[1]β-Damascenone-yielding precursor(s) from *Cabernet Sauvignon* grapes

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Abstract

β-Damascenone, a potent aroma component of in a variety of grapes and wines, has a sensory threshold of approximately 2ng/L in water. This C_{13} norisoprenoid ketone is principally generated from hydrolyzable precursors, which was glycosidically bound. Isolation of β-damascenone-yielding precursors from Cabernet Sauvignon grape skins using C_{18} reversed phase chromatography followed by droplet countercurrent chromatography yielded multiple chromatographically distinguishable precursor fractions. The existence of multiple precursors was further evident from the varying ratios of 3-hydroxy-β-damascone (the by-product):β-damascenone generated upon acid hydrolysis of the chromatographically distinguishable precursor fractions.

***Keywords**: β-damascenone precursors, C_{13} norisoprenoids*

1 Introduction

In contrast to the intensely flavored wines of Muscat and floral grapes, the aroma of which is dependent on monoterpenes, there exist a large number of non-Muscat and non-floral grape varieties. These varieties (such as Cabernet Sauvignon, Shiraz, Merlot and Chardonnay) contain glycosidic precursors of C_{13} norisoprenoid components, which are important sources of grape-derived flavor in the wines (Williams *et al*. 1989; Williams *et al*. 1992). Although these glycosides are non-volatile and flavorless, hydrolysis of the glycosides can release components that may contribute to flavor either directly or by subsequent rearrangement. Such release is believed to occur during winemaking and maturation. Direct analysis (such as mass spectrometry and nuclear magnetic resonance spectrophotometry) would provide knowledge on the actual chemical structure of the glycosidic precursors, thus its hydrolytic behavior could be studied independently. Based on sensory significance of the hydrolysates the appropriate treatment can be exploited in winemaking or maturation to "milk" potential reserve flavor.

The kerosene-like aroma commonly linked to heated or old Riesling wine is due to 1,1,6-trimethyl-1,2-dihydronapthalene (TDN), an example of a C_{13} norisoprenoid. Another example is the potent β-damascenone (**6**), which imparts a narcotic scent reminiscent of exotic flowers. β-Damascenone is particularly interesting, because of its very low sensory threshold of approximately 2 ng/L in water (Buttery *et al*. 1990), a threshold that indicates that even trace levels of it may be important to wine flavor. Both these compounds are highly volatile, extremely stable (pH 1.0 at 100^{o}C) and are products of the acid hydrolysis of their respective precursor forms in the grape berries and must (Naiker, 1997).

Norisoprenoid glycosides, in particular that of β-damascenone precursors are believed to arise through degradation of allenic carotenoids (such as neoxanthin, **1**) and the subsequent glycosylation of the derived alcohols. However, the formation of β-damascenone from glycosidic precursor must be a multi-step process, as β-damascenone does not posses a hydroxyl group through which glycoconjugation could occur. There is evidence, from studies with Riesling wine, that there are several precursors and that they exist as both glycoconjugate and non-glycosidic polar compounds (Winterhalter *et al*. 1990). Later work, which studied possible hydrolytic pathways, suggested that the allene triol (**3**) is a key intermediate in the formation of β-damascenone (Skouromounis *et al*. 1993) (Figure 1). In favor of this is an oxidized form of the compound, grasshopper ketone (**2**), the primary degradation product of neoxanthin which has been identified in grape juice, and which under enzymatic reduction gives rise to the important allene triol. The latter itself has now also been detected in grape juice (Naiker and Allen, 1997).

