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Two-Dimensional GC-DCCC Analysis of the Glycoconjugates of Monoterpenes, Norisoprenoids, and Shikimate-Derived Metabolites from Riesling Wine

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Two-dimensional mapping of glycoconjugates from Riesling wine was achieved by using droplet countercurrent chromatography to separate the glycosides followed by enzymic hydrolysis and GC–MS to characterize the volatile aglycons. This technique has allowed observation of 27 monoterpenes, 20 shikimate metabolites, and 40 norisoprenoids, with compounds in all classes apparently conjugated with mono- and disaccharides. The presence of conjugates more polar than disaccharides was also evident. New wine aglycons identified in this work include the uroterpenols (*p*-menth-1-ene-8,9-diols), four isomeric 3,4-dihydro-3-hydroxyactinidols, 9-hydroxymegastigma-5,7-dien-4-one, 9-hydroxymegastigm-5-en-4-one, and (tentatively) 4-hydroxy-3-methoxyphenylacetic acid ethyl ester, 2-(4-hydroxy-3-methoxyphenyl)ethyl acetate, and 8,9-dehydrotheaspirone. The relative abundance of norisoprenoid glycosides emphasizes the need to further study these compounds in relation to wine aroma.

Recent studies in these laboratories have shown that volatile secondary metabolites of grapes, including mevalonate- and shikimate-derived compounds, accumulate in these fruits as nonvolatile conjugates (Williams et al., 1989). The work also demonstrated the sensory significance of compounds released from the conjugates and indicated the benefits in analyzing these flavor precursors for determining varietal differences. In an analogous approach to this last aspect, Versini et al. (1988) have also attempted to relate bound monoterpenes and norisoprenoids to varietal and clonal differences among grapes.

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In our study (Williams et al., 1989) and in subsequent research directed specifically at norisoprenoid constituents (Sefton et al., 1989), the need was recognized to determine the structures of both the individual secondary metabolites and the conjugating moieties. All of the grape conjugates identified to date are glycosidic, and in the case of the monoterpenols as well as for benzyl alcohol and 2-phenylethanol, not only β-D-glucopyranosides but also β-rutinosides and 6-O-α-L-arabinofuranosyl-β-D-glucopyranosides are involved (Günata et al., 1988; Salles et al., 1988; Williams et al., 1982b, 1983). At least one other unidentified sugar also appears to be employed in conjugating grape monoterpenes (Günata et al., 1988). It is not known if other flavor precursor compounds in the shikimate or norisoprenoid classes are similarly present as both mono- and disaccharide glycosides.

The occurrence of different glycoconjugates of individual aglycons in the flavor precursor fraction of one other fruit, i.e., Jonathan apples, has been indicated from enzymic rate studies (Schwab and Schreier, 1988b).

Important reasons for fully elucidating the structures of the conjugates include (a) the need to gain an understanding of the dissimilar physical and chemical properties of a particular secondary metabolite when it is substituted by different conjugates and (b) the necessity of investigating the different susceptibilities to enzymic hydrolysis of variously substituted glycoconjugates.

In spite of these well-defined needs, progress in the analysis and structural determination of grape conjugates has been slow. This is because of the heterogeneity of the glycosyl portion and the structural diversity of the aglycons, which together make the isolates containing the conjugates highly complex and intractable.

In most recent analyses of monoterpene glycosides from grapes, both HPLC (Bitteur et al., 1989) and HPLC in conjunction with soft ionization MS (Salles et al., 1988) have been applied with some success. As an alternative approach, the all-liquid separation technique of droplet countercurrent chromatography (DCCC) was found to be valuable for the preparative-scale resolution of grape conjugates (Strauss et al., 1987a). This technique, when used in combination with NMR and MS, allowed complete elucidation of several glycosidic derivatives.

The present work further applies DCCC in this field and expands the number of compounds that are to be found in conjugated form in wine. Also, the DCCC partition data have been used to determine if glycoconjugation involving moieties other than simple monosaccharides is a general feature of these flavor precursors.

EXPERIMENTAL SECTION

General Procedures. All solvents were of high purity at purchase and were redistilled before use. Details of ^1H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy, preparation of C_{18} reversed-phase (C_{18} RP) isolates, and droplet countercurrent chromatography (DCCC) of the isolates were given previously (Strauss et al., 1987a). The wine was a Riesling from the 1988 vintage, and it was dealcoholized by concentration in vacuo at room temperature.

Enzymatic Hydrolysis and Quantification of the Aglycons. The organic solvent was evaporated from the pooled DCCC fractions, and a 1-mL aliquot of the aqueous residue from each group was dissolved in phosphate buffer (pH 5.0, 20 mL). To this were added *n*-octyl β-D-glucopyranoside (Sigma Chemical Co.) as an internal standard (10 μg) and Rohapect C solution (150 mg in 10 mL of water). This solution was incubated for 24 h at 37 °C and then continuously extracted with CH_2Cl_2 for 16 h. The solvent extract was concentrated by distillation through a column of Fenske's helices, and the isolated aglycons were analyzed by GC–MS. The mass of each aglycon in the aliquots taken was determined by comparing the MS ion current for the peak with that given for the *n*-octyl alcohol from the internal standard. Masses in each group of DCCC fractions were then estimated, summed up, and expressed as a total concentration in the wine taken for the experiment.

