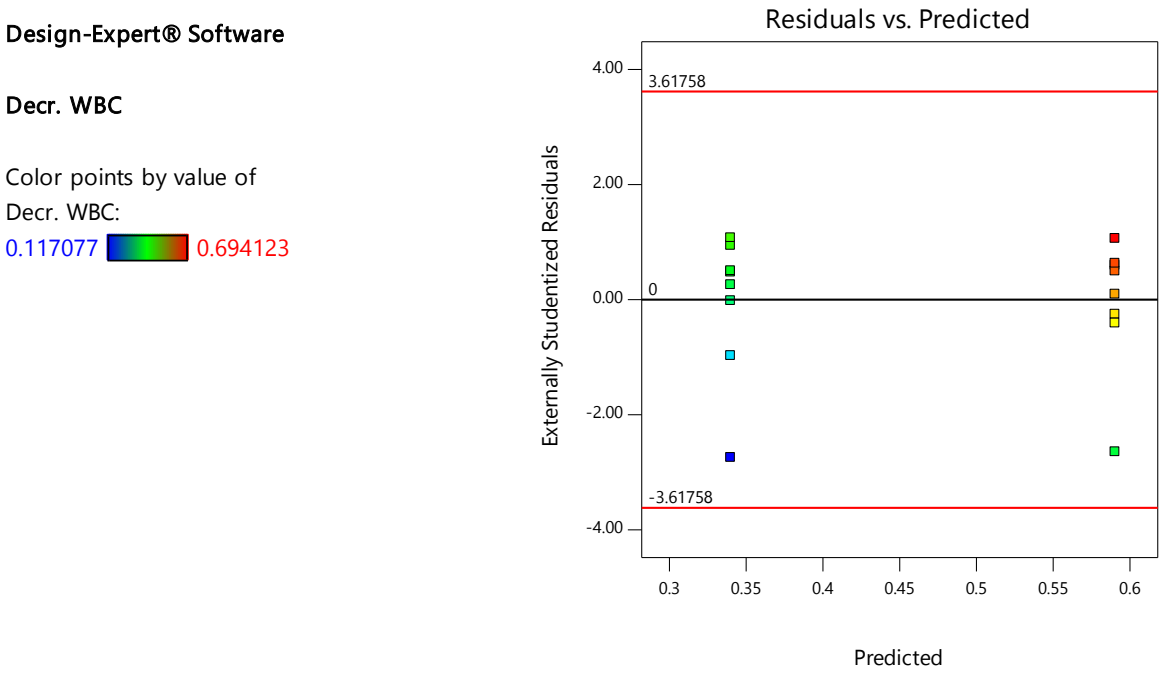
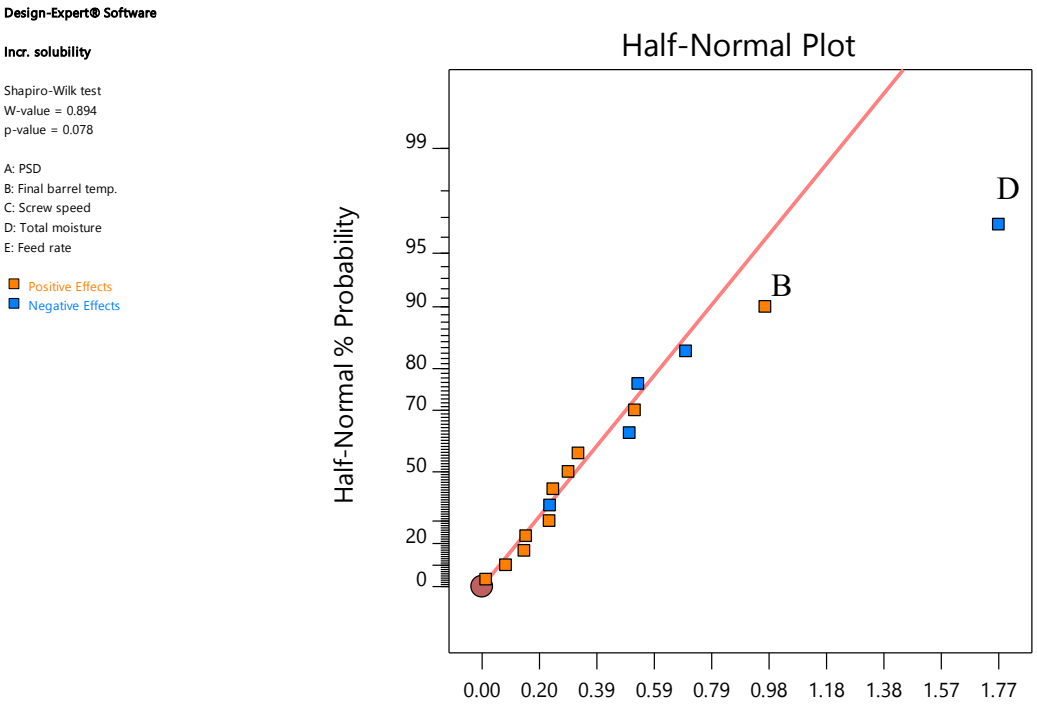


Figure S 4 Diagnostics graphs of the selected model for water binding capacity (WBC), (A) Half-Normal Probability plot of effects, and (B) Plot of residuals versus predicted response values

