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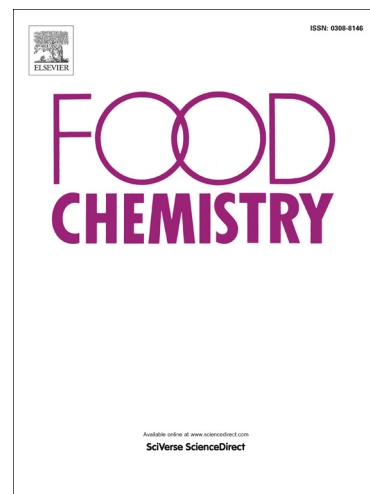
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1 **Original research article**

2 **Terpene evolution during the development of *Vitis vinifera* L. cv. Shiraz grapes**

3

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16

17 **Running title:** Terpene evolution in Shiraz grape development

**18 Abstract**

19 The flavour of wine is derived, in part, from the flavour compounds present in the  
20 grape, which change as the grapes accumulate sugar and ripen. Grape berry  
21 terpene concentrations may vary at different stages of berry development. This study  
22 aimed to investigate terpene evolution in grape berries from four weeks post-  
23 flowering to maturity. Grape bunches were sampled at fortnightly intervals over two  
24 vintages (2012-13 and 2013-14). In total, five monoterpenoids, 24 sesquiterpenes,  
25 and four norisoprenoids were detected in grape samples. The highest concentrations  
26 of total monoterpenoids, total sesquiterpenes, and total norisoprenoids in grapes  
27 were all observed at pre-veraison. Terpenes derived from the same biosynthetic  
28 pathway had a similar production pattern during berry development. Terpenes in  
29 grapes at harvest might not necessarily be synthesised at post-veraison, since the  
30 compounds or their precursors may already exist in grapes at pre-veraison, with the  
31 veraison to harvest period functioning to convert these precursors into final products.

32

33 **Keywords:** rotundone, terpene, sesquiterpene, grape ripening

34

**35 Highlights**

- 36 • Pre-veraison berries contain the highest total terpenoid concentrations
- 37 • Berries at different developmental stages have different terpene profiles
- 38 • Terpene biosynthesis pathways dictate production patterns during berry  
39 development
- 40 • Rotundone was present in Shiraz flower caps and grapes at both pre-veraison  
41 and post-veraison

42

## 43 1. Introduction

44 Grape berries contain hundreds of compounds that contribute to wine flavour and  
45 aroma. Most of these grape compounds exist as odourless, glycosidically-bound  
46 forms that are hydrolysed into active aroma compounds during fermentation or  
47 storage (Hjelmeland & Ebeler, 2015). However, some compounds in grapes, such as  
48 certain terpenes, may undergo minimal or no alteration and are directly linked to  
49 wine aroma (Dunlevy, Kalua, Keyzers, & Boss, 2009). The majority of identified  
50 terpenes that contribute to grape and wine flavour and aroma belong to the  
51 monoterpenes (monoterpenoids) sub-family, and only one sesquiterpene sub-family  
52 member is reported to be important for wine aroma and flavour (Dunlevy, et al.,  
53 2009). The oxygenated bicyclic sesquiterpene, named rotundone, gives grapes and  
54 wine a 'black pepper' character (Wood, et al., 2008) and is stylistically important to  
55 the 'terroir' of some cool climate Australian wine regions (Herderich, et al., 2012).  
56 Rotundone can be formed by aerial oxidation and biosynthesis from a precursor  
57 compound,  $\alpha$ -guaiene (Huang, Burrett, Sefton, & Taylor, 2014; Takase, et al., 2015).  
58 The discovery of rotundone has directed attention to the importance of  
59 sesquiterpene flavours; however, there is only limited research on sesquiterpene  
60 production in grapes.

61

62 Grape terpene production varies at different berry developmental stages: pre-  
63 veraison, the lag phase, and post-veraison (Kalua & Boss, 2009). In west Victoria  
64 (Australia), Shiraz grapevines start their annual cycle around late  
65 September/October, when dormant vines undergo bud burst and start to grow. In this  
66 region, Shiraz fruit set typically occurs from early to middle December, when berries  
67 experience a period of rapid cell division and slight cell expansion (pre-veraison)

68 before entering the lag phase of minimal berry growth during early January. At the  
69 end of the lag phase (late January to early February in this region), berries go  
70 through veraison with cell expansion, berry softening, and colour change  
71 (Gladstones, 2004). For Shiraz, harvest ripeness typically occurs from the middle of  
72 March to early April in this region, but usually in middle to late April in our study  
73 vineyard (P. Zhang, Barlow, Krstic, Herderich, Fuentes, & Howell, 2015; Pangzhen  
74 Zhang, Howell, Krstic, Herderich, Barlow, & Fuentes, 2015). The post-veraison to  
75 harvest period is critical for quality grape production, since grape berries rapidly  
76 accumulate sugar, change colour, shift metabolism, and accumulate flavour  
77 compounds (Gladstones, 2004).

78

79 Most previous studies on the development period-specific evolution of grape volatiles  
80 and terpenes have focused on the post-veraison to harvest period. A previous study  
81 reported that the total monoterpene concentration reaches its maximum at  
82 intermediate-ripe at 6-8° Baumé in Airen, and at around 11° Baumé in Chardonnay  
83 and Macabeo (García, Chacón, Martínez, & Izquierdo, 2003). However,  
84 norisoprenoids and monoterpenes concentrations have increase with grape  
85 maturation in Pinot noir grapes (Fang & Qian, 2006). In *Vitis vinifera* L. cv 'Fernão-  
86 Pires' grapes, 16 monoterpenes and two norisoprenoids were detected in grapes  
87 from veraison to harvest, which reached maximum concentrations at intermediate-  
88 ripe (Coelho, Rocha, Barros, Delgadillo, & Coimbra, 2007). Another study reported  
89 that bicyclic sesquiterpene concentrations increased from half-veraison to harvest in  
90 Riesling, Lemberger, Shiraz, and Yellow Muscat, while acyclic sesquiterpene  
91 concentrations decreased (May & Wüst, 2012). However, only 13 sesquiterpenes  
92 were identified in this study, with nine observed in Shiraz. A more comprehensive

93 study on sesquiterpene composition in *Vitis vinifera* L. cv. Baga found that most  
94 sesquiterpenes, including  $\alpha$ -guaiene, continuously increased from intermediate-ripe  
95 to post-maturation (Coelho, Rocha, Delgadillo, & Coimbra, 2006). In Cabernet  
96 Sauvignon, two monoterpenes and seven sesquiterpenes were identified at pre-  
97 veraison, but no terpenoids were observed at veraison and post-veraison (Kalua, et  
98 al., 2009; Kalua & Boss, 2010). The same study reported monoterpenes and  
99 sesquiterpenes in Riesling grapes at both pre-veraison and post-veraison, but not at  
100 veraison. Studies that have investigated rotundone accumulation in grapes after  
101 veraison demonstrated that it mainly accumulates after intermediate ripeness  
102 (Geffroy, Dufourcq, Carcenac, Siebert, Herderich, & Serrano, 2014; Pangzhen  
103 Zhang, et al., 2015). An understanding of sesquiterpene accumulation during berry  
104 development, especially prior to veraison, is lacking, and a detailed study on  
105 sesquiterpene evolution throughout the berry development stages is required.

106

107 Here, we fill this information gap and explore terpene evolution, especially the less  
108 well-studied but important sesquiterpenes, in grapes. Terpene profiles in Shiraz  
109 grapes were measured from early berry formation to late ripening. These data are  
110 useful for manipulation of compound production, such as rotundone, to improve wine  
111 aroma.

112

## 113 **2. Materials and methods**

### 114 *2.1. Chemicals*

115 Rotundone ((3S,5R,8S)-3,4,5,6,7,8-hexahydro-3,8-dimethyl-5-(prop-1-en-2-yl)-  
116 1(2H)-azulenone) and  $^2\text{H}_5$ -rotundone were synthesised as previously described  
117 (Siebert, Wood, Elsey, & Pollnitz, 2008; Wood, et al., 2008). A reference standard of

118  $\alpha$ -copaene was supplied by Sigma-Aldrich (Castile Hill, NSW, Australia). Working  
119 solutions of standards were prepared volumetrically in ethanol and stored at 4 °C until  
120 required. High-performance liquid chromatography (HPLC)-grade ethyl acetate, n-  
121 pentane, methanol, and ethanol were obtained from Rowe Scientific (Doveton, Vic,  
122 Australia). Analytical-grade potassium L-tartrate monobasic, tartaric acid, and other  
123 chemicals were obtained from Sigma-Aldrich. Water was purified using the Milli-Q  
124 system (Millipore Australia, Bayswater, Victoria, Australia).

125

## 126 2.2. Vineyard site

127 The study was conducted in a commercial vineyard (The Old Block, Mount Langi  
128 Ghiran 37.31°S, 143.15°E) located in the Grampians wine region of Victoria,  
129 Australia. The vineyard is approximately 15.5 km east of the nearest Bureau of  
130 Meteorology (BOM) weather station at Ararat Prison (Australian BOM Station No.  
131 089085). The long-term mean January temperature (MJT) recorded at this weather  
132 station is 19.1 °C, with annual average rainfall of 584.2mm by February 2015: it is  
133 classified as a cool climate wine region (Gladstones, 2004). The MJT and total  
134 rainfall from October to harvest for the two studied growing seasons (2012-13 and  
135 2013-14) were 20.0 °C, 124.1 mm and 21.7 °C, 140.9 mm, respectively (Table S-1).  
136 The vineyard was planted in 1968 with *Vitis vinifera*, cv. Shiraz on its own roots, 3.0  
137 m between rows and 1.8 m between vines, with rows oriented northeast to  
138 southwest. Grapevines were trained to a vertical shoot positioned (VSP) trellis, and a  
139 dripping irrigation system was installed along vineyard rows with a dripper spacing of  
140 0.5 m and dipper output of 1.5 litres per hour. Grapevines were irrigated when  
141 required at a rate of 5.76 L/(hr·vine). The total irrigation volumes from October to  
142 harvest in the two studied growing season seasons (2012-13 and 2013-14) were



143 84.3 mm and 60.8 mm, respectively. No significant pest or disease pressure was  
144 observed during the experimental seasons.

