Application of Droplet Countercurrent Chromatography to the Analysis of Conjugated Forms of Terpenoids, Phenols, and Other Constituents of Grape Juice

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Minor polar constituents isolated from juices of the *Vitis vinifera*, cv. Rhine Riesling, Muscat of Alexandria, and Chardonnay, were separated by droplet countercurrent chromatography (DCCC). Analyses were made of compounds in the DCCC fractions by GC–MS after enzymic hydrolysis and by NMR after acetylation and further liquid chromatography. These procedures have allowed identification of conjugated forms, including glycosides, of monoterpenoids, C_{13} norisoprenoids, phenols, and other compounds. Among the grape constituents identified were (*E*)-6-hydroxy-2,6-dimethylocta-2,7-dien-1-yl-β-D-glucopyranoside and the corresponding 6-*O*-α-arabinofuranosyl-β-D-glucopyranoside, 1-methyl-1-(*trans*-5′-methyl-*cis*-5′-vinyltetrahydrofuran-*r*-2′-yl)ethyl-6-*O*-α-arabinofuranosyl-β-D-glucopyranoside, astilbin, quercetin-3-*O*-β-glucopyranuronoside and roseoside. Additionally, conjugates of (*E*)-2,6-dimethylocta-3,7-diene-2,6-diol, (*E*)-2,6-dimethyloct-7-ene-2,6-diol, (*E*)-9-hydroxymegastigma-4,7-dien-3-one, (*E*)-3-hydroxymegastigma-5,8-dien-7-one, zingerone, 4-(4-hydroxy-3-methoxyphenyl)butan-2-ol, and 3-(4-hydroxy-3-methoxyphenyl)propan-1-ol, 2-(4-hydroxy-3-methoxyphenyl)ethanol, methyl vanillate, cinnamyl alcohol, and 3-phenylpropan-1-ol were found. The work demonstrates that DCCC will facilitate separation and analysis of polar, nonvolatile constituents of grapes, including precursors of volatile flavorants.

In earlier research into grape and wine flavor, precursors of volatile monoterpenes and volatile C_{13} norisoprenoids were recognized (Williams et al., 1982a). This, and later work (Strauss et al., 1984, 1986a, 1986b), has stimulated an interest in the role of flavor precursors in other fruits (Engel and Tressl, 1983; Heidlas et al., 1984), fruit products (Chen et al., 1986), and spices (Nitz et al., 1985). However, these precursor compounds are usually present in fruit juices or plant tissues as highly complex mixtures of polar substances, often conjugated as glycosides, and as such they are water soluble and difficult to isolate and separate. This problem has been overcome, in part, by selective retention of the precursors on C_{18} reversed-phase (C_{18} RP) adsorbent (Williams et al., 1982a) or on Amberlite XAD-2 resin (Gunata et al., 1985). Such procedures have allowed isolation, partial separation, and characterization of monoterpene glycosides from grapes (Williams et al., 1982b). However, the need to further separate individual constituents, particularly minor components, from the precursor concentrates still presents a formidable problem.

The development of all-liquid chromatographic systems, particularly droplet countercurrent chromatography (DCCC) (Tanimura et al., 1970), for the preparative-scale separation of mixtures of highly polar constituents (Hostettmann et al., 1984) appeared to offer a solution. This paper reports results of a study in which DCCC has been applied to precursor concentrates from juices of three *Vitis vinifera* grape varieties.

EXPERIMENTAL SECTION

General Procedures. All solvents were of analytical grade at purchase and were redistilled before use. GC–MS was carried out on a wide-bore (0.32-mm i.d.) fused silica column 25 m in length, with a 0.5-μm film thickness bonded phase of 7% cyanopropyl, 7% phenylmethyl siloxane (supplied as a BP10 column from SGE, Melbourne). The column was operated isothermally at 100 °C for 1 min, then programmed to 260 °C at 4 °C/min, and held at the upper temperature.