A



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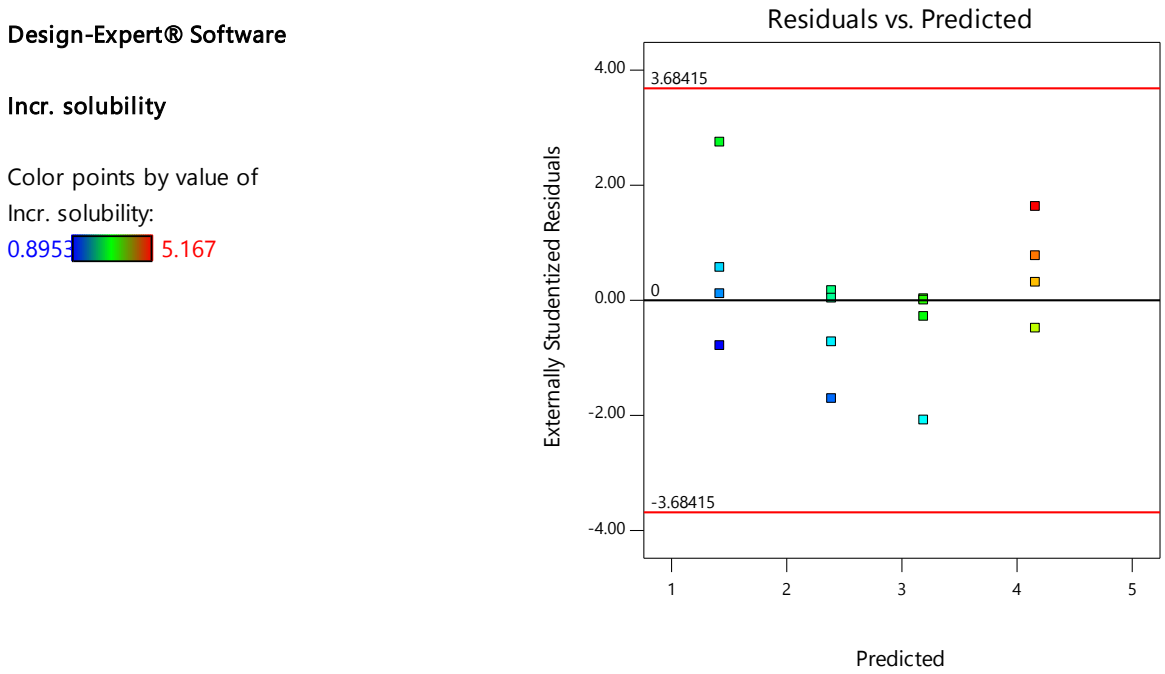
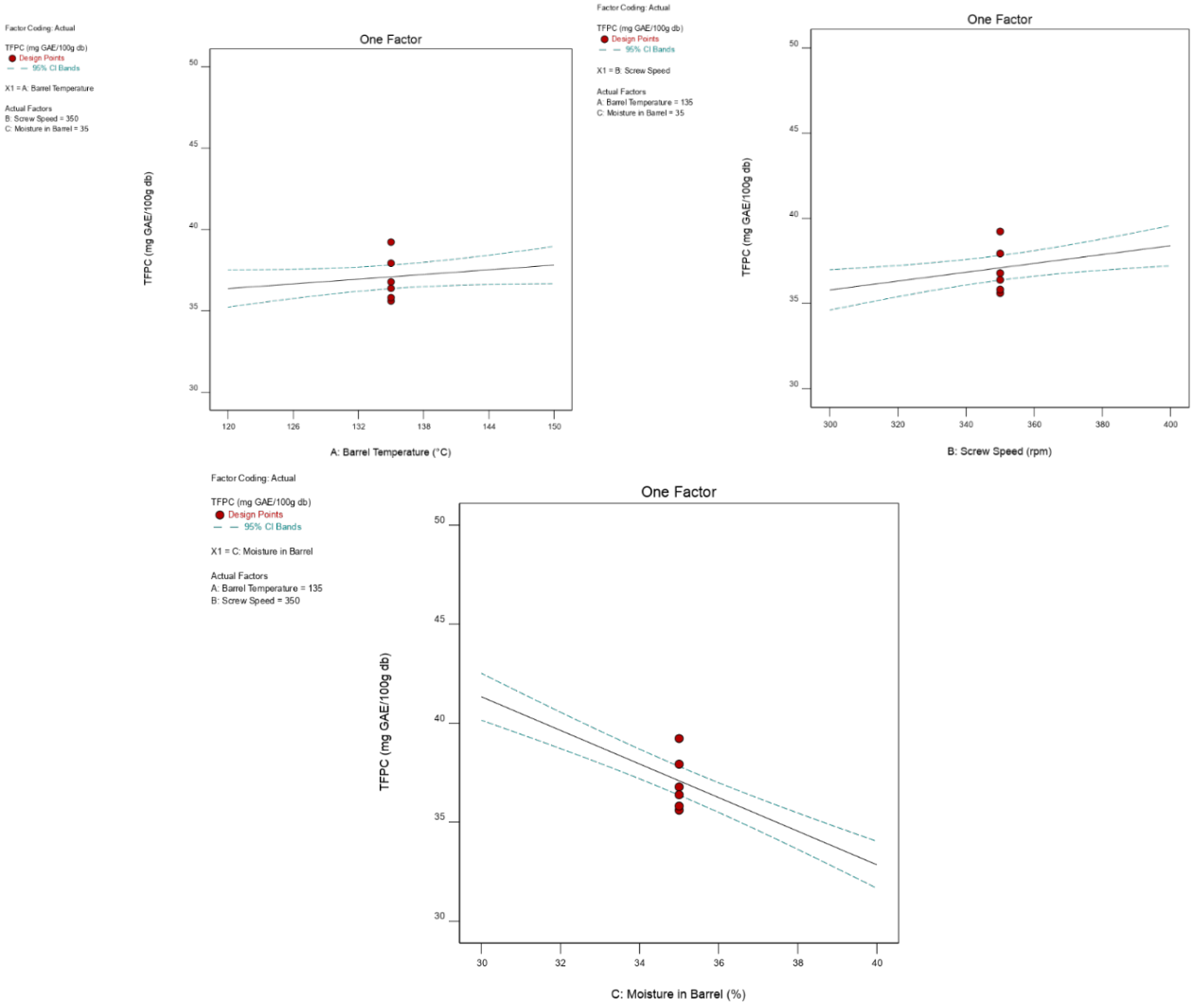


Figure S 5 Diagnostics graphs of the selected model for Solubility (SOL), (A) Half-Normal Probability plot of effects, and (B) Plot of residuals versus predicted response values

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



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

Figure S 6 One-factor plots of TFPC (mg GAE/100 g db) as a function of (A) barrel temperature (°C), (B) screw speed (rpm) and (C) moisture content in barrel (%)

