Detection and Partial Characterization of Eight β-Damascenone Precursors in Apples (*Malus domestica* Borkh. Cv. Empire)

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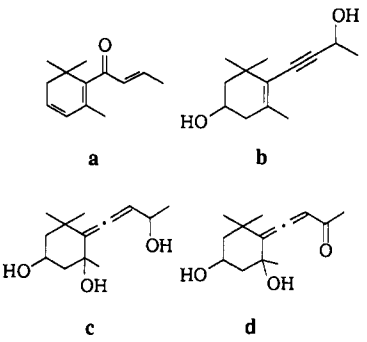
β-Damascenone, a potent aroma compound in a variety of natural products, is primarily liberated from precursors. Isolation of β-damascenone precursors from apples *Malus domestica* Borkh. cv. Empire, using Amberlite XAD-2 adsorption and C_{18} flash chromatography columns followed by C_{18} HPLC, yielded at least eight separate precursors, of which the most abundant was further purified and characterized. Mass spectra from ion spray tandem MS, GC/MS of TMS sugars, and GC/MS of the enzymatically released aglycon indicated a structure of 9(or 3)-α-L-arabinofuranosyl-(1,6)-β-D-glucopyranoside of the acetylenic diol. The sizes of the other precursors were estimated from their retention during size exclusion chromatography (GPC). The presence of two triglycosides, four or more diglycosides, and two or more free aglycons was consistent with the GPC data.

INTRODUCTION

β-Damascenone [**a**, 1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-2-buten-1-one] is a potent flavor compound in many natural products. Its characteristic floral odor contributes to the odor character of Bulgarian rose oil (Demole et al., 1970), tobacco (Wahlbert et al., 1977), tea (Renold et al., 1974), raspberry oil (Winter and Enggist, 1971), apple products (Schreier et al., 1978; Williams and Tucknott, 1978; Cunningham et al., 1986), various grape varieties and wine (Acree et al., 1981; Winterhalter, 1992), alcoholic beverages (Masuda and Nishimura, 1980), coffee, honey (Sen et al., 1991), Satsuma mandarin juice (Araki and Sakakibara, 1991), textured soy protein (Ames and MacLeod, 1984), tomatoes (Buttery et al., 1990a), purple passion fruit (Winterhalter et al., 1991), elderberries (Mikova et al., 1984), and starfruit (Herderich et al., 1992). Because of its low odor threshold, 2 pg/g in H_2O (Buttery et al., 1990b), only trace quantities are necessary for odor.

β-Damascenone is liberated from precursor compounds when heated in an acidic environment. It increases substantially when acidic natural products are heated to about 80 °C as in tomatoes (Buttery et al., 1990b), apples (Schreier et al., 1978; Zhou et al., 1993), and mandarin juice (Araki and Sakakibara, 1991). In vitro investigations have shown the presence of polyol β-damascenone precursors, namely 3,9-dihydroxymegastigm-5-en-7-yne (acetylenic diol) (**b**) and 3,5,9-trihydroxymetastigma-6,7-diene (allenic triol) (**c**) (Sefton et al., 1989; Skouroumounis et al., 1992). The grasshopper ketone **d** (3,5-dihydroxymetastigna-6,7-dien-9-one) has been implicated as a possible biosynthetic precursor (Winterhalter, 1992). In addition, hydrolysis of glycosidic extracts of wine, grapes, and tomatoes has demonstrated glycosidic precursor occurrence (Buttery et al., 1990a; Williams et al., 1992). An allenic triol glucoside precursor was identified in *Lycium halimifolium* Mil (Näf et al., 1990). The techniques of droplet countercurrent chromatography (DCCC) (Winterhalter et al., 1990) and HPLC (Braell et al., 1986) provided evidence for multiple precursors in grapes and wine. However, because of the trace quantities of the precursors in most natural products, actual structural identification of the glycosidic precursors has been difficult.