Unfortunately for flavor development, the allenic triol (**3**) or its glycosylated forms rearranges rapidly (at pH 3 and room temperature) yielding only a small proportion of β-damascenone (**6**). The major product is the flavor-less 3-hydroxy-β-damascone (**5**) and the next most abundant product is the acetylenic diol (**4**) (Figure 1). Typically, β-damascenone is 4-8 % of the product, and the ratio of 3-hydroxy-β-damascone to β-damascenone generated is 10:1. Although the acetylenic diol (**4**) and its side chain glucoside can also provide both β-damascenone and 3-hydroxy-β-damascone (Figure 1), the transformation is very much slower (at pH 3 and room temperature) than that of its formation from the allene triol. The ratio of 3-hydroxy-β-damascone to β-damascenone generated is comparable with that produced directly from the allene

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triol (Skouromounis *et al*. 1993). Furthermore, it has been reported that neither the component **5** nor its glucosidic precursor yielded β-damascenone under any conditions employed (pH 1 and 3 at 100oC, 8 hours) (Skouromounis *et al*. 1993). This latter finding in combination with the high stability of β-damascenone, suggests that inter-conversion of **5** to **6** and/or vice-versa will not proceed. In winemaking terms reaction [A] occurs during pre-harvest and maceration while reaction [B] occurs during post-vinification such as during barrel/bottle storage and maturation (Sefton *et al*. 1993).

