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

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23

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25

26 **Abstract**

27 Seeds of the legume lupin (*Lupinus* spp.) are becoming increasingly important as human food.
28 The seed coat, at ~25% of the whole seed of *Lupinus angustifolius* (Australian sweet lupin,
29 ASL), is the main by-product of lupin kernel flour production. The primary market for lupin
30 seed coat is low value feed with very limited use in foods. In this study, seed coats of six ASL
31 commercial varieties from two growing sites were sampled for identification and quantification
32 of polyphenols using a high-performance liquid chromatography (HPLC) with diode array
33 detector (DAD) and coupled with a triple quadrupole mass spectrometer which equipped with
34 electrospray ionization source (ESI-MS/MS). Three flavones (apigenin-7-*O*-β-apiofuranosyl-
35 6,8-di-*C*-β-glucopyranoside, vicenin 2, and apigenin-7-*O*-β-glucopyranoside), one isoflavone
36 (genistein) and one dihydroflavonol derivative (aromadendrin-6-*C*-β-D-glucopyranosyl-7-*O*-
37 [β-D-apiofuranosyl-(1→2)]-*O*-β-D-glucopyranoside), and several hydroxybenzoic and
38 hydroxycinnamic acid derivatives were identified. Considerable variations in levels of
39 individual polyphenols were found but apigenin-7-*O*-β-apiofuranosyl-6,8-di-*C*-β-
40 glucopyranoside was the predominant polyphenol in all samples accounting for 73.08 - 82.89 %
41 of the total free polyphenols. These results suggest that ASL seed coat could be valuable dietary
42 source of polyphenols.

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

List of Abbreviations

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

45 **1. Introduction**

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

57 The seed coat of pulses, including chickpea (Sreerama, Neelam, Sashikala, & Pratape,
58 2010), faba bean (Boudjou, Oomah, Zaidi, & Hosseinian, 2013), field pea (Marles, Warkentin,
59 & Bett, 2013), lentil (Oomah, Caspar, Malcolmson, & Bellido, 2011), and mung bean (Luo,
60 Cai, Wu, & Xu, 2016), is the predominant contributor of polyphenols to the whole seeds
61 (Zhong et al., 2018). For example, 80.3-84.2% of the total polyphenol and over 83.9% of total
62 flavonoid content of whole mung bean seed is present in the seed coat (Luo et al., 2016).
63 Additionally, significantly higher polyphenol levels are found in dark coloured (black and red)
64 chickpea and lentil seeds than those in lighter coloured (white and beige) counterparts (Xu,
65 Yuan, & Chang, 2007). In contrast, the total polyphenol content in seed coats of *L. mutabilis*,

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

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

77 **2. Materials and methods**

78 *2.1. Materials*

79 Whole seeds (ca 4 kg) of six ASL genotypes (Coromup, Mandelup, Jenabillup, PBA
80 Barlock, PBA Gunyidi and PBA Jurien) harvested from two locations within 2015 growing
81 season were obtained from the Department of Primary Industries and Regional Development -
82 Agriculture and Food (Kensington, WA, Australia). The lupins were grown in Wongan Hills
83 (WH; 30.54 °S, 116.43 °E) and Eradu (ER; 28.70 °S, 115.05 °E) WA. Wongan Hills has a
84 rainfall of 388.4 mm annually and 153.5 clear days with temperature ranging from 12 °C to
85 28 °C, whereas Eradu has a rainfall of 450.4 mm annually but a smaller temperature variation
86 (14.4 °C -24.7 °C) and less clear days (108.1 days) (BOM (Bureau of Meteorology), 2017).

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

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

97 *2.2. Reagents and polyphenols standards*

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

104 *2.3. Extraction of free and bound phenolic compounds*

105 ***Free polyphenols extraction:*** Polyphenols were extracted as described by our research
106 team (Wu et al., 2016). Duplicate 2 g samples of each ground lupin seed coat was mixed with
107 10 mL chilled 80% methanol (methanol/water, v/v) in a 15 mL tube with polyvinyl lined cap.

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

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

122 *2.4. HPLC-DAD-ESI-MS/MS analysis*

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

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

135 Two stage MS/MS spectra were acquired in the electrospray ionisation (ESI) negative ion
136 mode with full scan ranging from *m/z* 50 to 1300. 5 L/min of N_2 was employed as the nebulizing
137 gas at 45 psi, 300°C. Capillary voltage and nozzle voltage were set at 3.5 kV and -500 V
138 respectively. Sheath gas (N_2) was maintained at 11 L/min and 250 °C. Collision energy was
139 selected as 25 eV based on the abundance of the daughter ions. Data acquisition and analyses
140 were performed on the Agilent MassHunter workstation. The identification confidence level
141 of each compound was presented in table 1 following La Barbera et al. (2017) described.