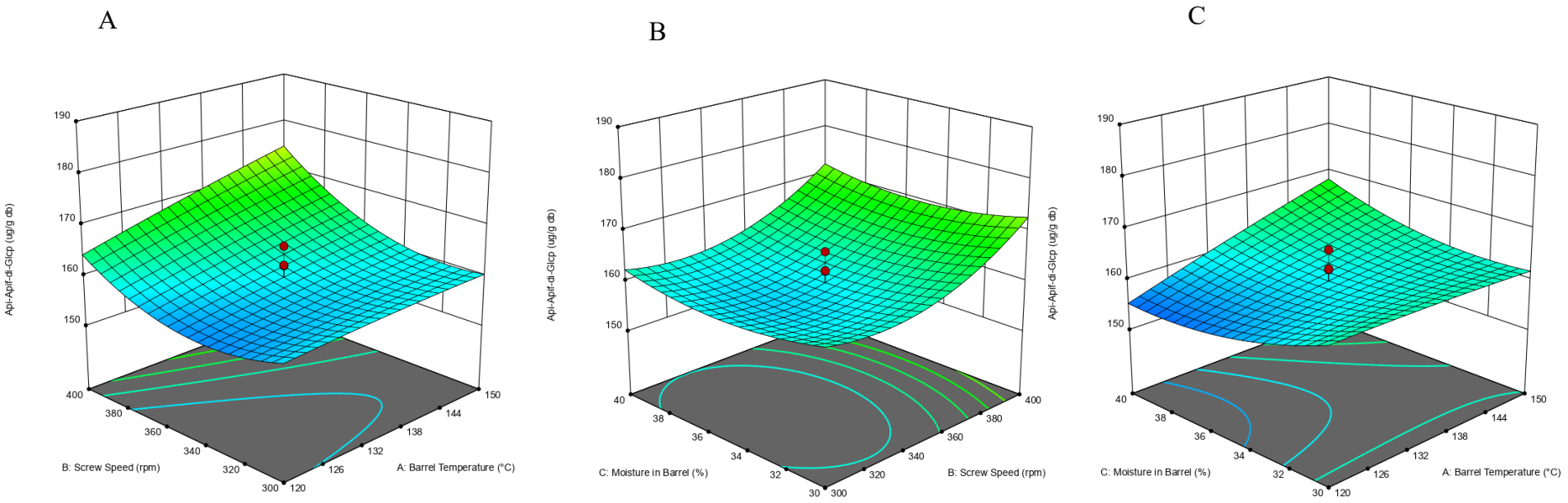


Figure S 7 3D surface plot of Api-Api*f*-di-Glc*p* demonstrating the effects (A) barrel temperature (°C) and screw speed (rpm) at moisture of 35%; (B) screw speed (rpm) and total moisture in barrel (%) at barrel temperature of 135 °C; (C) barrel temperature (°C) and total moisture in barrel (%) at a constant screw speed of 350 rpm

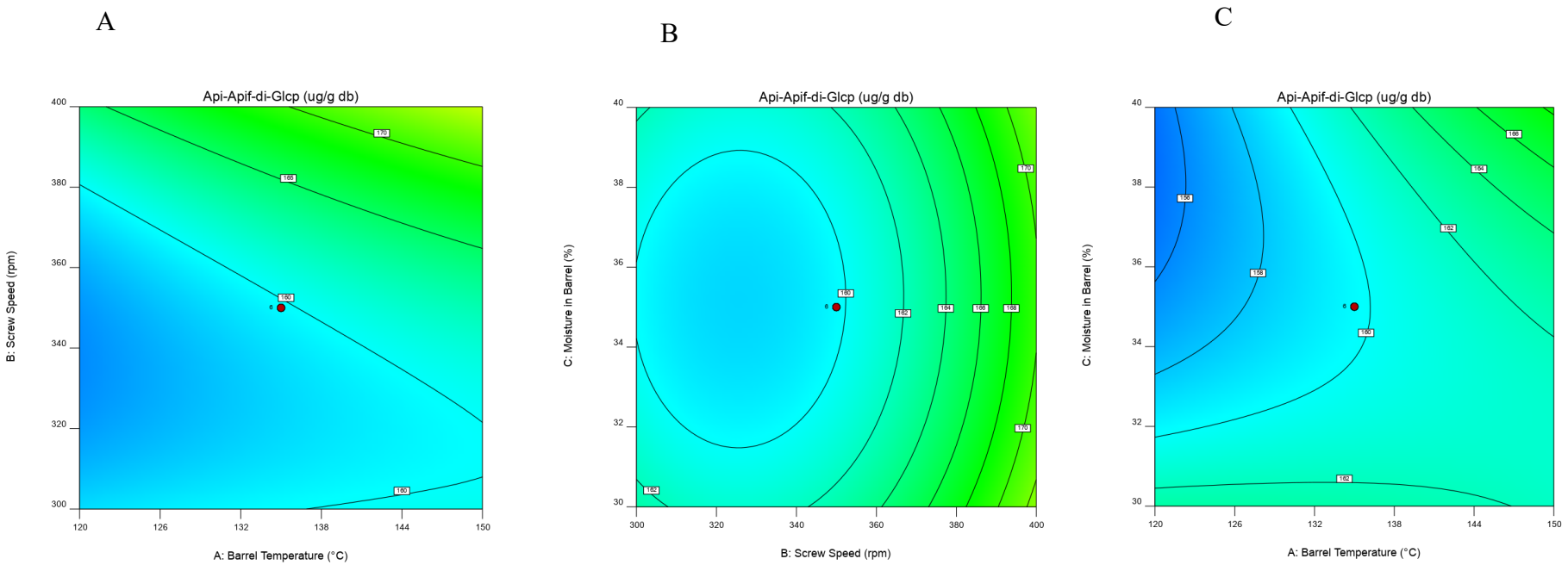


Figure S 8 Contour plot of Api-Api*f*-di-Glc*p* demonstrating the effects (A) barrel temperature (°C) and screw speed (rpm) at moisture of 35%; (B) screw speed (rpm) and total moisture in barrel (%) at barrel temperature of 135 °C; (C) barrel temperature (°C) and total moisture in barrel (%) at a constant screw speed of 350 rpm.

