Effect of cultivar and processing on anti-nutritional factors and bioaccessibility of minerals of Australian sweet lupin

(Lupinus angustifolius L.)

by

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STATEMENT OF AUTHENTICATION

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

Signature: …

Date: December 2016
ACKNOWLEDGEMENTS

During my PhD journey, I had to face a few unfortunate events which were out of my control. During the third year, I had to significantly change my research project and transferred from Curtin University to Western Sydney University. To complete my PhD within four years by overcoming the challenges could not have happened without help and support from my supervisors, colleagues, friends and family.

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Most importantly, I would like to express my heartfelt gratitude and appreciation to my beloved mother for her love, patience and support throughout this endeavor.
ABSTRACT

Australian sweet lupin (ASL) (*Lupinus angustifolius* L.) is an underutilised grain legume with a unique chemical composition. It contains high protein and dietary fibre and is a good source of vitamins, minerals and bioactive compounds. Due to various health benefits, there is an increasing interest in developing lupin incorporated functional foods. However, the presence of anti-nutritional factors is one of the reasons that limit commercial production of lupin based foods. Major anti-nutritional factors in lupin include raffinose family oligosaccharides (RFOs), phytate and polyphenols. Phytate and polyphenols have negative effects on the minerals bioavailability. RFOs also cause flatulence and abdominal discomfort.

Published information on the anti-nutritional factors and mineral bioavailability of ASL is limited. The present study aimed at investigating the effect of cultivar, cultivation year and dehulling on mineral (calcium, iron, magnesium, potassium and zinc), anti-nutritional factors (RFOs, phytate, total phenolics, total flavonoids and condensed tannins) and mineral bioaccessibility (calcium, iron and zinc) of ASL. The relationships between minerals or anti-nutritional factors, and mineral bioaccessibility were also determined.

Ten cultivars of ASL (Belara, Corumup, Gungurru, Jenabillup, Mandelup, PBA Barlock, PBA Gunyidi, Quilinock, Tanjil, and Walan 2385) cultivated at Wongan Hills Research Station in 2011, 2012 and 2013 were obtained from the Department of Agriculture and Food, Western Australia. Lupin samples were analysed for RFOs, phytate, total phenolics, total flavonoids, condensed tannins, calcium, iron, magnesium, potassium and zinc contents. Bioaccessibility of calcium, iron and zinc in heat treated lupin samples were determined using a dialysability method.

The results showed that most of ASL cultivars are good sources of RFOs. Average total RFOs content in ASL (dehulled seeds) was 10.5 g/100 g DM which is higher than most of the other pulses such as black gram and mung bean. Phytate content in lupin is similar to some other pulses such as chickpea and mung bean but lower than kidney bean and soybean. ASL cultivars had low levels of total phenolics (< 100 mg GAE/100 g DM), total flavonoids (< 20 mg CE/100 g DM) and condensed tannins (< 80 mg...
Dehulled seed of ASL contained relatively high amount of calcium (95 mg/100 g DM) and potassium (1120 mg/100 g DM). Iron (3 mg/100 g DM) and zinc (4 mg/100 g DM) contents in ASL (dehulled seed) were similar to some other grain legumes such as soybean and lentil.

The results showed that cultivar has a significant influence on RFOs, phytate, total phenolics, total flavonoids, condensed tannins, calcium, iron, magnesium, potassium and zinc contents in lupin. Belara and Mandelup contain high levels of total RFOs and recommended for prebiotic rich functional food product development. Gungurru and PBA Barlock had low RFOs and suitable for lupin-enriched foods with low flatulence effect. PBA Barlock contained higher level of polyphenols and flavonoids than most of the other lupin cultivars. Walan 2385 has the highest condensed tannin content and high in flavonoids contents. The highest iron (3.2 mg/100 g DM) and zinc (3.8 mg/100 g DM) contents were found in Belara and Quilinock, respectively. Belara and Quilinock also contain high calcium contents. These findings on the effect of cultivar on anti-nutritional factors and minerals are helpful in selecting suitable cultivars for particular food applications.

Lupin flour for food applications is commercially produced by the dry dehulling technique. Lupin flour can be incorporated into various foods. The effect of dry dehulling on iron, magnesium, zinc, RFOs, phytate, total phenolic and total flavonoid contents depends on the lupin cultivar. Dehulling increases condensed tannin and potassium contents in most lupin cultivars. Calcium contents in all lupin cultivars were reduced as a result of dehulling.

Average calcium, iron and zinc bioaccessibility values of ASL (dehulled seeds) were 11, 21 and 12%. Calcium and zinc bioaccessibility values of lupin were poor and lower than some other grain legumes such as cowpea and mung bean. The low calcium and zinc bioaccessibility values indicate that these minerals may form large complexes which are difficult to be digested by the human digestive system. In contrast, iron bioaccessibility of ASL is higher than values reported for other pulses including red grams and black grams. Cultivar had a significant effect on calcium and iron bioaccessibility of lupin but had no effect on zinc bioaccessibility. PBA Gunyidi had higher calcium bioaccessibility than some of the other cultivars. High iron
bioaccessibility values were found in Gungurru and Mandelup. Calcium bioaccessibility of most of the lupin cultivars were increased after dehulling.

Phytate to calcium molar ratios of dehulled lupin samples present poor calcium bioavailability. All tested lupin cultivars had high phytate to iron molar ratios indicating poor iron bioavailability. High phytate to zinc molar ratios were recorded in almost all of the lupin samples implying poor zinc bioavailability. Although the phytate to mineral molar ratios are widely used as predictors of mineral bioavailability, the result of the study shows that the phytate to mineral molar ratio is not correlated to the mineral bioaccessibility of lupin. Therefore, the phytate to mineral molar ratio may be not a suitable predictor of the mineral bioaccessibility of lupin.

Bioaccessibility was not directly related to calcium, iron or zinc contents of lupin which shows that high mineral content is not always related to high mineral bioaccessibility. Calcium content was negatively correlated to bioaccessibility of calcium of lupin. Results also showed negative trend between iron content and iron bioaccessibility as well as zinc content and zinc bioaccessibility. Poor correlations between minerals (calcium, iron and zinc) bioaccessibility values and anti-nutritional factors (RFOs, phytate, total phenolics, total flavonoids and condensed tannins) were found. This finding indicates that the anti-nutritional factors studied are not likely to significantly affect the minerals bioaccessibility of lupin.

A stepwise multiple regressions were performed to develop predictive equations to predict mineral bioaccessibility using the minerals and anti-nutritional factors contents. A predictive equation using calcium, phytate and RFOs contents can predict 69% of the calcium bioaccessibility of lupin. Iron, calcium, RFOs and polyphenols contents can be used to predict 70% of the iron bioaccessibility of lupin. A regression equation using zinc, calcium and condensed tannin contents can estimate 59% of zinc bioaccessibility of lupin. These predictive equations indicate that there are other factors affecting the minerals bioaccessibility of lupin in addition to the factors studied.
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1. INTRODUCTION

Lupin is an underutilised pulse which has been consumed as a food in the Mediterranean region and the Andean highlands for centuries. Traditional lupin varieties are high in alkaloids and bitter in taste and have limited acceptability as a food source. Low alkaloid lupin cultivars approved for human consumption have been developed by breeding programs. Australian sweet lupin (ASL), *Lupinus angustifolius* L., is the major lupin species with low alkaloid content cultivated in Australia. At present, Australia is the largest lupin producer in the world. Lupin flour has a unique chemicals composition with high protein and dietary fibre and a good source of vitamins, minerals and a range of bioactive compounds. Lupin flour is low glycaemic index and contains negligible amount of starch (Arnoldi et al. 2015).

Cardiovascular diseases are the main cause of deaths worldwide and diabetes is one of the top ten leading causes of death (WHO 2016a). Australian Institute of Health and Welfare (2014) reported that one-fourth of Australian adults had cardiovascular diseases and/or diabetes. Unhealthy diet is one of the important causative factors of cardiovascular diseases and diabetes. Regular consumption of unhealthy diet leads to high levels of blood pressure, glucose and cholesterol resulting in the development of non-communicable diseases (Mendis, Puska & Norrving 2011).

Health benefits of lupin are well documented. Consumption of lupin results in the reduction of risk factors of cardiovascular diseases and type 2 diabetes. Anti-diabetic effect, blood pressure-lowering effect and cholesterol-lowering effect of lupin have been reported (Arnoldi et al. 2015). Lupin is also a good prebiotic and improves bowel functions resulting in reducing the risk of colon cancer (Smith et al. 2006; Johnson et al. 2006).

Despite the health benefits, lupin is mainly used for livestock feed. There is an increasing interest in using lupin in human foods as a result of health benefits. A range of lupin incorporated foods such as breads, biscuits and pasta have been developed. There is a limited success in commercial production of lupin based foods, mainly due to poor texture (as a result of high dietary fibre), beany flavour (mainly due to
lipoxygenase enzymes activity), and the presence of anti-nutritional factors. Major anti-nutritional factors in lupin include raffinose family oligosaccharides (RFOs), phytate and polyphenols. RFOs cause flatulence and abdominal discomfort limiting the consumption of pulses including lupin. Phytate and phenolic compounds have strong negative effects on the mineral bioavailability (Strain & Cashman 2009).

Mineral deficiencies are major public health problems worldwide. Approximately one-third of the world population has iron and/or zinc deficiency (WHO 2016b). The global prevalence of inadequate calcium intake was higher than 50%. Iron deficiency causes anaemia and impairs function of endocrine and immune systems. Zinc deficiency leads to retarded physical growth, and impaired gastrointestinal and immune function. Inadequate dietary calcium increases the risk of osteoporosis. Although inadequate intake is a common cause of mineral deficiency, poor mineral bioavailability due to the presence of anti-nutritional factors is also play a crucial role (Bailey et al. 2015; Kumssa et al. 2015; Lazarte et al. 2015).

Limited published information on anti-nutritional factors and mineral bioavailability of ASL has been published. No studies on the effects of cultivar and processing on anti-nutritional factors and mineral bioavailability of ASL have been conducted. The study aimed at determining the minerals and anti-nutritional factors contents, and bioavailability of minerals in ASL cultivars. To support the use of lupin as a health enhancing food ingredient in various food and nutraceutical products, it is necessary to study the presence of anti-nutritional factors and mineral bioavailability. The outcomes of study will be helpful in understanding the effect of cultivar and processing on minerals, anti-nutritional factors and bioavailability of minerals in lupin. The outcome will also provide the information which is beneficial in selecting appropriate cultivars for particular food applications.
1.1 Hypotheses
Cultivar and processing affect the level of minerals, anti-nutritional factors and bioaccessibility of minerals in Australian sweet lupin.

1.2 Objectives
- To investigate the effect of cultivar on minerals and anti-nutritional factors contents, and mineral bioaccessibility of ASL
- To investigate the effect of cultivation year on minerals and anti-nutritional factors contents, and mineral bioaccessibility of ASL
- To investigate the effect of dehulling on minerals and anti-nutritional factors contents, and mineral bioaccessibility of ASL
- To determine the relationship between minerals and anti-nutritional factors contents, and mineral bioaccessibility of ASL
2. LITERATURE REVIEW

2.1 Lupin

2.1.1 Taxonomy

Lupin or lupine (*Lupinus* L.) is a plant species in the genus *Lupinus*, subfamily *Papilionaceae*, family *Fabaceae* or *Leguminosae*. Most lupin species are herbaceous, but some species are shrubs and trees. The attractive long flowering spikes formed above its leaves is one of dominant characteristics of lupin plant. Its long nodular root, found commonly in plants in the *Leguminosae* family, has an ability to fix atmospheric nitrogen which can improve the soil fertility. The pods are flat with hairs on the outside and 4–10 cm in length. The colour of endosperm is yellowish and most seeds are spherical or flat. Seed surface is smooth or fine-meshed (Office of the Gene Technology Regulator 2013; Trugo, von Baer, D & von Baer, E 2003).

Lupin plant growth is classified into seedling, vegetative, flowering and podding stages (Figure 1). The seedling stage starts at emergence showing at least one of the clearly visible cotyledons at the soil surface, and ends with the appearance of unfolded four leaves. The vegetative stage begins with unfolded five leaves, and ends with the occurrence of the flower buds on the main stem (after 18 to 20 leaves have been produced). The flowering stage starts with the visible flower buds (Budding point), and ends when the last petal is wither away. The podding stage starts when pods are thickened and developed rapidly (pod fill point). The pods become dry, and the seeds become hard with yellow cotyledons when matured (White, French & McLarty 2008).

There is no clear information about the number of species within the genus *Lupinus*. The number is considered to be in the range of 800–1000 species (Kurlovich et al. 2002). Though, up to 200 species in the genus *Lupinus* are known, only *L. albus* (white lupin), *L. angustifolius* (blue or narrow-leafed lupin), *L. luteus* (yellow lupin) and *L. mutabilis* (Andean or pearl lupin) are commercially cultivated. The seed characteristics of some domesticated species of lupins are shown in Table 1.
Figure 1: Stages of lupin plant growth
(Modified from Dracup & Kirby 1996; White, French & McLarty 2008)
Table 1: Seed characteristics of the main domesticated species of lupins

<table>
<thead>
<tr>
<th>Species</th>
<th>Seed weight (mg)</th>
<th>Seed colour</th>
<th>Per cent of hull (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. albus</td>
<td>180–950</td>
<td>White or pink</td>
<td>18</td>
</tr>
<tr>
<td>L. angustifolius</td>
<td>30–240</td>
<td>White or cream, sometimes speckled brown</td>
<td>24</td>
</tr>
<tr>
<td>L. luteus</td>
<td>70–150</td>
<td>White, sometimes speckled black</td>
<td>24</td>
</tr>
<tr>
<td>L. mutabilis</td>
<td>80–280</td>
<td>White or cream</td>
<td>13</td>
</tr>
</tbody>
</table>

(Source: Clements et al. 2008; Dracup & Kirby 1996)

2.1.2 History of lupin

Lupin has been consumed by people living in the Mediterranean region and the Andean highlands for centuries. However, wild types of lupin seed contain alkaloids which make the seed bitter and toxic to animal and human (Office of the Gene Technology Regulator 2013). The presence of alkaloids resulting in the bitterness of lupin was indicated as critical factor that limits lupin applications in feeds and/or foods. Lupin varieties containing high levels of alkaloids are called bitter lupin. In the late 1700s, lupins were moved from their origins to northern Europe, and were grown as green manuring to improve soils. In the early of 20th century, there was a success in developing sweet lupin varieties (low alkaloid content) by German scientists. The sweet lupin varieties were adopted and cultivated in Europe and introduced into Australia. Later Australia becomes the world largest producer of lupin (White, French & McLarty 2008).

2.1.3 Development of Australian sweet lupin cultivars

The development of lupin cultivars in Australia was pioneered by the Western Australian plant breeder, Dr John Gladstones. Australian sweet lupin (ASL) refers to sweet variety (low alkaloid content) of Lupinus angustifolius L. or narrow-leafed lupin which is the major lupins grown in Australia (Department of Agricultural and Food, Western Australia 2008). In Australia, ASL cultivars have been developed through breeding programmes targeting improvements in yield, disease resistance, seed quality and duration of vegetation (Office of the Gene Technology Regulator 2013). The seeds of ASL are spherical in shape composing of about 24% hull, 72.5% cotyledons and
3.5% seedling axis. Cross-section of ASL mature seed and the seed composition are shown in Figure 2.

![Cross-section of mature seed of Australian sweet lupin](Source: Dracup & Kirby 1996)

Some ASL cultivars developed in Australia and their agronomic features are shown in Table 2. Commercial cultivation of genetically modified (GM) lupins has not been reported. However, genetic engineering researches on production of GM lupin have been established in Australia, Poland and the USA (Office of the Gene Technology Regulator 2013).

<table>
<thead>
<tr>
<th>Cultivars</th>
<th>Year of release</th>
<th>Seed size</th>
<th>Seed colour</th>
<th>Seed protein</th>
<th>Seed alkaloids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belara</td>
<td>1997</td>
<td>medium</td>
<td>brown</td>
<td>medium</td>
<td>very low</td>
</tr>
<tr>
<td>Coromup</td>
<td>2006</td>
<td>large</td>
<td>light brown</td>
<td>high</td>
<td>low-medium</td>
</tr>
<tr>
<td>Jenabillup</td>
<td>2007</td>
<td>medium-large</td>
<td>light brown</td>
<td>medium</td>
<td>low</td>
</tr>
<tr>
<td>Mandelup</td>
<td>2004</td>
<td>medium-large</td>
<td>brown</td>
<td>medium</td>
<td>low-medium</td>
</tr>
<tr>
<td>Quilinock</td>
<td>1999</td>
<td>large</td>
<td>light brown</td>
<td>medium</td>
<td>low</td>
</tr>
<tr>
<td>Tanjil</td>
<td>1998</td>
<td>small-medium</td>
<td>dark brown</td>
<td>medium</td>
<td>low-medium</td>
</tr>
</tbody>
</table>

(Source: GRDC 2008)
2.1.4 Production

Lupins are cultivated for three main reasons: for livestock and aquaculture feeds, green manure, and a small quantity for human food. Approximately 980,000 tonnes of lupin seeds were produced worldwide in 2014. Australia is the largest lupin producer, accounted for around 59% of the total world production, followed by Poland (14%) and Russia (8%) in 2014 (FAOSTAT 2016). Table 3 shows the top 10 lupin producing countries in 2014. Animal feed industries, especially ruminants are the major usage of lupin, and there is an increasing demand for human foods.

Table 3: The top 10 lupin producing countries in 2014

<table>
<thead>
<tr>
<th>Country</th>
<th>Production (tonnes)</th>
<th>Percentage of the global production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australia</td>
<td>580,810</td>
<td>59</td>
</tr>
<tr>
<td>Poland</td>
<td>139,802</td>
<td>14</td>
</tr>
<tr>
<td>Russian Federation</td>
<td>75,690</td>
<td>8</td>
</tr>
<tr>
<td>Germany</td>
<td>40,800</td>
<td>4</td>
</tr>
<tr>
<td>Belarus</td>
<td>34,137</td>
<td>3</td>
</tr>
<tr>
<td>Ukraine</td>
<td>28,930</td>
<td>3</td>
</tr>
<tr>
<td>South Africa</td>
<td>18,770</td>
<td>2</td>
</tr>
<tr>
<td>Chile</td>
<td>16,781</td>
<td>2</td>
</tr>
<tr>
<td>France</td>
<td>15,020</td>
<td>2</td>
</tr>
<tr>
<td>Peru</td>
<td>12,156</td>
<td>1</td>
</tr>
</tbody>
</table>

(Source: FAOSTAT 2016)

2.1.5 Food applications

There is a growing interest of using lupin in food application mainly due to its potential health benefits (Arnoldi et al. 2015; Khan et al. 2015). Also, lupin has a potential as a future sustainable source of plant protein for human (Jayasena, Chih & Nasar-Abbas 2011; Lucas et al. 2015). Lupin flour is considered as an excellent ingredient for supplementing various food products due to its unique chemical composition (Jayasena, & Nasar-Abbas 2011; Sironi et al. 2005). Substitution of traditional wheat-based food products with lupin flour can improve nutritional quality such as protein, dietary fibre and mineral contents in the products (Jayasena, Leung & Nasar-Abbas 2010; Martínez-Villaluenga et al. 2010).
A range of lupin incorporated foods such as biscuit (Jayasena & Nasar-Abbas 2011), bread (Johnson et al. 2003; Villarino et al. 2014; Villarino et al. 2015), breakfast bars (Hall & Johnson 2004), crisp (Lampart-Szczapa et al. 1997), instant noodle (Jayasena, Leung, & Nasar-Abbas 2010), muffin (Nasar-Abbas & Jayasena 2012; Rumiyati, James & Jayasena 2015), pasta and spaghetti (Jayasena & Nasar-Abbas 2012; Lampart-Szczapa et al. 1997; Rayas-Duarte et al. 1996), and tofu (Jayasena, Khu & Nasar-Abbas 2010) have been developed.

Villarino et al. (2015) studied the effect of lupin flour substituted wheat flour on physical properties of bread. The results indicated that incorporation of 20% lupin flour provide comparable volume, crumb cell characteristics and instrumental textural properties to the control wheat bread. Jayasena & Nasar-Abbas (2011) reported that up to 20% of lupin flour can be replaced wheat flour with no significant changes in taste, flavour, texture and overall acceptability of biscuits. Nasar-Abbas & Jayasena (2012) reported that 30% incorporation of lupin flour had no significant effect on the density, height and most of the textural parameters of the muffins. The sensory evaluation also indicated the taste, flavour, texture and overall acceptability of 30% incorporated lupin muffin compared to the control.

Hall and Johnson (2004) indicated that the overall consumer acceptability of 10% lupin flour incorporated cookies, and 20% lupin flour incorporated breakfast bars were rated similarly to the control products. Jayasena, Leung, & Nasar-Abbas (2010) reported that 20% lupin flour can be incorporated to instant noodle with no significant difference on the sensory properties. The nutritional value of the lupin incorporated noodles was improved by the increasing of 42% protein and 200% dietary fibre compared to the control. Jayasena and Nasar-Abbas (2012) reported that pasta substituted semolina with 20% of lupin flour showed similar colour, appearance, taste, texture and overall acceptability to the control.

Lupin can be substituted soybean in a variety of food products. Jayasena, Khu & Nasar-Abbas (2010) reported that in tofu production, up to 40% lupin can substitute soybean with no effect on texture, flavour and overall acceptability. Priatni et al. (2013) also indicated that tempe produced from lupin was not significantly different in quality and sensory properties compared to traditional soybean tempe. Lupin can
also be incorporated or used in various kinds of functional foods for different purposes including appetite suppressant, low glycaemic index, weight loss diets and prebiotics (Martínez-Villaluenga et al. 2005; Sipsa 2008). The use of lupins in food applications may also include sprout, high protein drinks and gluten-free products (Sipsa 2008). Commercial and potential food applications of lupins are shown in Figure 3.

2.1.6 Limitations

Although lupin is a nutritious pulse exhibiting various health benefits, there are some limitations of using lupin in foods. Limited numbers of lupin incorporated foods are commercially available mainly due to poor texture, beany flavour, flatulence, and concern on allergenicity.

Lupin flour can be incorporated in food in a limited amount, since lupin contains high levels of dietary fibre, which may cause the undesirable texture of some food products (Jayasena & Nasar-Abbas 2011). Lupins, similar to other legumes, also exhibit undesirable beany flavour mainly due to lipoxygenase enzymes activity (Loiseau et al. 2001; Stephany et al. 2015). Lupin is one of the richest sources of raffinose family oligosaccharides (RFOs) (Martínez-Villaluenga et al. 2008). RFOs cannot be digested and absorbed in the human gastrointestinal tract. These oligosaccharides are passed through the large intestine and fermented into short-chain fatty acids and gas, causing flatulence and abdominal discomfort (Guillon & Champ 2002; Martínez-Villaluenga et al. 2008).

Similar to other legumes, allergic reactions to lupin have also been reported. Lupin allergy is induced either by primary sensitization or by cross-reactivity with peanut proteins particularly in individuals allergic to peanuts (Dooper et al. 2009; Reis et al. 2007). The prevalence of lupin primary sensitization is not known but appears low, while the prevalence of lupin allergy among peanut-allergic individuals has been estimated between 4% and 30% (Goggin et al. 2008). It has been reported that β-conglutin, a major storage protein, is a principal allergen in lupin (Goggin et al. 2008). For this reason, lupins have been included in the list of food allergens, and must be indicated on labelling according to the European Union food regulations. However, Food Standard Australia New Zealand (FSANZ 2016) has not yet established compulsory allergen labelling for lupin in 2015.
2.1.7 Nutritional composition

Lupin is a rich source of protein and dietary fibre, good source of some vitamins and minerals, low in fat content, and contains negligible amount of starch (Khan et al. 2015). Table 4 shows the chemical compositions of whole lupin seeds of different species.
Table 4: Chemical compositions of whole lupin seeds

<table>
<thead>
<tr>
<th>Composition</th>
<th>L. albus (g/100 g DM)</th>
<th>L. angustifolius</th>
<th>L. luteus</th>
<th>L. mutabilis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ash</td>
<td>3.1–3.9</td>
<td>2.6–3.7</td>
<td>3.1–5.3</td>
<td>3.0–3.2</td>
</tr>
<tr>
<td>Protein</td>
<td>30.6–39.5</td>
<td>28.9–35.2</td>
<td>36.8–49.2</td>
<td>44.0–52.6</td>
</tr>
<tr>
<td>Fat</td>
<td>9.5–14.6</td>
<td>6.3–7.1</td>
<td>4.8–8.8</td>
<td>15.0–16.2</td>
</tr>
<tr>
<td>Total dietary fibre</td>
<td>34.4–39.4</td>
<td>46.5</td>
<td>33.7–34.3</td>
<td>NA</td>
</tr>
<tr>
<td>- Insoluble</td>
<td>30.8–34.2</td>
<td>40.1</td>
<td>28.8–31.1</td>
<td>NA</td>
</tr>
<tr>
<td>- Soluble</td>
<td>3.6–5.2</td>
<td>6.4</td>
<td>3.2–4.9</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA = Not applicable


Lupin seeds are usually dehulled for human food; however, only a few studies have reported the chemical composition of dehulled lupin (Bähr et al. 2014). Table 5 shows the chemical compositions of dehulled lupin of different species.

Table 5: Chemical compositions of dehulled lupin

<table>
<thead>
<tr>
<th>Composition</th>
<th>L. albus (g/100 g DM)</th>
<th>L. angustifolius</th>
<th>L. luteus</th>
<th>L. mutabilis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ash</td>
<td>3.5–5.0</td>
<td>2.9–4.2</td>
<td>4.7–5.1</td>
<td>4.0</td>
</tr>
<tr>
<td>Protein</td>
<td>45.3–52.9</td>
<td>41.1–44.4</td>
<td>55.3–57.4</td>
<td>52.0</td>
</tr>
<tr>
<td>Fat</td>
<td>12.7</td>
<td>6.8–9.8</td>
<td>7.9–8.2</td>
<td>17.0</td>
</tr>
<tr>
<td>Total dietary fibre</td>
<td>28.4–32.5</td>
<td>36.3–40.1</td>
<td>21.0</td>
<td>NA</td>
</tr>
<tr>
<td>- Insoluble</td>
<td>NA</td>
<td>30.6</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>- Soluble</td>
<td>NA</td>
<td>6.3</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA = Not applicable


2.1.7.1 Protein

Lupin seed is one of the most abundant sources of plant proteins. Lupin seeds contain approximately 30–50% proteins which vary considerably between species. High
protein contents are found in *L. mutabilis* and *L. luteus*, whereas lower protein levels are found in *L. albus* and *L. angustifolius* (Table 4). Protein contents in dehulled lupin vary from 41 to 57% (Table 5). The major storage proteins are globulins (80–90%), and the minor storage protein are albumins (10–20%) (Kohajdová et al. 2011; Office of the gene Technology Regulator 2013). The globulins and albumins are classified into four groups including α-conglutin (11S globulin), β-conglutin (7S globulin), γ-conglutin (7S basic globulin) and δ-conglutin (2S sulphur-rich albumin) (Duranti et al. 2008; Foley et al. 2011). Lupins have high amounts of essential amino acids such as arginine, leucine, lysine and phenylalanine; however limited amount of sulphur containing amino acids such as methionine (Doxastakis et al. 2002; Glencross et al. 2011). Wheat flour is poorer in lysine and higher in the sulphur-containing amino acids (methionine and cysteine) than lupin flour. Therefore, wheat flour can be substituted by lupin flour in various kinds of food products to improve the amino acid balance of the product (Villarino et al. 2016). Essential amino acid profiles of different lupin species are shown in Table 6.

Table 6: Essential amino acid profiles of whole lupin seeds

<table>
<thead>
<tr>
<th>Amino acid</th>
<th><em>L. albus</em> (g/100 g DM)</th>
<th><em>L. angustifolius</em></th>
<th><em>L. luteus</em></th>
<th><em>L. mutabilis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>4.68</td>
<td>3.65</td>
<td>4.37</td>
<td>4.64</td>
</tr>
<tr>
<td>Cystine</td>
<td>0.50</td>
<td>0.46</td>
<td>0.88</td>
<td>0.82</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.65</td>
<td>0.76</td>
<td>1.05</td>
<td>1.63</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.41</td>
<td>1.23</td>
<td>1.42</td>
<td>1.99</td>
</tr>
<tr>
<td>Leucine</td>
<td>2.30</td>
<td>2.08</td>
<td>3.06</td>
<td>3.47</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.57</td>
<td>1.43</td>
<td>2.07</td>
<td>2.65</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.24</td>
<td>0.22</td>
<td>0.27</td>
<td>0.41</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.23</td>
<td>1.12</td>
<td>1.56</td>
<td>1.94</td>
</tr>
<tr>
<td>Threonine</td>
<td>1.19</td>
<td>1.04</td>
<td>1.36</td>
<td>1.79</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.37</td>
<td>0.32</td>
<td>0.84</td>
<td>0.41</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.71</td>
<td>1.07</td>
<td>1.12</td>
<td>2.35</td>
</tr>
<tr>
<td>Valine</td>
<td>1.36</td>
<td>1.18</td>
<td>1.33</td>
<td>1.89</td>
</tr>
</tbody>
</table>

(Adopted from Petterson et al. 1998; Gross et al. 1988)
2.1.7.2 Fat
Fat contents in whole seeds and dehulled lupins are in a range of 6–16% and 7–15%, respectively Table 4 and Table 5. Low levels of fat are detected in *L. angustifolius* and *L. luteus*, while high fat levels are found in *L. albus* and *L. mutabilis*. The fatty acid profile in lupin seeds vary largely among species. However, a high proportion of oleic acid and linoleic acid with low levels of saturated fatty acids are found commonly in all lupin species (Chiofalo et al. 2012; Trugo, von Baer, D & von Baer, E 2003).

2.1.7.3 Carbohydrates and dietary fibre
Lupin seeds are very low in starch (< 2%). The main carbohydrates in lupin seeds are oligosaccharides and non-starchy polysaccharides (dietary fibre). The major oligosaccharides found in the lupin seeds are sucrose and raffinose family oligosaccharides (Martínez-Villaluenga et al. 2008). The dietary fibre contents in lupin seeds vary significantly depending on the species. Lupin seed is an excellent source of dietary fibre with a range of 34–47% mainly consists of insoluble fibre (85–90% of total fibre) (Table 4).

2.1.7.4 Vitamins
Lupin seeds are good sources of B group vitamins especially thiamine (0.5 mg/100 g), riboflavin (0.4 mg/100 g) and niacin (4.0 mg/100 g) (Trugo, von Baer, D & von Baer, E 2003). Other vitamins presented in lupins include vitamin A (carotenoid) and vitamin E (tocopherol). Wang et al. (2008) reported the total carotenoid contents in lupins ranged from 53–229 μg/g. Lutein, zeaxanthin and beta-carotene are present, with lutein as the major carotenoid (Wang et al. 2008). Total tocopherol contents in lupins varied from 9.9–52.5 mg/100 g (Martínez-Villaluenga et al. 2006).

2.1.7.5 Minerals
The mineral contents in lupins depend on genotype and the environmental factors such as soil type and rainfall (Petterson et al. 1998). Lupin is a good source of some minerals such as calcium (134–225 mg/100 g), iron (3–9 mg/100 g), and zinc (3–8 mg/100 g) (Table 7).
Table 7: Minerals contents in whole lupin seeds

<table>
<thead>
<tr>
<th>Composition</th>
<th>L. albus</th>
<th>L. angustifolius</th>
<th>L. luteus</th>
<th>L. mutabilis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td>134–209</td>
<td>168–225</td>
<td>220</td>
<td>216</td>
</tr>
<tr>
<td>Iron</td>
<td>3.5–7.9</td>
<td>3.2–7.5</td>
<td>8.7–9.3</td>
<td>3.2</td>
</tr>
<tr>
<td>Zinc</td>
<td>3.0–5.3</td>
<td>3.4–5.5</td>
<td>5.6–8.3</td>
<td>5.1</td>
</tr>
</tbody>
</table>


2.1.8 Health benefits and biological activities of lupin

Lupin contains various nutrients and bioactive compounds with unique health benefits (Arnoldi et al. 2015; Khan et al. 2015). Various health benefits of lupin including anti-diabetic effect, cholesterol-lowering effect, blood pressure-lowering effect, bowel function improvement, prebiotic potential have been reported (Belski et al. 2011; Fornasini et al. 2012; Johnson et al. 2006; Parolini et al. 2012; Smith et al. 2006). Some biological activities of lupins related to their phytochemical compositions have been published by Khan, Kampanit, Nasar-Abbas, Huma and Jayasena (2015). A copy of the paper is presented in Appendix A1.

2.1.8.1 Anti-diabetic effect

Lupin seeds have been used as an anti-diabetic product in traditional medicine (Bertoglio et al. 2011; Capraro et al. 2011; Terruzzi et al. 2011). The glucose-lowering effect of aqueous extract of whole lupin seed and seed coat was investigated by Knecht et al. (2006). The extracts of two lupin species; L. albus and L. caudatus, were administered orally to mice fasted overnight. After one hour, glucose (2 g/kg) was administered orally or intraperitoneally. After glucose administration, blood was collected at 0, 30 and 90 minutes. Hypoglycaemic activity was shown only in the whole seed extract. The activity was present when glucose was administered orally but not intra-peritoneally. The anti-hyperglycaemic effect would be exhibited when intake lupin extract is administered prior to or with a meal (Knecht et al. 2006).

The effect of raw L. mutabilis consumption on plasma glucose and insulin concentrations in healthy and dysglycaemic volunteers was studied (Fornasini et al. 2015).
The consumption of raw lupin seed powder at a dose 98.4 mg (containing approximate 3.125 mg of alkaloid) by dysglycaemic subjects showed a significant reduction in blood glucose and insulin levels. However, consumption of raw lupin powder in the healthy volunteers did not affect blood glucose and insulin levels. Baldeon et al. (2012) also investigated the hypoglycaemic effects of cooked debittered lupin powder and lupin alkaloids in volunteers with diabetes. The intakes of cooked debittered lupin powder or lupin alkaloids by diabetic volunteers result in reducing blood glucose and insulin levels (Baldeon et al. 2012).

Magni et al. (2004) studied the glucose lowering effect of lupin $\gamma$−conglutin in hyperglycaemic rats. Lupin $\gamma$−conglutin from $L.\ albus$ was purified by using anion and cation exchange chromatography. Three doses of $\gamma$−conglutin (30, 60 or 120 mg/kg body weight) were administered orally 30 minutes before overloading of glucose (2 g/kg body weight). The reductions in plasma glucose levels in hyperglycaemic rats which are administered lupin $\gamma$−conglutin were found. At high dose of $\gamma$−conglutin (120 mg/kg body weight), the effect was comparable to metformin, a well-known glucose lowering drug.

Bertoglio et al. (2011) studied the hypoglycaemic effect of lupin $\gamma$-conglutin in animal and human models. Lupin $\gamma$-conglutin enriched powder was prepared from dehulled lupin seeds ($L.\ albus$). This powder contained approximately 21% of $\gamma$-conglutin. In animal study, three doses of test powder (50, 100, or 200 mg/kg body weight corresponding to 10.5, 21 or 42 mg $\gamma$-conglutin, respectively) were administered orally 30 minutes before glucose overload. In human study, fifteen adult healthy volunteers (>18 years old, BMI < 30 kg/m$^2$ and normal plasma glucose) were included. Three doses of test product (750, 1500 or 3000 mg corresponding to 157, 315 or 630 mg $\gamma$-conglutin, respectively) were administered orally 30 minutes before a carbohydrate meal (75 g carbohydrate). The lupin $\gamma$-conglutin enriched powder showed a dose-dependent decrease of blood glucose concentration in rats and healthy humans (Bertoglio et al. 2011).

Lovati et al. (2012) also studied the hypoglycaemic effect of lupin $\gamma$-conglutin in hyperglycaemic rats. Lupin $\gamma$-conglutin from protein isolate of $L.\ albus$ seeds at dose
28 mg/kg body weight was administered orally daily for three weeks. Results exhibited that lupin \( \gamma \)-conglutin treated group reduced significantly the increment in plasma glucose and insulin levels in glucose overloaded rats compared to non-treated group. These findings suggest the potential use of lupin \( \gamma \)-conglutin as a glucose lowering agent.

### 2.1.8.2 Cholesterol-lowering effect

The cholesterol-lowering effect from intake of lupin and its preparation in animal and human models have been reported. Hall et al. (2005) studied the effect of lupin kernel fibre incorporated food on serum lipids in men using randomized crossover dietary intervention. Lupin fibre incorporated foods and control foods were consumed by subjects for 1 month each. Lupin fibre incorporated foods with sensory acceptability included bread, muffin, chocolate brownie, chocolate milk drink, toasted muesli, pasta and instant mashed potato. The addition of lupin fibre to the diet resulted in a significant reduction in total cholesterol and LDL-cholesterol (bad cholesterol).

Fontanari et al. (2012) studied the cholesterol-lowering effect of whole lupin seed and its protein isolate in hypercholesteraemic hamsters. Hamsters were divided into three groups fed with the following diets for four weeks; control diet, diet containing whole lupin seed; and diet containing lupin protein isolate. A higher HDL-cholesterol level was found in whole lupin diet group. The lower levels of total cholesterol and LDL-cholesterol were found in both whole lupin diet and lupin protein isolate diet groups compared to the control diet group.

Parolini et al. (2012) studied the hypocholesterolemic effect of lupin protein isolate in rats. Protein isolate from \( L. \ angustifolius \) seeds was prepared by an extraction/precipitation process followed by spray drying. The percentage of protein was 91.15% DM of the dry powder. Rats were fed hypercholesterolaemic diet containing casein (control) or hypercholesterolaemic diet containing lupin protein isolate for four weeks. Total cholesterol level in rats fed with lupin protein isolate was lower than the control group, while HDL-cholesterol was not different.

Fechner et al. (2014) studied the cholesterol-lowering effect of lupin kernel fibre intake in 60 moderately hypercholesteraemic adults. Subjects consumed either a lupin fibre
incorporated diet (25 g lupin fibre/day) or a control diet for one month each. There were reductions in total cholesterol (9%) and LDL-cholesterol (12%) during the intake of lupin fibre incorporated diet period compared to the control diet period. However, HDL-cholesterol (good cholesterol) levels were not different between the two periods.

### 2.1.8.3 Blood pressure-lowering effect

The blood pressure-lowering effects of lupin and its preparations consumption have been reported. Pilvi et al. (2006) indicated that lupin protein isolated reduces blood pressure in a spontaneously hypertensive rat model. The rats were fed with a 6% NaCl diet containing lupin protein isolate for two weeks. A lower systolic blood pressure was found in the lupin group than in the control group. Lee et al. (2009) studied the effects of lupin flour incorporated bread on blood pressure in overweight and obese adults. Subjects were assigned to consume white bread (control group) or lupin flour incorporated bread at 15–20% of their usual daily energy intake for 16 weeks. Consumption of the lupin bread resulted in a reduction of systolic blood pressure, while diastolic blood pressure had not been changed.

Belski et al. (2011) investigated the effect of lupin flour incorporated food intake on cardiovascular disease risk factors in 131 overweight subjects using a 12-month parallel-design trial. Participants were divided into two groups, and given either control diet or lupin diet for 12 months. Bread, biscuits and pasta (with or without lupin incorporation) were provided to the subjects as a replacement of other cereal-based food products they normally consumed. The reductions in systolic and diastolic blood pressures were found in the lupin group. Fechner et al. (2014) also reported the blood pressure-lowering effect (systolic blood pressure) of lupin kernel fibre intake in moderately hypercholesterolaemic adults.

### 2.1.8.4 Bowel function improvement

Johnson et al. (2006) studied the effect of lupin fibre on bowel function in healthy men (n = 38). Participants consumed a control diet and lupin fibre diet for one month each. Bowel function markers such as frequency of defecation, transit time, faecal output, and faecal pH and moisture were determined. The increases in frequency of defecation, faecal output and faecal moisture, and the reductions in transit time and faecal pH were
found during lupin fibre intake period. Consumption of lupin fibre incorporated food could improve bowel function (Johnson et al. 2006).

2.1.8.5 Prebiotic potential
Lupin contains high levels of dietary fibre and raffinose family oligosaccharides (RFOs). Dietary fibre and RFOs are prebiotics since they can promote the growth of beneficial bacteria. Gulewicz et al. (2002) investigated the prebiotic potential of RFOs extracted from *L. angustifolius* in a rat model. Rats were given a solution of RFOs extract (15 mg per 100 g of body weight) daily. The rats’ faces were collected on the 1st, 10th, and 20th days of the experiment. An increase in bifidobacteria and decrease in coliform in the group of rats fed with RFOs extract was found (Gulewicz et al. 2002). Smith et al. (2006) investigated the prebiotic property of lupin fibre in 18 health men. Participants consumed a control diet and a lupin fibre incorporated diet for 28 days each. Consumption of lupin fibre incorporated diet increased levels of beneficial bacteria (*Bifidobacterium* spp.), and reduced levels of pathogenic bacteria (*Clostridium* spp.).

2.1.8.6 Anticarcinogenic effect
Johnson et al. (2006) indicated that lupin fibre can improve bowel function which could result in a reduction of colon cancer risk. In the same study, the authors also reported the reduction of β-glucuronidase activity (colon cancer risk biomarker) in the participants’ faeces. Liu (2009) investigated anticancer activity of lupin seed extracts and lupin alkaloids through cytotoxicity test against several cancer cell lines. Lupin seed extracts from one bitter lupin and three sweet lupins were studied. Lupin seed extracts and lupin alkaloids (angustifoline and lupanine) showed mild cytotoxicity (Liu 2009). Ramos Herrera et al. (2011) determined lunasin (bioactive peptides exhibiting anticancer effect) in protein extract of lupin seeds. Lunasin were found in prolamins fraction from whole seeds of *L. albus*, albumin fractions from dehulled seeds of *L. montanus*, and glutelin fractions from dehulled seeds of *L. campestris*. The results suggested the anticarcinogenic effect of lupin proteins (Ramos Herrera et al. 2011).
2.1.8.7 Antimutagenic effect (Anti-mutation)

Jiménez Martínez et al. (2003) reported the anti-mutagenic effects (inhibitory effect of spontaneous mutation) of phenolic compounds, oligosaccharides and quinolizidinic alkaloids isolated from *Lupinus campestris* seeds (bitter lupin). The mutagenic responses of *Salmonella typhimurium* tester strain YG1024 were determined using the Kado microsuspension assay, and 1-nitropyrene was used as a mutagen. Lupin phenolic compounds showed the inhibitory effects in a dose-dependent manner with a maximum inhibition of 86%. Lupin oligosaccharides from water and dimethyl sulfoxide (DMSO) extractions exhibited anti-mutagenic activity with the maximum inhibition of 32 and 76%, respectively. The anti-mutagenic effects of quinolizidinic alkaloids in lupin did not depend on the concentration. The highest inhibitory effect (75%) was found from the lowest dose of tested quinolizidinic alkaloids.

2.1.8.8 Anti-inflammatory effect

Inflammation is considered as one of the major risk factors for various chronic diseases in humans (Tabas & Glass 2013). Gamarra Castillo et al. (2005) investigated the anti-inflammatory capacity of aqueous extract from *L. mutabilis* seed in rats by measuring the inhibition of the oedema. The oral administration of lupin seed extracts at dose 4000 mg/kg body weight exhibited a significant reduction of oedema formation. The anti-inflammatory effect of lupin aqueous extract was significantly higher than that of the control group.

*In vitro* anti-inflammatory properties of protein hydrolysates from seeds of *L. angustifolius* have been investigated by using a THP-1-derived macrophage model and inhibition of enzymes involving in the inflammatory pathway (Millán-Linares et al. 2014a & 2014b). Lupin protein hydrolysates (LPHs) were attained by hydrolysis of lupin protein isolate (LPI) using Izyme and Alcalase. After treated THP-1-derived macrophages with LPHs, the expression of proinflammatory cytokines (tumor necrosis factor, IL-6, IL-1β) were reduced and the expression of anti-inflammatory cytokines (chemokine (C-C motif) ligand 18 (CCL18)) were increased. LPHs inhibited the nitric oxide production (inflammatory mediator) significantly. LPHs also inhibited the activities of four enzymes involving in the inflammatory pathway including phospholipase A2 (PLA2), cyclooxygenase 2 (COX-2), thrombin, and
transglutaminase (TG). The authors suggested the potential use of lupin protein hydrolysates as functional food to prevent chronic diseases related to inflammation.

2.1.8.9 Antioxidant activity
A few studies reported the correlation between total phenolic compound contents and antioxidant activity of lupin (Tsaliki et al. 1999; Siger et al. 2012). Tsaliki et al. (1999) reported the antioxidant activity of hot and cold methanolic extracts from \textit{L. albus} seeds using a spectrophotometric method. The hot methanolic extracts showed higher antioxidant activity than the cold methanol extracts. The antioxidant activity was correlated to the total phenolic contents in the lupin extracts (Tsaliki et al. 1999). Siger et al. (2012) reported the antioxidant activities of methanol extracts from seeds of \textit{L. albus}, \textit{L. luteus} and \textit{L. angustifolius} using DPPH radical scavenging and Total peroxyl radical-trapping potential (TRAP) methods.

However, several studies found that there were no association between phenolic compound contents and antioxidant activity of lupin (Dueñas et al. 2009; Martínez-Villaluenga et al. 2009; Oomah et al. 2006). Dueñas et al. (2009) reported the antioxidant activity of \textit{L. angustifolius} seed extracts using DPPH radical scavenging assay. Martínez-Villaluenga et al. (2009) reported the moderate antioxidant capacity of \textit{L. angustifolius} seeds which is comparable to other legumes. Oomah et al. (2006) reported the antioxidant activity of extract from \textit{L. angustifolius} seeds using photochemiluminescence (PCL) assay.

2.1.8.10 Antibacterial activity
The antibacterial properties of \textit{L. albus}, \textit{L. luteus} and \textit{L. angustifolius} seeds were studied by Lampart-Szczapa et al. (2003). The antibacterial activities of the ethanol extracts from cotyledons and seed coat were tested on Gram (+) \textit{Bacillus subtilis} ATCC6633 and Gram (–) \textit{Escherichia coli} ATCC25922. The extracts from the seed coat exhibited antibacterial activity but not the extracts of lupin from cotyledon. The inhibitory effect on the growth of bacteria depended on the content of total phenolic compounds. The extracts derived from \textit{L. albus} cv. Bac (bitter cultivar) presented the strongest antibacterial properties while the lowest activity was observed from \textit{L. luteus} cv. Popiel. Erdemoglu et al. (2007) also reported that the alkaloid extract from lupin
seeds presents significant antibacterial properties on *B. subtilis*, *S. aureus* and *P. aeruginosa*.

### 2.1.8.11 Antiviral activity

Barakat et al. (2010) studied the antiviral activity of *Lupinus termes* seed extract. Crude extracts of lupin seed (20 µg/mL; non-toxic dose from cytotoxic assay) were tested for antiviral activity against herpes simplex virus-1 (HSV-1) and hepatitis A virus-27 (HAV-27) using plaque infectivity count assay. The seed extracts of *L. termes* exhibited strong antiviral activity against HAV-27.

### 2.1.8.12 Antifungal activity

Woldemichael and Wink (2002) found that saponins from the methanolic extract of *L. angustifolius* seed exhibit antifungal activity against *C. albicans*. Three saponin compounds found in the lupin seed extract, showed moderate antifungal activity. Erdemoglu et al. (2007) also investigated antifungal activities of *L. angustifolius* alkaloid extract against *Candida albicans* and *C. krusei*. The authors reported that the alkaloid extract from lupin exhibited moderate antifungal activity against the test strains.

Various health benefits and biological activities of lupin have been reported (Arnoldi et al. 2015; Khan et al. 2015). Therefore, lupin can be incorporated into food products with enhanced health benefits. However, information on anti-nutritional factors which could have negative effects is limited. Hardly any published information on the effects of cultivar, growing season and dehulling on the composition and bioavailability of mineral of ASL have been reported.

