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Original research article

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

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Running title: Terpene evolution in Shiraz grape development

Abstract

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

Keywords: rotundone, terpene, sesquiterpene, grape ripening

Highlights

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

1. Introduction

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

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

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

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

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

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

2. Materials and methods

2.1. Chemicals

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

118 α-copaene was supplied by Sigma-Aldrich (Castile Hill, NSW, Australia). Working
119 solutions of standards were prepared volumetrically in ethanol and stored at 4℃ 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℃, 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℃, 124.1 mm and 21.7℃, 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

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

2.3. Evaluation of rotundone evolution during grape development

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

2.4. Preparation of samples and solid-phase microextraction-gas chromatography-multidimenstional mass spectrometery (SPME-MDGC-MS) rotundone analysis

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

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

2.5. Terpenoid analysis

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

The SPME fibre was desorbed in the pulsed splitless mode and the splitter, at 50:1, was opened after 30 s. The fibre was allowed to bake in the inlet for 10 min. The oven was started at 50°C, held for 1 min, increased to 230°C at 3°C/min, then increased to 280°C at 20°C/min and held at 280°C for 5 min. The MS transfer line was held at 250°C. The temperatures of the MS source and quadruple were230°C and 150°C, respectively. The MS was operated in positive EI mode at 70 eV with simultaneous selected ion monitoring (SIM) and scanning over a mass acquisition range of 35-280 m/z.

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

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

2.6. Statistical analysis

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

3. Results and discussion

3.1. Berry development pattern differentiation

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

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

3.2. Berry developmental stages and representative compounds

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

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

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

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

zonarene, ω-cadinene, and α-calacorene concentrations were also significantly higher at this stage than at all other stages. However, in Cabernet Sauvignon, only γ-muurolene was observed at early-stage berry development (Kalua, et al., 2009). Furthermore, *epi*-cubenol and cubenol were only observed at this stage. Four isoprenoids were observed at this stage and accounted for 8.44% of the total terpenoids.

3.2.2. Pre-veraison terpenoid profile (6 wpf)

At this stage, the terpenes were distant from those at 4 wpf in discriminant analysis (Fig. 2), indicating that the terpenoid profiles had significantly changed between these two time points. Significant decreases in total volatiles and terpenoid concentrations were observed at this stage (Table 1 in Pangzhen Zhang, et al.). The total monoterpenoids slightly decreased, while geranyl acetone remained the most abundant monoterpenoid. All monoterpenoid concentrations decreased except citronellol, which slightly increased. The total sesquiterpene concentration decreased dramatically to less than 1/6 that of the previous stage, with α-humulene and β-caryophyllene accounting for the majority of the decrease but remaining the most abundant sesquiterpenes. The concentrations of γ-muurolene, α-muurolene, δ-cadinene, zonarene, ω-cadinene, and α-calacorene all markedly decreased at this stage, while the concentration of α-guaiene slightly increased and calamenene started to appear in the berries. The highest total norisoprenoid concentrations were observed at this stage and accounted for 37.7% of the total terpenoids in terms of GC peak area. Theaspirane isomer A was the most abundant norisoprenoid in terms of GC peak area and accounted for 19.1% of the total terpenoids at this stage.

Compared to the previous stage, the concentration of all norisoprenoids increased except β-ionone, which slightly decreased.

3.2.3. Veraison terpenoid profile (9-13 wpf)

This developmental stage was characterised by lower concentrations of total volatiles and terpenoids (Table 1 in Pangzhen Zhang, et al.), suggesting that the berries at this stage might have lost the ability to produce volatile compounds or that previously formed compounds had been converted into non-volatile forms. This was consistent with previous observations in Cabernet Sauvignon, in which the fewest and lowest concentrations of volatiles were observed at similar stages (Kalua, et al., 2009). The 1,8-cineole, geraniol, and geranyl acetone concentrations continued to decrease and reached their lowest concentrations at wpf 11, while citronellol was not detected from wpf 11. Geraniol and geranyl acetone were previously observed at veraison in *Vitis vinifera* L. cv. Baga grapes (Coelho, et al., 2006) and, in the same study, limonene and citronellol were also detected at veraison. Geraniol, geranyl acetone, limonene, and citronellol were also observed in *Vitis vinifera* L. cv. 'Fernão-Pires' grapes at veraison (Coelho, et al., 2007). However, no monoterpenes were detected near veraison in Cabernet Sauvignon and Riesling (Kalua, et al., 2009, 2010). The total sesquiterpene concentration decreased to its lowest level during this period, accounting for only 5.75% of the total terpenoids in terms of GC peak area at wpf 13. β-Caryophyllene was not detected from wpf 11, while clovene re-appeared at wpf 11. The concentrations of α-guaiene, α-humulene, δ-cadinene, and calamenene decreased to very low levels, while γ-muurolene, α-muurolene, and ω-cadinene were not detected. The concentrations of zonarene and α-calacorene continued to 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 β-damascenone and β-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, α-guaiene, α-humulene, δ-
357 cadinene, and calamenene, remained low. α-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 β-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