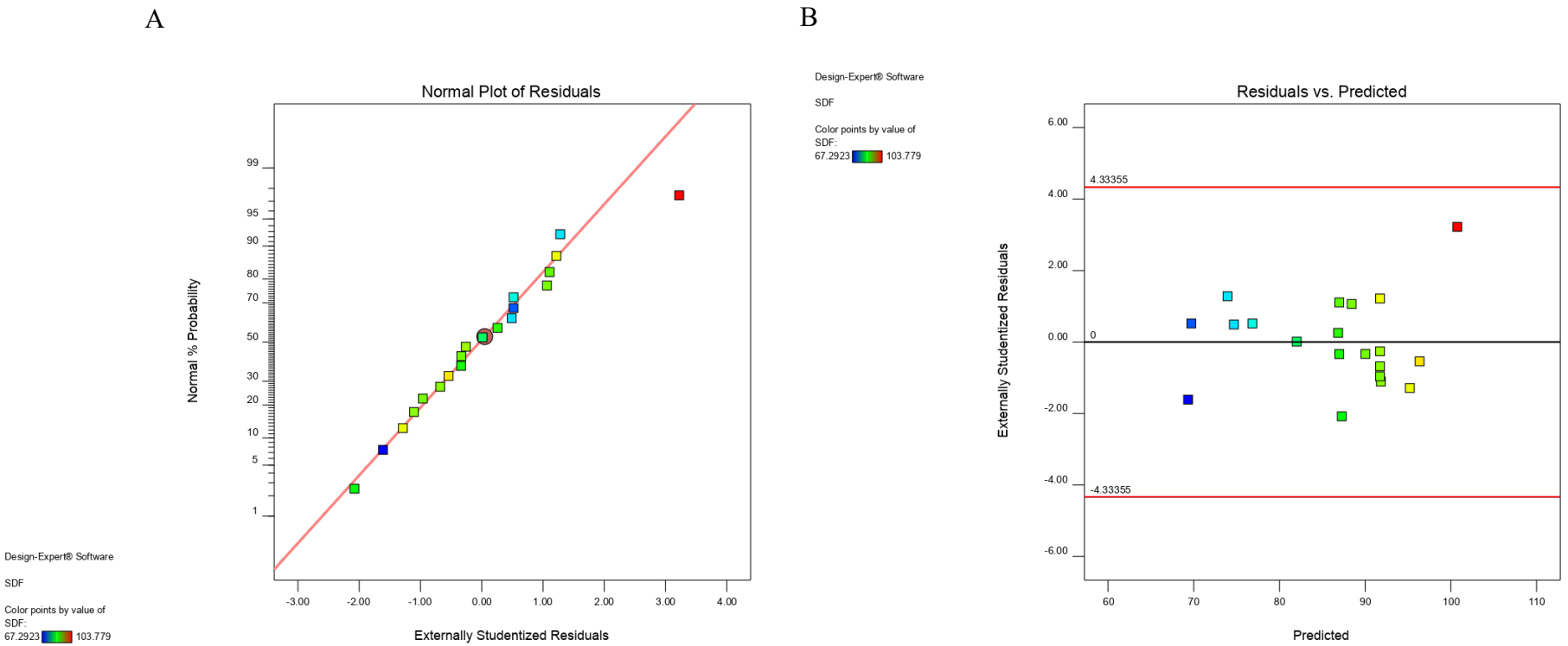


Figure S 9 Diagnostics graphs of the selected model for soluble dietary fibre (SDF): (A) normal probability plot and (B) residuals vs. predicted plot

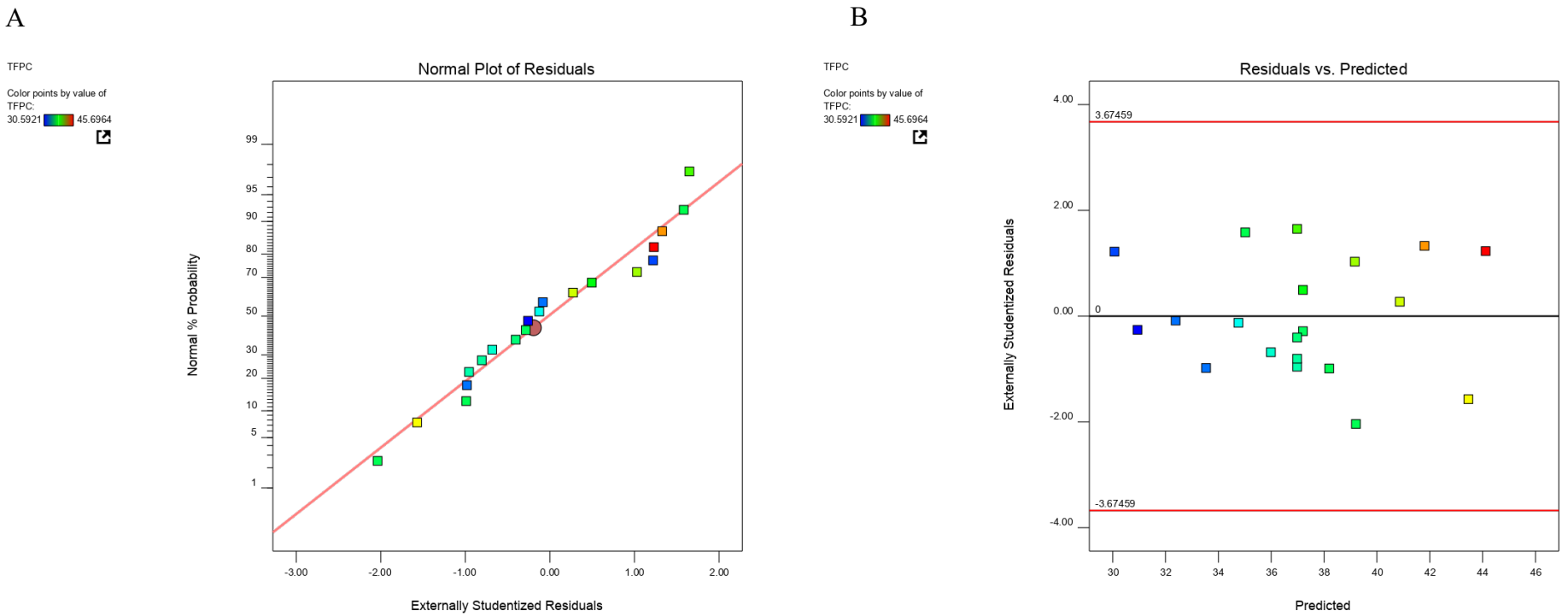


Figure S 10 Diagnostics graphs of the selected model for total free phenolic content (TFPC): (A) normal probability plot and (B) residuals vs. predicted plot