### 2.2 Anti-nutritional factors

Anti-nutritional factors are defined as compounds presented in food or feed which reduce nutrient utilisation including intake, digestion and absorption (D’Mello 2000; Soetan & Oyewole 2009). Its wider definition also includes compounds which may cause adverse effects on human or animal health (D’Mello 2000; Kiranmayi 2014). Plant species, variety, cultivar, agronomic practices and environmental factors influence the levels of these anti-nutritional factors (Soetan & Oyewole 2009).
Anti-nutritional factors can be divided into two main groups: heat-labile compounds and heat-stable compounds. Heat-labile anti-nutritional factors compose of saponins, lectins and proteinase inhibitor which are destroyed or reduced during thermal processing. Heat stable group includes phytic acid, polyphenol (mainly tannin), raffinose family oligosaccharides and quinolizidine alkaloids (D’Mello 2000). Main anti-nutritional factors in Australian sweet lupin are raffinose family oligosaccharides (RFOs), phytic acid and polyphenols. Lupin contains low amount of lectins and trypsin inhibitors compare to other legumes such as cowpea, kidney bean and soybean (Grant et al. 1995).

2.2.1 Raffinose family oligosaccharides (RFOs)

2.2.1.1 Chemical and physical property

RFOs are low molecular weight oligosaccharides that are soluble in water and water-alcohol solutions (Dey 1980). Raffinose, stachyose, verbascose and ajugose are mono-, di-, tri- and tetra-galactosyl derivatives of sucrose, respectively. Raffinose is a trisaccharide composed of galactose, glucose, and fructose. Stachyose is a tetrasaccharide composed of two galactose units, glucose, and fructose. Verbascose is a pentasaccharide composed of three galactose, one glucose and one fructose units. Ajugose is a hexasaccharide composed of four galactose, one glucose and one fructose units. The chemical structure of RFOs is shown in Figure 4.

![Figure 4: Chemical structure of raffinose family oligosaccharides](Source: Kotiguda et al. 2006)
2.2.1.2 Occurrence and content

In plants, RFOs are the most abundant soluble carbohydrates ranking next to sucrose (Martínez-Villaluenga et al. 2008). RFOs are synthesised and accumulated in storage organs, such as seeds and tubers during their development process (Frias et al. 1996; Martínez-Villaluenga, Frías & Vidal-Valverde 2005). Raffinose is found in all legume seeds to some extent. The major RFO in most legume seeds is stachyose, while verbascose is the predominant RFO in some species (Peterbauer & Richter 2001). Ajugose is not commonly found in legume but negligible amount have been reported in some species (Kotiguda et al. 2006). RFOs contents in legume seeds are presented in Table 8, which shows a wide variation of RFOs content and composition among different legumes species. Lupin is the richest source of RFOs ranging between 5.1–16.1% which varies depending on the species (Martínez-Villaluenga 2008).

Table 8: RFOs contents in legumes

<table>
<thead>
<tr>
<th>Legumes</th>
<th>RFOs content (% DM)</th>
<th>Raffinose</th>
<th>Stachyose</th>
<th>Verbascose</th>
<th>Total RFOs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black gram</td>
<td>0.3–0.8</td>
<td>0.3–0.8</td>
<td>1.1–3.3</td>
<td>1.8–4.7</td>
<td></td>
</tr>
<tr>
<td>Chickpeas</td>
<td>ND–2.4</td>
<td>0.4–2.6</td>
<td>ND–4.5</td>
<td>2.0–7.6</td>
<td></td>
</tr>
<tr>
<td>Common beans</td>
<td>0.2–2.5</td>
<td>0.2–4.2</td>
<td>0.1–4.0</td>
<td>0.4–8.0</td>
<td></td>
</tr>
<tr>
<td>Fababees</td>
<td>0.1–1.5</td>
<td>0.2–2.4</td>
<td>1.1–2.4</td>
<td>1.0–4.5</td>
<td></td>
</tr>
<tr>
<td>Lentils</td>
<td>0.1–1.0</td>
<td>1.1–4.0</td>
<td>ND–6.4</td>
<td>1.8–7.5</td>
<td></td>
</tr>
<tr>
<td>Mungbean</td>
<td>0.4–1.7</td>
<td>1.5–2.8</td>
<td>ND</td>
<td>1.9–4.5</td>
<td></td>
</tr>
<tr>
<td>Pea</td>
<td>0.4–2.3</td>
<td>0.3–5.5</td>
<td>ND–4.3</td>
<td>2.3–9.6</td>
<td></td>
</tr>
<tr>
<td>Pigeon peas</td>
<td>0.4</td>
<td>0.9</td>
<td>1.1</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>Soybean</td>
<td>1.0–2.0</td>
<td>2.2–4.9</td>
<td>ND–0.3</td>
<td>6.0–8.0</td>
<td></td>
</tr>
<tr>
<td>Lupin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. albus</em></td>
<td>0.3–0.6</td>
<td>5.0–7.2</td>
<td>ND–0.9</td>
<td>5.5–8.1</td>
<td></td>
</tr>
<tr>
<td><em>L. angustifolius</em></td>
<td>0.6–1.2</td>
<td>3.6–5.2</td>
<td>0.8–2.5</td>
<td>6.7–11.5</td>
<td></td>
</tr>
<tr>
<td><em>L. luteus</em></td>
<td>0.5–0.6</td>
<td>6.1–8.6</td>
<td>2.8–3.5</td>
<td>11–16.1</td>
<td></td>
</tr>
<tr>
<td><em>L. mutabilis</em></td>
<td>1.9</td>
<td>2.3</td>
<td>1.0</td>
<td>5.1</td>
<td></td>
</tr>
</tbody>
</table>

Physiological function of RFOs in plants includes transporting carbohydrates in the phloem and performing protective roles related to desiccation tolerance, frost resistance and storability of seeds (Obendorf et al. 1998). RFOs are present mainly in the cytosol and small amounts are presented in protein storage vacuoles (Obendorf & Górecki 2012).

2.2.1.3 Effect of genotype, cultivation conditions and environment

Genotypic variation (cultivar or variety) in RFOs contents in lupin have been reported in a few studies. Martínez-Villaluenga et al. (2005) reported a considerable variation in RFOs contents between Spanish lupin species. The highest total RFOs content was found in *L. luteus* (9.5–12.3%). Trugo et al. (1988) also reported that there was a large variation in RFOs composition and content between different species, superimposed on environmental factors.

Limited information on variations in RFOs content in lupin due to cultivation conditions, such as location, year and cultivation practice has been reported. A few studies have reported the effect of maturation temperature on RFOs contents in lupin which the information was discrepant. Górecki et al. (1996) found that there was no significant effect of maturation temperature (13 °C and 28 °C) during seed maturation on RFOs contents in lupin. In contrast, lupin matured at 18 °C had a twofold increase in stachyose and verbascose contents compared with seeds matured at 25 °C (Górecki et al. 1997). Piotrowicz-Cielak (2006) also reported that lupin seeds matured at high temperatures contain more raffinose and stachyose than seeds matured at lower temperatures.

Several studies reported the effect of environment on RFOs contents in other legumes. Nikolopoulou et al. (2006; 2007) indicated that cultivation year with different climatic conditions, especially rainfall, had significant effects on total RFOs contents in chickpea and peas. Johnson et al. (2013) reported that there was no significant difference on total RFOs contents in lentil grown in fields with different soil moisture contents. Tahir et al. (2011) concluded that although rainfall, soil type and temperature influenced the RFOs contents in lentil, the effect of cultivar is more dominant than the environmental factors.
2.2.1.4 Determination

RFOs have no chromophore groups which can be detected directly by UV-VIS spectrometer. There are several methods for RFOs determination including enzymatic assay, GC and HPLC. The most commonly used methods for RFOs is HPLC with various columns, mobile phases and detectors. This review focuses on HPLC application in RFOs analysis.

Sample preparation in RFOs analysis composes of two main steps including sample extraction and sample purification. Sample extraction is one of the most important steps in RFOs analysis. Major factors affecting the efficiencies of RFOs extraction include solvent, temperature and time (Gulewicz et al. 2000).

Widely used extraction solvents were water, ethanol, methanol solutions and mixtures of them at various concentrations. Several studies compared the effect of solvent on RFOs extraction which the results were discrepant. Knudsen et al. (1991) found that water is the most effective solution; however, 50% ethanol and 50% methanol solution showing comparable results with water, and the 80% of alcohol solutions were less efficient. The investigator reported 38% and 77% decreases in stachyose content using 80% methanol and 80% ethanol, respectively compared to water extraction. Kim et al. (2003) indicated that a 10% ethanol-water solution was the optimal extraction solvent for defatted soybean meal. Giannnoccaro et al. (2006) compared the extraction of RFOs by water and 10%, 50%, and 80% ethanol and concluded that water is the most effective solution for soybean.

From several studies, water and low concentration of alcohol solution seems appropriated for RFOs extraction. Water is more convenient and avoids the problem of solvent eluted from HPLC column at the similar time with RFOs (Kennedy et al. 1985). However, other water-soluble compounds such as protein and some polysaccharides may co-elute and interfere with the determination, therefore, sample purification is crucial.

Several studies have indicated the temperature and time influence on RFOs extraction. Kim et al. (2003) studied the effect of temperature and time at 25, 50, 65 and 80 °C, after 1, 2, 3 and 4 h on RFOs extraction from defatted soybean meal. It was found that
50 °C was the optimal temperature. The lower temperatures were less efficient, and the higher temperatures cause a decrease in RFOs contents. Longer extraction time increased the efficiency of RFOs extraction; therefore 4 h for RFOs extraction was recommended by Kim et al. (2003). Giannnoccaro et al. (2006) compared the extraction temperatures of 25 °C, 50 °C, and 80 °C for 15, 30, and 60 min. The investigators concluded that the optimum extraction temperature and time was 25 °C or 50 °C for 15 min. Though extraction conditions are optimized, qualitative analysis such as Molisch reaction test, a standard method to determine the presence of carbohydrate, should be conducted to ensure the complete extraction of carbohydrate from samples (Granito et al. 2002).

The main purpose of sample purification is to eliminate proteins which are commonly troublesome in oligosaccharides analysis by HPLC. Under certain chromatographic conditions amino acids in the sample may produce interfering peaks to the oligosaccharide of interest. Several approaches have been applied for sample purification in RFOs analysis. Organic solvents such as acetonitrile and ethanol have been used to remove soluble proteins in sample preparation for oligosaccharides determination (Valliyodan et al. 2015). Carrez reagents have also been used widely to remove proteins since they do not generate peak interfering the oligosaccharides chromatograms (Peris-Tortajada 2013). Ultrafiltration is one technique applied for protein removal in determination oligosaccharide contents (Kim et al. 2003). Ion-exchange has been used commonly in determination of oligosaccharides in fruit-based products. However, some ion-exchangers can adsorb oligosaccharides resulting in a lower recovery. Sep-Pak cartridge, chemically modified silica, is another widely used technique for sample purification because of its simplicity and good recovery. The disadvantages include high cost and limited useful life (Peris-Tortajada 2013).

The most widely used technique for RFOs determination is HPLC with various detectors. Refractive index detector (RID) has been applied commonly for oligosaccharides determination in various plant samples, in spite of its poor sensitivity and selectivity (Valliyodan et al. 2015). This detector is suitable for isocratic elution—constant composition of mobile phase during the chromatographic run, which is difficult to separate various oligosaccharides with similar properties at the same time (Peris-Tortajada 2013). Pulsed amperometric detector (PAD) is a universal detector
for oligosaccharides which it exhibits high sensitivity and selectivity. This detector is usually coupled with anion-exchange column which requires high purity and strong alkaline mobile phase (pH > 12). Contamination of carbon dioxide or carbonate in the mobile phase decrease column efficiency because of interfering anion occurring in the column. This detector is also not compatible with gradient elution—changes of mobile phase composition during chromatographic run. The evaporative light-scattering detector (ELSD) is another widely used detector. Unlike RID and PAD, this detector could attain high sensitivity and suitable with gradient elution (Peris-Tortajada 2013).

2.2.1.5 Anti-nutritional property
RFOs are considered as anti-nutritional factors in foods and feeds. Their negative nutritional effects include flatulence and abdominal discomfort due to the anaerobic fermentation in the large intestine, and interfering some nutrients digestion and absorption (Martínez-Villaluenga et al. 2008). RFOs are not digested and absorbed in the upper gastrointestinal tract of non-ruminants. They pass to the large intestine and ferment anaerobically by gut microflora into short-chain fatty acids and gas, which could result in flatulence and abdominal discomfort (Guillon & Champ 2002; Martínez-Villaluenga et al. 2008). Flatulence and abdominal discomfort are scrutinised as an important factor that discourage the consumption of legumes including lupin by humans (Han & Baik 2006). The interfering effects of RFOs on nutrients digestion and absorption have been reported. A few studies showed that RFOs from lupin reduce the protein digestibility (Glencross et al. 2003; Porres et al. 2005). Zdunczyk et al. (1999) indicated a lower intestinal absorption of glucose and methionine when RFOs from lupin seeds were incorporated in the perfusion fluid in animal study.

2.2.1.6 RFOs and mineral bioavailability
Limited information has been indicated the effect of RFOs on mineral bioavailability in lupin. Porres et al. (2005) studied in vitro minerals (Ca, Fe, Mg, Mn and Zn) digestibility of raw lupin flour compared to free RFOs lupin flour. Free RFOs lupin flour was prepared by soaking seeds in distilled water at 4 °C for 10–12 h and removing RFOs by two consecutive extractions with 50% ethanol at 40 °C overnight. The extracted seeds were then homogenised and lyophilised. The study found higher in vitro minerals (Ca, Fe, Mg, Mn and Zn) digestibility of raw lupin flour than free RFOs
lupin flour. It was suggested that extraction process could change dietary fibre composition and phytic acid of free RFOs lupin flour.

The information of RFOs on mineral bioavailability is scarce; however, several studies indicated that non-digestible oligosaccharides such as fructo-oligosaccharides and galacto-oligosaccharides increased the absorption of minerals (Kashimura et al. 1996; Lopez et al. 2000). The suggested mechanisms of these non-digestible oligosaccharides may involve the reduction of pH resulting in releasing of bound minerals and enlarge surface area in the colon (Bongers & van den Heuvel 2003).

2.2.1.7 Health benefits
Beneficial health effects, especially prebiotic property, of RFOs have been reported. Wongputtisin et al. (2015) indicated in vitro prebiotic activity of RFOs from soybean promoting the growth of Lactobacilli spp. but inhibiting the growth of Escherichia coli and Salmonella enterica. Several human studies also showed that RFOs promote the growth of beneficial bacteria (Bifidobacterium spp.) and decrease levels of pathogenic bacteria such as Clostridium spp. and coliforms (Benno et al. 1987; Fernando et al. 2010; Gibson & Roberfroid 1995; Guillon & Champ 2002). Therefore, there is an increasing interest in using RFOs as functional food ingredients. Functional food from RFOs preparation with prebiotic potential has been developed and commercially available (Li, Lu & Yang 2013).

Hepato-protective effect of RFOs by preventing reactive oxygen species (ROS)-related liver damage in animal model has been reported. Administration of RFOs extracted from Rehmannia glutinosa Libosch root orally daily for 21 days significantly reduced the serum markers of liver damage induced by carbon tetrachloride. RFOs also increased antioxidant levels of hepatic enzymes (Zhang et al 2013).

2.2.2 Phytic acid
2.2.2.1 Chemical and physical properties
Phytic acid or phytate in its salt form is the hexaphosphoric ester of inositol (Figure 5). Its common chemical names include inositol hexaphosphate (IP6), myo-inositol hexaphosphate and myo-inositol, hexakis (dihydrogen phosphate). Phytate is soluble
in water and slightly soluble in ethanol. The boiling point is 105 °C and melting point is more than 300 °C. Phytic acid is stable under ordinary conditions but incompatible with strong oxidizing agents. Phytate can be enzymatically hydrolyzed by phytase to lower inositol phosphates: inositol mono-, bi-, tri-, tetra-, and pentaphosphates (IP1, IP2, IP3, IP4, and IP5, respectively). Non-enzymatic hydrolysis can occur partially during thermal processing (Konietzny & Greiner 2003).

![Chemical structure of phytic acid](Source: Coulibaly et al. 2011)

### 2.2.2.2 Occurrence and content
Phytate is found commonly in cereal grains, legumes and oilseeds. Phytate is formed during seed maturation accompanied by other storage substances such as starch and lipids (Konietzny & Greiner 2003). Physiological functions of phytate in the seed include providing phosphorus, inositol phosphate and cations storage, relating to the formation of cell walls, and providing antioxidative potential against free radicals (Thavarajah 2014). In dormant seeds, phytate represents 60–90% of the total phosphate (Greiner, Konietzny & Jany 2006). Phytate is stored primarily as a complex salt of minerals (calcium, magnesium, potassium, etc.) and protein in globoids and aleurone particles of cereals and legumes (Loewus 2002). Phytic acid contents in legumes are shown in Table 9. Phytic acid contents in lupin vary between 0.1–1.7% DM depending on species.
Table 9: Phytate contents in legumes

<table>
<thead>
<tr>
<th>Legume</th>
<th>Phytate (% DM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black gram</td>
<td>0.80</td>
</tr>
<tr>
<td>Chickpeas</td>
<td>0.01–1.1</td>
</tr>
<tr>
<td>Common beans</td>
<td>0.2–1.9</td>
</tr>
<tr>
<td>Fababean</td>
<td>0.02–1.1</td>
</tr>
<tr>
<td>Lentils</td>
<td>0.06–2.3</td>
</tr>
<tr>
<td>Mungbean</td>
<td>1.1–1.2</td>
</tr>
<tr>
<td>Pigeon peas</td>
<td>1.3</td>
</tr>
<tr>
<td>Soybean</td>
<td>0.6–3.6</td>
</tr>
<tr>
<td>Lupin</td>
<td></td>
</tr>
<tr>
<td>L. luteus</td>
<td>0.1–0.8</td>
</tr>
<tr>
<td>L. albus</td>
<td>0.03–1.7</td>
</tr>
<tr>
<td>L. angustifolius</td>
<td>0.5–1.1</td>
</tr>
<tr>
<td>L. cosentinii</td>
<td>0.4–0.6</td>
</tr>
<tr>
<td>L. atlanticus</td>
<td>1.1</td>
</tr>
</tbody>
</table>


2.2.2.3 Effect of genotype, cultivation conditions and environment

Oatway et al. (2001) mentioned that phytate contents in plants are influenced by genotype (species, variety and cultivar), cultivation practices (e.g. irrigation conditions), type of soil, fertilizer application and environment conditions (rainfall, temperature, etc.). A few studies have reported effect of genotype on phytate contents in lupin. Burbano et al. (1995) report a great difference in phytate contents between L. albus (0.3 % DM) and L. luteus (0.8 % DM). Trugo et al. (1993) reported a wide variation of phytate contents in lupin ranged from 0.4 to 1.2 % DM which the lowest contents were found in L. albus and L. cosentinii. A significant difference in phytate content between L. albus (0.5 % DM) and L. mutabilis (0.8 % DM) was reported by De Carvalho (2005). However, no such research has been conducted for ASL which is the main lupin species grown in the world.
Limited published information on the effect of cultivation practice and environment on phytate contents in lupin is available. De Carvalho (2005) indicated that water stress had no effect on phytate contents in two lupin species grown in Portugal. However, the effect of cultivation practices on phytate contents in other cereals and legumes had been reported. An increase in phytate contents in oat grains was observed with increase nitrogen and phosphorus fertilizer application. During grain filling stage, high temperature increased phytate content, but high precipitation decreased phytate content in oat (Saastamoinen, Plaami & Kumpulainen 1992). Liu et al. (2005) indicated that the environmental effect superimpose genotype on phytate in rice. Dai et al. (2007) concluded that the effect of environment (location and year) is more dominant than cultivar on phytate content in barley.

2.2.2.4 Determination

Determination of phytate content is challenging since phytate has no peculiar molecular absorption spectra which can be detected by UV-VIS or fluorescence spectrometer. Derivatisation technique to produce compounds with molecular absorption spectra is also difficult since phytate is low reactive (Agostinho et al 2016). There are two main types of methods commonly used for phytate analysis—non-specific and specific methods.

Non-specific method was proposed firstly by Heubner and Stadler in 1914 based on the formation of a stable complex of ferric phytate (Benesova et al. 2013). Phytate was extracted by hydrochloric acid and the extracted solution was titrated with standardised ferric chloride solution. Ammonium thiocyanate was used as an indicator resulting in the formation of flesh-pink colour. The content of phytate was based on the iron content of ferric phytate precipitate. However, the end-point of this titration is not clear because the interference of colloidal precipitate of ferric phytate (Benesova et al. 2013). The non-specific method base on the reaction between ferric chloride and sulfosalicylic acid (wade reagent) in acidic conditions was developed in the 1950s. The reaction results in forming a complex with a peculiar pink colour with maximum absorbance at 500 nm. When phytate is present, ferric ion binds to phytate resulting in a decrease in absorbance (Benesova et al. 2013).
Since the stoichiometric ratios of ferric ion or phosphorus to phytic acid in the ferric phytate precipitate was inconsistent, ion-exchange chromatography technique was developed and modified (Oberleas & Harland 2007; Schlemmer et al. 2009). Harland & Oberleas (1977) proposed the use of AG1-X8 chloride form anion exchange chromatography to separate inorganic phosphorus from organic phosphate. The modify version of this method was accepted as an official AOAC method in 1986 (Oberleas & Harland 2007). Latta and Eskin (1980) modified the AOAC method by using AG1-X8 instead of AG1-X4, wade reagent to develop colour, and omitting the digestion step after ion-exchange chromatography (Park 2006; Agostinho 2016). This method has been modified and has become one of the most commonly used methods for phytate determination (Costa-Bauza et al. 2012; Park et al. 2006).

Non-specific method with modern instruments such as HPLC with UV-VIS detector (Dost & tokul 2006), HPLC with conductivity detector (Talamond et al. 1998), HPIC with UV and ELSD detectors (Phillippy et al 2003), ICP with atomic emission spectrometry (Grases et al. 2004) and ICP with mass spectrometer (Muñoz et al. 2003) were also used to determine phytate. Since phytate does not have characteristic molecular absorption spectrum, a colourimetric method to form a colour complex was required for the HPLC with UV-VIS detector.

Non-specific methods may overestimate the phytate content since it included other organic phosphates (lower phosphorylated inositol phosphates; IP1–IP5). Therefore, the non-specific methods are suitable for determination of phytate in raw food or food which low levels of IP1–IP3 are present. The major inositol phosphate in lupin seed was IP6, and negligible levels of lower inositol phosphates (IP1–IP5) were reported (de la Cuadra et al. 1994; Honke et al. 1998; Muzquiz et al. 2012).

The problem of overestimation in phytate content can be overcome by the specific methods. These methods are able to quantify phytate and other inositol phosphates separately. A number of HPLC and HPIC techniques with various columns, mobile phases and detectors were suggested for determination of phytate and inositol phosphates. Graf and Dintzis (1982) suggested HPLC-RI technique and ion-exchange chromatography to purify sample before injected into HPLC. The study showed good separation and detection of inositol phosphates in complex matrix. Hsu et al. (1990)
separated inositol phosphates (IP1, IP3, IP6) by anion-exchange chromatography and determined the compounds by a combination of thermospray liquid chromatographic and mass spectrometer. Skoglund et al. (1997) reported successful separation of inositol phosphate by anion-exchange chromatography and HPIC system with either UV detector or conductivity detector. Carlsson et al (2001) purposed the rapid analysis of inositol phosphates (IP2–IP6) using HPIC with UV detector after post-column reaction.

A few studies have used GC for inositol phosphates determination. March et al. (2001) reported the determination of phytate and other inositol phosphates in biological samples by GC-MS. Anion-exchange chromatography was used for purification. Phytate was hydrolysed to myo-inositol by phytase from *Aspergillus ficuum* and derivatised to trimethylsilyl derivative. However, this method is complicated because of the difficulties in finding highly active and stable phytase enzyme, and derivatisation process.

Specific methods are appropriate to determine phytate contents in processed foods since they can separate phytate and the lower phosphorylated derivatives decomposed by the food processing. However, these specific methods have not been used widely in food science and nutrition research, mainly used in cell biology studies. Main reason being the difficulty in finding suitable detectors because of the missing characteristic absorption spectra of and specific colourimetric reagents for inositol phosphates (Schlemmer et al. 2009). The complicated sample preparation, high costs of instrument and maintenance, and a long measuring time are also the disadvantages of these methods (Agostinho et al. 2016).

2.2.2.5 Anti-nutritional property

Phytate is considered an anti-nutritional factor because it can bind directly or indirectly with protein, starch and minerals, resulting in poor bioavailability of these nutrients (Muzquiz et al. 2012; Trugo, von Baer, D & von Baer, E 2003). The binding properties of phytate may change solubility, functionality, digestibility and absorption of the nutrients (Muzquiz et al. 2012). Phytate is indigestible by human and monogastric animals since they have no or limited intestinal phytase enzyme (Urbano et al. 2000).
Several mechanisms for reducing protein bioavailability by phytate have been reported (Selle et al. 2012). Phytate can form a complex with protein, and the protein–phytate complexes are indigestible by pepsin, and impede amino acid absorption in the small intestine (Kumar et al. 2010). Phytate may increase mucin secretions which increases endogenous amino acid flows because the protein component of mucin is indigestible (Selle et al. 2012). Phytate reduces Na$^+$ dependent transport systems and the activity of the Na$^+$ pump into the small intestine causing obstructive amino acid absorption. Phytate also has negative effects on several protein digestive enzymes including pepsin, trypsin and chymotrypsin (Greiner, Konietzny & Jany 2006; Urbano et al. 2000).

Phytate binds with starch through phosphate linkages. It may interact with amylase protein and/or binding with salivary minerals such as calcium which is a catalyst of amylase activity. Phytate could interact with a protein which is closely connected to the starch (Selle et al. 2012; Singh et al. 2010). Discrepant results on the effect of phytate on starch digestion were reported. Knuckles and Betschart (1987) reported the inhibitory effect of phytate on α-amylase with dose-dependent effect. Thompson et al. (1987) also found that phytate can reduce starch digestion in vitro and blood glucose response in humans. In contrast, Bjorck and Nyman (1987) studied the effect of phytate on starch digestion, and concluded that phytate had no effect on starch digestion.

Research on the direct effect of phytate itself on starch digestion is limited. Several studies reported the combined effect of anti-nutritional factors on starch bioavailability and/or the relationship between phytate contents in foods and the starch digestibility. Some authors indicated that the removal of anti-nutritional factors including phytate can improve starch digestibility (Alonso et al. 2000; Rehman & Shah 2005). However, Lee et al (2015) reported no significant relationship between phytate and in vitro starch digestibility of rice. Lupin contains very little starch and the role phytate plays in its digestion could be very different to most other plant material.

2.2.2.6 Phytate and mineral bioavailability

Phytate is a strong negatively charged compound which can form complexes with di-valent and tri-valent cations (Weaver & Kannan 2002; Urbano et al. 2000). In the
gastrointestinal tract of humans’, the insoluble phytate–minerals complexes are
digested and absorbed poorly because of the absence of phytase hydrolytic enzyme
(Urbano et al. 2000). The solubility and stability of the phytate–mineral complexes
depend on the size and valence of the mineral, pH, phytate-to-mineral molar ratio, and
other compounds in the matrix (Konietzny & Greiner 2003).

A mineral can bind to more than one phosphate group within or between phytate
molecules (Figure 6). Phytate forms complexes with minerals in the following order
of strength; Cu^{2+} > Zn^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+} > Fe^{3+} > Ca^{2+}. The following order of
stability of various phytate–mineral complexes at pH 7.4 was reported: Zn^{2+} > Cu^{2+} >
Ni^{2+} > Co^{2+} > Mn^{2+} > Ca^{2+}. Zn bioavailability is greatly reduced by the presence of
phytate. Secondary cations can present synergistic effect on increasing the insolubility
of phytate–mineral complexes; for example, Ca^{2+} enhances the adsorption of Zn^{2+} into
phytate molecules by forming of phytate-Ca-Zn complexes (Konietzny & Greiner
2003).

Figure 6: Interactions of phytate with minerals
(Source: Konietzny & Greiner 2003)

The negative effects of phytate on mineral bioavailability using in vitro studies have
been reported. Wolters et al. (1993) estimated the mineral bioavailability of bread with
various phytate contents. The authors found the decrease in calcium, iron and zinc
bioavailability with the increasing phytate contents. Porres et al. (2005) reported a
significant increase in mineral bioavailability of whole lupin flour after phytase
treatment. Sanz-Penella et al (2012) reported a negative effect of phytate on iron
bioavailability in bread supplemented with different percentage of whole amaranth
flour (0, 20 and 40%) using in vitro digestion/Caco-2 cell model.
Contrary results on the effect of phytate on mineral bioavailability in animal studies were reported. Zhou et al. (1992) reported the inhibitory effect of phytate on zinc bioavailability in rats. Hansen et al. (1996) also found that phytate reduced the absorption of calcium and zinc in the experiments using rat models. Saha et al. (1994) reported significantly lower absorptions of calcium, iron and zinc when rats were fed with high phytate content wheat compared to low phytate content wheat. In contrast, a few studies in rats indicated no inhibitory effect of phytate on mineral absorption (Beard et al. 1988; Mason et al. 1993). The results could be due to the effect of intestinal phytase that is naturally presented in rats which can hydrolyse phytate (Heaney et al. 1991; Iqbal et al. 1994).

In human studies, dose-dependent inhibitory effects of phytate on Ca, Fe and Zn absorption have been reported (Brune et al. 1992; Hurrell et al. 1992; Fredlund et al. 2002). Heany et al. (1991) reported the inhibitory effect of soybean phytate on calcium absorption. Hurrell et al. (1992) studied the effect of reducing the phytate in soy-protein isolates on non-haem iron absorption in 32 human subjects and concluded that phytate is a major inhibitor of iron absorption in soy-protein isolates. Couzy et al. (1998) studied the effect of phytate on zinc absorption in the healthy elderly (71–78 years) and young respondents (23–43 years) with similar zinc and protein status. The strong inhibitory effects of phytate on zinc absorption in both elderly and young subjects were reported.

Phytate to mineral molar ratio is commonly used to predict the bioavailability of minerals (Dahiya et al. 2013; Ma et al. 2005). The critical values of phytate to mineral molar ratio indicating poor bioavailability have been proposed; phytate:calcium > 0.24, phytate:iron > 1, phytate:zinc > 15, and phytate × calcium:zinc > 200 (Lazarte et al. 2015; Ma et al. 2005).

2.2.2.7 Health benefits
Beneficial health effects of phytate have been reported. Phytate can reduce the bioavailability of heavy metals (e.g. cadmium and lead) present in foods (Rimbach & Pallaufl 1997). Hypolipidaemic effect of phytate has been reported in several studies. Jariwalla et al. (1990) reported that addition of phytate to the cholesterol-enriched diet significantly lowered serum total cholesterol and triacylglycerols in rats. Katayama
(1997) found a significant reduction in hepatic concentrations of total lipids and triglycerides in rats fed with diet supplemented with phytate. Kang et al. (2012) also reported the anti-hyperlipidaemic effect in mice fed with high-fat diet supplemented with phytate. Several studies have reported anti-carcinogenic activity of phytate against colon cancer (Norazalina et al. 2010), breast cancer (Shamsuddin & Vucenik 1999) and prostate cancer (Zi et al. 2000).

2.2.3 Polyphenols

2.2.3.1 Chemical and physical properties

Polyphenols are compounds chemicals consist of a benzene ring with one or more hydroxyl groups (Dykes & Rooney 2007). The chemical structure of polyphenols is extremely varied. According to the chemical structures of the aglycones, polyphenols can be classified into four main groups: phenolic acids, flavonoids, polyphenol amides and other polyphenols (Tsao 2010). Phenolic acids (Figure 7) consist of two main groups of compounds, namely benzoic acid and cinnamic acid derivatives.

![Figure 7: Chemical structure of phenolic acids](Source: Tsao 2010)

Flavonoids are compound with a phenolic benzopyran structure (El Gharras 2009). According to the hydroxylation pattern and differences in the chromane ring, flavonoids can be divided into four sub-groups of numerous compounds (Figure 8). Isoflavones, neoflavonoids and chalcones are classified in the same subgroup of flavonoids. Flavones, flavonols, flavanones and flavanonols are in the same subgroup which is the most common group found in the plant kingdom. Other subgroups include flavanols (flavan-3-ols or catechins) and proanthocyanidins (condensed tannins).
Anthocyanidins are also a subgroup of flavonoids which exhibit blue, purple and red colours in plants.

<table>
<thead>
<tr>
<th>Chemical Structure of Important Flavonoids</th>
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</table>

(Source: Tsao 2010)

Polyphenol amides are polyphenols with N-containing functional substituents (Figure 9). Capsaicinoids in chili peppers avenanthramides in oats are examples of polyphenol amides. Other polyphenols subgroups (Figure 10) are important compounds found in plants such as resveratrol in grapes, ellagic acid in berries, lignans in flaxseed, and curcumin in turmeric (Tsao 2010).
2.2.3.2 Occurrence and content

Polyphenols are one of the largest groups of secondary metabolites in plants (Dykes & Rooney 2007). These compounds are found in numerous plants including cereals, fruits, legumes and vegetables (Bravo 1998). Polyphenols are synthesised in plants as responses to ecological and physiological stresses such as insect attack, pathogen...
infestation and UV radiation (Khoddami et al. 2013). In legumes, the main polyphenols are flavonoids, phenolic acids and tannins. Legumes with high polyphenol contents are dark in colour, such as black beans, black gram and red kidney beans (Campos-Vega et al. 2010).

The major polyphenols compounds identified in lupin are in the subgroup of flavonoids (mainly flavones and isoflavones) and phenolic acids (Khan et al. 2015). Oomah et al. (2006) reported total phenolic compounds in lupin varieties grown in France ranged between 1189–1465 mg/100 g. Total phenolic compounds in lupin grown in Western Australia ranged between 369–2660 mg/100 g (Wang & Clements 2008). Duenas et al. (2009) reported that flavones were the most abundant phenolic compounds in lupin seeds (76% of total polyphenols). Hydroxybenzoic compounds, isoflavones and hydroxycinnamic compounds contents are 18%, 4% and 1% of total polyphenols, respectively (Duenas et al. 2009). The main identified flavones were glycosides of luteolin, apigenin and diosmetin, while genistein derivatives and genistein aglycone are the main identified phenolic compounds in the isoflavone group (Duenas et al. 2009). Table 10 shows total phenolic compounds contents in legumes.

2.2.3.3 Effect of genotype, cultivation conditions and environment

Polyphenols compositions and contents in plant are highly influenced by genotype and environment (Bravo 1998). Polyphenols contents vary greatly between cultivars (Bravo 1998). Different types of stresses have considerable effects on many polyphenols, especially on phenolic acids (Manach et al. 2004). Polyphenol contents of plants cultivated with stress conditions are higher than that of plants grown without stress (Asami et al. 2003). Limited information on the impact of genotype, cultivation practice and environment on polyphenols in lupin has been published. Oohmah et al. (2006) indicated that genotype has a significant effect on polyphenols contents in lupin.
Table 10: Total phenolic compounds contents in legumes

<table>
<thead>
<tr>
<th>Legumes</th>
<th>Total phenolic compounds (mg/100 g DM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black gram</td>
<td>540–1200</td>
</tr>
<tr>
<td>Chickpeas</td>
<td>78–230</td>
</tr>
<tr>
<td>Common beans</td>
<td>34–280</td>
</tr>
<tr>
<td>Faba beans</td>
<td>280–1120</td>
</tr>
<tr>
<td>Lentils</td>
<td>376–417</td>
</tr>
<tr>
<td>Mungbean</td>
<td>440–800</td>
</tr>
<tr>
<td>Pigeon peas</td>
<td>380–1710</td>
</tr>
<tr>
<td>Soybean</td>
<td>81–589</td>
</tr>
<tr>
<td>Lupin</td>
<td></td>
</tr>
<tr>
<td>L. albus</td>
<td>452–1161</td>
</tr>
<tr>
<td>L. angustifolius</td>
<td>535–1465</td>
</tr>
<tr>
<td>L. luteus</td>
<td>369–731</td>
</tr>
<tr>
<td>L. mutabilis</td>
<td>799–2660</td>
</tr>
</tbody>
</table>


2.2.3.4 Determination

Extraction, separation and determination of polyphenols are challenging due to presence of various forms of chemical structures. Some of the polyphenols are sensitive to heat, light and oxygen, therefore sample collection, preparation and storage steps are critical to minimise degradation and loss of polyphenols (Tsao 2010). The assays used for determination of polyphenol can be classified into two groups (Shahidi & Naczk 2012).

1) Determination of group of compounds such as total phenolic, total flavonoid and condensed tannin contents.

2) Determination of specific compound such as cinnamic acid, resveratrol and curcumin.

Different extraction procedures are applied for polyphenols analysis; however, there is no satisfactory extraction procedure that can be used for the extraction of all polyphenols or a specific class of polyphenolic compounds in plant material (Shahidi...
Most of the polyphenolic compounds are hydrophilic and can be extracted using water and mixture of water and polar organic solvents such as acetone, ethanol and methanol (Stalikas 2007; Tsao 2010).

Polyphenols are stable under acidic conditions thus extraction is commonly performed using weak acids with organic solvents (Tsao 2010). Both free and bound forms of polyphenols exist. Ferulic acid and lignans are bound forms of polyphenols (often bound to structural materials); therefore, strong acids such as 2–4 M HCl are used to extract these compounds (Tsao 2010). The extraction time, temperature and the ratio of sample-to-solvent also impact on the recovery of polyphenols (Naczk & Shahidi 2004). Longer extraction time and higher temperature aid the solubility of polyphenols of the sample; however, some phenolic compounds can be degraded under the long extraction or high temperature conditions (Biesaga & Pyrzyńska 2013).

Determinations of group of phenolic compounds are commonly carried out using colourimetric methods with spectrophotometric detection. These methods are economical, rapid and simple for quantification of polyphenols. However, they cannot quantify individual polyphenol compounds (Khoddami et al. 2013). Light and temperature have to be controlled properly during the analysis, since these factors can affect the stability of the coloured complex.

Two widely used colourimetric methods for determination of total phenolic compounds in plant materials are Folin-Denis and Folin-Ciocalteu methods. Both assays are based on the reduction of reagents containing tungsten and molybdenum (Folin–Denis or Folin-Ciocalteu reagent) when phenolic compounds are present in an alkaline condition. The absorbance (a broad light absorption spectrum around 760 nm) of blue-coloured complex is measured using spectrophotometer compared to a calibration curve of standard solution (gallic acid equivalent) (Shahidi & Naczk 2012).

Total flavonoid contents are also determined by colourimetric method. Flavonoids can form a stable complex with aluminum chloride (AlCl₃) resulting in a yellow-coloured solution. The absorbance (a broad light absorption spectrum around 410 nm) of the complex is detected by a spectrophotometer compared to a calibration curve of standard solution (catechin equivalent) (Shahidi & Naczk 2012).
Condensed tannins (proanthocyanins) can also be determined by colourimetric methods. The vanillin assay is commonly used for the determination of condensed tannins in plant materials because of its sensitivity, simplicity and specificity (Shahidi & Naczk 2012). The assay is based on the condensation of the vanillin reagent with proanthocyanins in acidic conditions. The reaction gives a pink-coloured complex which can be detected by a spectrophotometer at a broad light absorption spectrum around 500 nm. Catechin is often used as a standard in the vanillin assay (Shahidi & Naczk 2012).

Determination of individual phenolic compound is performed by chromatographic techniques using advanced instruments. Although the chromatographic techniques can identify and quantify individual phenolic compounds, these methods are time consuming and various authentic standards (Chang et al. 2002).

High performance liquid chromatography (HPLC) is the most commonly used technique for both separation and quantitation of phenolic compounds (Shahidi & Naczk 2012). Purification step to remove the interferences from the crude extract is required prior to separation using HPLC. Acrylic resins (XAD-7, EXA-31), amberlite, polyamide, Sephadex LH-20, and solid phase extraction (SPE) cartridges are examples of materials applied to purify phenolic compounds from sample extracts (Khoddami et al. 2013).

Various types of stationary phases and mobile phases have been used for the analysis of polyphenols. Acetonitrile and methanol, or their aqueous forms, are commonly used mobile phases in HPLC determination of phenolic. To avoid the ionization of phenolic compounds during chromatographic separation, pH of the mobile phase in the range pH 2–4 is recommended (Khoddami et al. 2013). Because of the large variation in chemical structure of phenolic compounds, a gradient elution system is more applicable than an isocratic elution system (Robbins 2003). Selecting of an appropriate column is the other important procedure in identifying polyphenols. Column is generally selected based on the chemical structure of the group of phenolic compounds and their polarity. Both normal phase and reversed phase column have been reported for identification of polyphenols, but reversed phase is preferable in the separation and identification of phenolic compounds (Robbins 2003).
Commonly used detection techniques for HPLC characterisation of phenolic compounds are UV-visible and photodiode array detectors. Other detectors used for the detection of phenolic compounds include electrochemical coulometric array detector, fluorimetric detector, mass spectrometric detector and nuclear magnetic resonance detector (Shahidi & Naczk 2012).

Gas chromatography (GC) is another major chromatographic technique applied for the determination of plant polyphenols. Flame ionization detection (FID) and mass spectrometer are commonly used detectors. Phenolic compounds are low volatile, therefore derivatisation step is required. A variety of reagents used to modify and generate volatile derivatives in GC characterisation of phenolic compounds include diazomethane, dimethyl sulfoxide with methyl iodide, and ethyl and methyl chloroformate (Robbins 2003).

HPLC and GC are accurate and reliable methods to determine phenolic compounds. HPLC or GC coupled with mass spectrometric detectors is highly sensitive and specific technique due to the mass selectivity of detection (Khoddami et al. 2013). Recently, with the progress in the development of HPLC techniques, new trends in the determination of phenolic compounds are hydrophilic interaction liquid chromatography (HILIC) and 2-dimensional liquid chromatography (2-D LC). HILIC can be applied to higher compatibility of mobile phase when couple with MS. The accuracy is also enhanced when quantify polar compounds in complex food matrices. 2-D LC can achieve separation and identification of structurally similar and minor compounds from complex matrices (Khoddami et al. 2013; Tsao 2010).

### 2.2.3.5 Anti-nutritional property

Polyphenols can interact with proteins from food sources affecting the nutrients digestions and absorptions. Many *in vitro* studies have shown that proteins can bind to polyphenols (Frazier et al. 2010; Hasni et al. 2011; Nagy et al. 2012). The structural characteristics of polyphenols including molecular weight, structural flexibility and number of hydroxyl group (OH) play an important role in protein–polyphenol interactions (Jakobek 2015). Frazier et al (2010) indicated that high molecular weight polyphenols such as tannins are able to bond strongly to proteins. Polyphenols that are structurally flexible exhibited equal binding strength to different proteins (gelatin and
bovine serum albumin), while less structurally flexible polyphenols showed stronger binding to some proteins (gelatin) and weaker binding to others (bovine serum albumin) (Frazier et al. 2010). The increase in the number of OH groups on the polyphenol molecule enhances the order of binding to proteins (Hasni et al. 2011).

The interactions of polyphenols and proteins can affect nutritional quality of proteins. The availability of some amino acids can be affected by the interaction of polyphenols and proteins. The interaction of phenolic acids and flavonoids with soy proteins can affect the blocking of cysteine, lysine and tryptophan residues in the protein molecules, thus the availabilities of these amino acids are decreased (Rawel et al. 2002). Petzke et al. (2005) reported the reduction of whey proteins digestibility as a result of the interaction with chlorogenic acid. A decrease in protein absorption after their interaction with polyphenols has been reported (Aguié-Béghin et al. 2008).

Polyphenols affect fat bioavailability by interfering the emulsification process (increase droplet size and decrease surface area) resulting in decrease in lipase activity and fat absorption. In the fat absorption process, fats are first transformed into emulsions, and then transferred into the stomach and duodenum. The emulsions are exposed to the surface-active compounds in the gastrointestinal tract which help in emulsification. Lipolysis, a process which lipase breakdown lipids into smaller particles, is carried out to make lipids ready for absorption (Shishikura et al. 2006). Therefore, specific emulsion properties, namely droplet size and surface area are crucial for the lipase activity, which could affect the fat absorption process. Shishikura et al. (2006) reported polyphenols from green and black tea interfere emulsification by increasing droplet size and decreasing specific surface area in an in vitro model. A study on the effect of black tea polyphenols on diet-induced obesity in rat model also reported that black tea polyphenols inhibited the lipase activity resulting in inhibition of intestinal lipid absorption (Uchiyama et al. 2011). The inhibitory effects of apple polyphenols and procyanidins on lipase activity (in vitro) and triglyceride absorption (in vivo) were also reported (Sugiyama et al. 2007). It seems that polyphenols may have a beneficial health effect in terms of decreasing fat absorption.
2.2.3.6 Polyphenols and mineral bioavailability

Polyphenols can bind to positively charged minerals such as calcium, iron, magnesium and zinc in foods resulting in reduction in mineral bioavailability (Hambidge 2010). The inhibitory effect of polyphenols on iron absorption has widely published. Polyphenols are able to form complex with iron in the intestine and reduce the bioavailability of iron (Hambidge 2010). Black tea containing high levels of tannin showed a strong inhibitory effect on iron absorption (Hurrell et al. 1999). They also showed that tannins reduce iron absorption by binding to non-haem iron and Polyphenols in red wine and cocoa decrease iron bioavailability.

The capability of polyphenols to form complexes with iron depends on the structure of polyphenols. Brune et al. (1989) reported that the inhibitory effect of coffee polyphenols on iron bioavailability mainly depended on phenolic galloyl groups. The authors concluded that the group of condensed tannins may not inhibit the iron absorption. In contrast, Petry et al. (2010) reported that polyphenols with galloyl groups, such as hydrolysable tannins, and catechol groups, such as condensed tannins strongly inhibitor iron absorption. Ariza-Nieto et al. (2007) also reported a strong inhibitory effect of condensed tannin from bean on iron bioavailability.

Limited information on the individual inhibitory effect of polyphenols, as well as different group of polyphenols on mineral bioavailability in grains and legumes has been published. It is difficult to study the effect on polyphenols alone on mineral bioavailability in grains and legumes, since these food commodities also contain high levels of other inhibitors such as phytate. Few studies reported the inhibitory effect of polyphenol on mineral bioavailability in dephytinized grain and legume. Hurrell et al. (2003) indicated a significant increase in iron absorption of sorghum porridge prepared from low-polyphenols sorghum after removing phytate. However, iron absorption from porridge prepared from high-polyphenol sorghum was not improved.

2.2.3.7 Health benefits

Anti-carcinogenic, anti-diabetic, anti-inflammatory and antioxidant properties of polyphenols have been reported (Devi et al. 2014). Polyphenolic compounds have been promoted in the prevention of non-communicable diseases such as cardiovascular diseases, diabetes and cancers (Scalbert et al. 2005).
Epidemiological studies have indicated that consumption of diets rich in fruits and vegetables reduce the risk in developing cancers. Anti-carcinogenic effects of polyphenols in various foods/drinks such as green tea (Carvalho et al. 2010), mango (Noratto et al. 2010) and turmeric (Tokusoglu et al. 2015) have been reported. Mechanisms of anti-carcinogenic effects of polyphenols include modulation of carcinogen metabolism, prevention of oxidative stress, and prevention of DNA damage (Yang et al. 2009).

Anti-diabetic effects of polyphenols from plants such as brown algae (Lee & Jeon 2013), green tea (Sabu et al. 2002) and soybean (Ademiluyi & Oboh 2013) have been reported. Mechanisms of anti-diabetic effects of polyphenols include inhibition of α-glucosidase and α-amylase during enzymatic hydrolysis of carbohydrates to glucose, reduction in carbohydrate absorption, modulation of the enzymes related to glucose metabolism, improvement of β-cell function and insulin action, and stimulation of insulin secretion (Hanhineva et al. 2010; Iwai et al. 2006).