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Preparation of Isolates from Grape Juice. In a typical procedure, grape juice (in batches of ca. 1 L), which had been stored at −20 °C until required, was thawed, filtered, and passed through a column (35 × 1.5 cm (i.d.)) packed with C_{18} RP adsorbent. The column was washed with water (ca. 100 mL) and eluted with MeOH (ca. 50 mL). Eluates from several runs were pooled and concentrated to dryness in vacuo. The concentrate from Chardonnay juice (8 L) was subjected to DCCC without further treatment. The C_{18} RP isolate from Muscat of Alexandria juice (14 L) was given light treatment with polyvinylpolypyrrolidone (PVPP). The concentrate in water (20 mL) was stirred for 1 h at room temperature with PVPP (1 g), filtered, and evaporated in vacuo. For treatment of the Rhine Riesling C_{18} RP isolate, a column of PVPP (3 g; 6 × 1.5 cm (i.d.)) was prepared and washed with H_2O, MeOH, and H_2O. The concentrate from Rhine Riesling juice (11 L) in water (20 mL) was passed through the column under a slight positive pressure of N_2. The PVPP resin in the column was washed with water to remove material occluded (but not adsorbed), and the aqueous solutions were pooled and evaporated to dryness in vacuo.

DCCC of Grape Concentrates. An Eyela Model DCC-300 (Tokyo Rikakikai Co., Ltd.) unit, equipped with 300 tubes (400 × 3.4 mm (i.d.)) was used. The instrument was operated at 28 °C in the ascending mode, with the upper (more polar) layer from a mixture of $CHCl_3$–MeOH–H_2O (7:13:8) utilized as the mobile phase and the lower layer as the stationary phase (Hostettmann et al., 1984). The grape concentrate was dissolved in a 1:1 mixture of the two phases (20 mL) and applied to the DCCC via a 20-mL sample loop. Fractions were taken consecutively over intervals of 12–15 min at a flow rate of 12–20 mL/h. Progress of the separation was monitored by TLC (Si gel 60) with the DCCC stationary phase as developing solvent.

Enzymic Hydrolysis of DCCC Fractions. Enzymic hydrolyses were performed by incubation of solvent-free DCCC fractions, dissolved in phosphate buffer (pH 5.0) using the commercial pectinase Rohapect C as previously described (Wilson et al., 1984). Liberated aglycons were solvent extracted and analyzed by GC–MS.

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Table I. Conjugates Isolated by DCCC from Grape Juices

compound	evidence for assgnt[a]	ref[b] F	G	H	I	grape var[c]	DCCC fraction
Monoterpenes							
(*E*)-6-hydroxy-2,6-dimethylocta-2,7-dien-1-yl-β-D-glucopyranoside (**1a**)	A–D	*d*	*d*	*e*	*e, f*	RR	112
(*E*)-6-hydroxy-2,6-dimethylocta-2,7-dien-1-yl-6-*O*-α-arabinofuranosyl-β-D-glucopyranoside (**1b**)	A–D	*e*	*d*	*e*	*e, f*	RR	65
1-methyl-1-(*trans*-5′-methyl-*cis*-5′-vinyltetrahydrofuran-*r*-2′-yl)ethyl-6-*O*-α-arabinofuranosyl-β-D-glucopyranoside (**2b**)	A–D	*g*	*h*	*i*	*h*	RR	162
conjugate of (*E*)-2,6-dimethylocta-3,7-diene-2,6-diol (**3**)	D		*j*	*k*	*j*	RR	112
glycoside of linalool (**4**)	A, B, D		*h*	*l*	*h*	M	179
conjugate of (*E*)-3,7-dimethyloct-2-ene-1,7-diol (**5**)	D		*g*		*h*	RR	65
conjugate of α-terpineol (**6**)	D		*h*	*m*	*h*	RR	161
conjugate of 2,6-dimethyloct-7-ene-2,6-diol (**7**)	D		*j*	*m*	*j*	RR	65
conjugate of geraniol (**8**)	D		*h*	*m*	*h*	RR	>200
conjugate of nerol (**9**)	D		*h*	*m*	*h*	RR	>200
conjugate of a *p*-menthenediol (isomer 1) (**10**)	E					RR	85
conjugate of a *p*-menthenediol (isomer 2) (**10**)	E					RR	112
C_{13} Norisoprenoids							
roseoside (**11a**)	A–D	*n*	*o*	*o*	*o*	RR	112
conjugate of (*E*)-9-hydroxymegastigma-4,7-dien-3-one (**12c**)	D		*o*	*o*	*o*	RR	112
						Ch	120
conjugate of (*E*)-3-hydroxymegastigma-5,8-dien-7-one (**13**)	D		*p*		*q*	RR	40
conjugate of megastigma-4,8-diene-3,7-dione (**14**)	E		*p*			RR	40
conjugate of megastigma-5,8-diene-3,7-dione (**15**)	E					RR	40
conjugate of unknown (**16**)						RR	112
conjugate of unknown (**17**)						RR	161
Phenolics							
astilbin (**18d**)	A, B	*r*		*r*		Ch	138
quercetin-3-*O*-β-glucopyranuronoside (**19e**)	A, B	*s, t*		*u*		M	63
3-*O*-glycoside of kaempferol (**20**)	A	*s*		*v*		M	100
conjugate of 2-(4-hydroxy-3-methoxyphenyl)ethanol (**21**)	E				*w*	RR	65
conjugate of methyl vanillate (**22**)	D		*x*		*w*	RR	>200
conjugate of propiovanillone (**23**)	E				*y*	RR	>200
conjugate of zingerone (**24**)	D		*x*			RR	>200
conjugate of 4-(4-hydroxy-3-methoxyphenyl)butan-2-ol (**25**)	D		*g*			RR	112
conjugate of 3-(4-hydroxy-3-methoxyphenyl)propan-1-ol (**26**)	D		*g*		*q*	Ch	138
conjugate of a dimethoxyphenol (**27**)	E		*x*			RR	65
Miscellaneous							
conjugate of benzyl alcohol (**28**)	D		*m*	*z*	*m*	RR	53
conjugate of 2-phenylethanol (**29**)	D		*m*	*z*	*m*	RR	85
						Ch	138
conjugate of 1-hexanol (**30**)	D		*m*	*m*	*m*	RR	>200
conjugate of cinnamyl alcohol (**31**)	D		*g*			RR	>200
conjugate of 3-phenylpropan-1-ol (**32**)	D		*g*			RR	112
glucose derivative of indole-3-lactic acid (**33**)	A–C, E					RR	63
conjugate of unknown (**34**)						RR	53
conjugate of unknown (**35**)						RR	162