The determination of the number of β-damascenone precursors in apples as well as their partial characterization was the aim of this research. Eight β-damascenone precursors were found at levels of 0.5–4.6 ng/g in apples using C_{18} HPLC as the final chromatographic step. Despite these low levels, sufficient material was available after purification to characterize the most abundant precursor. Characterization was performed by ion spray mass spectrometry (Bruins et al., 1987), enzymatic hydrolysis followed by GC/MS, and trimethylsilation with GC/MS. Information about the sizes of the eight precursors was obtained using gel permeation chromatography.

MATERIALS AND METHODS

Fruit. Apples, *Malus domestica* Borkh. cv. Empire, were harvested in fall 1991 in central New York and obtained through local markets.

Juice Preparation. Apples (100 kg) were crushed in a Fitzpatrick hammer mill set at 4000 rpm, through a 1/4-in. screen. The apple mash was then pressed in a 36-in. hydraulic press at 2000 psi. After filtration through four layers of cheesecloth and centrifugation at 1300 rpm, below 20 °C in a Louis Allis centrifuge, 66 L of apple juice was obtained.

Precursor Assay. Throughout the purification scheme, gas chromatography mass fragmentometry (SIM, selected ion monitoring) was used to monitor β-damascenone liberated from precursors by acid hydrolysis. One milliliter of sample was mixed with 3 mL 0.1 M citric acid (pH 2), heated at 90 °C for 20 min,

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cooled in ice water for 2 min, and extracted with 2 mL of freon. One microliter of the freon extract was injected into a 0.22 mm × 25 m fused silica capillary column, coated with 0.33 μm of methyl silicone coupled to a HP 5970 MSD (mass selective detector). After 3 min at 35 °C, the temperature was programmed at 25 °C/min to 152 °C, at 4 °C/min to 170 °C while β-damascenone elutes, and at 30 °C/min to 225 °C, where it was held for 10 min. The mass fragments (*m/z* 69, 91, 105, 121, and 190) were generated at 70 eV by electron impact and were used to monitor β-damascenone. An external standard curve was produced using β-damascenone standards (Firmenich, Geneva, Switzerland) in Freon 113.

Flash Column Chromatography. The first separation was carried out by passing three 20-L samples of apple juice through a column (80 × 8 cm i.d.) of Amberlite XAD-2 resin (Gunata et al., 1985). The columns were rinsed with 10 L of water at a flow rate of 2 L/h and eluted sequentially with 5 L each of *n*-pentane, ethyl acetate, and methanol. The precursors were monitored in the rinses using the precursor assay. The absence of β-damascenone precursors in the water and pentane fractions was determined by the precursor assay. Ethyl acetate and methanol rinses were further purified by separately pouring the fractions, redissolved in water, onto a C_{18} flash glass column (60 × 3 cm i.d.). The column was washed with 3 volumes each of water, 10% methanol, and 100% methanol in succession. β-Damascenone precursors eluted with the first column volume of methanol.

C_{18} HPLC Separation. A Varian (Sugar Land, TX) liquid chromatograph equipped with a Star 9010 solvent delivery system, a Star 9095 autosampler, and a Star 9096 polychrome diode array detector operated in the range 190–367 nm was used. The semipreparative (10 mm i.d. × 25 cm) and guard (10 mm i.d. × 5 cm) columns were packed with Microsorb 5 μm C_{18} (Rainin, Woburn, MA). Elution was performed using a mobile-phase gradient from 10 to 100% methanol in water at a rate of 5 mL/min for 35 min.

The precursor samples injected are the two that eluted from Amberlite XAD-2 with ethyl acetate and methanol and were both further purified by C_{18} flash chromatography. To check for the presence of free β-damascenone in these fractions, 1 mL of eluate was taken from each fraction and used for the precursor assay. The HPLC fractions with β-damascenone activity were each separately rechromatographed under the same conditions as before to determine the number of unique precursors. Additionally, standard acetylenic diol **b** donated by Dr. P. J. Williams was chromatographed on the C_{18} HPLC.