145

### 146 *2.3. Evaluation of rotundone evolution during grape development*

147 Shiraz grapes were sampled in triplicate (2 kg per field replicate) at fortnightly  
148 intervals from four weeks post-flowering (wpf) until commercial harvest in two  
149 consecutive growing seasons (2012-13 and 2013-14). The wpf was counted from the  
150 time of a minimum of 80% cap-fall, grapevine growth stage E-L 25-26 (Pearce &  
151 Coombe, 2004). Grape bunch samples were randomly collected from different  
152 grapevines across the vineyard (n>30 grapevines) on each sampling date. In the  
153 2013-14 growing season, samples were also collected at 80% caps off (E-L25) on 11  
154 December 2013. Final sampling was conducted at the beginning of commercial  
155 harvest on 10 April 2013 and 8 April 2014, respectively. Commercial harvest was  
156 performed between 9 and 16 April 2013 and between 8 and 17 April 2014 with  
157 selective hand harvest first followed by machine harvest at later dates. Grape  
158 samples were collected in zip-lock plastic bags, frozen at -20°C, transferred to the  
159 laboratory in Styrofoam boxes on dry ice, and stored at -20°C before analysis. Total  
160 soluble solids (TSS), titratable acidity (TA), and the pH of bunch samples were  
161 analysed using a refractometer, alkaline titration, and a pH meter, respectively,  
162 following published protocols (Iland, 2004).

163

### 164 *2.4. Preparation of samples and solid-phase microextraction-gas chromatography-* 165 *multidimensional mass spectrometry (SPME-MDGC-MS ) rotundone analysis*

166 Grape samples were prepared for rotundone analysis based on a published protocol  
167 (Geffroy, et al., 2014). For each grape sample, 100 g of de-stemmed grapes were

168 sub-sampled before being homogenised with a hand-held blender. Sub-samples  
169 were centrifuged to separate solids and liquids. The solids were mixed with 30 ml  
170 ethanol, 30 ml water, and 100 µl d5-rotundone (516 ng/ml in ethanol) as internal  
171 standard, then shaken for 24 h at 22°C and sonicated before reintroducing the juice.  
172 Sub-samples were then centrifuged and filtered (1.6 µm glass fibre) to obtain berry  
173 extract filtrate, which was topped up to 200 ml with deionised water prior to solid  
174 phase extraction (SPE) performed as reported previously (Siebert, et al., 2008). The  
175 SPE residue supernatant was air dried with nitrogen and reconstituted in 0.5 ml  
176 ethanol and 9 ml deionised water. The samples were then analysed by SPME-GC-  
177 MS in the Australian Wine Research Institute (AWRI) following established protocols  
178 (Geffroy, et al., 2014).

179

### 180 *2.5. Terpenoid analysis*

181 Terpenoid analysis was conducted for grape samples of 2013-14 vintage based on a  
182 published protocol (Parker, Pollnitz, Cozzolino, Francis, & Herderich, 2007) with the  
183 following modifications. An Agilent Technologies 6890 gas chromatograph (GC;  
184 Agilent Technologies, Santa Clara, CA) was equipped with a Gerstel MPS2  
185 multipurpose sampler and coupled to an Agilent 5973 mass selective detector (MSD).  
186 The instruments were controlled using Agilent G1701EA MSD ChemStation software  
187 in conjunction with Gerstel Maestro software (version 1.4.20.0). The GC was fitted  
188 with a J&W DB-5ms capillary column measuring approximately 30 m × 0.25 mm,  
189 0.25 µm film df. The carrier gas was helium (ultrahigh purity, BOC, Adelaide, SA,  
190 Australia), and the flow rate was 1.0 ml/min in constant flow mode. The GC inlet was  
191 fitted with a resilanised borosilicate glass SPME inlet liner (Supelco, 6.5 mm o.d.,  
192 0.75mm i.d., 78.5 mm long) held at 220°C.

193

194 The SPME fibre was desorbed in the pulsed splitless mode and the splitter, at 50:1,  
195 was opened after 30 s. The fibre was allowed to bake in the inlet for 10 min. The  
196 oven was started at 50°C, held for 1 min, increased to 230°C at 3°C/min, then  
197 increased to 280°C at 20°C/min and held at 280°C for 5 min. The MS transfer line  
198 was held at 250°C. The temperatures of the MS source and quadruple were 230°C  
199 and 150°C, respectively. The MS was operated in positive EI mode at 70 eV with  
200 simultaneous selected ion monitoring (SIM) and scanning over a mass acquisition  
201 range of 35-280 m/z.

202

203 100 g of de-stemmed grapes were sub-sampled before being homogenised using a  
204 hand-held blender. 5 g of homogenised sample was transferred into a HS-SPME vial  
205 (Agilent Technologies, 20ml) and mixed with 500 µL  $\alpha$ -copaene (200.64 µg/L in  
206 ethanol) as internal standard. The samples were then shaken for 24 h at 22°C before  
207 adding 2 ml saturated brine and being subjected to SPME-GC-MS analysis. The vial  
208 and its contents were heated to 45°C. A polydimethylsiloxane/divinylbenzene  
209 (PDMS/DVB, Agilent) 65µm SPME fibre was exposed to the headspace for 60 min  
210 with agitation. Sesquiterpenes were identified by comparing the mass spectra and  
211 retention indices with the terpenoids library in MassFinder (version 4.1, Dr Hochmuth  
212 Scientific Consulting, Hamburg, Germany). All compounds except  $\alpha$ -guaiene were  
213 semi-quantified as  $\alpha$ -copaene equivalents, expressed as relative areas  $\times$  100. It is  
214 therefore difficult to compare these semi-quantitative values with values in other  
215 studies.  $\alpha$ -guaiene was determined by SIM with  $\alpha$ -copaene as internal standard; the  
216 ions monitored were: m/z 105, 133, 147, 161, and 204; dwell time 25ms each. The  
217 target ions were typically m/z 147 for  $\alpha$ -guaiene and 161 for  $\alpha$ -copaene with ions 105,

218 133, and 204 m/z used as qualifiers. Data were analysed using Agilent G1701DA  
219 MSD ChemStation software.  $\alpha$ -guaiene was expressed as the ratio of m/z 147:m/z  
220 161 multiplied by the concentration of  $\alpha$ -copaene internal standard. The assay  
221 precision was validated by a series of standard additions of internal standard as  
222 described previously (Parker, et al., 2007). Blank SPME runs and blank internal  
223 standards were checked regularly.

#### 224 *2.6. Statistical analysis*

225 TSS, TA, pH, and terpenoids in grape samples from different berry developmental  
226 stages were compared using one-way analysis of variance (ANOVA) at  $p < 0.05$   
227 (CoStat, version 6.4, CoHort Software, Monterey, USA). Terpenoid concentrations at  
228 different berry developmental stages were analysed by discriminant analysis using  
229 SPSS v.21 (SPSS Inc., Chicago, IL. USA).

230

### 231 **3. Results and discussion**

#### 232 *3.1. Berry development pattern differentiation*

233 Different grape berry developmental stages have previously been characterised  
234 using grape berry weight, °Brix, pH, and titratable acidity measurements (Table 1)  
235 (Coombe & Iland, 2004; Kalua, et al., 2009). In this study, veraison occurred  
236 between 10 and 11 wpf in both growing seasons. The 2013-14 harvest was brought  
237 forward due to heavy rain forecast in late April 2014 (harvest at 17 wpf) and, as a  
238 result, the grapes were less ripe than in the 2012-13 growing season (harvest at  
239 18 wpf). As expected, °Brix and pH were lower and TA was higher in 2013-14 grapes.  
240 Initial measurements showed that there were fewer terpenes in grape samples at  
241 veraison compared to both pre- and post-veraison (Fig. 1).

242

243 The differences in terpene profiles at different berry developmental stages were  
244 analysed using discriminant analysis to identify terpene production patterns and the  
245 most important compounds present at each developmental stage. Discriminant  
246 analysis biplots (Fig. 2) were used to visualise terpene concentration patterns during  
247 berry development, and most of the variance (91.2%) was explained by the first two  
248 discriminant functions. Fourteen variables were used in the classification functions,  
249 including limonene, 1,8-cineole, geraniol, citronellol, clovene,  $\alpha$ -ylangene,  $\beta$ -  
250 bourbonene, (E)-  $\beta$ -caryophyllene,  $\beta$ -copaene,  $\alpha$ -guaiene, theaspirane isomer A,  
251 theaspirane isomer B, (E)- $\beta$ -damascenone,  $\beta$ -lonone. These compounds were  
252 shown to vary significantly among different grape ripening stages using ANOVA (See  
253 Table 1 in Pangzhen Zhang, et al. ). Grape berries at 4 and 17 wpf were clearly  
254 separated from the other developmental stages, while those at 6, 9, 13, 11 and 15  
255 wpf clustered into one group. Within this cluster, 6 and 15 wpf tended to separate  
256 from 9, 11 and 13 wpf. This suggests that the terpenoid profiles changed rapidly and  
257 markedly after fruit set between 4 and 6 wpf, but only slightly from 6 wpf to veraison  
258 (9, 11, and 13 wpf). Terpene profiles changed slightly from veraison (9-13 wpf) to  
259 intermediate-ripe (15 wpf), but rapidly towards 17 wpf. Thus, discriminant analysis  
260 allowed us to categorise berry developmental stages into early, pre-veraison,  
261 veraison, intermediate-ripe, and harvest, a useful frame of reference for  
262 understanding terpene evolution during berry development.