[a] Structure assigned from the following data: A, ^{1}H NMR; B, ^{13}C NMR; C, MS on acetate derivative; D, product given by enzyme treatment identified by GC–MS and comparison with an authentic reference compound or data previously obtained in this laboratory; E, product of enzymic hydrolysis tentatively identified by interpretation of MS. [b] Key: F, for assignment of the conjugate; G, for assignment of the compound given by enzymic hydrolysis; H, for this or a related conjugate as a grape or wine product; I, for the compound given by enzymic hydrolysis, as a grape or wine product. [c] Key: Ch, Chardonnay; M, Muscat of Alexandria; RR, Rhine Riesling. [d] Tschesche et al., 1977. [e] Strauss et al., 1987b. [f] Rapp et al., 1986. [g] Established in this work. [h] For an appropriate reference see the review of Strauss et al. (1986a). [i] Strauss et al., 1984. [j] Williams et al., 1980. [k] Wilson et al., 1984. [l] Williams et al., 1982b. [m] Williams et al., 1982a. [n] Bhakuni et al., 1974. [o] Strauss et al., 1987a. [p] Ohloff et al., 1973. [q] Observed in this laboratory in acid-hydrolyzed isolates of grape juice. [r] Trousdale and Singleton, 1983. [s] Mabry et al., 1970. [t] Tsuchihashi et al., 1981. [u] Ribéreau-Gayon, 1964. [v] Egger et al., 1976. [w] Güntert et al., 1986. [x] Heller and Milne, 1978. [y] Etievant, 1981. [z] Williams et al., 1983.

Isolation of Compounds from DCCC Fractions. Selected fractions were pooled, and the solvent was evaporated in vacuo. Where appropriate, further separation of components was achieved directly by flash chromatography on SiO_2 (Still et al., 1978) using the DCCC stationary phase as eluting solvent. When derivatization of DCCC fractions was required to permit separation, acetylation of the solvent-free fractions was carried out at room temperature with Ac_2O–pyridine. The acetates, after workup, were isolated by flash chromatography, with Et_2O–pentane-based solvent systems. Pure compounds were subjected to EIMS, ^{1}H NMR, and ^{13}C NMR analyses.