Final purification of the precursor **6** (Figure 1) was achieved by using overload and recycle HPLC techniques. Smaller fractions (1 mL) than previously (5 mL) were collected in the region of precursor elution.

Enzymatic Hydrolysis. A nonselective pectinase containing β-glucosidase, β-apiosidase, β-arabinosidase, and β-rhamnosidase activities (Gist-brocades, Seclin Cedes, France) at a level of 0.05 g was added to 200 ng of β-damascenone equivalents of purified precursor **6**. The incubation was performed at pH 5 (phosphate buffer) for 24 h at 37 °C. A subsequent ethyl acetate extraction was analyzed by GC/MS for determination of the liberated aglycons. The column temperature was programmed at 4 °C/min from 35 to 225 °C.

Trimethylsilation (TMS). The purified precursor **6** was concentrated to dryness under a reduced pressure of 60 mmHg. For monosaccharide analysis, the precursor was hydrolyzed according to the precursor assay and concentrated to dryness prior to TMS (Sweeley et al., 1963). One hundred microliters of the TMS reagent (Sigma Sil A, containing trimethylchlorosilane, hexamethyldisilazane, and pyridine in a ratio of 1:3:9) was added to the sample with agitation, and after 20 min, 1 μL of the supernatant was injected onto the GC/MS. The column temperature was programmed at 3 °C/minute from 120 to 225 °C, and held at 225 °C. Monosaccharide standards (Sigma) were trimethylsilated according to the same procedure. *N*-Paraffin retention indices as well as *o*-nitrophenyl β-D-galactopyranoside (Sigma) were used to determine relative retention.

Ion Spray Tandem Mass Spectrometry. A Sciex (Thornhill, ON) TAGA 6000E upgraded to an API III atmospheric pressure ionization (API) ion spray tandem triple quadrupole mass spectrometer was used. The sample was precursor **6** present at 10 ng/μL β-damascenone equivalents, in 200 μL of water. A standard acetylenic diol **b** was also analyzed. The samples were introduced into the mass spectrometer using flow injection techniques (Bruins et al., 1987). Each sample was injected (Rheodyne fixed-loop A 520 injector) at 20 μL/min onto a continuous pumped (Applied Biosystems 140A solvent delivery system) methanol mobile phase containing 10 mM ammonium formate. Nitrogen, maintained at 60–65 psi, nebulized the liquid stream. Poly(propylene glycol) (PPG) was used to calibrate the mass axis of each mass resolving quadrupole (Q1 and Q3). The mass spectrometer was operated in single and tandem MS modes, scanning a mass range of *m/z* 100–700. Parent and daughter ion scans were also made.

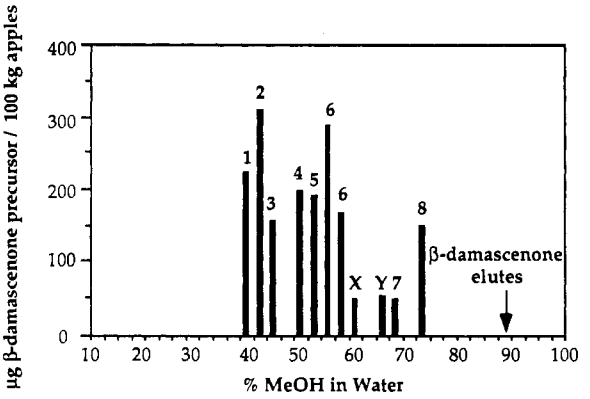

Figure 1. C_{18} HPLC characterization of eight precursors detected in apple juice. Reprinted with permission from Zhou et al. (1993). Copyright 1993 Allured Publishing.