Gas Chromatography–Mass Spectrometry (GC–MS). For aglycon analyses GC–MS was undertaken with a Finnigan 4021 mass spectrometer coupled to a Varian 3300 gas chromatograph. The chromatograph was equipped with a 30-m J&W DB1701 fused-silica column, 0.25-mm i.d. and 0.25-μm film thickness. Helium was used as the carrier gas at a linear velocity of 40 cm/s. Injections were made with a split injector at 200 °C and a split ratio of 1:10. The column was held at 60 °C for 1 min, programmed at 4 °C/min to 250 °C, and held at this temperature for 20 min. Electron impact mass spectra were taken at 70 eV.

Analysis of peracetylated glycosides was made with a Finnigan TSQ 70 GC–MS equipped with a 30-m J&W DB-5 fused-silica column, 0.25-mm i.d. and 0.25-μm film thickness. The injector temperature was 280 °C, and the column was held at 100 °C for 5 min, programmed at 5 °C/min to 320 °C, and held at that temperature for 20 min. Other conditions were as above.

Authentic Materials. A mixture of the diastereoisomeric *p*-menth-1-ene-8,9-diols (**35**) (Carman et al., 1986) was donated; 4-vinylguaiacol (**15**), 4-vinylphenol (**19**), eugenol (**20**), geranic acid (**23**), tyrosol (**45**), and raspberry ketone (**63**) were commercially available. Diastereoisomeric 3,4-dihydro-3-hydroxyactinidols (**59**, **61**, **67**, **71**) were prepared by lithium aluminum hydride reduction of the isomeric 3,4-dihydro-3-oxoactinidols (**47**, **49**, **51**, **54**) (Strauss et al., 1987b). The 4-oxo compounds **70** and **76** were prepared by the method of Kaiser and Lamparsky (1978) and gave mass and ^1H NMR spectra in close agreement with data published by those authors. ^{13}C NMR spectrum of **70** (22.49 MHz, $CDCl_3$): δ 13.3, 23.6, 27.3, 34.2, 35.5, 37.3, 68.5, 125.0, 129.9, 140.5, 160.7, 199.4. ^{13}C NMR spectrum of **76** (22.49 MHz, $CDCl_3$): δ 11.4, 23.3, 26.8, 34.1, 36.3, 37.3, 37.9, 68.2, 130.7, 164.7, 198.9.

Isolation and Analysis of Peracetylated Glycosides. Combined DCCC fractions 131–160 and combined fractions 221–280, separated from isolates obtained from 54 L of wine, were each acetylated with Ac_2O–pyridine at room temperature. The acetates were further separated by flash chromatography on SiO_2. In this way peracetylated fractions 131–160 yielded products that included 1-methyl-1-(*trans*-5-methyl-*cis*-5-vinyltetrahydrofuran-*r*-2-yl)ethyl 6-O-α-arabinofuranosyl-β-D-glucopyranoside, which gave an identical EIMS to, and was symmetrically peak enhanced by, that of an authentic sample (Strauss et al., 1987b) when the two were co-injected into the GC–MS. Similarly, acetylated DCCC fractions 221–280 gave inter alia 1-methyl-1-(*trans*-5-methyl-*cis*-5-vinyltetrahydrofuran-*r*-2-yl)-ethyl β-D-glucopyranoside (19.4 mg). Signals for the ^1H and ^{13}C NMR spectra of this product were essentially the same as those seen for the corresponding arabinoglucoside without the signals assigned to the arabinose moiety (Strauss et al., 1987b). EIMS: *m/z* (rel intens) 485 (0.5, M − 15), 331 (25), 271 (4), 211 (4), 170 (8), 169 (92), 153 (93), 151 (22), 145 (6), 139 (12), 135 (8), 127 (13), 115 (8), 111 (22), 109 (59), 97 (10), 93 (20), 81 (22), 71 (36), 69 (8), 55 (14), 43 (100).

RESULTS AND DISCUSSION

The use of C_{18} RP for isolation of material from dealcoholized wines has been shown previously to give components that are predominantly conjugated (Williams et al., 1982a). These components were separated by DCCC by employing a solvent system made up from the two phases produced by mixing $CHCl_3$–MeOH–H_2O (7:13:8) with the more dense, less polar layer used as the stationary phase. With this system more polar constituents emerged early, and less polar compounds were found in later, higher numbered fractions.

To facilitate the second step of the analysis, sequential fractions from the DCCC were pooled into eight groups. The organic solvent was removed from each group of fractions, and the residue was hydrolyzed with a nonselec-