263

### 264 *3.2. Berry developmental stages and representative compounds*

265 A visual inspection of terpenoid profiles indicated that berries contain different  
266 terpenoids at different developmental stages (Fig. 1). Grape berries are terpene-rich,  
267 and peaks were well separated at 4 wpf (Fig. 1a). The number and peak size

268 dropped dramatically towards veraison (11 wpf) (Fig. 1b). However, there were more  
269 compounds at relatively low abundance at harvest (17 wpf; Fig. 1c). We have  
270 quantified the production of terpenoids based on relative amounts per kg in this  
271 study. We note that there may be some effects on the terpenoids concentration  
272 caused by the dilution from increased berry size and skin area. However, these  
273 changes won't affect the comparison of accumulation pattern among different  
274 metabolites. It is, therefore, reasonable to explore terpene evolution in grapes based  
275 on their developmental stages.

276

### 277 *3.2.1. Early berry developmental stage terpenoid profile (4 wpf)*

278 At the early developmental stage, berries appeared to be very capable of terpene  
279 production, as evidenced by the large numbers and high concentrations of  
280 terpenoids (Fig. 3a, Table 1 in Pangzhen Zhang, et al. ). Berries at this stage  
281 contained the highest concentration of total volatiles and terpenoids compared to all  
282 other stages, with the total terpenoid concentration accounting for 24.22% of the total  
283 volatiles detected in terms of GC peak area (Table 1 in Pangzhen Zhang, et al. ).  
284 The highest total monoterpenoid concentration was also observed at this stage, with  
285 geranyl acetone the most abundant monoterpenoid. Limonene was only observed at  
286 this stage, while 1,8-cineole (eucalyptol) and geraniol concentrations were  
287 significantly higher at this stage compared to other stages. The major  
288 sesquiterpenes observed at this stage were  $\alpha$ -humulene and  $\beta$ -caryophyllene,  
289 accounting for 33.88% and 29.95% of the total terpenoids (by GC peak area)  
290 respectively. This is consistent with a previous study that reported the highest  
291 concentrations of these two sesquiterpenes at early berry development in Cabernet  
292 Sauvignon (Kalua, et al., 2009). In addition,  $\gamma$ -muurolene,  $\alpha$ -muurolene,  $\delta$ -cadinene,

293 zonarene,  $\omega$ -cadinene, and  $\alpha$ -calacorene concentrations were also significantly  
294 higher at this stage than at all other stages. However, in Cabernet Sauvignon, only  $\gamma$ -  
295 muurolene was observed at early-stage berry development (Kalua, et al., 2009).  
296 Furthermore, *epi*-cubenol and cubenol were only observed at this stage. Four  
297 isoprenoids were observed at this stage and accounted for 8.44% of the total  
298 terpenoids.

299

### 300 3.2.2. Pre-veraison terpenoid profile (6 wpf)

301 At this stage, the terpenes were distant from those at 4 wpf in discriminant analysis  
302 (Fig. 2), indicating that the terpenoid profiles had significantly changed between  
303 these two time points. Significant decreases in total volatiles and terpenoid  
304 concentrations were observed at this stage (Table 1 in Pangzhen Zhang, et al. ). The  
305 total monoterpenoids slightly decreased, while geranyl acetone remained the most  
306 abundant monoterpenoid. All monoterpenoid concentrations decreased except  
307 citronellol, which slightly increased. The total sesquiterpene concentration decreased  
308 dramatically to less than 1/6 that of the previous stage, with  $\alpha$ -humulene and  $\beta$ -  
309 caryophyllene accounting for the majority of the decrease but remaining the most  
310 abundant sesquiterpenes. The concentrations of  $\gamma$ -muurolene,  $\alpha$ -muurolene,  $\delta$ -  
311 cadinene, zonarene,  $\omega$ -cadinene, and  $\alpha$ -calacorene all markedly decreased at this  
312 stage, while the concentration of  $\alpha$ -guaiene slightly increased and calamenene  
313 started to appear in the berries. The highest total norisoprenoid concentrations were  
314 observed at this stage and accounted for 37.7% of the total terpenoids in terms of  
315 GC peak area. Theaspirane isomer A was the most abundant norisoprenoid in terms  
316 of GC peak area and accounted for 19.1% of the total terpenoids at this stage.

317 Compared to the previous stage, the concentration of all norisoprenoids increased  
318 except  $\beta$ -ionone, which slightly decreased.

319

### 320 3.2.3. Veraison terpenoid profile (9-13 wpf)

321 This developmental stage was characterised by lower concentrations of total  
322 volatiles and terpenoids (Table 1 in Pangzhen Zhang, et al. ), suggesting that the  
323 berries at this stage might have lost the ability to produce volatile compounds or that  
324 previously formed compounds had been converted into non-volatile forms. This was  
325 consistent with previous observations in Cabernet Sauvignon, in which the fewest  
326 and lowest concentrations of volatiles were observed at similar stages (Kalua, et al.,  
327 2009). The 1,8-cineole, geraniol, and geranyl acetone concentrations continued to  
328 decrease and reached their lowest concentrations at wpf 11, while citronellol was not  
329 detected from wpf 11. Geraniol and geranyl acetone were previously observed at  
330 veraison in *Vitis vinifera* L. cv. Baga grapes (Coelho, et al., 2006) and, in the same  
331 study, limonene and citronellol were also detected at veraison. Geraniol, geranyl  
332 acetone, limonene, and citronellol were also observed in *Vitis vinifera* L. cv. 'Fernão-  
333 Pires' grapes at veraison (Coelho, et al., 2007). However, no monoterpenes were  
334 detected near veraison in Cabernet Sauvignon and Riesling (Kalua, et al., 2009,  
335 2010). The total sesquiterpene concentration decreased to its lowest level during this  
336 period, accounting for only 5.75% of the total terpenoids in terms of GC peak area at  
337 wpf 13.  $\beta$ -Caryophyllene was not detected from wpf 11, while clovene re-appeared at  
338 wpf 11. The concentrations of  $\alpha$ -guaiene,  $\alpha$ -humulene,  $\delta$ -cadinene, and calamenene  
339 decreased to very low levels, while  $\gamma$ -muurolene,  $\alpha$ -muurolene, and  $\omega$ -cadinene were  
340 not detected. The concentrations of zonarene and  $\alpha$ -calacorene continued to  
341 decrease to zero by the end of this period. Sesquiterpenes were not detected at



342 veraison in Baga, Cabernet Sauvignon, and Riesling grapes (Coelho, et al., 2007;  
343 Kalua, et al., 2009, 2010). The norisoprenoid concentration also decreased at this  
344 stage, but not as much as monoterpenoids and sesquiterpenes. As a result, total  
345 norisoprenoids accounted for 71.54% of the total terpenoids in terms of GC peak  
346 area at 11 wpf. All four norisoprenoids were also detected in Baga grapes, while only  
347  $\beta$ -damascenone and  $\beta$ -ionone were observed in 'Fernão-Pires' grapes at this stage  
348 (Coelho, et al., 2007; Coelho, et al., 2006).

349

#### 350 *3.2.4. Intermediate ripeness terpenoid profile (15 wpf)*

351 At this stage, the total volatile and terpenoid concentrations slightly increased but  
352 remained relatively low (Table 1 in Pangzhen Zhang, et al. ). The concentrations of  
353 all three monoterpenoids, 1,8-cineole, geraniol, and geranyl acetone, slightly  
354 increased. A slight increase in geraniol was also observed in Baga and 'Fernão-Pires'  
355 grapes at intermediate ripeness (Coelho, et al., 2007; Coelho, et al., 2006). The  
356 sesquiterpene concentrations, including clovene,  $\alpha$ -guaiene,  $\alpha$ -humulene,  $\delta$ -  
357 cadinene, and calamenene, remained low.  $\alpha$ -ylangene and *epi*-zonarene first  
358 appeared at this stage, paralleling a previous report in Baga (Coelho, et al., 2006).  
359 The total norisoprenoid concentration slightly decreased, with  $\beta$ -damascenone not  
360 detected from this stage onwards. However, relatively higher norisoprenoid  
361 concentrations were observed at this stage in Baga and 'Fernão-Pires' grapes  
362 (Coelho, et al., 2007; Coelho, et al., 2006).

363

#### 364 *3.2.5. Harvest terpenoid profile (17 wpf)*

365 The terpene profile at harvest was distant from intermediate ripeness in discriminant  
366 analysis (Fig. 2), indicating that the terpenoid profile significantly changed from 15 to

367 17 wpf. The total volatile concentration slightly decreased, while total terpenoids  
368 increased during this period (Table 1 in Pangzhen Zhang, et al. ). This increase was  
369 mainly due to the sesquiterpenes, since total monoterpenoids and norisoprenoids  
370 slightly decreased at this stage compared to 15 wpf. This suggests that the late  
371 ripening period, rather than the whole post-veraison period, is important for  
372 sesquiterpene development at harvest.  $\beta$ -bourbonene,  $\beta$ -copaene, guaia-6,9-diene,  
373 selina-4(15),6-diene,  $\delta$ -selinene,  $\gamma$ -cadinene, 7-epi- $\alpha$ -selinene, and  $\alpha$ -cadinene first  
374 appeared, while  $\alpha$ -calacorene reappeared, at this stage. The concentration of  
375 clovene,  $\alpha$ -ylangene,  $\alpha$ -guaiene, rotundone, and calamenene significantly increased,  
376 while  $\alpha$ -humulene, epi-zonarene, and  $\delta$ -cadinene tended to increase. This was  
377 consistent with previous observations in Baga grapes, in which all sesquiterpenes  
378 were only observed at later stages of ripening (Coelho, et al., 2006). In another study  
379 in Shiraz, higher  $\alpha$ -ylangene,  $\beta$ -bourbonene,  $\gamma$ -cadinene, and  $\alpha$ -humulene  
380 concentrations were observed at harvest compared to veraison (May, et al., 2012),  
381 although  $\alpha$ -humulene remained low in the present study.  $\alpha$ -muurolene was observed  
382 in Riesling grapes, but not the current study, at harvest (Kalua, et al., 2010).