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

3.3. Terpenoid evolution

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

sesquiterpenes (C_{15}), and norisoprenoids (C_{13}), respectively. We discuss terpenoid biosynthesis in this study according to terpene sub-families and pathways.

3.3.1. Evolution of monoterpenoids

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

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

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

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

3.3.2. Sesquiterpene evolution

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

3.3.2.1. The humulyl carbocation pathway

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

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

3.3.2.2. The germacrene A pathway

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

The production of (**25**) at different berry developmental stages was further investigated in detail in two growing seasons (**Fig. 5**). Overall, grapes from the 2012-13 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

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

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

3.3.2.3. The germacrene C pathway

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

3.3.2.4. The germacrene D pathway

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

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

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

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

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

The amophenyl cation pathway starts with the amophenyl cation (**50**) and is mainly responsible for the biosynthesis of α-amorphene, γ-amorphene, δ- amorphene, and ω-amorphene (Bülow, et al., 2000). However, none of these compounds were detected in the present study, the only product detected being α-ylangene (**51**). In addition, the *VvTPS* gene responsible for amophenyl cation production remains elusive.

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

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

3.3.2.5. The nerolidol diphosphate pathway

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

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

3.3.3 Norisoprenoid evolution

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

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

4. Conclusions

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

Baumes, R., Wirth, J., Bureau, S., Gunata, Y., & Razungles, A. (2002). Biogeneration of C13-norisoprenoid compounds: experiments supportive for an apo-carotenoid pathway in grapevines. *Analytica Chimica Acta, 458*(1), 3-14.

Boland, W., Gäbler, A., Gilbert, M., & Feng, Z. (1998). Biosynthesis of C11 and C16 homoterpenes in higher plants; stereochemistry of the C • C-bond cleavage reaction. *Tetrahedron, 54*(49), 14725-14736.

Bülow, N., & König, W. A. (2000). The role of germacrene D as a precursor in sesquiterpene biosynthesis: investigations of acid catalyzed, photochemically and thermally induced rearrangements. *Phytochemistry, 55*(2), 141-168.

Chappell, J., & Coates, R. M. (2010). Sesquiterpenes. In L. Mander & H.-W. Liu (Eds.), *Comprehensive Natrual Products II*, vol. 1 (pp. 609-641). Kidlington, United Kingdom: Elsevier L.td.

Christmann, A., Moes, D., Himmelbach, A., Yang, Y., Tang, Y., & Grill, E. (2006). Integration of Abscisic Acid Signalling into Plant Responses. *Plant Biology, 8*(3), 314-325.

Coelho, E., Rocha, S. M., Barros, A. S., Delgadillo, I., & Coimbra, M. A. (2007). Screening of variety- and pre-fermentation-related volatile compounds during ripening of white grapes to define their evolution profile. *Analytica Chimica Acta, 597*(2), 257-264.

Coelho, E., Rocha, S. M., Delgadillo, I., & Coimbra, M. A. (2006). Headspace-SPME applied to varietal volatile components evolution during Vitis vinifera L. cv. 'Baga' ripening. *Analytica Chimica Acta, 563*(1–2), 204-214.

Coombe, B. G., & Iland, P. G. (2004). Grape berry development and winegrape quality. In P. R. Dry & B. G. Coombe (Eds.), *Viticulture* 2nd ed., vol. 1 (pp. 210-248). Adelaide: Winetitles.