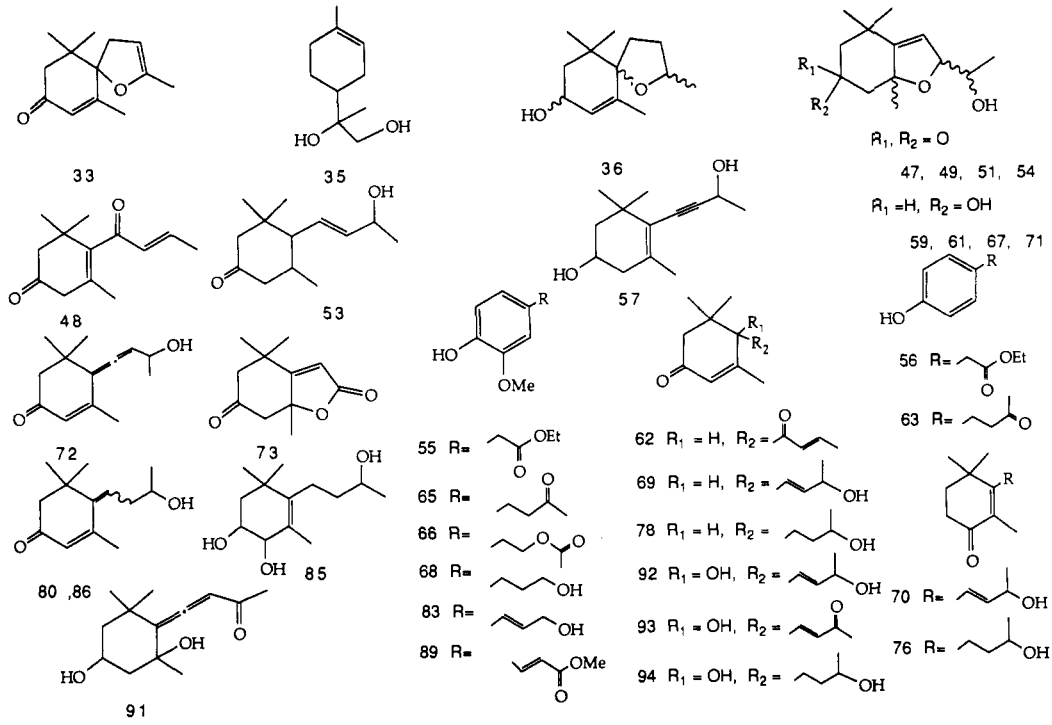

Figure 1. Structures of some compounds referred to in this work.

tive glycosidase, Rohapect C (Aryan et al., 1987). The aglycons obtained from this hydrolysis were then determined by GC–MS.

As an alternative to enzymic hydrolysis, some groups of DCCC fractions were acetylated and the products so formed were examined by GC–MS directly. Data from these investigations will be the subject of a separate study. Importantly, however, from the characteristic fragmentation patterns observed for tetraacetyl glucosides and heptaacetyl disaccharides (Williams et al., 1982b, 1983), it was evident that the compounds present in the DCCC fractions were glycosidic.

Table I shows the observed occurrence of individual aglycons in the eight groups of DCCC fractions. Aglycons are listed in order of increasing retention time on the GC system used in the analysis, and their total concentrations are given in the table as well as their relative proportions found conjugated in the combined fractions. Table I thus represents a plot of the GC partition function for the aglycons against the DCCC partition function of the intact glycosides. Presented in this way the range of glycoconjugates for each volatile aglycon is apparent at a glance.

Also included in Table I is the evidence for compound assignments as well as literature reports of the products as grape or wine conjugates. In those cases where assignments were not made, EIMS data for the unknowns, as well as spectral data for previously unreported grape constituents, are gathered in Table II.

Recognition that both aglycon and glycon structures influence the efficiency of glycosidase action (Aryan et al., 1987; Günata et al., 1988; Hösel and Conn, 1982; Schwab and Schreier, 1988b) made it necessary to determine the extent of liberation of the different aglycons by Rohapect C under the conditions employed here. Obviously, any significant discrimination against an aglycon or one of its glycosides would lead to distorted patterns of occurrence in Table I. Accordingly, a control experiment was undertaken involving acid hydrolysis of the aqueous residue remaining after solvent removal of those aglycons liberated by Rohapect C hydrolysis of a total C_{18} RP isolate from the Riesling wine. Examination of this acid hydrolysate by GC–MS revealed insignificant concentrations of volatile products, indicating that no intact glycosides remained in the aqueous phase after the enzyme treatment. Thus, the distribution map of products seen in Table I was not influenced by the specificity of the enzyme employed.

The identified aglycons in Table I fall into three structural categories, i.e., monoterpenes, norisoprenoid compounds, and shikimate metabolites.

Monoterpenes. In *Vitis vinifera* grapes such as Riesling, monoterpenes are major contributors to varietal aroma (Strauss et al., 1986b). It is unsurprising then that the Riesling wine studied here yielded such a large number of different monoterpenols, which together make up more than 30% of the total concentration of conjugated volatiles found in the sample.

Of the monoterpene aglycons in Table I, diols **26**, **28**, and **35** have not been observed previously as conjugates in grapes. The first two of these have been reported as free compounds of grape must (Rapp et al., 1983; Strauss et al., 1986b), while uroterpenol(*p*-menth-1-ene-8,9-diol) (**35**) has not been recognized as a grape constituent. Uroterpenol (**35**) is a mixture of two diastereoisomers (Carman et al., 1986) that were inseparable by GC. Uroterpenol glycosides could have been formed in this wine during its conservation, by acid-catalyzed cyclization of glycosides of the major monoterpene diols **24** and **27**.