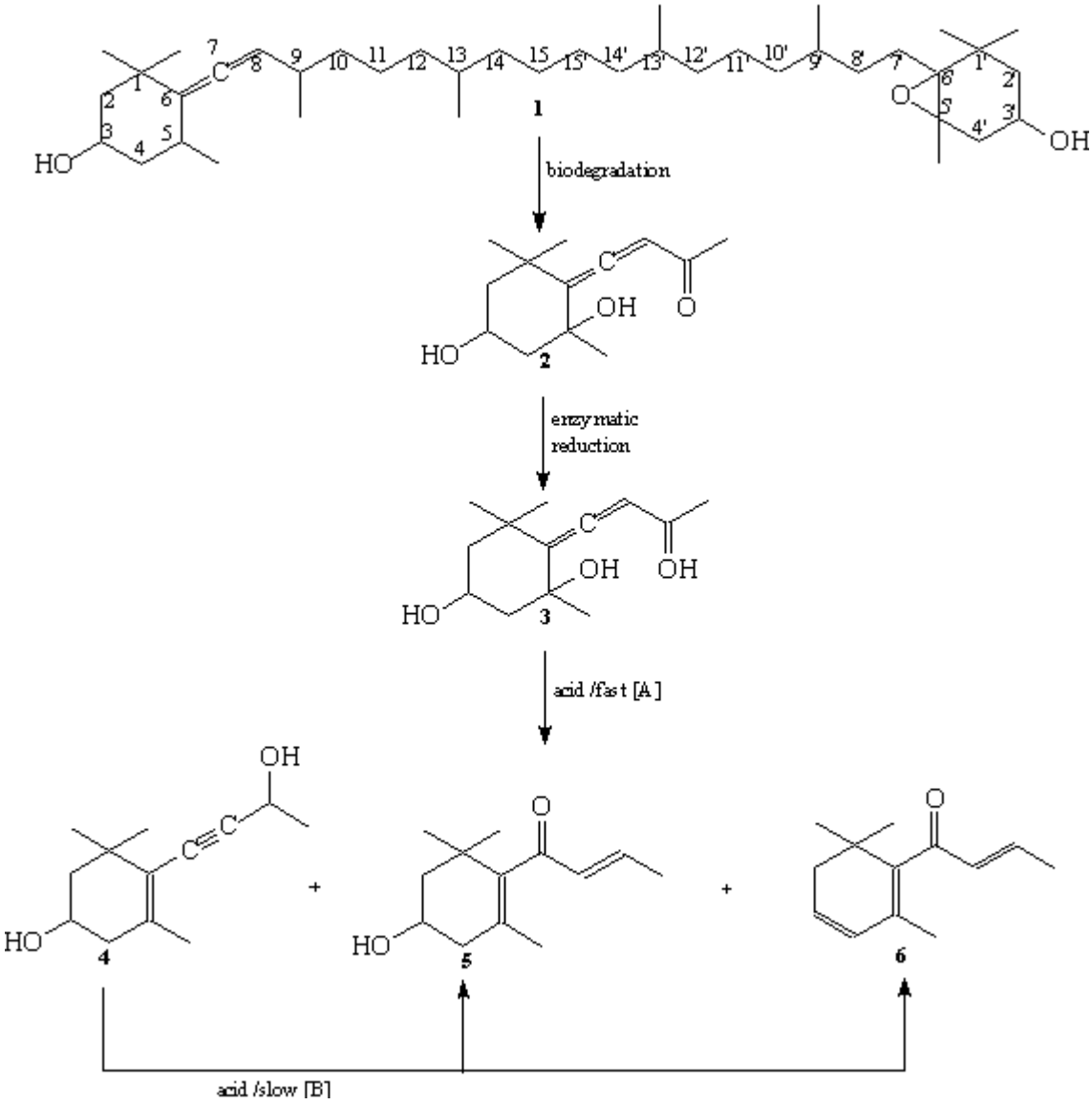

Figure 1. Proposed pathway for β-damascenone (**6**) formation from an allenic carotenoid (**1**).

Wines made from Cabernet Sauvignon grapes can exhibit a flavor complexity and intensity that makes the study of such a variety attractive as a source of flavor precursors. The ability of Cabernet Sauvignon grapes to blend with other black grape varieties (such as Shiraz and Merlot) further highlights the importance of choosing this variety for investigation. However, the concentration of flavor precursors may be as low as µg/kg or even ng/kg, and a disincentive to their study is that their extraction, particularly if under conditions comparable to that occurring during winemaking, is accompanied by an abundance of phenolic material of considerable complexity. Many grape phenolic compounds are glycosidic, adding to the separation difficulties, and some phenolic components readily oxidize or polymerize, contributing to increased matrix complexity.

Work presented here explores the potential for identifying flavor component precursors in Cabernet Sauvignon grape skins. The isolation, purification and chromatographic separation of the precursors followed by detection and quantification of β-damascenone (**6**) and 3-hydroxy-β-damascone (**5**) released upon acid hydrolysis is reported.

2 Experimental Methodology

2.1 Grape berry skin extraction

Cabernet Sauvignon grape berries were hand picked from Charles Sturt University vineyard (stage 1, rows 1-2)

Wagga Wagga, NSW (1995 vintage). Fruit (130 kg) was lightly pressed using a water bag presser and destemmed to give 55 kg of presscake, which was frozen until required for extraction.

Frozen presscake containing skins (55 Kg) was extracted in a 44-gallon steel drum with chilled 50 % aqueous ethanol (40 L) stirring with an electric propeller. Extraction was stopped after 48 hrs and the mixture pressed using a water bag press to give 55 L of ethanolic extract. Extraction was carried out at 15 ^{O}C and the ethanolic extracts were stored in a 4 ^{O}C cold room prior to centrifuging and concentrating it under vacuum (water bath temperature at 28 ^{O}C) to *ca*. 15 L and frozen until further treatment.

2.2 Polyvinylpolypyrrolidone (PVPP) treatment of the crude extract

In a typical procedure concentrated skin crude extracts (500 mL) were diluted to 2 L using Milli Q water. The diluted extract was stirred with PVPP (175 g) for 1 hr at 4 ^{O}C, then filtered through a Whatman filter paper (11.0 cm) under vacuum. The filtrate was subjected to a second PVPP (175 g) treatment at 4 ^{O}C, then filtered through a Whatman filter paper (11.0 cm) under vacuum. The filtrate was frozen until further required.

2.3 C_{18}-Reverse phase (C_{18}-RP) isolation of glycosides

Bio-Rex MT glass column (700 x 60 mm i.d.) was packed with slurry of octadecyl-functionalized silica gel (350.0 g) in 100 % MeOH. The column was washed with water until all MeOH was washed from the column material.