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

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

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

163 *2.5. Statistical analysis*

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

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

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

174 **3. Results and discussion**

175 *3.1. Identifications of individual polyphenols*

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

181 *3.1.1 Flavones in free faction*

182 **F3** was the dominant individual polyphenol in FP fraction. The UV-Vis absorbance at 275
183 nm and 340 nm of **F3** (Fig 2.) showed the characteristic UV absorption bands of flavones (Wu
184 et al., 2016). The absorption properties were largely the same as that of the authentic apigenin-
185 8-*C*-β-D-glucopyranoside (vitexin) standard (270 nm, 332 nm). The mass spectra of the **F3**
186 revealed the $[M-H]^-$ ion at *m/z* 725. The yield of two-fold neutral 120 amu fragments (*m/z* 725
187 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)
188 and *m/z* 353 (apigenin + 83) are characteristics of apigenin-di-*C*-glycosyl flavone
189 fragmentations (Fig 2.&3.) (Ferreres, Silva, Andrade, Seabra, & Ferreira, 2003). In addition,
190 fragments at *m/z* 593 ($[(M-H)-132]^-$, Y_0), *m/z* 575 ($[(M-H)-150]^-$, $Z_{0,1}$) and *m/z* 455 ($[(M-H)-$
191 $120-150]^-$, $^{0,2}X_{1,1}Z_{0,2}$) suggested the presence of *O*-pentose which was supported by diagnostic

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

202 The UV-Vis absorption properties and fragment performances of **F4** were very similar
203 with those of **F3**, with typical UV absorption at 273 nm (Band II) indicating the introduction
204 of di-*C*-glycosides substituents at C6 or C8 positions (Santos-Buelga, García-Viguera, &
205 Tomás-Barberán, 2003). Likewise, the same two successive losses of 120 amu neutral portions
206 (*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*-
207 glycosides either. Moreover, the $[M\text{-}H]^-$ of **F4** (*m/z* 593) was 132 amu smaller than that of **F3**
208 (*m/z* 725) but accompanied by the same fragment ions at *m/z* 383 ($[(M\text{-}H)\text{-}120\text{-}90]^-$) and *m/z*
209 353 ($[(M\text{-}H)\text{-}120\text{-}120]^-$), indicating it was apigenin di-*C*-glycosides but was not conjugated by
210 the pentose, i.e., apiose. Ions at *m/z* 297 $[(M\text{-}H)\text{-}120\text{-}120\text{-}56]^-$ corresponded to the further losses
211 of 2×CO. As such, **F4** was tentatively identified as apigenin-6,8-di-*C*-β-D-glucopyranoside
212 (vicenin 2). This was consistent with the mass spectra of the standard (Cao, Yin, Qin, Cheng,

213 & Chen, 2014), and the compound was previously reported in seeds of lupins (Siger et al.,
214 2012).
215 Both **F5** and **B5** had λ_{max} at 278 nm and 320 nm. The deprotonated molecular ion $[M-H]^-$
216 of the compound was *m/z* 431 but this study failed to obtain its ion fragments (MS^2). However,
217 based on the UV absorption and deprotonated molecular ion, as well as the slightly longer
218 elution time (33.94 min) then the authentic standard vitexin (33.75 min), it could be tentatively
219 identified as apigenin-7-*O*-β-glucopyranoside (Api-7-*O*-Glc*p*) (Santos-Buelga et al., 2003).
220 The longer retention time of **F4** (di-*C*-glycosides) than that of **F3** (*O*-pentosyl-di-*C*-glycosides)
221 in our study could be explained by the induction of a third sugar moiety in **F3** which can
222 increase polarity and thus shorter retention time (Santos-Buelga et al., 2003). A longer retention
223 time of vitexin (mono-*C*-glycosides) than **F4** supported the elution order. In contrast, Siger et
224 al. (2012) reported a reverse elution order of the two compounds (**F3** and **F4**). This
225 inconsistency could be attributed to the different HPLC conditions and column used. In the
226 case of the longer retention time of **F5**/**B5** (mono-*O*-glycosides) than vitexin (mono-*C*-
227 glycosides), Santos-Buelga et al. (2003) revealed that flavone *O*-glycosides generally elute
228 after the corresponding *C*-glycosides.
229 Besides the compound **F3**, **F4** and **F5**/**B5** found in our ASL seed coat samples, much more
230 complex but similar flavone-glycosides were also reported, e.g., apigenin-7-*O*-β-
231 apiofuranosyl-6-*C*-β-glucopyranosyl-8-*C*- (6''''-*O*-*E*-feruloyl)-β-glucopyranoside (molecular
232 weight (Mw) = 902) in *L. hartwegii* (Kamel, 2003) ; apigenin-6-*C*-β-D-glucopyranosyl-8-*C*-
233 [α-L-rhamnopyranosyl-(1→2)]-β-glucopyranoside (Mw = 740) and apigenin-6-*C*-β-D-