Shikimate-Derived Aglycons. Most of the shikimate-derived products in Table I have been identified in earlier studies on grape conjugated fractions. However, phenols **15**, **19**, and **20** and the tentatively identified **55**, **56**, **66**, and **89** have not been reported as volatile grape aglycons. Both 4-vinylguaiacol (**15**) and 4-vinylphenol (**19**) were studied by Versini (1985) as significant constituents of wines, but not of musts, of the variety Gewürztraminer. These two compounds (**15** and **19**) and eugenol (**20**) were also discussed by Dubois (1983) as volatile phenols of wines. Unfortunately, the facile thermal decarboxylation of ferulic and *p*-coumaric acids to give the volatile phenols **15** and **19**, respectively (Kovacik et al.,

Table I. Distribution of Enzyme-Liberated Aglycons in DCCC Fractions from a Riesling Wine C_{18} RP Isolate

compd. no.	compd	concn,[a] ppb	DCCC fractions[f]								evidence for assgnt[g]	ref[h]	
			60–75	76–90	91–100	101–130	131–160	161–190	191–220	221–280		E	F
1	*trans*-furan linalool oxide	76	+			+	+++	+		+++	A	*i*	*j*
2	*cis*-furan linalool oxide	133	++			+	+++	+		+++	A	*i*	*j*
3	linalool	2						+			A	*i*	*i*
4	benzyl alcohol	38	+	+	+++	+	++	+		+	A	*k*	*l*
5	unknown (2 isomers)	3						+	+				
6	2-phenylethanol	136	+	+	+	+++	+	+++	+	+	A	*k*	*l*
7	pyran linalool oxide, isomer 1	20	+	+		++		+	++		A	*i*	*m*
8	α-terpineol	57			+		+++	++		+	A	*i*	*i*
9	pyran linalool oxide, isomer 2	5	+	+		+		+	++		A	*i*	*m*
10	nerol	8						++	+		A	*i*	*i*
11	geraniol	20						++	++		A	*i*	*i*
12	2,6-dimethylocta-3,7-diene-2,6-diol	75		++	++	++	++			+	A	*i*	*m*
13	2,6-dimethyloct-7-ene-2,6-diol	10		+	++		+				A	*i*	*m*
14	2,6-dimethylocta-1,7-diene-3,6-diol	4		+	+		+				A	*i*	*m*
15	4-vinylguaiacol	176	+++	++	++	+++	+	+	++	++	B		
16	unknown	3				+		++	+				
17	unknown monoterpenoid	1			+			+					
18	*cis*-1,8-terpin	9		++		++					A	*i*	
19	4-vinylphenol	43	+++	+	+	+		+	+	+	B		
20	eugenol	3					+			+	B		
21	*trans*-1,8-terpin	3		+		+					A	*i*	
22	2,6-dimethyloct-7-ene-1,6-diol	10			++	+	+				C	*n*	*n*
23	geranic acid	4						+	+		B		*o*
24	(*Z*)-2,6-dimethylocta-2,7-diene-1,6-diol	36		+	++		++				A	*p*	*p*
25	unknown	3	+	+		+			+	+			
26	3,7-dimethyloctane-1,7-diol	8			++	+	+	+			C	*q*	
27	(*E*)-2,6-dimethylocta-2,7-diene-1,6-diol	214		++	+++	++	+++				A	*p*	*p*
28	(*Z*)-3,7-dimethyloct-2-ene-1,7-diol	2			+						A	*i*	
29	unknown *p*-menthenediol, isomer 1	3			+		+				D		*j*
30	(*E*)-3,7-dimethyloct-2-ene-1,7-diol	66		++	+++	+	++				A	*i*	*j*
31	unknown *p*-menthenediol, isomer 2	9		+		++					D		*j*
32	unknown	18	+	+	++	+	+						
33	8,9-dehydrotheaspirone[b]	trace	+		+	+	+	+	+		C	*r*	
34	vanillin	11	+	+		+	+	+	+	+	A	*s*	*s*
35	*p*-menth-1-ene-8,9-diol	25				++		++	++		B	*t*	
36	3-hydroxytheaspiranes (4 isomers)[b]	24	+	++		++		+	+		A	*u*	*v*
37	unknown *p*-menthenediol, isomer 3	84	++	++		+++					D		*j*
38	methyl vanillate	13					+	++	+		A	*j*	*j*
39	unknown norisoprenoid (MW = 224), isomer 1	12	+	++	+	++	+	++			D		
40	isomer of 2,6-dimethylocta-2,6-diene-1,8-diol	8			++		+				D	*p*	*p*
41	unknown norisoprenoid (MW = 224), isomer 2	13	++	++	+	++	+	++	+		D		
42	unknown norisoprenoid (MW = 210), isomer 1[c]	16								++	D		
43	unknown norisoprenoid (MW = 210), isomer 2[c]	17								++	D		
44	(*E*,*E*)-2,6-dimethylocta-2,6-diene-1,8-diol	46			++		++				A	*p*	*p*
45	tyrosol	35							+++		B		*s*
46	2-(4-hydroxy-3-methoxyphenyl)-ethanol	12		+	+	+				++	D	*w*	*j*
47	3,4-dihydro-3-oxoactinidol, isomer 1[b,d]	119	+	+		+	+	+		+	A	*x*	
48	megastigma-5,8-diene-3,7-dione[d]		++	+		+	+		+	+++	A	*y*	*y*
49	3,4-dihydro-3-oxoactinidol, isomer 2[b]	36	+	+		+	++	++		+	A	*x*	
50	propiovanillone	2	+			+			+		D		*j*
51	3,4-dihydro-3-oxoactinidol, isomer 3[b]	30	+	+		+	++			+	A	*x*	
52	unknown norisoprenoid	33		+	+		++	++			D		
53	9-hydroxymegastigm-7-en-3-one	53					++			+++	A	*y*	
54	3,4-dihydro-3-oxoactinidol, isomer 4[b]	5	+			+	+			+	A	*x*	
55	4-hydroxy-3-methoxyphenylacetic acid ethyl ester	2								+	D		
56	4-hydroxyphenylacetic acid ethyl ester	2							+		C	*z*	
57	megastigm-5-en-7-yne-3,9-diol	4	+	+							A	*y*	*y*
58	unknown norisoprenoid	107		+			++	+++			D		
59	3,4-dihydro-3-hydroxyactinidol, isomer 1[b]	trace					+				B	*aa*	
60	unknown norisoprenoid	46	+	+++		+		+++			D		
61	3,4-dihydro-3-hydroxyactinidol, isomer 2[b]	trace					+				B	*aa*	
62	megastigma-4,8-diene-3,7-dione	82	++	+	+	+	+	+	+	+++	A	*y*	*y*
63	raspberry ketone	14	+				+	++			B		*s*
64	unknown norisoprenoid	66	++	+++	+++	++	+	++			D		