383

### 384 3.3. Terpenoid evolution

385 All plant terpenoids are biosynthesised from their universal  $C_5$  precursor isopentenyl  
386 diphosphate (IPP) (1) and its allylic isomer, dimethylallyl pyrophosphate (DMAPP)  
387 (2), via the mevalonate (MVA) and methylerythritol-phosphate (MEP) pathways (Fig.  
388 4a). All terpenes are synthesised by repetitive addition of  $C_5$  isoprenoid units, with  
389 isoprene (3), the smallest compound in this family. (1) and (2) elongate to form  
390 geranyl diphosphate (GPP) (4), while farnesyl diphosphate (FPP) (5) and  
391 geranylgeranyl diphosphate (GGPP) (6) are the precursors of monoterpenes ( $C_{10}$ ),

392 sesquiterpenes (C<sub>15</sub>), and norisoprenoids (C<sub>13</sub>), respectively. We discuss terpenoid  
393 biosynthesis in this study according to terpene sub-families and pathways.

394

### 395 3.3.1. Evolution of monoterpenoids

396 The total free monoterpenoid concentration decreased from early berry development  
397 onwards (Fig. 3a). The four detected free monoterpenes are biosynthesised via  
398 different pathways from **(4)**, while geranyl acetone **(7)**, a monoterpenoid, is mainly  
399 derived from  $\zeta$ -carotene (Not shown in Fig. 4) (Simkin, Schwartz, Auldridge, Taylor,  
400 & Klee, 2004). However, there is still a possibility that **(7)** could be produced from **(5)**  
401 via nerolidol **(8)** (Boland, Gäbler, Gilbert, & Feng, 1998) (Fig. 4b). Geraniol **(9)**,  
402 limonene **(10)**, and linalool **(11)** are synthesised directly from **(4)** (Davis, 2010; Davis  
403 & Croteau, 2000; Hsiao, et al., 2006; Luan, Mosandl, Münch, & Wüst, 2005). 1,8-  
404 cineole **(12)** is synthesised via the  $\alpha$ -terpinyl cation **(13)** and  $\alpha$ -terpineol **(14)**, while  
405 citronellol **(15)** is synthesised via **(9)** and mainly exist in S-enantiomer form (Davis,  
406 2010; Davis, et al., 2000; Hsiao, et al., 2006; Luan, et al., 2005). Biosynthesis of the  
407 four detected monoterpenes are regulated by different terpenoid synthase genes  
408 (*VvTPS*), with some overlap (Martin, et al., 2010).

409

410 The production of **(10)** and **(12)** is similar (Fig. 3a) due to the common *VvTPS* genes  
411 responsible for their biosynthesis. **(10)** is the product of *VvGwaPhe*, *VvPNaPin*, and  
412 *VvCsbOciM*, with the first two genes also responsible for the biosynthesis of **(14)** and,  
413 therefore, related to **(12)** (Martin, et al., 2010). One *VvPNaPin* isoform, *VvPNaPin1*,  
414 is mainly expressed during flowering, early berry development, and maturity, but not  
415 around veraison in Moscato Bianco (Matarese, Cuzzola, Scalabrelli, & D'Onofrio,  
416 2014). This partially explains the detection of **(10)** and **(12)** at wpf 4 in the present

417 study. Biosynthesis of (14) is also related to another gene, *VvTer*, which is mainly  
418 expressed at flowering and early berry development in Moscato Bianco (Matarese, et  
419 al., 2014), which also explains the relatively higher concentration of (12) at wpf 4.

420

421 The production of (9) and (15) is also similar (Fig. 3a), which may due to their  
422 biosynthetic relationship (Fig. 4b). (9) is regulated by *VvGwGer*, *VvCSGer*, and  
423 *VvPNGer* of the *VvTPS-g* subfamily of *VvTPS*, and *VvPNGer* is likely to be the main  
424 enzyme responsible for the biosynthesis of (9) (Martin, et al., 2010; Matarese,  
425 Scalabrelli, & D'Onofrio, 2013). *VvPNGer* is mainly expressed in flowers and berries  
426 in early development (Matarese, et al., 2014), which explains the higher  
427 concentration of (9) at wpf 4. Since (15) is a downstream product of (9), *VvGwGer*,  
428 *VvCSGer*, and *VvPNGer* may also be related to the biosynthesis of (15). In addition,  
429 (15) can be produced from (9) and (11) during fermentation (Luan, et al., 2005). (11)  
430 is a product of many *VvTPS* genes including *VvPNaPin*, *VvPNRLin*, *VvPNLinNer*,  
431 and *VvPNLNGI1* (Martin, et al., 2010), of which *VvPNRLin* has the highest  
432 transcription levels in Moscato Bianco and Aleatico (Matarese, et al., 2013). Absence  
433 of (11) in the present study indicates possible differences between floral and non-  
434 floral cultivars in expression of certain gene, and warrants further investigation. The  
435 production of (7) is different from the four detected monoterpenes (Fig. 3a), which  
436 may be due to its synthesis from  $\zeta$ -carotene and/or (5) rather than (4). Carotenoid  
437 cleavage dioxygenase genes, *LeCCD1A* and *LeCCD1B*, are responsible for the  
438 biosynthesis of (7) from  $\zeta$ -carotene (Simkin, et al., 2004). Overall, the production of  
439 (9), (10), (12), and (15) have similar trends of evolution during berry development,  
440 which reflects their possible genetic interactions.

441

### 442 3.3.2. Sesquiterpene evolution

443 The total sesquiterpene concentration decreased from early berry development to  
444 veraison and slightly increased at harvest. Although present at significantly lower  
445 total concentrations, the number of sesquiterpenes at wpf 17 was similar to that at  
446 wpf 4. However, sesquiterpene profiles at these two stages were different, which  
447 may be due to the activation of different biosynthetic pathways at different berry  
448 developmental stages. All sesquiterpenes are synthesised from (5) via farnesyl  
449 carbocation (16) (Fig. 4). The downstream biosynthetic pathways of the compounds  
450 detected in this study can be classified into five categories: the humulyl carbocation  
451 pathway (17-20) (Fig. 4c), the germacrene A pathway (21-26) (Fig. 4d), the  
452 germacrene C pathway (27-29) (Fig. 4e), the germacrene D pathway (32-54) (Fig.  
453 4f), and the nerolidol diphosphate pathway (55-58) (Fig. 4g) (Bülow & König, 2000;  
454 Chappell & Coates, 2010; Davis, 2010; Davis, et al., 2000; May, Lange, & Wüst,  
455 2013)

456

#### 457 3.3.2.1. The humulyl carbocation pathway

458 The humulyl carbocation pathway starts with humulyl carbocation (17), which is  
459 derived from (16) (Fig. 4c) (Chappell, et al., 2010; Davis, et al., 2000). The main  
460 pathway products include  $\beta$ -caryophyllene (18), clovene (19), and  $\alpha$ -humulene (20),  
461 which were most abundant at the early stage of berry development (Table 1 in  
462 Pangzhen Zhang, et al. ). The biosynthesis of (18) and (20) is regulated by three  
463 common *VvTPS* genes, *VvGwECar*, *VvPNECar*, and *VvPNaHum*, which explains  
464 their similar production trends during berry development (Fig. 3a) (Martin, et al.,  
465 2010). Previous study have demonstrated that *VvGwECar* and *VvPNaHum* are  
466 mainly expressed in flower buds and open flowers in Moscato Bianco (Matarese, et

467 al., 2014). Higher concentrations of **(18)** and **(20)** observed at wpf 4 could have been  
468 synthesised during flowering, and gradually decrease afterward similar as that of  
469 Moscato Bianco (Matarese, et al., 2014). Further study is required to understand the  
470 differences between floral and non-floral cultivars in the production of **(18)** and **(19)**.  
471 In addition, **(18)** is also regulated by a fourth gene, *VvPNEb2epi Car*, which helps to  
472 explain their different production patterns after veraison. The concentration of **(19)**  
473 was extremely low compared to **(18)** and **(20)** (Fig. 3a), and the gene responsible for  
474 its production has yet to be identified. **(19)** is only remotely related to **(18)**, and  
475 therefore its production pattern is different (Chappell, et al., 2010; Davis, et al.,  
476 2000).

477

#### 478 3.3.2.2. The germacrene A pathway

479 The germacrene A pathway starts with germacrene A **(21)**, which is derived from **(16)**  
480 (Fig. 4d). The primary pathway products include  $\alpha$ -guaiene **(22)** and  $\alpha$ -selinene **(23)**.  
481 A very recent study reported aerial oxidation of **(22)** into rotund-2-ol **(24)** and  
482 rotundone **(25)** via hydroperoxy-guaiene **(26)** (Huang, et al., 2014; Huang, Sefton,  
483 Sumbly, Tiekink, & Taylor, 2015). It is possible that **(22)** was released by grape  
484 berries and aeri ally oxidised into **(25)** (Fig. 4d). However, this chemical synthesis  
485 does not explain the storage mechanism of **(25)**. A recent study reported the  
486 identification of *VvSTO2*, which is responsible for the biosynthesis of a cytochrome  
487 P450 CYP71BE5 enzyme that oxidize **(22)** into **(25)** (Takase, et al., 2015).