Gel Permeation Chromatography. Gel permeation purification was performed on the sample which had previous purification through XAD-2 and C_{18} column chromatography. Both the methanol and ethyl acetate XAD-2 elutants were combined and applied to a column of Bio-Gel P-2 (2.5 cm i.d. × 75 cm) with water as the running buffer at a flow rate of 25 mL/hr. Five-milliliter fractions were collected after the void volume eluted to collect and separate the precursors. The precursor assay was performed on the fractions to determine where the precursors eluted.

Precursors eluted in four GPC fractions were chromatographed on the C_{18} HPLC column, with the same conditions as before. The last two fractions required concentration under reduced pressure of 60 mmHg before injection, 2× for the third and 40× for the fourth. The precursor assay was performed on the HPLC eluting fractions.

RESULTS AND DISCUSSION

Detection of Eight Precursors by C_{18} HPLC. Eight separate β-damascenone precursors (1–8) have been detected in apples (Figure 1) and are characterized by their percent methanol in water elution on C_{18} HPLC. The precursor levels, expressed as micrograms of β-damascenone released under hydrolysis, are the sum of the values from the ethyl acetate and methanol XAD-2 fractions and represent the total amounts found in apples. In total, β-damascenone precursors were present at a level of 18 ng/g apple, with the most abundant precursor at about 4.6 ng/g apple (Table 2). Free β-damascenone in unheated apples has been reported at a level of less than 1 ng/g (Shure, 1992). Liberation of the precursors would generate a β-damascenone level of 9000× its odor threshold (2 pg/g) or 9000 odor units. Thus, although 4.6 ng/g is a very low amount for chemical characterization, it produces a large sensory response.

The C_{18} HPLC rechromatography step determined the presence of eight unique precursors, illustrated by Figure 2. It can be seen, for example, that when fraction 1 was injected, the majority of β-damascenone precursors eluted in fraction 1. However, when fraction 6′ was injected, the majority of precursors eluted in fraction 6, hence the

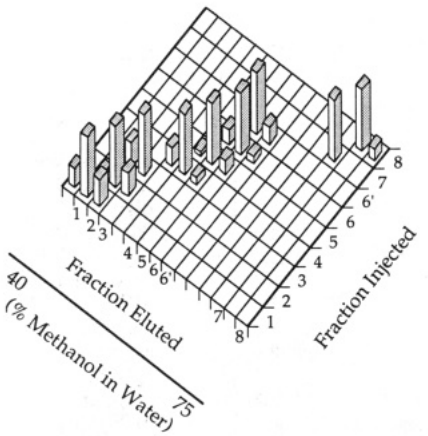

Figure 2. HPLC rechromatography of precursor fractions showing liberated β-damascenone. Fraction numbers correspond to Figure 1.

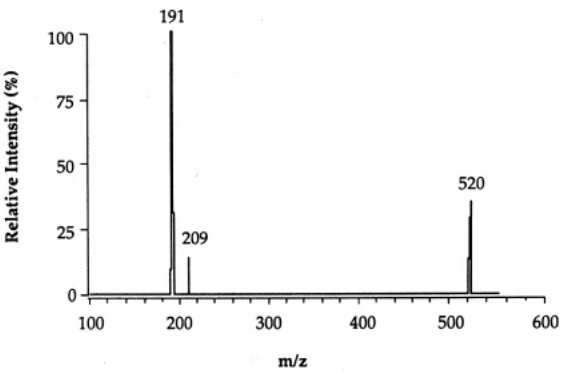

Figure 3. Ion spray mass spectrum of precursor **6** showing an ammoniated molecular ion *m/z* 520.

labeling 6′. Although fraction 8 upon rechromatography showed a precursor maximum in its preceding fraction, the initial HPLC separation did not show a precursor there. Hence, 8 was judged to be a unique precursor. Eight of the 11 fractions containing β-damascenone precursors are unique and distinct because the main precursor fraction was the same as that injected. The percent methanol at the elution of each precursor (Table 2) was used as an indicator of the presence of that precursor in any fraction. It is possible that greater than eight precursors were present because fractions can contain more than one precursor and fractions **X** and **Y** were not available for this rechromatography step. Although as many as three β-damascenone precursors have been demonstrated in the past (Braell et al., 1986; Winterhalter et al., 1990), the presence of at least eight indicates a complex biogenesis.