Table I. (Continued)

compd. no.	compd	concn,[a] ppb	DCCC fractions[f] 60–75	76–90	91–100	101–130	131–160	161–190	191–220	221–280	evidence for assgnt[g]	ref[h] E	F
65	zingerone	16							++		A	*j*	*j*
66	2-(4-hydroxy-3-methoxyphenyl)ethyl acetate	15		++		+				+	D		
67	3,4-dihydro-3-hydroxyactinidol, isomer 3[b]	trace					+				B	*aa*	
68	dihydroconiferyl alcohol	38	++	+	++	+++	+		+	+	A	*j*	*j*
69	(*E*)-9-hydroxymegastigma-4,7-dien-3-one	127	+	+	+	+++	+++		+	+++	A	*x*	*x*
70	(*E*)-9-hydroxymegastigma-5,7-dien-4-one	5								++	B	*ab*	
71	3,4-dihydro-3-hydroxyactinidol, isomer 4[b]	trace				+					B	*aa*	
72	9-hydroxymegastigma-4,6,7-trien-3-one	23	++	++	+		++			+	A	*y*	*y*
73	dehydrololiolide	trace				+	+				A	*y*	
74	unknown norisoprenoid	7							+	++	D		
75	unknown	2				+		+	+				
76	9-hydroxymegastigm-5-en-4-one	22					++	+		+	B	*ab*	
77	syringaldehyde	5				+	+				A	*s*	
78	9-hydroxymegastigm-4-en-3-one[d]		++	+		++	+++			+++	A	*y*	*y*
79	unknown norisoprenoid[d]	260	+	+		+				++	D		
80	9-hydroxymegastigma-4,6-dien-3-one, isomer 1[d]					+	+			+	A	*y*	*y*
81	unknown norisoprenoid	10						++	+		D		
82	unknown norisoprenoid	4		+		+				+	D		
83	coniferyl alcohol	31		+	++	++	+			+	A	*j*	*j*
84	unknown	20				++		++	++				
85	megastigm-5-ene-3,4,9-triol	11		+		+		+	++		A	*u*	*u*
86	9-hydroxymegastigma-4,6-dien-3-one, isomer 2	12				+	+			++	A	*y*	*y*
87	unknown isomers	3					+			+			
88	unknown	28				+++		+	++				
89	methyl ferulate	20	++			+					C	*z*	
90	unknown norisoprenoid[e]	148		+	++	+	+++	+++			A		
91	3,5-dihydroxymegastigma-6,7-dien-9-one	26		+		+++					A	*y*	*y*
92	6,9-dihydroxymegastigma-4,7-dien-3-one	67	+	++	+	+++	++				A	*x*	*x*
93	6-hydroxymegastigma-4,7-diene-3,9-dione	28	+	+		+			+	++	A	*y*	*y*
94	6,9-dihydroxymegastigm-4-en-3-one	6		+		+	+				A	*y*	*y*

[a] Concentration in the wine. [b] Megastigmane numbering. [c] This is unknown 8 in Strauss et al. (1986a). [d] Compounds not resolved by GC. [e] This is unknown 17 in Strauss et al. (1987a). [f] Relative proportions were ranked as follows: +, 0.1–2 ppb; ++, 2–20 ppb; +++, 20–100 ppb. [g] Key: A, previously identified in this laboratory; B, symmetrical peak enhancement on coinjection with an authentic sample and comparison of mass spectrum with that of an authentic sample recorded under the same conditions; C, comparison of mass spectrum with a published spectrum; D, tentatively assigned from interpretation of the mass spectrum. [h] Key: E, for assignment of the aglycon; F, for the aglycon occurring in a conjugated form in grapes or wine. [i] Strauss et al., 1986b. [j] Strauss et al., 1987a. [k] Williams et al., 1982a. [l] Williams et al., 1983. [m] Wilson et al., 1984. [n] Versini et al., 1988. [o] Grossmann et al., 1987. [p] Strauss et al., 1988. [q] Rapp et al., 1983. [r] Fujimori et al., 1981. [s] Williams et al., 1989. [t] Carman et al., 1986. [u] Strauss et al., 1986a. [v] Strauss et al., 1984. [w] Güntert et al., 1986. [x] Strauss et al., 1987b. [y] Sefton et al., 1989. [z] Heller and Milne, 1978. [aa] Enzell and Wahlberg, 1986. [ab] Kaiser and Lamparsky, 1978.