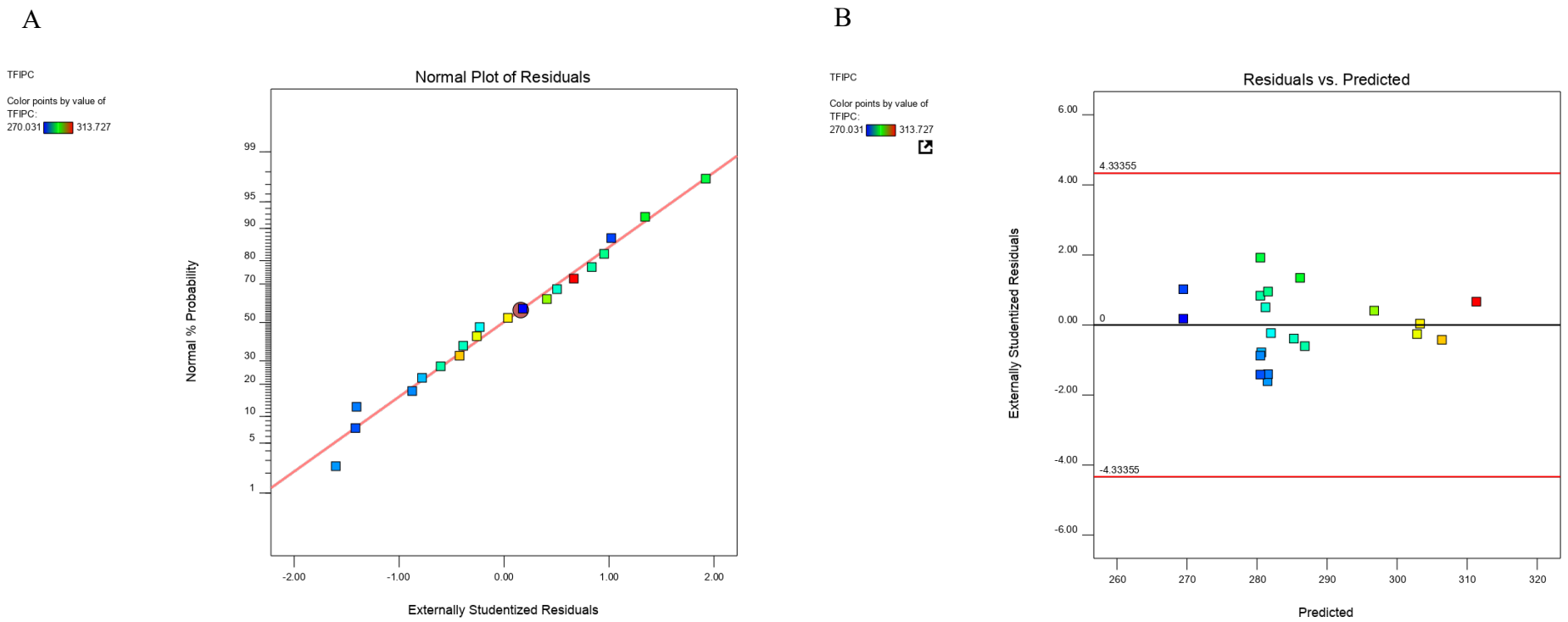
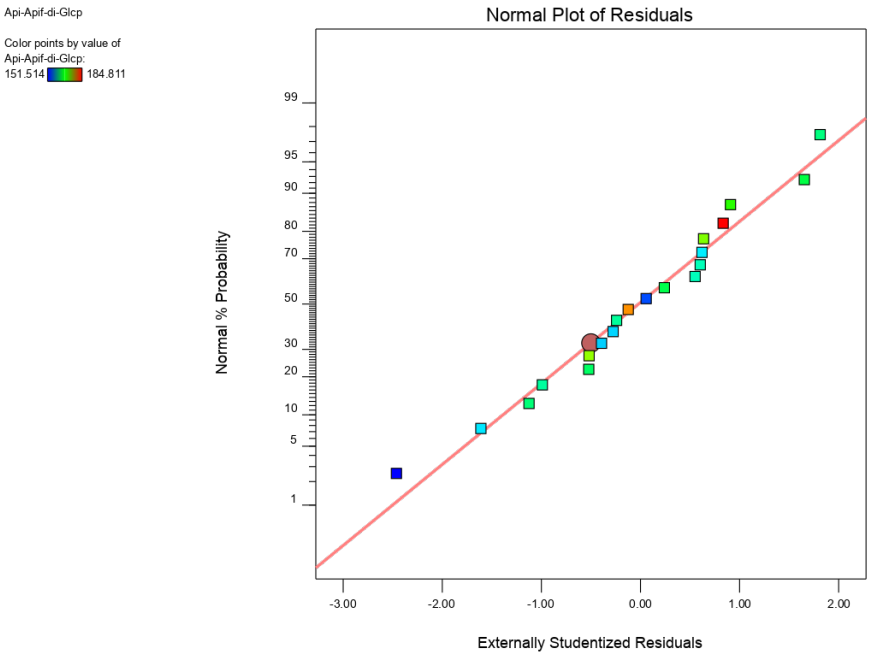


Figure S 11 Diagnostics graphs of the selected model for total free individual phenolic content (TFIPC): (A) normal probability plot and (B) residuals vs. predicted plot

A



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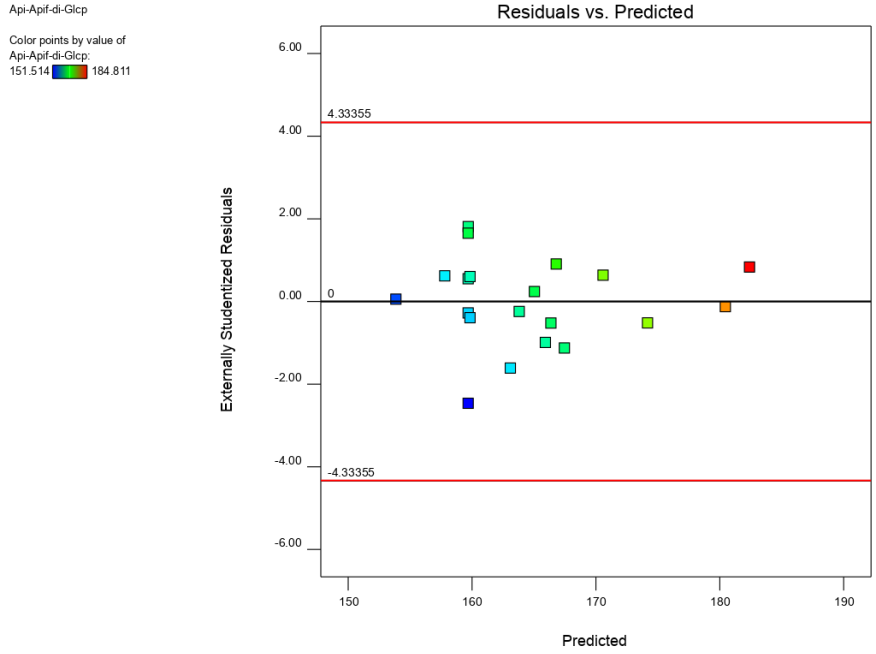