Characterization of Precursor 6. Precursor **6**'s characterization as the 9(or 3)-α-L-arabinofuranosyl (1,6)-β-D-glucopyranoside of the acetylenic diol **b** was made using ion spray mass spectrometry, enzymatic hydrolysis aglycon analysis, and TMS sugar analysis.

Enzymatic hydrolysis of precursor **6** yielded the acetylenic diol **b** (Table 1) as determined by mass spectrum and retention time matches with an authentic standard. The aglycon for precursor **6** is the acetylenic diol **b**.

TMS and GC/MS of precursor **6** determined which mono- or disaccharides were attached. The hydrolyzed precursor produced monosaccharides that matched in retention time and mass spectra those of arabinose and glucose authentic standards (Table 1). Additionally, the intact precursor showed arabinoglucoside which matched in mass spectrum and retention time those of the α-arabinofuranosyl-α(β)-glucopyranose previously reported (Voirin et al., 1990). Although the mass spectra of arabinoglucoside and apioglucoside are very similar, the abundance of *m/z* 191 is a distinguishing feature (Voirin et al., 1990). A low abundance of ion 191 indicated that the disaccharide contained arabinose instead of apiose.

Molecular weight and structural information was obtained by ion spray mass spectrometry. Ion spray is a gentle technique in which liquid held in a charged capillary is nebulized by a stream of nitrogen to produce ions by evaporation (Bruins et al., 1987). Fragmentation in ion spray, like FAB, is simpler than EI. It has been shown that glycosides fragment after protonation of the glycosidic oxygen by breaking the oxygen–sugar bond and, after hydrogen transfer, leaving behind a protonated alcohol (Crow et al., 1986). This fragmentation mechanism is probably what is occurring in ion spray mass spectrometry, as a protonated aglycon (*m/z* 209 in Figure 3) is present.

Figure 3 shows the ion spray mass spectrum of precursor **6**. Ions *m/z* 520, 209, and 191 have relative intensities of 55, 19, and 100, respectively. These ions correspond to the glycosidic ammoniated precursor, *m/z* 520, and its daughter ions: protonated β-damascenone, *m/z* 191, and protonated acetylenic diol **b**, *m/z* 209. Ammoniation of molecular ions results because of the presence of ammonium formate in the solvent. Only an ammoniated molecular ion (*m/z* 226) was present in the mass spectrum of the standard acetylenic diol **b**.

The presence of the ion *m/z* 209 (protonated acetylenic diol) from parent *m/z* 520 further confirms the enzymatic hydrolysis results that the molecule is a sugar adduct of the acetylenic diol. A molecular weight of 502 corresponds to $C_{24}O_{11}H_{38}$. The TMS identification of arabinosyl glucoside supports the identification of the precursor as the acetylenic diol diglycosylated by arabinosylglucoside (Figure 4). The sugars could be attached at either hydroxyl group of the acetylenic diol. α-L-arabinofuranosyl (1,6)-β-D-glucopyranoside is the common form of this disaccharide in natural products and is a common diglycoside

Table 1. Mass Spectra of Enzymatic Hydrolysis Liberated Aglycon and TMS Sugar Derivatives from β-Damascenone Precursor 6