Authentic Materials. Compounds **1c** (Hase et al., 1982), **5** (Mousseron-Canet et al., 1964), and **12c** (Aasen et al., 1973) were prepared by the methods cited. Megastigmadienones **11c** and **13** were donated to us. Zingerone (**24**) was commercially available (Aldrich), and $NaBH_4$ reduction of this afforded the alcohol **25**. Coniferyl alcohol and dihydroconiferyl alcohol (**26**) were prepared by $LiAlH_4$ reduction of ferulic and 3-(4-hydroxy-3-methoxyphenyl)propanoic acids, respectively. 3-Phenylpropan-1-ol (**32**) was obtained by hydrogenation of cinnamyl alcohol (**31**) (10% Pd on carbon catalyst). Methylation of excess vanillic acid with diazomethane gave ester **22**. Other reference materials utilized according to Table I were available either through commercial means or through the references cited in the table.

Spectral Data for Isolated Compounds. Mass

Table II. Mass Spectral Data

compound	EIMS at 70 eV, m/z (rel intens)
(*E*)-2,6-dimethylocta-2,7-diene-1,6-diol (**1c**)	152 (0.2), 137 (2), 119 (3), 110 (3), 109 (2), 96 (4), 93 (5), 79 (7), 71 (34), 68 (14), 67 (28), 55 (26), 53 (10), 43 (100), 41 (43)
(*E*)-3,7-dimethyloct-2-ene-1,7-diol (**5**)	136 (3), 121 (4), 93 (5), 83 (20), 81 (21), 70 (20), 69 (42), 68 (30), 59 (58), 55 (22), 43 (100)
unknown *p*-menthenediol[a] (isomer 1) (**10**)	137 (2), 109 (20), 94 (28), 79 (45), 59 (82), 43 (100)
unknown *p*-menthenediol[a] (isomer 2) (**10**)	152 (3), 121 (3), 109 (10), 94 (8), 93 (7), 91 (8), 79 (77), 59 (100), 43 (72)
vomifoliol (**11c**)	168 (3), 150 (5), 135 (5), 125 (10), 124 (100), 121 (8), 111 (6), 79 (13), 55 (12), 45 (18), 43 (50), 41 (13)
(*E*)-9-hydroxymegastigma-4,7-dien-3-one (**12c**)	208 (1), 152 (13), 134 (8), 109 (23), 108 (100), 95 (11), 91 (15), 79 (9), 45 (23), 43 (32)
(*E*)-3-hydroxymegastigma-5,8-dien-7-one (**13**)	208 (17), 193 (18), 175 (29), 149 (10), 121 (40), 105 (14), 91 (11), 69 (100), 55 (14), 43 (15), 41 (57)
megastigma-4,8-diene-3,7-dione[a] (**14**)	206 (0.5), 191 (1), 138 (8), 123 (12), 79 (5), 77 (3), 69 (100), 41 (42)
megastigma-5,8-diene-3,7-dione[a] (**15**)	206 (12), 191 (13), 163 (10), 149 (26), 121 (29), 107 (18), 105 (20), 77 (22), 69 (95), 55 (20), 53 (18), 41 (100)
C_{13} norisoprenoid unknown[a] (**16**)	209 (2), 191 (1), 163 (3), 123 (5), 121 (3), 109 (3), 77 (2), 43 (100)
C_{13} norisoprenoid unknown[a] (**17**)	208 (1), 165 (2), 141 (7), 128 (11), 124 (13), 109 (16), 85 (72), 71 (68), 55 (25), 43 (100), 41 (60)
2-(4-hydroxy-3-methoxyphenyl)ethanol[a] (**21**)	168 (12), 137 (100), 122 (5), 94 (5), 77 (5), 65 (3), 51 (6)
methyl vanillate (**22**)	182 (48), 167 (3), 151 (100), 123 (15), 108 (8), 79 (7), 65 (12), 52 (30), 51 (28)
propiovanillone[a] (**23**)	180 (18), 151 (100), 123 (23), 108 (12), 77 (13), 65 (23), 52 (40), 41 (37)
zingerone (**24**)	194 (19), 151 (10), 137 (75), 119 (13), 91 (25), 77 (25), 65 (11), 51 (15), 43 (100)
4-(4-hydroxy-3-methoxyphenyl)butan-2-ol (**25**)	196 (33), 178 (2), 163 (4), 138 (45), 137 (100), 131 (30), 123 (10), 106 (9), 91 (25), 77 (15), 65 (12), 51 (13), 45 (48)
coniferyl alcohol	180 (43), 152 (5), 138 (10), 137 (100), 124 (80), 122 (22), 109 (18), 94 (20), 91 (35), 78 (21), 77 (43), 65 (31), 55 (27), 53 (30), 52 (25), 51 (45)
3-(4-hydroxy-3-methoxyphenyl)propan-1-ol (**26**)	182 (32), 138 (37), 137 (100), 122 (14), 94 (11), 91 (16), 77 (23), 65 (13), 55 (12), 51 (17)
dimethoxyphenol[a] (**27**)	154 (85), 139 (72), 138 (25), 111 (100), 93 (35), 69 (32), 65 (38), 56 (27), 55 (33)
cinnamyl alcohol (**31**)	134 (38), 115 (5), 105 (15), 92 (100), 91 (77), 78 (45), 77 (25), 55 (5), 51 (10)
3-phenylpropan-1-ol (**32**)	136 (27), 118 (68), 117 (100), 105 (15), 103 (18), 92 (59), 91 (93), 78 (25), 77 (20), 65 (30), 51 (26)
unknown (**34**)	137 (8), 123 (5), 95 (6), 85 (57), 81 (20), 71 (13), 69 (15), 57 (13), 55 (28), 45 (100), 43 (38), 41 (43)
unknown (**35**)	184 (62), 169 (100), 141 (55), 126 (16), 111 (28), 69 (53), 55 (13)