In a typical procedure, PVPP treated grape skin extracts were thawed, and pumped through the column using a Gilsons Miniplus 2 peristaltic pump at 4 ^{O}C. The column was washed with enough water to remove any free sugars and organic acids prior to eluting with 100 % MeOH. The column was then washed with large amounts of water to remove any traces of MeOH bound to the C_{18} material before the next batch of skin extract was pumped through it. All MeOH eluants obtained were pooled, concentrated and frozen until further required.

2.4 Droplet counter-current chromatography (DCCC) of C_{18}-RP isolates from skin extracts

An Eyela Model DCC-300 (Tokyo Rikakikai Co., Ltd.) unit equipped with 300 tubes (400 x 3.4 mm i.d.) was used. The DCCC instrument was operated in the descending mode, with the lower (more polar) layer from a mixture of distilled BuOH:MeOH:H_2O (5:1:5) utilized as the mobile phase and the upper layer as the stationary phase (Hostettmann *et al*. 1986).

In a typical DCCC separation, C_{18}-RP isolates (1.0-1.5 g) from grape skin extracts were dissolved in a mixture (1:1) of the two phases (20 mL) and applied to the DCCC instrument via a 20 mL sample loop. Consecutive fractions of 600 drops/tube at a flow rate of 2-4 mL/hr were collected. DCCC fractions collected were transferred into screw capped borosilicate tubes and stored in the freezer.

2.5 Precursor assay

GC-MS was used to monitor β-damascenone (**6**) and 3-hydroxy-β-damascone (**5**) liberated from precursors by acid hydrolysis. A known quantity of the selected DCCC fraction was evaporated to dryness before redissolving it in a known volume of tartrate buffer (prepared by adjusting a solution of saturated potassium hydrogen tartrate to pH 3.2 with 100 g/L tartaric acid solution). The aqueous sample (in sealed ampoule) was heated at 100 ± 2 ^{O}C (oven) for 16 hrs, cooled, a solution of 3-hexadecanone, the internal standard (known amount) in EtOH (known volume) then added, and the hydrolysate extracted with Et_2O (5 x 1 mL). The organic extract was dried ($MgSO_4$) and concentrated by fractional distillation through a Vigreux column packed with Fenskes helices prior to GC-MS analysis.

2.6 Gas chromatography mass spectrometry (GC-MS) calibration

A VG Trio 2 GC-MS system was used. The gas chromatograph was equipped with a DBWax fused silica column (30 m x 0.32 mm i.d. and 0.25 μm film thickness), and operated with helium carrier gas at a linear velocity of 15 cm/s. GC-MS analysis was performed with a splitless injection mode for 2.0 min, after which the injection mode was changed to split. The injector temperature was kept constant at 250 ^{O}C and the source temperature at 180 ^{O}C. The column was held at 60 ^{O}C for 1 min followed by a gradient from 60 ^{O}C to 150 ^{O}C at 5 ^{O}C/min, from 150 ^{O}C to 250 ^{O}C at 10 ^{O}C/min, then held at this temperature for 2 min. Positive ion electron impact mass spectra were taken at 70 eV, scanning from 35 to 400 dalton scan with a 0.1 s inter-scan delay.

A standard mixture containing β-damascenone (**6**) (1.0 mg/mL), 3-hydroxy-β-damascone (**5**) (1.0 mg/mL) and 3-hexadecanone as internal standard (IS) (1.1 mg/mL) was prepared in dichloromethane. Aliquots from this standard mixture were diluted to provide concentrations of 4, 50, 100, 154, 200 and 500 ng/μL for **6** & **5** and 4.4, 55, 110, 167, 220 and 550 ng/μL for 3-hexadecanone. GC-MS analysis of the diluted standard mixtures was performed in random order with 3-5 replicate injections. Blank injections of dichloromethane were made before analysis and between each analysis of standard mixture. The ion chromatographic peak area for each component was obtained by the summation of the peak areas corresponding to ions at m/z 105, 121, 175 and 190 (**6**) (R_t 16.0-17.0 min), m/z 105, 121, 175, 190, 193 and 208 (**5**) (R_t 30.0-31.0 min), and m/z 211 (IS) (R_t 22.0-23.0 min).