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

240 *3.1.2. Isoflavones in free (FP) and bound polyphenol (BP) faction*

241 **F8/B8** had a deprotonated ion at *m/z* 269, and showed a characteristic ion at *m/z* 133 which
242 was the same as that of the authentic genistein standard but different from those of the apigenin
243 standard (*m/z* 151 and *m/z* 117) (Vukics & Guttman, 2010). It was therefore identified as
244 genistein and this identification was also supported by its UV spectra (λ_{max} = 267 nm). Some
245 studies reported that no isoflavones were detected in *L. angustifolius* species, whereas high
246 level of isoflavones (e.g., mutabilein, mutabilin) were found in *L. mutabilis* (Dini, Schettino,
247 & Dini, 1998; Ranilla et al., 2009). Other researchers, however, found genistein and its
248 derivatives in raw and/or germinated *L. angustifolius* seeds. Examples include genistein
249 apiofuranosyl diglycoside (Mw = 726), genistein diglucoside (Mw = 594), genistein-7-*O*-β-
250 glucopyranoside (Mw = 432), genistein diglycoside (Mw = 564, attaching pentose-hexose
251 moiety) and several types of genistein acetyl glycosides (Dueñas et al., 2009). The first three
252 compounds which had the same deprotonated molecular ions as **F3**, **F4** and **F5**/**B5** in the
253 current study, however, were identified as flavones, with apigenin being the corresponding
254 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*-[β-D-apiofuranosyl-(1→2)]-*O*-β-D-glucopyranoside (Aro-6-*C*-Glc*p*-7-*O*-Api*f* Glc*p*).

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

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

285 In terms of phenolic acid hexosides in bound fraction, *p*-coumaric acid glucoside (**B3**) and
286 ferulic acid glucoside (**B4**) showed diagnostic 162 amu losses ($[M-H-Glu]^-$) with resulting in
287 the corresponding ions at deprotonated phenolic acids. Deprotonated protocatechuic acid (**B2**,
288 $[M-H]^-$ at *m/z* 153) and *p*-coumaric acid moiety ($[M-H-162]^-$ at *m/z* 163) of **B3** showed
289 characteristic 44 amu carboxyl group losses and then giving signals at $[M-H-COO]^-$ and [M-
290 H-162-COO]$^-$, namely ions at *m/z* 109 and *m/z* 119 respectively (Gruz, Novák, & Strnad, 2008;
291 Wu et al., 2016). Moreover, fragment at *m/z* 178 of **B4** was due to a methyl radical loss ([M-
292 H-162-CH_3]$^-$, 15 amu) of the ferulic acid moiety and then a further 44 amu loss to *m/z* 134 (Wu
293 et al., 2016). We failed to get MS data for **B6**, but it had same retention time and UV-Vis
294 absorption properties with the authentic ferulic acid standard. **B1** showed deprotonated ion at
295 *m/z* 405 but produced fragments at *m/z* 191 and *m/z* 111 which are characteristic for quinic acid

296 (Clifford et al., 2005). Therefore, the compound was denoted as quinic acid derivative. Due to
297 the lack of MS^2 data for either, **F7** and **B7** were designated as cinnamic acid derivative mainly
298 basing on their characteristic maximum UV absorption at 340 nm. Protocatechuic acid (**B2**),
299 ferulic acid glycoside (**B4**), ferulic acid (**B6**), *p*-coumaric acid and other hydroxybenzoic and
300 hydroxycinnamic compounds previously found in *L. angustifolius* seeds (Dueñas et al., 2009).

301 *3.2. Quantification of individual polyphenols.*

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

309 *3.2.1. Quantifications of individual polyphenols in free fraction.*

310 Of all individual polyphenols of the 12 lupin seed coat samples (6 genotypes by 2
311 locations), apigenin-7-*O*-β-apiofuranosyl-6,8-di-*C*-β-D-glucopyranoside (Api-7-*O*-Api*f*-6,8-
312 di-*C*-Glc*p,* **F3**) was the dominant compound in the free fraction, ranging from 697.85 μg/g d.b.
313 to 1011.82 μg/g d.b. (as vitexin equivalent), which accounted for 73.08 - 82.89 % of the total
314 polyphenols in free fraction (Table 3). High contents of this compound have also been found
315 in Polish grown *L. angustifolius*, *L. luteus* and *L. albus* whole seeds, 409.6-428.8 μg/g d.b.,
316 709.8-876.9 μg/g d.b. and 257.5-259.5μg/g d.b. respectively (Siger et al., 2012). Interestingly,