[a] Tentative assignment only.

spectral data for selected compounds are presented in Table II. The 6-*O*-α-arabinofuranosyl-β-D-glucopyranoside **2b** of the *cis*-furan linalool oxide was isolated and purified as the hexa-*O*-acetyl derivative, which had the following spectral properties: EIMS (70 eV, probe) m/z (rel intens) 547 (0.2), 428 (0.1), 331 (0.9), 259 (52), 169 (9), 157 (8), 154 (16), 153 (61), 152 (15), 139 (65), 136 (17), 135 (17), 129 (17), 127 (12), 111 (23), 109 (28), 97 (32), 95 (10), 93 (27), 85 (20), 81 (43), 71 (54), 69 (30), 68 (14), 67 (13), 55 (36), 43 (100), 41 (34); ^{13}C NMR spectrum (22.49 MHz, $CDCl_3$) δ 20.6 (acetate methyls), 21.8, 24.1, and 25.4 (monoterpenyl methyls), 26.8 ($CH_2CH(i\text{-}Pr)O$), 37.4 ($CH_2C(Me,vinyl)O$), 63.0 (arabinosyl C5 i.e. A5), 66.8 (glucosyl C6 i.e., G6), 69.3 (G4), 71.6 (G2), 72.4 (G3), 72.8 (Me_2CO), 73.2 (G5), 76.1 (A3), 79.6 (A2), 82.9 ($CH_2{=}CHC(Me)O$), 83.9 (A4), 84.1 ($CH_2CH(i\text{-}Pr)O$), 95.3 (G1), 105.9 (A1), 111.2 ($CH_2{=}CH$), 144.3 ($CH_2{=}CH$), 169.0, 169.2, 169.5, 169.7, 170.2, and 170.3 (6 acetate CO); ^1H NMR spectrum (90 MHz, $CDCl_3$) δ 1.14, 1.19, and 1.23 (9 H, 3 s, 3 CH_3), 1.7–1.9 (4 H, m, 2 monoterpenyl CH_2), 1.97, 1.99, 2.00, 2.01, 2.05, and 2.08 (18 H, 6 s, 6 CH_3COO), 3.4–4.0 (5 H, m, monoterpenyl CH_2CHO; H on A4, G5, and G6), 4.16 (1 H, d, J = 7.8 Hz, H on G1), 4.3–5.4 (8 H, m, $CH_2{=}CH$, H on A1, A2, A3, G2, G3, G4), 5.92 (1 H, dd, $CH_2{=}CH$).