2.7 GC-MS analysis of DCCC separated skin C18-RP isolate fractions

Known quantities of a range of DCCC fractions were subjected to acid hydrolysis (pH 3.2, 100 ^{O}C, 16 hrs) prior to addition of 3-hexadecanone (IS) and solvent extraction with diethyl ether. The β-damascenone precursor assay protocol, as described above, was used to prepare these samples. GC-MS was used to quantify β-damascenone (**6**) and 3-hydroxy-β-damascone (**5**) in the organic extract, liberated from glycosylated precursor(s) prior to and after acid hydrolysis. Organic extracts (200 μL, stored at 0 ^{O}C) obtained for these DCCC fractions were further concentrated (50-100 μL) under a stream of nitrogen gas at room temperature prior to GC-MS analysis for increased

sensitivity in the mass spectrometric detection. Every sample analysis (2.0 µL injected) was acquired before and after injecting a blank (dichloromethane).

2.8 Ultraviolet-visible spectra

UV-vis spectra were recorded using a Varian 200 UV-Vis spectrophotometer.

3 Results and Discussion

Prior knowledge of the occurrence of β-damascenone-yielding precursors in trace concentrations prompted a large-scale extraction initially. This would allow the isolation and purification of an ample quantity of precursor so that direct analysis with mass spectrometry and nuclear magnetic resonance spectrophotometry would be feasible. Cabernet Sauvignon grape skin samples were extracted with 50 % aqueous ethanol, concentrated and treated consecutively with PVPP to preferentially remove the abundant phenolic material in the skin crude extracts. Consecutive PVPP treatments removed approximately 97-99 % of both 280 and 520 nm light absorbing components from the aqueous ethanolic extracts. These initial purification of the total extract was achieved with a minimal loss (1.6 %) of total available β-damascenone-yielding precursors as determined by the GC-MS analysis for β-damascenone generated before and after PVPP treatments of the crude extracts. So, at least at that early stage in the purification process, significant loss of β-damascenone-yielding precursors through adsorption to PVPP was not evident.

Isolation of glycosidic material from the PVPP treated extract was carried out by reversed phase C_{18} chromatography. Loss of β-damascenone-yielding precursors upon such treatment was even lower (0.3 %) than that observed earlier for the PVVP treatment of the crude extracts. Further separation of these C_{18}-RP isolates was achieved through droplet counter current chromatography. Prior to commencing with DCCC separation it was crucial to select an appropriate solvent system which would improve the resolution of the desired β-damascenone precursors from each other, from other non-phenolic glycosidic material and importantly from the presence of residual but comparatively dominant interfering phenolic material. From an evaluation of several solvents systems (Naiker, 1997), BuOH:MeOH:H_2O was selected as the most appropriate. After continuos DCCC separation for 120 hrs, 250 fractions were collected. Selected fractions were measured for phenolic content and screened for the presence of β-damascenone-yielding precursor(s) by quantitative GC-MS analysis of both β-damascenone (**6**) and 3-hydroxy-β-damascone (**5**). The ratio of 3-hydroxy-β-damascone to β-damascenone (**5**:**6**), generated upon acid hydrolysis of selected fractions was also determined.

Figures 2-3 shows the distribution of **5** and **6** across the range of DCCC fractions assayed. In each case the line is drawn through data points that represent concentrations determined using the mean response factor for each of **5** and **6**. Figure 3 indicates the presence of at least four chromatographically distinguishable precursor fractions of 3-hydroxy-β-damascone. The situation with β-damascenone is less defined, but two chromatographically distinguishable precursor fractions can be identified (Figure 2).

