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

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Abstract

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

Keywords: *Lupinus angustifolius*; Australian sweet lupin; seed coat; polyphenols; genotype by environment; by-product; HPLC-DAD-ESI-MS/MS

List of Abbreviations

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

1. Introduction

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

The seed coat of pulses, including chickpea (Sreerama, Neelam, Sashikala, & Pratapa, 2010), faba bean (Boudjou, Oomah, Zaidi, & Hosseinian, 2013), field pea (Marles, Warkentin, & Bett, 2013), lentil (Oomah, Caspar, Malcolmson, & Bellido, 2011), and mung bean (Luo, Cai, Wu, & Xu, 2016), is the predominant contributor of polyphenols to the whole seeds (Zhong et al., 2018). For example, 80.3-84.2% of the total polyphenol and over 83.9% of total flavonoid content of whole mung bean seed is present in the seed coat (Luo et al., 2016). Additionally, significantly higher polyphenol levels are found in dark coloured (black and red) chickpea and lentil seeds than those in lighter coloured (white and beige) counterparts (Xu, Yuan, & Chang, 2007). In contrast, the total polyphenol content in seed coats of *L. mutabilis*,

L. albus, and *L. angustifolius* grown in Brazil is reported to be 1.15 - 4.49 mg catechin equivalents (CE)/g dry basis which is much lower than in cotyledons (7.38 - 12.42 mg CE/g dry basis) (Ranilla, Genovese, & Lajolo, 2009). A majority of tannins of lupin, however, is present in the seed coat (Petterson, 1998).

Despite these conflicting results, to the best of our knowledge, no work on identification and quantification of individual polyphenols in lupin seed coats have been reported. Moreover, effects of genotype and environment on the polyphenols in the ASL seed coat has not been investigated. To this end, in this paper, individual polyphenols in ASL seed coat were identified and quantified. Six commercial varieties of ASL grown in two locations in WA were used to evaluate the effects of genotype, environment, and their interaction ($G \times E$) on contents of the individual polyphenolics.

2. Materials and methods

2.1. Materials

Whole seeds (ca 4 kg) of six ASL genotypes (Coromup, Mandelup, Jenabillup, PBA Barlock, PBA Gunyidi and PBA Jurien) harvested from two locations within 2015 growing season were obtained from the Department of Primary Industries and Regional Development - Agriculture and Food (Kensington, WA, Australia). The lupins were grown in Wongan Hills (WH; 30.54 °S, 116.43 °E) and Eradu (ER; 28.70 °S, 115.05 °E) WA. Wongan Hills has a rainfall of 388.4 mm annually and 153.5 clear days with temperature ranging from 12 °C to 28 °C, whereas Eradu has a rainfall of 450.4 mm annually but a smaller temperature variation (14.4 °C -24.7 °C) and less clear days (108.1 days) (BOM (Bureau of Meteorology), 2017).

The two locations belong to lupin Agzone 5 and Agzone 2 respectively based on their rainfalls (White, French, McLarty, & Grains Research and Development Corporation, 2008). The seeds were cleaned manually, vacuum-packed in polyethylene bags and stored in the dark at 4 °C until use.

The seeds were dehulled by a laboratory-scale AMAR dehuller (NSIC.SSI, India). Coarse seed coats were separated by a vacuum separator (Kimseed Pty Ltd, WA, Australia) with manual removal of any remaining broken cotyledons. The seed coats were dried at 50 °C for overnight and milled to flours using a ZM 200 Retch Mill (Retsch GmbH & Co, Haan, Germany), then passed through (> 97 %) a 500 µm screen. The resulting seed coat flours were thereafter vacuum-packed in polyethylene bags and stored at 4 °C in the dark until analysis.

2.2. Reagents and polyphenols standards

Hydrochloric acid, sodium hydroxide, ethyl acetate, and LC-MS grade acetonitrile, formic acid and methanol were purchased from Thermo Fisher Scientific (Scoresby, Vic, Australia). Authentic standards, including caffeic acid, *trans*-cinnamic acid and ferulic acid were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Vitexin (apigenin-8-C-glucoside), *p*-coumaric acid, protocatechuic acid and genistein were purchased from Cayman Chemical (Redfern, NSW, Australia).

2.3. Extraction of free and bound phenolic compounds

Free polyphenols extraction: Polyphenols were extracted as described by our research team (Wu et al., 2016). Duplicate 2 g samples of each ground lupin seed coat was mixed with 10 mL chilled 80% methanol (methanol/water, v/v) in a 15 mL tube with polyvinyl lined cap.