Davis, E. M. (2010). Advances in the Enzymology of Monoterpenoid Cyclization Reactions. In L. Mander & H.-W. Liu (Eds.), *Comprehensive Natrual Products II*, vol. 1 (pp. 585-608). Kidlington, United Kingdom: Elsevier L.td.

Davis, E. M., & Croteau, R. (2000). Cyclization enzymes in the biosynthesis of monoterpenes, sesquiterpenes, and diterpenes. In *Biosynthesis*, (pp. 53-95). Berlin Heidelberg: Springer.

Drew, D. P., Andersen, T. B., Sweetman, C., Møller, B. L., Ford, C., & Simonsen, H. T. (2015). Two key polymorphisms in a newly discovered allele of the Vitis vinifera TPS24 gene are responsible for the production of the rotundone precursor α-guaiene. *Journal of Experimental Botany*, erv491.

Dunlevy, J. D., Kalua, C. M., Keyzers, R. A., & Boss, P. K. (2009). The Production of Flavour & Aroma Compounds in Grape Berries. In K. Roubelakis-Angelakis (Ed.), *Grapevine Molecular Physiology & Biotechnology* 2 ed., (pp. 293-340): Springer Netherlands.

Fang, Y., & Qian, M. C. (2006). Quantification of Selected Aroma-Active Compounds in Pinot Noir Wines from Different Grape Maturities. *Journal of Agricultural and Food Chemistry, 54*(22), 8567-8573.

García, E., Chacón, J. L., Martínez, J., & Izquierdo, P. M. (2003). Changes in Volatile Compounds during Ripening in Grapes of Airén, Macabeo and Chardonnay White Varieties Grown in La Mancha Region (Spain). *Food Science and Technology International, 9*(1), 33-41.

Geffroy, O., Dufourcq, T., Carcenac, D., Siebert, T., Herderich, M., & Serrano, E. (2014). Effect of ripeness and viticultural techniques on the rotundone concentration in red wine made from Vitis vinifera L. cv. Duras. *Australian Journal of Grape and Wine Research, 20*(3), 401-408.

Gladstones, J. S. (2004). Climate and Australian Viticulture. In P. R. Dry & B. G. Coombe (Eds.), *Viticulture* 2nd ed., vol. 1 (pp. 90-118). Adelaide: Winetitles.

Grimplet, J., Deluc, L. G., Tillett, R. L., Wheatley, M. D., Schlauch, K. A., Cramer, G. R., & Cushman, J. C. (2007). Tissue-specific mRNA expression profiling in grape berry tissues. *BMC Genomics, 8*, 187-123.