Flavonoids have a major contribution to anti-inflammatory effect compared to other phenolic compounds (González et al. 2011). Anti-inflammatory properties of polyphenols from plants have been reported widely. Grape phenolic compounds especially flavonoids (flavonols, flavanols and procyanidins) exhibited significant anti-inflammatory properties (Panico et al. 2006; Terra et al. 2009). The suggested anti-inflammatory mechanisms of flavonoids include inhibition of the synthesis and activities of different pro-inflammatory mediators, inhibition of transcription factors, and activation of nuclear factor-erythroid 2-related factor 2 (Nrf2) (Serafini et al. 2010).

Antioxidant property is the most remarkable biological activity of polyphenols which has been reported widely in various plants. The anti-oxidative mechanisms of polyphenols involve chelating with metal ions resulting in decreasing the pro-oxidant activity of these metal ions, inhibiting enzymes involved in the oxidative processes, inhibiting lipid oxidation, reducing hydrogen peroxide formation, and scavenging of free radicals (Xia et al. 2010). The free radical scavenging potency depends on chemical structures of polyphenols such as the degree of methoxylation and the number of hydroxyl groups (Halliwell 2008; Yordi et al. 2012).
2.3 Bioavailability and bioaccessibility

The bioavailability and bioaccessibility terms have been used interchangeably; however, they are not the same. There are different definitions of the term bioavailability across various disciplines including nutrition, pharmacology and toxicology (Ng et al. 2009). US Food and Drug Administration defined bioavailability as “the rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of action” (USFDA 2016).

In nutrition area, various definitions of bioavailability have been proposed. The similar concept of the term bioavailability refers to the fraction of ingested nutrient or compound that reaches the systemic circulation and is utilised for physiological functions (Cardoso et al. 2015). In fact, there are practical and ethical difficulties in determination of delivery and utilisation of compounds. Therefore, the term “bioavailability is usually defined as “the fraction of dose of compounds or their active metabolite that reaches the systemic circulation” (Carbonell-Capella et al. 2014; Cardoso et al. 2015).

Bioaccessibility is defined as “the fraction of a compound that is released from its matrix in the gastrointestinal tract and thus becomes available for intestinal absorption” (Fernández-García et al. 2009). Bioaccessibility of a compound depends only on the digestion and release from the matrix (Etcheverry et al. 2012). The concept of bioaccessibility assessment is to evaluate nutritional potency of food or food formula developed with the aim of improving human health (Fernández-García et al. 2009).

For this literature review, the terms bioavailability and bioaccessibility are used interchangeably in order to maintain the terminology cited from other authors. However, according to the definition, bioavailability has to be evaluated by in vivo methods in animals or humans, and the results from in vitro studies are considered as bioaccessibility (Carbonell-Capella et al. 2014; Cardoso et al. 2015).
2.3.1 Factors affecting mineral bioavailability

2.3.1.1 Physiological factors

Physiological factors including age, sex, nutritional status, physiological state, diseases and infection can influence absorption and utilisation of minerals (Fairweather-Tait & Hurrell 1996; Gibson 2007). High physiological requirement of mineral during rapid growth, pregnancy and lactation results in physiological adaptations to increase the mineral absorption and utilisation. On the other hand, when physiological requirement of a mineral is low, the decreased intestinal absorption and/or the increased renal excretion may occur (Gibson 2007). Due to the high physiological requirement of calcium, iron and zinc in infants, young children, pregnant and lactating women increased the absorption of these minerals is observed in this cohort. Higher calcium and iron absorptions are also found in individuals with the minerals deficiencies. In the elderly, the bioavailability of certain mineral is affected by the changes in the secretory and absorptive capacity of the intestine (Gibson 2007). Lower absorption of calcium is indicated in the elderly because of the changes in vitamin D metabolism involving vitamin D receptors in the intestinal mucosa (Ebeling et al. 1992). Habitual dietary intakes of minerals also affect the bioavailability of minerals. Low habitual calcium intake increases the absorption of calcium, while the decreased calcium absorption is found in individuals with high habitual calcium intake.

Some diseases, disorders and infections also influence on the mineral bioavailability. Hypochlorhydria (state of absence or low gastric acid) can decrease the absorption of calcium, iron and zinc. The solubilisation and absorption of these minerals depends on pH. Infections with microorganism such as *Ascaris lumbricoides*, *Giardia lamblia*, rotavirus and salmonella can alter the characteristic of intestinal mucosa causing the increases in intestinal permeability and reductions in mineral absorption. For example, abnormal intestinal permeability interferes zinc homeostasis by causing the large endogenous faecal losses of zinc (Brewster et al. 1997). Changes in the intestinal mucosa structure also interfere the iron homeostasis, since it is regulated by uptake and transfer by the intestinal mucosa (Gibson 2007). Physiological factors affecting calcium, iron and zinc bioavailability are shown in Table 11.
Table 11: Physiological factors affecting calcium, iron and zinc bioavailability

<table>
<thead>
<tr>
<th>Mineral</th>
<th>Increased bioavailability</th>
<th>Decreased bioavailability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td>Vitamin D adequacy</td>
<td>Vitamin D deficiency</td>
</tr>
<tr>
<td></td>
<td>Increased mucosal mass</td>
<td>Decreased mucosal mass</td>
</tr>
<tr>
<td></td>
<td>Calcium deficiency</td>
<td>Menopause</td>
</tr>
<tr>
<td></td>
<td>Phosphorus deficiency</td>
<td>Old age</td>
</tr>
<tr>
<td></td>
<td>Pregnancy</td>
<td>Decreased gastric acid</td>
</tr>
<tr>
<td></td>
<td>Lactation</td>
<td>Rapid intestinal transit time</td>
</tr>
<tr>
<td></td>
<td>Low habitual calcium intake</td>
<td>High habitual calcium intake</td>
</tr>
<tr>
<td></td>
<td>Diseases (e.g. hyperparathyroidism, sarcoidosis, idiopathic hypercalciuria)</td>
<td>Diseases (e.g. malabsorption syndrome, celiac disease, Crohn’s disease, chronic renal failure, diabetes, hypoparathyroidism, primary biliary cirrhosis)</td>
</tr>
<tr>
<td>Iron</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Haem</td>
<td>Low iron status</td>
<td>High iron status</td>
</tr>
<tr>
<td>- Non-haem</td>
<td>Low iron status</td>
<td>High iron status</td>
</tr>
<tr>
<td></td>
<td>Pregnancy</td>
<td>Hypochlorhydria</td>
</tr>
<tr>
<td></td>
<td>Diseases (aplastic anemia, haemolytic anemia, haemochromatosis)</td>
<td></td>
</tr>
<tr>
<td>Zinc</td>
<td>Low zinc status</td>
<td>High zinc status</td>
</tr>
<tr>
<td></td>
<td>Disease (acrodermatitis enteropathica)</td>
<td></td>
</tr>
</tbody>
</table>

(Modified from Strain & Cashman 2009)

2.3.1.2 Dietary factors

Bioavailability of minerals is also depended on dietary factors. Amount of mineral load, chemical form of the mineral, interactions between minerals and other food components can affect the bioavailability of minerals. Table 12 shows dietary factors affecting calcium, iron and zinc bioavailability.
Table 12: Dietary factors affecting calcium, iron and zinc bioavailability

<table>
<thead>
<tr>
<th>Mineral</th>
<th>Increased bioavailability</th>
<th>Decreased bioavailability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td>Lactose (in infants)</td>
<td>Phytate</td>
</tr>
<tr>
<td></td>
<td>Non-digestible oligosaccharides</td>
<td>Oxalate</td>
</tr>
<tr>
<td></td>
<td>Low calcium intake</td>
<td>High calcium intake</td>
</tr>
<tr>
<td></td>
<td>Ingestion with a meal</td>
<td>Ingestion without a meal</td>
</tr>
<tr>
<td>Iron</td>
<td>Low haem iron intake</td>
<td>High haem iron intake</td>
</tr>
<tr>
<td></td>
<td>Meat</td>
<td>Calcium</td>
</tr>
<tr>
<td></td>
<td>Ascorbic acid</td>
<td>Phytate</td>
</tr>
<tr>
<td></td>
<td>Meat, fish, seafood</td>
<td>Iron-binding phenolic</td>
</tr>
<tr>
<td></td>
<td>Certain organic acids</td>
<td>Compounds</td>
</tr>
<tr>
<td></td>
<td>Calcium</td>
<td>Calcium</td>
</tr>
<tr>
<td>Zinc</td>
<td>Low zinc intake</td>
<td>High zinc intake</td>
</tr>
<tr>
<td></td>
<td>Certain organic acids</td>
<td>Phytate</td>
</tr>
<tr>
<td></td>
<td>Certain amino acids</td>
<td>Certain metals</td>
</tr>
</tbody>
</table>

(Source: Strain & Cashman 2009)

Amount of the mineral load has a significant effect on the bioavailability of mineral. Ireland and Fordtran (1973) reported a decrease in calcium absorption when increasing calcium load administered via intestinal perfusion system in human subjects. Heaney et al. (1991) studied the effect of calcium load on the absorption in healthy women, and reported an inverse correlation between the logarithm of calcium load and its absorption. Sian et al. (1993) found decrease in zinc absorptions when higher zinc contents were administered to the subjects.

Chemical form of mineral is one of the most important factors affecting its bioavailability. Two forms of iron including haem iron and non-haem iron presented in food. Haem iron is bound in a porphyrin ring which found in haemoglobin and myoglobin in food from animal origin. Non-haem iron, mostly in salt forms, is commonly found in a wide range of foods from both animal and plant origins (Gibson 2007). Non-haem iron is also found as a contaminant from soil or during preparation, processing and storage of foods (Harvey et al. 2000). Haem iron is more available absorbed and less affected by other dietary components than non-haem iron. When
haem iron is released from the food matrix, the haem molecule shields the iron atom from interactions with other food components. Thus, the iron can be soluble and absorbed through the gut. Chemical form of zinc also affect its bioavailability. The organic forms of zinc tend to be more readily absorbed and less influenced by other components than the inorganic forms (Solomons et al. 1979). The chemical form of mineral is related to its solubility, therefore the absorption efficiency is higher for mineral compounds well soluble in water or diluted acids.

Competitive interactions between minerals affect their absorption and bioavailability (Sandström 2001). Minerals with similar physicochemical properties sharing the same uptake pathways can compete for transport proteins or other uptake mechanisms (Gibson 2007). The extents of the mineral interactions depend on the contents of these minerals. The interactions of minerals are high in foods with mineral supplements or fortificants. The discrepant results of the interaction between certain minerals on the bioavailability have been reported. Some studies indicated that the iron supplement or fortification in the diets reduced zinc absorption or plasma zinc concentration (Power et al. 1991; Sandström 2001). Conversely, no effect of iron supplements on serum zinc concentration has been reported by Sheldon et al. (1985). The findings of the interactions between calcium and iron were also inconsistent. However, most studies reported that calcium supplement has no negative effect on iron status (Ilich-Ernst et al. 1998; Minihane & Fairweather-Tait 1998; Yan et al. 1996). These inconsistent findings may be due to the different physiological factors of the subjects used in the studies as well as the forms and approaches of minerals provided to the subjects. Some minerals can form complex with other food components which may inhibit the absorption of minerals (Sandström 2001). For example, calcium does not have a direct inhibitory effect on zinc absorption, but it can form insoluble complexes with phytate and zinc which impairs zinc absorption (Sandström 2001) as described in section 2.2.2.6.

Certain food components have significant impact on the mineral bioavailability because they can interact with minerals. Some dietary components have the ability to form insoluble complexes with minerals in the gut or compete using the same uptake pathway, resulting in the decreased bioavailability. Whereas several compounds can enhance the absorption or utilisation of the minerals (Strain & Cashman 2009).
Food components which have been studied widely of the inhibitory effects on the mineral bioavailability include oxalate, phytate and polyphenolic compounds. Oxalate is found in many plants such as spinach, rhubarb, taro and sweet potatoes. High contents of oxalate are also found in some animals such as snails and mollusks (Gibson 2007). Oxalate can form insoluble complexes with calcium, thus reducing the calcium bioavailability. The strong inhibitory effect of oxalate on calcium bioavailability in various foods have been reported (Amalraj & Pius 2015; Kamchan et al. 2004; Weaver et al. 1999). However, limited information on the inhibitory effects of oxalate on iron and zinc has been published. Gillooly et al (1983) found a reduction in iron absorption of human subjects when the additional amount of oxalate was included in the diet. Kelsay et al (1988) reported a decrease in zinc balance in the subjected who consumed high fibre and high oxalate foods. The effects of phytate and polyphenolic compounds on the mineral bioavailability are described in section 2.2.2.6 and 2.2.3.6, respectively.

The inhibitory effects of some anti-nutritional factors on mineral bioavailability can be modified by food preparation and processing techniques (Platel & Srinivasan 2015). Diets rich in mineral inhibitors such as unrefined cereals and legumes can reduce the absorption of minerals. Food preparation such as dehulling, milling, and soaking seeds before cooking can reduce the anti-nutrients resulting increased bioavailability of minerals. Food processing such as fermentation, germination and enzymatic treatment also increase the bioavailability of minerals (Platel & Srinivasan 2015).

Some food components enhance the bioavailability of minerals. Ascorbic acid (vitamin C) can form complexes with iron in the stomach, preventing iron forming complexes with inhibitors such as phytates and tannins (Teucher et al. 2004). The degree of the enhancing effect depends on ascorbic acid content as well as the composition and properties of the diet. Some organic acids (e.g. acetic, butyric, citric, formic, lactic and propionic acids) present naturally or as food additives can also form soluble complexes with minerals in the gut enhancing the absorption of non-haem iron and zinc. The enhancing effects of these organic acids on iron absorption depend on the type of organic acid, the molar ratio of organic acid to iron and the iron source (Salovaara et al. 2002).
2.3.2. Determination of mineral bioaccessibility and bioavailability
Various approaches are applied to determine the mineral bioaccessibility and bioavailability. Two major methodologies are classified as *in vitro* and *in vivo* methods. *In vitro* methods include the *in vitro* digestion with or without Caco-2 cell model. *In vivo* studies of the mineral bioavailability determination are assessed by animal experiments or human studies.

2.3.2.1 *In vitro* methods
*In vitro* methodologies are based on the simulation of physiological conditions and the digestion sequences occurred in the gastrointestinal tract. Figure 11 shows the digestion system of the human gastrointestinal tract. There are four main *in vitro* methods to determine the bioaccessibility of minerals: (1) solubility (2) dialysability (3) gastrointestinal model (4) Caco-2 cell model (Salovaara et al. 2002).

Figure 11: Digestion system of the human gastrointestinal tract
(Source: Guerra et al. 2012)

2.3.2.1.1 Solubility method
Solubility method has been developed for measuring mineral bioaccessibility based on the concept that only soluble mineral can be absorbed (Sahuquillo et al. 2003). The fraction of the total mineral in the diet after the digestion processes released from the food matrix and ready for absorption is measured. Pepsin and hydrochloric acid mixture is added to mimic the gastric digestion. The pH of the gastric digest is
neutralised by adding sodium bicarbonate before the intestinal digestion step. The pancreaticin and bile salt mixture is added, and the digested sample is incubated for two hours. After completing the simulated digestion processes, the digested sample is centrifuged and the supernatant (soluble fraction) is collected. Percent solubility is calculated as the amount of soluble compound after digestion relative to the total amount of compound in the test sample (Etcheverry et al. 2012). This method can be applied to determine a large number of samples with good reproducibility. However, the solubility assay does not simulate physiological conditions of human (Wienk et al. 1999).

2.3.2.1.2 Dialysability method
Dialysability assay measures the amount of soluble mineral after digestion and the dialysis equilibrium. This method also measures the available mineral for absorption in the small intestine (Etcheverry et al. 2012). Unlike the solubility assay, dialysis bag or tubing with a certain molecular weight cut off (MWCO) is added to the gastric digested sample during the intestinal phase. The dialysis bag contains sodium bicarbonate diffusing slowly through the bag which is simulated the bicarbonate secretion in the intestine to neutralise the gastric acids. During incubation, pancreaticin and bile salt mixture is added. After completing the simulated digestion processes, the dialysis bag is collected to measure the mineral content. Percent dialysability is calculated as the amount of mineral present in the dialysis bag relative to the total amount of mineral found in the test sample. This assay has been applied widely as a reliable predictor for assessing mineral bioavailability due to its good correlation with the in vivo studies (Bueno et al. 2013; Gupta et al. 2006; Wienk et al. 1999).

2.3.2.1.3 Sophisticated gastrointestinal model
Sophisticated gastrointestinal models for simulating the human digestive system have been developed and are commercially available. These models can simulate many physiological conditions which the solubility and dialysability assays cannot perform such as peristalsis and churning during the intestinal phase, and gastrointestinal transit times. For example, the Netherlands organisation’s gastrointestinal model (TIM) consists of two computer-controlled chambers: TIM1 (represent the digestive systems occurred in the stomach and small intestine) and TIM2 (represent the fermentation process in the large intestine). The advantages of this method include various digestion
parameters and the digested sample can be collected at any step of the digestive system. However, these models are expensive and only a few validated studies have been published (Etcheverry et al. 2012).

2.3.2.1.4 Caco-2 cell model

The Caco-2 cell model has been applied to assess the mineral uptake or absorption. The Caco-2 cell is referred to an epithelial cell line obtained from a human colon adenocarcinoma which has been widely used as a model of intestinal barrier. Under specific conditions of cell culture process, the cells can differentiate and form a cell monolayer which exhibit similar morphological and functional characteristics to the mature enterocyte presented in the small intestine (Glahn et al. 2002). The example diagram of in vitro digestion/Caco-2 cell culture model is shown in Figure 12. It should be noted that the terms absorption and bioavailability are commonly interchanged, but they are not the same. In other words, the fraction of iron absorption does not indicate the iron amount utilised in the body (Wienk et al. 1999). The greater zinc absorption does not relate to the greater amount of zinc utilised at the tissue level (Ammerman 1995). The Caco-2 cell model is complicated and requires trained personnel with intensive knowledge of cell culture methods.

![In vitro digestion/Caco-2 cell culture model](Source: Glahn et al. 1998)

**Figure 12: The diagram of in vitro digestion/Caco-2 cell culture model**
(Source: Glahn et al. 1998)
The advantages and limitations of each *in vitro* method applied for mineral bioavailability determination are shown in Table 13. *In vitro* methods are inexpensive, rapid, less labour intensive, and no ethical constraints. The conditions of the experiment can be controlled easily and optimally as well as no confounding factors such as inter-individual differences in mineral status, resulting in high repeatability of results (Wienk et al. 1999). However, several physiological factors such as peristalsis and churning, transit time, and diffusion barriers cannot be simulated efficiently (Miller & Burner 1989).

**Table 13: Advantages and limitations of *in vitro* methods applied for mineral bioavailability determination**

<table>
<thead>
<tr>
<th><em>In vitro</em> method</th>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solubility</td>
<td>- Simple</td>
<td>- Cannot assess rate of uptake or absorption or transport kinetics</td>
</tr>
<tr>
<td></td>
<td>- Inexpensive</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Need only basic equipment</td>
<td>- Cannot measure food component competition at the site of absorption</td>
</tr>
<tr>
<td></td>
<td>- Easily controlled conditions</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- High reproducibility</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- No ethical constraints</td>
<td>- Sometimes not a reliable indicator for bioavailability</td>
</tr>
<tr>
<td>Dialysability</td>
<td>- Simple</td>
<td>- Cannot assess rate of uptake or absorption or transport kinetics</td>
</tr>
<tr>
<td></td>
<td>- Inexpensive</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Need only basic equipment</td>
<td>- Cannot measure food component competition at the site of absorption</td>
</tr>
<tr>
<td></td>
<td>- Easily controlled conditions</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- High reproducibility</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- No ethical constraints</td>
<td></td>
</tr>
<tr>
<td>Gastrointestinal model</td>
<td>- Can incorporate many digestion parameters such as peristalsis and churning</td>
<td>- Expensive</td>
</tr>
<tr>
<td></td>
<td>- Allows the collection of digest at any Step of the digestive system</td>
<td>- Few validated studies</td>
</tr>
<tr>
<td>Caco-2 cell model</td>
<td>Allows the study of nutrient or food component competition at the site of absorption</td>
<td>Requires trained personnel with knowledge of cell culture methods</td>
</tr>
</tbody>
</table>

(Modified from Etcheverry et al. 2012)
In vitro bioaccessibility methods are useful to provide information on the possible interactions between food components, the effects of digestion conditions (pH and enzymes) and the effects of food preparation and processing conditions (Fernández-García et al. 2009). These assays are also useful to compare foods based on the bioaccessibility of certain minerals (Sahuquillo et al. 2003).

2.3.2.2 In vivo methods

Mineral bioavailability has been measured using animal experiment and human study. In vivo methods commonly used for assessing mineral bioavailability are classified into two major techniques; non-isotopic and isotopic techniques.

2.3.2.2.1 Non-isotopic techniques

In vivo studies on mineral solubility are conducted by measuring the mineral content in the gastrointestinal tract in animal models and mostly are performed in rats. After feeding the diet for two to three hours, the gastrointestinal tract of a studied animal is removed. The contents in the gastrointestinal tract are centrifuged to separate soluble and insoluble fractions. The mineral bioavailability is calculated from the relative content of mineral in the soluble fraction to the total mineral content in the diet (Wienk et al. 1999).

Chemical balance techniques implicate assessments of mineral intakes and excretions over a certain period of time (Fairweather-Tait 1992). The amount of mineral absorbed can be calculated by subtract the mineral contents found in faeces and urine from the total mineral intake (Ammerman 1995). The chemical balance technique is suitable for studying minerals which are well absorbed. This technique is not suitable for measuring individual foods or meals. The technique is also not representing the bioavailability of many minerals since this method does not consider the endogenous mineral excreted in the faeces (from abrasion of mucosal cells) or back excretion of minerals into the gastrointestinal tract.

Rate of repletion method involves measuring the repletion rate of a studied mineral provided to deficient or depleted animals or subjects. For example, iron bioavailability can be determined by measuring the haemoglobin repletion rate in anaemic subjects; one group intake normal diets and another group obtained iron supplement. The use of
animal model in this approach for assessing certain mineral bioavailability is no longer acceptable because of the species differences. The ethical constraint to deplete animals and finding deficient subjects is a limitation of this technique (Fairweather-Tait & Dainty 2002).

Plasma appearance method is performed by measuring level of minerals in plasma relative to the total mineral intake. The oral dose of the studied mineral should be sufficient to create an observable plasma tolerance curve, i.e. the mineral level appear in plasma must be greater than the amount of mineral disappear over the period of absorption from the gastrointestinal tract. This technique is suitable for assessing bioavailability of mineral in fortified foods or supplements (Fairweather-Tait 1992).

2.3.2.2.2 Isotopic technique
Radioisotopes and stable isotopes are commonly applied to measure bioavailability of minerals. The fates of absorption and metabolism of a certain labelled mineral in diets can be tracked by various approaches similar to non-isotopic techniques. Earlier, radioisotopes were used for assessing mineral bioavailability. The radioisotopes can be added directly to the studied food (extrinsic labelling) or biosynthetic incorporation, i.e. introduced to plants or animals resulting in labelled mineral in tissues (intrinsic labelling). The extrinsic labelling is easier and less expensive than intrinsic labelling (Ammerman 1995; Fairweather-Tait & Dainty 2002).

Since the use of radioisotopes in human studies can pose a health risk to subjects, thus it should not be administered to pregnant, lactating women and children (Fairweather-Tait & Dainty 2002). Therefore, stable isotopes have been applied widely instead of radioisotopes to study mineral bioavailability. However, some minerals such as aluminium and cobalt are mono-isotope which cannot be studied by this technique. Other limitations of using stable isotopes include complicated and difficult procedures, expensive, and require advance instruments such as mass spectrometry and neutron activation analysis (Fairweather-Tait & Dainty 2002).

Similar to non-isotopic techniques, isotopes balance technique can be used to assess mineral bioavailability. The studied diet, enriched with known amount of isotopes, is fed to subjects, and urine and faeces are collected over a period of time to measure
isotopic excretion (Fairweather-Tait & Dainty 2002). The retention and absorption of mineral is calculated from the subtraction of excreted isotopes from the total amount of isotopes consumed. In this approach, the endogenous excretion of mineral and unabsorbed mineral (labelled) can be distinguish. Plasma appearance approach using isotopes can also be applied to assess mineral bioavailability (Fairweather-Tait 1992; Wienk et al. 1999).

Ideally, the study of mineral bioavailability should be performed using *in vivo* experiments. However, human studies are expensive, provide limited data, and can perform on a small scale. While animal studies are limited by some metabolism differences between animals and humans (Hansen et al. 1996). It is also difficult to study the bioavailability of nutrient of a particular food using *in vivo* experiments, since human or animal requires sufficient nutrients from daily diet. Generally, the ingredient/food of interest has to be incorporated into diet to meet all nutrients requirements. Consequently, *in vitro* methods are commonly used as a reliable indicator for mineral bioavailability of a particular ingredient/food (Hemalatha, et al. 2009).

### 2.3.3 Bioaccessibility and bioavailability of minerals in legumes

Bioaccessibility of calcium, iron and zinc in raw legumes using *in vitro* methods have been reported (Table 14). Among legumes, chickpeas and lentils were studied widely on the mineral bioaccessibility using various *in vitro* methods. Considering the bioaccessibility reported in the same legume, the highest mineral bioaccessibility were found from the solubility method, followed by the dialysability approach, and Caco-2 cell model (Table 14). Limited studies on the bioaccessibility of minerals of lupin using *in vitro* method have been published. Suliburska et al. (2009) studied the mineral bioaccessibility of three Polish lupin species namely *L. albus*, *L. angustifolius* and *L. luteus* using solubility method. The authors found that the bioaccessibility of iron and zinc was in a range of 9.1–18.4% and 9.4–36.7%, respectively. Lombardi-Boccia et al. (2003) reported that the iron and zinc bioaccessibility of lupin were 7.8% and 9.0%, respectively by using dialysability method. However, the mineral bioaccessibility of ASL cultivars has not been studied.
Table 14: The mineral bioaccessibility of raw legumes using in vitro methods

<table>
<thead>
<tr>
<th>Legume</th>
<th>In vitro method</th>
<th>Bioaccessibility of minerals (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ca</td>
</tr>
<tr>
<td>Black gram</td>
<td>Dialysability</td>
<td>NA</td>
</tr>
<tr>
<td>Chick peas</td>
<td>Solubility</td>
<td>19.3–29.1</td>
</tr>
<tr>
<td></td>
<td>Dialysability</td>
<td>20.0</td>
</tr>
<tr>
<td></td>
<td>Digestion/Caco-2 cell</td>
<td>14.6</td>
</tr>
<tr>
<td>Common beans</td>
<td>Dialysability</td>
<td>15.0</td>
</tr>
<tr>
<td>Faba bean</td>
<td>Solubility</td>
<td>NA</td>
</tr>
<tr>
<td>Kidney bean</td>
<td>Solubility</td>
<td>27.5</td>
</tr>
<tr>
<td>Lentils</td>
<td>Solubility</td>
<td>46.6–59.0</td>
</tr>
<tr>
<td></td>
<td>Dialysability</td>
<td>16.0</td>
</tr>
<tr>
<td></td>
<td>Digestion/Caco-2 cell</td>
<td>2.5</td>
</tr>
<tr>
<td>Lupin</td>
<td>Solubility</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Dialysability</td>
<td>NA</td>
</tr>
<tr>
<td>Mungbean</td>
<td>Solubility</td>
<td>15.7–30.3</td>
</tr>
<tr>
<td></td>
<td>Dialysability</td>
<td>NA</td>
</tr>
<tr>
<td>Pea</td>
<td>Solubility</td>
<td>42.0</td>
</tr>
<tr>
<td>Pigeon pea</td>
<td>Dialysability</td>
<td>NA</td>
</tr>
<tr>
<td>Soybean</td>
<td>Solubility</td>
<td>8.1–9.1</td>
</tr>
<tr>
<td>White beans</td>
<td>Solubility</td>
<td>33.7</td>
</tr>
<tr>
<td></td>
<td>Digestion/Caco-2 cell</td>
<td>3.6</td>
</tr>
</tbody>
</table>

NA = Not applicable

(Adopted from Ghavidel & Prakash 2007; Grewal & Jood 2006; Hemalatha et al. 2007; Kumari et al. 2014; Lombardi-Boccia et al. 2003; Luo et al. 2010; Martínez 2010; Sahuquillo et al. 2003; Sebastiá et al. 2001; Suliburska & Krejpcio 2014; Viadel et al. 2006)

Bioavailability of calcium, iron and zinc of various raw legumes using in vivo methods have been reported (Table 15). A few studies on the bioavailability of minerals of raw lupin incorporated diet have been published. Rubio et al. (1994) reported bioavailability of calcium and zinc at 39.8% and 5.9%, respectively from lupin meal incorporated diet using chemical balance method with non-isotopic technique in rat model. The authors found the higher bioavailability of zinc at 22.2% when using isotopic technique. Porres et al. (2006) reported calcium bioavailability of raw lupin incorporated diet in rat using chemical balance method with non-isotopic technique at 80.6%. However, rat is not an appropriate model for assessing mineral bioavailability.
of legumes due to the presence of intestinal phytase which can degrade phytate (anti-nutritional factor of minerals) (Iqbal et al. 1994).

Table 15: The mineral bioavailability of raw legumes using in vivo methods

<table>
<thead>
<tr>
<th>Legume</th>
<th>In vivo method/subject</th>
<th>Bioavailability of minerals (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ca</td>
</tr>
<tr>
<td>Faba bean</td>
<td>Non-isotopic technique, chemical balance, rats</td>
<td>84.6</td>
</tr>
<tr>
<td>Kidney bean</td>
<td>Non-isotopic technique, chemical balance, rats</td>
<td>52.1</td>
</tr>
<tr>
<td>Lentils</td>
<td>Non-isotopic technique, chemical balance, rats</td>
<td>84.4</td>
</tr>
<tr>
<td>Lupin</td>
<td>Non-isotopic technique, chemical balance, rats</td>
<td>39.8</td>
</tr>
<tr>
<td></td>
<td>Isotopic technique, chemical balance, rats</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Non-isotopic technique, chemical balance, rats</td>
<td>80.6</td>
</tr>
<tr>
<td>Pea</td>
<td>Non-isotopic technique, chemical balance, rats</td>
<td>62.6</td>
</tr>
</tbody>
</table>

NA = Not applicable

(Adopted from Alonso et al. 2001; Aranda et al. 2004; Porres et al. 2006; Rubio et al. 1994; Urbano et al. 1999)

The bioavailability of minerals of cooked legumes and products using in vivo methods have been reported (Table 16). Since legumes are usually cooked prior to consumption, some studies reported the mineral bioavailability of cooked legumes instead of raw legumes. Petterson et al. (1994) studied the bioavailability of zinc from lupin (L. angustifolius) incorporated milk, bread and sauce labelling zinc radioisotope using whole body retention technique in humans. The authors found that the bioavailability of zinc from consumption of lupin milk, bread, sauce were 16.2%, 27.0% and 28.2%, respectively.
### Table 16: The mineral bioavailability of cooked legumes and products using *in vivo* methods

<table>
<thead>
<tr>
<th>Legume</th>
<th><em>In vivo</em> method/subject</th>
<th>Detail of the consumed legume</th>
<th>Bioavailability of minerals (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ca</td>
</tr>
<tr>
<td>Chickpeas</td>
<td>Non-isotopic technique,</td>
<td>Cooked</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>rate of repletion, rats</td>
<td>Extruded</td>
<td>NA</td>
</tr>
<tr>
<td>Common beans</td>
<td>Non-isotopic technique,</td>
<td>Water soaking, boiled</td>
<td>84.1</td>
</tr>
<tr>
<td></td>
<td>chemical balance, rats</td>
<td>Acid soaking, boiled</td>
<td>68.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Basic soaking, boiled</td>
<td>85.9</td>
</tr>
<tr>
<td>Lupin</td>
<td>Isotopic technique,</td>
<td>Lupin milk</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>whole body retention,</td>
<td>Lupin bread</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>humans</td>
<td>Lupin incorporated sauce</td>
<td>NA</td>
</tr>
<tr>
<td>Soybean</td>
<td>Isotopic technique,</td>
<td>Low phytate soybean, boiled</td>
<td>41.4</td>
</tr>
<tr>
<td></td>
<td>plasma appearance,</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>humans</td>
<td>High phytate soybean, boiled</td>
<td>31.0</td>
</tr>
</tbody>
</table>

NA = Not applicable

(Adopted from Heany et al. 1991; Nestares et al. 2003; Petterson et al. 1994; Poltronieri et al. 2000)

### 2.3.4 Effect of processing on bioaccessibility of minerals in legumes

The effects of various processing techniques on the bioaccessibility of minerals in legumes using *in vitro* methods have been reported. The discrepant results on the effects of processing on bioaccessibility of legumes have been reported. One of the main reasons for the contrary results is different conditions for processing method, for example, different time/temperature conditions for soaking and cooking (Hotz & Gibson 2007; Jha et al. 2015). Another reason is the variation in genotype, i.e. variety or cultivar, which contribute to the differences of legumes components, especially mineral inhibitors and enhancers.

Many studies reported that soaking legume seeds, such as faba beans, mung beans, soybeans and white beans improved their mineral bioaccessibility (ElMaki et al. 2007;
Grewal & Jood 2006; Kumari et al. 2014; Luo & Xie 2014). Some anti-nutritional factors such as phytate and polyphenols are washed out during soaking (Hotz & Gibson 2007). The levels of phytates and polyphenols were also reduced by endogenous enzymes during soaking (Lorri & Savberg 1995; Sandberg 2002). However, Hemalatha et al. (2007) found a reduction in zinc bioaccessibility of mung beans after soaking seeds.

There are two types of dehulling; wet and dry techniques. Wet dehulling includes a step of soaking seeds to loosen the hull before dehulling. Dry dehulling is done by using a mechanical dehuller without soaking seeds (Tiwari et al. 2011). Several studies have shown that both wet and dry dehulling improve mineral bioaccessibility of various legumes. Grewal and Jood (2006) reported that wet dehulling improves calcium, iron and zinc bioaccessibility of mung beans. The increase in calcium and iron bioaccessibility of pigeon peas after wet dehulling has also been reported (Duhan et al. 2002). Barakoti and Bains (2007) reported that dry dehulling enhanced iron bioaccessibility of mung beans.

Many studies have reported that germination improved bioaccessibility of minerals. Duhan et al. (2002) reported that germination improved calcium and iron bioaccessibility of pigeon peas. Ghavidel & Prakash (2007) reported that germination of legume seeds (chickpea, cowpea, lentil and mung beans) improved their calcium and iron bioaccessibility. Hemalatha et al. (2007) also reported an increase in iron bioaccessibility of germinated chickpea and mung beans. Barakoti and Bains (2007) reported an increase in iron bioaccessibility of mung beans after germination. Kumari et al. (2014) also reported that germination improve calcium, iron and zinc bioaccessibility of soybeans. In contrast, a reduction of zinc bioaccessibility after germination of mung beans has been reported (Hemalatha et al. 2007). Luo et al. (2009) indicated that germination did not affect the iron bioaccessibility of faba beans.

Fermentation has been found to be a processing technique which improves mineral bioaccessibility of grains and legumes (Gupta et al. 2015). The effect of fermentation on mineral bioaccessibility varied considerably, depending on the plant species and conditions of fermentation (Raes et al. 2014). Several studies reported the effect of fermentation on mineral bioaccessibility of legumes. Mamiro et al. (2001) reported
that fermentation enhanced calcium, iron and zinc bioaccessibility of kidney beans. Luo et al. (2009) indicated that fermentation did not impact on the iron bioaccessibility of faba beans. Porres et al. (2003) reported that natural fermentation (without any inoculum) improved iron bioaccessibility, while decreased calcium and zinc bioaccessibility of beans. The authors also found that using a controlled fermentation (using an inoculum of *L. plantarum*) did not affect iron and calcium bioaccessibility, while reduced zinc bioaccessibility of beans.

The effects of heat treatments such as traditional cooking, microwave cooking and pressure cooking on mineral bioaccessibility of legumes have been reported. Duhan et al. (2002) reported increase in calcium and iron bioaccessibility of pigeon peas after ordinary cooking (boiling until seed soften) and pressure cooking (at 1.5 kg/cm² pressure for 5 min). The authors found that ordinary and pressure cooking improved calcium and iron bioaccessibility of pigeon peas. Hemalatha et al. (2007) studied the effect of cooking on iron and zinc bioaccessibility of whole and dehulled legumes. The authors reported that microwave cooking caused the increases in iron bioaccessibility of dehulled black beans, chick peas, mung beans and pigeon peas, while did not affected iron bioaccessibility of dehulled mung beans. Whereas, pressure cooking increased iron bioaccessibility of dehulled mung beans and pigeon peas, but did not affect those of black beans and chick peas. The authors also found that microwave and pressure cooking reduced zinc bioaccessibility of black beans, chick peas, cowpeas, mung beans and pigeon peas.

Hassan et al. (2005) studied the effect of soaking, wet dehulling and cooking on mineral availability of two lupin cultivars, *L. termis* cv. Dongola and Golo. The minerals in samples were extracted using 0.03 M HCl at 37 °C for 3 hrs, and reported as the mineral availability (instead of bioaccessibility). The authors reported increase in iron availability after soaking, cooking and dehulling in Dongola cultivar, while decrease in iron availability in Golo cultivar. The decreases in zinc availability were found in both lupin cultivars after soaking, cooking and dehulling.

Suliburska et al. (2009) studied the effects of dehulling, fermentation and extrusion on iron and zinc bioaccessibility (solubility method) of three lupin species in Poland. The discrepant results on the effect of processing techniques on the mineral bioaccessibility
among lupin species were reported. They found that dehulling improved iron bioaccessibility of *L. luteus* but not *L. albus* and *L. angustifolius*. Dehulling improved zinc bioaccessibility of *L. luteus* and *L. albus* but not *L. angustifolius*. However, the authors did not mention the dehulling method (wet or dry) used in the study. Fermentation improved the iron bioaccessibility of all three lupin species. Fermentation enhanced zinc bioaccessibility in *L. luteus* but not *L. albus* and *L. angustifolius*. Extrusion improved the iron bioaccessibility of *L. albus* and *L. angustifolius* but not *L. luteus*. Extrusion did not improve zinc bioaccessibility of all three lupin species.
3. MATERIALS AND METHODS

3.1 Lupin seeds
Ten cultivars of lupin (*Lupinus angustifolius* L.) seed samples (Belara, Corumup, Gungurru, Jenabillup, Mandelup, PBA Barlock, PBA Gunyidi, Quilinock, Tanjil, and Walan 2385) cultivated in 2011, 2012 and 2013 were obtained from the Department of Agriculture and Food, Western Australia. Walan 2385 cultivar was available only from two cultivation years (2012 and 2013). All lupin cultivars were cultivated in Wongan Hills research station (30.54°S, 116.43°E), Western Australia, Australia.

The lupin seed samples were cleaned using a vacuum separator (Kimseed, Perth, WA, Australia) at medium setting (Figure 13). Immature and damaged seeds were removed manually. All seed samples were packed in polyethylene bags and stored in a refrigerator at 4 °C until further analysis.

Figure 13: Vacuum separator
3.2 Processing

3.2.1 Dehulling

Lupin seeds were dehulled using dry dehulling technique by a dehuller (Amar industries, Punjab, India) (Figure 14). After dehulling, kernels and hulls were separated using a vacuum separator at medium setting (Kimseed, Perth, WA, Australia).

![Dehuller](image)

**Figure 14: Dehuller**

The dehuller was thoroughly cleaned using a vacuum cleaner between dehulling of different samples to minimise cross contamination. After separating hulls and kernels by the vacuum separator, the undehulled seeds and hulls were manually separated to ensure samples are free from hulls. Figure 15 shows a lupin seed sample after dehulling.

![Lupin seed sample after dehulling](image)

**Figure 15: Lupin seed sample after dehulling**
3.2.2 Milling
Both whole seed and dehulled lupin samples were milled using a sample mill (1090 Cemotec, Foss Tecator AB, Hoganas, Sweden) at the finest setting (Figure 16). The samples were poured into the feeder of the mill at a rate of around 75 g/min. The mill was thoroughly cleaned using a vacuum cleaner between milling of different samples to minimise cross contamination. All milled samples were packed in polyethylene bags and stored in a freezer at $-18 \pm 2$ °C until further analysis.

![Sample mill](image)

Figure 16: Sample mill

3.2.3 Heat treatment
It is a common practice that lupin seeds or flour undergoes cooking process prior to consumption. Therefore, the mineral bioaccessibility was assessed in cooked lupin. Heat treatment of milled lupin samples was conducted according to Sirtori et al. (2010). Milled whole lupin or milled dehulled lupin sample (50 g) was placed in a 500 ml glass beaker and mixed with deionized water at a ratio of 1:2. The mixture was placed in a water bath (SWB20, Ratek, Melbourne, VIC, Australia) at 95 °C for 15 min. Each sample was cooled to room temperature and lyophilised in a freeze dryer (Alpha 1-2 LD plus, John Morris Scientific, Sydney, NSW, Australia). All lyophilised samples were packed in polyethylene bags and stored in a freezer at $-18 \pm 2$ °C until further analysis.
3.3 Determination of physical properties

3.3.1 Colour measurement

Only dehulled lupin samples were used for the colour measurement. The colours of the samples were measured using a spectrophotometer (CM-508i, Minolta Co. Ltd., Osaka, Japan) which employs the CIELAB colour system ($L^* a^* b^*$). The parameter $L^*$ represents to brightness with a range value of 0–100 (black to white). The positive values of $a^*$ contribute to red colours and negative values represent to the green ones ($−120$ to $120$). The $b^*$ positive values represent to yellow colours and negative values for the blue colours with a range value of $−120$ to $120$ (Pathare et al. 2013). The instrument was calibrated using the white-coloured disc supplied with the instrument prior to measurement. The milled sample was put into a clear plastic dish with 2.5 cm diameter and 1 cm height. The $L^*$, $a^*$ and $b^*$ values were measured in duplicate. Total colour difference values ($\Delta E^*$) of samples compared to white colour standard were calculated using the equation 3.1 (Pathare et al. 2013).

\[
\Delta E^* = \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2}
\]  
(Eq. 3.1)

Where:
$L_0^*$, $a_0^*$, $b_0^*$ are reference values of white colour standard ($L_0^* = 97.64$, $a_0^* = −0.29$, $b_0^* = −0.23$)
$L^*$, $a^*$, and $b^*$ are the colour parameter values of dehulled lupin samples

3.3.2 Particle size analysis

Only dehulled lupin samples were used for the particle size analysis. A laser diffraction particle size analyser (Mastersizer 2000, Malvern instruments Ltd., Worcestershire, UK) equipped with sample cell (Hydro 2000S) was used to determine the particle size of the samples. Water was used as a dispersant and obscuration was set between 0.10 and 0.20. Median particle size ($d(0.5)$) of lupin sample was measured. Analysis was performed in duplicate.
3.4 Chemicals analysis

3.4.1 Moisture
Moisture content was determined using AOAC method 925.10 (AOAC 2005). A metal dish (55 mm diameter and 15 mm height) was dried at 105°C and cooled in an air-tight desiccator with silica gel as a drying agent. The metal dish was weighed soon after reaching room temperature and the weight was recorded as W0. Sample (approximately 2 g) was weighed accurately in the metal dish and recorded as W1. The metal dish with the sample was dried at 105°C in a ventilated drying oven (Contherm digital series, Contherm Scientific Ltd., Wellington, New Zealand) to constant weight. Then, the metal dish was closed with the cover (that was previously dried) and transferred to a desiccator to cool down to room temperature. The metal dish with dried sample was weighed soon after reaching room temperature and recorded as W2. Moisture content was calculated using the equation 3.2.

\[
\text{Moisture content (\%)} = \frac{(W_1 - W_2)}{(W_1 - W_0)} \times 100 \quad \text{(Eq. 3.2)}
\]

Where:
- \( W_0 \): Weight of empty dish (g)
- \( W_1 \): Weight of dish and sample before drying (g)
- \( W_2 \): Weight of dish and sample after drying (g)

3.4.2 Ash
Ash content was determined using AOAC method 923.03 (AOAC 2005). Empty crucible (35 mL) was placed in a muffle furnace (Thermolyne 48000, Thermo Scientific, Waltham, MA, USA) at 525 °C (until weight is constant), cooled in a desiccator, weighed soon after reaching room temperature, and record as W0. Sample (approximately 1 g) was weighed accurately in a crucible and recorded as W1. Crucible with sample was placed in the muffle furnace at 525 °C until white ash results which means free from carbon. The crucible was transferred to a desiccator and weighed soon after reaching room temperature and recorded as W2. Ash content was calculated using the equation 3.3.

\[
\text{Ash content (\%)} = \frac{(W_1 - W_2)}{(W_1 - W_0)} \times 100 \quad \text{(Eq. 3.3)}
\]
Where:

\[ W_0 = \text{Weight of empty crucible (g)} \]
\[ W_1 = \text{Weight of crucible and sample before burning (g)} \]
\[ W_2 = \text{Weight of crucible and sample after burning (g)} \]

### 3.4.3 Minerals

Calcium, iron, magnesium, potassium and zinc contents were determined using AOAC method 985.35 (AOAC 2005). To prevent the minerals contaminations, all glasswares were cleaned, soaked in 10% HNO₃ (v/v) overnight, rinsed three times with deionised water and dried before use. Standard solution 1000 µg/mL of calcium, iron, magnesium, potassium and zinc were purchased from Fisher Scientific (Leicestershire, UK). Nitric acid and lanthanum chloride were purchased from Ajax Finechem (Sydney, NSW, Australia).

Sample (1 g) was weighed accurately in a crucible which was previously dried and cooled in a desiccator. Sample was dried on the hot plate (Thermoline Scientific, Sydney, NSW, Australia) until smoking ceased. Then, sample was placed in the muffle furnace (Thermolyne 48000, Waltham, MA, USA) at 525°C until white ash results (free from carbon). The crucible was transferred to a desiccator for cooling down to room temperature. If the colour of ash was grey, 3 mL of 1 M HNO₃ was added, dried on the hot plate and placed in the muffle furnace at 525°C for 2 h. Ash was dissolved in 1 M HNO₃ and the solution was transferred into a 25-mL volumetric flask. The volume was made up to 25 mL with 1 M HNO₃. Flame Atomic Absorption Spectrometer (AvantaΣ, GBC Scientific Equipment Pty Ltd, Melbourne, VIC, Australia) was used to determined calcium, iron, magnesium, potassium and zinc in the sample.