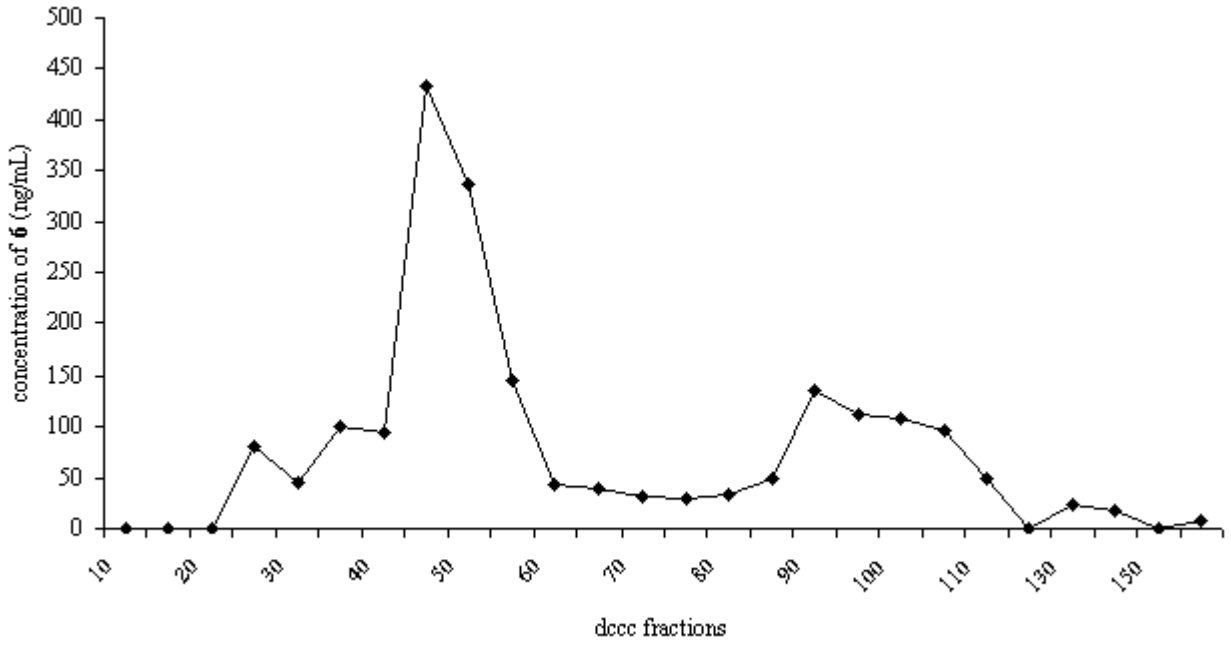

Figure 2. Concentration of β-damascenone (**6**) across the DCCC fractions assayed.