767 Herderich, M. J., Siebert, T. E., Parker, M., Capone, D. L., Jeffery, D. W., Osidacz, P., & Francis, I. L.
768 (2012). Spice Up Your Life: Analysis of Key Aroma Compounds in Shiraz. In *Flavor Chemistry*
769 *of Wine and Other Alcoholic Beverages*, vol. 1104 (pp. 3-13): American Chemical Society.
770 Hjelmeland, A. K., & Ebeler, S. E. (2015). Glycosidically Bound Volatile Aroma Compounds in Grapes
771 and Wine: A Review. *American Journal of Enology and Viticulture, 66*(1), 1-11.
772 Hsiao, Y. Y., Tsai, W. C., Kuoh, C. S., Huang, T. H., Wang, H. C., Wu, T. S., Leu, Y. L., Chen, W. H., &
773 Chen, H. H. (2006). Comparison of transcripts in Phalaenopsis bellina and Phalaenopsis
774 equestris (Orchidaceae) flowers to deduce monoterpene biosynthesis pathway. *BMC Plant*
775 *Biol, 6*, 14.
776 Huang, A.-C., Burrett, S., Sefton, M. A., & Taylor, D. K. (2014). Production of the Pepper Aroma
777 Compound, (−)-Rotundone, by Aerial Oxidation of α-Guaiene. *Journal of Agricultural and*
778 *Food Chemistry, 62*(44), 10809-10815.
779 Huang, A.-C., Sefton, M. A., Sumby, C. J., Tiekink, E. R. T., & Taylor, D. K. (2015). Mechanistic Studies
780 on the Autoxidation of α-Guaiene: Structural Diversity of the Sesquiterpenoid Downstream
781 Products. *Journal of Natural Products, 78*(1), 131-145.
782 Iland, P. (2004). *Chemical analysis of grapes and wine : techniques and concepts*. Campbelltown, SA:
783 Patrick Iland Wine Promotions.
784 Kalua, C. M., & Boss, P. K. (2009). Evolution of Volatile Compounds during the Development of
785 Cabernet Sauvignon Grapes (Vitis vinifera L.). *Journal of Agricultural and Food Chemistry,*
786 *57*(9), 3818-3830.
787 Kalua, C. M., & Boss, P. K. (2010). Comparison of major volatile compounds from Riesling and
788 Cabernet Sauvignon grapes (Vitis vinifera L.) from fruitset to harvest. *Australian Journal of*
789 *Grape and Wine Research, 16*(2), 337-348.
790 Luan, F., Mosandl, A., Münch, A., & Wüst, M. (2005). Metabolism of geraniol in grape berry
791 mesocarp of Vitis vinifera L. cv. Scheurebe: demonstration of stereoselective reduction, E/Z-
792 isomerization, oxidation and glycosylation. *Phytochemistry, 66*(3), 295-303.
793 Lücker, J., Bowen, P., & Bohlmann, J. (2004). Vitis vinifera terpenoid cyclases: functional
794 identification of two sesquiterpene synthase cDNAs encoding (+)-valencene synthase and
795 (−)-germacrene D synthase and expression of mono- and sesquiterpene synthases in
796 grapevine flowers and berries. *Phytochemistry, 65*(19), 2649-2659.
797 Martin, D. M., Aubourg, S., Schouwey, M. B., Daviet, L., Schalk, M., Omid, T., Lund, S. T., & Bohlmann,
798 J. (2010). Functional Annotation, Genome Organization and Phylogeny of the Grapevine
799 (Vitis vinifera) Terpene Synthase Gene Family Based on Genome Assembly, FLcDNA Cloning,
800 and Enzyme Assays. *BMC Plant Biology, 10*, 226-247.
801 Matarese, F., Cuzzola, A., Scalabrelli, G., & D'Onofrio, C. (2014). Expression of terpene synthase
802 genes associated with the formation of volatiles in different organs of Vitis vinifera.
803 *Phytochemistry, 105*(0), 12-24.
804 Matarese, F., Scalabrelli, G., & D'Onofrio, C. (2013). Analysis of the expression of terpene synthase
805 genes in relation to aroma content in two aromatic Vitis vinifera varieties. *Functional Plant*
806 *Biology, 40*(6), 552-565.
807 Mathieu, S., Terrier, N., Procureur, J., Bigey, F., & Günata, Z. (2005). A Carotenoid Cleavage
808 Dioxygenase from Vitis vinifera L.: functional characterization and expression during grape
809 berry development in relation to C13-norisoprenoid accumulation. *Journal of Experimental*
810 *Botany, 56*(420), 2721-2731.
811 May, B., Lange, B. M., & Wüst, M. (2013). Biosynthesis of sesquiterpenes in grape berry exocarp of
812 Vitis vinifera L.: Evidence for a transport of farnesyl diphosphate precursors from plastids to
813 the cytosol. *Phytochemistry, 95*(0), 135-144.
814 May, B., & Wüst, M. (2012). Temporal development of sesquiterpene hydrocarbon profiles of
815 different grape varieties during ripening. *Flavour and Fragrance Journal, 27*(4), 280-285.
816 Mendes-Pinto, M. M. (2009). Carotenoid breakdown products the—norisoprenoids—in wine aroma.
817 *Archives of Biochemistry and Biophysics, 483*(2), 236-245.