1969), raises the possibility that where observed here by GC these two phenols may be artifacts, arising in part, or totally, from the corresponding cinnamic acids present in the DCCC fractions.

4-Hydroxyphenylacetic acid ethyl ester (**56**) was reported as a wine constituent by Güntert et al. (1986), who also observed **89** and several compounds related to **55** in a GC study of free volatile phenols in a California Riesling. Nevertheless, compounds **55**, **56**, and **89** are recorded here for the first time as phenols present in a conjugated form in wine.

Norisoprenoid Aglycons. The norisoprenoids of grapes have been the subject of a recent investigation (Sefton et al., 1989), and many of the compounds in Table I were discussed in that report. Five members in that category, however, are worthy of further comment, i.e., the diastereoisomers **47**, **49**, **51**, and **54** and the lactone **73**. The four isomeric 3,4-dihydro-3-oxoactinidols (**47**, **49**, **51**, **54**) were observed in earlier studies in these laboratories as enzyme-liberated products from Gewürztraminer juice (Strauss et al., 1987b), and they have now been similarly generated from the C_{18} RP isolate from Riesling wine. These four isomers were not recorded among the aglycons from Sauvignon Blanc, Semillon, or Chardonnay juices, although they were found in free forms or as mild acid generated products from these nonfloral varieties (Sefton et al., 1989). The lactone **73** presumably arises by oxidative degradation of the 3,4-dihydro-3-oxoactinidols (Uegaki et al., 1979).

Two other norisoprenoid structures not recorded in the study of the three nonfloral varieties (Sefton et al., 1989), i.e., isomers **36** and triol **85**, are confirmed as aglycons in the Riesling wine. The role of **36** and **85** in the hydrolytic generation of the grape volatile vitispirane has been discussed, as has their earlier observation in grapes (Strauss et al., 1984, 1986a).

Seven norisoprenoids in Table I are new to grapes, i.e., isomers **59**, **61**, **67**, and **71** as well as ketones **33**, **70**, and **76**. The bicyclic skeleton of the first quartet of diastereoisomers is presumably formed in the same way as that of the 3-oxo analogues **47**, **49**, **51**, and **54**. The tentatively assigned ketone **33** may be formed from 6-hydroxymegastigm-4-ene-3,9-dione as found by Fujimori et al. (1981), although the latter compound was not found among the aglycons. Oxidation of diol **94** with Jones reagent gave a mixture of products, the major compo-