After vortexed for 10 s, the tube was covered by aluminium foil and mixed by rotational-shaking for 2 h in the dark followed by centrifugation at 3220 ×g, 4 °C for 10 min. The residue was extracted twice more with 7 mL of 80% methanol, the supernatants were then collected, pooled and made up to 25 mL with 80% methanol. Extracts were stored at -80 °C before free phenolics analysis (FP fraction).

Bound polyphenols extraction: The seed coat residue from free fraction extraction was transferred to 50 mL tube and mixed with 15 mL 2 mol/L NaOH. The mixture was vortexed for 10 s; then the tube was flushed with N₂, capped and sealed. After covered by aluminium foil and rotational-shaken for 2 h at ambient temperature, the mixture was acidified to pH 2 with 12 mol/L HCl. Alkaline hydrolysed previously-matrix bound phenolics (BP fraction) was extracted with 15 mL ethyl acetate, intermittently shaken for 10 min then centrifuged at 3220 ×g, 4 °C for 5 min. Organic layer was collected and the extraction was repeated for 5 times. The combined extract was evaporated at 30 °C under N₂ to dryness. Extract was re-suspended with 10 mL of 100% methanol and stored at -80 °C before use.

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

Individual polyphenols were identified and quantified according to the procedure developed by our research group (Wu et al., 2016) with minor modifications. An Agilent 1200 auto-sampler HPLC system was coupled to diode array detector (DAD) and an MS/MS system (Agilent 6460 LC-QQQ, Agilent Technologies, Palo Alto, CA, USA). The DAD was set to monitor signals at 190-600 nm with resolution of 2 nm. After filtering through a millipore membrane (0.22 µm), samples (20 µL) were injected into the Kinetex XB-C 18 reversed phase-

HPLC column (5 μ m, 250 $\times$ 4.6 mm, Phenomenex, Torrance, CA, USA) at 25 °C. Solvent A consisted of 0.05% formic acid in water; solvent B was acetonitrile. Flow rate was set at 0.5 mL/min to facilitate the coupling to mass spectrometer. A linear gradient elution was conducted: linear to 8% B from 0% B in 18 min, and changed to 18% B in 2 min, to 20% B in 15 min, then linear to 80% B in 10 min, and finally to 100% B in 2 min. This was followed by extra 8 min for column washing (100% B) and re-equilibration (100% A) respectively.

Two stage MS/MS spectra were acquired in the electrospray ionisation (ESI) negative ion mode with full scan ranging from m/z 50 to 1300. 5 L/min of N₂ was employed as the nebulizing gas at 45 psi, 300°C. Capillary voltage and nozzle voltage were set at 3.5 kV and -500 V respectively. Sheath gas (N₂) was maintained at 11 L/min and 250 °C. Collision energy was selected as 25 eV based on the abundance of the daughter ions. Data acquisition and analyses were performed on the Agilent MassHunter workstation. The identification confidence level of each compound was presented in table 1 following La Barbera et al. (2017) described.

For quantification of individual phenolic compounds, only those peaks with signal/noise (S/N) >10 were selected. Due to the lack of available standards, individual phenolic compound (except protocatechuic acid, ferulic acid and genistein) were semi-quantified using compounds with similar chemical structures and UV absorption. Apigenin-7-*O*- β -apiofuranosyl-6,8-di- β -D-glucopyranoside (**F3**), vicianin 2 (**F4**) and apigenin-7-*O*- β -glucopyranoside (**F5/B5**) were quantified using vitexin as standard (Siger et al., 2012). Phenolic acid hexosides, including cinnamic acid glucoside (**F1**), *p*-coumaric acid glucoside (**B3**) and ferulic acid glucoside (**B4**) were quantified using their corresponding phenolic acids. Dicafeoylquinic acid (**F6**) content

was calculated using caffeic acid standard curve. Aromadendrin-6-C- β -D-glucopyranosyl-7-O-[β -D-apiofuranosyl-(1 $\rightarrow$ 2)]-O- β -D-glucopyranoside (**F2**) was quantified using dihydroquercetin (or taxifolin). **B1** and **F7/B7** were not quantified since the peaks were not confidently identified. All results for the compounds above were expressed as μ g standards equivalent per g of dry sample (μ g vitexin equivalent per g of dry sample for **F3** and **F4**, for example).

Linearity of the selected seven standards, and their spike recovery which was performed using lupin (Coromup, ER) seed coat and detected in free fraction are presented in table 2. The limit of detection (LOD) and quantification (LOQ) were calculated at S/N ratios of 3 and 10 respectively. Six replicates of a mixed standards solution at a same concentration were freshly prepared and analysed in a single day and six separate days to evaluate the intraday and inter-day precision respectively. The intra- and inter-day variations were calculated as relative standard deviation (RSD) of the peak area.