818 Parker, M., Pollnitz, A. P., Cozzolino, D., Francis, I. L., & Herderich, M. J. (2007). Identification and
819 Quantification of a Marker Compound for 'Pepper' Aroma and Flavor in Shiraz Grape Berries
820 by Combination of Chemometrics and Gas Chromatography−Mass Spectrometry. *Journal of*
821 *Agricultural and Food Chemistry, 55*(15), 5948-5955.
822 Pearce, I., & Coombe, B. G. (2004). Grapevine phenology. In P. R. Dry & B. G. Coombe (Eds.),
823 *Viticulture* 2nd ed., vol. 1 (pp. 210-248). Adelaide: Winetitles.
824 Siebert, T. E., Wood, C., Elsey, G. M., & Pollnitz, A. P. (2008). Determination of Rotundone, the
825 Pepper Aroma Impact Compound, in Grapes and Wine. *Journal of Agricultural and Food*
826 *Chemistry, 56*(10), 3745-3748.
827 Simkin, A. J., Schwartz, S. H., Auldridge, M., Taylor, M. G., & Klee, H. J. (2004). The tomato carotenoid
828 cleavage dioxygenase 1 genes contribute to the formation of the flavor volatiles β-ionone,
829 pseudoionone, and geranylacetone. *The Plant Journal, 40*(6), 882-892.
830 Sweetman, C., Wong, D. C., Ford, C. M., & Drew, D. P. (2012). Transcriptome analysis at four
831 developmental stages of grape berry (Vitis vinifera cv. Shiraz) provides insights into
832 regulated and coordinated gene expression. *BMC Genomics, 13*(1), 691.
833 Takase, H., Sasaki, K., Shinmori, H., Shinohara, A., Mochizuki, C., Kobayashi, H., Ikoma, G., Saito, H.,
834 Matsuo, H., & Suzuki, S. (2015). Cytochrome P450 CYP71BE5 in grapevine (Vitis vinifera)
835 catalyzes the formation of the spicy aroma compound (−)-rotundone. *Journal of*
836 *Experimental Botany*, erv496.
837 Taylor, I. B., Burbidge, A., & Thompson, A. J. (2000). Control of abscisic acid synthesis. *Journal of*
838 *Experimental Botany, 51*(350), 1563-1574.
839 Wirth, J., Guo, W., Baumes, R., & Günata, Z. (2001). Volatile Compounds Released by Enzymatic
840 Hydrolysis of Glycoconjugates of Leaves and Grape Berries from Vitis vinifera Muscat of
841 Alexandria and Shiraz Cultivars. *Journal of Agricultural and Food Chemistry, 49*(6), 2917-
842 2923.
843 Wood, C., Siebert, T. E., Parker, M., Capone, D. L., Elsey, G. M., Pollnitz, A. P., Eggers, M., Meier, M.,
844 Vössing, T., Widder, S., Krammer, G., Sefton, M. A., & Herderich, M. J. (2008). From Wine to
845 Pepper: Rotundone, an Obscure Sesquiterpene, Is a Potent Spicy Aroma Compound. *Journal*
846 *of Agricultural and Food Chemistry, 56*(10), 3738-3744.
847 Zhang, P., Barlow, S., Krstic, M., Herderich, M., Fuentes, S., & Howell, K. (2015). Within-Vineyard,
848 Within-Vine, and Within-Bunch Variability of the Rotundone Concentration in Berries of Vitis
849 vinifera L. cv. Shiraz. *Journal of Agricultural and Food Chemistry, 63*(17), 4276-4283.
850 Zhang, P., Fuentes, S., Siebert, T., Krstic, M., Herderich, M., Barlow, E. W. R., & Howell, K. Terpene
851 production pattern during the development of Vitis vinifera L. cv. Shiraz grapes. *Data in*
852 *Brief*, submitted.
853 Zhang, P., Howell, K., Krstic, M., Herderich, M., Barlow, E. W. R., & Fuentes, S. (2015). Environmental
854 Factors and Seasonality Affect the Concentration of Rotundone in *Vitis vinifera* L. cv. Shiraz
855 Wine. *PLoS ONE, 10*(7), e0133137.