Operating parameters for flame atomic absorption spectrometer used for minerals determination are shown in Table 17. Calibration curves were plotted for each mineral based on the correlations between absorption and concentration. Working standards of each mineral were prepared by serial dilutions of the stock standard (1000 µg/mL) with 1% HNO₃. Concentrations of individual mineral used to prepare a calibration curve were as follow:
1) Calcium (Ca): 5, 10, 20, 30 and 50 µg/mL
2) Iron (Fe): 1, 2, 3, 4 and 5 µg/mL
3) Magnesium (Mg): 5, 10, 15, 20 and 25 µg/mL
4) Potassium (K): 10, 20, 30, 40 and 50 µg/mL
5) Zinc (Zn): 0.4, 0.6, 0.8, 1.0 and 1.5 µg/mL

Table 17: Operating parameters for flame atomic absorption spectrometer

<table>
<thead>
<tr>
<th>Mineral</th>
<th>Wavelength, (nm)</th>
<th>Slit width, (nm)</th>
<th>Flame</th>
<th>Range, µg/mL</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca</td>
<td>422.7</td>
<td>0.5</td>
<td>N₂O - C₂H₂</td>
<td>5–50</td>
<td>Add 0.1% LaCl₃</td>
</tr>
<tr>
<td>Fe</td>
<td>248.3</td>
<td>0.2</td>
<td>Air - C₂H₂</td>
<td>1–5</td>
<td>-</td>
</tr>
<tr>
<td>K</td>
<td>769.9</td>
<td>0.5</td>
<td>Air - C₂H₂</td>
<td>10–50</td>
<td>Add 0.1% LaCl₃</td>
</tr>
<tr>
<td>Mg</td>
<td>202.6</td>
<td>1.0</td>
<td>Air - C₂H₂</td>
<td>5–25</td>
<td>Add 0.1% LaCl₃</td>
</tr>
<tr>
<td>Zn</td>
<td>213.9</td>
<td>0.5</td>
<td>Air - C₂H₂</td>
<td>0.4–1.5</td>
<td>-</td>
</tr>
</tbody>
</table>

Individual mineral contents (µg/g) were calculated using the equation 3.4.

\[
\text{Mineral content} = \frac{X \times DF \times V}{W} \quad \text{(Eq. 3.4)}
\]

Where:
- \( X \) = Concentration of mineral calculated from calibration curve (µg/mL)
- \( DF \) = Dilution factor
- \( V \) = Final volume (mL)
- \( W \) = Sample weight (g)

3.4.4 Raffinose family oligosaccharides (RFOs)

3.4.4.1 Reagents and standard solutions

Analytical grade D-(+)-raffinose pentahydrate (≥ 98.0%), stachyose hydrate from *Stachys tuberifera* (≥ 98.0%), verbascose (≥ 97.0%) and acetonitrile were purchased from Sigma-Aldrich (Sydney, NSW, Australia). Ultrapure water was prepared by a filtration system with a resistivity of 18.2 MΩcm (Millipore Corporation, Bedford, MA, USA). Molisch’s reagent was prepared by dissolving 0.5 g of α-naphthol (Sigma-Aldrich, Sydney, NSW, Australia) in 10 mL of 95% ethanol (Thermo Scientific, ...
Sydney, NSW, Australia). Carrez I reagent was prepared by dissolving 21.9 g zinc acetate (Asia Pacific Speciality (APS) Chemical, Sydney, NSW, Australia) and 3 g glacial acetic acid (Thermo Scientific, Sydney, NSW, Australia), and made up to 100 mL with water in a volumetric flask. Carrez II reagent was prepared by dissolving 10.6 g potassium ferrocyanide (Ajax Finechem, Sydney, NSW, Australia) in 100 mL water. Stock standard solutions of raffinose, stachyose and verbascose at concentrations of 1000 µg/mL were prepared in ultrapure water and stored at 4 °C in a refrigerator. Standard solutions of 100, 200, 300, 400 and 500 µg/mL concentration were prepared by appropriate dilution of the stock standard solutions in ultrapure water. All standard solutions were filtered through a 0.45 µm Millipore membrane filter.

3.4.4.2 Determination
Raffinose, stachyose and verbascose contents were determined following the sample preparation process described by Giannoccaro et al. (2008) with some modifications. Each lupin sample (0.1 g) was mixed with 4 mL of ultrapure water and shaken in a shaking water bath (SWB20, Ratek, Melbourne, VIC, Australia) at 50 °C for 1 h. The solution was then centrifuged (5810R, Eppendorf AG, Hamburg, Germany) at 4000 rpm for 10 min. The residue was re-extracted until the Molisch reaction test (standard method to determine the presence of carbohydrate) showed a negative result (took 2–3 extractions). The supernatants from each extraction were combined in a 50-mL centrifuge tube. Protein precipitation was conducted by adding 100 µL of Carrez I reagent and mixing by vortex for 1 min followed by adding 100 µL of Carrez II reagent and mixing by vortex for 1 min. The volume was made up to 15 mL with water. The tube was allowed to stand for 30 min to complete the protein precipitation. The solution was then centrifuged at 4000 rpm for 15 min at 4°C to minimise protein degradation. The supernatant was collected and filtered through 0.45 µm Millipore membrane filter.

High-performance liquid chromatography with evaporative light scattering detector (HPLC-ELSD) was used for RFOs determination. The conditions of HPLC-ELSD followed the methods described by Shanmugavelan et al. (2013) with some modifications. Hewlett Packard Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) with a degasser (G1322A), a quaternary pump (G1311A), and
a Rheodyne model 7000 sample injector was used for the analysis. The detector was an evaporative light scattering detector (ELSD 2000ES, Grace Davison Discovery Sciences, Deerfield, IL, USA). Data processing was carried out with a Chemstation software (Agilent Technologies, Santa Clara, CA, USA). Chromatography was performed on a Prevail carbohydrate column; 5 µm, 250 x 4.6 mm (Grace Davison Discovery Sciences, Deerfield, IL, USA). Under isocratic elution, acetonitrile: ultrapure water (55:45 v/v) was used as the mobile phase at a flow rate of 0.8 mL/min. ELSD used nitrogen as nebulizing gas at a flow rate 2.0 L/min, and the temperature of drift tube was fixed at 40 °C. Injection volumes were 20 µL at ambient temperature. The total runtime of the HPLC analysis was 20 min. Calibration curves were plotted for each RFO based on the correlations between the logarithm of peak areas and the logarithm of concentrations. RFO content (µg/g) was calculated using the equation 3.5.

\[
\text{RFO} = \frac{X \times DF \times V}{W} \quad \text{(Eq. 3.5)}
\]

Where:
- \(X\) = Concentration of RFO calculated from calibration curve (µg/mL)
- \(DF\) = Dilution factor
- \(V\) = Final volume (mL)
- \(W\) = Sample weight (g)

### 3.4.5 Phytate

#### 3.4.5.1 Reagent and standard solutions

Phytic acid sodium salt hydrate from rice and sulfosalicylic acid were purchased from Sigma-Aldrich (Sydney, NSW, Australia). AG1-X8 anion exchange resin 200–400 mesh was purchased from Bio-Rad Laboratories (Sydney, NSW, Australia). Iron (III) chloride hexahydrate was bought from Thermo Fisher Scientific (Melbourne, VIC, Australia). Hydrochloric acid and Sodium chloride were purchased from Ajax Finechem (Sydney, NSW, Australia).
3.4.5.2 Determination

Phytate was determined using a colourimetric method, described by Fruhbeck, et al. (1995) with slight modifications. Sample (0.4 g) was extracted with 8 mL of 2.4% HCl for 1 h at room temperature in an ultrasonic bath (Type PXA, Unisonics, Sydney, NSW, Australia). The mixture was centrifuged (5810R, Eppendorf AG, Hamburg, Germany) at 4000 rpm for 20 min at 20°C and the supernatant was collected. Two millilitres of supernatant was diluted with deionised water and pH was adjusted to 6 with 1 M NaOH, then the final volume was adjusted to 10 mL (1:5 dilution) in a volumetric flask. The aliquot (10 mL) was passed through a 200–400 mesh AG1-X8 anion exchange column (0.5 g). Then the column was eluted with 15 mL of 0.1 M NaCl to remove inorganic phosphorous and other interfering compounds, and the eluate was discarded. Phytate was eluted with 15 mL of 0.7 M NaCl and the eluate was collected. Three millilitres of the eluate or deionised water (blank) or phytate standard solutions (20, 40, 60, 80, 100 µg/mL) were pipetted into a 15-mL amber centrifuge tube. Then, 50 µL of 0.2% HCl was added to adjust pH of the solution between 3.0–3.5. One millilitre of wade reagent (0.03% FeCl₃.6H₂O and 0.3% sulfosalicylic acid in deionised water) was added to each tube and mixed. All solutions were then centrifuged at 4000 rpm for 5 min at 15°C. The absorbance was measured using a UV-VIS spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan) at 500 nm. Phytate content (µg/g) was calculated using the equation 3.6.

\[
\text{Phytate} = \frac{X \times \text{DF} \times V}{W} \quad \text{(Eq. 3.6)}
\]

Where:

- \(X\) = Concentration of phytate calculated from calibration curve (µg/mL)
- \(\text{DF}\) = Dilution factor
- \(V\) = Final volume (mL)
- \(W\) = Sample weight (g)
3.4.6 Total phenolics, total flavonoids and condensed tannins

3.4.6.1 Reagents and standard solutions

Catechin hydrate, gallic acid, Folin-Ciocalteu reagent, formic acid and vanillin were purchased from Sigma-Aldrich (Sydney, NSW, Australia). Ethanol, methanol, sodium carbonate (NaCO₃) and sodium hydroxide (NaOH) were purchased from Thermo Scientific (Sydney, NSW, Australia). Sodium nitrite (NaNO₂), Aluminum chloride hexahydrate (AlCl₃.6H₂O) and hydrochloric acid (HCl) were purchased from Ajax Finechem (Sydney, NSW, Australia). Standard solutions of gallic acid were prepared for total phenolic compound analysis; stock standard solution 1000 µg/mL, and working standard solutions 25, 50, 75, 100 and 125 µg/mL were prepared. Standard solutions of catechin hydrate were prepared for total flavonoid and condensed tannin analysis. Stock standard solutions of catechin hydrate and gallic acid at 1000 µg/mL were prepared. Working standard solutions of 20, 30, 40, 50 and 100 µg/mL were prepared for calibration curve of total flavonoid determination. Working standard solutions of 20, 30, 40, 50 and 100 µg/mL were prepared for calibration curve of condensed tannin determination. Methanol vanillin solution (4%) was prepared freshly by dissolving 2 g vanillin in a 50-mL volumetric flask and the volume was made up with methanol.

3.4.6.2 Sample extraction

Sample extraction for determination of total phenolics, total flavonoids and condensed tannins followed the method of Heimler et al. (2005) with slight modifications. Sample (0.2 g) was weighed accurately into a 10-mL centrifuge tube. Then, 4 mL of 80% methanol was added and pH was adjusted to 2.0 with formic acid. The sample was then placed in an ultrasonic bath (Unisonics, Type PXA, Sydney, NSW, Australia) at room temperature for 2 h. The extracted solution was centrifuged (5810R, Eppendorf AG, Hamburg, Germany) at 4000 rpm for 15 min at room temperature and the supernatant was collected. The volume was adjusted to 5 mL with 80% methanol.

3.4.6.3 Total phenolics

The extracted solution (250 µL) or working standard solutions of gallic acid were mixed with 4 mL of deionised water, 250 µL of Folin-Ciocalteu reagent (two-fold dilution) and 0.5 mL of saturated Na₂CO₃ (7%). The mixture was allowed to stand for
25 min at room temperature. The absorbance was measured at 760 nm in a UV-VIS spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan) and deionised water was used as a blank. The total phenolic compounds content was expressed as gallic acid equivalent (µg GAE/g) through the calibration curve of gallic acid (Heimler et al. 2005). Total phenolic content (µg GAE/g) was calculated using the equation 3.7.

\[
\text{Total phenolics} = \frac{X \times DF \times V}{W}
\]  

(Eq. 3.7)

Where:

- \(X\) = Concentration of total phenolics calculated from calibration curve (µg GAE/mL)
- \(DF\) = Dilution factor
- \(V\) = Final volume (mL)
- \(W\) = Sample weight (g)

### 3.4.6.4 Total flavonoids

The extracted solution (500 µL) or working standard solutions of catechin hydrate was added to an amber test tube. Then, 75 µL of NaNO₂, 150 µL of 10% AlCl₃ (freshly prepare), 500 µL of 1 M NaOH, 1,525 µL of deionised water and 500 µL of saturated Na₂CO₃ (7%) were added respectively. The mixture was allowed to stand for 5 min at room temperature. The absorbance was measured at 510 nm in a UV-VIS spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan) and deionised water was used as a blank. The amount of total flavonoid content is expressed as catechin equivalent (µg CE/g) through the calibration curve of catechin (Heimler et al. 2005). Total flavonoid content (µg CE/g) was calculated using the equation 3.8.

\[
\text{Total flavonoid} = \frac{X \times DF \times V}{W}
\]  

(Eq. 3.8)

Where:

- \(X\) = Concentration of total flavonoids calculated from calibration curve (µg CE/mL)
- \(DF\) = Dilution factor
- \(V\) = Final volume (mL)
- \(W\) = Sample weight (g)
3.4.6.5 Condensed tannins
The extracted solution (0.5 mL) or working standard solutions of catechin hydrate was added to an amber test tube. Then, 3 mL of methanol vanillin solution (4 %) and 1.5 mL of concentrated hydrochloric acid were added. The mixture was allowed to stand for 15 min at room temperature. The absorbance was measured at 500 nm in a UV-VIS spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan) and methanol was used as a blank. The amount of condensed tannins is expressed as catechin equivalents (µg CE/g) through the calibration curve of catechin (Heimler et al. 2005). Condensed tannin content (µg CE/g) was calculated using the equation 3.9.

\[
\text{Condensed tannins} = \frac{X \times DF \times V}{W} \quad \text{(Eq. 3.9)}
\]

Where:
- \( X \) = Concentration of condensed tannins calculated from calibration curve (µg CE/mL)
- \( DF \) = Dilution factor
- \( V \) = Final volume (mL)
- \( W \) = Sample weight (g)

3.4.7 Method validation
Method validations of minerals, raffinose family oligosaccharides, phytate, total phenolic, total flavonoid and condensed tannin contents determination were conducted. Typical method validation of accuracy, repeatability and calibration were performed to confirm the suitability of the methods used in this study.

3.4.7.1 Accuracy (Recovery)
According to the International Accreditation Service (IAS), accuracy is defined as “the closeness of agreement between a measured quantity value and a true quantity value of a measurand” (IAS 2015). A common method in food analysis for determining of accuracy is the recovery test. A recovery test was performed in every sample batch analysis by measuring an analyte in a sample and a spiked sample (a sample with a known amount of a standard solution added). Recovery as a percentage was calculated using the equation 3.10.
Recovery (%) = \frac{\text{amount of analyte in a spiked sample} - \text{amount of analyte in a sample}}{\text{amount of analyte added}} \times 100 \quad \text{(Eq. 3.10)}

The acceptance criteria of percentage of recovery defined by the Australian Pesticides and Veterinary Medicines Authority (APVMA 2004) are shown in Table 18. Acceptance criteria of percentage of recovery depend on the concentration of analyte. Based on the average contents of the analytes found in the present study, the acceptance criteria for percentage of recovery were 90–110% for RFOs, 80–120% for calcium, magnesium, potassium and phytate, and 75–125% for iron, zinc and phenolic compounds (total phenolics, total flavonoids and condensed tannins).

<table>
<thead>
<tr>
<th>Analyte content (%)</th>
<th>Acceptable recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 10</td>
<td>98–102</td>
</tr>
<tr>
<td>&gt; 1–10</td>
<td>90–110</td>
</tr>
<tr>
<td>0.1–1</td>
<td>80–120</td>
</tr>
<tr>
<td>&lt; 0.1</td>
<td>75–125</td>
</tr>
</tbody>
</table>

(Adapted from APVMA 2004)

3.4.7.2 Repeatability (Precision)

Repeatability is defined as “the closeness of agreement between mutually independent test results obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time” (IAS 2015). Repeatability was measured by determinations of six replicates of an identical analyte. An average and standard deviation of the analyte content were calculated. The relative standard deviation (RSD) is commonly used to represent repeatability, and was calculated using the equation 3.11.

\[
\text{RSD} \% = \frac{\text{Standard deviation}}{\text{Average}} \times 100 \quad \text{(Eq. 3.11)}
\]

Acceptance criteria of RSD depend on the concentration of analyte as shown in Table 19. Based on the average contents of the analytes, the acceptance criteria were 5% for RFOs, 10% for calcium, magnesium, potassium and phytate, and 20% for iron, zinc and phenolic compounds (total phenolics, total flavonoids and condensed tannins).
Table 19: Acceptance criteria of precision

<table>
<thead>
<tr>
<th>Analyte content (%)</th>
<th>Acceptable RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 10</td>
<td>2</td>
</tr>
<tr>
<td>&gt; 1–10</td>
<td>5</td>
</tr>
<tr>
<td>0.1–1</td>
<td>10</td>
</tr>
<tr>
<td>&lt; 0.1</td>
<td>20</td>
</tr>
</tbody>
</table>

(Adapted from APVMA 2004)

3.4.7.3 Calibration

Calibration is referred to “the ability of a detection system to produce an acceptable, well defined, correlation between the instrumental response and the concentration of the analyte in the sample” (IAS 2015). When instrumental responses and analyte concentrations demonstrate linear correlation, the linear regression analysis is performed. However, some analytical detectors such as ELSD do not demonstrate linear correlation; therefore, mathematical transformation and/or non-linear regression analysis need to be applied (USFDA 1996). To determine the calibration of analysis, five concentrations of an analyte were used, and regression analysis was performed in every sample batch analysis. The coefficient of determination \( R^2 \) from regression analysis (linear or non-linear) was calculated. The acceptable criterion of calibration used in the study was \( R^2 > 0.98 \) (IAS 2015).

3.5 Bioaccessibility of minerals

For the determinations of bioaccessibility of minerals, heat treated lupin samples were used. Bioaccessibility of calcium, iron and zinc in lupin samples were determined using a dialysability method described by Cámara et al. (2005).

3.5.1 Reagents

Pepsin and pancreatin were purchased from Chem-supply (Adelaide, SA, Australia). Bile salt was purchased from Sigma-Aldrich (Sydney, NSW, Australia). Sodium bicarbonate (\( \text{NaHCO}_3 \)) and sodium hydroxide (\( \text{NaOH} \)) were purchased from Thermo Fisher Scientific (Melbourne, VIC, Australia). Hydrochloric acid (\( \text{HCl} \)) was purchased from Ajax Finechem (Sydney, NSW, Australia). The dialysis tubing (SnakeSkinTM,
Thermo Fisher Scientific, Melbourne, VIC, Australia) with molecular weight cutoff (MWCO) of 10,000 Dalton was used. Pepsin solution was prepared freshly by dissolving 16 g of pepsin in 100 mL of 0.1 M HCl. Pancreatin-bile salt solution was prepared freshly by dissolving 0.4 g pancreatin and 2.5 g of bile salt in 100 mL of 0.1 M NaHCO$_3$.

3.5.2 Determination

3.5.2.1 Gastric stage

Sample (10 g) was homogenised with 80 g of deionised water, and the pH was adjusted to 2.0 with 6 M HCl. Freshly prepared pepsin solution (3 g) was added, and the mixture was made up to 100 g with deionised water. Then the mixture was incubated at 37 °C for 2 h in a shaking water bath (SWB20, Ratek, Melbourne, VIC, Australia) setting at 100–120 strokes/min. After incubation, the gastric digest mixture was kept in an ice bath until ready to conduct an intestinal stage (Cámara et al. 2005).

3.5.2.2 Titratable acidity

Freshly prepared pancreatin – bile salt mixture (5 g) was added to the gastric digest mixture (20 g). The pH was adjusted to 7.5 with 0.5 M NaOH. After 30 min, the pH was checked and readjusted to 7.5. Titratable acidity was defined as the amount of 0.5 M NaOH required titrating the gastric digest and pancreatin-bile salt mixture to pH 7.5. The amount of NaOH in this titratable acidity was used to calculate the amount of NaHCO$_3$ (mole equivalent) for preparing dialysis tubing (Cámara et al. 2005).

3.5.2.3 Intestinal stage

Gastric digest mixture (20 g) was weighed into a wide-necked 250-mL Erlenmeyer flask. Dialysis tubing contained 25 g deionised water and NaHCO$_3$ (mole equivalent to NaOH used in the titratable acidity) was put in the flask. The mixture was incubated at 37 °C for 30 min in a shaking water bath (SWB20, Ratek, Melbourne, VIC, Australia). Then pancreatin-bile salt mixture (5 g) was added, and the mixture was incubated at 37 °C for 2 h. After incubation, the dialysis tubing was removed from the flasks, washed by deionised water, dried by paper towel and weighed. The content of dialysis tubing was analysed for mineral levels by flame atomic absorption spectrophotometry (AvantaΣ, GBC Scientific Equipment Pty Ltd, Melbourne, VIC,
Australia) as described in the section 3.4.3. Bioaccessibility (%) of mineral was calculated using the equation 3.10 (Cámara et al. 2005).

\[
\text{Bioaccessibility} = \frac{100 \times D}{C}
\]  
(Eq. 3.10)

Where:

\(D\) = Dialysed mineral content (µg/g)

\(C\) = Total mineral content (µg/g).

3.6 Phytate to mineral molar ratio as a predictor of mineral bioavailability

The molar of phytate was calculated by dividing the phytate content (mg/100 g DM) by its molecular weight (660.04 g/mol). The molar of each mineral was obtained by dividing the mineral concentration (mg/100 g DM) by atomic weight (calcium 40.08 g/mol, iron 55.85 g/mol and zinc 65.38 g/mol). The molar ratio of phytate to mineral was obtained by dividing the molar of phytate with the molar of mineral (Ma et al. 2005).

3.7 Statistical analysis

All determination was carried out in duplicate. Statistical tests were conducted using the SPSS statistical analysis software program version 22 (IBM Corporation, Armonk, NY, USA). One-way analysis of variance (ANOVA) with Tukey’s post-hoc test \((P < 0.05)\) was used to determine the effects of cultivar and cultivation year on analyte content. Paired sample t-test \((P < 0.05)\) was used to compare analyte content between whole seed and dehulled lupin. Correlations between mineral bioaccessibility, and minerals and anti-nutritional factors were estimated using a stepwise multiple regression.
4. RESULTS AND DISCUSSIONS

4.1 Physical properties

4.1.1 Colour

Colour scores of dehulled lupin of different cultivars are shown in Table 20. The average scores of brightness ($L*$), redness ($a*$), yellowness ($b*$) and total colour difference compared to white colour standard ($\Delta E*$) were 84.57, 5.28, 36.81 and 39.67, respectively. There were significant differences in brightness and redness between lupin cultivars. However, there was no significant difference in yellowness and total colour difference between lupin cultivars. Oomah et al. (2006) reported that flour of eight lupin cultivars ($L$. angustifolius grown in Canada) had average scores of brightness (90.29), redness (2.35) and yellowness (19.12). Oomah et al. (2006) found that cultivar had a significant effect on individual colour score of lupin.

Table 20: Colour of milled dehulled lupin

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Brightness ($L*$)</th>
<th>Redness ($a*$)</th>
<th>Yellowness ($b*$)</th>
<th>Total colour difference ($\Delta E*$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belara</td>
<td>84.70 ± 0.44$^a_b$</td>
<td>5.61 ± 0.29$^a_b$</td>
<td>36.14 ± 0.98$^a$</td>
<td>39.05 ± 1.05$^a$</td>
</tr>
<tr>
<td>Corumup</td>
<td>84.37 ± 0.79$^a_b$</td>
<td>5.77 ± 0.83$^a_b$</td>
<td>37.49 ± 3.44$^a$</td>
<td>40.45 ± 3.58$^a$</td>
</tr>
<tr>
<td>Gungurru</td>
<td>84.72 ± 1.07$^a_b$</td>
<td>4.48 ± 0.72$^b$</td>
<td>37.02 ± 2.10$^a$</td>
<td>39.72 ± 2.40$^a$</td>
</tr>
<tr>
<td>Jenabillup</td>
<td>85.02 ± 0.75$^a$</td>
<td>4.66 ± 0.95$^a_b$</td>
<td>35.41 ± 4.01$^a$</td>
<td>38.14 ± 4.11$^a$</td>
</tr>
<tr>
<td>Mandelup</td>
<td>83.29 ± 0.91$^b$</td>
<td>5.84 ± 0.76$^a_b$</td>
<td>38.79 ± 3.00$^a$</td>
<td>42.03 ± 3.19$^a$</td>
</tr>
<tr>
<td>PBA Barlock</td>
<td>84.13 ± 1.06$^a_b$</td>
<td>5.28 ± 1.02$^a_b$</td>
<td>37.24 ± 3.36$^a$</td>
<td>40.22 ± 3.63$^a$</td>
</tr>
<tr>
<td>PBA Gunyidi</td>
<td>84.04 ± 1.16$^a_b$</td>
<td>5.91 ± 0.54$^a$</td>
<td>37.79 ± 4.15$^a$</td>
<td>40.86 ± 4.32$^a$</td>
</tr>
<tr>
<td>Quilinock</td>
<td>85.61 ± 0.84$^a$</td>
<td>4.87 ± 0.84$^a$</td>
<td>35.08 ± 3.69$^a$</td>
<td>37.66 ± 3.84$^a$</td>
</tr>
<tr>
<td>Tanjil</td>
<td>84.87 ± 0.73$^a_b$</td>
<td>5.41 ± 0.28$^a_b$</td>
<td>37.23 ± 3.11$^a$</td>
<td>39.99 ± 3.14$^a$</td>
</tr>
<tr>
<td>Walan 2385</td>
<td>85.19 ± 0.77$^a$</td>
<td>4.81 ± 0.72$^a_b$</td>
<td>35.41 ± 0.85$^a$</td>
<td>38.10 ± 1.15$^a$</td>
</tr>
<tr>
<td>Average</td>
<td>84.57 ± 1.03</td>
<td>5.28 ± 0.84</td>
<td>36.81 ± 3.10</td>
<td>39.67 ± 3.29</td>
</tr>
</tbody>
</table>

For mean ± SD, N = 6 except Walan 2385 N = 4. Mean values with different superscript in the same column are significantly different ($P < 0.05$).
4.1.2 Particle size

Table 21 shows the median particle sizes of dehulled lupin samples of different cultivars. The milling technique used in the present study resulted in the average median particle size of dehulled lupin samples to be 332 µm and ranged from 206–421 µm. There was no significant difference in median particle size between lupin cultivars except Quilinock had significantly higher median particle size than Gungurru and PBA Barlock. This could be an indication that seeds of Quilinock are harder than the seeds of Gungurru and PBA Barlock.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Median particle size (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belara</td>
<td>323.61 ± 173.78&lt;sup&gt;ac&lt;/sup&gt;</td>
</tr>
<tr>
<td>Corumup</td>
<td>318.55 ± 92.21&lt;sup&gt;ac&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gungurru</td>
<td>205.74 ± 89.39&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Jenabillup</td>
<td>295.28 ± 18.30&lt;sup&gt;ac&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mandelup</td>
<td>401.24 ± 35.17&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>PBA Barlock</td>
<td>236.52 ± 40.12&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>PBA Gunyidi</td>
<td>415.61 ± 169.68&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Quilinock</td>
<td>421.31 ± 69.30&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tanjil</td>
<td>356.02 ± 32.73&lt;sup&gt;ac&lt;/sup&gt;</td>
</tr>
<tr>
<td>Walan 2385</td>
<td>358.30 ± 34.97&lt;sup&gt;ac&lt;/sup&gt;</td>
</tr>
<tr>
<td>Average</td>
<td>332.35 ± 111.69</td>
</tr>
<tr>
<td>Range</td>
<td>205.74–421.31</td>
</tr>
</tbody>
</table>

For mean ± SD, N = 6 except Walan 2385 N = 4. Median values with different superscript are significantly different ($P < 0.05$).

Lupin has high fibre content and negligible amount of starch which could make the seeds difficult to be milled into small particles. Majzoobi et al. (2014) reported that wheat bran fraction with higher particle size contain higher fibre content. Kerr et al. (2000) reported that starch content relates to the particle size of cowpea and higher starch content resulted in smaller particle sizes.
All dehulled lupin samples showed trimodal distributions. The particle size distribution of Belara cultivar grown in 2012 is presented in Figure 17 as an example. The multimodal particle size distribution of grains could be due to the heterogeneous structure of the tissues composing of different contents of protein, fibre, starch, etc. Only a few studies on particle size distribution of lupin have been published. Villarino et al. (2015) reported that Belara, Coromup, Jenabillup and Mandelup flour samples had trimodal distributions which are similar to the results of present study. However, the authors found that flour of Gungurru and Tanjil had bimodal distributions. Particle size distributions of other grain legumes such as cowpea, faba bean and pea were bimodal distributions (Kerr et al. 2000; Petitot et al. 2010).

**Figure 17: Particle size distribution of dehulled lupin**

The fractions of milled lupin sample were classified into flour, fine, medium and coarse using the same classification used for soybean flour and grits as defined by Berk (1992).

1) Flour: Particle size less than 180 µm or pass through sieve no. 100.
2) Fine: Particle sizes between 180–420 µm or pass through sieve no. 80
3) Medium: Particle sizes between 420–840 µm or pass through sieve no. 40
4) Coarse: Particle sizes greater than 840 µm or pass through sieve no. 20

The percentage of flour, fine, medium and coarse particles of different lupin cultivars are presented in Table 22. The milling technique used in the present study resulted in 40% flour, 14% of fine particles, 22% of medium particles and 24% of coarse particles. There were no significant differences in percentage of flour between lupin cultivars...
except between Gungurru and PBA Gunyidi. Belara had a significantly higher percentage of fine particles than those of PBA Barlock, PBA Gunyidi, Quilinock and Tanjil. PBA Gunyidi had a significantly higher percentage of medium particles than those of Gungurru and PBA Barlock. Percentage of coarse particle of PBA Gunyidi was significantly higher than those of Gungurru and Belara.

Table 22: Percentage of flour, fine, medium and coarse particle of lupin

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Flour</th>
<th>Fine</th>
<th>Medium</th>
<th>Coarse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belara</td>
<td>38.81 ± 10.04&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>17.62 ± 4.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.25 ± 4.58&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>20.32 ± 7.60&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Corumup</td>
<td>40.27 ± 6.79&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>14.47 ± 2.28&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>21.94 ± 2.53&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>23.31 ± 2.24&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gungurru</td>
<td>47.08 ± 5.92&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.15 ± 1.61&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>18.61 ± 1.95&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.17 ± 2.96&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Jenabillup</td>
<td>42.27 ± 1.13&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>14.18 ± 0.84&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>21.37 ± 0.94&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>22.18 ± 0.63&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mandelup</td>
<td>37.22 ± 2.48&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>13.60 ± 1.11&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>23.30 ± 1.09&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>25.88 ± 1.82&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>PBA Barlock</td>
<td>45.62 ± 1.87&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>13.12 ± 1.38&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.62 ± 0.82&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.65 ± 2.84&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>PBA Gunyidi</td>
<td>35.57 ± 10.49&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.74 ± 0.78&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>23.86 ± 5.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.83 ± 5.65&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Quilinock</td>
<td>37.04 ± 2.89&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>12.63 ± 1.49&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.92 ± 1.56&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>27.42 ± 2.59&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tanjil</td>
<td>40.13 ± 1.46&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>13.00 ± 1.18&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>21.69 ± 0.80&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>25.20 ± 2.37&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Walan 2385</td>
<td>39.06 ± 1.68&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>14.38 ± 4.08&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>21.37 ± 2.52&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>25.18 ± 5.18&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Average</td>
<td>40.31 ± 17.94</td>
<td>13.99 ± 9.43</td>
<td>21.69 ± 9.57</td>
<td>24.01 ± 11.76</td>
</tr>
</tbody>
</table>

For mean ± SD, N = 6 except Walan 2385 N = 4. Mean values with different superscript in the same column are significantly different (P < 0.05).

Villarino et al. (2015) indicated that Australian sweet lupin fine and coarse particles related to protein and dietary fibre contents. Fine particles (< 100 µm) contained high protein content and coarse particles (>100 µm) were rich in dietary fibre. The main dietary fibre of lupin kernel is insoluble non-starch polysaccharides which could be difficult to be milled resulting in higher amount of large particle size fractions.
4.2 Chemical composition

4.2.1 Moisture

The moisture contents in whole seed and dehulled lupin samples are shown in Table 23. The average moisture contents in whole and dehulled lupin samples were 9.6 and 9.7%, respectively. In whole lupin samples, there was no significant difference in moisture content between different cultivars. The moisture contents in dehulled lupin samples of different cultivars were not significantly different except between Belara and Gungurru.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Moisture content (%)</th>
<th>Whole seed</th>
<th>Dehulled lupin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belara</td>
<td>8.1 ± 0.3\textsuperscript{a}</td>
<td>8.1 ± 0.2\textsuperscript{b}</td>
<td></td>
</tr>
<tr>
<td>Corumup</td>
<td>9.6 ± 1.1\textsuperscript{a}</td>
<td>9.9 ± 1.0\textsuperscript{ab}</td>
<td></td>
</tr>
<tr>
<td>Gungurru</td>
<td>11.1 ± 2.0\textsuperscript{a}</td>
<td>11.3 ± 1.9\textsuperscript{a}</td>
<td></td>
</tr>
<tr>
<td>Jenabillup</td>
<td>10.1 ± 1.5\textsuperscript{a}</td>
<td>10.2 ± 1.5\textsuperscript{ab}</td>
<td></td>
</tr>
<tr>
<td>Mandelup</td>
<td>9.7 ± 1.1\textsuperscript{a}</td>
<td>9.6 ± 1.2\textsuperscript{ab}</td>
<td></td>
</tr>
<tr>
<td>PBA Barlock</td>
<td>9.5 ± 1.5\textsuperscript{a}</td>
<td>9.6 ± 1.5\textsuperscript{ab}</td>
<td></td>
</tr>
<tr>
<td>PBA Gunyidi</td>
<td>9.7 ± 1.8\textsuperscript{a}</td>
<td>9.5 ± 1.8\textsuperscript{ab}</td>
<td></td>
</tr>
<tr>
<td>Quilinock</td>
<td>10.1 ± 1.5\textsuperscript{a}</td>
<td>10.1 ± 1.4\textsuperscript{ab}</td>
<td></td>
</tr>
<tr>
<td>Tanjil</td>
<td>9.4 ± 1.0\textsuperscript{a}</td>
<td>9.5 ± 1.1\textsuperscript{ab}</td>
<td></td>
</tr>
<tr>
<td>Walan 2385</td>
<td>9.3 ± 0.2\textsuperscript{a}</td>
<td>9.4 ± 0.3\textsuperscript{ab}</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>9.6 ± 1.5</td>
<td>9.7 ± 1.5</td>
<td></td>
</tr>
</tbody>
</table>

For mean ± SD, N = 6 except Walan 2385 N = 4. Mean values with different superscript in the same column are significantly different ($P < 0.05$).
4.2.2 Raffinose family oligosaccharides (RFOs)

4.2.2.1 Method validation

To calculate the concentration of raffinose, stachyose and verbascose, calibration curves of standards with known concentrations were prepared. Chromatograms of mixed standard RFOs at concentration 100, 200, 300, 400 and 500 µg/mL are shown in Figure 18. Since lupin samples contain sucrose and the peak of sucrose is close to raffinose, standard of sucrose was also mixed to the RFOs standard solutions. The chromatogram result demonstrates that the determination of RFOs under the modified conditions (section 3.4.4) shows good separation of individual RFO within a short HPLC runtime (20 min).

Method validation of RFOs determination was performed as explained in section 3.4.7. The accuracy of the determination was assessed by spiking a sample with known amounts (50 µg/mL) of raffinose, stachyose and verbascose. The recoveries of raffinose, stachyose and verbascose were 95.7–106.5%, 91.2–107.8% and 95.4–103.6%, respectively complying with the acceptance criteria of 90–110% (APVMA 2004). The precision was assessed by inter-day determinations of six replications of the known concentrations of each RFO. The percentage of relative standard deviation was 3.9% for raffinose, 2.1% for stachyose and 3.2% for verbascose which were in the acceptable range (APVMA 2004).

Evaporative light scattering detector (ELSD) is a non-linear detector and the correlation between peak area and concentration is not linear. Generally, the relationship between peak area and concentration detected from ELSD is exponential (Dvořáčková et al. 2014). Therefore, the second polynomial regression of correlation between the logarithm of peak area and the logarithm of concentration was applied to achieve a good correlation.
Figure 18: Chromatogram of mixed standard solution (sucrose, raffinose, stachyose and verbascose)
The second order polynomial equations of correlations between the logarithm of peak areas and the logarithm of concentrations of sucrose, raffinose, stachyose and verbascose are shown in Figure 19. All calibration curves showed $R^2 > 0.99$.

**Figure 19: Calibration curves of sucrose, raffinose, stachyose and verbascose**

### 4.2.2.2 RFOs content

Raffinose, stachyose, verbascose and total RFOs contents in whole seed and dehulled lupin samples are presented in Tables 24 and 25, respectively. Raffinose, stachyose and verbascose were found in all lupin samples. In whole seeds, the average raffinose, stachyose, verbascose and total RFOs content were 1.3, 5.3, 1.9 and 8.5 g/100 g DM, respectively. Dehulled lupin had average raffinose, stachyose, verbascose and total RFOs content of 1.5, 7.2, 1.9 and 10.5 g/100 g DM, respectively.
Table 24: RFOs contents in whole seeds of lupin cultivars

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Raffinose</th>
<th>Stachyose</th>
<th>Verbascose</th>
<th>Total RFOs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belara</td>
<td>1.6 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.1 ± 0.8&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.5 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.1 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Corumup</td>
<td>1.3 ± 0.2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>5.7 ± 0.2&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>2.0 ± 0.2&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>9.1 ± 0.4&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gungurru</td>
<td>1.2 ± 0.2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>4.5 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.7 ± 0.2&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>7.3 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Jenabillup</td>
<td>1.0 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.9 ± 0.3&lt;sup&gt;de&lt;/sup&gt;</td>
<td>1.5 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.4 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mandelup</td>
<td>1.4 ± 0.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>6.5 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.2 ± 0.2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>10.0 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PBA Barlock</td>
<td>1.0 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.6 ± 0.5&lt;sup&gt;de&lt;/sup&gt;</td>
<td>2.0 ± 0.4&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>7.6 ± 0.9&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>PBA Gunyidi</td>
<td>1.3 ± 0.2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>5.2 ± 0.2&lt;sup&gt;ce&lt;/sup&gt;</td>
<td>1.9 ± 0.2&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>8.4 ± 0.4&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Quilinock</td>
<td>1.6 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.4 ± 0.4&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>2.0 ± 0.7&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>9.0 ± 1.1&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tanjil</td>
<td>1.2 ± 0.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>4.9 ± 0.3&lt;sup&gt;ce&lt;/sup&gt;</td>
<td>1.7 ± 0.2&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>7.9 ± 0.2&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Walan 2385</td>
<td>1.6 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.3 ± 0.7&lt;sup&gt;bce&lt;/sup&gt;</td>
<td>1.7 ± 0.2&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>8.6 ± 1.0&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td>1.3 ± 0.3</td>
<td>5.3 ± 0.7</td>
<td>1.9 ± 0.4</td>
<td>8.5 ± 1.2</td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td>1.0–1.6</td>
<td>4.5–6.5</td>
<td>1.5–2.5</td>
<td>7.3–10.1</td>
</tr>
<tr>
<td>% of total RFOs</td>
<td>15.0%</td>
<td>62.5%</td>
<td>22.5%</td>
<td></td>
</tr>
</tbody>
</table>

For mean ± SD, N = 6 except Walan 2385 N = 4. Mean values with different superscript in the same column are significantly different (P < 0.05).

The results show that Australian sweet lupins are good sources of RFOs (Tables 24 and 25). Stachyose was the major RFO in ASL accounting for 63% in whole seed and 68% in dehulled lupin which is in agreement with results of other studies (Frias et al. 1996; Martínez-Villaluenga, Frías & Vidal-Valverde 2005).

Martínez-Villaluenga et al. (2008) indicated that European and South American lupin were the richest source of RFOs (5.1–16.1 g/100 g DM) compare to other grain legumes such as chickpea (2.0–7.6 g/100 g DM), faba beans (1.0–4.5 g/100 g DM) and lentil (1.8–7.5 g/100 g DM). European lupins contained stachyose 60–70% of total RFOs contents (Frias et al. 1996; Martínez-Villaluenga, Frías & Vidal-Valverde 2005). Stachyose were the main RFO in most legumes such as lentils, peas and soybeans (Martínez-Villaluenga et al. 2008). However, verbascose was the major RFO in some legumes such as black grams and mungbeans (Fan et al. 2015; Souframanien et al. 2014).
<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Raffinose</th>
<th>Stachyose</th>
<th>Verbascose</th>
<th>Total RFOs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belara</td>
<td>2.2 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.0 ± 0.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.9 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.0 ± 0.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Corumup</td>
<td>1.4 ± 0.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6.7 ± 0.2&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>1.7 ± 0.1&lt;sup&gt;de&lt;/sup&gt;</td>
<td>9.8 ± 0.3&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gungurru</td>
<td>1.0 ± 0.4&lt;sup&gt;e&lt;/sup&gt;</td>
<td>5.6 ± 1.5&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>1.2 ± 0.2&lt;sup&gt;f&lt;/sup&gt;</td>
<td>7.8 ± 1.9&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Jenabillup</td>
<td>1.5 ± 0.2&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>6.7 ± 0.5&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>2.0 ± 0.2&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>10.2 ± 0.9&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mandelup</td>
<td>1.5 ± 0.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>13.0 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.3 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.8 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PBA Barlock</td>
<td>0.9 ± 0.0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>5.2 ± 0.2&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1.5 ± 0.4&lt;sup&gt;e&lt;/sup&gt;</td>
<td>7.6 ± 0.1&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>PBA Gunyidi</td>
<td>1.9 ± 0.3&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>6.7 ± 0.6&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>2.1 ± 0.4&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>10.8 ± 1.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Quilinock</td>
<td>1.8 ± 0.1&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>6.2 ± 0.3&lt;sup&gt;de&lt;/sup&gt;</td>
<td>1.9 ± 0.3&lt;sup&gt;cde&lt;/sup&gt;</td>
<td>9.8 ± 0.5&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tanjil</td>
<td>1.1 ± 0.3&lt;sup&gt;e&lt;/sup&gt;</td>
<td>7.1 ± 1.0&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>1.6 ± 0.2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>9.8 ± 1.2&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td>Walan 2385</td>
<td>1.6 ± 0.1&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>6.0 ± 0.3&lt;sup&gt;def&lt;/sup&gt;</td>
<td>1.6 ± 0.3&lt;sup&gt;e&lt;/sup&gt;</td>
<td>9.3 ± 0.4&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Average</td>
<td>1.5 ± 0.5</td>
<td>7.2 ± 2.3</td>
<td>1.9 ± 0.5</td>
<td>10.5 ± 2.8</td>
</tr>
<tr>
<td>Range</td>
<td>0.9–2.2</td>
<td>5.2–13.0</td>
<td>1.2–2.9</td>
<td>7.6–16.8</td>
</tr>
<tr>
<td>% of RFOs</td>
<td>14.0%</td>
<td>68.0%</td>
<td>18.0%</td>
<td></td>
</tr>
</tbody>
</table>

For mean ± SD, N = 6 except Walan 2385 N = 4. Mean values with different superscript in the same column are significantly different (P < 0.05).

### 4.2.2.3 Effect of cultivar

Cultivar had a significant effect (P < 0.05) on raffinose, stachyose, verbascose and total RFOs contents (Tables 24 and 25). In whole seed samples, high levels of total RFOs were recorded in Belara and Mandelup. Low levels of total RFOs were found in Gungurru, Jenabillup and PBA Barlock. In dehulled lupin samples, high levels of total RFOs were found in Belara and Mandelup. The lowest levels of total RFOs were found in Gungurru and PBA Barlock. Overall, the results from both whole seed and dehulled lupin samples showed that Belara and Mandelup cultivars contained high levels of total RFOs, and Gungurru and PBA Barlock contained low levels of total RFOs.

A few studies reported RFOs contents in some ASL cultivars. Evans et al. (1993) showed that the total RFOs contents in dehulled seed of ASL cultivar Gungurru was 7.7 g/100 g DM which is similar to the result recorded for this study (7.8 g/100 g DM). RFOs contents in dehulled lupin of two ASL cultivars, Danja and Yorrel (no longer commercially cultivated) were 7.4 and 8.0 g/100g DM, respectively (Evans et al. 1993). Saini and Gladstones (1986) found that RFOs contents in whole seeds of Marri,
Illyarrie, Unicrop and Uniharvest cultivars of ASL (no longer commercially cultivated) ranged from 9.9–10.6 g/100 g DM. Up-to-date information on RFOs contents in current ASL cultivar is important in selecting suitable cultivars for food applications. Based on the results of this study, Gungurru and PBA Barlock cultivars are recommended for lupin-enriched foods with low flatulence effect due to their lower RFO contents. Belara and Mandelup cultivars are suitable for functional foods with prebiotic potential due to their high RFOs contents which can promote the growth of beneficial bacteria and decrease levels of pathogenic bacteria.

### 4.2.2.4 Effect of cultivation year

The average raffinose, stachyose, verbascose and total RFO contents in whole seed and dehulled lupin samples from 2011, 2012 and 2013 cultivation years are presented in Figure 20. Cultivation year had no significant effect on individual RFO and total RFOs contents in both whole seed and dehulled lupin samples. This could be due to the similar climatic conditions reported during the three cultivation years of the study. As shown in Figure 21, there were no significant differences in mean monthly maximum temperature, mean monthly minimum temperature and mean monthly rainfall of cultivated area (Wongan Hills research station) between 2011, 2012 and 2013 as reported by the Australian Bureau of Meteorology (2015).

A limited number of studies have been conducted on the effect of cultivation year or climatic conditions on RFOs contents in lupin. However, there are a few studies on the effect of cultivation year on RFOs contents in other grain legumes (Johnson et al. 2013; Nikolopoulou et al. 2006; Tahir et al. 2011). Cultivation year with different climatic conditions, especially rainfall, had significant effects on total RFOs contents in chickpea and peas (Nikolopoulou et al. 2006; 2007). In contrast, Johnson et al. (2013) reported that cultivation year had no significant effect on RFOs in lentil without specifying the similarity or difference of climatic conditions. However, the authors stated that soil moisture contents in the fields between two cultivation years were different. Tahir et al. (2011) concluded that although rainfall, soil type and temperature influenced the RFOs contents in lentil, the effect of cultivar is more dominant than the environmental effects.
Figure 20: Effect of cultivation year on raffinose, stachyose, verbascose and total RFOs contents

For mean values, N = 18 for 2011, N = 20 for 2012 and 2013. Error bar shows the standard deviation of the means. Mean values between cultivation years with the same letter indicate no significant difference (P ≥ 0.05).

RFOs are accumulated in legume seeds during seed maturation and their physiological functions are associated with the desiccation tolerance and frost resistance (Martínez-Villaluenga et al. 2008). A few studies have reported conflicting information on the effect of maturation temperature on RFOs contents in lupin. Górecki et al. (1996) found there was no significant effect of maturation temperature (13 °C and 28 °C) on RFOs contents in lupin. In contrast, Górecki et al. (1997) reported that lupin matured
at 18 °C had a twofold increase in stachyose and verbascose contents compared with seeds matured at 25 °C. Piotrowicz-Cielak (2006) also reported that lupin seeds matured at high temperatures contained more raffinose and stachyose than seeds matured at lower temperatures. The current study showed that cultivation year with similar climatic conditions had no impact on RFOs content in lupin. Similar mean temperature and rainfall conditions were reported over the three growing periods.

4.2.2.5 Effect of dehulling

The effects of dehulling on raffinose, stachyose, verbascose and total RFOs contents in different lupin cultivars are shown in Figure 22. Dehulling had no effect on raffinose contents in Coromup, Gungurru, PBA barlock, PBA Gunyidi, Quilinock, Tanjil and Walan 2385. In contrast, Raffinose contents in Belara, Jenabillup and Mandelup were significantly increased after dehulling.

Dehulling increased stachyose contents in all studied cultivars except Gungurru. As a result of dehulling, verbascose contents increased in Belara, Mandelup and PBA

Figure 21: Main climate statistics of cultivated area (Wongan Hills research station) in 2011, 2012 and 2013.
(Modified from data from the Australian Bureau of Meteorology 2015)
Mean values between cultivation years with the same letter indicate no significant difference ($P \geq 0.05$).
Gunyidi, decreased in Coromup, Gungurru and Walan 2385, and had no effect on Jenabillup, PBA Barlock, Quilinock and Tanjil.