2.5. Statistical analysis

Fragmentations of flavonoid aglycones and glycosides were designated using nomenclature systems proposed by Ma, Li, Vanden Heuvel, and Claeys (1997) and Domon and Costello (1988) respectively. Proposed mass spectrum fragmentation pathways were drawn using ChemDraw Prime software (V 16.0, PerkinElmer, VIC, Australia).

All the results were reposted on dry basis (d.b.) and expressed as mean $\pm$ standard deviation ($n \geq 2$). Two-way ANOVA with Tukey post hoc tests were conducted to investigate the main effects of genotype (G), location (E) and their interaction (G $\times$ E). One-way ANOVA by Tukey

test was performed to find any significant differences ($P < 0.05$) between means of genotypes within locations. Independent samples t-test was used to compare across different locations. All analyses were performed on SPSS Statistics V22 (SPSS Inc., Chicago, Illinois, US).

3. Results and discussion

3.1. Identifications of individual polyphenols

The HPLC-DAD chromatograms of free polyphenol extract (FP) and bound polyphenol extract (BP) of PBA Barlock are shown in Fig 1. Peaks were labelled as **F1-8** (FP) and **B1-8** (BP) respectively. HPLC retention time (R_t), UV-Vis absorption maximum wavelength ($\lambda_{\max}$), deprotonated molecular ions and fragment ions of the 13 individual polyphenols are listed in table 1.

3.1.1 Flavones in free fraction

F3 was the dominant individual polyphenol in FP fraction. The UV-Vis absorbance at 275 nm and 340 nm of **F3** (Fig 2.) showed the characteristic UV absorption bands of flavones (Wu et al., 2016). The absorption properties were largely the same as that of the authentic apigenin-8-C- β -D-glucopyranoside (vitexin) standard (270 nm, 332 nm). The mass spectra of the **F3** revealed the $[M-H]^-$ ion at m/z 725. The yield of two-fold neutral 120 amu fragments (m/z 725 to m/z 605, $^{0,2}X_{1,1}$; and m/z 455 to m/z 335, $^{0,2}X_{1,2}$), as well as ions at m/z 383 (apigenin + 113) and m/z 353 (apigenin + 83) are characteristics of apigenin-di-C-glycosyl flavone fragmentations (Fig 2.&3.) (Ferrerres, Silva, Andrade, Seabra, & Ferreira, 2003). In addition, fragments at m/z 593 ($[(M-H)-132]^-$, Y_0), m/z 575 ($[(M-H)-150]^-$, $Z_{0,1}$) and m/z 455 ($[(M-H)-120-150]^-$, $^{0,2}X_{1,1}Z_{0,2}$) suggested the presence of *O*-pentose which was supported by diagnostic

ion pairs at m/z 575 and at m/z 455 (Cuyckens & Claeys, 2004; Vukics & Guttman, 2010). The pentose can be attached either to the flavone aglycone giving an -*O*-glycoside or one of the two glucoses forming a *C,O*-glycoside, which is difficult to distinguish by MS/MS (Cavaliere, Foglia, Pastorini, Samperi, & Lagana, 2005). However, this compound was tentatively identified as apigenin-7-*O*- β -apiofuranosyl-6,8-di-*C*- β -D-glucopyranoside (Api-7-*O*-Apif-6,8-di-*C*-Glc_p), mainly because it was previously isolated and identified using MS and nuclear magnetic resonance (NMR) spectra in the whole seeds of *L. hartwegii* by Kamel (2003) and *L. termis* by Elbandy and Rho (2014). The identification is further supported by the results of Siger et al. (2012) who identified the compound in *L. angustifolius*, *L. luteus* and *L. albus* seeds grown in Poland, and reported a highly similar mass spectra.

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

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

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

Besides the compound **F3**, **F4** and **F5/B5** found in our ASL seed coat samples, much more complex but similar flavone-glycosides were also reported, e.g., apigenin-7-*O*- β -apiofuranosyl-6-*C*- β -glucopyranosyl-8-*C*-(6''''-*O*-*E*-feruloyl)- β -glucopyranoside (molecular weight (Mw) = 902) in *L. hartwegii* (Kamel, 2003) ; apigenin-6-*C*- β -D-glucopyranosyl-8-*C*- $[\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 2)]- β -glucopyranoside (Mw = 740) and apigenin-6-*C*- β -D-

glucopyranosyl-8-C-[β -D-apiofuranosyl-(1 $\rightarrow$ 2)]- β -glucopyranoside (Mw = 726) in *L. termis* (Elbandy & Rho, 2014); apigenin-7-neohesperidoside (Mw = 578), apigenin-7-apioglucoside (Mw = 564) and several unidentified apigenin derivatives in raw and germinated *L. angustifolius* seeds (Dueñas, Hernandez, Estrella, & Fernandez, 2009). Apart from apigenin as the aglycone, luteolin derivatives and diosmetin derivatives are also found in *L. angustifolius* seeds. All of these studies suggest the high complexity of flavones in lupins.