488

489 The production of **(25)** at different berry developmental stages was further  
490 investigated in detail in two growing seasons (**Fig. 5**). Overall, grapes from the 2012-  
491 13 growing season contained significantly higher concentrations of **(25)** (2.06 ng/100

492 berries) compared to those from the 2013-14 growing season (0.90 ng/100 berries),  
493 while grapes at harvest (E-L38) had a significantly higher concentration of **(25)** (3.19  
494 ng/100 berries) than all other groups, followed by E-L 31 (2.12 ng/100 berries), E-L  
495 32 (1.28 ng/100 berries), E-L 37 (1.10 ng/100 berries), E-L 36 (0.72 ng/100 berries),  
496 and E-L 35 (0.44 ng/100 berries) (two-way ANOVA,  $p < 0.05$ ) (**Fig. 5a**). In the 2012-13  
497 growing season, the grape concentration of **(25)** gradually decreased from pre-  
498 veraison (E-L 31: 3.24 ng/100 berries, E-L 32: 1.52 ng/100 berries) to 80% veraison  
499 (E-L 35: 0.44 ng/100 berries), and then gradually increased until harvest (E-L36:  
500 1.04 ng/100 berries, E-L 37:1.61 ng/100 berries, E-L: 4.48 ng/100 berries) (**Fig. 5b**).  
501 A similar trend was observed in the 2013-14 growing season. The pre-veraison  
502 groups (E-L31: 1.00 ng/100 berries, E-L 32: 1.04 ng/100 berries) contained  
503 significantly higher concentrations of **(25)** compared to E-L 35 (0.45ng/100 berries),  
504 E-L36 (0.41 ng/100 berries), and E-L 37 (0.59 ng/100 berries). Grapes at harvest (E-  
505 L 38: 1.89 ng/100 berries) had significantly higher concentrations of **(25)** than all  
506 other groups. The concentration of **(25)** in flower caps were also analysed in the  
507 2013-14 growing season. Flower caps (1.62 ng/100 flowers) contained significantly  
508 higher concentrations of rotundone than grape berries at all stages before harvest,  
509 and were similar to those in harvested grapes. Previous studies only reported  
510 identification of rotundone in grape exocarp at veraison and post-veraison periods  
511 (Geffroy, et al., 2014; Takase, et al., 2015). This is the first time rotundone has been  
512 reported in flower caps and pre-veraison grape berries.

513

514 In the present study, **(22)** and **(25)** had similar 'U' shaped production patterns as  
515 expected, which further demonstrated our hypothesis that sesquiterpenes from a  
516 similar pathway had a similar production pattern. **(25)** has been converted into a

517 similar unit when comparing its production pattern with other terpenes compounds in  
518 Fig. 3 and Table 1 in Pangzhen Zhang, et al. . A newly discovered allele of *VvTPS24*  
519 encodes the *TvGuaS* enzyme that is mainly responsible for the production of **(22)**  
520 (Drew, Andersen, Sweetman, Møller, Ford, & Simonsen, 2015). The timing of  
521 *TvGuaS* production during grape ripening remains unclear, but is likely to be high at  
522 early and late berry development stages when **(22)** was observed (Table 1 in  
523 Pangzhen Zhang, et al. ). Previous study reported transcription of *VvSTO2* from 8  
524 wpf until harvest (18 wpf) with the peak transcription level at 14 wpf (Takase, et al.,  
525 2015). This explains the fast accumulation of rotundone at post-veraison period  
526 observed in the current and previous studies (Fig. 5) (Geffroy, et al., 2014).  
527 However, whether the same gene is expressed in flower and pre-veraison grape  
528 berries remains unclear, warrants further investigation.

529 Apart from the *TvGuaS* enzyme encoded by *VvTPS24* allele, *VvTPS24* could  
530 produce the *VvPNSeInt* enzyme, which is also responsible for the production of **(22)**,  
531 but at small percentage (3.5%) (Martin, et al., 2010). The same enzyme is mainly  
532 responsible for the biosynthesis of  $\delta$ -selinene **(27)** and 7-*epi*- $\alpha$ -selinene **(28)**, which  
533 were only observed at wpf 17 in the current study. This suggested that *VvPNSeInt*  
534 enzyme may only be produced at a late stage of berry development, which was  
535 inconsistent to a previous study showing transcription corresponding to *VvTPS24*  
536 mainly at veraison but not at harvest (Sweetman, Wong, Ford, & Drew, 2012).  
537 Therefore, other mechanism/enzymes may exist to regulate the production of **(27)**  
538 and **(28)**.

539

540 *3.3.2.3. The germacrene C pathway*



541 The germacrene C pathway starts with germacrene C (**29**), which is derived from  
542 (**16**) (Fig. 4e). Major pathway products include guaia-6,9-diene (**30**) and (**27**), which  
543 had a similar production pattern (Fig. 3b). It is unknown exactly how (**28**) and (+)-  
544 valencene (**31**) are synthesised, but they appear to be derived from (**16**) (Lücker,  
545 Bowen, & Bohlmann, 2004). However, they may be derived from the germacrene A  
546 and/or C pathways, since (**28**) is a product of the same *VvPNSeInt* enzyme as (**22**)  
547 and (**27**) (Martin, et al., 2010). It has previously been shown that *VitisM4670* cDNA  
548 expression is mainly confined to late-stage berry development, and encodes the  
549 *VvVal VvTPS* responsible for the biosynthesis of (**28**) and (**31**) (Lücker, et al., 2004).  
550 This is consistent with our finding that (**28**) is only present at wpf 17. In addition, (**31**)  
551 could be further oxidized into  $\beta$ -nootkatol by the same cytochrome P450 enzyme that  
552 turns (**22**) into (**25**) (Takase, et al., 2015).

553

#### 554 3.3.2.4. The germacrene D pathway

555 The germacrene D pathway is the most complicated pathway implicated in the  
556 synthesis of the products detected in the present study, and is thought to be a major  
557 sesquiterpene synthesis pathway (Fig. 4f) (Bülow, et al., 2000; Chappell, et al., 2010;  
558 Davis, et al., 2000). Germacrene D (**32**) only presents in (R)-enantiomer form in  
559 grape berries and is mainly regulated by *VvGwGerD* and *VvPNGerD* and is also a  
560 by-product of *VvGwECar* and *VvPNECar* (Martin, et al., 2010; May, et al., 2013).  
561 Three major sub-pathways could explain our current findings: the cadinenyl cation  
562 pathway (**33-36**), the muurolenyl cation pathway (**37-49**), and the amophenyl cation  
563 pathway (**50-51**).

564

565 The cadinenyl cation pathway starts with the cadinenyl cation (**33**), which is derived  
566 from (**32**) (Bülow, et al., 2000; Chappell, et al., 2010; Davis, et al., 2000). Its major  
567 products are  $\gamma$ -cadinene (**34**) and  $\alpha$ -cadinene (**35**), both of which were only observed  
568 at wpf 17 (Table 1 in Pangzhen Zhang, et al. ). (**34**) is a major product of *VvGwgCad*  
569 and a minor product of *VvPNCuCad* (Martin, et al., 2010). It is uncertain how (**35**) is  
570 synthesised.  $\omega$ -cadinene (**36**) may also be derived from (**35**) (Bülow, et al., 2000). In  
571 the present study, (**36**) showed a different pattern of production during berry  
572 development compared to (**34**) and (**35**) (**Fig. 3b**). Therefore, the pathway converting  
573 (**35**) to (**36**) might not be activated at the berry development stages studied, and the  
574 latter may be produced via an alternative pathway, as discussed below.

575

576 The muurolenyl cation pathway is important to discuss in the context of the present  
577 study, and starts with the muurolenyl cation (**37**), derived from (**32**) (Bülow, et al.,  
578 2000; Chappell, et al., 2010; Davis, et al., 2000). The direct products of (**37**) are  $\alpha$ -  
579 muurolene (**38**),  $\delta$ -cadinene (**39**),  $\gamma$ -muurolene (**40**), and  $\alpha$ -copaene (**41**).  $\alpha$ -  
580 calacorene (**42**), (**36**), and *cis*-muurola-4(15),5-diene (**43**) are synthesised directly  
581 from (**39**). Two intermediates (A: **44** and B: **45**) are also synthesised from (**39**), then  
582 converted into *epi*-zonarene (**46**) and zonarene (**47**), respectively. (**46**) and (**47**) can  
583 be further converted into different calamenenes ((R): **48**, (S): **49**), which can also be  
584 synthesised from (**43**). In the present study, (**43**) was not detected at any  
585 developmental stages, indicating that the pathway between (**43**) and (**48**, **49**) may  
586 not be activated in Shiraz grapes at the berry developmental stages studied.

587

588 The majority of sesquiterpenes produced by the muurolenyl cation pathway had  
589 highly similar production patterns (Fig. 3a) and were predominantly produced at early

590 berry developmental stages before veraison (wpf 4-11). The production pattern of  
591 **(39)** was similar to **(47)** pre-veraison, and to **(46)** from wpf 15 to 17(Fig. 3a),  
592 suggesting that major downstream product of **(39)** could switch from **(47)** to **(46)** after  
593 veraison. **(48)** and **(49)** are downstream products of **(46)** and **(47)**, respectively. The  
594 **(48, 49)** observed in this study is more like to be **(49)** before veraison and **(48)** post-  
595 veraison. Other sesquiterpenes from muurolenyl cation pathway, **(36)**, **(38)**, **(40)**,  
596 and **(42)** had similar production patterns, and were only observed pre-veraison(Fig.  
597 b). The *VvPNCuCad* gene product is responsible for the production of **(39)** and **(41)**,  
598 but no other enzymes have been identified as responsible for the production of  
599 sesquiterpenes from the muurolenyl cation pathway (Martin, et al., 2010).