317 those authors also reported high levels of vicenin 2 (**F4** in the present study) in the whole seeds
318 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
319 μg/g d.b. respectively. In contrast, much lower contents of vicenin 2 (**F4**) were found in our
320 seed coats, from 24.65 μg/g d.b. up to 59.53 μg/g d.b. The differences might partly be related
321 to the genetic (different genotypes) and environmental (different grown locations) differences,
322 because considerable variability was also apparent in our collection as will be discussed below.
323 In addition, the differences in distributions of the two compounds in the seed coat and
324 cotyledon may have also contributed. Luo et al. (2016) reported provocatively high vitexin
325 (apigenin-8-*C*-glucoside) and isovitexin (apigenin-6-*C*-glucoside) contents (37,430 μg/g d.b.
326 and 47,180 μg/g d.b., respectively) in mung bean seed coat, while the two compounds were
327 not detected in mung bean cotyledons. In the current study the high percentage of api-7-*O*-
328 Api*f*-6,8-di-*C*-Glc*p* (**F3**) suggested that lupin seed coat could be a good plant source of the
329 compound. For example, raw celery which is regularly the main dietary source of apigenin
330 usually contains between 28.5 and 240.2 μg/g, but up to 786.5 μg/g in the seeds (Bhagwat &
331 Haytowitz, 2014). Total flavone intakes have been associated with lower risk for all-cause
332 mortality (Mink, 2007). Followed by purification and investigations of the potential
333 bioactivities of **F3**, the compound also could be used to produce other apigenin derivatives
334 (e.g., vitexin and isovitexin) by removing particular glycosides (e.g., by using acid hydrolysis)
335 resulting in compounds that may have multiple pharmacological effects (He et al., 2016).
336 Phenolic acids (in free or esterified form) of lupins seeds, including *p*-hydroxybenzoic acid,
337 procatechuic acid, chlorogenic acid, vanillin acid, *p*-coumaric acid and ferulic acid, have

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

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

359 Tiger, Olson, and Balasubramanian (2006) reported that only negligible anthocyanins were
360 found in Australian sweet lupin seed using UV-Vis method. However, positive ion mode is
361 generally used to identify the anthocyanins using mass spectrometry (Motilva, Serra, & Macia,
362 2013). Therefore, further investigations should be performed. Nevertheless, the results of this
363 study indicated that the polyphenols in lupin seed coats were primary flavonoids (flavones plus
364 isoflavone), 90.64 % to 94.41 % of total free polyphenol content, a finding that in agreement
365 with that of the lentil seed coat polyphenol profile (Dueñas, Hernández, & Estrella, 2002), and
366 that of whole seeds of *L. angustifolius*, *L. luteus* and *L. albus* (Siger et al., 2012).

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

380 but were in genotype-dependent manner; genotype was the determining contributor of the

381 observed variations.

382 *3.2.2. Quantification of individual polyphenols in bound fraction.*

383 It was the first time to evaluate bound polyphenols in lupin seed coat. Bound polyphenols

384 could reach colon then largely be metabolized by gut bacteria and show physiological benefits

385 (Saura-Calixto, 2012). Although acid hydrolysis was used to hydrolyse the polyphenols from

386 seed coats of chickpea (Sreerama, Sashikala, & Pratape, 2010) and lentil (Dueñas, Sun,

387 Hernández, Estrella, & Spranger, 2003), alkaline hydrolysis was used in our study since

388 alkaline hydrolysis was reported to be a better procedure to release polyphenols from

389 polysaccharides than acid hydrolysis because it (1) can reduce polyphenols (especially

390 flavonoids and phenolic acids) losses; (2) is an effective method of cleavage of ester bonds

391 which bind polyphenols to the cell wall (Acosta-Estrada, Gutierrez-Uribe, & Serna-Saldivar,

392 2014). This approach has been employed to release polyphenols of 10 legumes seed coats

393 (Sosulski & Dabrowski, 1984), and more recently lentil seed coat (Dueñas et al., 2002). As

394 shown in table 4, the majority of phenolic individual types in bound fraction were phenolic

395 acid derivatives. No api-7-*O*-Api*f*-6,8-di-*C*-Glc*p* (**F3**) and vicenin 2 (**F4**) were detected in the

396 bound fraction. Ferulic acid derivatives widely occur in plant cell walls, and contribute to cell

397 wall rigidity by crosslinking polysaccharides and lignin (Rosazza, Huang, Dostal, Volm, &

398 Rousseau, 1995). In addition, they also esterify with various compounds (e.g., flavonoids,

399 sterols and hydroxycarboxylic acids), that can be cleaved by alkaline hydrolysis.