Figure 22: Effect of dehulling on RFOs contents in lupin cultivars
For mean values, N = 6 except Walan 2385 N = 4. Error bar shows the standard deviation of the means. Mean values between whole seed and dehulled lupin samples with different letters indicate significant difference ($P < 0.05$).

Dehulling increased or had no effect on the total RFOs contents in lupins. Dehulling increased the total RFOs contents in Belara, Coromup, Jenabillup, Mandelup, PBA Gunyidi and Tanjil. Dehulling had no significant effect on the total RFOs contents in Gungurru, PBA Barlock, Quilinock and Walan 2385. This result showed that the effect of dehulling on RFOs contents depends on the lupin cultivar and type of RFO. Overall, dehulling resulted in a significant increase in raffinose, stachyose and total RFOs contents but had no significant effect on verbascose content (Figure 23).

![Figure 23: Effect of dehulling on RFOs contents in lupin](image)

For mean values, N = 58. Error bar shows the standard deviation of the means. Mean values between whole seed and dehulled lupin samples with different letters indicate significant difference ($P < 0.05$).

There are some discrepancies between the results of studies on the effect of dehulling on RFOs contents in other grain legumes. These could be due to the use of different dehulling methods. Some studies have employed wet dehulling and others have used dry dehulling. Wet dehulling includes a step of soaking seeds in water to loosen the hull before dehulling. A mechanical dehuller (without soaking) is used for dry dehulling. Since RFOs are water soluble compounds, wet dehulling results in a significant decrease in RFOs contents as they could be solubilised and removed with soaked water. In contrast, the results showed that dry dehulling increases RFOs
contents in lupin (Figure 23). Brenes et al. (2003) also reported higher contents of total RFOs in dehulled lupin (6.3 g/100 g DM) than that of whole seeds (5.9 g/100 g DM). The authors used a commercial pea splitter with a plate-type grinder, and removed the hulls mechanically. Wang et al. (2008 & 2009) reported that dry dehulling by using the Satake TM05C Grain Testing Mill resulted in a significant increase of stachyose and verbascose contents (two major RFOs) in field pea and lentils. The results of current study clearly indicated that dry dehulling resulted in a significant increase in total RFOs contents in lupin. Commercial lupin flour is produced by dry dehulling method and the flour is incorporated in to various foods. Therefore, use of dry dehulling technique is appropriate to determine the effect of dehulling on RFOs contents in lupin.

4.2.3 Phytate
4.2.3.1 Method validation
Calibration curves of phytate standard at 20, 40, 60, 80 and 100 µg/mL were prepared. Spiking samples with known amounts of phytate (30 µg/mL) were employed to determine the accuracy. The recoveries of phytate were ranged from 91.1 to 107.2% which were in the acceptable range of 80–120% (APVMA 2004). The precision was assessed by inter-day determinations of six replications of the known concentrations of phytate. The result showed that the percentage of relative standard deviation of phytate was 3.8% which was in the acceptable range of less than 10% (APVMA 2004).

The method of phytate determination was based on the reaction between ferric chloride and sulfosalicylic acid (wade reagent) in an acid condition resulting in forming a pink colour complex with maximum absorbance at 500 nm. When phytate is present, ferric ion binds to phytate resulting in a decrease in absorbance. A negative correlation between absorbance of the complex and phytate concentration was observed. The calibration curve between absorbance of the complex and phytate concentration (Figure 24) showed $R^2 > 0.99$.

4.2.3.2 Phytate content
The average phytate content in whole seed was 0.80 g/100 g DM and dehulled lupin was 0.78 g/100 g DM (Table 26). Petterson et al. 1998 and Trugo et al. 1993 reported
that phytate contents in whole seeds of lupins were ranged from 0.50–1.10 g/100 g DM. Porres et al. (2005) reported phytate content in whole seed flour of *L. albus* of 0.83 g/100 g DM. Omer et al. (2016) reported the phytate content in whole seeds of *L. albus* of 0.28 g/100 g. The phytate contents in lupin were similar to various grain legumes such as chickpeas (0.3–1.3 g/100 g DM), lentil (0.3–1.1 g/100 g DM) and mung bean (0.6–1.1 g/100 g DM) (Reddy 2002). Lupin contains lower phytate level than some of the other grain legumes such as kidney bean (0.9–1.6 g/100 g DM), red kidney beans (1.2–2.1 g/100 g DM) and soybean (1.0–2.2 g/100 g DM) (Reddy 2002).

![Figure 24: Calibration curve of phytate](image)

**4.2.3.3 Effect of cultivar**

Cultivar had a significant effect on phytate contents (Table 26). In whole seed samples, high levels of phytate were recorded in Coromup and Gungurru. Low levels of phytate were found in PBA Gunyidi and Walan 2385. In dehulled lupin samples, high levels of phytate were found in Gungurru, Jenabillup and Quilinock. Low levels of phytate were found in PBA Gunyidi and PBA Barlock. The results of both whole seed and dehulled lupin samples showed that Gungurru and Quilinock contained high level of phytate, and PBA Gunyidi and PBA Barlock contained low levels of phytate.

Burbano et al. (1995) studied phytate contents in Spanish lupin and reported low level in *L. albus* (0.3 g/100 g DM) and high level in *L. luteus* (0.8 g/100 g DM). De Carvalho (2005) studied the phytate contents in lupins grown in Portugal and reported significant differences between *L. albus* (0.5 g/100 g DM) and *L. mutabilis* (0.8 g/100 g DM).
Trugo et al. (1993) recorded phytate contents in six ASL cultivars namely Marry, Yandee, Illyarrie, Chittick, Wandoo and Danja (no longer commercially cultivated) ranged from 0.7–1.1 g/100 g DM. The authors have not reported significant differences between cultivars.

Table 26: Phytate contents in whole and dehulled seeds of lupin cultivars

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Phytate (g/100 g DM)</th>
<th>Whole seeds</th>
<th>Dehulled seeds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belara</td>
<td>0.72 ± 0.13&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.75 ± 0.18&lt;sup&gt;bd&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Corumup</td>
<td>0.98 ± 0.12&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.80 ± 0.19&lt;sup&gt;ad&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Gungurru</td>
<td>1.02 ± 0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.98 ± 0.17&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Jenabillup</td>
<td>0.80 ± 0.07&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>1.02 ± 0.07&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Mandelup</td>
<td>0.70 ± 0.06&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.66 ± 0.19&lt;sup&gt;de&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>PBA Barlock</td>
<td>0.74 ± 0.29&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.58 ± 0.10&lt;sup&gt;de&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>PBA Gunyidi</td>
<td>0.65 ± 0.12&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.43 ± 0.07&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Quilinock</td>
<td>0.91 ± 0.11&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>1.08 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Tanjil</td>
<td>0.79 ± 0.13&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>0.63 ± 0.24&lt;sup&gt;de&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Walan 2385</td>
<td>0.69 ± 0.03&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.94 ± 0.13&lt;sup&gt;abc&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>0.80 ± 0.18</td>
<td>0.78 ± 0.25</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>0.65–1.02</td>
<td>0.43–1.08</td>
<td></td>
</tr>
</tbody>
</table>

For mean ± SD, N = 6 except Walan 2385 N = 4. Mean values with different superscript in the same column are significantly different (P < 0.05).

It is commonly believed that phytate has a negative effect on mineral bioavailability. However, health benefits of phytate including anti-carcinogenic, anti-oxidative and anti-hyperlipidaemic effects have been reported (Kang et al. 2012; Raboy 2002). The information on phytate contents in current ASL cultivar is important in selecting suitable cultivars for food applications. Lupin cultivars with high phytate content may be used for producing functional foods with health benefits.

4.2.3.4 Effect of cultivation year

The average phytate contents in whole seed and dehulled lupin samples from 2011, 2012 and 2013 cultivation years are presented in Figure 25. Cultivation year had no significant effect on phytate contents in both whole seed and dehulled lupin samples.
This could be due to the similar temperature and rainfall of the growing area during the three cultivation years (Figure 21).

**Figure 25: Effect of cultivation year on phytate contents**

For mean values, N = 18 for 2011, N = 20 for 2012 and 2013. Error bar shows the standard deviation of the means. Mean values between cultivation years with the same letter indicate no significant difference ($P \geq 0.05$).
A limited number of studies on the effect of cultivation year or climatic conditions on phytate contents in lupin have been published. The effect of water stress on phytate contents in *L. albus* and *L. mutabilis* was studied by De Carvalho (2005). During the seed formation stage, water stress was created by withholding watering for 20 days compare to the well-watered plants. The author concluded that water stress had no significant effect on phytate contents in lupin.

The effect of environmental conditions on phytate contents in other cereals and grain legumes have been documented. Li et al. (2013) studied the effect of environmental conditions on phytate contents in soybean. Soybeans were grown at five locations in Henan province, China. Daytime temperatures (during blooming and podding stages) and mean temperature (during seed filling and maturity) were correlated with phytate contents in soybean. Saastamoinen, Plaami & Kumpulainen (1992) reported that phytate content in oats was increased by high temperature and decreased by high rainfall during grain filling stage. Liu et al. (2005) studied the effect of environment on phytate contents in rice. Twenty-four rice cultivars were grown at four locations with different ecological conditions. The authors reported a significant difference in phytate content from different growing locations; however, no information on the effect of environment such as temperature and rainfall was reported.

### 4.2.3.5 Effect of dehulling

The effects of dehulling on phytate contents in different lupin cultivars are shown in Figure 26. Dehulling had no significant effect on phytate contents in Belara, Coromup, Gungurru, Mandelup, PBA Barlock and Tanjil. Phytate contents in Jenabillup, Quilinock and Walan 2385 were significantly increased after dehulling. In contrast, dehulling significantly decreased phytate content in PBA Gunyidi. Results showed that the effect of dehulling on phytate contents depends on the lupin cultivar.

Wet dehulling has been applied as an effective processing step to reduce phytate content in various grain legumes (Ertaş & Bilgiçli 2014). Phytate is water soluble compound and soaking seeds before dehulling result in the leaching of phytate into soaking water. Dehulled lupin and lupin flour are commercially produced by dry dehulling; however, the information on the effect of dry dehulling on different
cultivars of lupin is limited. Embaby (2010) reported significant increases in phytate contents in lupins (*L. albus* and *L. termis* from Egypt) after dry dehulling.

The effect of dehulling on phytate contents in different cultivars of other legumes (not lupin) has been reported. Wang et al. (2009) reported that dry dehulling increased phytate contents significantly in all studied lentil cultivars. The average phytate content in whole seeds and dehulled seeds of eight lentil cultivars were 0.67 and 0.84 g/100 g DM, respectively. Wang et al. (2008) also reported that dry dehulling results in a significant increase in phytate contents in six field pea cultivars. The average phytate content in whole seeds was 0.73 g/100 g DM and increased to 0.77 g/100 g DM after dehulling.

![Figure 26: Effect of dehulling on phytate contents in lupin cultivars](image)

For mean values, N = 6 except Walan 2385 N = 4. Error bar shows the standard deviation of the means. Mean values between whole seed and dehulled lupin samples with different letters indicate significant difference (*P* < 0.05).
4.2.4 Total phenolics

4.2.4.1 Method validation

A calibration curve using gallic acid at 25, 50, 75, 100 and 125 µg/mL as a reference standard was prepared to calculate the concentration of total phenolic compounds. The accuracy of the determination was assessed by spiking samples with known amounts of gallic acid (40 µg/mL). The recoveries of total phenolic compounds ranged from 102.0–105.9% which was in the acceptable range (APVMA 2004). The repeatability was assessed by inter-day determinations of six replications of the known concentrations of gallic acid. The percentage of relative standard deviation of total phenolic ranged from 1.8–4.2% complying with the acceptance criteria (APVMA 2004). The calibration curve between absorbance and gallic acid concentration (Figure 27) showed $R^2 > 0.99$.

![Calibration curve of total phenolic contents](image)

**Figure 27: Calibration curve of total phenolic contents**

4.2.4.2 Total phenolic content

The total phenolic contents (TPC) in whole seed and dehulled lupin samples are presented in Table 27. The average total phenolic content of 94.66 mg GAE/100 g DM was reported for whole seeds. Dehulled lupin had an average total phenolic content of 110.96 mg GAE/100 g DM.

It is difficult to compare the total phenolic contents in lupin with other studies due to differences in extraction solvents, methods and/or reference standards used in different
studies. Aqueous methanol has been used widely for phenolic compounds extraction; however, other solvents such as acetone, ethanol and tris buffer have also been used (Stalikas 2007; Tsao 2010). Gallic acid is often used as a reference standard for determination of total phenolic content. However, various other standard solutions such as caffeic acid, chlorogenic acid, ferrulic acid and tannic acid have been used as reference standards in determination of total phenolic contents (Prior et al. 2005).

Compare to the other studies with the similar conditions of analysis (aqueous methanol as extract solvent, using the Folin–Ciocalteu method and gallic acid as a reference standard), the total phenolic contents found in this study is similar to or lower than the values reported in other studies. Rumiyati et al. (2013) reported similar total phenolic contents in lupin flour of L. angustifolius at 95.4 mg GAE/100 g DM. Sbihi et al. (2013) found that the total phenolic contents in whole seeds of L. albus collected in Saudi Arabia ranged from 105.6 to 115.1 mg GAE/100 g DM. Siger et al. (2012) reported average total phenolic contents, using vitexin for generating calibration curve and converting to gallic acid equivalent, in whole seed samples of L. albus, L. angustifolius and L. luteus of 241.7, 264.1 and 283.6 mg GAE/100 g DM, respectively.

Some studies have reported total phenolic contents in lupins using other standard compounds such as catechin and ferrulic acid as reference standards. Oomah et al. (2006) reported that total phenolic contents in L. angustifolius cultivated in Canada ranged from 1190–1470 mg CE/100 g. Martinez-Villaluenga et al. (2009) reported the total phenolic contents in L. albus and L. angustifolius of 182 and 238 mg ferrulic acid/100 g DM, respectively. Fernandez-Orozco et al. (2006) reported the total phenolic content in L. angustifolius from Spain at 143 mg CE/100 g DM.

### 4.2.4.3 Effect of cultivar

Cultivar had a significant effect ($P < 0.05$) on total phenolic content (Table 27). In whole seed samples, Tanjil cultivar contained significantly higher level of total phenolic contents than Belara, Coromup, Gungurru, Quilinock and Walan 2385. The total phenolic contents in other cultivars were not significantly different. In dehulled seed samples, PBA Barlock contained higher level of TPC than other cultivars except Jenabillup.
Table 27: Total phenolic contents in whole and dehulled seeds of lupin cultivars

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Total phenolic content (mg GAE/100 g DM)</th>
<th>Whole seeds</th>
<th>Dehulled seeds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belara</td>
<td>87.66 ± 7.00ᵇ</td>
<td>110.22 ± 11.02ᵇ</td>
<td></td>
</tr>
<tr>
<td>Corumup</td>
<td>90.45 ± 10.58ᵇ</td>
<td>106.82 ± 10.24ᵇ</td>
<td></td>
</tr>
<tr>
<td>Gungurru</td>
<td>88.31 ± 5.34ᵇ</td>
<td>97.07 ± 9.34ᵇ</td>
<td></td>
</tr>
<tr>
<td>Jenabillup</td>
<td>98.69 ± 12.50ᵃᵇ</td>
<td>116.05 ± 3.60ᵃᵇ</td>
<td></td>
</tr>
<tr>
<td>Mandelup</td>
<td>90.81 ± 9.23ᵃᵇ</td>
<td>110.62 ± 8.92ᵇ</td>
<td></td>
</tr>
<tr>
<td>PBA Barlock</td>
<td>101.26 ± 9.61ᵃᵇ</td>
<td>134.60 ± 14.69ᵃ</td>
<td></td>
</tr>
<tr>
<td>PBA Gunyidi</td>
<td>101.07 ± 4.35ᵃᵇ</td>
<td>109.64 ± 7.67ᵇ</td>
<td></td>
</tr>
<tr>
<td>Quilinock</td>
<td>93.15 ± 6.63ᵃᵇ</td>
<td>112.66 ± 7.91ᵇ</td>
<td></td>
</tr>
<tr>
<td>Tanjil</td>
<td>106.03 ± 5.03ᵃ</td>
<td>111.84 ± 13.54ᵇ</td>
<td></td>
</tr>
<tr>
<td>Walan 2385</td>
<td>86.44 ± 3.21ᵇ</td>
<td>94.66 ± 10.42ᵇ</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>94.66 ± 9.81</td>
<td>110.96 ± 13.78</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>86.44–106.03</td>
<td>94.66–134.60</td>
<td></td>
</tr>
</tbody>
</table>

For mean ± SD, N = 6 except Walan 2385 N = 4. Mean values with different superscript in the same column are significantly different (P < 0.05).

Wang and Clements (2008) reported the total phenolic contents in three ASL cultivars—Belara (578.4 mg GAE/100 g), Kalya (578.0 mg GAE/100 g) and Wongan Hill Telerack (535.1 mg GAE/100 g). The authors have not reported significant differences between cultivars. The total phenolic content in whole seeds of Belara cultivar found in the present study was lower than the content recorded by Wang and Clements (2008). This could be due to different methods (e.g. sample extraction duration, reaction duration with Folin-Ciocalteu reagent and wavelength used for measuring maximum absorbance) used in the determinations.

A few studies reported total phenolic contents in different cultivars of lupin grown in different countries. Oomah et al. (2006) reported that cultivar had a significant effect on total phenolic contents in eight cultivars of *L. angustifolius* grown in Canada. Ranilla et al. (2009) reported the significant effect of cultivar on total phenolic contents in six cultivars of *L. mutabilis* grown in Peru. Siger et al. (2012) showed that different cultivars of three lupin species (*L. albus*, *L. angustifolius* and *L. luteus*) contained significantly different contents of total phenolic compounds.
4.2.4.4 Effect of cultivation year

The average contents of total phenolics in whole seed and dehulled lupin samples from 2011, 2012 and 2013 cultivation years are presented in Table 28. Cultivation year had no significant effect on the total phenolic contents in both whole seed and dehulled lupin samples. This could be an indication that similar climatic conditions (Figure 21) during the cultivation result in similar total phenolic contents.

Although limited study on the effect of cultivation year on total phenolic content in lupin has been published, there are several studies on the effect of cultivation year on total phenolic contents in other legumes. Oomah et al. (2005) reported that cultivation year had a significant effect on the total phenolic content in bean. In contrast, Wang et al. (1998) reported that environmental condition (different cultivation years and locations) had no significant effect on the total phenolic contents in field pea and grass pea. However, these authors have not mentioned the similarities or differences in climatic conditions of the cultivation years or locations.

Table 28: Total phenolic, total flavonoid and condensed tannin contents in lupin from different cultivation years

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sample</th>
<th>Cultivation year</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2011</td>
</tr>
<tr>
<td>Total phenolic</td>
<td>Whole seed</td>
<td>94.33 ± 9.16a</td>
</tr>
<tr>
<td>(mg GAE/100 g DM)</td>
<td>Dehulled seed</td>
<td>109.63 ± 16.15a</td>
</tr>
<tr>
<td>Total flavonoid</td>
<td>Whole seed</td>
<td>21.34 ± 7.88a</td>
</tr>
<tr>
<td>(mg CE/100 g DM)</td>
<td>Dehulled seed</td>
<td>18.24 ± 5.35a</td>
</tr>
<tr>
<td>Condensed tannin</td>
<td>Whole seed</td>
<td>48.60 ± 16.52a</td>
</tr>
<tr>
<td>(mg CE/100 g DM)</td>
<td>Dehulled seed</td>
<td>71.38 ± 19.94a</td>
</tr>
</tbody>
</table>

For mean ± SD, N = 18 for 2011, N = 20 for 2012 and 2013. Mean values with the same superscript in the same row are not significantly different (P ≥ 0.05).

4.2.4.5 Effect of dehulling

The effects of dehulling on the total phenolic contents in different lupin cultivars are shown in Figure 28. Dehulling significantly increased the total phenolic contents in Belara, Coromup, Jenabillup, Mandelup, PBA Barlock and Quilinock. Dehulling had no significant effect on the total phenolic contents in Gungurru, PBA Gunyidi, Tanjil
and Walan 2385. The result showed cultivar influenced the total phenolic contents in lupin after dehulling.

**Figure 28: Effect of dehulling on total phenolic contents in lupin cultivars**

For mean values, N = 6 except Walan 2385 N = 4. Error bar shows the standard deviation of the means. Mean values between whole seed and dehulled lupin samples with different letters indicate significant difference ($P < 0.05$).

Several studies reported the total phenolic contents in whole seeds of lupins (Oomah et al. 2006; Siger et al. 2012; Wang & Clements 2008) but not after dehulling. Only a few studies showed total phenolic contents in lupin seed fractions but not the effect of dehulling. Ranilla et al. (2009) studied the total phenolic contents in hull, cotyledon, hypocotyl and whole seed of six cultivars of *L. mutabilis*. Hull fractions of all cultivars contained much lower total phenolic contents than cotyledon and hypocotyl fractions. Whole seed samples of all cultivars contained lower total phenolic contents than cotyledon and hypocotyl fractions. However, the authors have not reported differences in total phenolic contents between whole seed and dehulled seed samples.

Discrepant results on the effect of dehulling on the total phenolic contents have been reported among other grain legumes. Oomah et al. (2005) reported a significant decrease in the total phenolic contents in bean after dehulling. Han & Baik (2008)
reported that dehulling decreased the total phenolic contents in chickpea, lentil, yellow pea and soybean, but had no effect on the total phenolic contents in green pea. Boudjou et al (2013) reported that dehulling increased the total phenolic contents in faba bean, but decreased the total phenolic contents in lentil. Alonso et al. (1998) reported that dehulling had no effect on the total phenolic contents in pea.

4.2.5 Total flavonoids

4.2.5.1 Method validation

Calibration curve of standard catechin hydrate at 20, 30, 40, 50 and 100 µg/mL was used to calculate the concentration of total flavonoids. The accuracy of the determination was assessed by spiking samples with 40 µg/mL of catechin hydrate. The total flavonoid recoveries were ranged from 94.8–107.6% which was in the acceptable range of 75–125% (APVMA 2004). Inter-day determinations of six replications of the known concentrations of catechin hydrate were used to assess the repeatability. The percentage of relative standard deviation was ranged from 0.9–4.5% which was in the acceptable range of less than 20% (APVMA 2004). The calibration curve between absorbance and catechin hydrate concentration showed $R^2 > 0.99$ (Figure 29).

![Figure 29: Calibration curve of total flavonoids](image)
4.2.5.2 Total flavonoid content
The average total flavonoid content in whole seed was 21.11 mg CE/100 g DM and in dehulled seeds was 19.87 mg CE/100 g (Table 29). A few other studies have reported the total flavonoid contents in lupins. Sbihi et al. (2013) reported that the total flavonoid contents in whole seeds of *L. albus* ranged from 45.3 to 49.7 mg GAE/100 g DM. Martínez-Villaluenga et al. (2009) reported the total flavonoid content in *L. albus* and *L. angustifolius* of 110 and 25 mg CE/100 g DM, respectively. The total flavonoid contents in *L. angustifolius* cultivated in Canada ranged from 415–495 mg rutin equivalent/100 g were reported by Oomah et al. (2006); however, the author used rutin instead of catechin as the reference standard.

Table 29: Total flavonoid contents in whole and dehulled seeds of lupin cultivars

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Total flavonoid content (mg CE/100 g DM)</th>
<th>Whole seeds</th>
<th>Dehulled seeds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belara</td>
<td>17.63 ± 1.67&lt;sup&gt;de&lt;/sup&gt;</td>
<td>16.51 ± 5.86&lt;sup&gt;cd&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Corumup</td>
<td>30.72 ± 4.09&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>15.47 ± 3.25&lt;sup&gt;cd&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Gungurru</td>
<td>22.65 ± 2.95&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>12.43 ± 2.81&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Jenabilup</td>
<td>16.47 ± 3.11&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>22.41 ± 4.31&lt;sup&gt;ac&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Mandelup</td>
<td>33.96 ± 2.52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.36 ± 2.86&lt;sup&gt;ac&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>PBA Barlock</td>
<td>16.74 ± 2.42&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>27.97 ± 2.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>PBA Gunyidi</td>
<td>26.46 ± 3.14&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>18.37 ± 3.96&lt;sup&gt;cd&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Quilinock</td>
<td>15.75 ± 1.35&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>18.24 ± 3.52&lt;sup&gt;cd&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Tanjil</td>
<td>11.98 ± 1.77&lt;sup&gt;f&lt;/sup&gt;</td>
<td>20.07 ± 2.75&lt;sup&gt;bc&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Walan 2385</td>
<td>17.67 ± 2.04&lt;sup&gt;de&lt;/sup&gt;</td>
<td>27.33 ± 3.44&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>21.11 ± 7.38</strong></td>
<td><strong>19.87 ± 5.73</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td>11.98–33.96</td>
<td>12.43–27.97</td>
<td></td>
</tr>
</tbody>
</table>

For mean ± SD, N = 6 except Walan 2385 N = 4. Mean values with different superscript in the same column are significantly different (*P* < 0.05).

4.2.5.3 Effect of cultivar
Cultivar had a significant effect on the total flavonoid contents (Table 29). High levels of total flavonoids in whole seed samples were recorded in Mandelup and Coromup, while low levels were found in Tanjil, PBA Barlock and Quilinock. High contents of
total flavonoids in dehulled seed samples were found in PBA Barlock and Walan 2385, low level of total flavonoids was found in Gungurru cultivar. A significant effect of cultivar on total flavonoid contents in eight cultivars of L. angustifolius grown in Canada was also recorded by Oomah et al. (2006).

The effect of cultivar on the total flavonoid contents in other grain legumes has been reported. Heiras-Palazuelos et al. (2013) reported that chickpea cultivar had a significant effect on the total flavonoid contents. However, the chickpea samples were obtained from different locations which the environmental factor may also affect the flavonoid content. Oomah et al. (2005) studied the total flavonoid contents in six bean cultivars grown in the same location and found a significant difference between bean cultivars.

4.2.5.4 Effect of cultivation year
Cultivation year had no significant effect on total flavonoid contents in lupin (Table 28). The information on the effect of cultivation year or climatic conditions on the total flavonoid contents in lupin has not been published. A few studies have reported the effect of cultivation year on the total flavonoid contents in other grain legumes. A significant effect of cultivation year on the total flavonoid content in bean has been reported by Oomah et al. (2005). The authors recorded the average flavonoid contents in six bean cultivars grown in 1999 and 2000 of 56 and 80 mg rutin equivalent/100 g, respectively. Wang and Murphy (1994) reported that cultivation year had a significant effect on isoflavones contents (flavonoids subgroup) in soybean.

4.2.5.5 Effect of dehulling
The effect of dehulling on the total flavonoid contents showed significant difference between cultivars (Figure 30). Dehulling had no significant effect on the total flavonoid contents in Belara, Jenabillup and Quilinock. The total flavonoid contents in Coromup, Gungurru, Mandelup and PBA Gunyidi decreased after dehulling. In contrast, the total flavonoid contents in PBA Barlock, Tanjil and Walan 2385 were increased as a result of dehulling.

Limited information on the effect of dehulling on flavonoid contents in lupin has been published. A discrepant result on isoflavone contents in different seed fractions were
found between lupin cultivars (Ranilla et al. 2009). Isoflavones contents in whole seed and hull fractions were lower than cotyledon and hypocotyl fractions of most *L. mutabilis* cultivars. However, one cultivar contained higher isoflavone content in hull fraction than other fractions. The authors have not reported significant differences between whole seed and dehulled seed fractions.

Oomah et al. (2005) reported a significant decrease in flavonoid content in bean after dehulling. The average flavonoid contents in whole bean and dehulled bean were 68 and 51 mg rutin equivalent/100 g, respectively (Oomah et al. 2005). Gujral et al. (2013) studied the total flavonoid contents in whole seeds and dehulled seeds of various legumes. The authors found similar total flavonoid contents between whole seeds and dehulled seeds of chickpea, soybean and moth bean. They also found that whole seeds of kidney bean and mung bean had higher total flavonoid contents than those of dehulled seeds.

![Figure 30: Effect of dehulling on total flavonoid contents in lupin cultivars](image)

For mean values, N = 6 except Walan 2385 N = 4. Error bar shows the standard deviation of the means. Mean values between whole seed and dehulled lupin samples with different letters indicate significant difference (*P* < 0.05).
4.2.6 Condensed tannins

4.2.6.1 Method validation

To calculate the concentration of condensed tannin, calibration curve was prepared by using catechin hydrate at 20, 30, 40, 50 and 100 µg/mL as a reference standard. The accuracy test was performed by spiking samples with known amounts of catechin hydrate (40 µg/mL). The recoveries of condensed tannin ranged from 95.6–109.8% complying with the acceptance criteria (APVMA 2004). Inter-day determinations of six replications of the known concentrations of catechin hydrate were performed to assess the repeatability. The percentage of relative standard deviation of condensed tannin ranged from 0.9–3.5% which was less than 20% (APVMA 2004). The calibration curve between absorbance and catechin hydrate concentration showed $R^2 > 0.99$ (Figure 31).

![Figure 31: Calibration curve of condensed tannins](image)

\[ y = 0.0015x + 0.0108 \]
\[ R^2 = 0.9976 \]

4.2.6.2 Condensed tannin content

Condensed tannin contents in whole seed and dehulled lupin samples are presented in Table 30. The average condensed tannin content was 46.41 mg CE/100 g DM and ranged from 23.89–59.94 mg CE/100 g DM in whole seed samples. Dehulled lupin had average condensed tannin content of 78.90 mg CE/100 g DM and ranged from 53.98–120.98 mg CE/100 g DM.
There are two types of tannins namely hydrolysable tannin and condensed tannin. Condensed tannin is the most common tannin found in legumes, and hydrolysable tannin is not detected in many legumes (Madsen & Brinch-Pedersen 2016; Reed 1995). Some authors reported tannin contents in lupins (Guemes-Vera et al. 2012; Hassan et al. 2005; Lampart-Szczapa et al. 2003; Omer et al. 2016) but they have used the vanillin assay which actually measures only the condensed tannin content.

A considerable variation in condensed tannin contents in lupins has been reported. Ranilla et al. (2009) reported that condensed tannins were not detected in all studied lupin samples (L. mutabilis from Peru, and L. albus and L. angustifolius from Brazil). Lampart-Szczapa et al. (2003) reported low levels of condensed tannin in L. albus, L. angustifolius and L. luteus at 5.40, 1.45 and 2.45 mg CE/100 g, respectively. The average condensed tannin content in L. termis at 94.10 mg CE/100 g DM was reported by Hassan et al. (2005). Omer et al. (2016) reported the condensed tannin content in whole seeds of L. albus at 100.02 mg CE/100 g. Guemes-Vera et al. (2012) reported the condensed tannin content in L. albus at 74.3 mg CE/100 g.

Some studies (Eggum et al. 1993; Embaby 2010) have used the Folin-Denis method (not the vanillin assay) and tannic acid instead of catechin as the reference standard, for determining tannin content in lupin. Eggum et al. (1993) reported high contents of tannin in lupins grown in Australia and France ranging from 310–360 and 550–690 mg/100 g DM, respectively. Embaby (2010) recorded high tannin contents in whole seeds of L. termis and L. albus at 753 and 384 mg/100 g DM, respectively.

4.2.6.3 Effect of cultivar
As shown in Table 30, cultivar had a significant effect ($P < 0.05$) on condensed tannin content. In whole seed samples, Belara, Quilinock and Walan 2385 had significantly lower contents of condensed tannin than the other cultivars. In dehulled seed samples, the highest condensed tannin level was recorded in Walan 2385 cultivar.
Table 30: Condensed tannin contents in whole and dehulled seeds of lupin cultivars

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Condensed tannin content (mg CE/100 g DM)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole seeds</td>
<td>Dehulled seeds</td>
<td></td>
</tr>
<tr>
<td>Belara</td>
<td>31.09 ± 6.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>62.01 ± 10.77&lt;sup&gt;de&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Corumup</td>
<td>57.84 ± 10.92&lt;sup&gt;a&lt;/sup&gt;</td>
<td>53.98 ± 7.70&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Gungurru</td>
<td>48.55 ± 8.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>58.10 ± 12.98&lt;sup&gt;de&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Jenabillup</td>
<td>52.57 ± 8.96&lt;sup&gt;a&lt;/sup&gt;</td>
<td>95.03 ± 10.38&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Mandelup</td>
<td>59.94 ± 8.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>75.33 ± 5.72&lt;sup&gt;cd&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>PBA Barlock</td>
<td>48.38 ± 5.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>92.92 ± 5.34&lt;sup&gt;bc&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>PBA Gunyidi</td>
<td>52.58 ± 8.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>71.16 ± 7.57&lt;sup&gt;de&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Quilinock</td>
<td>23.89 ± 7.44&lt;sup&gt;b&lt;/sup&gt;</td>
<td>73.91 ± 11.18&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Tanjil</td>
<td>57.55 ± 6.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>99.63 ± 9.87&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Walan 2385</td>
<td>24.41 ± 2.60&lt;sup&gt;b&lt;/sup&gt;</td>
<td>120.98 ± 15.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>46.41 ± 14.72</strong></td>
<td><strong>78.90 ± 21.22</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td><strong>23.89–59.94</strong></td>
<td><strong>53.98–120.98</strong></td>
<td></td>
</tr>
</tbody>
</table>

For mean ± SD, N = 6 except Walan 2385 N = 4. Mean values with different superscript in the same column are significantly different (P < 0.05).

Eggum et al. (1993) reported tannin contents in six lupin cultivars namely Danja, Geebungs, Gungarry, Peter, Wando and Yandee (no longer commercially cultivate) grown in Australia and five cultivars cultivated in France. However, the authors have not reported significant differences between cultivars.

Some studies reported significant differences in condensed tannin contents between cultivars of other grain legumes such as cowpeas (Plahar et al. 1997), lentils, common beans and soybeans (Xu et al. 2007). The legume samples were collected from markets or different origins. It should be noted that to determine the effect of cultivar on chemical composition, different cultivars have to be grown in similar conditions.

A few studies reported significant differences in condensed tannin contents between cultivars of grain legumes under similar growing conditions. de Mejia et al. (2003) determined condensed tannin contents in five cultivars of common bean and found that cultivar had a significant effect on condensed tannin contents. Ranilla et al. (2007)
recorded a significant difference in condensed tannin contents in 25 Brazilian bean cultivars. The condensed tannin contents in whole seeds of Brazilian beans ranged from 100 to 4100 mg CE/100 g.

### 4.2.6.4 Effect of cultivation year

The average contents of condensed tannin in whole seed and dehulled lupin samples from 2011, 2012 and 2013 cultivation years are presented in Table 28. Cultivation year had no significant effect on condensed tannin contents in both whole seed and dehulled lupin samples. The climatic conditions recorded during the three cultivation years of the study were not significantly different (Figure 21).

Wang et al. (1998) indicated that condensed tannin contents in grass pea were not affected by different cultivation years and locations. However, the authors did not mention the similarities or differences in climatic conditions of the cultivation years and/or locations. de Mejia et al. (2003) reported that growing location with different soil types had a significant effect on condensed tannin content in beans. Beans were cultivated in five different sites in Mexico and the average condensed tannin contents ranged from 2160 to 3290 mg CE/100 g.

### 4.2.6.5 Effect of dehulling

The effects of dehulling on condensed tannin contents in different lupin cultivars are shown in Figure 32. Dehulling increased condensed tannin contents in all lupin cultivars except Corumup and Gungurru. Walan 2385 showed the highest increase in condensed tannin content after dehulling.

Embaby et al. (2010) reported that dehulling increased tannin content in *L. termis* and *L. albus* similar to the result of the present study. The author showed that dehulling significantly increased tannin contents in *L. termis* from 753 to 816 mg/100 g DM, and *L. albus* from 384 to 428 mg/100 g DM. This could be due to condensed tannin of lupin seeds mainly occurred in the cotyledon (Lampart-Szczapa et al. 2003). In contrast, some studies reported that dehulling results in a decrease in condensed tannin contents in other grain legumes. Alonso et al. (1998) reported that dehulling resulted in a decrease in condensed tannin contents in pea. Wang (2008) found a significant decrease in condensed tannin contents in red lentils from 600 to 20 mg CE/100 g DM.
after dehulling. Wang et al. (2009) reported that dehulling resulted in a decrease in condensed tannin content in lentils.

**Figure 32:** Effect of dehulling on condensed tannin contents in lupin cultivars

For mean values, $N = 6$ except Walan 2385 $N = 4$. Error bar shows the standard deviation of the means. Mean values between whole seed and dehulled lupin samples with different letters indicate significant difference ($P < 0.05$).

4.2.7 Ash

4.2.7.1 Effect of cultivar

The average ash content in whole seed was 3.06 g/100 g DM and ranged from 2.73–3.23 g/100 g DM (Table 31). Coromup, PBA Gunyidi and Quilinock were some of the cultivars having high ash contents. PBA Barlock and Belara were some of the cultivars had low ash contents.

Dehulled lupin samples had an average ash content of 3.23 g/100 g DM and ranged from 2.86–3.63 g/100 g DM. High ash contents were found in some of the cultivars such as Jenabillup, Quilinock and Coromup. Walan 2385 and Belara were some of the cultivars low in ash contents (Table 31).

Villarino et al. (2015) reported that the average ash content in flours of six ASL cultivars was 3.0 g/100 g. The authors reported that there was no significant difference
in ash contents between cultivars. Bartkiene et al. (2016) reported that the average ash content in whole seeds of seven cultivars of *L. angustifolius* grown in Lithuania was 3.0 g/100 g DM. Bähr et al. (2014) found an average ash content in dehulled seeds of four cultivars of *L. angustifolius* from Germany was 4.0 g/100 g DM. The average ash contents found in the previous studies were similar to the values recorded in the present study.

**Table 31: Ash contents in whole seed and dehulled lupin**

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Ash content (g/100 g DM)</th>
<th>Whole seed</th>
<th>Dehulled lupin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belara</td>
<td>2.81 ± 0.17&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>2.94 ± 0.35&lt;sup&gt;cd&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Corumup</td>
<td>3.23 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.40 ± 0.22&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Gungurru</td>
<td>3.17 ± 0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.33 ± 0.30&lt;sup&gt;abc&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Jenabilup</td>
<td>3.10 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.63 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Mandelup</td>
<td>3.14 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.27 ± 0.08&lt;sup&gt;ad&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>PBA Barlock</td>
<td>2.73 ± 0.17&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.10 ± 0.37&lt;sup&gt;bd&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>PBA Gunyidi</td>
<td>3.19 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.18 ± 0.19&lt;sup&gt;bd&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Quilinock</td>
<td>3.18 ± 0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.44 ± 0.11&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Tanjil</td>
<td>3.04 ± 0.06&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.21 ± 0.08&lt;sup&gt;ad&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Walan 2385</td>
<td>3.01 ± 0.20&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>2.86 ± 0.23&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>3.06 ± 0.21</td>
<td>3.23 ± 0.31</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>2.73–3.23</td>
<td>2.86–3.63</td>
<td></td>
</tr>
</tbody>
</table>

For mean ± SD, N = 6 except Walan 2385 N = 4. Mean values with different superscript in the same column are significantly different (*P* < 0.05).

### 4.2.7.2 Effect of cultivation year

The average ash contents in whole and dehulled lupin samples from 2011, 2012 and 2013 cultivation years are presented in Figure 33. Cultivation year had no significant effect on ash contents in both whole and dehulled lupin samples. Saastamoinen et al. (2013) studied the effect of cultivation year on ash contents in lupins grown in Finland and reported that cultivation year (2010 and 2011) had no significant effect on ash contents in lupin. However, the authors have not reported the similarity or difference of climatic conditions of the two cultivation years.
4.2.7.3 Effect of dehulling

The effects of dehulling on ash contents in different lupin cultivars are shown in Figure 34. Dehulling had no significant effect on ash contents in Belara, Coromup, Gungurru, Mandelup, PBA Barlock, PBA Gunyidi and Walan 2385. Dehulling significantly increased ash contents in Jenabillup, Quilinock and Tanjil. The result showed that the effect of dehulling on ash contents depends on the lupin cultivar.

Figure 34: Effect of dehulling on ash contents in lupin cultivars
For mean values, N = 6 except Walan 2385 N = 4. Error bar shows the standard deviation of the means. Mean values between whole seed and dehulled lupin samples with different letters indicate significant difference ($P < 0.05$).
Večerek et al. (2008) studied the effect of dehulling (manual dehulling) on ash contents in nine cultivars from three lupin species (*L. albus*, *L. angustifolius* and *L. luteus*). The authors reported that dehulling had no significant effect on ash contents in lupins.

### 4.2.8 Minerals

Cultivation year had no significant effect on calcium, iron, magnesium, potassium and zinc contents in lupin (Table 32). This could be due to all cultivars were grown in the same area with similar agricultural practices. Moreover, the climatic conditions of the cultivated area between the three cultivation years were similar (Figure 21). Therefore, only the effect of cultivar and dehulling on the minerals contents will be discussed in this section.

#### Table 32: Calcium, iron, magnesium, potassium and zinc contents in lupins grown in different cultivation years

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sample</th>
<th>Cultivation year</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2011</td>
<td>2012</td>
<td>2013</td>
<td></td>
</tr>
<tr>
<td>Calcium</td>
<td>Whole seed</td>
<td>266.45 ± 38.10a</td>
<td>268.17 ± 42.28a</td>
<td>266.97 ± 71.42a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dehulled seed</td>
<td>100.38 ± 22.96a</td>
<td>93.09 ± 20.66a</td>
<td>90.18 ± 28.16a</td>
<td></td>
</tr>
<tr>
<td>Iron</td>
<td>Whole seed</td>
<td>2.92 ± 0.75a</td>
<td>2.70 ± 0.78a</td>
<td>2.61 ± 0.56a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dehulled seed</td>
<td>3.38 ± 0.87a</td>
<td>3.12 ± 0.94a</td>
<td>3.14 ± 1.00a</td>
<td></td>
</tr>
<tr>
<td>Magnesium</td>
<td>Whole seed</td>
<td>136.96 ± 14.10a</td>
<td>136.14 ± 23.03a</td>
<td>146.32 ± 18.00a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dehulled seed</td>
<td>153.97 ± 17.60a</td>
<td>152.44 ± 17.87a</td>
<td>167.59 ± 25.08a</td>
<td></td>
</tr>
<tr>
<td>Potassium</td>
<td>Whole seed</td>
<td>670.94 ± 127.11a</td>
<td>705.58 ± 96.68a</td>
<td>660.41 ± 118.75a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dehulled seed</td>
<td>1151.21 ± 190.34a</td>
<td>1133.02 ± 244.15a</td>
<td>1081.75 ± 285.76a</td>
<td></td>
</tr>
<tr>
<td>Zinc</td>
<td>Whole seed</td>
<td>3.13 ± 1.14a</td>
<td>3.53 ± 1.51a</td>
<td>3.70 ± 1.79a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dehulled seed</td>
<td>3.91 ± 0.74a</td>
<td>3.78 ± 0.66a</td>
<td>3.75 ± 0.73a</td>
<td></td>
</tr>
</tbody>
</table>

For mean ± SD, N = 18 for 2011, N = 20 for 2012 and 2013. Mean values with the same superscript in the same row are not significantly different (*P* ≥ 0.05).

#### 4.2.8.1 Calcium

#### 4.2.8.1.1 Method validation

Calibration curve of calcium at 5, 10, 20, 30 and 50 µg/mL was used to calculate the concentration of calcium. To determine the percentage of recovery as an accuracy of the determination, 10 µg/mL of calcium was added. The recoveries of calcium were ranged from 96.8–103.7% which was in the acceptance criteria of 80–120% (APVMA
Repeatability was assessed by inter-day determining six replications of the known concentration of calcium. The percentage of relative standard deviation ranged from 5.3–7.4% which was in the acceptable range of less than 10% (APVMA 2004). The coefficient of determination ($R^2$) of calibration curve between absorbance and calcium concentration was 0.99 (Figure 35).

\[
y = 0.0061x + 0.0033
\]

\[R^2 = 0.9995\]

**Figure 35: Calibration curve of calcium**

### 4.2.8.1.2 Calcium contents

The average calcium contents in whole seed and dehulled lupin were 267.22 and 94.35 mg/100 g DM, respectively (Table 33). Trugo et al. (1993) recorded that calcium contents in whole seed of *L. albus* from Brazil and *L. angustifolius* from Australia ranged from 134–198 and 168–225 mg/100 g DM, respectively. Bhardwaj et al. (1998) found that the average calcium content in *L. albus* grown in the USA was 340 mg/100 g seeds. A study by Porres et al. (2006) showed that calcium content in *L. albus* was 139 mg/100 g DM. Whole seeds of *L. albus* cultivated in Sudan had average calcium content of 173 mg/100 g DM (Omer et al. 2016). The average calcium contents in whole and dehulled seeds of *L. angustifolius* of 211 and 84 mg/100 g, respectively were reported by FSANZ (2015).
Table 33: Calcium contents in whole seeds and dehulled seeds of lupin cultivars

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Calcium content (mg/100 g DM)</th>
<th>Whole seeds</th>
<th>Dehulled seeds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>296.32 ± 24.87^b</td>
<td>121.63 ± 11.67^a</td>
</tr>
<tr>
<td>Belara</td>
<td></td>
<td>280.66 ± 57.74^b</td>
<td>88.93 ± 8.95^b</td>
</tr>
<tr>
<td>Corumup</td>
<td></td>
<td>371.73 ± 48.23^a</td>
<td>86.61 ± 11.94^bc</td>
</tr>
<tr>
<td>Gungurru</td>
<td></td>
<td>253.85 ± 7.56^bc</td>
<td>119.96 ± 15.81^a</td>
</tr>
<tr>
<td>Jenabillup</td>
<td></td>
<td>251.49 ± 26.91^bc</td>
<td>90.66 ± 8.96^b</td>
</tr>
<tr>
<td>Mandelup</td>
<td></td>
<td>201.58 ± 25.30^c</td>
<td>63.45 ± 9.46^c</td>
</tr>
<tr>
<td>PBA Barlock</td>
<td></td>
<td>245.95 ± 25.00^bc</td>
<td>73.50 ± 19.50^bc</td>
</tr>
<tr>
<td>PBA Gunyidi</td>
<td></td>
<td>247.40 ± 20.99^bc</td>
<td>127.98 ± 10.70^a</td>
</tr>
<tr>
<td>Quilinock</td>
<td></td>
<td>249.16 ± 28.02^bc</td>
<td>77.16 ± 8.32^bc</td>
</tr>
<tr>
<td>Tanjil</td>
<td></td>
<td>277.52 ± 9.56^b</td>
<td>93.32 ± 13.39^b</td>
</tr>
<tr>
<td>Walan 2385</td>
<td></td>
<td>267.22 ± 52.24</td>
<td>94.35 ± 24.12</td>
</tr>
</tbody>
</table>

Range

|               | 201.58–371.73 | 63.45–127.98 |

For mean ± SD, N = 6 except Walan 2385 N = 4. Mean values with different superscript in the same column are significantly different (P < 0.05).

4.2.8.1.3 Effect of cultivar

Calcium content was significantly affected by the lupin cultivar. In whole seed samples, the highest calcium content of 371 mg/100 g DM was found in Gungurru (Table 33). In dehulled seed samples, Belara, Jenabillup and Quilinock had higher calcium contents compared to other cultivars. Mandelup which is one of the most commonly grown ASL cultivars had 32% less calcium content compared to Gungurru.

Calcium contents in lupin found in the study were lower than or similar to values reported in previous studies (Bartkiene et al. 2016; Straková et al. 2006). Bhardwaj et al. (1998) studied the effect of cultivar on calcium contents in 12 cultivars of *L. albus* grown in the USA. The calcium contents were ranged from 270–400 mg/100 g. However, the authors have not reported the statistical differences between cultivars. Večerek et al. (2008) reported calcium contents in nine cultivars of three lupin species; *L. albus* (Amiga, LAL and Ole2ka), *L. angustifolius* (APR 82, Boruta and Probor), and *L. luteus* (Boregine, Bormal and Wodjil). The average calcium contents in whole seeds
and dehulled seeds were 480 and 310 mg/100 g, respectively. The authors have not reported the source of lupin seeds (i.e. growing area or market) and statistical difference between cultivars.