600

601 The amophenyl cation pathway starts with the amophenyl cation **(50)** and is mainly  
602 responsible for the biosynthesis of  $\alpha$ -amorphene,  $\gamma$ -amorphene,  $\delta$ -amorphene, and  
603  $\omega$ -amorphene (Bülow, et al., 2000). However, none of these compounds were  
604 detected in the present study, the only product detected being  $\alpha$ -ylangene **(51)**. In  
605 addition, the *VvTPS* gene responsible for amophenyl cation production remains  
606 elusive.

607

608 Apart from the cadinenyl cation, muurolenyl cation, and amophenyl cation sub-  
609 pathways, the germacrene D pathway is also responsible for the biosynthesis of **(27)**,  
610 selina-4(15),6-diene **(52)**,  $\beta$ -bourbonene **(53)**, and  $\beta$ -copaene **(54)** (Bülow, et al.,  
611 2000); these compounds were only observed at harvest in the present study (Table 1  
612 in Pangzhen Zhang, et al. ). **(52)** can be further modified into **(27)**, which is also a  
613 product of the germacrene C pathway, as discussed earlier. Therefore, it is unclear  
614 which pathway produces **(27)**.

615

616 Previous studies have reported that the *VvTPS* gene responsible for the production  
617 of **(32)**, *VvGwGerD*, is highly expressed in flowers but has low expression in grape  
618 berries (Matarese, et al., 2014). However, no **(32)** was observed in this study. **(32)**  
619 might have already been converted to downstream products, such as those in the  
620 muurolenyl cation pathway, after flowering. It is also reasonable to conclude that the  
621 cadinenyl cation and amophenyl cation pathways are regulated by different *VvTPS*  
622 genes than the muurolenyl cation pathway, since their end-product production  
623 patterns were completely different during berry development (Fig. 3).

624

#### 625 *3.3.2.5. The nerolidol diphosphate pathway*

626 The nerolidol diphosphate pathway significantly overlaps with the germacrene D  
627 pathway (Fig. 4g). **(37)**, **(51)**, and **(54)** may be synthesised from **(16)** through the  
628 nerolidol diphosphate pathway via nerolidol diphosphate (NPP) **(55)** and  
629 intermediate C **(56)**, rather than from the germacrene D pathway (Chappell, et al.,  
630 2010; Davis, et al., 2000). Since **(37)** was the precursor for the muurolenyl cation  
631 pathway, the whole pathway may not necessarily be derived from the germacrene D  
632 pathway but from the nerolidol diphosphate pathway **(56)**. Furthermore, the starting  
633 point of the germacrene D pathway, **(32)**, may derive from **(56)** (i.e., the nerolidol  
634 diphosphate pathway) (Chappell, et al., 2010; Davis, et al., 2000). 1-*epi*-cubenol **(57)**  
635 is another nerolidol diphosphate pathway product via intermediate D **(58)**. This  
636 compound and its isomer, cubenol, had similar production patterns (**Fig. 3b**), and  
637 their biosynthesis may, therefore, likely be related. **(57)** was only observed at wpf 4,  
638 which was consistent with timing of production of most compounds derived from the  
639 muurolenyl cation pathway **(37-49)**. Since both **(57)** and muurolenyl cation pathway

640 sesquiterpenes were mainly detected at wpf 4, it would be reasonable to assume  
641 that (56) was abundant at the same time. At wpf 4, the whole muurolenyl cation  
642 pathway (37-49) was likely produced from (56) instead of (32), which could explain  
643 the absence of (27), (34), (35), (51), (52), (53) and (54) at wpf 4. This also suggests  
644 that the pathway between (56) and (32) may not be activated at wpf 4. (51) and (54)  
645 were only observed at late stages of berry development, and therefore the pathway  
646 producing (51) and (54) from (56) might not be activated at wpf 4. In addition, the  
647 germacrene D pathway (32-54) derived from (16), except muurolenyl cation sub-  
648 pathway (37-49), may only be activated near harvest to produce (27), (34), (35),  
649 (51), (52), (53) and (54).

650

### 651 3.3.3 Norisoprenoid evolution

652 The total norisoprenoid concentration reached a maximum at wpf 6 and decreased  
653 thereafter until wpf 17. All norisoprenoids detected in the present study are  
654 synthesised from (6) via  $\beta$ -carotene (59) (Fig. 4h) (Mendes-Pinto, 2009).  
655 Theaspirane (60) and  $\beta$ -ionone (61) are derived from (59) via different pathways,  
656 while  $\beta$ -damascenone (62) is derived from (59) via neoxanthin (63) (Baumes, Wirth,  
657 Bureau, Gunata, & Razungles, 2002; Mendes-Pinto, 2009). In grape berries, the  
658 genes encoding carotenoid biosynthesis and catabolism are skin-specific (Grimplet,  
659 et al., 2007). Previous studies found that carotenoids were mainly synthesised pre-  
660 veraison and degraded after veraison to produce norisoprenoids (Grimplet, et al.,  
661 2007). However, the norisoprenoids formed from carotenoids are mainly in  
662 glycoconjugated forms (Wirth, Guo, Baumes, & Günata, 2001). Another study  
663 reported that the concentration of total glycoconjugated norisoprenoids gradually  
664 increases from veraison to harvest in Shiraz, with no free norisoprenoids produced at

665 any point (Mathieu, Terrier, Procureur, Bigey, & Günata, 2005). Clearly, the free  
666 norisoprenoids observed in the present study reached maximum level before  
667 veraison, and no significant increase in free norisoprenoids was observed after wpf  
668 11. This suggests that, even if glycoconjugated norisoprenoids were produced after  
669 veraison, the glycosidase enzyme may not be available in Shiraz to release free  
670 norisoprenoids during post-veraison stages (Mathieu, et al., 2005). In addition,  
671 abscisic acid (**64**), a sesquiterpene, can be biosynthesised from (**63**) rather than (**5**)  
672 (Taylor, Burbidge, & Thompson, 2000). It is well known that biosynthesis of this  
673 compound is related to plant water availability (Christmann, Moes, Himmelbach,  
674 Yang, Tang, & Grill, 2006). A recent study reported that the biosynthesis of (**25**) was  
675 closely related to vineyard water availability (Geffroy, et al., 2014; Pangzhen Zhang,  
676 et al., 2015). Therefore, the biosynthesis of (**64**) may be used as an indicator of (**25**).

677

#### 678 **4. Conclusions**

679 Monoterpenoids, sesquiterpenes, and norisoprenoids have different production  
680 patterns during berry development from pea-size to commercial harvest. Clear  
681 differences in berry terpene profiles at different berry developmental stages indicate  
682 that the mechanisms of terpene biosynthesis may be different at different ripening  
683 stages. The decrease in the number and concentration of terpenes from pre-  
684 veraison to veraison suggests that a number of their biosynthetic pathways could be  
685 inactivated. Alternatively, terpenes produced during early berry development may be  
686 degraded into other compounds or converted into non-volatile forms. Sharp  
687 increases in sesquiterpene concentrations during the last two weeks of berry  
688 development indicate that the later ripening stages, rather than the whole veraison to

689 maturity period, is more critical in defining the final concentration of sesquiterpenes,  
690 such as rotundone, in harvested grapes and, therefore, wine.

691

692 Our analysis of terpene compound evolution during berry development suggests that  
693 terpene biosynthesis is more dependent on the activation of the pathway it belongs  
694 to, and terpenes synthesised via similar pathways tend to appear at similar berry  
695 developmental stages. Categorisation of terpenes based on their biosynthetic  
696 pathway, especially sesquiterpenes, helps to understand pathway activation and  
697 regulation and can be used to predict the production patterns for other, less well-  
698 characterised compounds in the same pathway. Furthermore, highly similar  
699 production patterns in the same pathway will help future molecular classification  
700 studies that investigate the genes that regulate the whole pathway rather than a  
701 single product, and more pathway interactions are likely to be present. Even though  
702 some compounds can be synthesised by multiple pathways, not all pathways are  
703 active simultaneously.

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856

857

858 **Figure legends**

859 **Fig. 1.** Chromatograms showing the differences in terpenoids at different berry development stages:  
 860 (a) pre-veraison, 4 weeks post flowering, E-L 31; (b) 80% veraison, 11 weeks post flowering, E-L 35-  
 861 36; (c) post-veraison, 17 weeks post flowering, E-L 38. Peaks: (1) Limonene; (2) 1,8-Cineole; (3)  
 862 Geraniol; (4) Theaspirane isomer A; (5) Theaspirane isomer B; (6) Clovene; (7)  $\alpha$ -Ylangene; (8)  $\alpha$ -  
 863 copaene (internal standard); (9) (E)- $\beta$ -Damascenone; (10)  $\beta$ -Bourbonene; (11) (E)-  $\beta$ -Caryophyllene;  
 864 (12)  $\beta$ -Copaene; (13)  $\alpha$ -Guaiene; (14) Guaia-6,9-diene; (15) Selina-4(15),6-diene; (16) Geranyl  
 865 acetone; (17)  $\alpha$ -Humulene; (18)  $\gamma$ -Muurolene; (19)  $\beta$ -Ionone; (20)  $\delta$ -Selinene; (21) epi-Zonarene; (22)  
 866  $\alpha$ -Muurolene; (23)  $\gamma$ -Cadinene; (24)  $\delta$ -Cadinene; (25) Cis/trans-Calamenene; (26) Zonarene; (27)  
 867 Citronellol; (28) 7-epi- $\alpha$ -Selinene; (29)  $\omega$ -Cadinene; (30)  $\alpha$ -Cadinene; (31)  $\alpha$ -Calacorene; (32) 1-epi-  
 868 Cubenol; (33) Cubenol

869

870 **Fig. 2.** Discriminant analysis biplots illustrating the pattern of terpenoids production at different berry  
 871 development stages in Shiraz grapes. Numbers in the biplots represent the weeks post-flowering  
 872 (wpf) for Shiraz grapes in the 2013-14 growing season.