400 Following the trends in the results of polyphenol contents in the free fraction, the total
401 polyphenol contents varied among the genotypes and locations, but the results in bound fraction
402 showed much larger standard deviations. Generally, the effects of genotype, location and their
403 interaction on bound polyphenol quantity were found in great similarities with free fraction,
404 but with location and genotype × location showing no influences on levels of both api-7-*O*-
405 Glc*p* (**B5**, P = 0.117 and 0.269 respectively) and genistein (**B8**, P = 0.613 and 0.717
406 respectively). The hydroxycinnamics and api-7-*O*-Glc*p* were the dominant bound phenolic
407 individuals, totally accounted up to 96.58 % of total bound phenolic compounds. In addition,
408 individual and total bound polyphenol content of PBA Jurien of the two locations were the
409 lowest among the genotypes ($P < 0.001$). Conversely, PBA Barlock of the two locations had
410 the highest total bound polyphenol content which was mainly contributed by the highest levels
411 of total hydroxycinnamics. Particularly, the protocatechuic acid levels in PBA Jurien, 5.07 ±
412 2.99 μg/g d.b. (ER) and 5.68 ± 0.42 μg/g d.b. (WH) respectively, were much lower than PBA
413 Barlock (51.45 ± 4.96 μg/g d.b. and 63.38 ± 2.03 μg/g d.b. respectively). In contrast to free
414 fraction with respect to total bound polyphenol content, only PBA Gunyidi and Mandelup seeds
415 from WH showed statistically significantly higher than those of ER, but the remaining did not.

416 **4. Conclusions**

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

421 [β-D-apiofuranosyl-(1→2)]-*O*-β-D-glucopyranoside), together with several hydroxybenzoic
422 and hydroxycinnamic acid derivatives were, for the first time, identified in ASL seed coats
423 using HPLC-ESI-MS/MS. Mass spectrum fragmentation pathways for apigenin-7-*O*-β-
424 apiofuranosyl-6,8-di-*C*-β-glucopyranoside (**F3**) and vicenin 2 were also proposed. The
425 remarkable concentration of **F3** in the free polyphenol extracts highlighted the potential that
426 lupin seed coat could be good source for the compound. Taken together, these results of this
427 study support and promote the idea that ASL seed coat could be further value added by
428 exploring the potential for it as a fibre-polyphenol bioactive ingredient, manufacturing
429 flavonoid-fortified high fibre foods and flavonoid-based nutraceuticals, for example. However,
430 future studies are required to further optimize the phenolic extraction method and MS/MS
431 conditions (like using positive ion mode and higher collision energy); and isolate and
432 standardize **F3** to confirm structures (especially its glycosylation patterns) using higher
433 resolution MS spectrometry and NMR; investigate the influences of processing, including
434 harvest, storage, dehulling, milling and food development technologies (e.g., baking, boiling,
435 extrusion) on polyphenol composition and bioavailability in ASL seed coat.
436 This study has also examined the impacts of genotype, environment and their interaction
437 on ASL seed coat polyphenols. The results demonstrated that both free and bound polyphenols
438 in ASL seed coat were significantly affected by all of genotypic and environmental factors.
439 However, the observed variation was largely attributed to genotype. Notwithstanding, only two
440 growing sites were selected in this study. Their environmental conditions were similar and

441 potential seasonal effects were not included. Further screenings, using a broader range of
442 environmental conditions, are important to obtain more comprehensive insights on this matter.
443
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461

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610

611

612 Figure Caption

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615 Figure 1. UV chromatogram at 280 nm (DAD) and base peak chromatogram (BPC, all) of the

616 free (A) and bound fraction (B) of PBA Barlock (ER) seed coat.

617

618 Figure 2. ESI-MS/MS spectra (a) and UV-Vis absorption profile (b) of aromadendrin-6-*C*-β-

619 D-glucopyranosyl-7-*O*-[β-D-apiofuranosyl-(1→2)]-*O*-β-D-glucopyranoside (**F2**) and

620 apigenin-7-*O*-β-apiofuranosyl-6,8-di-*C*-β-D-glucopyranoside (**F3**).

621

622 Figure 3. Proposed mass spectrum fragmentation pathway for apigenin-7-*O*-β-apiofuranosyl-

623 6,8-di-*C*-β-D-glucopyranoside (**F3**) and apigenin-6,8-di-*C*-β-D-glucopyranoside (**F4**, dashed

624 box).