Straková et al. (2006) studied calcium contents in whole seeds of 12 lupin cultivars (Amiga, Anda, Atu, Boruta, Butan, Dieta, Juno, Karo, Prima, Rose, Sonet and Watt) grown in Europe and found that calcium contents ranged from 229 mg/100 g DM in Karo to 510 mg/100 g DM in Butan. Bartkiene et al. (2016) reported a significant difference in calcium contents in six cultivars of *L. angustifolius* from Lithuanian research centre for agriculture and forestry (ranged from 165 to 253 mg/100g DM).

### 4.2.8.1.4 Effect of dehulling

Dehulling decreased calcium contents in all lupin cultivars (Figure 36). The maximum reduction in calcium content after dehulling was reported in Gungurru. Večerek et al. (2008) reported that dehulling resulted in significant decreases in calcium contents in nine lupin cultivars. In contrast, Hassan et al. (2005) reported a significant increase in calcium content of *L. termis* seeds after wet dehulling (soaking seeds before dehulling).

![Figure 36: Effect of dehulling on calcium contents in lupin cultivars](image)

For mean values, N = 6 except Walan 2385 N = 4. Error bar shows the standard deviation of the means. Mean values between whole seed and dehulled lupin samples with different letters indicate significant difference (*P* < 0.05).
The effect of dehulling on calcium contents has been reported in other grain legumes. Saharan et al. (2001) found that wet dehulling caused a decrease in calcium content in faba beans from 201 to 179 mg/100 g DM. Duhan et al. (2002) reported a decrease in calcium content (25% decreases) in pigeon pea after wet dehulling. Wang et al. (2009) reported a significant decrease in calcium contents in eight lentil cultivars after dry dehulling. Moraghan et al. (2006) studies the accumulation of calcium in seeds of common beans and soybeans. The authors found that hull of common beans and soybeans contained significantly higher calcium contents than those of kernels.

4.2.8.2 Iron

4.2.8.2.1 Method validation

A calibration curve was prepared by using iron at 1, 2, 3, 4 and 5 µg/mL to determine the iron content. The percentage of recovery was determined by adding a sample with 2 µg/mL of iron. The iron recovery was ranged from 90.1 to 98.5% which represented acceptable accuracy of the determination (APVMA 2004). Inter-day determinations of six replications of the known concentration of iron were applied to calculate the percentage of relative standard deviation as repeatability. The percentage of relative standard deviation of iron determination ranged from 2.9–7.2 which in the acceptable level of less than 10% (APVMA 2004). Calibration curves between absorbance and iron concentration showed coefficient of determination ($R^2$) of 0.99 (Figure 37).

Figure 37: Calibration curve of iron

\[
y = 0.149x - 0.002 \\
R^2 = 0.999
\]
4.2.8.2.2 Iron content

Iron contents in whole and dehulled lupin samples are shown in Table 34. The average iron contents in whole and dehulled seeds were 2.74 and 3.21 mg/100 g DM, respectively. Iron contents found in lupin samples in the present study were similar to or lower than the values reported by Hassan et al. (2005), Krejpcio et al. (2006), and Yorgancilar and Bilgiçli (2014). FSANZ (2015) has reported iron contents in whole seed and dehulled Australian sweet lupin at 3.9 and 4.3 mg/100 g, respectively. According to a study conducted by Hassan et al. (2005), the average iron content in whole seeds of *L. termis* at 2.7 mg/100 g. Yorgancilar and Bilgiçli (2014) found an iron content in whole seed of *L. albus* at 4.3 mg/100 g. Iron contents in whole seeds of *L. albus* and *L. angustifolius* from Poland were 3.8 and 4.8 mg/100 g DM, respectively (Krejpcio et al. 2006). High average iron content at 12.51 mg/100 g DM of *L. albus* collected from markets in Ethiopia was reported by Tizazu and Emire (2010).

Table 34: Iron contents in whole seeds and dehulled seeds of lupin cultivars

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Iron content (mg/100 g DM)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole seed</td>
<td>Dehulled seed</td>
<td></td>
</tr>
<tr>
<td>Belara</td>
<td>3.92 ± 0.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.76 ± 0.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Corumup</td>
<td>2.14 ± 0.29&lt;sup&gt;de&lt;/sup&gt;</td>
<td>3.36 ± 0.23&lt;sup&gt;bc&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Gungurru</td>
<td>2.31 ± 0.12&lt;sup&gt;cde&lt;/sup&gt;</td>
<td>1.82 ± 0.18&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Jenabillup</td>
<td>2.57 ± 0.30&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>3.59 ± 0.41&lt;sup&gt;bc&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Mandelup</td>
<td>3.10 ± 0.25&lt;sup&gt;bd&lt;/sup&gt;</td>
<td>2.05 ± 0.34&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>PBA Barlock</td>
<td>2.46 ± 0.46&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.89 ± 0.50&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>PBA Gunyidi</td>
<td>2.75 ± 0.16&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>3.65 ± 0.34&lt;sup&gt;bc&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Quilinock</td>
<td>3.72 ± 0.30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.36 ± 0.27&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Tanjil</td>
<td>2.29 ± 0.27&lt;sup&gt;cde&lt;/sup&gt;</td>
<td>3.08 ± 0.16&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Walan 2385</td>
<td>1.81 ± 0.37&lt;sup&gt;e&lt;/sup&gt;</td>
<td>3.66 ± 0.58&lt;sup&gt;bc&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>2.74 ± 0.70</strong></td>
<td><strong>3.21 ± 0.93</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td><strong>1.81–3.92</strong></td>
<td><strong>1.82–4.76</strong></td>
<td></td>
</tr>
</tbody>
</table>

For mean ± SD, N = 6 except Walan 2385 N = 4. Mean values with different superscript in the same column are significantly different (*P* < 0.05).
4.2.8.2.3 Effect of cultivar

The highest iron contents in whole seed samples were found in Belara (3.92 mg/100 g DM) and Quilinock (3.72 mg/100 g DM). Low level of iron in whole seed sample was recorded in Walan 2385 (1.81 mg/100 g DM). In dehulled lupin samples, Belara had the highest iron content (4.76 mg/100 g DM), while the lowest iron levels were found in Gungurru, Mandelup and Quilinock (Table 34).

A few studies have reported the effect of cultivar on iron content in lupin. Bartkiene et al. (2016) reported that cultivar had a significant effect on iron content in lupin. The authors found that the iron contents in whole seeds of seven *L. angustifolius* cultivars from Lithuania (research centre for agriculture and forestry) ranged from 5.3–6.4 mg/100 g DM. Bhardwaj et al. (1998) determined the effect of cultivar on iron contents in twelve *L. albus* cultivars (Kali, Kalina, L1027N, L127N, L133N, L139N, L251N, L389N, PI469095, PI481545, PI483074 and Ultra) grown in the USA. The average iron content in whole seeds of 12 lupin cultivars was 11.8 mg/100 g and ranged from 7.4–18.9 mg/100 g. However, significant differences between cultivars have not been reported.

4.2.8.2.4 Effect of dehulling

The effect of dehulling on iron contents in lupin cultivars is shown in Figure 38. Dehulling significantly increased iron contents in Belara, Coromup, Jenabillup, PBA Barlock, PBA Gunyidi, Tanjil and Walan 2385. In contrast, dehulling resulted in decrease in iron contents in Gungurru, Mandelup and Quilinock indicating the effect of dehulling on iron contents depends on the lupin cultivar.

Limited information on the effect of dehulling, especially dry dehulling, on iron content in lupin has been published. Hassan et al. (2005) reported that wet dehulling caused significant increases in iron contents in two lupin cultivars (*L. termis* cv. Dongola and Golo). The lupin seeds were obtained from a local market in Khartoum, Sudan. Lupin seeds were soaked in distilled water for three days, water was changed every 8 h, and hulls were removed manually. Suliburska et al. (2009) reported the discrepant results of the effect of dehulling on iron contents in different lupin species. Dehulling significantly decreased iron contents in *L. luteus* and *L. albus* from 8.72 to 5.25 and 3.66 to 2.84 mg/100 g DM, respectively. In contrast, dehulling had no
significant effect on iron content in *L. angustifolius* (4.80 mg/100 g DM in whole seeds and 4.10 mg/100 g DM in dehulled seeds). However, the authors have not specified the dehulling technique.

![Figure 38: Effect of dehulling on iron contents in lupin cultivars](image)

For mean values, N = 6 except Walan 2385 N = 4. Error bar shows the standard deviation of the means. Mean values between whole seed and dehulled lupin samples with different letters indicate significant difference (*P* < 0.05).

### 4.2.8.3 Magnesium

#### 4.2.8.3.1 Method validation

Calibration curve for magnesium determination was prepared by using magnesium standard at 0.1, 0.2, 0.3, 0.4 and 0.5 µg/mL. The accuracy test was performed by spiking samples with known amounts of magnesium (0.2 µg/mL). The recoveries of magnesium ranged from 92.7–103.5% complying with the acceptance criteria (APVMA 2004). Inter-day determinations of six replications of the known concentrations of magnesium were performed to assess the repeatability. The percentage of relative standard deviation of magnesium ranged from 0.9–4.0% which was in the acceptable range (APVMA 2004). The calibration curve between absorbance and magnesium concentration showed *R*² of 0.99 (Figure 39).
4.2.8.3.2 Magnesium content

The average magnesium content in whole lupin was 139.90 mg/100 g DM and ranged from 114.51–164.94 mg/100 g DM. Dehulled lupin recorded average magnesium content of 158.14 mg/100 g DM and ranged from 126.06–174.10 mg/100 g DM (Table 35). Similar magnesium contents in lupin have been reported by FSANZ (2015), Porres et al. (2006), and Yorgancilar and Bilgiçli (2014). Porres et al. (2006) showed that magnesium content in whole seeds of *L. albus* was 145 mg/100 g DM. Yorgancilar and Bilgiçli (2014) found that the magnesium contents in whole seeds of bitter and sweet *L. albus* were 190 and 160 mg/100 g DM, respectively. Magnesium contents in whole seeds and dehulled seeds of Australian sweet lupin of 171 and 189 mg/100 g were reported by FSANZ (2015).

Studies conducted by Bhardwaj et al. (1998) and Bartkiene et al. (2016) showed higher magnesium contents in lupin than that of lupin used in the study. The average magnesium content in whole seeds of *L. albus* in the USA was 260 mg/100 g (Bhardwaj et al. 1998). Whole seeds of *L. luteus* and *L. angustifolius* from Lithuania had magnesium content of 344 and 230 mg/100 g DM, respectively (Bartkiene et al. 2016).
Table 35: Magnesium contents in whole and dehulled seeds of lupin cultivars

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Magnesium content (mg/100 g DM)</th>
<th>Whole seed</th>
<th>Dehulled seed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belara</td>
<td>164.94 ± 10.64&lt;sup&gt;a&lt;/sup&gt;</td>
<td>162.21 ± 15.62&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Corumup</td>
<td>140.45 ± 17.29&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>174.10 ± 20.89&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Gungurru</td>
<td>123.77 ± 14.25&lt;sup&gt;c&lt;/sup&gt;</td>
<td>162.44 ± 10.70&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Jenabillup</td>
<td>143.52 ± 16.16&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>164.01 ± 22.88&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Mandelup</td>
<td>136.29 ± 16.38&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>164.06 ± 26.55&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>PBA Barlock</td>
<td>128.38 ± 12.76&lt;sup&gt;c&lt;/sup&gt;</td>
<td>152.78 ± 20.61&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>PBA Gunyidi</td>
<td>140.22 ± 5.05&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>144.32 ± 15.50&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Quilinock</td>
<td>159.87 ± 19.58&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>148.54 ± 6.34&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Tanjil</td>
<td>138.62 ± 6.94&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>172.18 ± 23.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Walan 2385</td>
<td>114.51 ± 16.23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>126.06 ± 10.67&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>139.90 ± 19.14</td>
<td>158.14 ± 21.37</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>114.51–164.94</td>
<td>126.06–174.10</td>
<td></td>
</tr>
</tbody>
</table>

For mean ± SD, N = 6 except Walan 2385 N = 4. Mean values with different superscript in the same column are significantly different (P < 0.05).

4.2.8.3.3 Effect of cultivar

Cultivar had a significant effect (P < 0.05) on magnesium content. In whole seed samples, high magnesium level was recorded in Belara. Low magnesium contents were found in Gungurru, PBA Barlock and Walan 2385. Similar magnesium contents were found among cultivars except Coromup and Tanjil had higher magnesium contents than that of Walan 2385 (Table 35).

Bartkiene et al. (2016) found a significant effect of cultivar on magnesium content in *L. angustifolius*. The magnesium contents in whole seeds of seven cultivars of *L. angustifolius* from Lithuania were ranged from 200–248 mg/100 g DM. The magnesium content was ranged from 230–290 mg/100 g based on a study conducted by Bhardwaj et al. (1998) using 12 cultivars of *L. albus* grown in the USA. However, the statistical differences between cultivars have not been indicated.
4.2.8.3.4 Effect of dehulling

The effect of dehulling on magnesium contents showed significant differences among cultivars (Figure 40). Dehulling had no significant effect on magnesium contents in Belara, Jenabilup, Mandelup, PBA Gunyidi and Quilinock. The magnesium contents in Coromup, Gungurru, PBA Barlock, Tanjil and Walan 2385 decreased after dehulling.

Večerek et al. (2008) reported that dehulling of lupin seeds had no significant effect on magnesium content. Nine cultivars of three lupin species (*L. albus, L. angustifolius* and *L. luteus*) had average magnesium contents of 235 mg/100 g DM in whole seeds and 270 mg/100 g DM in dehulled seeds. Porres et al. (2007) studied the distribution of magnesium in different parts of seeds (embryo, cotyledon and hull) of three lupin species (*L. albus* var. multolupa, *L. angustifolius* var. troll and *L. luteus* var. 4492). The authors found higher magnesium levels in cotyledon than those of embryo and hulls.

Figure 40: Effect of dehulling on magnesium contents in lupin cultivars

For mean values, N = 6 except Walan 2385 N = 4. Error bar shows the standard deviation of the means. Mean values between whole seed and dehulled lupin samples with different letters indicate significant difference ($P < 0.05$).
4.2.8.4 Potassium

4.2.8.4.1 Method validation

Calibration curve for potassium standard at 10, 20, 30, 40 and 50 µg/mL was used to calculate the concentration of potassium. The accuracy of the determination was assessed using the recovery test by spiking samples with 20 µg/mL of potassium. The recoveries of potassium were ranged from 98.8–105.6% which was in the acceptable range of 80–120% (APVMA 2004). Inter-day determinations of six replications of the known concentrations of potassium were used to assess the repeatability. The percentage of relative standard deviation representing repeatability was ranged from 0.5–3.0% which was in the acceptable range of less than 10% (APVMA 2004). The calibration curve between absorbance and potassium concentration showed $R^2 > 0.99$ (Figure 41).

![Figure 41: Calibration curve of potassium](image)

4.2.8.4.2 Potassium content

Potassium contents in whole seed and dehulled lupin samples are shown in Table 36. The average potassium content was 679.25 mg/100 g DM and ranged from 580.74–798.31 mg/100 g DM in whole seeds. Dehulled lupin had an average potassium content of 1120.98 mg/100 g DM and ranged from 860.15–1488.88 mg/100 g DM.
The average potassium content in whole seeds of *L. albus* and *L. angustifolius* grown in Lithuania at 1323 mg/100 g DM was reported by Bartkiene et al. (2016). Yorgancilar and Bilgiçli (2014) showed potassium contents in whole seeds of bitter and sweet lupin (*L. albus*) of 760.1 and 1300.3 mg/100 g DM, respectively. According to FSANZ (2015), potassium contents in whole seeds and dehulled lupin were 570 and 730 mg/100 g, respectively. Similar or lower potassium contents in lupin samples were found in the present study compare to the previous studies.

Table 36: Potassium contents in whole and dehulled seeds of lupin cultivars

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Whole seed (mg/100 g DM)</th>
<th>Dehulled seed (mg/100 g DM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belara</td>
<td>580.74 ± 112.89&lt;sup&gt;c&lt;/sup&gt;</td>
<td>901.13 ± 147.89&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Corumup</td>
<td>754.40 ± 121.03&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>1297.90 ± 131.50&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gungurru</td>
<td>602.10 ± 115.22&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>967.08 ± 143.80&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Jenabillup</td>
<td>765.29 ± 107.03&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>957.51 ± 129.98&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mandelup</td>
<td>655.11 ± 39.37&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>1045.63 ± 80.98&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PBA Barlock</td>
<td>798.31 ± 128.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1324.50 ± 140.11&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PBA Gunyidi</td>
<td>669.74 ± 63.89&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>1488.88 ± 101.13&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Quilinock</td>
<td>591.86 ± 51.09&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>925.94 ± 100.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tanjil</td>
<td>671.56 ± 58.26&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>1354.16 ± 51.46&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Walan 2385</td>
<td>715.55 ± 97.61&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>860.15 ± 80.65&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td>679.25 ± 114.12</td>
<td>1120.98 ± 242.43</td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td>580.74–798.31</td>
<td>860.15–1488.88</td>
</tr>
</tbody>
</table>

For mean ± SD, N = 6 except Walan 2385 N = 4. Mean values with different superscript in the same column are significantly different (*P* < 0.05).

### 4.2.8.4.3 Effect of cultivar

Cultivar had a significant effect (*P* < 0.05) on potassium contents (Table 36). In whole seed samples, high level of potassium was found in PBA Barlock (798 mg/100 g DM) and low level of potassium was recorded in Belara (580 mg/100 g DM). Corumup, PBA Barlock, PBA Gunyidi and Tanjil had higher potassium levels than the other cultivars in dehulled lupin samples.
Bartkiene et al. (2016) reported that cultivar had a significant effect on potassium contents in *L. angustifolius*. The authors reported that an average potassium content in seven lupin cultivars from Lithuania was 1315 mg/100 g DM. Bhardwaj et al. (1998) studied the effect of cultivar on potassium content in *L. albus* cultivated in the USA. The authors found that the average potassium content in whole seeds of 12 lupin cultivars was 1060 mg/100 g. Although the authors stated that they studied the effect of cultivar, the statistical differences in potassium contents among lupin cultivars have not been reported.

### 4.2.8.4.4 Effect of dehulling

Figure 42 demonstrates the effects of dehulling on potassium contents in different lupin cultivars. Dehulling significantly increased potassium contents in all lupin cultivars except Walan 2385. The results indicated that potassium is mainly deposited in the kernel and not in the hull of lupin.

![Figure 42: Effect of dehulling on potassium contents in lupin cultivars](image)

For mean values, N = 6 except Walan 2385 N = 4. Error bar shows the standard deviation of the means. Mean values between whole seed and dehulled lupin samples with different letters indicate significant difference ($P < 0.05$).

Hassan et al. (2005) found that wet dehulling significantly increased potassium contents in two lupin cultivars (*L. termis* cv. Dongola and Golo). Porres et al. (2007) studied the distribution of potassium in seeds of two Spanish lupin species (*L. albus*...
var. multolupa, and L. luteus var. 4492) and L. angustifolius var. troll from Poland. The authors found that hulls of the three lupin species contain lower potassium contents than those of cotyledon. The potassium contents as a percentage of whole seeds in the hulls of L. albus, L. angustifolius and L. luteus were 11.3, 19.7 and 12.2, respectively.

4.2.8.5 Zinc
4.2.8.5.1 Method validation
To calculate the concentration of zinc, a calibration curve was prepared by using zinc standard at 0.4, 0.6, 0.8, 1.0 and 1.5 µg/mL. The accuracy test was performed by spiking samples with known amounts of zinc (0.6 µg/mL). The recoveries of zinc ranged from 90.6–94.8% complying with the acceptance criteria of 75–125% (APVMA 2004). Inter-day determinations of six replications of the known concentrations of zinc were performed to assess the repeatability. The percentage of relative standard deviation of zinc ranged from 6.3–8.9 % which was in the acceptable range of less than 20% (APVMA 2004). The calibration curve between absorbance and zinc concentration showed R² of 0.99 (Figure 43).

![Figure 43: Calibration curve of zinc](image_url)
4.2.8.5.2 Zinc content

Zinc contents in whole seed and dehulled lupin samples are shown in Table 37. The average zinc content was 3.46 mg/100 g DM and ranged from 1.40–5.09 mg/100 g DM in whole seed samples. Dehulled lupin had an average zinc content of 3.81 mg/100 g DM and ranged from 3.18–5.37 mg/100 g DM.

Lupin samples in the present study had zinc contents similar to or lower than the values reported by other researchers. Bartkiene et al. (2016) reported that the average zinc content in whole seeds of two lupin species (*L. luteus* and *L. angustifolius*) was 4.39 mg/100 g DM. Whole seeds and dehulled seeds of Australian sweet lupin had zinc contents of 3.4 and 3.6 mg/100 g (FSANZ 2015). Yorgancilar and Bilgiçli (2014) reported zinc contents in whole seeds of bitter and sweet *L. albus* of 7.4 and 7.6 mg/100 g DM, respectively. The average zinc content of 6.8 mg/100 g in whole seeds of *L. albus* was reported by Bhardwaj et al. (1998).

Table 37: Zinc contents in whole and dehulled seeds of lupin cultivars

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Zinc content (mg/100 g DM)</th>
<th>Whole seed</th>
<th>Dehulled seed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belara</td>
<td></td>
<td>3.78 ± 1.90&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>3.58 ± 0.41&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Corumup</td>
<td></td>
<td>4.98 ± 0.62&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.17 ± 0.17&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gungurru</td>
<td></td>
<td>5.09 ± 0.52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.18 ± 0.50&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Jenabillup</td>
<td></td>
<td>2.23 ± 0.42&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>3.57 ± 0.10&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mandelup</td>
<td></td>
<td>2.39 ± 0.67&lt;sup&gt;bd&lt;/sup&gt;</td>
<td>3.50 ± 0.24&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>PBA Barlock</td>
<td></td>
<td>2.74 ± 0.39&lt;sup&gt;bd&lt;/sup&gt;</td>
<td>3.76 ± 0.33&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>PBA Gunyidi</td>
<td></td>
<td>1.40 ± 0.43&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.74 ± 0.26&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Quilinock</td>
<td></td>
<td>3.42 ± 0.87&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>5.37 ± 0.52&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tanjil</td>
<td></td>
<td>4.13 ± 1.51&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.77 ± 0.76&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Walan 2385</td>
<td></td>
<td>5.01 ± 0.57&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.31 ± 0.28&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td></td>
<td>3.46 ± 1.51</td>
<td>3.81 ± 0.70</td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td></td>
<td>1.40–5.09</td>
<td>3.18–5.37</td>
</tr>
</tbody>
</table>

For mean ± SD, N = 6 except Walan 2385 N = 4. Mean values with different superscript in the same column are significantly different (*P* < 0.05).
4.2.8.5.2 Effect of cultivar
Zinc contents in lupin cultivars are shown in Table 37. Cultivar had a significant effect ($P < 0.05$) on zinc contents. High levels of zinc in whole seed samples were recorded in Coromup, Gungurru and walan 2385, while low levels were found in PBA Gunyidi. The highest zinc content in dehulled seed samples was found in Quilinock (5.37 mg/100 g DM).

Bartkiene et al. (2016) found a significant effect of cultivar on zinc content in whole seeds of *L. angustifolius* grown in Lithuania. Seven cultivars of *L. angustifolius* had average zinc contents of 4.16 mg/100 g DM and ranged from 3.52–5.37 mg/100 g DM. Bhardwaj et al. (1998) studied the effect of cultivar on zinc contents of 12 cultivars of *L. albus* cultivated in the USA. However, statistical differences between cultivars have not been reported. The average zinc content was 6.8 mg/100 g and ranged from 5.5–7.8 mg/100 g.

4.2.8.5.3 Effect of dehulling
The effect of dehulling on zinc contents showed significant differences between cultivars (Figure 44). Dehulling had no effect on zinc contents in Belara and Tanjil. The zinc contents in Coromup and Gungurru decreased after dehulling. In contrast, zinc contents in Jenabillup, Mandelup, PBA Barlock, PBA Gunyidi and Quilinock were increased as a result of dehulling.

Suliburska et al. (2009) reported that dehulling (the dehulling technique was not specified) significantly increased zinc contents in three lupin species. After dehulling, the zinc contents were increased from 5.18 to 5.43 mg/100 g DM in *L. albus*, from 4.84 to 5.02 mg/100 g DM in *L. angustifolius* and from 8.26 to 10.46 mg/100 g DM in *L. luteus*. Hassan et al. (2005) found that the effects of wet dehulling on zinc contents in *L. termis* were discrepant among different cultivars. There was a significant decrease in zinc content in *L. termis* cv. Dongola after wet dehulling. In contrast, wet dehulling significantly increased zinc content in *L. termis* cv. Golo.
For mean values, \( N = 6 \) except Walan 2385 \( N = 4 \). Error bar shows the standard deviation of the means. Mean values between whole seed and dehulled lupin samples with different letters indicate significant difference \( (P < 0.05) \).

4.3 Bioaccessibility of minerals

The effects of lupin cultivars and dehulling on mineral bioaccessibility were evaluated. The possibility of using phytate to mineral molar ratio as a predictor of mineral bioaccessibility was also investigated. Simple linear regression was performed to determine the correlations between mineral bioaccessibility and minerals (calcium, iron, magnesium and zinc) and anti-nutritional factors (RFOs, phytate, polyphenols, flavonoids and condensed tannins). A stepwise multiple regression analysis was conducted to predict mineral bioaccessibility using combined effects of the minerals and anti-nutritional factors.

To study the correlations, only minerals with di-valent cations (calcium, iron, magnesium and zinc) were included, since these minerals can form complexes with phytate \( (\text{Konietzny & Greiner 2003}) \) and act as mineral competitors \( (\text{Gibson 2007}) \) which affect the mineral bioaccessibility. Anti-nutritional factors (phytate, RFOs, polyphenols, flavonoids and condensed tannins) were used in correlation analyses. The minerals, RFOs and phytate contents in raw lupin samples were used, since the heating process does not change the contents (dry matter) of these compounds. However, total
phenolics, total flavonoids and condensed tannin contents in heat treated lupin samples were used for calculating correlations.

### 4.3.1 Bioaccessibility of calcium

The bioaccessibility values of calcium in heat treated whole and dehulled lupin samples of different cultivars are presented in Table 38. The average calcium bioaccessibility of whole lupin was 6.02% and ranged from 3.66% to 7.02%. Dehulled lupin had average calcium bioaccessibility of 10.59% and ranged from 7.29–13.64%.

Among whole seed samples, there were no significant differences in calcium bioaccessibility between cultivars except between PBA Gunyidi and Gungurru. In dehulled lupin, PBA Gunyidi, PBA Barlock and Coromup were some of the cultivars high in calcium bioaccessibility. Quilinock, Jenabillup and Belara were some of the cultivars having low calcium bioaccessibility. The low calcium bioaccessibility of lupin samples indicates that calcium in ASL is bound to other compounds resulting in less accessible.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Bioaccessibility of calcium (%)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole seeds</td>
<td>Dehulled seeds</td>
<td></td>
</tr>
<tr>
<td>Belara</td>
<td>5.75 ± 1.17&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>9.22 ± 0.82&lt;sup&gt;bd&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Corumup</td>
<td>6.17 ± 1.41&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>12.06 ± 0.87&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Gungurru</td>
<td>3.66 ± 0.66&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.70 ± 2.75&lt;sup&gt;abc&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Jenabillup</td>
<td>6.53 ± 1.07&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>7.85 ± 1.16&lt;sup&gt;cd&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Mandelup</td>
<td>6.30 ± 2.00&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>9.71 ± 1.48&lt;sup&gt;ad&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>PBA Barlock</td>
<td>6.44 ± 1.00&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>12.41 ± 3.58&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>PBA Gunyidi</td>
<td>7.02 ± 1.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.64 ± 2.64&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Quilinock</td>
<td>6.40 ± 2.16&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>7.29 ± 1.33&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Tanjil</td>
<td>5.97 ± 1.83&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>11.49 ± 2.10&lt;sup&gt;abc&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Walan 2385</td>
<td>5.90 ± 2.14&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>10.45 ± 2.91&lt;sup&gt;ad&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>6.02 ± 1.64</strong></td>
<td><strong>10.59 ± 2.79</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td><strong>3.66–7.02</strong></td>
<td><strong>7.29–13.64</strong></td>
<td></td>
</tr>
</tbody>
</table>

For mean ± SD, N = 6 except Walan 2385 N = 4. Mean values with different superscript in the same column are significantly different (P < 0.05).
Porres et al. (2005) reported an unusually high calcium bioaccessibility of 91.5% in whole lupin flour (*L. albus*) using the dialysability method (the same method as this study). The very high calcium bioaccessibility showed by the authors could be attributed to the use of higher molecular weight cutoff (12,000–14,000 Dalton) dialysis tubing which allows larger complexes pass through the dialysis membrane. In addition, lupin flour in the study by Porres et al. (2005) were ground and sieved resulting in particle size of less than 180 µm. The sieving process may have removed dietary fibre and other compounds which affect calcium bioaccessibility. In the present study, lupin samples were used for analyses without sieving.

Calcium bioaccessibility using different method (solubility) for whole seeds of *L. albus* (23%) and *L. angustifolius* (31.7%) were shown by Krejpcio et al. (2006). Hassan et al (2005) reported the availability (not the bioaccessibility) of calcium in lupin (*L. termis*) by using a different method. They extracted lupin samples with hydrochloric acid at 37 °C for 3 h, and determined calcium content before and after extraction to calculate calcium availability. The authors reported very high calcium availability (approximately 71%) of whole lupin.

Calcium bioaccessibility of other grain legumes (not lupin) using dialysability method (similar to the method followed in the present study) have been published. Sebastiá et al. (2001) reported that calcium bioaccessibility of raw seeds of chickpea, common bean and lentils were 20%, 15% and 16%, respectively. Kamchan et al. (2004) reported that calcium bioaccessibility of cooked soybean (seeds were soaked overnight and boiled) was 11.1%. Amalraj and Pius (2015) studied calcium bioaccessibility of various grain legumes commonly consumed in India. The authors reported calcium bioaccessibility of cooked seeds of black gram (25.4%), cowpea (34.7%), mung bean (32.0%), pigeon pea (37.0%) and soybean (27.1%). The calcium bioaccessibility values of lupin samples found in the present study were similar to or lower than those of other grain legumes found in the literature.

A few *in vivo* studies using rat models have been conducted to determine the calcium absorption (an indicator of bioavailability) of lupin incorporated diets. A study by Rubio et al. (1994) showed 39.8% calcium absorption in rats fed with seven grams of lupin meal incorporated diets for ten days. The lupin meal incorporated diet contained 36% of lupin meal and had 576 mg/100 g calcium content. The calcium absorption as
a percentage was calculated from the amount of calcium ingested and excreted. Porres et al. (2006) reported high calcium absorption at 66.2% in rats fed with lupin based diets. The diet contained 47.2 g of lupin flour and 508 mg of calcium per 100 g. It should be emphasised that the enzyme phytase is presented in the rat intestine; therefore, rat is not a suitable model for determining mineral bioavailability of phytate containing foods such as cereals and grain legumes (Iqbal et al. 1994).

4.3.1.1 Effect of dehulling on calcium bioaccessibility

Dehulling resulted in a significant increase in calcium bioaccessibility of all lupin cultivars except Quilinock (Figure 45). Whole lupin samples had significantly higher calcium contents but lower calcium bioaccessibility than those of dehulled samples. This could be an indication that lupin hulls contain higher contents of compounds such as cellulose, hemicellulose and pectin that may interfere with calcium bioaccessibility. The effect of wet dehulling on calcium availability of lupin has been reported by Hassan et al. (2005). They found a significant decrease in calcium availability of lupin (L. termis) as a result of wet dehulling.

Figure 45: Effect of dehulling on calcium bioaccessibility of lupin cultivars

For mean values, N = 6 except Walan 2385 N = 4. Error bar shows the standard deviation of the means. Mean values between whole seed and dehulled lupin samples with different letters indicates significant differences ($P < 0.05$).
The effects of dehulling on calcium bioaccessibility (dialysability method) of other pulses have been reported by Lombardi-Boccia et al. (1998). Dehulling increased calcium bioaccessibility of mottled and white beans. Calcium bioaccessibility of whole mottled bean was 13.8% and was increased to 24.2% after dehulling. Calcium bioaccessibility was increased from 14.3 to 28.2% as a result of dehulling of white bean. However, the authors did not mention the technique used for dehulling (e.g. wet or dry).

4.3.1.2 Phytate to calcium molar ratio as a predictor of calcium bioavailability

Phytate to calcium molar ratio is widely used as a predictor of calcium bioavailability. Phytate to calcium molar ratio of greater than 0.24 indicates poor calcium bioavailability (Dahiya et al. 2013; Lazarte et al. 2015; Ma et al. 2007).

Table 39 shows phytate to calcium molar ratios in whole and dehulled lupin cultivars. Whole lupin had an average phytate to calcium molar ratio of 0.19. Phytate to calcium molar ratios of whole seeds were not affected by the cultivar. Whole seed of all lupin cultivars had phytate to calcium molar ratio less than 0.24 indicating good calcium bioaccessibility. The molar ratios of phytate to calcium of lupin found in the present study were comparable to the results reported by Trugo et al. (1993). They found that phytate to calcium molar ratios of whole seeds of *L. albus* ranged from 0.2–0.3 and phytate to calcium molar ratios of *L. angustifolius* ranged from 0.2–0.4.

The average phytate to calcium molar ratio was increased from 0.19 to 0.51 as a result of dehulling (Table 39). Dehulled seed of all cultivars had no significant differences in phytate to calcium molar ratio, except Gungurru had a higher phytate to calcium molar ratio than those of Belara, Mandelup and PBA Gunyidi. Dehulled lupin of all cultivars had phytate to calcium molar ratio greater than 0.24 indicating poor calcium bioaccessibility.
Conflicting result on calcium bioaccessibility was found in the study. Based on the phytate to calcium molar ratio, dehulling reduced the calcium bioaccessibility. The calcium bioaccessibility values recorded from the dialysability method showed that dehulling increased calcium bioaccessibility (Figure 45).

The regression equation and coefficient of determination ($R^2$) from regression analysis between calcium bioaccessibility and phytate to calcium molar ratio of lupin is shown in Figure 46. Results show a trend of increasing calcium bioaccessibility with increasing phytate to calcium molar ratio, however, the degree of correlation is low ($R^2 = 0.39$). This suggests that phytate to calcium molar ratio is not a suitable predictor for calcium bioaccessibility of lupin. Other inhibitory factors of lupin may have a greater influence on calcium bioaccessibility than phytate.

Liang et al. (2010) studied calcium bioaccessibility of rice-based products using solubility method. The authors concluded that phytate to calcium molar ratio was not correlated to calcium bioaccessibility. In contrast, Dahiya et al. (2013) reported a
downward trend between calcium bioaccessibility (solubility method) and phytate to calcium molar ratio of mung beans. However, the authors have not reported coefficient of determination (a degree of correlation) between calcium bioaccessibility and phytate to calcium molar ratio.

**Figure 46: Linear regression between calcium bioaccessibility and phytate to calcium molar ratio**

4.3.1.3 Linear correlations between minerals, anti-nutritional factors and calcium bioaccessibility

Linear regression analyses were performed to determine the correlations between minerals, anti-nutritional factors and calcium bioaccessibility. Linear regression equations, correlation coefficients and correlation of determination of individual factors and calcium bioaccessibility of lupin are shown in Table 40. The highest coefficient of determination was found between the calcium bioaccessibility and calcium content ($R^2 = 0.62$) indicating calcium content is correlated to calcium bioaccessibility of lupin.

Poor correlations between calcium bioaccessibility and iron ($R^2 = 0.04$), magnesium ($R^2 = 0.06$), zinc ($R^2 < 0.01$), RFOs ($R^2 = 0.04$), phytate ($R^2 = 0.12$), polyphenols ($R^2 < 0.01$), flavonoids ($R^2 = 0.02$) and condensed tannin ($R^2 = 0.24$) in lupin samples were found (Table 40). Amalraj and Pius (2015) studied the linear correlations between calcium bioaccessibility and inhibitory factors (phytate, oxalate, dietary fibre and
tannin) of nine pulses commonly consume in India. Low degree of negative correlations were found between calcium bioaccessibility and phytate ($R^2 = 0.39$), oxalate ($R^2 = 0.31$), dietary fibre ($R^2 = 0.27$) and tannin ($R^2 = 0.07$).

Table 40: Linear regression equations, correlation coefficients and correlation of determination between factors influence calcium bioaccessibility

<table>
<thead>
<tr>
<th>Factors</th>
<th>Equation</th>
<th>$R$</th>
<th>$R^2$</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (g/100 g)</td>
<td>$y = 13.17 - 26.92x$</td>
<td>−0.79</td>
<td>0.63</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Iron (mg/100 g)</td>
<td>$y = 6.06 + 0.75x$</td>
<td>0.20</td>
<td>0.04</td>
<td>0.03</td>
</tr>
<tr>
<td>Magnesium (g/100 g)</td>
<td>$y = 3.14 + 34.17x$</td>
<td>0.24</td>
<td>0.06</td>
<td>0.01</td>
</tr>
<tr>
<td>Zinc (mg/100 g)</td>
<td>$y = 9.14 - 0.23x$</td>
<td>−0.08</td>
<td>&lt; 0.01</td>
<td>0.37</td>
</tr>
<tr>
<td>RFOs (g/100 g)</td>
<td>$y = 5.78 + 0.27x$</td>
<td>0.19</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>Phytate (g/100 g)</td>
<td>$y = 12.30 - 5.05x$</td>
<td>−0.34</td>
<td>0.12</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Polyphenols (mgGAE/100 g)</td>
<td>$y = 9.23 - 0.01x$</td>
<td>−0.06</td>
<td>&lt; 0.01</td>
<td>0.51</td>
</tr>
<tr>
<td>Flavonoids (mgCE/100 g)</td>
<td>$y = 9.71 - 0.04x$</td>
<td>−0.13</td>
<td>0.02</td>
<td>0.16</td>
</tr>
<tr>
<td>Condensed tannins (mgCE/100 g)</td>
<td>$y = 6.21 + 0.05x$</td>
<td>0.49</td>
<td>0.24</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

There was a negative correlation between calcium bioaccessibility and calcium content (Figure 47) indicating the higher calcium content results in a lower calcium bioaccessibility in lupin. The negative correlation between calcium bioaccessibility and calcium content is in agreement with the results of the study conducted by Cámara et al. (2005). Using an exponential model, they reported a strong negative correlation ($R = −0.91$, $R^2 = 0.83$) between calcium bioaccessibility (dialysability method) and calcium contents in thirteen dishes in Spain. Amalraj and Pius (2015) reported that pulses with high calcium contents (black gram, horse gram and soy bean) had low calcium bioaccessibility values. They also found that pulses with low calcium contents (green bean, green pea and red gram) had high calcium bioaccessibility values. However, the authors have not reported the correlations between bioaccessibility and calcium contents. A few studies conducted in human models reported a negative correlation between calcium intake and calcium absorption (Heaney et al. 1991; Ireland & Fordtran 1973).
4.3.1.4 Multiple regression between minerals, anti-nutritional factors and calcium bioaccessibility

A predictive equation to forecast calcium bioaccessibility using minerals and anti-nutritional factors was developed using stepwise multiple regression. Calcium, phytate and RFOs contents had negative correlations with calcium bioaccessibility (Predictive Equation 1, 2 & 3). The result showed that calcium content is the strongest predictive factor of calcium bioaccessibility in lupin.

By using Predictive Equation 1, calcium content can be used to explain 63% of the calcium bioaccessibility of lupin. Using calcium and phytate contents in the Predictive Equation 2 can determine 67% of the calcium bioaccessibility. Predictive Equation 3 indicates that calcium, phytate and RFOs contents can predict 69% of the calcium bioaccessibility. These Predictive Equations indicate that there are other factors affecting calcium bioaccessibility of lupin in addition to the factors studied. Dietary fibre and oxalate are examples of these factors.

\[
y = 13.17 - 26.92x_1 \quad (R^2 = 0.63)
\] Predictive Equation 1

\[
y = 15.38 - 25.69x_1 - 3.07x_2 \quad (R^2 = 0.67)
\] Predictive Equation 2

\[
y = 17.99 - 27.43x_1 - 3.44x_2 - 0.21x_3 \quad (R^2 = 0.69)
\] Predictive Equation 3
Where:
\[
\begin{align*}
  y & = \text{Calcium bioaccessibility (\%)} \\
  x_1 & = \text{Calcium content (g/100 g DM)} \\
  x_2 & = \text{Phytate content (g/100 g DM)} \\
  x_3 & = \text{RFOs content (g/100 g DM)}
\end{align*}
\]

Lack of published information on the factors affects the calcium bioaccessibility of lupin. However, a few studies (Jyothi lakshmi & Kaul 2011; Krishnan et al. 2012) have investigated the combined effect of nutrients and anti-nutritional factors on calcium bioaccessibility in other foods such as finger millet products and water melon seeds. Krishnan et al. (2012) developed a multiple regression equation to predict calcium bioaccessibility using phytate, polyphenol, soluble dietary fibre and insoluble dietary fibre of finger millet products showing high coefficient of determination (\(R^2 = 0.92\)). Jyothi lakshmi and Kaul (2011) found a high degree of multiple correlation (\(R^2 = 0.93\)) between calcium bioaccessibility and oxalate, condensed tannin and phytate of decorticated water melon seeds.

As reported by Jyothi lakshmi & Kaul (2011) and Krishnan et al. (2012) using multiple regressions, both dietary fibre and oxalate contents have influence on calcium bioaccessibility. However, Amalraj and Pius (2015) showed a poor correlation between calcium bioaccessibility and dietary fibre content (\(R^2 = 0.27\)), and between calcium bioaccessibility and oxalate content (\(R^2 = 0.31\)) of various grain legumes by using linear regressions.

Lupin seeds contain high amount of dietary fibre (43.6 g/100 g) (FSANZ 2015). Oxalate content in lupin has been reported only in one study conducted by Gad et al. (1982) showing oxalate content in whole lupin (\textit{L. termis}) was 0.17 g/100 g DM. The oxalate content in lupin was similar to or lower than other grain legumes; faba bean (0.56 g/100 g DM), lentil (0.40 g/100 g DM), pea (0.28 g/100 g DM) and chickpea (0.18 g/100 g DM) (Gad et al. 1982). Therefore, the effects of dietary fibre and oxalate contents on calcium bioaccessibility of ASL cultivars need to be investigated in future studies.
4.3.2 Bioaccessibility of iron

Table 41 shows the iron bioaccessibility values of whole and dehulled samples of different lupin cultivars. Whole lupin had an average iron bioaccessibility of 17.33% and values ranged from 10.30–25.87%. The average iron bioaccessibility of dehulled lupin was 20.89% and ranged from 12.55–35.23%. There were significant differences in iron bioaccessibility among some lupin cultivars. In whole seed samples, Walan 2385 and PBA Barlock were some of the cultivars with high iron bioaccessibility. Lower iron bioaccessibility values were found in Quilinock and Belara. In dehulled seed samples, the highest iron bioaccessibility was recorded in Gungurru. Low iron bioaccessibility values were found in some cultivars including Belara and PBA Barlock.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Bioaccessibility of iron (%)</th>
<th>Whole seeds</th>
<th>Dehulled seeds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belara</td>
<td>12.53 ± 0.89fg</td>
<td>12.55 ± 2.02c</td>
<td></td>
</tr>
<tr>
<td>Corumup</td>
<td>19.62 ± 4.39bd</td>
<td>19.47 ± 1.11cd</td>
<td></td>
</tr>
<tr>
<td>Gungurru</td>
<td>17.79 ± 3.40cde</td>
<td>35.23 ± 3.94a</td>
<td></td>
</tr>
<tr>
<td>Jenabillup</td>
<td>14.93 ± 1.52dg</td>
<td>19.05 ± 1.94cd</td>
<td></td>
</tr>
<tr>
<td>Mandelup</td>
<td>16.98 ± 3.14cdf</td>
<td>29.21 ± 1.72b</td>
<td></td>
</tr>
<tr>
<td>PBA Barlock</td>
<td>23.57 ± 3.10ab</td>
<td>15.33 ± 2.29de</td>
<td></td>
</tr>
<tr>
<td>PBA Gunyidi</td>
<td>14.46 ± 1.82efg</td>
<td>17.44 ± 3.15de</td>
<td></td>
</tr>
<tr>
<td>Quilinock</td>
<td>10.30 ± 1.39g</td>
<td>23.33 ± 3.03c</td>
<td></td>
</tr>
<tr>
<td>Tanjil</td>
<td>20.10 ± 2.33bce</td>
<td>19.84 ± 1.66cd</td>
<td></td>
</tr>
<tr>
<td>Walan 2385</td>
<td>25.87 ± 1.58a</td>
<td>15.71 ± 4.49de</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>17.33 ± 5.05</td>
<td>20.89 ± 7.06</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>10.30–25.87</td>
<td>12.55–35.23</td>
<td></td>
</tr>
</tbody>
</table>

For mean ± SD, N = 6 except Walan 2385 N = 4. Mean values with different superscript in the same column are significantly different (P < 0.05).

Iron bioaccessibility of lupin (not ASL) using the same in vitro method (dialysability) has been investigated. Lombardi-Boccia et al. (2003) found a low level of iron bioaccessibility of 7.8% for whole lupin collected from Italy. According to a study by Porres et al. (2005), whole lupin flour (L. albus, in Spain) had higher iron
bioaccessibility of 27.8%. The use of dialysis tubing with different molecular weight cutoff could be a reason for difference in iron bioaccessibility between studies.

A few researchers have conducted studies on iron bioaccessibility of lupin, but by using different methods. Krejpcio et al. (2006) reported that the iron bioaccessibility values of whole seeds of *L. albus* and *L. angustifolius* from Italy were 25.5% and 19.1%, respectively by using the solubility method. Suliburska et al. (2009) reported the iron bioaccessibility values (solubility method) of whole seeds of *L. albus* (18.43%), *L. angustifolius* (13.95%) and *L. luteus* (9.05%) grown in Poland. Hassan et al. (2005) reported high value of iron availability (not the bioaccessibility) of lupin seeds (*L. termis*) of 70% by using hydrochloric acid extraction.

The iron bioaccessibility values of other pulses using the same method followed by the current study have been reported. Hemalatha et al. (2007) showed that iron bioaccessibility values of chickpea, green gram, red gram, black gram and cow pea were 6.89, 2.25, 3.06, 2.76 and 1.77%, respectively. The result of a study conducted by Sebastiá et al. (2001) found that iron bioaccessibility of raw seeds of chickpea, common bean and lentils were 3.2, 3.2 and 4.8%, respectively. The iron bioaccessibility of other grain legumes are lower than that of lupin detected in the present study.

4.3.2.1 Effect of dehulling on iron bioaccessibility

The effects of dehulling on iron bioaccessibility of different lupin cultivars are shown in Figure 48. Dehulling had no significant effect on iron bioaccessibility of Belara, Coromup, PBA Gunyidi and Tanjil. As a result of dehulling, iron bioaccessibility values of Gungurru, Jenabillup, Mandelup and Quilinock were significantly increased. In contrast, a decrease in iron bioaccessibility as an effect of dehulling was found in PBA barlock and Walan 2385. The results clearly showed that the effect of dehulling on iron bioaccessibility greatly depends on the lupin cultivar.
Suliburska et al. (2009) studied the effect of dehulling on the iron bioaccessibility (solubility method) of different lupin species; however, they have not reported the technique used for dehulling (wet or dry). The authors found that dehulling significantly increased iron bioaccessibility of *L. luteus* but had no effect on *L. albus* and *L. angustifolius*. The results indicate that the effect of dehulling depends on lupin species. Hassan et al. (2005) showed that wet dehulling resulted in a significant decrease in iron availability (not bioaccessibility) of lupin (*L. termis*).