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Figure legends

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

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

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

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

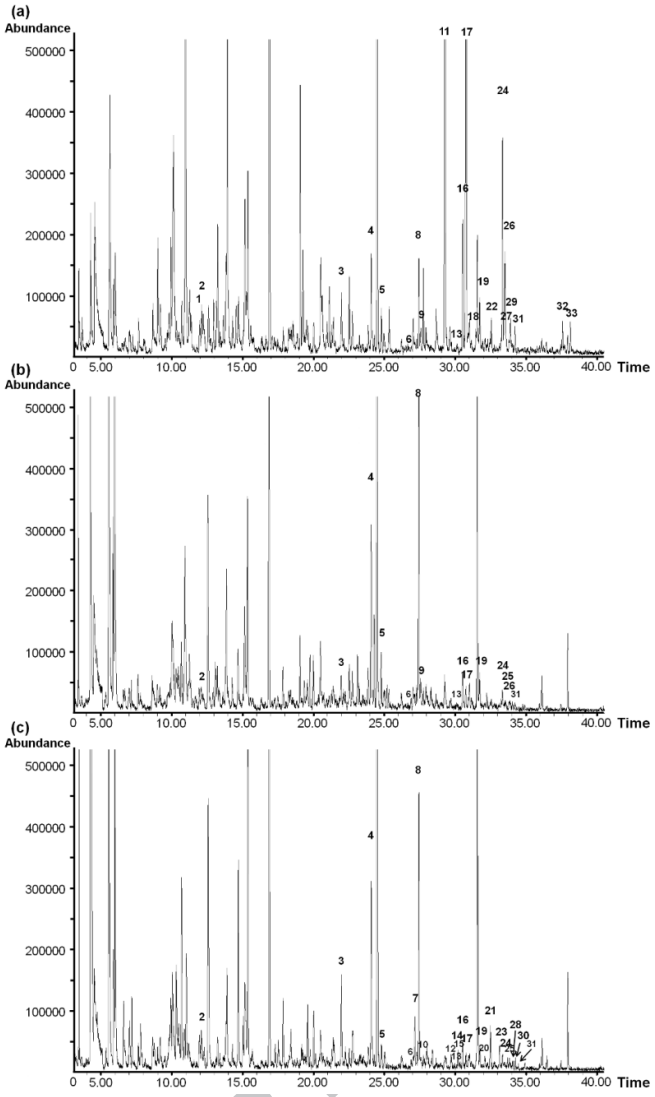
886

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; α, β 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

Table legend

Table 1. Sampling details and grape composition at different phenological stages of grape ripening[a]



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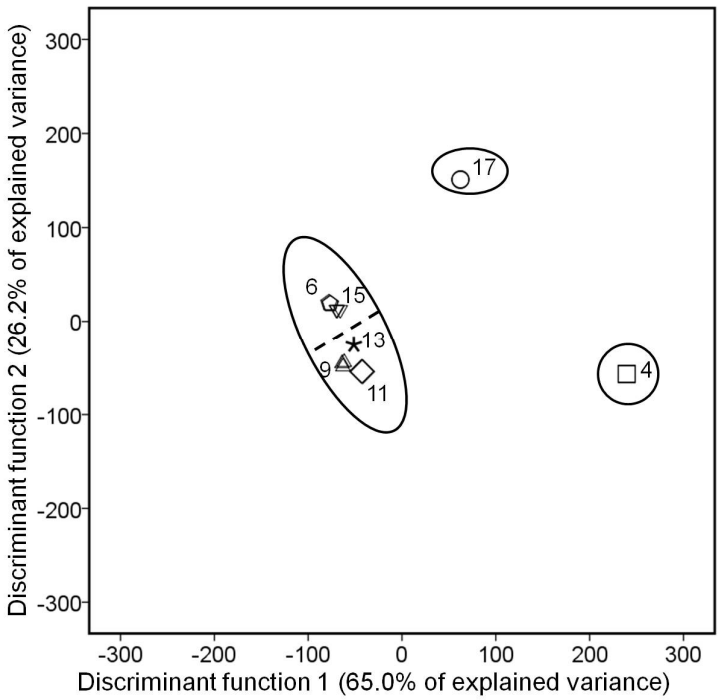
903 **Fig. 1.**