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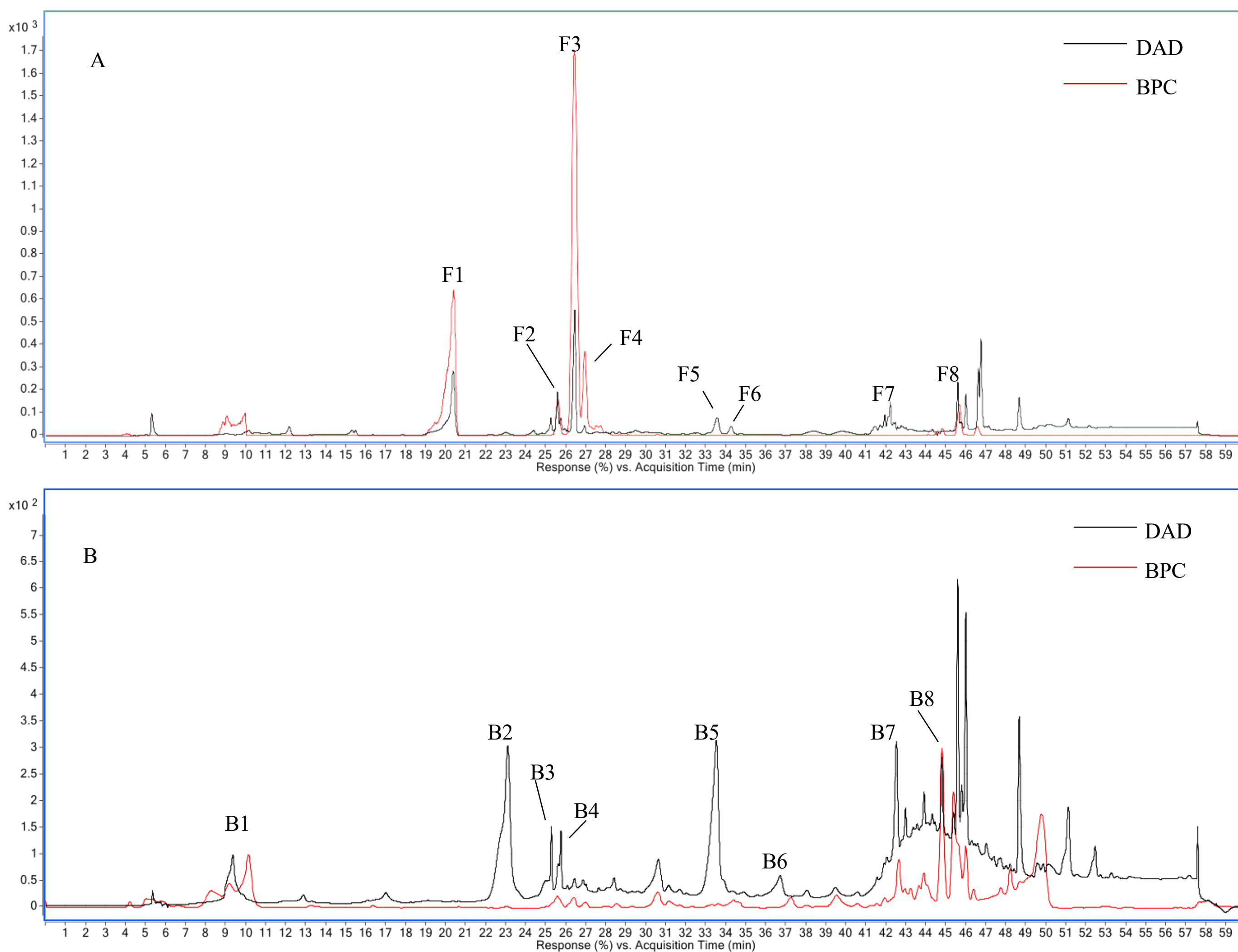

627 Figure 1. UV chromatogram at 280 nm (DAD) and base peak chromatogram (BPC, all) of the

628 free (A) and bound fraction (B) of PBA Barlock (ER) seed coat.