Table II. Mass Spectral Data

compd	EIMS at 70 eV, m/z (rel intens)
5, isomer 1	180 (53), 165 (100), 124 (70), 109 (19), 95 (90), 81 (32), 69 (43), 55 (47), 43 (74)
5, isomer 2	180 (10), 165 (92), 124 (9), 109 (100), 96 (16), 81 (66), 68 (80), 55 (87), 43 (32)
16	226 (1), 211 (5), 179 (1), 158 (4), 143 (4), 131 (100), 121 (13), 105 (10), 94 (32), 75 (20), 43 (13)
17	121 (5), 111 (3), 98 (10), 89 (10), 83 (16), 71 (100), 56 (16), 43 (62)
25	182 (18), 167 (100), 151 (34), 135 (6), 119 (7), 107 (5), 91 (13), 77 (8), 65 (5), 43 (10)
32	166 (3), 151 (5), 148 (3), 138 (8), 121 (20), 117 (23), 97 (24), 71 (100), 68 (23), 55 (36), 43 (83)
33	206 (27, M^+), 191 (7), 173 (3), 150 (33), 136 (23), 121 (21), 108 (93), 93 (47), 77 (24), 53 (16), 43 (100)
35	152 (20, $M - H_2O$), 139 (25), 121 (55), 105 (6), 95 (33), 94 (25), 93 (23), 75 (29), 71 (35), 57 (25), 43 (100)
39, 41	224 (12), 209 (5), 196 (2), 182 (3), 168 (20), 140 (35), 126 (100), 111 (14), 98 (43), 83 (28), 55 (28), 43 (30)
52	194 (3), 179 (4), 153 (2), 145 (3), 136 (7), 123 (9), 120 (8), 112 (12), 97 (21), 95 (20), 85 (20), 68 (15), 55 (17), 43 (100)
55	210 (19, M^+), 138 (6), 137 (100), 122 (7), 94 (4), 66 (3), 51 (3), 39 (4)
56	180 (10, M^+), 108 (7), 107 (100), 79 (3), 77 (14), 51 (6)
58	208 (4), 193 (3), 190 (20), 175 (12), 151 (21), 135 (100), 123 (15), 107 (41), 93 (30), 79 (21), 67 (13), 55 (10), 43 (83)
59, 61, 67, 71	181 (90, M - 45), 163 (23), 125 (100), 107 (8), 95 (30), 83 (29), 57 (69), 43 (48)
60	196 (1), 181 (7), 178 (1), 163 (9), 155 (6), 137 (36), 123 (28), 96 (29), 85 (100), 81 (50), 69 (38), 55 (53), 41 (56)
64	210 (18), 195 (3), 192 (1), 177 (17), 153 (64), 135 (100), 121 (57), 107 (65), 93 (70), 81 (48), 67 (48), 55 (50), 41 (81)
66	210 (23, M^+), 179 (3), 150 (25), 137 (100), 122 (6), 107 (26), 91 (10), 77 (18), 65 (8), 43 (21)
70	208 (3, M^+), 193 (20), 175 (3), 165 (100), 147 (8), 137 (37), 123 (23), 109 (13), 91 (21), 77 (15), 55 (26), 43 (57)
74	208 (4), 193 (3), 166 (82), 153 (38), 137 (29), 125 (76), 109 (28), 95 (37), 82 (29), 55 (49), 43 (100)
75	280 (8), 209 (10), 181 (100), 129 (27), 91 (14), 75 (42), 59 (14), 43 (34)
76	210 (3, M^+), 195 (7), 177 (5), 165 (12), 152 (27), 137 (38), 121 (23), 109 (50), 93 (27), 79 (30), 67 (33), 55 (56), 43 (100)
79	206 (2), 191 (14), 173 (8), 163 (58), 145 (13), 135 (57), 121 (38), 105 (37), 91 (44), 77 (30), 67 (25), 43 (100)
81	190 (2), 175 (4), 152 (78), 137 (42), 121 (23), 109 (34), 95 (22), 79 (14), 55 (17), 43 (100)
82	208 (49), 193 (7), 175 (10), 161 (5), 147 (13), 137 (25), 135 (100), 121 (30), 105 (37), 91 (50), 79 (23), 55 (21), 43 (48)
84	222 (5), 204 (6), 194 (2), 189 (4), 179 (4), 164 (87), 151 (17), 136 (37), 121 (33), 107 (8), 91 (18), 77 (20), 65 (10), 55 (16), 43 (100)
87	222 (10), 207 (3), 138 (31), 123 (100), 95 (23)
88	224 (3), 206 (25), 191 (3), 181 (26), 163 (6), 152 (18), 137 (30), 122 (43), 109 (47), 91 (13), 79 (27), 67 (18), 55 (18), 43 (100)
89	208 (100, M^+), 193 (5), 177 (70), 150 (12), 145 (38), 133 (16), 117 (19), 105 (8), 89 (26), 77 (15), 51 (13)

nent of which had a GC retention time and mass spectrum identical with those given by the aglycon assigned as **33**.

Ketones **70** and **76** are the first megastigmanes with a carbonyl function at position 4 to be recorded in grapes. Such compounds have been found recently in quince fruit (Winterhalter and Schreier, 1988) and earlier from *Osmanthus* absolute (Kaiser and Lamparsky, 1978).

Lastly from the MS data in Table II for the unknown constituents, at least 13 of these appear to be norisoprenoid compounds, i.e., **39**, **41–43**, **52**, **58**, **60**, **64**, **74**, **79**, **81**, **82**, and **90**. Thus the norisoprenoids are the most numerous, and with a total concentration of over 1300 ppb, they are also the most abundant volatile aglycons in this Riesling wine.

Distribution of Aglycons. A few individual compounds in the three structural categories were found in a single group of DCCC fractions or in a pair of consecutive groups. However, the majority of aglycons in all categories were found in two or more nonconsecutive groups of fractions. This confirms that most of the compounds, irrespective of their biosynthetic origin, exist in more than one conjugated form and that these different conjugates have different mobilities through the DCCC system.

Comparison of compositional data obtained in the previous DCCC study (Strauss et al., 1987a) with that from the present work permits an assignment of some of the separated conjugates observed here. Thus, for example, it was demonstrated that the 6-*O*-α-arabinofuranosyl-β-D-glucopyranoside of *cis*-furan linalool oxide (**2**) was a significant monoterpene component of Riesling. This compound has again been confirmed in fractions 131–160 in the present study, while the β-D-glucopyranoside of the same monoterpene **2** was found in fractions 221–280. Accordingly, substitution of a glucosyl moiety with a second carbohydrate residue accounts in part for the separation of the conjugates of this particular monoterpene. It is reasonable to assume from the wide distribution of most compounds recorded in Table I that in addition to the monoterpenoids, the majority of the shikimate-derived metabolites, and norisoprenoids, are similarly present as glucosides and as disaccharide conjugates.

Nevertheless, other highly polar conjugates must also exist. This is demonstrated again in the case of monoterpene oxide **2**, where a mobile conjugate was found in fractions 60–75. A recent report (Schwab and Schreier, 1988a) has established the occurrence in papaya fruit of four isomeric malonated benzyl β-D-glucosides. If such malonyl substitution of glucose is involved in these *V. vinifera* conjugates, it would contribute to the large number of polar derivatives that were observed. Additionally, there are increasing reports of glycosides of plant secondary metabolites in which the sugar moiety is esterified by a cinnamic acid (Shimomura et al., 1987) or a monoterpene acid (Gering-Ward, 1989). Such mixed conjugation could be the case here and so account for the finding of geranic acid (**23**) or the ubiquitous occurrence of the vinyl phenols **15** and **19** in the DCCC fractions, the latter pair arising by decarboxylation of ferulic and *p*-coumaric acids as discussed above.