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

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

3.1.3. Dihydroflavonols in free faction

The strong UV-Vis absorption peak at 295 nm and the small peak of lower intensity (shoulder) at 325 nm implied that the **F2** could be flavanone or dihydroflavonol (Fig 2.) (Mabry, Markham, & Thomas, 1970). However, flavanones almost only occur in *Citrus* genus, but dihydroflavonols are ubiquitously distributed in legumes (Santos-Buelga et al., 2003). Dueñas et al. (2009) have found 4 dihydroflavonols, namely 3 dihydroquercetin (taxifolin) derivatives and dihydrokaempferol (aromadendrin) acetylglycoside in *L. angustifolius* seeds.

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

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

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

In terms of phenolic acid hexosides in bound fraction, *p*-coumaric acid glucoside (**B3**) and ferulic acid glucoside (**B4**) showed diagnostic 162 amu losses ($[M-H-Glu]^-$) with resulting in the corresponding ions at deprotonated phenolic acids. Deprotonated protocatechuic acid (**B2**, $[M-H]^-$ at m/z 153) and *p*-coumaric acid moiety ($[M-H-162]^-$ at m/z 163) of **B3** showed characteristic 44 amu carboxyl group losses and then giving signals at $[M-H-COO]^-$ and $[M-H-162-COO]^-$, namely ions at m/z 109 and m/z 119 respectively (Gruz, Novák, & Strnad, 2008; Wu et al., 2016). Moreover, fragment at m/z 178 of **B4** was due to a methyl radical loss ($[M-H-162-CH_3]^-$, 15 amu) of the ferulic acid moiety and then a further 44 amu loss to m/z 134 (Wu et al., 2016). We failed to get MS data for **B6**, but it had same retention time and UV-Vis absorption properties with the authentic ferulic acid standard. **B1** showed deprotonated ion at m/z 405 but produced fragments at m/z 191 and m/z 111 which are characteristic for quinic acid

(Clifford et al., 2005). Therefore, the compound was denoted as quinic acid derivative. Due to the lack of MS² data for either, **F7** and **B7** were designated as cinnamic acid derivative mainly basing on their characteristic maximum UV absorption at 340 nm. Protocatechuic acid (**B2**), ferulic acid glycoside (**B4**), ferulic acid (**B6**), *p*-coumaric acid and other hydroxybenzoic and hydroxycinnamic compounds previously found in *L. angustifolius* seeds (Dueñas et al., 2009).

3.2. Quantification of individual polyphenols.

Results for HPLC-DAD method validation are shown in Table 2. Briefly, the R² of the 7 analysed standards were all greater than 0.99, indicating good linearities within the ranges used. The intra- and inter-day variations of all the standards were lower than 0.48% and 2.74% respectively. Moreover, the percentage of recovery of these standards which spiked in lupin (Coromup, ER) seed coat ranged from 97.61% to 104.38% with acceptable precision. The results suggested that the HPLC-DAD method is adequate for quantifying the selected phenolics.

3.2.1. Quantifications of individual polyphenols in free fraction.

Of all individual polyphenols of the 12 lupin seed coat samples (6 genotypes by 2 locations), apigenin-7-*O*- β -apiofuranosyl-6,8-di-*C*- β -D-glucopyranoside (Api-7-*O*-Apif-6,8-di-*C*-Glc_p, **F3**) was the dominant compound in the free fraction, ranging from 697.85 μ g/g d.b. to 1011.82 μ g/g d.b. (as vitexin equivalent), which accounted for 73.08 - 82.89 % of the total polyphenols in free fraction (Table 3). High contents of this compound have also been found in Polish grown *L. angustifolius*, *L. luteus* and *L. albus* whole seeds, 409.6-428.8 μ g/g d.b., 709.8-876.9 μ g/g d.b. and 257.5-259.5 μ g/g d.b. respectively (Siger et al., 2012). Interestingly,