Astilbin (**18d**): ^1H NMR (90 MHz, $Me_2SO\text{-}d_6$) δ 1.06 (d, J = 6.1 Hz, rhamnosyl CH_3), 4.11 (s, rhamnosyl H1), 4.60 (d, J = 9.5 Hz, H3), and 5.24 (d, J = 9.5 Hz, H2), assignment confirmed by comparison with ^1H NMR data for the isomeric astilbins as reported by Gaffield et al. (1975); ^{13}C NMR (22.49 MHz, CD_3OD–$CDCl_3$ (4:1)) δ 194.1 (C4), 166.6 (C7), 163.4 (C5), 162.1 (C9), 145.4 (C4′), 144.5 (C3′), 127.3 (C1′), 118.8 (C6′), 114.9 and 113.9 (C2′ and C5′), 100.7 (C10), 100.3 (rhamnosyl C1; i.e. R1), 95.9 (C6), 94.8 (C8), 82.0 (C2), 76.8 (C3), 71.9 (R4), 70.3 (R3), 69.8 (R2), 68.7 (R5), 16.3 (R6). The ^{13}C assignments for the aglycon were taken from Markham et al. (1982) and for the rhamnosyl moiety from Pozsgay et al. (1981).

Quercetin-3-*O*-β-glucopyranuronoside (**19e**): ^1H NMR (90 MHz, CD_3OD) δ 7.62 (2 H, sextet, H2′ and H6′), 6.85 (1 H, d, J = 8.3 Hz; H5′), 6.38 (1 H, d, J = 2 Hz, H8), 6.19 (1 H, d, J = 2 Hz, H6), 5.33 (1 H, d, J = 6.9 Hz, glucuronosyl H1; i.e. G1), 3.4–3.9 (4 H, m, G2, G3, G4, G5); ^{13}C NMR (22.49 MHz, CD_3OD) δ 179.3 (C4), 172.1 (G6), 166.0 (C7), 163.0 (C9), 159.1 (C2), 158.5 (C5), 149.9 (C4′), 145.9 (C3′), 135.5 (C3), 123.6 (C6′), 122.9 (C1′), 117.3 and 116.0 (C5′ and C2′), 105.7 (C10), 104.3 (G1), 100.0 (C6), 94.8 (C8), 77.6 (G5), 77.0 (G3), 75.4 (G2), 72.9 (G4). The UV spectrum was in close agreement with that published by Ribéreau-Gayon (1964).

Glycoside of kaempferol (**20**): ^1H NMR (90 MHz, D_2O) δ 8.09 (2 H, d, J = 9 Hz, H2′ and H6′), 6.88 (2 H, d, J = 9 Hz, H3′ and H5′), 6.41 (1 H, d, J = 2 Hz, H8), 6.21 (1 H, d, J = 2 Hz, H6), 5.14 (1 H, d, J = 7.6 Hz, glycosidic H1), plus other carbohydrate protons at 3.3–4.0 [cf. data of Mabry et al. (1970)].

RESULTS AND DISCUSSION

Analysis of C_{18} RP Isolates. Figure 1 shows thin-layer chromatograms (TLC) of fractions separated by DCCC from a C_{18} RP concentrate isolated from Rhine Riesling juice. A TLC of the PVPP-treated starting material before application of the DCCC technique is also shown in Figure 1. PVPP treatment had been used in an effort to simplify the composition of the C_{18} RP isolate by removing constituents with phenolic properties (Jones et al., 1965).

Nevertheless, a great number of components, many of them conjugated as glycosides, were still present in the starting material. The complexity of such mixtures frustrated all previous attempts at separation and analysis of the numberous minor components of these concentrates.

TLC analysis of the DCCC fractions (Figure 1) demonstrates that the all-liquid chromatographic technique can

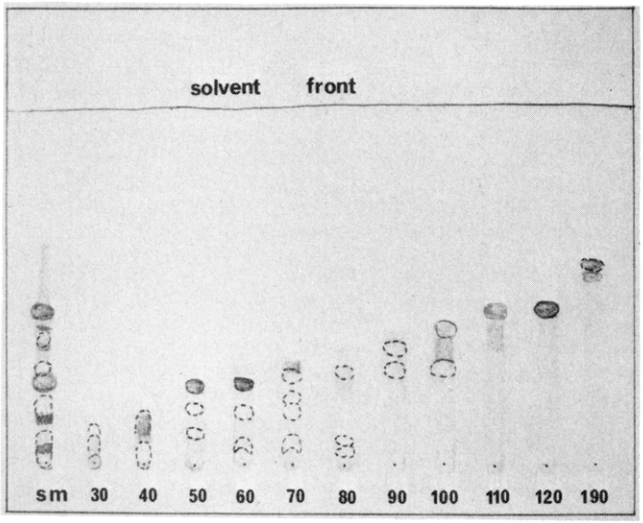


Figure 1. Thin-layer chromatogram of PVPP-treated C_{18} RP isolate from Rhine Riesling grape juice (sm) and of various fractions separated by DCCC from this material. Components indicated by broken lines were visualized by UV before the plate was sprayed.

resolve the constituents of C_{18} RP concentrates into classes of different polarity.