The effect of dehulling on iron bioaccessibility of other pulses (not lupin) has been reported. DellaValle et al. (2013) studied the effect of dry dehulling on iron bioaccessibility of lentils using Caco-2 cell culture model. They found that dehulling significantly increased iron bioaccessibility but decreased iron contents in lentils. The authors stated that the increase in iron bioaccessibility could be due to the removal of the polyphenols rich seed coat. Liu et al. (2015) used Caco-2 cell culture model and reported that the effect of dry dehulling on iron bioaccessibility depend on pea cultivar, increase in some and had no effect on others cultivars.
4.3.2.2 Phytate to iron molar ratio as a predictor of iron bioavailability

The molar ratio of phytate to iron is commonly used to predict the iron bioavailability (Gibson et al. 2010). The phytate to iron molar ratio greater than 1 is considered as an indicator of poor iron bioavailability (Dahiya et al. 2013; Ma et al. 2007).

Table 42 shows the phytate to iron molar ratios in whole and dehulled lupin. There was a significant difference in phytate to iron molar ratio of different lupin cultivars. Whole lupin samples had an average phytate to iron molar ratio of 26.66 and ranged from 15.58–39.51. In whole seed samples, Coromup, Gungurru and Walan 2385 were some of the cultivars high in phytate to iron molar ratio. Belara and Mandelup were some of the cultivars low in phytate to iron molar ratio. The average phytate to iron molar ratio in dehulled lupin samples was 23.33 and ranged from 10.20–45.74. The highest ratios (the lowest iron bioaccessibility) were found in Gungurru and Quilinock. PBA Barlock and PBA Gunyidi were some of the cultivars having low phytate to iron molar ratios. All tested lupin samples had phytate to iron molar ratio much higher than 1 indicating very poor iron bioavailability.

Although limited information on phytate to iron molar ratio of Australian grown *L. angustifolius* has been published, a few researchers have studied on phytate to iron molar ratio of other grains and legumes, such as mung bean, millet and sorghum. Dahiya et al. (2013) reported that the average phytate to iron molar of mung beans was 17. A study by Lestienne et al. (2005) showed the phytate to iron molar ratios for millet (5.3), maize (34.4), sorghum (22.8), rice (55.5), soybean (10.1), cowpea (7.8) and mung bean (2.8). Ma et al. (2005) found that phytate to molar ratios of oat flakes, millet and sorghum were 31.6, 17.5 and 38.6, respectively. Based on a study conducted by Luo et al. (2009), the phytate to iron molar ratio of faba bean was 20.2. Similar to lupin, the iron bioavailability values of other grains and legumes seem to be very poor since the phytate to iron molar ratios were greater than 1.
Table 42: Phytate to iron molar ratio in whole and dehulled seeds of lupin cultivars

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Phytate to iron molar ratio</th>
<th>Whole seeds</th>
<th>Dehulled seeds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belara</td>
<td>15.58 ± 3.13&lt;sup&gt;e&lt;/sup&gt;</td>
<td>13.21 ± 2.38&lt;sup&gt;cd&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Corumup</td>
<td>39.51 ± 8.52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.37 ± 5.57&lt;sup&gt;bd&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Gungurru</td>
<td>37.48 ± 6.73&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>45.74 ± 6.77&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Jenabillup</td>
<td>26.78 ± 5.02&lt;sup&gt;bce&lt;/sup&gt;</td>
<td>24.18 ± 3.09&lt;sup&gt;bd&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Mandelup</td>
<td>19.19 ± 2.58&lt;sup&gt;de&lt;/sup&gt;</td>
<td>27.88 ± 10.38&lt;sup&gt;bd&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>PBA Barlock</td>
<td>26.11 ± 11.48&lt;sup&gt;bce&lt;/sup&gt;</td>
<td>12.64 ± 1.84&lt;sup&gt;cd&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>PBA Gunyidi</td>
<td>20.26 ± 4.43&lt;sup&gt;ce&lt;/sup&gt;</td>
<td>10.20 ± 2.52&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Quilinock</td>
<td>20.87 ± 4.24&lt;sup&gt;ce&lt;/sup&gt;</td>
<td>39.04 ± 4.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Tanjil</td>
<td>29.57 ± 5.99&lt;sup&gt;acd&lt;/sup&gt;</td>
<td>17.27 ± 6.93&lt;sup&gt;bd&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Walan 2385</td>
<td>33.51 ± 8.48&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>22.48 ± 6.53&lt;sup&gt;bc&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td>26.66 ± 9.81</td>
<td>23.33 ± 12.40</td>
<td></td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td>15.58–39.51</td>
<td>10.20–45.74</td>
<td></td>
</tr>
</tbody>
</table>

For mean ± SD, N = 6 except Walan 2385 N = 4. Mean values with different superscript in the same column are significantly different (P < 0.05).

Figure 49 presents the regression equation and coefficient of determination (R²) between iron bioaccessibility and phytate to iron molar ratio of lupin samples. An increasing trend was found between iron bioaccessibility and phytate to iron molar ratio. Poor correlation (R² = 0.34) indicates that phytate to iron molar ratio is not a good predictor of the iron bioavailability of lupin.

Dahiya et al. (2013) showed that iron bioaccessibility (solubility method) was not correlated to phytate to the iron molar ratio of mung beans. Using rice based products, Liang et al. (2010) also reported that iron bioaccessibility (solubility method) was not correlated to the phytate to iron molar ratio. The authors indicated that mineral bioaccessibility of rice based products is not exclusively determined by the molar ratio of phytate to iron.
4.3.2.3 Linear correlations between minerals, anti-nutritional factors and iron bioaccessibility

To determine the correlations between minerals, anti-nutritional factors and iron bioaccessibility, linear regression analyses were employed. Table 43 shows linear regression equations, correlation coefficients and correlation of determination of individual factors and iron bioaccessibility of lupin. The highest coefficient of determination was found between the iron bioaccessibility and iron content ($R^2 = 0.46$) indicating iron content has a moderate correlation with iron bioaccessibility of lupin. Poor correlations between iron bioaccessibility and other factors; calcium ($R^2 = 0.08$), magnesium ($R^2 < 0.01$), zinc ($R^2 = 0.03$), RFOs ($R^2 = 0.02$), phytate ($R^2 = 0.02$), polyphenols ($R^2 = 0.09$), flavonoids ($R^2 = 0.01$) and condensed tannin ($R^2 = 0.02$) were found (Table 43).

![Graph showing linear regression between iron bioaccessibility and phytate to iron molar ratio](image)

**Figure 49:** Linear regression between iron bioaccessibility and phytate to iron molar ratio
Table 43: Linear regression equations, correlation coefficients and correlation of determination between factors influence iron bioaccessibility

<table>
<thead>
<tr>
<th>Factors</th>
<th>Equation</th>
<th>R</th>
<th>R²</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (g/100 g)</td>
<td>$y = 22.59 - 19.22x$</td>
<td>−0.29</td>
<td>0.08</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Iron (mg/100 g)</td>
<td>$y = 34.15 - 5.06x$</td>
<td>−0.68</td>
<td>0.46</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Magnesium (g/100 g)</td>
<td>$y = 20.06 - 6.35x$</td>
<td>−0.02</td>
<td>&lt;0.01</td>
<td>0.82</td>
</tr>
<tr>
<td>Zinc (mg/100 g)</td>
<td>$y = 15.52 + 0.99x$</td>
<td>0.18</td>
<td>0.03</td>
<td>0.05</td>
</tr>
<tr>
<td>RFOs (g/100 g)</td>
<td>$y = 15.96 + 0.33x$</td>
<td>0.12</td>
<td>0.02</td>
<td>0.19</td>
</tr>
<tr>
<td>Phytate (g/100 g)</td>
<td>$y = 15.50 + 4.56x$</td>
<td>0.16</td>
<td>0.02</td>
<td>0.1</td>
</tr>
<tr>
<td>Polyphenols (mgGAE/100 g)</td>
<td>$y = 28.16 - 0.07x$</td>
<td>−0.30</td>
<td>0.09</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Flavonoids (mgCE/100 g)</td>
<td>$y = 21.54 - 0.07x$</td>
<td>−0.12</td>
<td>0.01</td>
<td>0.22</td>
</tr>
<tr>
<td>Condensed tannins (mgCE/100 g)</td>
<td>$y = 18.04 + 0.03x$</td>
<td>0.13</td>
<td>0.02</td>
<td>0.18</td>
</tr>
</tbody>
</table>

There was an inverse relationship between iron bioaccessibility and iron content (Figure 50) indicating the higher iron content results in a lower iron bioaccessibility of lupin. Cámara et al. (2005) also found a negative correlation between iron bioaccessibility and iron content in school meals. The authors reported a negative correlation ($R = −0.80$, $R^2 = 0.64$) between iron bioaccessibility (dialysability method) and iron contents by using an exponential model.

![Figure 50: Linear regression between iron bioaccessibility and iron content](image-url)
4.3.2.4 Multiple correlations between minerals, anti-nutritional factors and iron bioaccessibility

To predict iron bioaccessibility of lupin using minerals and anti-nutritional factors, a stepwise multiple regression was performed to generate a predictive equation. Iron, calcium and polyphenols contents had negative correlations with iron bioaccessibility, while RFOs content had a positive correlation (Predictive Equations 4, 5, 6 & 7).

Predictive Equation 4 can estimate 46% of the iron bioaccessibility of lupin using iron content. Both iron and calcium contents together can explain 68% of the iron bioaccessibility of lupin (Predictive Equation 5) which is 22% improvement compare to the Predictive Equation 4. Predictive Equation 6 indicates that iron, calcium, and RFOs contents can predict 69% of the iron bioaccessibility. Predictive Equation 7 shows that iron, calcium, RFOs and polyphenols contents can predict 70% of the iron bioaccessibility of lupin. The results indicate that among the factors studied, iron and calcium contents are the most significant predictors of iron bioaccessibility of lupin. RFOs and polyphenols also have effects on iron bioaccessibility of lupin. These Predictive Equations indicate that there are other factors affecting iron bioaccessibility of lupin in addition to the factors studied.

\[
y = 34.15 - 5.06x_1 \quad (R^2 = 0.46) \quad \text{Predictive Equation 4}
\]

\[
y = 42.53 - 5.93x_1 - 32.07x_2 \quad (R^2 = 0.68) \quad \text{Predictive Equation 5}
\]

\[
y = 39.20 - 6.03x_1 - 29.35x_2 + 0.33x_3 \quad (R^2 = 0.69) \quad \text{Predictive Equation 6}
\]

\[
y = 41.68 - 5.87x_1 - 27.74x_2 + 0.33x_3 - 0.02x_4 \quad (R^2 = 0.70) \quad \text{Predictive Equation 7}
\]

Where:
\[
y = \text{Iron bioaccessibility (\%)}
\]
\[x_1 = \text{Iron content (mg/100 g DM)}
\]
\[x_2 = \text{Calcium content (g/100 g DM)}
\]
\[x_3 = \text{RFOs content (g/100 g DM)}
\]
\[x_4 = \text{Polyphenols content (mg GAE/100 g DM)}
\]

Lombardi-Boccia et al. (2003) studied iron bioaccessibility (dialysability method) of whole seeds and globulin fraction of lupin collected in Italy. They found a surprise
result that iron bioaccessibility of whole lupin was higher than that of globulin fraction. The authors suggested that intrinsic characteristics of lupin protein structure were the main factor causing the low iron bioaccessibility of lupin. The interactions of peptides and iron could lead to insoluble complexes resulting in low iron bioaccessibility of lupin.

Although no information on multiple regression between various factors on iron bioaccessibility of lupin has been published, multiple regression analysis between iron bioaccessibility, and nutrients and anti-nutritional factors in other foods have been studied (Hemalatha et al. 2007; Jha et al. 2015; Jyothi lakshmi & Kaul 2011; Krishnan et al. 2012). Hemalatha et al. (2007) investigated the multiple regression between iron bioaccessibility, and zinc, calcium, phytic acid, soluble dietary fibre, insoluble dietary fibre and condensed tannin of pulses commonly consumed in India. They developed a predictive equation from the multiple regression analysis that can explain 75% of iron bioaccessibility of pulses. However, the authors mentioned that only calcium and insoluble dietary fibre contents were significantly correlated to iron bioaccessibility.

Jha et al. (2015) showed that multiple regression equation with zinc, polyphenols, flavonoids, phytate, soluble dietary fibre and insoluble dietary fibre contents can explain up to 92% of iron bioaccessibility in pearl millet. The combined effect of phytate, condensed tannin and oxalate using multiple regression determined up to 97% of iron bioaccessibility of watermelon seeds as reported by Jyothi lakshmi and Kaul (2011). In contrast, a low coefficient of determination ($R^2 = 0.25$) from multiple regression of iron bioaccessibility and phytate, polyphenols, soluble dietary fibre and insoluble dietary fibre of finger millet products was reported by Krishnan et al. (2012).

Based on the results of the present study, RFOs, phytate, polyphenols, flavonoids and condensed tannins may be not the main factors influencing iron bioaccessibility of lupin. Protein, dietary fibre and other components in lupin may play important roles in iron bioaccessibility. Thus, further studies should be conducted aim at evaluating the effects of protein and dietary fibre and other compounds on iron bioaccessibility of lupin.
4.3.3 Bioaccessibility of zinc

Zinc bioaccessibility values of whole and dehulled lupin samples of different cultivars are shown in Table 44. The average zinc bioaccessibility of 8.88% was recorded for whole seed samples. There were significant differences in zinc bioaccessibility among lupin cultivars of whole lupin samples. The highest zinc bioaccessibility was found in PBA Gunyidi. Walan 2385, Gungurru and Coromup were some of the cultivars low in zinc bioaccessibility values.

Dehulled lupin samples had an average zinc bioaccessibility of 11.91% and ranged from 8.70–14.18%. There was no significant difference in zinc bioaccessibility of dehulled lupin samples between cultivars, indicating that any of the ASL cultivar can be used for food applications with the same zinc bioaccessibility.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Bioaccessibility of zinc (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole seeds</td>
</tr>
<tr>
<td>Belara</td>
<td>8.59 ± 6.53( ^bc )</td>
</tr>
<tr>
<td>Corumup</td>
<td>5.65 ± 1.27( ^bc )</td>
</tr>
<tr>
<td>Gungurru</td>
<td>5.41 ± 1.78( ^bc )</td>
</tr>
<tr>
<td>Jenabillup</td>
<td>10.90 ± 3.90( ^bc )</td>
</tr>
<tr>
<td>Mandelup</td>
<td>11.16 ± 3.83(^b)</td>
</tr>
<tr>
<td>PBA Barlock</td>
<td>9.54 ± 1.28( ^bc )</td>
</tr>
<tr>
<td>PBA Gunyidi</td>
<td>18.49 ± 3.78(^a)</td>
</tr>
<tr>
<td>Quilinock</td>
<td>7.65 ± 2.30( ^bc )</td>
</tr>
<tr>
<td>Tanjil</td>
<td>5.86 ± 2.26( ^bc )</td>
</tr>
<tr>
<td>Walan 2385</td>
<td>3.96 ± 1.36(^c)</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td>8.88 ± 5.01</td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td>5.41–18.49</td>
</tr>
</tbody>
</table>

For mean ± SD, N = 6 except Walan 2385 N = 4. Mean values with different superscript in the same column are significantly different (\( P < 0.05 \)).

Zinc bioaccessibility of lupin (not ASL) using the same method followed by the present study has been investigated by a few studies. Lombardi-Boccia et al. (2003) reported that whole lupin collected in Italy had zinc bioaccessibility of 9% which is
similar to the present study. Porres et al. (2005) reported high value of 27.5% for zinc bioaccessibility in whole lupin flour (L. albus, in Spain). The difference in zinc bioaccessibility of lupin could be due to the different lupin species and/or molecular weight cutoff of dialysis tubing used between studies. Dialysis tubing with molecular weight cutoff off 6,000–8,000 Dalton was employed by Lombardi-Boccia et al. (2003). Dialysis tubing with molecular weight cutoff of 10,000 Dalton was used in the present study and 12,000–14,000 Dalton was used by Porres et al. (2005).

Zinc bioaccessibility values of lupin using different in vitro methods have been reported. Krejpcio et al. (2006) reported the zinc bioaccessibility values (solubility method) of 31.6% for L. albus and 42.2% for L. angustifolius. Suliburska et al. (2009) reported that zinc bioaccessibility values (solubility method) of whole seeds of L. albus, L. angustifolius and L. luteus from Poland were 26.55, 36.70 and 9.43%, respectively. Hassan et al. (2005) found that two lupin cultivars (L. termis, in Sudan) had very different values of zinc availability (not the bioaccessibility). The authors reported that zinc availability of Dongola cultivar was 20.8% and that of Golo cultivar was 64.9%.

The zinc bioaccessibility values of other grain legumes have been studied. Hemalatha et al. (2007) found that zinc bioaccessibility values of chickpea, green gram, red gram, black gram and cow pea from India were 44.9, 27.0, 45.7, 33.4 and 53.0%, respectively. The zinc bioaccessibility values of 32% for chickpea, 32% for common bean and 48% for lentils were reported by Sebastiá et al. (2001). The results from the other studies indicate that the zinc bioaccessibility values of other grain legumes are higher than that of lupin found in the present study.

4.3.3.1 Effect of dehulling on zinc bioaccessibility

Figure 51 shows the effects of dehulling on zinc bioaccessibility of different lupin cultivars. Dehulling had no significant effect on zinc bioaccessibility of Belara, Jenabillup, PBA Barlock and Quilinock. A significant increase in zinc bioaccessibility of Coromup, Gungurru, Mandelup and Tanjil were found after dehulling. In contrast, dehulling decreased zinc bioaccessibility of PBA Gunyidi.
For mean values, $N = 6$ except Walan 2385 $N = 4$. Error bar shows the standard deviation of the means. Mean values between whole seed and dehulled lupin samples with different letters indicates significant differences ($P < 0.05$).

Suliburska et al. (2009) studied the effect of dehulling on the zinc bioaccessibility (solubility method) of different lupin species grown in Poland. The authors found that the effect of dehulling on zinc bioaccessibility depends on the lupin species. They reported that dehulling significantly increased zinc bioaccessibility values of $L.\ albus$ and $L.\ luteus$ but had no effect on the value of $L.\ angustifolius$. However, the authors have not reported the technique used for dehulling. Hassan et al. (2005) found that wet dehulling resulted in a decrease in zinc availability (not bioaccessibility) of lupin ($L.\ termis$).

### 4.3.3.2 Phytate to zinc molar ratio as a predictor of zinc bioavailability

Phytate to zinc molar ratio have been used as an indicator of zinc bioavailability (Gibson et al. 2010). Phytate to zinc molar ratio greater than 15 indicate poor zinc bioavailability (Ma et al. 2005).

Phytate to zinc molar ratios in whole and dehulled lupin of different cultivars are shown in Table 45. Whole seed samples showed the average phytate to zinc molar ratio of 27.44. PBA Gunyidi had significantly higher zinc bioaccessibility than the other cultivars except Jenabillup.
Dehulled lupin samples had an average phytate to zinc molar ratio of 20.70 (Table 45). Gungurru, Jenabillup and walan 2385 were some of the cultivars with high phytate to zinc molar ratios. Low phytate to zinc molar ratios were found in some cultivars such as PBA Gunyidi, PBA Barlock and Tanjil. All lupin samples except whole seeds Walan 2385 and dehulled PBA Gunyidi had average phytate to zinc molar ratios greater than 15 indicating poor zinc bioavailability. Trugo et al. (1993) reported the average phytate to zinc molar ratios of whole seeds of *L. albus* (two cultivars from Brazil) and *L. angustifolius* (six cultivars from Australia which are no longer commercially grown) of 11.4 and 16.7, respectively.

Table 45: Phytate to zinc molar ratio in whole and dehulled seeds of lupin cultivars

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Phytate to zinc molar ratio</th>
<th>Whole seeds</th>
<th>Dehulled seeds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belara</td>
<td>21.22 ± 6.19b</td>
<td>20.51 ± 2.90bd</td>
<td></td>
</tr>
<tr>
<td>Corumup</td>
<td>19.92 ± 4.58b</td>
<td>19.00 ± 4.58de</td>
<td></td>
</tr>
<tr>
<td>Gungurru</td>
<td>19.76 ± 2.31b</td>
<td>30.54 ± 0.90a</td>
<td></td>
</tr>
<tr>
<td>Jenabillup</td>
<td>36.24 ± 5.23ab</td>
<td>28.23 ± 2.39ab</td>
<td></td>
</tr>
<tr>
<td>Mandelup</td>
<td>31.56 ± 11.68b</td>
<td>18.75 ± 6.17de</td>
<td></td>
</tr>
<tr>
<td>PBA Barlock</td>
<td>26.49 ± 10.05b</td>
<td>15.36 ± 2.95de</td>
<td></td>
</tr>
<tr>
<td>PBA Gunyidi</td>
<td>53.29 ± 28.30a</td>
<td>11.43 ± 1.56c</td>
<td></td>
</tr>
<tr>
<td>Quilinock</td>
<td>27.10 ± 4.46b</td>
<td>20.02 ± 1.77cd</td>
<td></td>
</tr>
<tr>
<td>Tanjil</td>
<td>20.50 ± 5.99b</td>
<td>17.50 ± 8.44de</td>
<td></td>
</tr>
<tr>
<td>Walan 2385</td>
<td>13.74 ± 1.72b</td>
<td>28.14 ± 2.84abc</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>27.44 ± 14.80</td>
<td>20.70 ± 6.95</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>13.74–53.29</td>
<td>11.43–30.54</td>
<td></td>
</tr>
</tbody>
</table>

For mean ± SD, N = 6 except Walan 2385 N = 4. Mean values with different superscript in the same column are significantly different (*P* < 0.05).

Hemalatha et al. (2007) studied the molar ratios of phytate to zinc in other pulses; chickpea (12.9), green gram (22.7), red gram (16.4), black gram (23.4) and cow pea (14.2). Ma et al. (2005) found high molar ratios of phytate to zinc of various grains including wheat flour (80.23), oat flake (68.14) and sorghum (103.21). A study by Lazarte et al. (2015) showed the phytate to zinc molar ratios of rice (8.5), quinoa
(56.5), fava beans (25.3) and lentils (24.2). The phytate to zinc molar ratios of most grain legumes reported by the previous studies are similar to values reported for ASL cultivars in the present study. Some grains such as wheat, sorghum and quinoa have considerably higher phytate to zinc molar ratios than ASL samples.

Regression equation and coefficient of determination \((R^2)\) from regression analysis between zinc bioaccessibility and phytate to zinc molar ratio of lupin are shown in Figure 52. Results show a low coefficient of determination \((R^2 = 0.10)\) indicating the degree of correlation is poor. Phytate to zinc molar ratios cannot be used to predict zinc bioaccessibility of lupin.

![Figure 52: Linear regression between zinc bioaccessibility and phytate to zinc molar ratio](image)

\[y = 0.16x + 6.55\]
\[R^2 = 0.10\]

**4.3.3.3 Phytate \times calcium to zinc molar ratio as a predictor of zinc bioavailability**

Calcium presents synergistic effect on decreasing the solubility of phytate–zinc complexes (Konietzny et al. 2003). High calcium levels in food can reduce the zinc bioavailability (Konietzny et al. 2003). Therefore, phytate \(\times\) calcium to zinc molar ratio could be a more useful predictor of zinc bioavailability than the molar ratio of phytate to zinc. Phytate \(\times\) calcium to zinc molar ratio higher than 200 is considered to impair zinc bioavailability (Lazarte et al. 2015).
Table 46 shows phytate × calcium to zinc molar ratio in whole and dehulled lupin samples. Whole lupin samples had an average phytate × calcium to zinc molar ratio of 176.71 and ranged from 95.40–313.95. PBA gunyidi had significantly higher phytate × calcium to zinc molar ratio than that of other cultivars except Jenabillup. All whole seed samples except Jenabillup and PBA Gunyidi had phytate × calcium to zinc molar ratios lower than 200 indicating good zinc bioavailability.

### Table 46: Phytate × calcium to zinc molar ratio in whole and dehulled seeds of lupin cultivars

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Phytate × calcium to zinc molar ratio</th>
<th>Whole seeds</th>
<th>Dehulled seeds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belara</td>
<td>157.96 ± 53.22&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>62.69 ± 14.08&lt;sup&gt;ac&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Corumup</td>
<td>135.27 ± 20.27&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>42.02 ± 9.75&lt;sup&gt;cd&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Gungurru</td>
<td>181.86 ± 19.80&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>66.17 ± 10.68&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Jenabillup</td>
<td>229.92 ± 38.03&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>85.18 ± 17.42&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Mandelup</td>
<td>195.75 ± 65.87&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>42.95 ± 16.48&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>PBA Barlock</td>
<td>130.93 ± 44.39&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>24.02 ± 4.45&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>PBA Gunyidi</td>
<td>313.95 ± 134.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.42 ± 3.41&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Quilinock</td>
<td>168.17 ± 36.66&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>63.67 ± 4.76&lt;sup&gt;ac&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Tanjil</td>
<td>130.81 ± 49.36&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>34.64 ± 19.40&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Walan 2385</td>
<td>95.40 ± 14.78&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>65.08 ± 7.71&lt;sup&gt;ac&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td>176.71 ± 79.84</td>
<td>50.19 ± 23.18</td>
<td></td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td>95.40–313.95</td>
<td>20.42–85.18</td>
<td></td>
</tr>
</tbody>
</table>

For mean ± SD, N = 6 except Walan 2385 N = 4. Mean values with different superscript in the same column are significantly different (<i>P</i> < 0.05).

The average phytate × calcium to zinc molar ratio of dehulled lupin samples were 50.19 (Table 46). Jenabillup, Gungurru and Walan 2385 were some of the cultivars had high phytate × calcium to zinc molar ratios. Low phytate × calcium to zinc molar ratios were found in PBA Gunyidi, PBA Barlock and Tanjil. All dehulled lupin samples had phytate × calcium to zinc molar ratios lower than 100 indicating better zinc bioavailability.
Compared to the lupin samples, Ma et al. (2005) reported lower phytate × calcium to zinc molar ratios of millet (7.63) and sorghum (16.72). Considerably high phytate × calcium to zinc molar ratios of wheat flour (226), quinoa (2470), fava beans (717) and lentils (791) were reported by Lazarte et al. (2015). According to the phytate × calcium to zinc molar ratios, all tested lupin samples had better zinc bioaccessibility (< 200) compare to wheat flour, quinoa and lentils.

Based on the phytate to zinc molar ratio, most of the studied lupin samples have poor zinc bioavailability (Table 45). In contrast, phytate × calcium to zinc molar ratios of almost all lupin samples showed good zinc bioavailability (Table 46).

Figure 53 shows regression equation and coefficient of determination between zinc bioaccessibility and phytate × calcium to zinc molar ratio of lupin. Similar to the phytate to zinc molar ratios (Figure 52), the results clearly shows that phytate × calcium to zinc molar ratio could not be used to predict zinc bioaccessibility of lupin.

Liang et al. (2010) reported that zinc bioaccessibility (solubility method) of rice-based products was not correlated to their molar ratios of phytate to zinc. Dahiya et al. (2013) also reported that zinc bioaccessibility (solubility method) of mung beans was not correlate to their phytate to zinc molar ratio.
4.3.3.4 Linear correlations between minerals, anti-nutritional factors and zinc bioaccessibility

Linear regression analyses were performed to determine the correlations between minerals, anti-nutritional factors and zinc bioaccessibility. Linear regression equations, correlation coefficients and correlation of determination of individual factors and zinc bioaccessibility of lupin are shown in Table 47. The highest coefficient of determination ($R^2 = 0.40$) was found between the zinc bioaccessibility and zinc content. Poor correlations between zinc bioaccessibility and calcium ($R^2 = 0.18$), iron ($R^2 = 0.02$), magnesium ($R^2 = 0.05$), RFOs ($R^2 = 0.07$), phytate ($R^2 = 0.06$), polyphenols ($R^2 < 0.01$), flavonoids ($R^2 = 0.01$) and condensed tannin ($R^2 = 0.08$) in lupin samples were found.

Table 47: Linear regression equations, correlation coefficients and correlation of determination between factors influence zinc bioaccessibility

<table>
<thead>
<tr>
<th>Factor</th>
<th>Equation</th>
<th>R</th>
<th>$R^2$</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (g/100 g)</td>
<td>$y = 13.98 - 19.81x$</td>
<td>-0.43</td>
<td>0.18</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Iron (mg/100 g)</td>
<td>$y = 8.00 + 0.81x$</td>
<td>0.16</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>Magnesium (g/100 g)</td>
<td>$y = 3.89 + 43.66x$</td>
<td>0.22</td>
<td>0.05</td>
<td>0.02</td>
</tr>
<tr>
<td>Zinc (mg/100 g)</td>
<td>$y = 19.02 - 2.37x$</td>
<td>-0.63</td>
<td>0.40</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>RFOs (g/100 g)</td>
<td>$y = 5.48 + 0.52x$</td>
<td>0.27</td>
<td>0.07</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Phytate (g/100 g)</td>
<td>$y = 14.41 - 5.07x$</td>
<td>-0.25</td>
<td>0.06</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Polyphenols (mgGAE/100 g)</td>
<td>$y = 11.68 - 0.01x$</td>
<td>-0.06</td>
<td>&lt; 0.01</td>
<td>0.51</td>
</tr>
<tr>
<td>Flavonoids (mgCE/100 g)</td>
<td>$y = 11.94 - 0.05x$</td>
<td>-0.11</td>
<td>0.01</td>
<td>0.26</td>
</tr>
<tr>
<td>Condensed tannins (mgCE/100 g)</td>
<td>$y = 8.74 + 0.04x$</td>
<td>0.28</td>
<td>0.08</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

The results showed a negative correlation between zinc bioaccessibility and zinc content (Figure 54) indicating the higher zinc content results in a lower zinc bioaccessibility of lupin. Lönnerdal (2000) conducted a critical review on dietary factors affecting zinc absorption and concluded that zinc contents had negative correlations to zinc absorptions in animal and human studies. In contrast, Cámara et al. (2005) showed that zinc bioaccessibility had no correlation to zinc contents in the Spanish school meals ($R = -0.14$, $R^2 = 0.02$).
4.3.3.5 Multiple regression between minerals, anti-nutritional factors and zinc bioaccessibility

A stepwise multiple regression was conducted to generate a regression equation to predict zinc bioaccessibility using minerals (Ca, Fe, Mg and Zn) and anti-nutritional factors (RFOs, phytate, polyphenols, flavonoids and condensed tannins). Zinc and calcium contents had negative correlations, and condensed tannin content had a positive correlation with zinc bioaccessibility. It was found that zinc content is the strongest predictive factor for zinc bioaccessibility of lupin (Predictive Equation 8, 9 & 10).

By using Predictive Equation 8, zinc content can be used to explain 40% of the zinc bioaccessibility. Using zinc and calcium contents, and the Predictive Equation 9 can determine 56% of the zinc bioaccessibility. Predictive Equation 10 indicates that zinc, calcium and condensed tannins can explain 59% of the zinc bioaccessibility. Limited predictability of zinc bioaccessibility using developed predictive equations indicate that there are other factors (e.g. protein and dietary fibre) affect zinc bioaccessibility of lupin in addition to the factors studied.

\[ y = 19.02 - 2.37x_1 \quad (R^2 = 0.40) \] …………………………… Predictive Equation 8
\[ y = 22.25 - 2.32x_1 - 18.86x_2 \quad (R^2 = 0.56) \] …………………………… Predictive Equation 9
y = 20.46 − 2.44x₁ − 12.89x₂ + 0.03x₃ (R² = 0.59) ............ Predictive Equation 10

Where:

y = Zinc bioaccessibility (%)

x₁ = Zinc content (mg/100 g DM)

x₂ = Calcium content (g/100 g DM)

x₃ = Condensed tannin content (mgCE/100 g DM)

Duranti et al. (2001) reported that conglutin γ of lupin had a strong interaction with zinc resulting in an insoluble complex which could reduce zinc bioaccessibility. Lombardi-Boccia et al. (2003) indicated that low zinc bioaccessibility of lupin could be due to inherent properties of lupin proteins.

Hemalatha et al. (2007) reported a moderate coefficient of determination (R² = 0.47) of multiple regression between zinc bioaccessibility and iron, calcium, phytate, dietary fibre and condensed tannins contents in chick pea, green gram, red gram, black gram, cowpea and French beans. The authors indicated that only phytate and insoluble dietary fibre had significant negative correlations with zinc bioaccessibility of pulses. They also found a downward trend between zinc bioaccessibility and calcium contents indicating that pulses with high calcium contents could have low zinc bioaccessibility.

Jha et al. (2015) reported that multiple regression between zinc bioaccessibility and polyphenols, flavonoids, phytate, soluble dietary fibre and insoluble dietary fibre and iron contents in pearl millet showing high coefficient of determination (R² = 0.98). The authors found that flavonoids, phytate, insoluble dietary fibre and iron contents had negative correlations with zinc bioaccessibility. Polyphenols and soluble dietary fibre had positive correlations with zinc bioaccessibility. However, the authors have not reported which factors had significant effect on the zinc bioaccessibility.

Jyothi lakshmi and Kaul (2011) studied the combined effect of phytate, condensed tannin and oxalate on zinc bioaccessibility of watermelon seeds. They reported a high coefficient of determination (R² = 0.97) from the multiple regression. Negative correlations were found between zinc bioaccessibility and condensed tannins and oxalate. Although it is commonly accepted that phytate has negative effect on zinc
bioaccessibility, the authors found a positive relation between zinc bioaccessibility and phytate.

The average condensed tannin in tested lupin samples (whole seeds) was 46 mgCE/100 g DM which is considerably lower than the values reported for watermelon seeds (336 mgCE/100 g DM) by Jyothi Lakshmi and Kaul (2011). Lupin seeds and watermelon seeds have similar phytate contents. However, the correlation coefficient between condensed tannins and zinc bioaccessibility of lupin was only $R^2 = 0.08$ and between phytate and zinc bioaccessibility ($R^2 = 0.06$) indicating poor predictability (Table 47).

Krishnan et al. (2012) studied the effect of phytate, polyphenols, soluble dietary fibre and insoluble dietary fibre on zinc bioaccessibility of finger millet products using a multiple regression. They reported a high coefficient of determination ($R^2 = 0.84$) between zinc bioaccessibility and the factors studied. Polyphenols and insoluble dietary fibre showed negative correlations to zinc bioaccessibility. Similar to the results reported by Jyothi Lakshmi and Kaul (2011) for watermelon seeds, Krishnan et al. (2012) showed that phytate had a positive correlation with zinc bioaccessibility.

The inhibitory effects of phytate on zinc bioavailability/bioaccessibility are well established in several in vivo and in vitro studies (Couzy et al. 1998; Hemalatha et al. 2007; Morris and Ellis 1989; Sandberg et al. 1987). However, results in the present study using a stepwise multiple regression showed that phytate had no significant influence on zinc bioaccessibility of lupin. Lupin contains high level of protein (approximately 40%) and it is possible that protein could form complexes with phytate resulting in less inhibitory effect on zinc bioaccessibility of lupin.
5. CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER STUDIES

5.1 Conclusions

- Year of cultivation has no effect on calcium, iron, magnesium, potassium, zinc, raffinose family oligosaccharides, phytate, total phenolic, total flavonoid and condensed tannin contents in ASL. This indicates that the impact of environmental factors on the minerals and anti-nutritional factors in ASL is negligible.

- ASL cultivar has a significant influence on calcium, iron, magnesium, potassium, zinc, raffinose family oligosaccharides, phytate, total phenolic, total flavonoid and condensed tannin contents. The cultivar has greater impact on the contents of minerals and anti-nutritional factors than the environmental conditions.

- Most of the ASL cultivars are good sources of raffinose family oligosaccharides. Belara and Mandelup cultivars contain high raffinose family oligosaccharides contents and are suitable for functional food applications with prebiotic potential. Gungurru and PBA Barlock cultivars have relatively low levels of raffinose family oligosaccharides and are recommended for lupin-enriched foods with less flatulence effects.

- PBA Barlock seeds (dehulled) contain higher level of total phenolics and total flavonoids than most of the other lupin cultivars. Walan 2385 has high total flavonoid and condensed tannin contents. PBA Barlock and Walan 2385 cultivars are recommended for food formulations with enhanced health benefits such as antioxidative and anti-inflammatory properties.

- Belara has the highest iron contents and Quilinock has the highest zinc content. Both Belara and Quilinock cultivars are also high in calcium contents. These two cultivars would be ideal for the production of foods rich in iron, zinc and calcium.
The effects of dehulling on iron, magnesium, zinc, raffinose family oligosaccharides, phytate, total phenolic and total flavonoid contents depend on the lupin cultivar. Dehulling increases condensed tannins and potassium contents in most lupin cultivars and decreases calcium contents in all lupin cultivars.

Calcium and zinc bioaccessibility values of dehulled ASL are poor and lower than some other grain legumes such as cowpea and mung bean. The low calcium and zinc bioaccessibility indicates that these minerals are bound and not easily digestible in the human gut. In contrast, iron bioaccessibility of ASL is around 20% which is higher than values reported for other pulses including chickpea, red grams and black grams.

Calcium bioaccessibility value of PBA Gunyidi (dehulled seeds) is higher than some of the other cultivars. Gungurru and Mandelup have high iron bioaccessibility. Zinc bioaccessibility values of dehulled lupins are not affected by the cultivar.

Dehulling increases calcium bioaccessibility in most of the lupin cultivars. The effects of dehulling on iron and zinc bioaccessibility depend on the lupin cultivar.

Although phytate to mineral molar ratio is widely used as a predictor of mineral bioavailability, the results of the present study indicate that phytate to mineral molar ratio is not correlate to the mineral bioaccessibility in lupin. It is not recommended using the phytate to mineral molar ratio to predict mineral bioaccessibility in lupin.

In lupin, bioaccessibility is not directly related to the mineral contents. Bioaccessibility of calcium is negatively correlated to calcium content. Lupins also have negative trends between iron content and iron bioaccessibility, and zinc content and zinc bioaccessibility.

A predictive equation can be used to estimate calcium bioaccessibility of ASL by using calcium, phytate and raffinose family oligosaccharides contents as variables. Similarly, iron, calcium, raffinose family oligosaccharides and polyphenols
contents can be used to predict the iron bioaccessibility in ASL. Zinc bioaccessibility in ASL also can be estimated by a regression equation involving zinc, calcium and condensed tannin contents.

- Raffinose family oligosaccharides, phytate, total phenolics, total flavonoids and condensed tannins have poor correlations to calcium, iron and zinc bioaccessibility values in lupin. This indicates that raffinose family oligosaccharides, phytate, total phenolics, total flavonoids and condensed tannins are not likely to be major factors affecting the minerals bioaccessibility in lupin.

5.2 Recommendations for further studies

- Oxalate has a negative effect on calcium bioavailability. Information on oxalate content in lupin is limited and it is recommended further study to determine the effect of oxalate on calcium bioaccessibility in lupin to be carried out.

- Lupin contains high amount of protein and dietary fibre which could affect the mineral bioaccessibility. Therefore, the effect of protein and dietary fibre (both soluble and insoluble) on the mineral bioaccessibility of lupin should be investigated.

- Researchers have no control over the climatic conditions during a field study. Similar climatic conditions were reported during three cultivation years and this could have affected the results of the study. A study aims at collecting samples from cultivation years with different climatic conditions is recommended.
REFERENCES


Cámara, F, Amaro, MA, Barberá, R & Lagarda, MJ 2005, ‘Speciation of bioaccessible (heme, ferrous and ferric) iron from school menus’, *European Food Research and Technology*, vol. 221, no. 6, pp. 768-73.


Dracup, M & Kirby, E 1996, *Lupin development guide*, University of Western Australia Press. Western Australia.


Dvořáčková, E, Šnóblová, M & Hrdlička, P 2014, ‘Carbohydrate analysis: From sample preparation to HPLC on different stationary phases coupled with evaporative light-scattering detection’, *Journal of separation science*, vol. 37, no. 4, pp. 323-37.


Glencross, BD 2001, Feeding lupins to fish: A review of the nutritional and biological value of lupins in aquaculture feeds, Department of Fisheries-Research Division, Government of Western Australia.


Grant, G, Dorward, PM, Buchan, WC, Armour, JC & Pusztai, A 1995, ‘Consumption of diets containing raw soya beans (Glycine max), kidney beans (Phaseolus vulgaris), cowpeas (Vigna unguiculata) or lupin seeds (Lupinus angustifolius) by rats for up to 700 days: Effects on body composition and organ weights’, British Journal of Nutrition, vol. 73, no. 1, pp. 17-29.


Gupta, RK, Gangoliya, SS & Singh, NK 2015, ‘Reduction of phytic acid and enhancement of bioavailable micronutrients in food grains’, *Journal of Food Science and Technology*, vol. 52, no. 2, pp. 676-84.


Halliwell, B 2008, ‘Are polyphenols antioxidants or pro-oxidants? What do we learn from cell culture and in vivo studies?’, *Archives of Biochemistry and Biophysics*, vol. 476, no. 2, pp. 107-12.


Han, IH & Baik, BK 2006, ‘Oligosaccharide content and composition of legumes and their reduction by soaking, cooking, ultrasound, and high hydrostatic pressure’, *Cereal Chemistry*, vol. 83, no. 4, pp. 428-33.


Hemalatha, S, Platel, K & Srinivasan, K 2007, ‘Zinc and iron contents and their bioaccessibility in cereals and pulses consumed in India’, *Food Chemistry*, vol. 102, no. 4, pp. 1328-36.


Karkle, ENL & Beleia, A 2010, ‘Effect of soaking and cooking on phytate concentration, minerals, and texture of food-type soybeans’, *Food Science and Technology (Campinas)*, vol. 30, pp. 1056-60.


Knecht, KT, Nguyen, H, Auker, AD & Kinder, DH 2006, ‘Effects of extracts of lupine seed on blood glucose levels in glucose resistant mice: Antihyperglycemic effects of *Lupinus albus* (White Lupine, Egypt) and *Lupinus caudatus* (Tailcup Lupine, Mesa Verde National Park)’, *Journal of Herbal Pharmacotherapy*, vol. 6, no. 3-4, pp. 89-104.


Moraghan, JT, Etchevers, JD & Padilla, J 2006, ‘Contrasting accumulations of calcium and magnesium in seed coats and embryos of common bean and soybean’, *Food Chemistry*, vol. 95, no. 4, pp. 554-61.


Obendorf, RL & Górecki, RJ 2012, ‘Soluble carbohydrates in legume seeds’, Seed Science Research, vol. 22, no. 4, pp. 219-42.


Pathare, PB, Opara, UL & Al Said, FAJ 2013, ‘Colour measurement and analysis in fresh and processed foods: A review’, Food and Bioprocess Technology, vol. 6, no. 1, pp. 36-60.


White, P, French, B & McLarty, A 2008, Producing lupins, Department of Agriculture and Food, Western Australia.


APPENDIX A - Copies of publications


Review
Phytochemical composition and bioactivities of lupin: a review

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Summary Lupin is a nonstarchy grain legume with high protein, dietary fibre and low fat contents. The industrial shift of lupin seed utilisation from feed to food has recently increased the scientific interest to explore its phytochemical composition and biological activities. Lupin seeds contain significant amounts of polyphenols, carotenoids, phytosterols, tocopherols, alkaloids and peptides with antioxidant, antimicrobial, anticarcinogenic and anti-inflammatory activities. Among polyphenols, genistein and their derivatives (isoflavones) are of great importance because of their phytoestrogenic potential. This comprehensive review will help out the readers in understanding the phytochemicals present in lupin and their benefits.

Keywords Anti-inflammatory, antioxidant, carotenoids, flavone, isoflavone, lupin, β-sitosterol.

Introduction
As the old ages, the world population is relying on the three major crops (wheat, rice and maize) for basic nutrients needs. The increase in population, changing weather conditions and reduction of fertile land for cultivation have lead the significant proportion of world population to hunger and malnutrition urging the scientists to find out the novel and affordable food sources (Small, 2012). Lupin, a crop that can grow on marginal agricultural lands in diverse environmental conditions, has high potential to add significantly into the existing food sources (Nelson & Hawthorne, 2000).

About 400 species of lupin (genus: Lupinus) have been found in nature. Among them, only few species, white lupin (Lupinus albus), blue lupin (L. angustifolius), yellow lupin (L. luteus) and pearl or Torri lupin (L. mutabilis) have been extensively studied for their agronomical characteristics and nutritional values. For the most parts of the world, lupins have traditionally been used primarily to feed livestock, especially cows, pigs, poultry and aquaculture and its principal usage still persist as feed (White et al., 2007). Interest in lupin as food has increased as people have become progressively more aware of its unique nutritional values and health benefits. Lupin, a nonstarchy grain legume, contains lower amounts of fat (~6%), high number of essential amino acids, important dietary minerals, higher protein (~40%) and dietary fibre (~28%) contents which attract it as a great food ingredient (Guemes-Vera et al., 2012). Lupin is considered a cheap alternative to other legume crops importantly soya bean as it contains comparable quantities of proteins with similar amino acid profile. However, lupin, as a food ingredient, has an advantage over soybean that it contains higher dietary fibre contents (~28%) compared to soya bean (~19%) (Bähr et al., 2014. Lupin-enriched noodles (Jayasena et al., 2010), pasta (Jayasena & Nasar-Abbas, 2012), bread (Villarino et al., 2015b), tofu (Jayasena et al., 2014), muffins (Runyati et al., 2015), tempe (Priatni et al., 2015) and biscuits (Jayasena & Nasar-Abbas, 2011) with improved nutritional value, have been developed. Lupin flour has been fractionated into dietary fibre, and lupin protein isolates have good emulsions and foam-forming properties which are important aspects of food formulation (Porras-Suavedra et al., 2013).

Various reviews on lupin species have recently been published covering their nutritional composition (Kohajdová et al., 2011), geographical distribution (Small, 2012), possible usages (Villarino et al., 2015a), allergenicity (Verma et al., 2013) and nutraceutical properties associated with proteins (Bouchenak &
Lamti-Senhadji, 2013). However, a comprehensive compilation of lupin in terms of their phytochemical composition, antioxidant potential and biological activities is still lacking and is imperative for future research and industrial applications. This review covers the aforesaid aspects of lupin species. Review information on some bioactive compounds such as alkaloids and peptides in lupin species has already been published and is not covered in this review.

Phytochemicals

Phytochemicals are a large group of plant-derived compounds assumed to be responsible for much of the disease protection conferred from diets rich in fruits, vegetables, beans, cereals, and plant-based beverages such as tea and wine. Based on the chemical structure, they are classified into various groups, for instance, polyphenols, phytosterogens, terpenoids, carotenoids, limonoids, phytosterols and phytohemaglutinins (Rochfort & Panozzo, 2007). Nowadays, scientific community is not only relying on the protein contents of legume crops but their phytochemical composition is considered equally important to consume them as food and feed. Lupin seeds have significant amounts of phytochemicals importantly polyphenols, phytosterols and squalene (triterpene) in comparison with other legume crops (Table 1) (Kalogeropoulos et al., 2010). Although soya bean contains higher amounts of isoflavones (Rochfort & Panozzo, 2007) and tocopherols (Boschini & Arnoldi, 2011), lupin can be an ingredient of choice by the food industry because of its overall nutritional profile and lower price.