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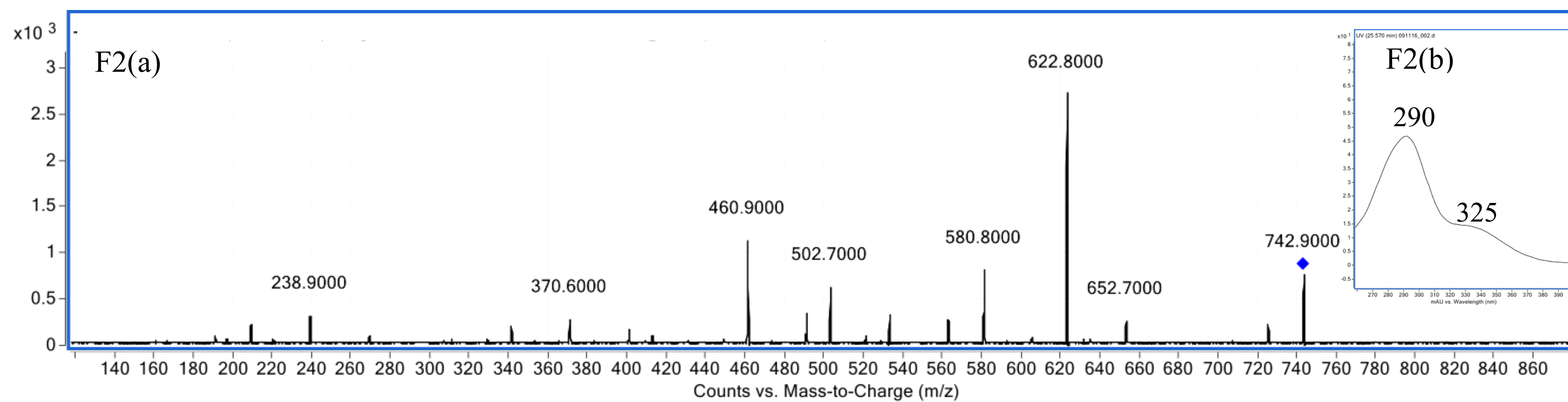
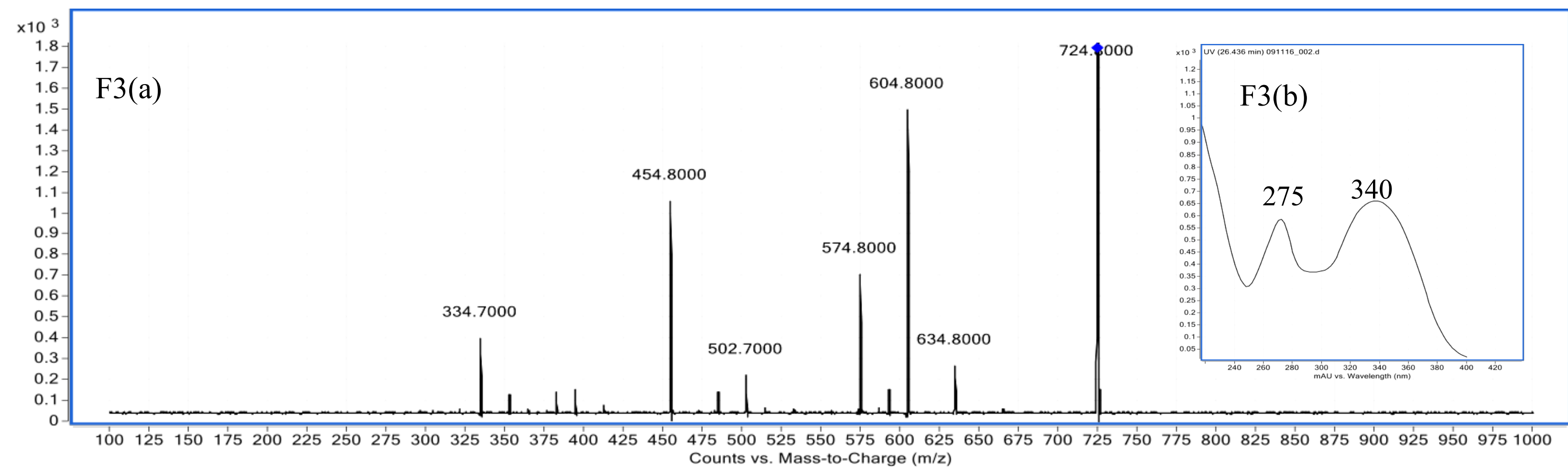
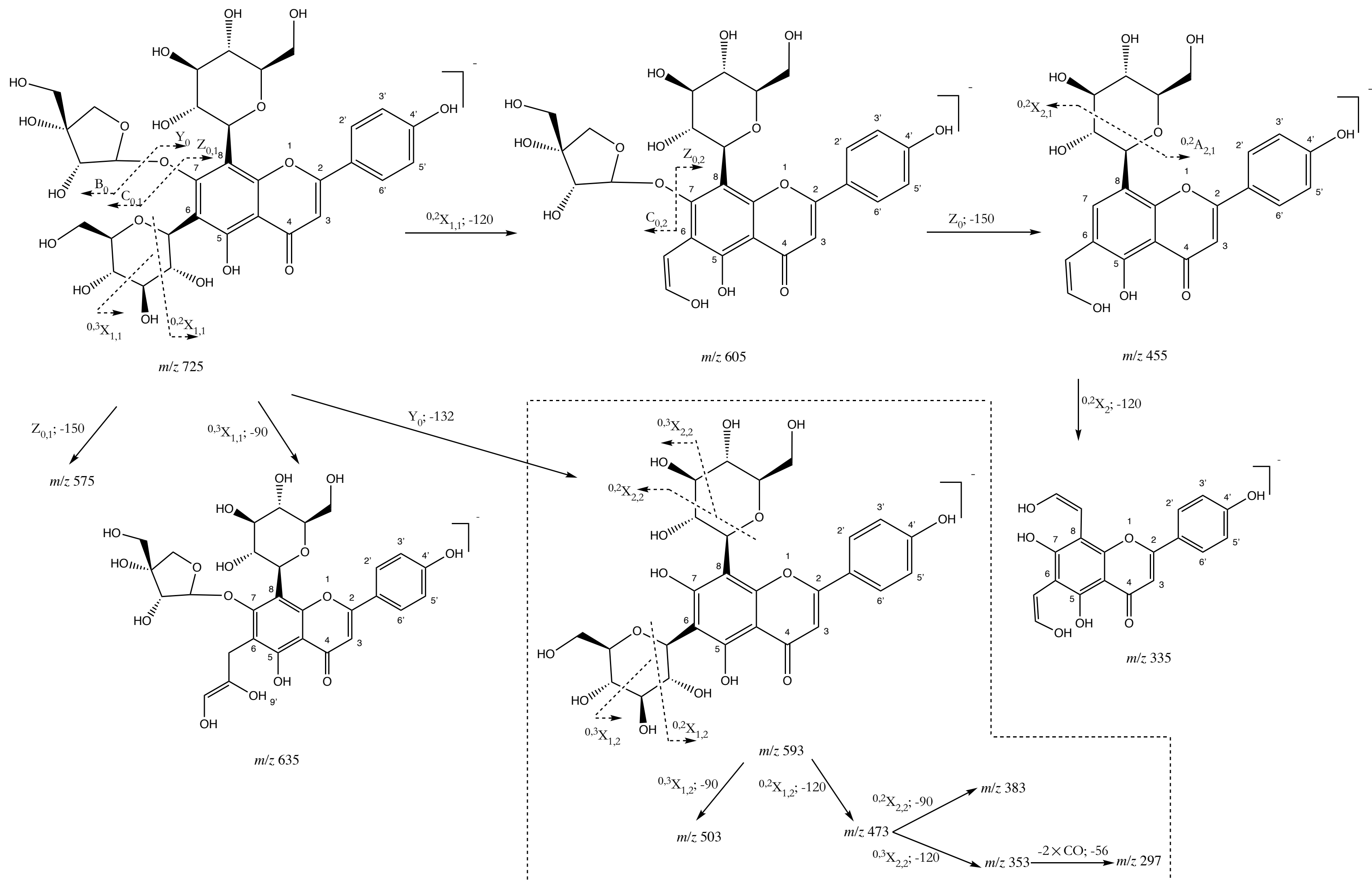