904

905 **Fig. 2.**

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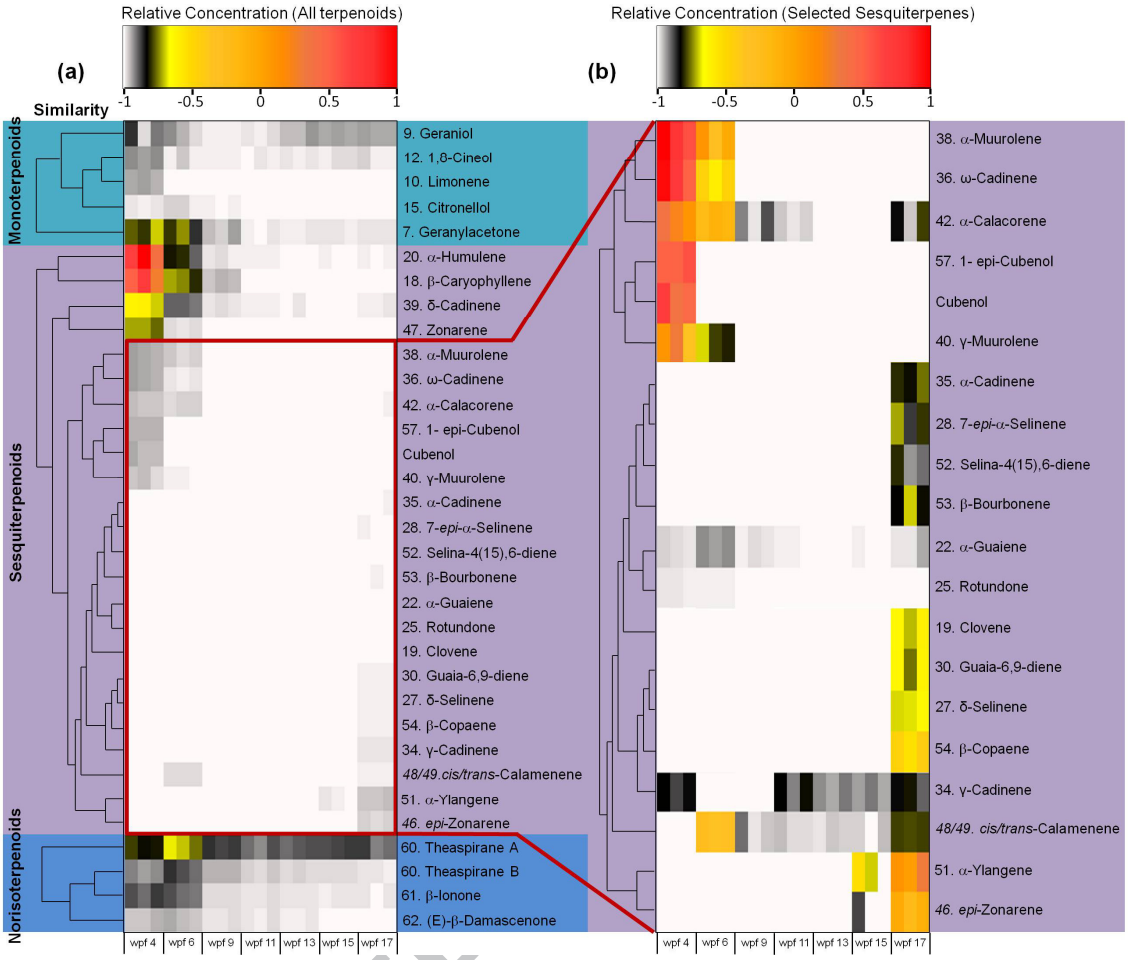


Fig. 3.