Figure S 12 Diagnostics graphs of the selected model for of Api-Api*f*-di-Glc*p* content: (A) normal probability plot and (B) residuals vs. predicted plot

Appendix B Statement of Contribution of Others

To Whom It May Concern，

I, Liezhou Zhong, am the major contributor to this thesis titled "Exploring and Improving Functional Properties of Underutilised Lupin Seed Coat". The thesis and all publications and presentations listed in "PUBLICATIONS" section and "CONFERENCE PRESENTATIONS" section, and a submitted manuscript entitled: "*Multi-response surface optimisation of extrusion cooking to modify functional properties of lupin seed coat*" were primarily executed, interpreted, discussed, written and edited by Liezhou Zhong under the main supervision and guidance of A/Prof Stuart K. Johnson and co-supervision of Dr Zhongxiang Fang, Prof Mark L. Wahlqvist and Prof Jonathon M. Hodgson.

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

Stuart K. Johnson	Signed:
Zhongxiang Fang	Signed:
Mark L. Wahlqvist	Signed:
Jonathon M. Hodgson	Signed:
Hayder Al-Ali	Signed:
Gangcheng Wu	Signed:
Michael W. Clarke	Signed:
Edwin Junaldi	Signed:

Appendix C The First Pages of Publications

Trends in Food Science & Technology 80 (2018) 35–42

Contents lists available at ScienceDirect

Trends in Food Science & Technology

journal homepage: www.elsevier.com/locate/tifs

Review

Seed coats of pulses as a food ingredient: Characterization, processing, and applications

Liezhou Zhong[a], Zhongxiang Fang[b], Mark L. Wahlqvist[c,d], Gangcheng Wu[a], Jonathan M. Hodgson[e,f], Stuart K. Johnson[a,*]

[a] School of Public Health, Curtin University, Perth, Western Australia, 6102, Australia
[b] Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Parkville, Victoria, 3010, Australia
[c] Fuli Institute of Food Science, Zhejiang University, Hangzhou, 310058, China
[d] Monash Asia Institute, Monash University, Melbourne, Victoria, 3800, Australia
[e] School of Medical and Health Sciences, Edith Cowan University, Joondalup, Western Australia, 6027, Australia
[f] Medical School, Royal Perth Hospital Unit, The University of Western Australia, Perth, Western Australia, 6000, Australia

ARTICLE INFO

Keywords:
Pulses
Seed coat
Dietary fibre
Minerals
By-product
Food ingredient

ABSTRACT

Background: In recognition of their multiple benefits on environment, food security, and human health, pulses are attracting worldwide attention. The seed coat is a major by-product of pulse processing, and its only markets are as low value ruminant feed and very limited use in high fibre foods. Recently, accumulating studies have suggested that this underutilised by-product has greater potential as a novel natural "nutritious dietary fibre" which can be used as a functional food ingredient.

Scope and approach: This review discusses biochemical and physicochemical functionalities of seed coats of six globally important pulses: chickpea, field pea, faba/broad bean, lentil and mung bean with a special emphasis on the emerging food pulse lupin. Food process modification and recent human food applications of the seed coats are summarized. Bio-availability of the seed coat compounds, and phomopsins contaminated lupin seed coats as a typical example of safety issue are discussed.

Key findings and conclusions: High levels of dietary fibre, minerals and potential health-promoting phytochemicals in the seed coats indicate their great potential to be used as a natural "nutritious dietary fibre". However, further in-depth studies are required to improve their desirable nutritional, physiological and techno-functional properties whilst minimizing any undesirable ones.

1. Introduction

"Pulses" refers to those low-fat content leguminous seeds which are harvested for dry grain (FAO, 1994). So, oilseeds (e.g. soybean and peanut), leguminous green vegetables (e.g. green peas and green beans) and leguminous fodder plants (e.g. clover and alfalfa) are traditionally excluded. Pulses are historically important in both the human diet and cropping systems as crop rotations, due to their rich-protein and biological nitrogen fixation ability. Although most pulses are not traditionally typical Western-style foodstuffs, international events like "International Year of Pulses 2016" and "Global Pulses Day" suggest that they are being promoted to be important human food world widely (Foyer et al., 2016).

As shown in Table 1, six of the 11 pulses which are covered in the FAO list, chickpea (*Cicer arietinum*), lupin (*Lupinus*), field pea (*Pisum sativum*), faba/broad bean (*Vicia fabae*), lentil (*Lens culinaris*) and mung bean (*Vigna radiate*), are the most important pulses globally, totally accounting for 79.89% of the world pulse production (81.8 million tonne) in 2016 (FAOSTAT, 2018). India is the largest pulse producer globally, followed by Canada, Myanmar and China. However, Australia is the largest lupin producer in the world, contributing an average of 58.22% of the world production in 2012–2016 (ABARES, 2018). Australian sweet lupin (ASL, *L. angustifolius*), which is also named "narrow-leafed lupin", is the most important lupin specie, constituting 93% of Australian lupin production and 52% of the world production (Pulse Australia, 2016). However, chickpea has overtaken lupin as Australia's largest pulse crop since 2011-12, with a production estimated at over 2 million tonne in 2016–17 (ABARES, 2018). As a leading pulse exporter, Australia exports over 90% of its chickpeas, faba beans, lentils and mung beans, and 60% of field peas were exported, being the largest

* Corresponding author.
E-mail address: S.Johnson@curtin.edu.au (S.K. Johnson).