identity	EIMS	retention index	rrt[a]
acetylenic diol **b**	43 (100), 193 (58), 208 (55), 157 (56), 131 (53), 91 (51), 105 (47), 175 (46), 142 (39), 77 (38), 115 (29)	1595	
TMS-glucose	204 (100), 73 (95), 191 (69), 147 (24), 217 (21), 205 (14), 192 (14), 75 (14), 74 (14), 89 (11), 117 (10), 193 (7), 133 (7), 103 (7), 129 (6)	α 1938 β 2040	α 0.544 β 0.609
TMS-arabinose	73 (100), 217 (90), 204 (72), 91 (66), 75 (32), 129 (21), 147 (17), 192 (14), 133 (13), 117 (11), 101 (10), 111 (9), 102 (9), 218 (8), 189 (8), 161 (8), 219 (7), 143 (7), 127 (7), 333 (5)	α 1651 β 1678	α 0.352 β 0.372
TMS-arabinosylglucose	204 (100), 73 (54), 205 (19), 69 (16), 217 (14), 147 (13), 103 (12), 361 (8), 206 (8), 319 (7), 191 (7), 129 (6), 111 (5), 201 (5), 275 (2), 305 (2), 451 (2), 435 (1), 563 (0.3)		0.939

[a] Relative retention time to *o*-nitrophenyl β-D-galactopyranoside.

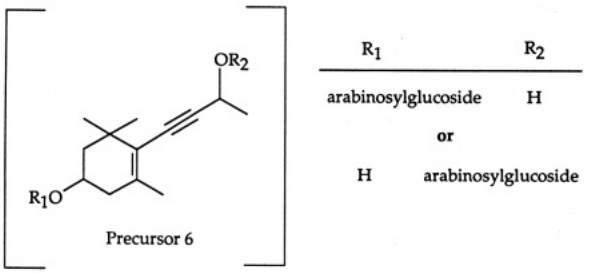

Figure 4. Structural characterization of precursor 6: arabinosyl glucoside of the acetylenic diol **b** (MW 502, $C_{24}O_{11}H_{38}$).

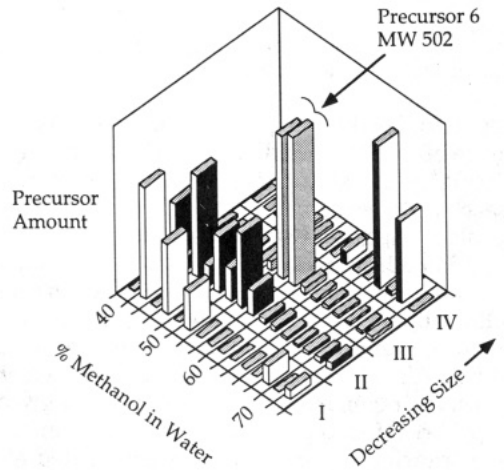

Figure 5. β-Damascenone precursor distribution across the C_{18} HPLC gradient for four fractions (I–IV) taken from the GPC precursor elution. The precursor amounts for GPC 1 and GPC 4 were multiplied by 25 for distribution comparison.

found with norisoprenoids in grapes (Voirin et al., 1990). This α-L-(1,6)-β-D stereochemistry of the arabinosyl glucoside acetylenic diol precursor is assumed, although not proven.

The acetylenic diol has been identified in tobacco (Fujimori et al., 1975), in purple passion fruit (Winterhalter et al., 1991), and in a free and unspecified bound form in wine and grapes (Sefton et al., 1989; Sefton and Williams, 1991). The postulated pathway of β-damascenone formation and the role of acetylenic diol were recently reviewed (Winterhalter, 1992). The acetylenic diol has been shown to produce β-damascenone as a minor product when heated at 80–100 °C under pH 1–3 for 20 min to several hours (Sefton et al., 1989). The mechanism for this transformation can be explained by acid-catalyzed Meyer–Schuster rearrangement, implying a 1,3-hydroxy shift (Swaminathan and Narayanan, 1971; Olsson et al., 1973). This previous research implicating acetylenic diol as a β-damascenone precursor supports the identification of its arabinosyl glucoside as a precursor.