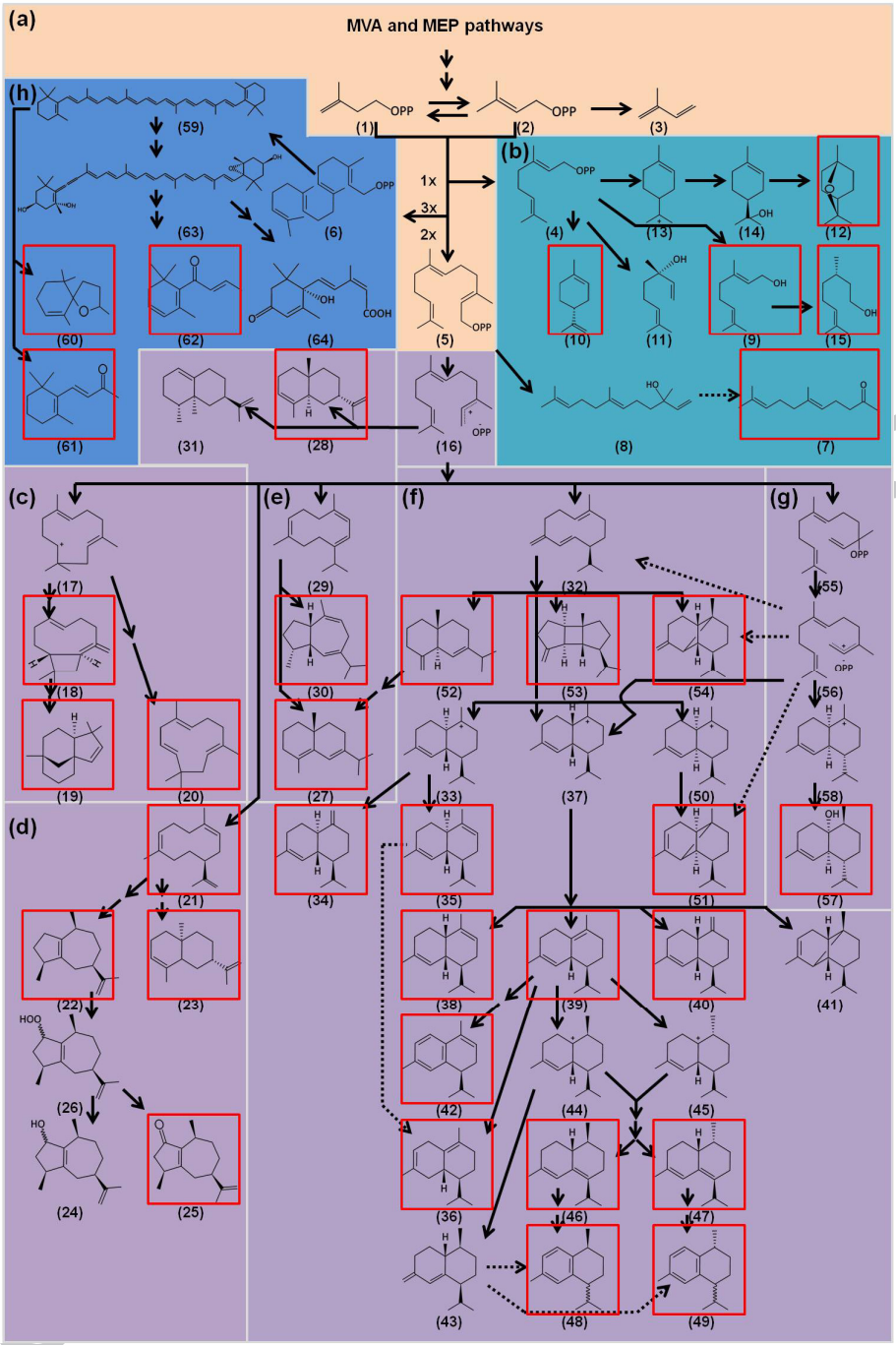
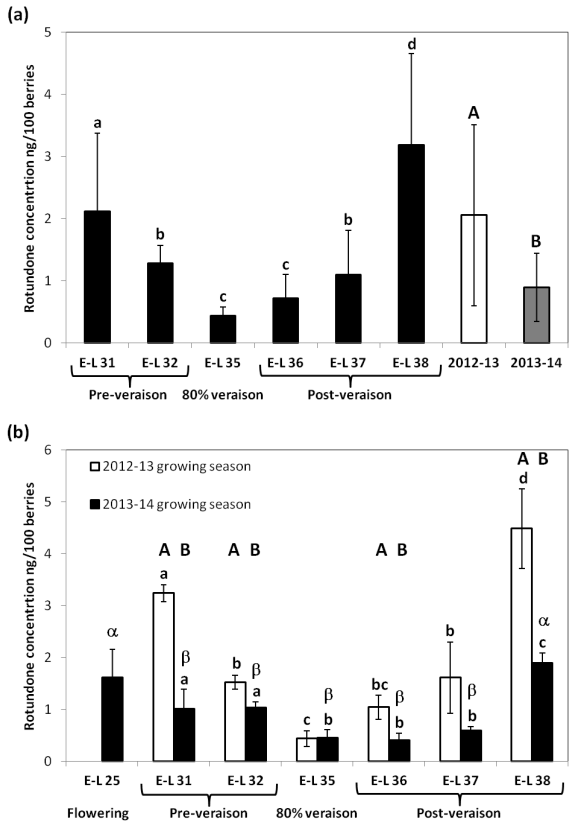


Fig. 4.



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917 **Fig. 5.**

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923 **Table 1.**

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

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

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Highlights

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