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Available online 26 July 2018
0924-2244/

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Food Research International

journal homepage: www.elsevier.com/locate/foodres

Characterization of polyphenols in Australian sweet lupin (*Lupinus angustifolius*) seed coat by HPLC-DAD-ESI-MS/MS

Liezhou Zhong[a], Gangcheng Wu[b,c], Zhongxiang Fang[d], Mark L. Wahlqvist[e,f], Jonathan M. Hodgson[g,h], Michael W. Clarke[i], Edwin Junaldi[a], Stuart K. Johnson[j,*]

[a] School of Public Health, Curtin University, Bentley, Western Australia 6102, Australia
[b] State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi 214122, China
[c] School of Food Science and Technology, Jiangnan University, Wuxi 214122, China
[d] Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Parkville, Victoria 3010, Australia
[e] Fuli Institute of Food Science, Zhejiang University, Hangzhou 310058, China
[f] Monash Asia Institute, Monash University, Melbourne, Victoria 3800, Australia
[g] School of Medical and Health Sciences, Edith Cowan University, Joondalup, Western Australia 6027, Australia
[h] Medical School, Royal Perth Hospital Unit, The University of Western Australia, Perth, Western Australia 6000, Australia
[i] Metabolomics Australia, Centre for Microscopy, Characterisation and Analysis, The University of Western Australia, Perth, Western Australia 6009, Australia.
[j] School of Molecular and Life Sciences, Faculty of Science and Engineering, Curtin Health Innovation Research Institute, Curtin University, Bentley, Western Australia 6102, Australia

ARTICLE INFO

Keywords:
Lupinus angustifolius
Australian sweet lupin
Seed coat
Polyphenols
Genotype by environment
By-product
Hplc-DAD-ESI-MS/MS

ABSTRACT

Seeds of the legume lupin (*Lupinus* spp.) are becoming increasingly important as human food. The seed coat, at ~25% of the whole seed of *Lupinus angustifolius* (Australian sweet lupin, ASL), is the main by-product of lupin kernel flour production. The primary market for lupin seed coat is low value feed with very limited use in foods. In this study, seed coats of six ASL commercial varieties from two growing sites were sampled for identification and quantification of polyphenols using a high-performance liquid chromatography (HPLC) with diode array detector (DAD) and coupled with a triple quadrupole mass spectrometer which equipped with electrospray ionization source (ESI-MS/MS). Three flavones (apigenin-7-*O*-β-apiofuranosyl-6,8-di-*C*-β-glucopyranoside, vicenin 2, and apigenin-7-*O*-β-glucopyranoside), one isoflavone (genistein) and one dihydroflavonol derivative (aromadendrin-6-*C*-β-D-glucopyranosyl-7-*O*-[β-D-apiofuranosyl-(1 → 2)]-*O*-β-D-glucopyranoside), and several hydroxybenzoic and hydroxycinnamic acid derivatives were identified. Considerable variations in levels of individual polyphenols were found but apigenin-7-*O*-β-apiofuranosyl-6,8-di-*C*-β-glucopyranoside was the predominant polyphenol in all samples accounting for 73.08–82.89% of the total free polyphenols. These results suggest that ASL seed coat could be valuable dietary source of polyphenols.

1. Introduction

Seeds of the legume lupin are attracting worldwide attention as a potential future staple food (Johnson, Clements, Villarino, & Coorey, 2017). This is due to their multiple roles in farming systems (through nitrogen fixation ability), and their likely benefits for human nutrition and health contributed by the high dietary fibre and protein content. Western Australia (WA) is the world's largest lupin producer, with *Lupinus angustifolius* (Australian sweet lupin, ASL) being the major species under production. However, ASL has a relatively higher percentage of seed coat, generally 25% of the whole seed, than most of other pulses like soybean (5–8%) and pea (*Pisum sativum* L.) (9–14%). As a result, flour production from the dehulled kernels for human consumption has a high proportion of commercial loss (~31% in Australia). This represents a tough disposal problem for the industry, since the seed coat has little market value or demand; it is primarily a low value animal feed (Sipsas, 2008).

The seed coat of pulses, including chickpea (Sreerama, Neelam, Sashikala, & Pratape, 2010a), faba bean (Boudjou, Oomah, Zaidi, & Hosseinian, 2013), field pea (Marles, Warkentin, & Bett, 2013), lentil

Abbreviations: ASL, Australian sweet lupin; BP, Bound polyphenols; ER, Eradu; FP, Free polyphenols; Mw, Molecular weight; WA, Western Australia; WH, Wongan Hills

* Corresponding author.
E-mail address: S.Johnson@curtin.edu.au (S.K. Johnson).

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Extrusion cooking increases soluble dietary fibre of lupin seed coat

Liezhou Zhong[a], Zhongxiang Fang[b], Mark L. Wahlqvist[c,d], Jonathan M. Hodgson[e,f], Stuart K. Johnson[g,*]

[a] School of Public Health, Curtin University, Perth, Western Australia, 6102, Australia
[b] Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Parkville, Victoria, 3010, Australia
[c] Fuli Institute of Food Science, Zhejiang University, Hangzhou, 310058, China
[d] Monash Asia Institute, Monash University, Melbourne, Victoria, 3800, Australia
[e] School of Medical and Health Sciences, Edith Cowan University, Joondalup, Western Australia, 6027, Australia
[f] Medical School, Royal Perth Hospital Unit, The University of Western Australia, Perth, Western Australia, 6000, Australia
[g] School of Molecular and Life Sciences, Faculty of Science and Engineering, Curtin Health, Australia

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ABSTRACT

Extrusion cooking is widely used to improve the functional properties of dietary fibre of food processing by-products. This study investigated the capacity of extrusion cooking to increase the proportion of dietary fibre in the Australian sweet lupin seed coat which is soluble and modify its physicochemical properties (water binding capacity and water solubility). Fractional factorial design was used to identify which of the extrusion cooking factors most affected seed coat dietary fibre composition and physicochemical properties. Extrusion cooking significantly increased soluble dietary fibre content of lupin seed coat, from 29.03 g/kg dry basis to a maximum of 90.28 g/kg dry basis, while decreasing insoluble dietary fibre content from 898.91 g/kg dry basis to 828.85 g/kg dry basis. Water solubility increased (4.02–4.47% vs. 5.37–9.64% dry basis) whereas water binding capacity slightly decreased (3.84–3.88 g/g vs. 3.15–3.73 g/g dry basis) after extrusion cooking. The screw speed, total moisture content in barrel and barrel temperature were identified as the most important processing factors. These findings suggest that extrusion cooking could be a practical technology to increase the soluble dietary fibre content in lupin seed coat and enhance health benefits of the seed coat.