those authors also reported high levels of vicenin 2 (**F4** in the present study) in the whole seeds of the three-lupin species, being 277.8-302.5 µg/g d.b., 536.3-631.4 µg/g d.b. and 119.0-143.0 µg/g d.b. respectively. In contrast, much lower contents of vicenin 2 (**F4**) were found in our seed coats, from 24.65 µg/g d.b. up to 59.53 µg/g d.b. The differences might partly be related to the genetic (different genotypes) and environmental (different grown locations) differences, because considerable variability was also apparent in our collection as will be discussed below. In addition, the differences in distributions of the two compounds in the seed coat and cotyledon may have also contributed. Luo et al. (2016) reported provocatively high vitexin (apigenin-8-*C*-glucoside) and isovitexin (apigenin-6-*C*-glucoside) contents (37,430 µg/g d.b. and 47,180 µg/g d.b., respectively) in mung bean seed coat, while the two compounds were not detected in mung bean cotyledons. In the current study the high percentage of api-7-*O*-Api-6,8-di-*C*-Glc (F3) suggested that lupin seed coat could be a good plant source of the compound. For example, raw celery which is regularly the main dietary source of apigenin usually contains between 28.5 and 240.2 µg/g, but up to 786.5 µg/g in the seeds (Bhagwat & Haytowitz, 2014). Total flavone intakes have been associated with lower risk for all-cause mortality (Mink, 2007). Followed by purification and investigations of the potential bioactivities of F3, the compound also could be used to produce other apigenin derivatives (e.g., vitexin and isovitexin) by removing particular glycosides (e.g., by using acid hydrolysis) resulting in compounds that may have multiple pharmacological effects (He et al., 2016).

Phenolic acids (in free or esterified form) of lupins seeds, including *p*-hydroxybenzoic acid, procatechuic acid, chlorogenic acid, vanillin acid, *p*-coumaric acid and ferulic acid, have

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

It was also somewhat surprising that high levels of isoflavones (genistein) were found in the free fraction in our study at 22.30 - 62.60 µg/g d.b., which differed from some previous studies that reported very low levels of isoflavones (0.9 µg/g d.b.) in whole *L. angustifolius* seeds and did not detect them in its seed coat (Dueñas et al., 2009; Ranilla et al., 2009). However compared to the results of the present study, much higher levels of genistein and its derivative were previously reported in *L. mutabilis* seed coats, 98.09-870 µg/g fresh weight (Ranilla et al., 2009). Not unexpectedly, no anthocyanins were found in the lupin seed coats by the HPLC-MS/MS, which could be supported by the results at 520 nm of the DAD. Oomah,

Tiger, Olson, and Balasubramanian (2006) reported that only negligible anthocyanins were found in Australian sweet lupin seed using UV-Vis method. However, positive ion mode is generally used to identify the anthocyanins using mass spectrometry (Motilva, Serra, & Macia, 2013). Therefore, further investigations should be performed. Nevertheless, the results of this study indicated that the polyphenols in lupin seed coats were primary flavonoids (flavones plus isoflavone), 90.64 % to 94.41 % of total free polyphenol content, a finding that in agreement with that of the lentil seed coat polyphenol profile (Dueñas, Hernández, & Estrella, 2002), and that of whole seeds of *L. angustifolius*, *L. luteus* and *L. albus* (Siger et al., 2012).

As mentioned, the quantity of individual phenolic compounds varied across the 6 genotypes and the 2 locations, whereas the profile was found to be constantly stable. Statistical analysis revealed that the levels of free individuals and total free polyphenol content of ASL seed coats were significantly affected by genotype, location and their interaction, with all of the effects being significant at $P < 0.01$. In contrast, location exhibited no effects on total flavones ($P = 0.189$). Among the 6 genotypes, the lowest **F3** level, and accordingly, the lowest total flavone content and total polyphenol content were measured in PBA Jurien ($P < 0.01$), which followed by PBA Gunyidi ($P < 0.01$) and consistently so for both ER and WH. Comparisons across the two locations, seeds of all genotypes harvested from WH accumulated lower level of total hydroxycinnamics than that of the counterparts from ER ($P < 0.001$). The higher temperature and UV radiation, but lower rainfall at WH tended to decrease free phenolic acid derivatives levels in the lupin seed coats. A reverse trend, however, was observed in genistein content. Collectively, location exerted effects on the levels of phenolic individuals

but were in genotype-dependent manner; genotype was the determining contributor of the observed variations.