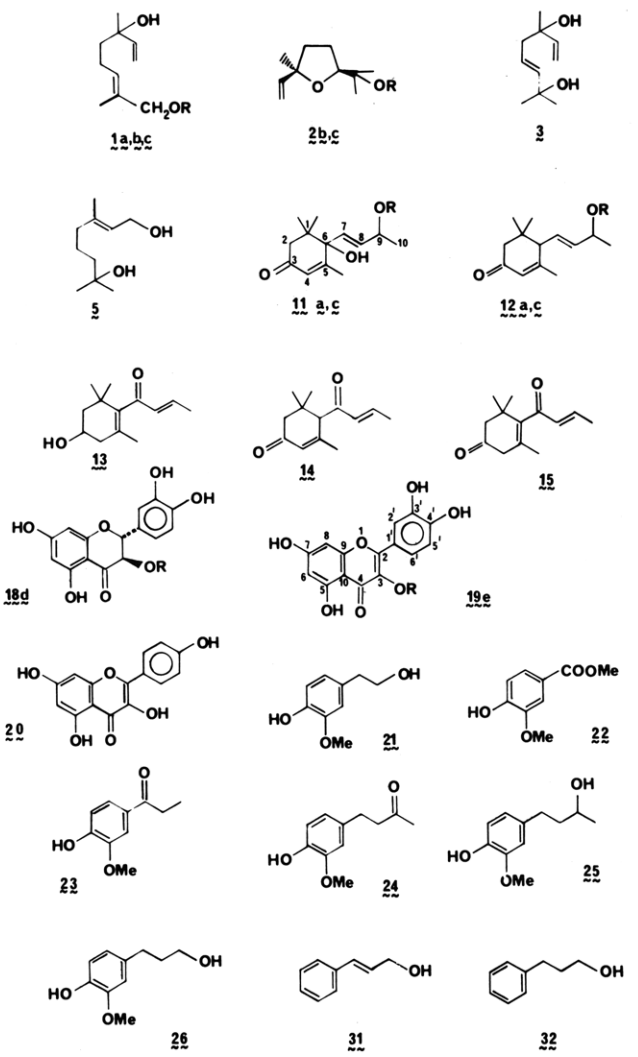
C_{18} RP concentrates from Muscat of Alexandria and Chardonnay juices, which had received respectively only light treatment, and no treatment with PVPP, were also separated by DCCC. The concentrates from both of these juices contained derivatives with phenolic properties as well as conjugates of nonphenols, including glycosidic materials.

For preliminary screening of components present in the DCCC fractions, enzymic hydrolysis was employed. Selected fractions were concentrated to remove organic solvent, and the residue was incubated with a commercial pectic enzyme preparation. The enzyme used for this purpose was known to have a nonspecific glycosidase function (Aryan et al., 1987). Volatile products liberated by the enzyme were then analyzed by GC–MS. This procedure allowed initial assignment of constituents in the various fractions as conjugates of the identified volatiles.

For more detailed analysis of individual constituents, selected groups of consecutive DCCC fractions were pooled and the combined fractions individually flash chromatographed (Still et al., 1978). Acetylation of flash column fractions was employed if required, followed by further partition liquid chromatography of the acetates, to give products in high purity, so allowing spectral data to be obtained. Thus, in favorable cases, total assignment of individual glycosidic moieties as well as of aglycons was made.

Table I gives data for partially and totally assigned conjugates in the C_{18} RP isolates from Chardonnay, Rhine Riesling, and Muscat of Alexandria juices. These compounds were present in a concentration range of 1–10 ppm in the juices examined. Structures for some of the compounds referred to in Table I are shown in Figure 2. The degree of certainty for each assignment was dependent on the spectral and chromatographic data available, and this information, along with the grape varieties and the DCCC fractions in which the conjugates were found, is also included in the Table. For convenience, the compounds in Table I are collected under different headings, i.e. conjugates of monoterpenoids, norisoprenoids, phenols, and a miscellaneous group.