1. Introduction

Due to multiple health benefits of dietary fibre (DF), efforts have been made to incorporate high fibre food processing by-products into human diets (Elleuch et al., 2011). These may be derived from vegetables (e.g. onion and carrot wastes), fruits (e.g. residues after juice or wine productions), cereals (e.g. wheat bran and rice bran), oil cake, pulses (e.g. seed coat), etc. (Zhong et al., 2018). Legume seeds *Lupinus angustifolius* (Australian sweet lupin), which are high in protein and DF, are increasingly important as human food (Foyer et al., 2016). The seed-coat, at ~25% of the whole seed, is the main by-product of lupin kernel flour production and has great potential to be a source of DF and phytochemicals such as phenolic antioxidants. It is well established that physiological benefits of different DF fractions are determined by their chemical structure (Wahlqvist, 2016). For example, it has been generally assumed that insoluble dietary fibre (IDF) shows no or slight effect on digesta viscosity and is poorly fermentable in colon whereas soluble dietary fibre (SDF) can significantly increase digesta viscosity and be well-fermented to produce short chain-fatty acids (Kumar, Sinha, Makkar, Boeck, & Becker, 2012). Given that over 95 percent of DF fraction in lupin seed coat is IDF, optimized technologies are required to increase SDF level to enhance the health benefits of lupin seed coat.

Extrusion cooking is the most widely used technology to increase SDF content in high IDF food material (Rashid, Rakha, Anjum, Ahmed, & Sohail, 2015; Wolf, 2010). During extrusion cooking, food materials are cooked in a sealed barrel under high pressure, high temperature and high mechanical shear in a short time. The process results in various chemical reactions, disruption of cell wall structures and rheological changes (Singh, Gamlath, & Wakeling, 2007). Extrusion cooking increased SDF content of a range of high IDF sources such as pea seed coat and sugar-beet pulps, soybean residue (Jing & Chi, 2013), carrot residue (Gao, Yan, Xu, Ye, & Chen, 2015), orange pomace (Huang & Ma, 2016), wheat bran (Rashid et al., 2015), rice and rye bran (Andersson, Andersson, Jonsall, Andersson, & Fredriksson, 2017), along with beneficially modified physico-chemical properties (e.g., viscosity, solubility

* Corresponding author.
E-mail address: S.Johnson@curtin.edu.au (S.K. Johnson).

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Lupin seed coat as a promising food ingredient: physicochemical, nutritional, antioxidant properties, and effect of genotype and environment

Liezhou Zhong,[1] Hayder Ali,[1] Zhongxiang Fang,[2] Mark L. Wahlqvist,[3,4] Jonathan M. Hodgson[5,6] & Stuart K. Johnson[7]*

1 School of Public Health, Curtin University, Perth, Western Australia 6102, Australia
2 Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Parkville, Victoria 3010, Australia
3 Monash Asia Institute, Monash University, Melbourne, Victoria 3800, Australia
4 Division of Population Health Science, National Health Research Institutes, Taipei 11503, Taiwan
5 School of Medical and Health Sciences, Edith Cowan University, Joondalup, Western Australia 6027, Australia
6 Medical School, Royal Perth Hospital Unit, The University of Western Australia, Perth, Western Australia 6000, Australia
7 School of Molecular and Life Sciences, Faculty of Science and Engineering, Curtin Health Innovation Research Institute, Curtin University, Bentley, Western Australia 6102, Australia

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Summary The high proportion of seed coat of legume lupins results in big milling lose during kernel flour production, though the seed coat could be value-added as human food. The physicochemical and nutritional properties and antioxidant capacities of seed coats of six Australian sweet lupin cultivars grown at two locations were evaluated. Results showed that genotype, environment and their interaction were significant for seed coat percentage, proximate composition, dietary fibre content, polyphenols and antioxidant capacities. Strong correlations between seed coat lightness and polyphenol content were found. A comparison using multivariate analysis of the seed coat properties showed clear separation based on growing sites. This study indicates the enormous potential of Australian sweet lupin seed coat as an 'antioxidant dietary fibre' food source. The results could also benefit to breed varieties with desirable levels of nutrients and phytochemicals.

Keywords Antioxidant capacity, Australian sweet lupin, dietary fibre, environment, genotype, physicochemical properties, seed coat.

Introduction

The legume lupins play an essential role in Australian and European farming systems through wheat: lupin rotation. Narrow-leafed lupin or Australian sweet lupin (ASL, *Lupinus angustifolius*) is the most worldwide grown domesticated lupin, followed by white lupin (*L. albus*) and yellow lupin (*L. luteus*) (Gresta *et al.*, 2017). ASL cultivars have been continuously bred to improve ASL performance, such as yield, seed qualities, adaptation, disease-resistant and herbicide tolerance (DPIRD, 2018). Recently, the ASL varieties that have dominated Australian production are Mandelup (released in 2004), Coromup (2006), Jenabillup (2007), PBA Gunyidi (2010), PBA Barlock (2013), PBA Jurien (2015) and PBA Leeman (2017) (DPIRD, 2018).

*Correspondent: E-mail: S.Johnson@curtin.edu.au

Lupin is a promising protein crop alternative to soybean and its use in human food is expanding rapidly. The high protein and high fibre lupin kernel flour have been widely used in bakery and meat products (Lucas *et al.*, 2015; Leonard *et al.*, 2019). However, the high proportion of lupin seed coat, which comprises 24% in average of the seed and hence results in high milling loss, complicates its economical usage in human food (Zhong *et al.*, 2018b). Recently, a renewed intention has been paid to value-add the by-product. For example, albus lupin seed coat has been commercially developed as an insoluble fibre ingredient (Vitafiber®, Avelup Ltd., Chile). Seed coat flour of ASL and yellow lupin (*L. luteus*) were incorporated into bakery foods as a source of dietary fibre (Tucek, 2009; Wandersleben *et al.*, 2018) and used to produce cellulose nanofiber aerogel (Ciftci, 2017). In contrast, only a few studies have investigated the nutritional and physicochemical properties of seed coats of several

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