Polyphenols

Work on the identification of phenolic compounds in lupin has been performed only in few species. Understanding of the structural diversity of total phenolics is of great interest to demonstrate various related biological activities. Generally, the major phenolic compounds identified in lupin species belong to subclass flavones, phenolic acids and isoflavones (Fig. 1 and Table 2). For instance, in L. angustifolius seeds, the flavones, phenolic acids and isoflavones are present in 76%, 19% and 4% of the total identified phenolics, respectively (Dueñas et al., 2009). The main identified flavones in the group are aglycone and/or glycosides of luteolin, apigenin and diosmetin, while the principal contribution in the isoflavone group is from genistein and its derivatives. In phenolic acids, protocatechueic acid (hydroxyquinamic acid) and p-hydroxybenzoic acid are the major representative components (Siger et al., 2012). Although flavones are found in higher quantities, many of the isoflavones present in lupin species get more importance because of their nonsteroidal phytoestrogenic activity in mammals (Ramilla et al., 2009). In addition, similar to flavones, they are good antioxidants because of their ability to trap singlet oxygen. According to the published information, the first identification of isoflavone in lupin species was reported by Fukui et al. (1973) in immature seeds of L. luteus.

Phenolic compounds can be found in all parts of lupin plant, that is stem, leaves, roots and seeds, and their concentrations vary depending on the plant part. A study on L. exaltatus has shown higher contents of isoflavones and their conjugates in roots than in stems, while free aglycones were identified in roots and inflorescences (Garcia-Lopez et al., 2006). Variations of lupin species in phenolic contents have been observed among different cultivars and growth locations. It has been found that total phenolic (expressed in gallic acid) and procyanidin (expressed as (+)-catechin) contents of aqueous acetone (70% v/v) extracts of lupin cultivars (L. albus, L. angustifolius, L. luteus, L. mutabilis and L. hispanicus) varied from 7 to 70 g kg$^{-1}$ and 70 to 530 mg kg$^{-1}$ seed, respectively (Ricardo-da-Silva et al., 1993). The study has been extended to find out that total phenolic and flavonoid contents of eight

<table>
<thead>
<tr>
<th>Legume</th>
<th>Total phenolic contents mg GAE per 100 g fresh weight</th>
<th>Phytosterols mg per 100 g fresh weight</th>
<th>Tocopherols mg per 100 g fresh weight</th>
<th>Squalene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lupin</td>
<td>19.4</td>
<td>53.6</td>
<td>0.47</td>
<td>1.74</td>
</tr>
<tr>
<td>Black-eyed pea</td>
<td>15.4</td>
<td>13.5</td>
<td>0.65</td>
<td>0.12</td>
</tr>
<tr>
<td>Broad beans</td>
<td>19.6</td>
<td>36.1</td>
<td>0.53</td>
<td>0.32</td>
</tr>
<tr>
<td>Chick peas</td>
<td>20.5</td>
<td>48.9</td>
<td>1.79</td>
<td>0.24</td>
</tr>
<tr>
<td>Lentils</td>
<td>25.85</td>
<td>27.25</td>
<td>0.68</td>
<td>0.15</td>
</tr>
<tr>
<td>Pinto beans</td>
<td>18.1</td>
<td>21.5</td>
<td>0.61</td>
<td>0.23</td>
</tr>
<tr>
<td>Split peas</td>
<td>12.7</td>
<td>38.25</td>
<td>0.64</td>
<td>0.21</td>
</tr>
<tr>
<td>White beans</td>
<td>15.1</td>
<td>28</td>
<td>0.30</td>
<td>0.64</td>
</tr>
</tbody>
</table>

Adapted from Kalogeropoulos et al. (2010).
genotypes of *L. angustifolius* seeds varied from 11.9 to 14.7 mg g$^{-1}$ (catechin equivalent) and 4.15 to 4.95 mg g$^{-1}$ (rutin equivalent), respectively (Oomah *et al.*, 2006). This study has not shown any correlation between antioxidant activity and phenolic contents, suggesting that genotype may not affect the antioxidant-related biological activity of phenolic compounds. Changes in phenolic contents during the growth cycle of plant are obvious as every stage has different biological requirements, faces diverse environmental conditions and shows various adaptive plant defence mechanisms. In a study on *L. albus*, the higher content of aglycone isoflavones in autumn-sown plants (77% of total isoflavones) compared to early spring-sown ones (4% of total isoflavones) was found consistent with the pragmatic increase of these compounds during performed experiments, supporting the proposition that autumn-sown plants experience greater stress (D’Agostina *et al.*, 2008).

As in other plant species, lupin phenolic compounds also greatly respond to processing conditions. Germination is being used as a process to increase the nutritional value of legumes with the fact that vegetable sprouts occupy a significant market in developed countries. Germination produces considerable variations in flavonoids and nonflavonoid phenolic compounds. The analysis of lupin (*L. angustifolius*) germinated seeds have indicated that there were quantitave and qualitative changes in polyphenolic composition during the course of germination, with a major increase of (iso) flavonoids. An augment in the antioxidant activity was also noted as a consequence of the germination process (Rumiyati *et al.*, 2013). One of the reasons for the increase in antioxidant potential in germinated seeds is the formation of aglycones, free forms of phenolic compounds like genistein in case of soya bean and lupin. It is well cited that during germination, enzymatic activity boosts up, such as that of glucosidases (Ribeiro *et al.*, 2006) which results in an increase in aglycone content. In addition, isoflavones are formed during germination through the malonate and phenylpropanoid pathways (Hahlbrock & Scheel, 1989) which proves the rapid increase in their concentration compared to flavones and phenolic acids in lupin sprouts after 9 days (Dueñas *et al.*, 2009).

Most of the grain legumes undergo the process of cooking (100 °C for several minutes) to make them palatable. As some polyphenols are sensitive to high temperature, cooking may significantly affect the phenolic contents in the cooked beans. Kalogeropoulos *et al.* (2010) studied the bioactive microconstituents such as polyphenols, phytosterols, tocopherols and triterpenic acids in cooked dry legumes including white lupin (*L. albus*) usually consumed in the Mediterranean countries. The study found a significant decrease in total phenolic contents during cooking. The obvious explanation for this decrease is the soaking and boiling of legumes, which result in partial leaching and thermal/oxidative decomposition of thermolabile phenolics.

The development of LC-MS has significantly enhanced the performance of separation by providing greater efficiency and drastically reducing analytical time. This technique proved to be potential mean for the identification of phenolic compounds in lupin (Wejaktowska *et al.*, 2013) but the quantification of the most of the phenolic compounds in lupin yet to be completed. Only a few lupin species (*L. angustifolius*, *L. luteus*, *L. albus*, *L. mutabilis*, *L. hispanicus* and *L. exaltatus*) have been explored for their phenolic compounds while other species are on their way to be explored (Elbandy & Rho, 2014), and, to the best of our knowledge, most of the species are still unexplored.
Table 2 Phenolic contents in different lupin species

<table>
<thead>
<tr>
<th>No.</th>
<th>Species per plant part</th>
<th>Flavones</th>
<th>Apigenin derivative 1</th>
<th>Apigenin derivative 2</th>
<th>Isoflavones</th>
<th>Genistein derivative</th>
<th>p-hydroxybenzoic acid</th>
<th>p-hydroxyphenolic acid</th>
<th>Tannins</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>L. luteus Seed (mg per 100 g dry wt.)</td>
<td>53-63&lt;sup&gt;a&lt;/sup&gt;</td>
<td>71-88&lt;sup&gt;b&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.11-0.23</td>
<td>0.003-0.068</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>L. albus Seed (mg per 100 g dry wt.)</td>
<td>12-14&lt;sup&gt;c&lt;/sup&gt;</td>
<td>25-26&lt;sup&gt;d&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>2.3-2.8</td>
<td>0.011-0.018</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>L. angustifolius Seed (mg per 100 g dry wt.)</td>
<td>28-30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>41-43&lt;sup&gt;a&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>4.3-4.4</td>
<td>0.034-0.042</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>L. albus Cooked dry seeds (µg per 100 g fresh wt.)</td>
<td>–</td>
<td>–</td>
<td>57.8</td>
<td>–</td>
<td>17.8</td>
<td>127</td>
<td>–</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>L. angustifolius Seed (µg g&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>19</td>
<td>–</td>
<td>–</td>
<td>0.9</td>
<td>–</td>
<td>4.6</td>
<td>0.3</td>
<td>–</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>L. luteus Flour (mg per g catechin)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.022-0.027</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>L. albus Flour (mg per g catechin)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.031-0.077</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>L. angustifolius Flour (mg per g catechin)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.013-0.016</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>L. mutabilis Cotyledon (mg genistein per 100 g fresh wt.)</td>
<td>–</td>
<td>–</td>
<td>16-31</td>
<td>5.5-7</td>
<td>9-25</td>
<td>–</td>
<td>–</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>L. albus Hypocotyl (mg genistein per 100 g fresh wt.)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>2</td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>L. albus&lt;sup&gt;2&lt;/sup&gt; Leaves (mg g&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>25-35</td>
<td>21-25</td>
<td>3.8&lt;sup&gt;f&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
<td>6</td>
</tr>
<tr>
<td>12</td>
<td>L. albus&lt;sup&gt;2&lt;/sup&gt; stems (mg g&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>–</td>
<td>–</td>
<td>23-32</td>
<td>20-27</td>
<td>1.5-3&lt;sup&gt;e&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>L. albus&lt;sup&gt;2&lt;/sup&gt; Roots (mg g&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>–</td>
<td>–</td>
<td>1.5-3</td>
<td>0.15-0.25</td>
<td>0.7-1&lt;sup&gt;f&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Apigenin-8,8-d-C-glucopyranoside (Visoehin).
<sup>b</sup>Apigenin-7-O-β-apiofuranosyl-8,8-d-C-glucopyranoside.
<sup>c</sup>Autumn sowing.
<sup>d</sup>Genistein. 

1 – Siger et al. (2012); 2 – Kalogeropoulos et al. (2010); 3 – Dueñas et al. (2009); 4 – Lampart-Sceppa et al. (2003); 5 – Ramilla et al. (2009); 6 – D’Agostina et al. (2008).
Phytosterols

Phytosterols (including plant sterols and stanols) are the analogous compounds of cholesterol in animals, with wide occurrence in vegetable oils, nuts, cereals and grain legumes (Ryan et al., 2007). Structural similarity of phytosterols with cholesterol permits them to relocate low-density lipoprotein cholesterol in the human intestine (Chen et al., 2014). Among more than 250 different phytosterols, the most common ones are β-sitosterol, campesterol, stigmasterol and their saturated stanols.

There is limited information available on phytosterol components in different lupin species. However, composition of phytosterols present in *L. albus* has been reported to demonstrate the effect of genotype and growing environment (Hamama & Bhardwaj, 2004). Depending on the studied factors, they found 19.9–28.7% sterols in unsaponifiable lipid fraction of lupin oil with major contribution by β-sitosterol, campest erol and stigmasterol. White lupin (*L. termis*) was found to be rich in sterols (1.2% of total lipids) when compared with the herbal oils of black cumin (*Nigella sativa*) (0.2% of total lipids) and artichoke (*Cynara scolymus*) (0.58% of total lipids) (Hassanein et al., 2011). As is the case with other legumes, cooking can significantly reduce the phytosterol content in lupin species. However, the effect may vary among the different phytosterols. In a study by Kalogeropoulos et al. (2010), stigmasterol (4.98 mg per 100 g fresh weight) was found more vulnerable to 80-min cooking than delta-avenasterol (5.89 mg per 100 g fresh weight) when compared with the previous studies (Hamama & Bhardwaj, 2004). On the other hand, sprouting significantly increase the nutritional value of lupin (Rumiyati et al., 2012) including increase in phytosterol contents (Rumiyati et al., 2013). In the study, the total phytosterol concentration in the germinated *L. angustifolius* [Australian Sweet Lupin (ASL)] seed flour increased by over threefold in 9 days (expressed per weight of oil basis) compared to the concentration in oil extracted from ungerminated ASL seed flour. Quantitatively, β-sitosterol was found to be the major phytosterol in the oil of both raw and germinated ASL seed flours, followed by campesterol and stigmasterol.

Tocopherols

Tocopherols, commonly referred as vitamin E, are believed as the most effectual lipid-phase natural antioxidants, which inhibit lipid peroxidation by acting as peroxyl radical scavengers that stop the chain reactions in membranes and lipoprotein particles (Traber & Atkinson, 2007). The determination of tocopherol content in several foods is of significance to establish the biological importance.

Lupin species mainly contain α-tocopherol, γ-tocopherol and δ-tocopherol. The presence of β-tocopherol is not yet detected in any species. The tocopherol contents vary among the different lupin species. γ-T is the most abundant isomer in all lupin species, whereas α-T and δ-T found to be species specific: for example, δ-T was not identified in *L. angustifolius*, and α-T and δ-T were not detected in *L. mutabilis* (Boschin & Arnoldi, 2011). In another study, the concentrations of α-tocopherol, γ-tocopherol and δ-tocopherol in raw *L. albus* seed were found to be 0.19, 20.1 and 0.25 mg 100 g⁻¹ of dry matter, respectively. This concentration significantly changed upon germination of *L. albus* seeds which have shown an increase in the α-tocopherol content by 20-folds while sharp decrease was observed in γ-tocopherol content (Frias et al., 2005). In addition, cooking of *L. albus* seed also causes significant loss of tocopherols, resulting in concentrations of δ-tocopherol as low as 0.02 mg per 100 g fresh weight (Kalogeropoulos et al., 2010). Lupin seeds are found to contain high concentrations of total tocopherols when compared with other folk medicinal oils such as artichoke and Nigella (Hassanein et al., 2011). Annicchiarico et al. (2014) have found unpredictable variations in tocopherol content, being largely inconsistent both across locations in different years and across genotypes in different environments.

Other important phytochemicals

Triterpenes are present abundantly in plants and animals with more than 20 000 compounds reported to date (Thimmappa et al., 2014). They are comparatively nontoxic and have pharmacological potential wielding anti-inflammatory, hepatoprotective, antitumour, antiviral, anti-HIV, antimicrobial, antifungal, antidiabetic, gastroprotective and antihyperlipidemic actions. Despite their beneficial properties, there is relatively little information available on their distribution in lupin species. Recently, the determination of triterpenic acids was reported in cooked lupin seeds (Kalogeropoulos et al., 2010). Ursolic acid (1.14 mg per 100 g fresh weight) was found in higher concentrations, followed by oleanolic acid (0.35 mg per 100 g fresh weight). Maslinic acid could not be detected in lupin samples while it was present in some other legumes such as chickpeas and lentils. Contrary to other phytochemicals, the genotypic effects on the content and the level of triterpene alcohol classes in lupin species were not found to be significant (Hamama & Bhardwaj, 2004). Lupenol was the most predominant (73.8–92.0% with a mean of 86.8% of total dimethyl sterols), and b-amin (4.8–11.1% with a mean of 6.8% of total dimethyl sterols) was the second-most abundant triterpene alcohol in lupin.
The carotenoids content (both of isochromic red and yellow fractions) of *L. barkeri* was higher as compared to *L. montana* and *L. albus* (Guemés-Vera et al., 2012). The difference in carotenoids contents observed among Lupinus samples agrees with interspecies variation, where characteristics pigments in this kind of legumes include lutein, zeaxanthin and carotene (Wang et al., 2008).

**Bioactivity**

Phytochemicals may have adverse effects such as limiting the digestibility of proteins, interfere with mineral bioavailability, induce pathological changes in intestine and liver tissues affecting metabolism, inhibit enzymes and bind nutrients making them unavailable. On the other hand, phytochemicals have a remarkable impact on the health and may provide therapeutic health benefits, including the prevention and/or treatment of diseases and physiological disorders (Prakash & Gupta, 2011) due to their antioxidant, anti-inflammatory activities, etc. This part of review comprehensively elaborates the various biological activities associated with lupin phytochemicals.

**Antioxidant activity**

The antioxidant activity of lupin seed is similar (Martínez-Villaluenga et al., 2009) or lower (Wang et al., 2008) compare to other grain legumes. Lupins contain high levels of phenolic compounds, mainly tannins and flavonoids (Oomah et al., 2006). Some studies reported the positive correlation between total phenolic contents and antioxidant activity (Siger et al., 2012). However, many studies found that there is no association between total phenolic content and antioxidant activity in lupin, as other compounds such as carotenoids, tocopherols and peptides present in lupin seeds may also contribute to the antioxidant activity (Ramíyay et al., 2013). The extraction process conditions also play important role in the antioxidant power of plant extracts. The hot methanol extract showed higher antioxidant activity than that of the cold extract (Tsai and et al., 1999) while the aqueous extract (phosphate buffer saline or tris buffer) contained higher level of phenolic compounds and showed higher antioxidant activity than those of the methanol extract (Fernandez-Orozco et al., 2006).

**Anti-inflammatory effect**

Anti-inflammatory activity of aqueous extract from bitter *L. mutabilis* seed was explored by measuring the inhibition of the oedema caused by the application of 2% carrageenan lambda in Sprague-Dawley rats (Gamarra-Castillo et al., 2006). The effect of three doses of lupin seed extracts (1000, 2000 and 4000 mg kg⁻¹ body weight) via oral administration was studied. It was found that oral administration of lupin seed extracts at dose 4000 mg kg⁻¹ exhibited a considerable reduction of oedema formation. The anti-inflammatory effect of lupin aqueous extract was significantly higher than that of the control group.

Recently, *in vitro* anti-inflammatory properties of protein hydrolysates from seeds of *L. angustifolius* have been investigated using a THP-1-derived macrophage model and inhibition of enzymes involving in the inflammatory pathway (Millán-Linares et al., 2014). Lupin protein hydrolysate (LPH) was attained by hydrolysis of lupin protein isolate (LPI) using two proteases,zyme and alcalase. The authors concluded that after treated THP-1-derived macrophages with LPH, the expression of proinflammatory cytokines (tumour necrosis factor, IL-6 and IL-1β) was reduced, and the expression of anti-inflammatory cytokines (chemokine C-C motif ligand 18) was increased. Moreover, LPHs inhibited the nitric oxide production (inflammatory mediator) significantly. Furthermore, LPHs inhibited the activities of four enzymes involving in the inflammatory pathway including phospholipase A2, cyclooxygenase 2, thrombin and transglutaminase. It suggested the potential use of LPHs as functional food to prevent chronic diseases related to inflammation.

**Anticarcinogenicity**

Advances in cancer research have been spectacular during the past decade. However, it is unfortunate that the rate of cancer incidence is increasing at an alarming rate. While it cannot be concluded that technological progress is promoting cancer rate, it is clear that research aim at combating cancer is essential.

Liu (2009) investigated anticancer activity of lupin seed extracts of sweet lupin (*L. angustifolius var. Fest*) and bitter lupins (*L. albus var. Wodjil, L. angustifolius var. Merrit* and *L. albus var. Minibeau*), and lupin alkaloids through cytotoxicity test against several cancer cell lines. The cytotoxicity was assessed against a P388 mouse cell line and a variety of human cell lines including prostate cancer (PC3), skin cancer (A-375), liver cancer (Hep-G2), colon cancer (Caco-2), foreskin fibroblast (HS27) and kidney epithelial (SV7t) cell lines. The cytotoxicity of alkaloid-rich fractions and pure alkaloids extracted from bitter lupin was also investigated. It was found that the methanol extract of bitter lupin exhibited higher cytotoxic activity against the P388 mouse lymphoblast cell line than the other three sweet lupins. In addition, the methanol extract of bitter lupin showed cytotoxic activity against P388 mouse lymphoblast and SV7t rert human kidney epithelial cell lines at high concentrations but no cytotoxicity.
against other cell lines including human breast, prostate, skin, liver, colon and foreskin cell lines. Alkaloid-rich fractions of bitter lupin exhibited cytotoxicity against P388, MCF-7, HS27 and SV77tert cell lines at concentration 1000 μg mL⁻¹; however, at concentration 500 μg mL⁻¹, the inhibition reduced below 30%. Moreover, the alkaloid-rich fraction showed little cytotoxicity against HepG2, Caco-2 and A375 cell lines. Two lupin alkaloids, angustifoline and lupanine were determined against the P388 mouse lymphoblast cell lines. Both of the alkaloids showed 30%–50% inhibition at a range of test concentration (0.78–100 μg mL⁻¹). Authors concluded that lupin seeds, especially sweet varieties, show low cytotoxicity. The main lupin alkaloids, angustifoline and lupanine, also have low cytotoxicity.

Antimutagenic effects of phenolic compounds, oligosaccharides and quinolizidinic alkaloids isolated and quantified from L. campestris seeds were observed by Martinez et al. (2003). The mutagenic activity of 1-nitropyrene (1-NP) as a model mutagen and the antimutagenic activities of phenolic compounds, oligosaccharides and quinolizidinic alkaloids from L. campestris seeds on Salmonella typhimurium tester strain YG1024 were determined using the Kado microsuspension assay. The results showed inhibitory effect of phenolic compounds from L. campestris seeds in a dose-dependent manner between 0 and 200 mg phenolic extract per tube with a maximum inhibition of 86%. Lupin carbohydrates from water and dimethyl sulfoxide (DMSO) extracts exhibited antimutagenic activity with the maximum inhibition of 32% and 76%, respectively. The higher percentage of inhibition from DMSO solvent may be owing to higher solubility of carbohydrate in DMSO than in water. In contrast to phenolic compounds and carbohydrates, the antimutagenic effects exhibited by quinolizidinic alkaloids in lupin did not depend on the concentration. The highest inhibitory effect (75%) on 1-NP mutagenicity was found from the lowest dose of tested quinolizidinic alkaloids (13.6 mg per tube).

Antimicrobial activity

The antibacterial properties of L. albus, L. hureus and L. angustifolius were studied by Lampart-Szcapa et al. (2003). The antibacterial activities of the ethanol extracts from cotyledons and seed coat of the three lupin species were tested on two species of bacteria, namely Gram (+) Bacillus subtilis ATCC6633 and Gram (+) Escherichia coli ATCC25922. It was found that the extracts from the lupin hull exhibit antibacterial activity against the indicator strains, but not from the extracts of lupin cotyledon. Inhibitory effect on the growth of test bacteria depended mainly on the content of total phenolic compound. The extracts derived from L. albus cv. Bac (bitter cultivar) presented the strongest antibacterial properties while the lowest activity was observed from L. hureus cv. Popiel. Moreover, Erdoganoglu et al. (2007) found that the alkaloid extract from lupin seeds presented significant antibacterial property on B. subtilis, S. aureus and P. aeruginosa while it showed weak activity on E. coli.

Several studies reported antifungal activity of lupin seed extract. Woldemichad & Wink (2002) found that saponins from the methanolic extract of L. angustifolius seed exhibited antifungal activity against Candida albicans. Erdoganoglu et al. (2007) investigated antifungal activities of L. angustifolius alkaloid extract against C. albicans and C. krusei. The alkaloid extract exhibited moderate antifungal activity against the test strains at minimum inhibitory concentrations of 250 μg mL⁻¹.

Barakat et al. (2010) studied the antiviral activity of Lupinus termes seed extract. Crude extracts of lupin seed (20 μg mL⁻¹; nontoxic dose from cytotoxic assay) were tested for antiviral activity against herpes simplex virus-I (HSV-1) and hepatitis A virus-27 (HAV-27) using plaque infectivity count assay. It was found that the seed extracts of L. termes exhibit strong antiviral activity; however, only the antiviral property against HAV-27 was observed from the extracts.

Conclusion

Phytochemicals possess potential biological properties and the consumption of sources containing them may play an important role to maintain long-term health. Lupin, a crop with high protein and fibre contents, has found with a rich source of phytochemicals importantly bioactive peptides, alkaloids, polyphenols, phytoestrogens, tocopherols, etc. The significant concentrations of these phytochemicals make the lupin flour a potential food ingredient especially for bakery products. As on the one hand, high fibre and protein contents help to control obesity and diabetes associated with the increase in body mass index, while on the other hand, the antioxidant, antihyperlipidemic and anti-inflammatory activities of phytochemicals of lupin act against various chronic diseases. Although significant literature is available on lupin phytochemicals and their associated health benefits, but still intensive work is required on their bioavailability after gastrointestinal digestion, their release from the complex plant matrix, clinical studies with authentic standards and molecular mechanisms of their integrated actions.

Acknowledgments

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Lupin bioactive and bioactivities: M. K. Khan et al. 2011

References


Ribeiro, M.L.L., Mandarino, J.M.G., Carro-Antez, M.C. et al. (2006). β-glucosidase activity and isoflavone content in germinated...


Effect of cultivar, cultivation year and dehulling on raffinose family oligosaccharides in Australian sweet lupin (*Lupinus angustifolius* L.).

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**Summary**

The aim of this study was to determine the effect of cultivar, cultivation year and dehulling on raffinose family oligosaccharides (RFOs) in current cultivars of Australian sweet lupin. Seed samples of ten cultivars grown in 2011, 2012 and 2013 were used in the study. Both whole seed and dehulled lupin samples were analysed for RFOs by high-performance liquid chromatography with evaporative light scattering detector. Lupin cultivar had a significant effect on RFO contents. Total RFO contents in whole seed and dehulled lupin samples varied between 7.3–10.1 g/100 g DM and 7.6–16.8 g/100 g DM, respectively. Belara and Mandelup cultivars had high levels of RFOs indicating the suitability for functional foods with probiotic effect. Gungurru and PBA Barlock contained low levels of RFOs and recommended for lupin-enriched foods with low flatulence effect. Cultivation year with similar climatic conditions had no significant effect on RFO contents. Dehulling increases raffinose, stachyose and total RFO contents in lupin.

**Keywords**

Australian sweet lupin, cultivation year, dehulling, lupin cultivar, raffinose family oligosaccharides.

**Introduction**

Lupin is a pulse containing high levels of protein and dietary fibre, low fat content and negligible amount of starch. It is also a good source of vitamins, minerals and bioactive compounds (Arnoldi et al., 2015; Khan et al., 2015). Australia accounts for approximately 85% of the world lupin production; lupin is mainly used as a feed ingredient (Lawrence, 2007).

There is a growing interest of using lupin in food application mainly due to its health benefits (Arnoldi et al., 2015). A range of lupin-incorporated foods such as biscuit (Jayasena & Nasar-Abbas, 2011), bread (Johnson et al., 2003; Villarino et al., 2014, 2015), instant noodle (Jayasena et al., 2010a), muffin (Nasar-Abbas & Jayasena, 2012; Rumiya et al., 2015), pasta (Lampart-Szczapa et al., 1997; Jayasena & Nasar-Abbas, 2012) and tofu (Jayasena et al., 2010b) have been developed.

RFOs are considered as antinutritional factors in human foods. Consumption of high levels of RFOs reduces absorption of glucose and methionine (Martínez-Villaluenga et al., 2008). RFOs are not digested and absorbed in the upper gastrointestinal tract of nonruminants. They pass to the large intestine and ferment anaerobically by gut microflora into short-chain fatty acids and gas which could result in flatulence and abdominal discomfort (Guillon & Champ, 2002; Martínez-Villaluenga et al., 2008). Flatulence and abdominal discomfort are scrutinised as an important factor that discourage human consumption of legumes including lupin (Han & Baik, 2006).

Beneficial health effects, especially prebiotic property, of RFOs have been reported. Wongputtisin et al. (2015) indicated *in vitro* prebiotic activity of RFOs from soya bean promoting the growth of *Lactobacilli* spp. but inhibiting the growth of *Escherichia coli* and *Salmonella enterica*. Several human studies also showed that RFOs promote the growth of beneficial bacteria (*Bifidobacterium* spp.) and decrease levels of...
pathogenic bacteria such as *Clostridium* spp. and coliforms (Benno et al., 1987; Gibson & Roberfroid, 1995; Guillou & Champ, 2002; Fernando et al., 2010). Therefore, there is an increasing interest in using RFOs as functional food ingredients. Recently, functional food from RFO preparation with prebiotic potential has been developed and commercially available (Li et al., 2013; Coorey et al. (2013)) showed that processing of lupin has an effect on its dietary fibre profile, indicating that dehulling can change the RFO profile of lupin.

Several studies conducted in European and South American lupin cultivars have indicated that lupin is a good source of RFOs. However, there is hardly any published information on RFO contents of current Australian sweet lupin (ASL) cultivars, even it is the major lupin produced in the world. The aim of this study was to determine the effect of lupin cultivar, cultivation year and dehulling on RFO (raffinose, stachyose, verbascose) contents. The findings are beneficial in selecting appropriate cultivars for food applications, that is low RFO cultivars for lupin-enriched foods with low flatulence effect and high RFO cultivars for functional foods with prebiotic potential.

**Materials and methods**

**Lupin samples**

Lupin seed samples of ten cultivars (Belara, Corumup, Gungarlu, Jenabilup, Mandelup, PBA Barlock, PBA Gunyiri, Quillnock, Tanjil and Walan 2385), from three cultivation years (2011, 2012 and 2013), grown at Wongan Hills research station (Western Australia), were obtained from the Department of Agriculture and Food Western Australia. Samples of Walan 2385 were available only from 2012 and 2013 cultivation years. Lupin seeds were dehulled by dry milling technique using a mechanical dehuller (Amar industries, Punjab, India). Hull was separated from the kernel using a vacuum separator (Kimseed, Perth, WA, Australia). Both whole seed and dehulled lupin samples were finely milled using a sample miller (Cemotec 1090; Foss, Mulgrave, Vic., Australia) at setting No.1 (the finest mill setting). All samples were packed in sealed polyethylene bags and stored in a freezer at −18 ± 2 °C until further analysis.

**Reagent and standard solutions**

Analytical grade D-(+)-raffinose pentahydrate (98.0%), stachyose hydrate from *Stachys tuberifera* (98.0%), verbascose (97.0%), acetonitrile and *ω*-naphthol were purchased from Sigma-Aldrich (Sydney, NSW, Australia). Ethanol (95%) and acetic acid were purchased from Thermo Scientific (Sydney, NSW, Australia). Zinc acetate was bought from Asia Pacific Speciality (APS) Chemical (Sydney, NSW, Australia). Potassium ferrocyanide was purchased from Ajax Finechem (Sydney, NSW, Australia). Ultra pure water was prepared by a filtration system with a resistivity of 18.2 MΩcm (Millipore, Billerica, MA, USA). Molisch’s reagent was prepared by dissolving 0.5 g of *ω*-naphthol in 10 mL of 95% ethanol. Carrez I reagent was prepared by dissolving 21.9 g zinc acetate and 3 g glacial acetic acid and made up to 100 mL with ultra pure water. Carrez II reagent was prepared by dissolving 10.6 g potassium ferrocyanide in 100 mL ultra pure water. Stock standard solutions of raffinose, stachyose and verbascose at concentrations of 1000 µg mL⁻¹ were prepared in ultra pure water and stored at 4 °C in a refrigerator. Standard solutions at concentration 100-500 µg mL⁻¹ were prepared by appropriate dilution of the stock standard solutions in ultra pure water. All standard solutions were filtered through a 0.45-µm Millipore membrane filter.

**Determination of raffinose family oligosaccharides**

Raffinose, stachyose and verbascose contents were determined using high-performance liquid chromatography with evaporative light scattering detector (HPLC-ELSD) following the sample preparation process described by Giannoccaro et al. (2008), and HPLC-ELSD conditions described by Shanmugasenan et al. (2013) with some modifications (Sanz & Martínez-Castro, 2007; Dvořáková et al., 2014). Each lupin sample (0.1 g) was mixed with 4 mL of ultra pure water and shaken in a shaking water bath (SWB20; Ratek, Melbourne, Vic, Australia) at 50 °C for 1 hr. The solution was then centrifuged (5810R; Eppendorf, Hamburg, Germany) at 3200 g for 10 min. The residue was re-extracted until the Molisch reaction test (standard method to determine the presence of carbohydrate) showed a negative result (approximately took 2–3 extractions). The supernatants from each extraction were combined in 50-mL centrifuge tube. Protein precipitation was conducted by adding 100 µL of Carrez I reagent and mixing for 1 min followed by adding 100 µL of Carrez II reagent and mixing for 1 min. The volume was made up to 15 mL with water. The tube was allowed to stand for 30 min to complete the protein precipitation. The solution was then centrifuged at 3200 g for 15 min at 4 °C. The cold temperature (4 °C) was used to minimise protein degradation. The supernatant was collected and filtered through 0.45-µm Millipore membrane filter.

Hewlett Packard Agilent 1100 HPLC system (Agilent, Santa Clara, CA, USA) with a degasser (G1322A), a quaternary pump (G1311A) and a Rheodyne model 7000 sample injector was used. The detector was an evaporative light scattering detector (ELSD 2000ES;
Grace Davison Discovery Sciences, Deerfield, IL, USA). Data processing was carried out with a Chemstation software (Agilent). Chromatography was performed on a Prevail carbohydrate column; 5 μm, 250 × 4.6 mm (Grace Davison Discovery Sciences). Under isocratic elution, acetonitrile:ultrapure water (55:45 v/v) was used as the mobile phase at a flow rate of 0.8 mL min⁻¹. ELSD used nitrogen as nebulising gas at a flow rate 2.0 L min⁻¹, and the temperature of drift tube was fixed at 40 °C. Injection volumes were 20 μL at ambient temperature. The total run-time of the HPLC analysis was 20 min. Calibration curves were plotted for each analyte based on the correlations between the logarithm of peak areas and the logarithm of concentrations. The second-order polynomial equation and log-log plotting of all analytes showed good R² values (0.99). Total RFO content was a summation of raffinose, stachyose and verbascose contents. Determination of RFO contents was performed in duplicate.

Statistical analysis

Statistical analysis was conducted using the SPSS statistical software version 22 (IBM Corporation, Armonk, NY, USA). One-way analysis of variance (ANOVA) with Tukey’s post hoc test (P < 0.05) was used to determine the effects of cultivar and cultivation year on RFO contents. Paired sample t-test (P < 0.05) was used to compare RFO contents between whole seed and dehulled lupin.

Results and discussion

RFO content

RFO contents in whole seed and dehulled lupin are presented in Tables 1 and 2, respectively. Raffinose, stachyose and verbascose were found in all samples. In whole seeds, the average total RFO content was 8.5 g/100 g DM and ranged from 7.3 to 10.1 g/100 g DM. Dehulled lupin had average total RFO contents of 10.5 g/100 g DM and ranged from 7.6 to 16.8 g/100 g DM. Martinez-Villaluenga et al. (2008) indicated that European and South American lupin cultivars were the richest source of RFOs (5.1–16.1 g/100 g DM) compared with other legumes such as chickpea (2.0–7.6 g/100 g DM), lentil (1.8–7.5 g/100 g DM) and soya bean (6.0–8.0 g/100 g DM).

Stachyose was the major RFO in lupin accounting for 63% in whole seed and 68% in dehulled lupin. These values were in agreement with the results indicating lupin contains stachyose approximately 60–70% of total RFO content (Saini & Lymberry, 1983; Frias et al., 1996; Martinez-Villaluenga et al., 2005). This study found current ASL cultivars were abundant sources of RFOs, especially stachyose.

Effect of lupin cultivar on RFO contents

Lupin cultivar had a significant effect (P < 0.05) on raffinose, stachyose, verbascose and total RFO contents (Tables 1 and 2). The composition of RFOs in lupin is influenced by the species and cultivar (Martinez-Villaluenga et al., 2008). There was a significant variation of RFO contents between cultivars in whole seed and dehulled lupin. High levels of total RFOs in dehulled lupin were found in Mandelup (16.8 g/100 g DM) and Belara (13.0 g/100 g DM). The lowest levels of RFOs in dehulled lupin were found in Gungurru (7.8 g/100 g DM) and PBA Barlock (7.6 g/100 g DM). Evans et al. (1993) showed that total RFO contents in dehulled seed of ASL cultivar Gungurru was 7.7 g/100 g DM which is similar to the result recorded for this study (7.8 g/100 g DM). Saini

<table>
<thead>
<tr>
<th>Cultivars</th>
<th>Raffinose</th>
<th>Stachyose</th>
<th>Verbascose</th>
<th>Total RFOs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belara</td>
<td>1.6 ± 0.3*</td>
<td>6.1 ± 0.8**</td>
<td>2.5 ± 0.2*</td>
<td>10.1 ± 0.8*</td>
</tr>
<tr>
<td>Corunup</td>
<td>1.3 ± 0.2**</td>
<td>5.7 ± 0.2**</td>
<td>2.0 ± 0.2**</td>
<td>9.1 ± 0.4**</td>
</tr>
<tr>
<td>Gungurru</td>
<td>1.2 ± 0.2**</td>
<td>4.5 ± 0.3**</td>
<td>1.7 ± 0.2**</td>
<td>7.3 ± 0.3**</td>
</tr>
<tr>
<td>Jenabilup</td>
<td>1.0 ± 0.1**</td>
<td>4.9 ± 0.3**</td>
<td>1.5 ± 0.2**</td>
<td>7.4 ± 0.4**</td>
</tr>
<tr>
<td>Mandelup</td>
<td>1.4 ± 0.1**</td>
<td>6.5 ± 0.3**</td>
<td>2.2 ± 0.2**</td>
<td>10.0 ± 0.5**</td>
</tr>
<tr>
<td>PBA Barlock</td>
<td>1.0 ± 0.2**</td>
<td>4.6 ± 0.8**</td>
<td>2.0 ± 0.4**</td>
<td>7.6 ± 0.9**</td>
</tr>
<tr>
<td>PBA Gumpy</td>
<td>1.3 ± 0.2**</td>
<td>5.2 ± 0.2**</td>
<td>1.9 ± 0.2**</td>
<td>8.4 ± 0.4**</td>
</tr>
<tr>
<td>Quillinock</td>
<td>1.8 ± 0.4**</td>
<td>5.4 ± 0.4**</td>
<td>2.0 ± 0.7**</td>
<td>9.0 ± 1.1**</td>
</tr>
<tr>
<td>Tanjil</td>
<td>1.2 ± 0.1**</td>
<td>4.9 ± 0.3**</td>
<td>1.7 ± 0.2**</td>
<td>7.9 ± 0.2**</td>
</tr>
<tr>
<td>Walan 2385</td>
<td>1.6 ± 0.1**</td>
<td>5.3 ± 0.3**</td>
<td>1.7 ± 0.2**</td>
<td>8.6 ± 1.0**</td>
</tr>
<tr>
<td>Average</td>
<td>1.3 ± 0.3</td>
<td>5.3 ± 0.7</td>
<td>1.8 ± 0.4</td>
<td>8.5 ± 1.2</td>
</tr>
<tr>
<td>Range</td>
<td>1.0-1.6</td>
<td>4.5-6.5</td>
<td>1.5-2.5</td>
<td>7.2-16.1</td>
</tr>
</tbody>
</table>

% of RFOs: 15.0% - 62.5% 22.0%

N = 6 for means and SD calculations.
Mean values with different superscripts in the same column are significantly different (P < 0.05).

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& Gladstones (1986) indicated RFO contents in whole seeds of Marri, Illyarrie, Unicrop and Uniharvest cultivars of ASL (No longer cultivated) ranged from 9.9 to 10.6 g/100 g DM. Evans et al. (1993) reported RFO contents in dehulled lupin of two obsolete ASL cultivars including Danja and Yorrel were 7.4 and 8.0 g/100 g DM, respectively. Up-to-date information on RFO contents in current ASL cultivar is important in selecting suitable cultivars for food applications. Based on the results of this study, Gungurru and PBA Barlock cultivars are recommended for lupin-enriched foods with low flatulence effect. Belara and Mandelup cultivars are suitable for functional foods with prebiotic potential.

**Effect of cultivation year on RFO contents**

Average contents of raffinose, stachyose, verbascose and total RFOs in whole seed and dehulled lupin samples from 2011, 2012 and 2013 cultivation years are presented in Figs 1 and 2, respectively. Cultivation year had no significant effect on individual RFO and total RFO contents in both whole seed and dehulled lupin. This could be due to the similar climatic conditions reported during the three cultivation years of the study. There were no significant differences in mean monthly maximum temperature (°C), mean monthly minimum temperature (°C) and mean monthly rainfall (mm) of cultivated area (Wongan Hills research station) between 2011, 2012 and 2013 (Fig. 3).

A limited number of studies have been conducted on the effect of cultivation year or climatic conditions on RFO contents in lupin. However, there are several studies on the effect of cultivation year on RFO contents in other legumes. Cultivation year with different climatic conditions, especially rainfall, had significant effects on total RFO contents in chickpeas and peas (Nikolopoulou et al., 2006, 2007). Johnson et al. (2013) reported that cultivation year had no significant effect on RFOs in lentil without specifying the similarity or difference of climatic conditions. However, the authors stated that soil moisture contents in the fields between two cultivation years were different. Tahir et al. (2011) concluded that although rainfall, soil type

| Table 2 RFO contents (g/100 g DM) in dehulled lupin of 10 lupin cultivars |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Cultivars       | Raffinose       | Stachyose       | Verbascose      | Total RFOs      |
| Bolar           | 2.2 ± 0.4d      | 8.0 ± 0.8b      | 2.9 ± 0.2d      | 13.0 ± 0.9d     |
| Conamup         | 1.4 ± 0.2f      | 6.7 ± 0.2 c     | 1.7 ± 0.1ef     | 9.8 ± 0.3de     |
| Gungurru        | 1.0 ± 0.4f      | 5.8 ± 1.5a      | 1.2 ± 0.2f      | 8.4 ± 1.9ef     |
| Jandabillup     | 1.5 ± 0.2 ef    | 6.7 ± 0.5 b     | 2.0 ± 0.2ae     | 10.2 ± 0.9cd    |
| Mandelup        | 1.5 ± 0.1f      | 13.0 ± 1.2f     | 2.3 ± 0.1f      | 18.8 ± 1.2f     |
| PBA Barlock     | 0.9 ± 0.0     | 5.2 ± 0.2c      | 1.5 ± 0.4b      | 7.6 ± 0.5f      |
| PBA Gunyidi     | 1.9 ± 0.3mp     | 6.7 ± 0.6 m     | 2.1 ± 0.4pa     | 10.8 ± 1.3m     |
| Quillonk        | 1.8 ± 0.1m      | 6.2 ± 0.3n     | 1.9 ± 0.3np     | 9.8 ± 0.5np     |
| Tanjil           | 1.1 ± 0.3n      | 7.1 ± 0.7o     | 1.6 ± 0.3n      | 9.8 ± 1.2oc     |
| Wulan 2315      | 1.6 ± 0.1n      | 6.0 ± 0.3m      | 1.6 ± 0.3n      | 9.3 ± 0.4od     |
| Average         | 1.5 ± 0.5       | 7.2 ± 2.3       | 1.9 ± 0.5       | 10.5 ± 2.8      |
| Range           | 9.9-22.2        | 5.2-10.0        | 1.5-2.9         | 7.6-16.8        |

N = 6 for means and SE calculations.

Mean values with different superscripts in the same column are significantly different (P < 0.05).

**Figure 1** Effect of cultivation year on raffinose, stachyose, verbascose and total RFO contents (g/100 g DM) in whole seeds. Mean values between cultivation years with different letters indicate significant difference (P < 0.05).
and temperature influenced the RFO contents in lentil, the effect of cultivar is more dominant than the environmental factors.

RFOs are accumulated in legume seeds during seed maturation and their physiological functions are associated with the desiccation tolerance and frost resistance (Martínez-Villaluenga et al., 2008). A few studies have reported conflicting information on the effect of maturation temperature on RFO contents in lupin. Górecki et al. (1996) found there was no significant effect of maturation temperature (13 and 28 °C) during seed maturation on RFO contents in lupin. In contrast, lupin matured at 18 °C had a twofold increase in stachyose and verbascose contents compared with seeds matured at 25 °C (Górecki et al., 1997). Piotrowsicz-Cielak (2006) also reported that lupin seeds matured at high temperatures contain more raffinose and stachyose than seeds matured at lower temperatures. The current study showed that cultivation year with similar climatic conditions had no impact on RFO content in lupin.

**Effect of dehulling on RFO contents**

Dehulling of legumes improves palatability, cooking quality and digestibility while reducing some of the antinutritional factors (Olayinka & Olayinka, 2011). The effects of dehulling on RFO contents of lupin are shown in Fig. 4. Dehulling resulted in a significant increase in raffinose, stachyose and total RFO contents (P < 0.05). Average total RFO content in dehulled lupin was significantly higher than that of whole seeds.
There are some discrepancies between the results of studies on the effect of dehulling on RFO contents in legumes. These could be due to the type of dehulling. Some studies have employed wet dehulling and others have used dry dehulling. Wet dehulling includes a step of soaking seeds in water to loosen the hull before dehulling and dry method is done using a mechanical dehuller without soaking (Tiwari et al., 2011). As RFOs are water-soluble compounds, wet dehulling results in a significant decrease in RFO contents as they could be solubilised and washed away (Mubarak, 2005; Omoikhoje et al., 2006). In contrast, dry dehulling increases RFO contents in legumes. Brenes et al. (2003) reported higher contents of total RFOs in dehulled lupin (6.3 g/100 g DM) than those of whole seeds (5.9 g/100 g DM). The authors used a commercial pea splitter with a plate-type grinder and removed the hulls mechanically. Wang et al. (2008) reported that dehulling using the Satake TM05C Grain Testing Mill resulted in a significant increase in stachyose and verbascose contents (two major RFOs) in all studied field pea cultivars, and increase in raffinose contents in some cultivars. The results of current study clearly indicated that dry dehulling resulted in a significant increase in total RFO contents in lupin. Commercial lupin flour is produced by dry dehulling method, and the flour is incorporated into various foods. Therefore, use of dry dehulling technique is appropriate to determine the effect of dehulling on RFO contents in lupin.

Conclusions

RFO content of lupin depends on the cultivar. Some cultivars are good sources of RFOs. Belara and Mandelup cultivars contain high amounts of RFOs and can be used to manufacture functional foods with prebiotic potential. Gungurru and PBA Barlock contain low levels of RFOs and are suitable for manufacturing lupin-enriched foods with low flatulence effect. Cultivation year with similar climatic conditions may not have significant impact on RFO contents. Food manufacturers will be able to select high or low RFO cultivars for particular food applications regardless of the cultivation year. Raffinose, stachyose and total RFO contents in lupin can be increased by dehulling.

References

RAFFINOSE FAMILY OLIGOSACCHARIDES CONTENTS IN
AUSTRALIAN SWEET LUPIN (\textit{LUPINUS ANGUSTIFOLIUS}) CULTIVARS

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Australian sweet lupin is a non-starchy grain legume which is high in protein and dietary fibre. Raffinose family oligosaccharides (RFOs) are the major water-soluble carbohydrates in legumes. These oligosaccharides are good source of prebiotics; however, high level of these compounds can cause flatulence and abdominal discomfort. The aim of this study was to determine RFOs contents in whole seed and dehulled lupin flour of ten cultivars grown in 2011, 2012 and 2013. Dehulled lupin flour samples contained significantly higher amount of RFOs than those of whole seed flour samples. RFOs in whole seed flour and dehulled lupin flour samples in different lupin cultivars varied between 7.3–10.1 g/100 g dry matter (DM) and 7.6–16.8 g/100 g DM, respectively. Belara and Mandelup had the highest levels of RFOs in both whole seed flour and dehulled lupin flour samples. Gunguru, Jenabillup and PBA Barlock contained low level of RFOs. The major RFO present in all lupin cultivars was stachyose (4.4–6.5 g/100 g DM in whole seed flour and 5.2–13.0 g/100 g DM in dehulled lupin flour). Year of cultivation had no significant effect on RFOs contents in both whole seed flour and dehulled lupin flour. It can be concluded that there is a wide variation of RFOs contents between different lupin cultivars.
Lupin, a non-starchy grain legume, is a rich source of protein and dietary fibre. Lupin is also a good source of essential minerals especially iron, zinc and calcium. Similar to other grain legumes, lupin contains phytate which may reduce the bioavailability of minerals. The phytate/mineral molar ratio was used to predict the bioavailability of minerals (iron, zinc and calcium). Whole seed and dehulled lupin flour of ten lupin cultivars from three years of cultivation were used. Cultivar had a significant effect on phytate, iron, zinc and calcium contents. High molar ratio of phytate/iron, phytate/zinc, phytate/calcium and phytate × calcium/zinc were found indicating poor bioavailability of minerals. To improve the bioavailability of minerals means of reducing or removing phytate need to be considered.