3.2.2. Quantification of individual polyphenols in bound fraction.

It was the first time to evaluate bound polyphenols in lupin seed coat. Bound polyphenols could reach colon then largely be metabolized by gut bacteria and show physiological benefits (Saura-Calixto, 2012). Although acid hydrolysis was used to hydrolyse the polyphenols from seed coats of chickpea (Sreerama, Sashikala, & Pratape, 2010) and lentil (Dueñas, Sun, Hernández, Estrella, & Spranger, 2003), alkaline hydrolysis was used in our study since alkaline hydrolysis was reported to be a better procedure to release polyphenols from polysaccharides than acid hydrolysis because it (1) can reduce polyphenols (especially flavonoids and phenolic acids) losses; (2) is an effective method of cleavage of ester bonds which bind polyphenols to the cell wall (Acosta-Estrada, Gutierrez-Urbe, & Serna-Saldivar, 2014). This approach has been employed to release polyphenols of 10 legumes seed coats (Sosulski & Dabrowski, 1984), and more recently lentil seed coat (Dueñas et al., 2002). As shown in table 4, the majority of phenolic individual types in bound fraction were phenolic acid derivatives. No *api-7-O-Apif-6,8-di-C-Glcp* (**F3**) and vicenin 2 (**F4**) were detected in the bound fraction. Ferulic acid derivatives widely occur in plant cell walls, and contribute to cell wall rigidity by crosslinking polysaccharides and lignin (Rosazza, Huang, Dostal, Volm, & Rousseau, 1995). In addition, they also esterify with various compounds (e.g., flavonoids, sterols and hydroxycarboxylic acids), that can be cleaved by alkaline hydrolysis.

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

4. Conclusions

Up to the present time, lupin seed coat is a low value animal feed and a waste disposal issue for lupin flour millers. In this study, three flavones (*apigenin-7-O- β -apiofuranosyl-6,8-di-C- β -glucopyranoside*, *vicenin 2* and *apigenin-7-O- β -glucopyranoside*), one isoflavone (genistein) and one dihydroflavonol derivative (*aromadendrin-6-C- β -D-glucopyranosyl-7-O-*

[β -D-apiofuranosyl-(1 $\rightarrow$ 2)]-O- β -D-glucopyranoside), together with several hydroxybenzoic and hydroxycinnamic acid derivatives were, for the first time, identified in ASL seed coats using HPLC-ESI-MS/MS. Mass spectrum fragmentation pathways for apigenin-7-O- β -apiofuranosyl-6,8-di-C- β -glucopyranoside (**F3**) and vicenin 2 were also proposed. The remarkable concentration of **F3** in the free polyphenol extracts highlighted the potential that lupin seed coat could be good source for the compound. Taken together, these results of this study support and promote the idea that ASL seed coat could be further value added by exploring the potential for it as a fibre-polyphenol bioactive ingredient, manufacturing flavonoid-fortified high fibre foods and flavonoid-based nutraceuticals, for example. However, future studies are required to further optimize the phenolic extraction method and MS/MS conditions (like using positive ion mode and higher collision energy); and isolate and standardize **F3** to confirm structures (especially its glycosylation patterns) using higher resolution MS spectrometry and NMR; investigate the influences of processing, including harvest, storage, dehulling, milling and food development technologies (e.g., baking, boiling, extrusion) on polyphenol composition and bioavailability in ASL seed coat.

This study has also examined the impacts of genotype, environment and their interaction on ASL seed coat polyphenols. The results demonstrated that both free and bound polyphenols in ASL seed coat were significantly affected by all of genotypic and environmental factors. However, the observed variation was largely attributed to genotype. Notwithstanding, only two growing sites were selected in this study. Their environmental conditions were similar and

potential seasonal effects were not included. Further screenings, using a broader range of environmental conditions, are important to obtain more comprehensive insights on this matter.

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Conflict of interest statement

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610

Figure Caption

Figure 1. UV chromatogram at 280 nm (DAD) and base peak chromatogram (BPC, all) of the free (A) and bound fraction (B) of PBA Barlock (ER) seed coat.

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

Figure 3. Proposed mass spectrum fragmentation pathway for apigenin-7-*O*- β -apiofuranosyl-6,8-di-*C*- β -D-glucopyranoside (**F3**) and apigenin-6,8-di-*C*- β -D-glucopyranoside (**F4**, dashed box).

Figure 1. UV chromatogram at 280 nm (DAD) and base peak chromatogram (BPC, all) of the free (A) and bound fraction (B) of PBA Barlock (ER) seed coat.

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

633 Figure 3. Proposed mass spectrum fragmentation pathway for apigenin-7-O-β-apiofuranosyl-6,8-di-C-β-D-glucopyranoside (F3) and apigenin-
 634 6,8-di-C-β-D-glucopyranoside (F4, dashed box).