Gel Permeation Size Analysis. The β-damascenone precursors eluted from the large GPC column in the first two-thirds column volume after the void volume. The C_{18} HPLC separation from four regions on the GPC precursor elution curve yielded size information about particular precursors. Figure 5 shows the differing precursor distribution seen for the different regions of the GPC elution. The postulate that the GPC column could separate precursors from each other was proven to be true. Bio-Rad states that Bio-Gel P-2, at best resolution, can separate molecules which differ by at least 10% in size, so it should be able to separate aglycons from monoglycosides from diglycosides. The GPC column indeed separated not only precursors from other larger and smaller substances but different precursors eluted at the beginning, middle, and end of the GPC precursor elution curve. Precursor **6** has

Table 2. Size, Amount, and HPLC Elution Parameters of β-Damascenone Precursors

β-damascenone precursor	% methanol in water C_{18} HPLC elution	MW	amt in apples (ng/g)
1	39.6	diglycoside+	2.2
Z	40.8–42.1	triglycoside+	*a*
2	42.1–43.4	diglycoside+	3.1
3	44.7–46.0	triglycoside+	1.5
4	49.9	diglycoside+	2.0
5	52.4	di- or triglycoside	1.9
6	55.0	MW 502	4.6
7	67.9	aglycon	0.5
8	73.0	aglycon	1.5
X	60.1	aglycon	0.5
Y	65.3	aglycon	0.5
free β-damascenone	88.4	MW 190	<1.0

[a] Contains a small portion of precursor **2**'s 3.1 ng/g.

a molecular weight of 502 and was used as an internal standard for comparative size analysis of the other precursors. Therefore, the GPC fractions I–IV are compared to the known precursor of molecular weight 502 in Figure 5.

The largest size precursors are those from I: precursors **Z**, **3**, and **5** (Table 2). Precursor **Z** was originally not seen because it eluted in the same fraction as precursor **2** on the HPLC. The precursors that eluted between 70 and 80% methanol in water (more nonpolar region) are probably not part of this largest group if we assume that sugar adducts are the cause for larger size. They are more likely hydrolysis products (aglycons or β-damascenone) from the precursors **Z**, **3**, and **5**. These precursors are probably the size of triglycosides because precursor **2** of MW 502, a diglycoside, was not present in I. If not triglycosides, they are at least diglycosides with a different structural arrangement of the sugar groups, different sugars, or a different aglycon than the smaller groups of precursors.

The fraction II precursors from Figure 5 are the next "largest in size" group of precursors. Precursors **1**, **2**, and **4** are seen uniquely in this chromatogram. These precursors appear to be intermediate in weight between precursor **6** and the group from fraction I. They are probably diglycosides with a larger Stokes radius than precursor **6**.

Only one precursor was detected in fraction III (precursor **6**), with a molecular weight of 502, indicating an arabinosyl glucoside of the acetylenic diol. A different position of sugar attachment to the aglycon could account for the different GPC retentions of **1**, **2**, and **4** from **6**.

Lastly, the smallest precursors are seen in IV: **X**, **7**, and **8**. When a synthetic sample of the acetylenic diol was chromatographed on the C_{18} HPLC under the same conditions, it eluted in the fraction **7**. In addition, a diglycoside of the acetylenic diol **b** has been implicated as a precursor from apples. Precursor **7** has polarity similar to that of the acetylenic diol **b** and may be an aglycone. Precursors **X** and **8**, also aglycons, could be the allenic triol or acetylenic triol, both of which have been postulated as natural β-damascenone precursors (Winterhalter, 1992; Sefton et al., 1989). The precursors 1–8 were assigned sizes, as shown in Table 2. Interestingly, no monoglycoside precursors were found in the apple.

The complexity of β-damascenone biogenesis was indicated by the detection of at least eight acid-hydrolyzable precursors. The structural characterization of the eight precursors was consistent with the presence of at least two triglycosides, four diglycosides, and two aglycons. The most abundant precursor was identified as the arabinofuranosyl glucopyranoside of the acetylenic diol **b**. To

increase the amount of precursors available for detailed characterization, a larger scale isolation is being planned.

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