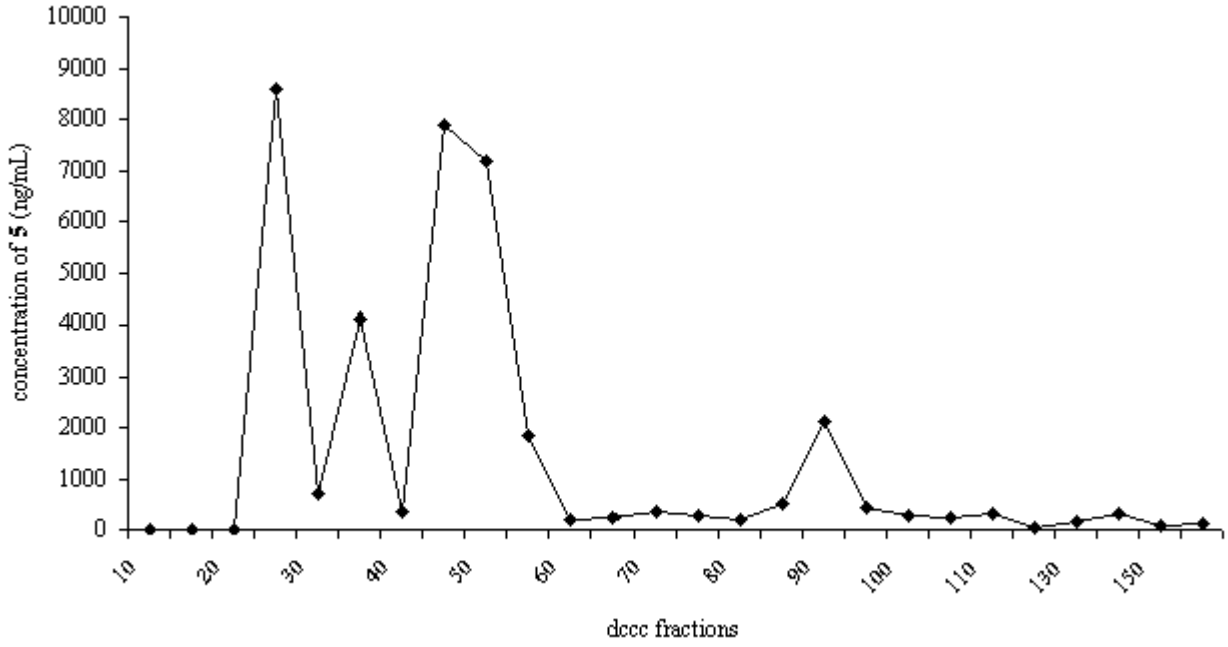

Figure 3. Concentration 3-hydroxy-β-damascone (**5**) across the DCCC fractions assayed.

These data indicate the occurrence of β-damascenone-yielding precursors with differing polarities. The existence of multiple precursors with widely differing polarities is consistent with previous findings in wine (Winterhalter *et al*. 1990). A similar situation has been found in apples (Roberts *et al*. 1994), for which at least eight precursors with abilities to generate β-damascenone were identified with one characterized as a disaccharide glycoside of the acetylenic diol (**4**). The direct analysis of the chromatographically distinguishable precursors identified in this work was not feasible. This was mainly due to the presence of residual phenolic material still present in the precursor eluting fractions particularly in the early eluting polar ones. Furthermore greater resolution of precursors from themselves and from co-eluting precursors of other norisoprenoids (Naiker, 1997) needs to be achieved. Separation of pure precursor(s) to allow direct analysis could be achieved through developing a more efficient solvent system and through employment of sophisticated countercurrent chromatography techniques (such as rotational locular countercurrent chromatography) (Herderich *et al*. 1992). Given the diversity of possible glycosides that could be derived from the allenic triol (**3**) and the acetylenic diol (**4**), through difference in size of the saccharide moiety (mono-, di, or trisaccharide etc.), the type of sugar (glucose, arabinose or apiose), or the position of glycosylation, the occurrence of multiple precursors is quite feasible.

Figure 4 shows the variation of the ratio of **5**:**6** in the same DCCC fractions, based upon the mean response factor ratio found with the standard mixture. Table 1 shows the respective ratios of **5**:**6**, in pooled DCCC fractions. Variation in the ratio of **5**:**6** yielded further supports the occurrence of multiple precursors capable of generating β-damascenone in Cabernet Sauvignon grapes. The variation in the ratio of **5**:**6** yielded further suggests that each of this chromatographically distinguishable precursor group(s) must be different to one another mainly in glycosylation patterns. These subtle differences allows each group of precursors (or the dominant precursor within these) to release β-damascenone and 3-hydroxy-β-damascone through varying pathways upon acid hydrolysis.

Table 1. Sum of DCCC precursor peak for ratios of **5**:**6** generated.