631 Figure 2. ESI-MS/MS spectra (a) and UV-Vis absorption profile (b) of aromadendrin-6-*C*-β-D-glucopyranosyl-7-*O*-[β-D-apiofuranosyl-(1→2)]-

632 *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)	*p*-coumaric acid glucoside	2a	Gruz, Novák, & Strnad, 2008
F2	25.57	290; 325sh	743b	653(17); 623(100); 581(32); 563 (13); 533(31); 503 (22); 461(54); 371(15)	Aromadendrin-6-*C*-β-D-glucopyranosyl-7-*O*-[β-D-apiofuranosyl-(1→2)]-*O*-β-D-glucopyranoside	2b	Dueñas et al., 2009
B4	25.73	290	355b	193(34);178(30);134(100)	Ferulic acid glucoside	2a	Gruz, Novák, & Strnad, 2008
F3	26.43	275; 340	725a	635(2); 605(10); 593(5); 575(7); 503(1); 455(7); 383(1); 353(1); 335 (3)	Apigenin-7-*O*-β-apiofuranosyl-6,8-di-*C*-β-glucopyranoside	2a	Siger et al., 2012

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

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

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

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

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
trans-Cinnamic acid	0.19-4.80	y=358.73+2.3522	1	1	4	99.32	2.30	0.11	0.34
Genistein	0.12-3.00	y=105.96x+1.5452	0.9997	4	13	97.89	4.42	0.23	0.37

647

648 Table 3. Individual polyphenol profile (μg/g dry basis) in free fraction of Australian sweet lupin seed coats[1]

			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-Glc*p*[4]	ER	717.28±2.22^{aA}	960.32±6.33^{cA}	764.60±3.47^{aA}	958.7±14.76^{cA}	883.18±19.52^{bA}	900.52±16.15^{bcA}
		WH	697.85±10.96^{aA}	954.85±7.87^{cdA}	804.18±6.21^{bB}	977.00±9.73^{deA}	1011.82±8.25^{eB}	928.95±18.85^{cA}
F4	Vicenin 2	ER	36.02±1.81^{bA}	25.70±0.06^{aA}	26.38±0.14^{aA}	33.68±0.92^{bA}	44.02±1.17^{dA}	28.48±0.05^{aA}
		WH	34.51±0.53^{bA}	24.65±0.20^{aB}	25.78±0.28^{aA}	33.84±0.06^{bA}	59.53±1.05^{cB}	32.62±2.35^{bA}
F5	Api-*O*-Glc*p*[5]	ER	80.21±8.62^{abcA}	74.80±7.51^{abcA}	81.96±17.59^{bcA}	46.50±1.20^{aA}	91.67±0.61^{cA}	57.21±0.59^{abA}
		WH	40.76±2.27^{aB}	32.54±11.15^{aB}	22.42±0.03^{aB}	23.65±2.31^{aB}	48.02±11.77^{aB}	36.36±0.21^{aB}
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-Glc*p*-Api*f*Glc*p*[6]	ER	35.83±1.08^{aA}	56.57±0.16^{dA}	42.88±0.01^{bA}	40.92±0.54^{bA}	50.87±1.05^{cA}	61.03±0.74^{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 ± 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→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 (μg/g dry basis) in bound fraction of Australian sweet lupin seed coats[1]

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

657 [1] Means ± 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).