873

874 **Fig. 3.** Heat map of terpenoids compounds in grape berries at different grape development stages in  
 875 the 2013-14 growing season, and heat map legends show the relative concentration range of (a) all  
 876 terpenoids detected and (b) selected sesquiterpenes. Number in front of each compound represents  
 877 the compound number in the **Fig. 4.** The Biosynthesis of cubenol is unclear, therefore number was  
 878 not assigned to this compound.

879

880 **Fig. 4.** Biosynthesis of selected monoterpenoids, sesquiterpenes and norisoprenoids. Arrow line  
 881 indicates the biosynthesis pathways, while the biosynthesis pathways labelled by dash line may not  
 882 be activated in the present study. "1 $\times$ , 2 $\times$ , 3 $\times$ " represent the unit of isopentenyl diphosphate  
 883 (IPP) to synthesis different compounds. Compounds detected in this study are enclosed in red boxes.  
 884 The enantiomeric purity of most chiral compounds is unknown, here, we use a single enantiomer to  
 885 represent all variations of a particular compound.

886

887 **Fig. 5.** Comparison of grape berry rotundone concentration (ng/100 berries) at different phenological  
888 stages of ripening in the 2012-13 and 2013-14 growing seasons. (a) Two-way ANOVA ( $p<0.05$ ) was  
889 conducted to compare grape rotundone concentration among ripening stages and between two  
890 growing seasons. (b) One-way ANOVA ( $p<0.05$ ) was conducted to compare grape rotundone  
891 concentration among different ripening stages in each growing seasons separately. a, b, c, d were  
892 used to label significant differences ( $p<0.05$ ) among ripening stages; A, B were used to label  
893 significant differences ( $p<0.05$ ) between growing seasons;  $\alpha$ ,  $\beta$  were used to label significant  
894 differences ( $p<0.05$ ) in rotundone among flower and grape berries of different ripening stages in the  
895 2013-14 growing season.

896

ACCEPTED MANUSCRIPT

897 **Table legend**

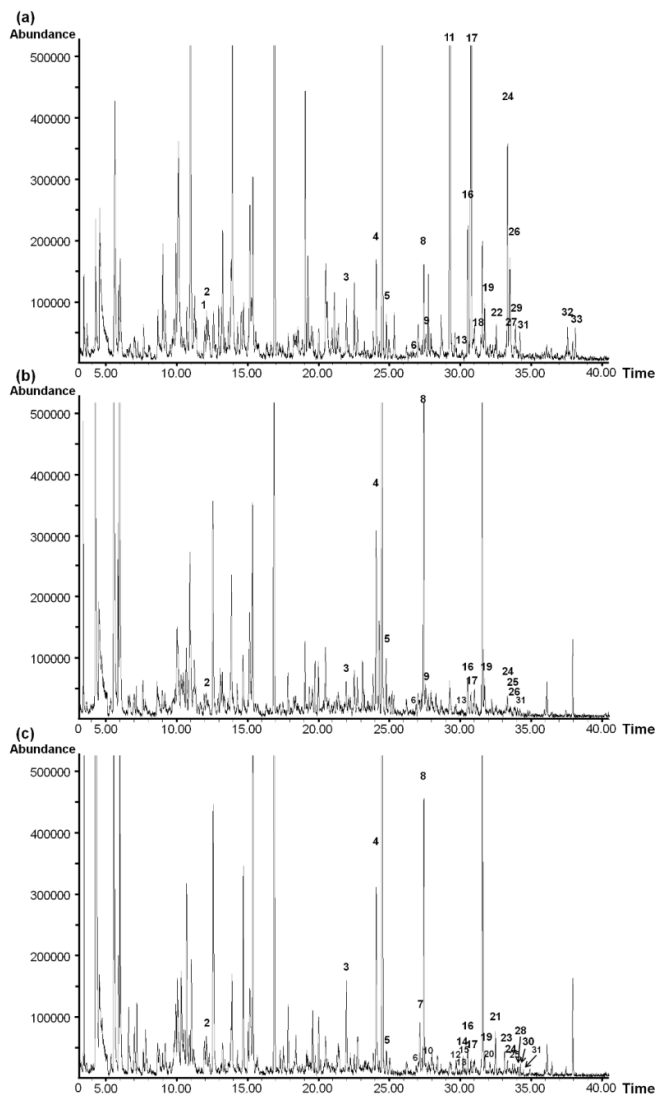
898 **Table 1.** Sampling details and grape composition at different phenological stages of grape ripening<sup>a</sup>

899

900

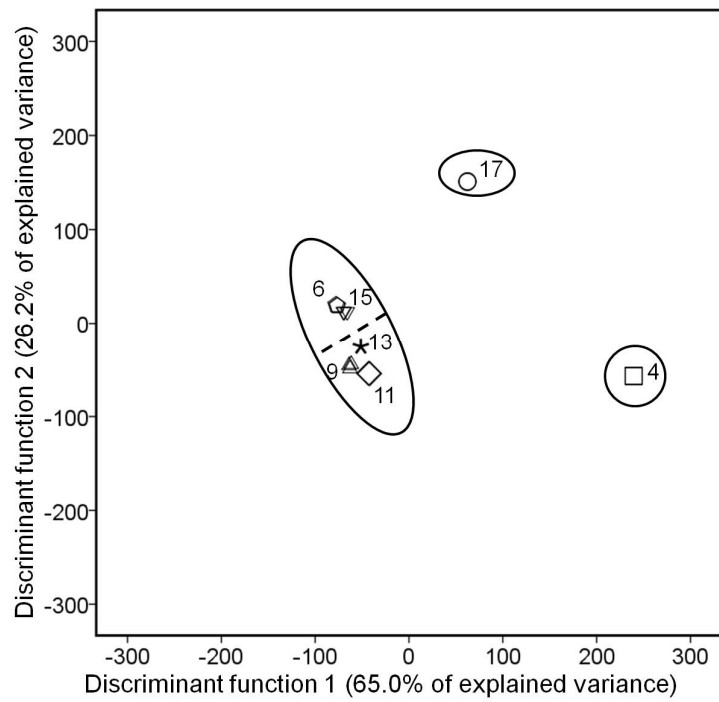
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902