Fraction peak no.	Fractions eluted in	Ratio (**5**:**6**)
1	20-30	83.1
2	31-40	26.5
3	41-60	17.5
4	80-95	11.1
5	96-120	3.7

What is the implication of the findings reported here towards winemaking? Cabernet Sauvignon fruits that consists of precursor(s) that generate more of the favorable and potent β-damascenone in comparison to the flavorless 3-hydroxy-β-damscone would be regarded as of higher quality. If a direct link between fruit quality in terms of β-damascenone generating ability is considered necessary, total grape berry analysis for the ratio of **5**:**6** yielded maybe a more sensible and practical approach as reported elsewhere (Naiker, 1997).

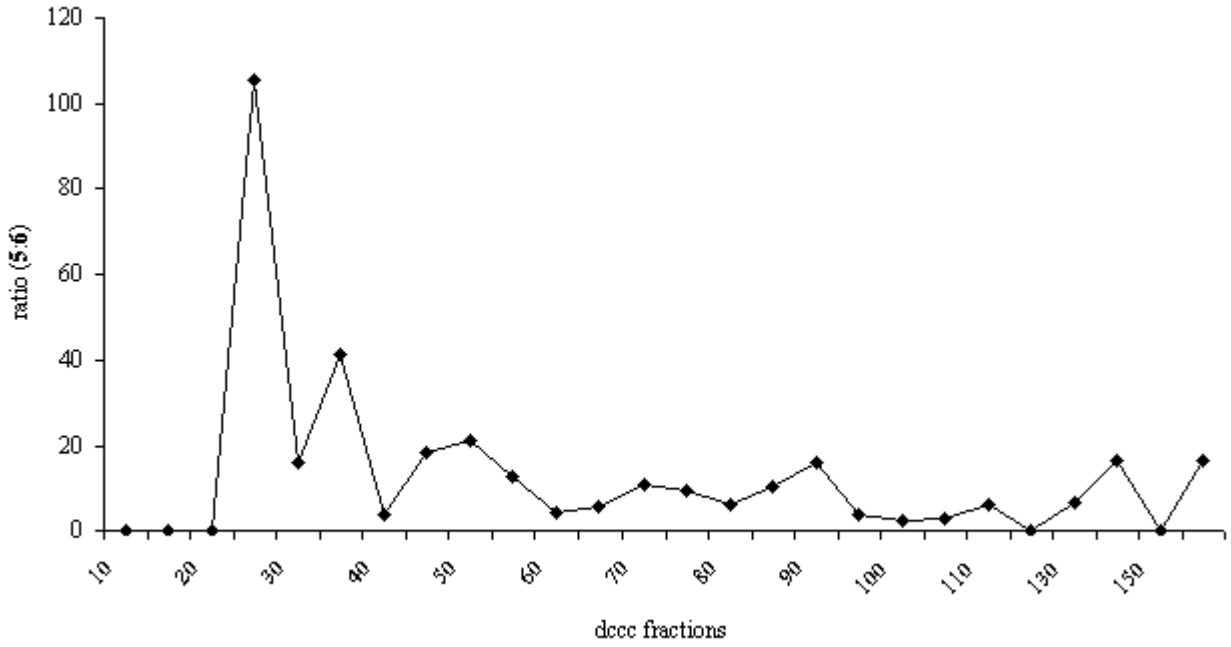

Figure 4. Ratio of **5**:**6** generated across the DCCC fractions assayed.

4 Conclusions

The findings reported here indicate the existence of multiple β-damascenone-yielding precursors in Cabernet Sauvignon grape skins. They possibly differ from each other in glycosylation pattern as are evident by the DCCC separation of skin glycosides into chromatographically distinguishable precursor fractions based on ratio of **5**:**6** generated.

The extent and position of glycosylation of these precursors may be a determining factor in the yield of β-damascenone upon acid hydrolysis. This is evident from the various ratios of **5**:**6** generated by various groups of precursor fractions. Thus if each DCCC peak arises from a single precursor, β-damascenone-yielding precursors exist in multiple forms that generate different ratios of **5**:**6** in these Cabernet Sauvignon grapes.

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