Finally, some of the aglycons have more than one hydroxyl function, thus presenting opportunities for polyglycosylation which could also contribute to the separations observed.

CONCLUSION

This study has employed a two-dimensional analytical approach to examine the range of bound volatile constituents present in wine. In the first step DCCC was used to resolve the conjugates into groups of different polarities. Aglycons were then released from the conjugates in the various DCCC fractions by using a nonselective glycosidase enzyme. Lastly, the volatile aglycons were analyzed by GC–MS and the compounds so determined were then plotted against the DCCC separations, thus allowing individual glycoconjugates to be mapped.

When applied to a Riesling wine this approach revealed more than 90 aglycons, conjugated with glycosidic and apparently substituted glycosidic moieties. Of the agly-

cons 27 were monoterpenes, 20 were shikimate-derived metabolites, and 40 were norisoprenoids.

In early studies on grape precursor fractions, norisoprenoids were recognized as significant components (Williams et al., 1982a), and the abundance and diversity of these compounds present in wine are clearly apparent from this present work. Although Riesling is a floral variety in which monoterpenes are major volatile aroma compounds (Strauss et al., 1986b), volatile norisoprenoids were found in the conjugated fraction at a concentration 40% higher than that of the monoterpenes. The well-recognized aroma properties of volatile norisoprenoids (Ohloff, 1978) mean that greater attention should be directed at this group of compounds, and the full extent of their contribution to grape and wine aroma should be investigated.

Further application of this technique of conjugate mapping will permit complete identification of individual glycosides. This process can be expedited by applying EIMS to the intact conjugates after derivatisation or by use of soft-ionization MS on the DCCC fractions directly. Research in this area is in progress.

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Comparison of Volatile Flavor Components in Fresh and Processed Orange Juices

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Fresh juices from Hamlin, Pineapple, and Valencia oranges and different commercial brands of processed orange juices were analyzed for volatile flavor components by a headspace analysis technique. Twenty components including eight alcohols, four aldehydes, three esters, and five hydrocarbons were identified and quantified. Unpasteurized and pasteurized single-strength juices not made from concentrate did not show marked changes in the profile of flavor components when compared to fresh juice. In contrast, pasteurized reconstituted juices from concentrate showed decreases in acetaldehyde, methyl acetate, methyl butyrate, and ethyl butyrate with increases in decanal, octanal, and linalool. Aseptically packaged single-strength juice, canned juice, and a 10% juice drink exhibited increased α-terpineol. Canned juice and the 10% juice drink also exhibited low levels of ethyl butyrate, acetaldehyde, hexanal, and limonene and total disappearance of ethyl acetate. This procedure has potential for routine monitoring of quality of processed citrus products.

The delicate fresh flavor of orange juice is easily changed by heat treatment during processing or by storage (Shaw, 1986). The juice undergoes compositional changes that invariably cause an alteration in the original flavor and aroma of the fresh juice. In order for processors to better understand the changes that take place during processing and storage of orange juices, quantitative information on the important volatile flavor components present in both fresh and processed orange juices is needed. Such information will enable processors to alter processing conditions and amounts of volatile flavor fractions added to produce products with flavor profiles more closely resembling those in fresh juice than is currently possible.

Of the volatile components important to flavor, esters and aldehydes are the primary contributors to fresh orange flavor (Bruemmer, 1975), although other components could also be important (Shaw, 1977). Other factors that influence the flavor are correct proportions of the different compounds (Shaw, 1979), taste threshold values of volatiles (Patton and Josephson, 1957), synergistic effects between volatiles (Shaw and Wilson, 1980), and the interaction of nonvolatile with volatile flavor components (Ahmed et al., 1978b).

Only recently have analytical methods become available to accurately quantify trace volatile constituents in citrus juices. Schreier (1981) quantified 29 volatile constituents of one fresh orange juice and showed quantitative changes in some constituents as well as the appearance of two new constituents after heat treatment of the juice. Solvent extraction and column chromatography were necessary steps prior to gas chromatographic (GC) analysis. Moshonas and Shaw (1987) quantified 24 volatile components of one fresh sample each of Valencia and Temple orange juices that had been distilled prior to GC analysis. Marsili (1986) used headspace analysis of a diluted orange juice sample to quantify nine volatile components of one processed juice sample. Rodriguez and Culbertson (1983) quantified eight volatile components of one fresh, one freeze-concentrated, and one heat-concentrated orange juice sample by GC analysis using a radioactive detector. Because only a single fresh or processed juice sample was analyzed in each of the above cases, none of those studies showed a range of quantitative values for individual flavor components present in either fresh or processed juices. Such a range of values determined on a variety of juice samples is needed to assess effects of quantitative changes due to processing on the loss of fresh flavor quality.

In the current report, quantitative values for 20 volatile flavor components of 15 fresh orange juices and 14 juices from major types of processed orange juice products were compared. By determing quantitative values for volatile components from several juice samples, we now have a better perspective on the quantities present in both fresh and processed juices.

MATERIALS AND METHODS

Juice Samples. Fresh juice samples were hand-extracted from Pineapple, Hamlin, and Valencia oranges (*Citrus sinensis* (L.) Osbeck) with use of a domestic mixer fitted with a reamer.