635

636 **TABLES**

637

638 Table 1. Polyphenols identified by HPLC-ESI-MS/MS in samples

Peak No.	t _R (min)	λ _{max}	[M-H] ⁻ m/z	m/z MS/MS (Abundance %)	Assigned identity	Identification confidence level	Reference
F1	20.63	275	309a	291(64); 247 (3); 180(30); 128(99)	Cinnamic acid glucoside	2a	Gruz, Novák, & Strnad, 2008
B1	9.89	202	405a	191(44);111(100)	Quinic acid derivative		Clifford, Knight, & Kuhnert, 2005
B2	23.08	275	153b	135(40); 109(80)	Protocatechuic acid	1	Gruz, Novák, & Strnad, 2008
B3	25.25	226; 310	325b	163 (87); 119(64)	<i>p</i> -coumaric acid glucoside	2a	Gruz, Novák, & Strnad, 2008
F2	25.57	290; 325sh	743b	653(17); 623(100); 581(32); 563 (13); 533(31); 503 (22); 461(54); 371(15)	Aromadendrin-6- <i>C</i> -β-D-glucopyranosyl-7- <i>O</i> -[β-D-apiofuranosyl-(1→2)]- <i>O</i> -β-D-glucopyranoside	2b	Dueñas et al., 2009
B4	25.73	290	355b	193(34);178(30);134(100)	Ferulic acid glucoside	2a	Gruz, Novák, & Strnad, 2008
F3	26.43	275; 340	725a	635(2); 605(10); 593(5); 575(7); 503(1); 455(7); 383(1); 353(1); 335 (3)	Apigenin-7- <i>O</i> -β-apiofuranosyl-6,8-di- <i>C</i> -β-glucopyranoside	2a	Siger et al., 2012

F4	26.92	273; 340	593b	503 (9); 473 (16); 383(11); 353(22); 297(3)	Vicenin 2	2a	Cao, Yin, Qin, Cheng, & Chen, 2014
F5/B5	33.94	278; 320	431c	n.d.	Apigenin-7- <i>O</i> - β -glucopyranoside	2a	Santos-Buelga et al., 2003
F6	34.58	327	515c	249(5); 179(2); 135(6)	Dicaffeoylquinic acid	2a	Clifford, Knight, & Kuhnert, 2005
B6	36.94	300sh; 330	n.d.	n.d.	Ferulic acid	1	Gruz, Novák, & Strnad, 2008
F7/B7	42.93	300sh; 335	361c	n.d.	Hydroxycinnamic acid derivative		Gruz, Novák, & Strnad, 2008
F8&B8	44.97	267; 330sh	269a	269(100); 195(25); 133 (52)	Genistein	1	Vukics & Guttman, 2010

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

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

642 Identification confidence level: Level 1, confirmed structures where a reference standard is available; level 2a, evidence by spectrum matching
643 with a spectrum from the literature; level 2b, diagnostic evidence where no other structure fits the experimental MS² information (La Barbera et
644 al., 2017).

645 Table 2. Method validation parameters of the seven selected standards and their recovery in lupin (Coromup, ER) seed coat using the HPLC-
646 DAD.