903 Fig. 1.

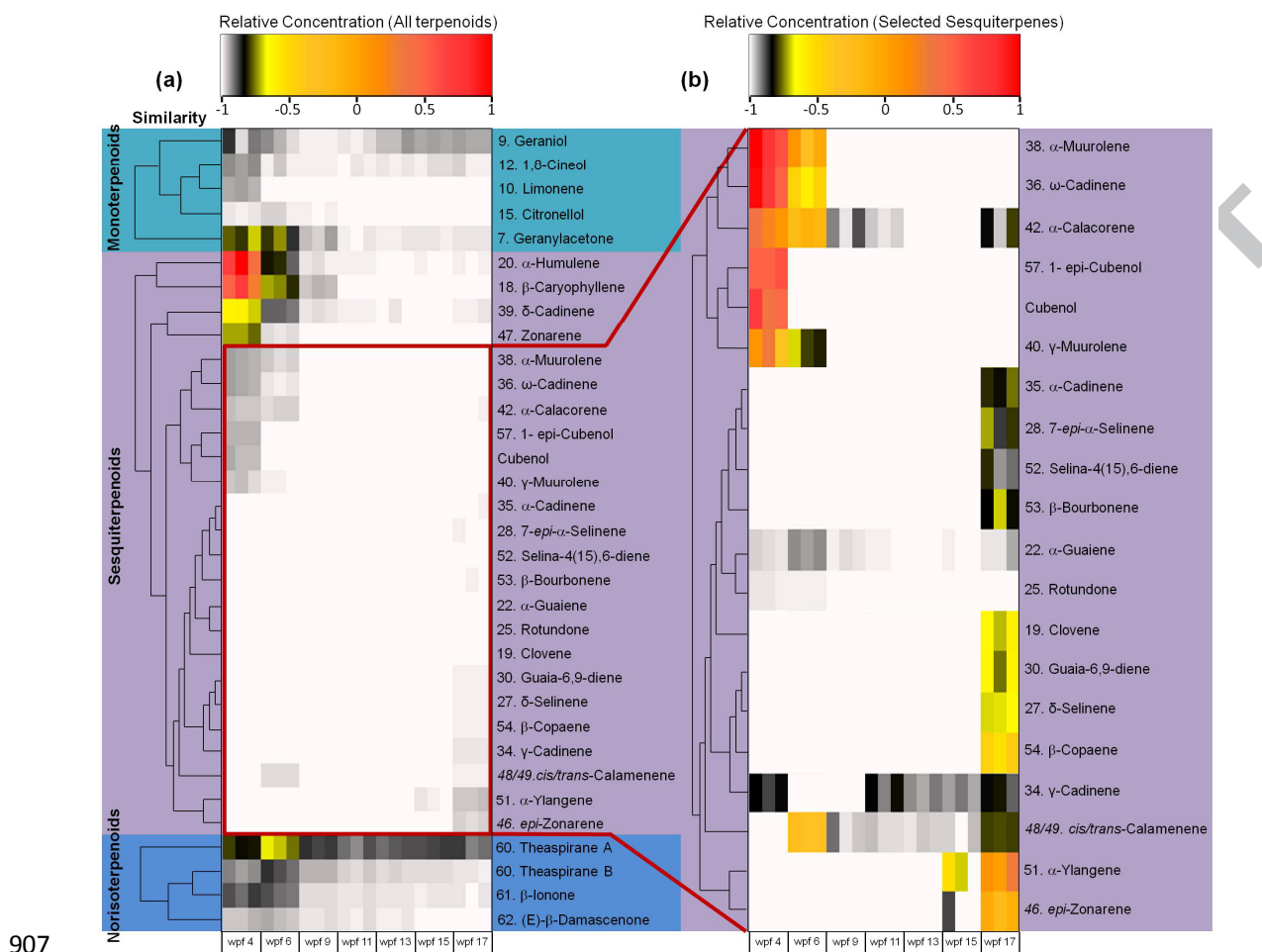


904

905 **Fig. 2.**

906





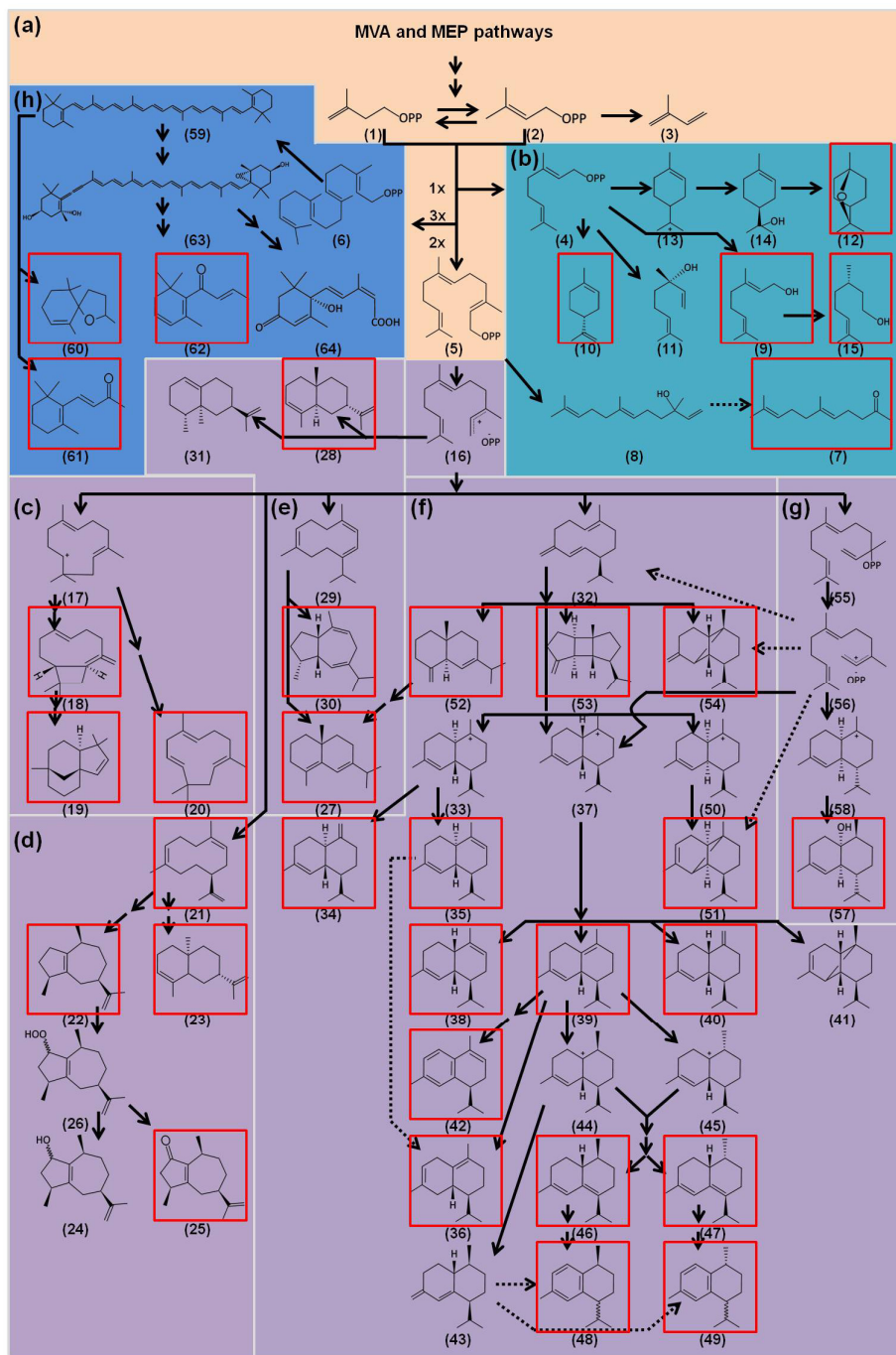
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Fig. 3.



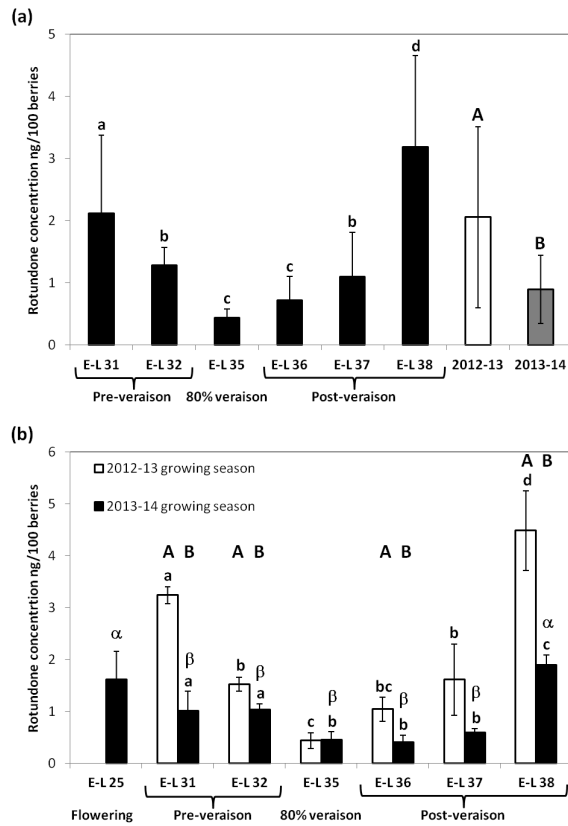
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913 Fig. 4.

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Fig. 5.

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Table 1.

The 2012-13 growing season <sup>a</sup>						
wpf <sup>b</sup>	Berry mass (g)	TSS <sup>c</sup> (°Brix)	pH	TA <sup>d</sup> (g/L)	Phenological stages <sup>e</sup>	Sample description
4	0.27	6.0±0.2a	2.37±0.02a	31.90±0.80a	E-L 31	Green pea size berries
6	0.40	5.4±0.2a	2.39±0.01a	33.58±0.95b	E-L 32	Green pea size berries
8	0.45	5.8±0.0a	2.52±0.01b	34.40±0.45b	EL 33-34	Most still hard and green
11	0.98	14.5±0.2b	3.05±0.03c	10.38±0.47c	E-L 35-36	80% veraison
13	1.02	20.1±0.3c	3.40±0.02d	5.51±0.30d	E-L 36	Light purple berries
14	1.12	22.1±0.4d	3.73±0.02e	4.05±0.31e	E-L 37	Purple berries
16	1.01	24.5±0.3e	3.81±0.03f	4.12±0.10e	E-L 37-38	Dark purple berries
18	0.99	26.3±1.0f	3.90±0.07g	4.00±0.15e	E-L 38	Some berries slightly shrank
The 2013-14 growing season						
wpf	Berry mass (g)	°Brix	pH	TA (g/L)	Phenological stages	Sample description
0 <sup>f</sup>	0.0049	n/a <sup>g</sup>	n/a	n/a	E-L 25-26	Flowering 80% caps off
4	0.18	6.3±0.2a	2.82±0.08a	30.58±0.26a	E-L 31	Green pea size berries
6	0.28	5.3±0.2b	2.76±0.03a	32.45±0.84b	E-L 32	Green pea size berries
9	0.41	7.1±0.5c	2.90±0.03a	26.70±0.36c	EL 34-35	Begin to colour and enlarge
11	0.69	15.9±0.7d	3.17±0.10b	11.62±2.33d	E-L 35-36	80% veraison
13	0.85	19.9±0.2e	3.46±0.05c	6.45±0.52e	E-L 36	Light purple berries
15	0.95	22.9±0.6f	3.72±0.05d	4.87±0.35ef	E-L 37	Purple berries
17	0.90	24.9±0.3g	3.74±0.09d	4.20±0.32f	E-L 38	Dark purple berries

924 <sup>a</sup>Different letters in the column represent significantly ( $p < 0.05$ ) different mean  $\pm$  standard error ( $n=3$   
 925 field replicates). <sup>b</sup>weeks post-flowering. <sup>c</sup>total soluble solid. <sup>d</sup>titratable acidity. <sup>e</sup>E-L system was used to  
 926 determine phenological stages (Pearce and Coombe 2004). <sup>f</sup>flower samples collected at around 80%  
 927 caps off, composition not compared with grape samples. <sup>g</sup>not applicable.

928

929

930

931 **Highlights**

- 932       • Pre-veraison berries contain the highest total terpenoid concentrations
- 933       • Berries at different developmental stages have different terpene profiles
- 934       • Terpene biosynthesis pathways dictate production patterns during berry
- 935       development
- 936       • Rotundone was present in Shiraz flower caps and grapes at both pre-veraison
- 937       and post-veraison

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ACCEPTED MANUSCRIPT