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

647

648 Table 3. Individual polyphenol profile (µg/g dry basis) in free fraction of Australian sweet lupin seed coats¹

			PBA Jurien	Coromup	PBA Gunyidi	Mandelup	PBA Barlock	Jenabillup
Hydroxycinnamics								
F1	CIA ²	ER	19.51±1.69 ^{aA}	31.14±0.54 ^{dA}	28.15±0.52 ^{cdA}	27.12±0.38 ^{bcA}	24.32±0.82 ^{bA}	31.40±0.09 ^{dA}
		WH	17.10±0.11 ^{aA}	27.83±0.77 ^{bcB}	26.88±0.01 ^{bcA}	26.49±1.44 ^{bA}	25.63±0.51 ^{bA}	29.68±0.35 ^{cB}
F6	diCQA ³	ER	67.88±2.60 ^{abA}	89.03±15.01 ^{bA}	71.50±2.69 ^{abA}	57.35±0.48 ^{aA}	63.18±0.51 ^{aA}	65.25±0.08 ^{abA}
		WH	49.08±0.20 ^{bB}	55.52±0.35 ^{cA}	42.31±0.02 ^{aB}	43.62±1.63 ^{aB}	46.56±2.66 ^{abB}	46.90±0.02 ^{abB}
Σ Subgroup		ER	87.40±0.91 ^{aA}	120.18±15.55 ^{bA}	99.65±3.21 ^{abA}	84.47±0.85 ^{aA}	87.50±1.32 ^{aA}	96.65±0.01 ^{abA}
		WH	66.18±0.09 ^{aB}	83.35±1.13 ^{cA}	69.19±0.03 ^{abB}	70.11±3.07 ^{abB}	72.19±3.17 ^{abB}	76.58±0.32 ^{bcB}
Flavones								
F3	Api-Api ^f -di-Glcp ⁴	ER	717.28±2.22 ^{aA}	960.32±6.33 ^{cA}	764.60±3.47 ^{aA}	958.7±14.76 ^{cA}	883.18±19.52 ^{bA}	900.52±16.15 ^{bcA}
		WH	697.85±10.96 ^{aA}	954.85±7.87 ^{cdA}	804.18±6.21 ^{bB}	977.00±9.73 ^{deA}	1011.82±8.25 ^{eB}	928.95±18.85 ^{cA}
F4	Vicenin 2	ER	36.02±1.81 ^{bA}	25.70±0.06 ^{aA}	26.38±0.14 ^{aA}	33.68±0.92 ^{bA}	44.02±1.17 ^{dA}	28.48±0.05 ^{aA}
		WH	34.51±0.53 ^{bA}	24.65±0.20 ^{aB}	25.78±0.28 ^{aA}	33.84±0.06 ^{bA}	59.53±1.05 ^{cB}	32.62±2.35 ^{bA}
F5	Api- <i>O</i> -Glcp ⁵	ER	80.21±8.62 ^{abcA}	74.80±7.51 ^{abcA}	81.96±17.59 ^{bcA}	46.50±1.20 ^{aA}	91.67±0.61 ^{cA}	57.21±0.59 ^{abA}
		WH	40.76±2.27 ^{aB}	32.54±11.15 ^{aB}	22.42±0.03 ^{aB}	23.65±2.31 ^{aB}	48.02±11.77 ^{aB}	36.36±0.21 ^{aB}
Σ Subgroup		ER	833.51±9.04 ^{aA}	1060.82±13.9 ^{cA}	872.94±21.21 ^{aA}	1038.88±16.88 ^{bcA}	1018.86±21.3 ^{bcA}	986.21±15.51 ^{bA}
		WH	773.12±13.76 ^{aB}	1012.04±3.08 ^{cB}	852.38±6.46 ^{bA}	1034.49±12.11 ^{cA}	1119.37±2.47 ^{dB}	997.93±20.99 ^{cA}
Isoflavones								
F8	Genistein	ER	24.89±0.39 ^{abA}	25.01±0.72 ^{bA}	30.79±0.25 ^{cA}	22.30±1.35 ^{aA}	34.01±0.17 ^{dA}	25.82±0.36 ^{bA}
		WH	41.79±4.22 ^{abB}	47.92±6.03 ^{abcB}	32.53±0.45 ^{aB}	33.13±2.19 ^{aB}	51.00±5.59 ^{bcA}	62.60±5.19 ^{cB}
Dihydroflavonols								
F2	Aro-Glcp-Api ^f Glcp ⁶	ER	35.83±1.08 ^{aA}	56.57±0.16 ^{dA}	42.88±0.01 ^{bA}	40.92±0.54 ^{bA}	50.87±1.05 ^{cA}	61.03±0.74 ^{cA}
		WH	37.52±0.75 ^{aA}	38.83±2.91 ^{aB}	34.20±0.13 ^{aB}	40.93±3.65 ^{aA}	48.29±0.14 ^{aA}	39.01±13.8 ^{aA}
Total		ER	986.63±15.55 ^{aA}	1262.58±28.89 ^{cA}	1046.26±24.66 ^{aA}	1186.58±16.93 ^{bcA}	1191.25±23.85 ^{bcA}	1169.71±15.89 ^{bA}
		WH	918.6±10.38 ^{aB}	1182.14±5.08 ^{cA}	988.3±6.76 ^{bA}	1178.66±3.19 ^{cA}	1290.84±6.44 ^{dB}	1176.12±1.68 ^{cA}

649 ¹ Means $\pm$ standard deviation (n=2).
 650 ² cinnamic acid glucoside (as *trans*-cinnamic acid equivalent); ³ dicaffeoylquinic acid (as caffeic acid equivalent); ⁴ apigenin-7-*O*- β -apiofuranosyl-
 651 6,8-di-*C*-glucopyranoside (as vitexin equivalent); ⁵ apigenin-7-*O*- β -glucopyranoside (as vitexin equivalent); ⁶ aromadendrin-6-*C*- β -D-
 652 glucopyranosyl-7-*O*-[β -D-apiofuranosyl-(1 $\rightarrow$ 2)]-*O*- β -D-glucopyranoside glucopyranoside (as taxifolin equivalent).
 653 ER, Eradu; WH, Wongan Hills.
 654 Means assigned with different small letters in the same row, and capital letters in the same column within each dependent variable indicate
 655 significant differences ($P < 0.05$).

656 Table 4. Individual polyphenol profile ($\mu\text{g/g}$ dry basis) in bound fraction of Australian sweet lupin seed coats¹

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

657 ¹ Means $\pm$ SD (n=2); ² *p*-coumaric acid equivalent; ³ as ferulic acid equivalent; ⁴ apigenin-7-*O*- β -glucopyranoside (as vitexin equivalent).

658 ER, Eradu; WH, Wongan Hills.

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