Mass spectral data for unknown products and some previously unrecognized grape constituents, all of which



a,R = β-D-glucopyranosyl c,R = H

b,R = 6-O-α-arabinofuranosyl-β-D-glucopyranosyl

d,R = β-rhamnopyranosyl e,R = β-glucopyranuronosyl

Figure 2. Compounds referred to in this work.

were released by enzymic hydrolysis of the DCCC fractions, are given in Table II.

Monoterpene Conjugates. Of the monoterpene conjugates fully characterized in this work, the β-D-glucopyranoside **1a** and 6-*O*-α-arabinofuranosyl-β-D-glucopyranoside **1b** of (*E*)-2,6-dimethylocta-2,7-diene-1,6-diol (**1c**) are of particular interest. The glucoside **1a**, known as betulalbuside A, has been previously identified in leaf extracts of *Betula alba* and fruits of *Chaenomeles japonica* (Tschesche et al., 1977). Recently Rapp et al. (1986) identified the aglycon **1c** and its *Z* isomer as natural components of Morio-Muskat grapes and wine. These authors along with Bock et al. (1986) have also shown that the diol **1c** is the major transformation product of linalool (**4**) by the fungus *Botrytis cinerea*, which can infect grapes. The diol **1c** has also been found in papaya fruit (Winterhalter et al., 1986) and tobacco (Behr et al., 1978).

The identification of glycosides **1a** and **1b** in this work has facilitated recognition of the free aglycon **1c** together with its *Z* isomer in juices of several grape varieties. Furthermore, the identification of **1b** confirms that monoterpene polyols, in addition to the previously recognized monofunctional monoterpene glycosides (Williams et al., 1982b), exist in grapes as disaccharide glycosides.

Details of **1a** and **1b** and discussion of the significance of the free diols to grape and wine varietal characterization will be presented elsewhere (Strauss et al., 1987b).

The isolation and identification of the 6-*O*-α-arabinofuranosyl-β-D-glucopyranoside **2b** of *cis*-furan linalool oxide confirms the previous tentative assignment of the glycosyl conjugation of this oxide **2c** (Strauss et al., 1984).

The other monoterpene conjugates reported in Table I are most likely to be glycosides, although data confirming the sugar moieties were not obtained. Disaccharide glycosides of linalool, geraniol, nerol, and α-terpineol have been previously reported in *V. vinifera* grapes (Williams et al., 1982b).

From the mass spectral data for the products released by enzymic treatment of the Rhine Riesling DCCC fractions 85 and 112, the unknowns **10** are considered to be an isomeric pair of *p*-menthenediols (see Table II). Such diols have not been seen previously among the monoterpene polyols of grapes.

C_{13} Norisoprenoid Conjugates. Roseoside (**11a**), the glucoside of vomifoliol (**11c**), has only recently been found for the first time in grapes (Strauss et al., 1987a). It appears to be widespread in nature however, having been isolated from leaves of *Vinca rosea* (Bhakuni et al., 1974) as well as from leaves of *B. alba* and fruit of *Cydonia oblonga* (Tschesche et al., 1976). It also occurs in fluecured tobacco, *Nicotiana tabacum* (Kodama et al., 1981), and in other species of the genus *Nicotiana* along with the β-glucoside of 3-oxo-α-ionol, i.e. (*E*)-9-hydroxymegastigma-4,7-dien-3-one (**12a**) (Kodama et al., 1984). Conjugates of the damascone derivatives **13–15** have not been described, although Kodama et al. (1984) suggested that a glucoside of (*E*)-3-hydroxymegastigma-5,8-dien-7-one (**13**) was present in *Nicotiana repanda*. The role of compounds **11a–17** in the flavor of grapes and wine is a subject of continuing investigation.

Phenolic Conjugates. Astilbin (**18d**) and quercetin-3-*O*-β-glucopyranuronoside (**19e**) were obtained in high purity when they each crystallized out of their respective DCCC fractions. Astilbin (**18d**) has been identified recently in Chardonnay grapes (Trousdale and Singleton, 1983), while the glucuronoside **19e** and glycosides of kaempferol (**20**) are well-recognized components of *V. vinifera* fruit (Ribéreau-Gayon, 1964) and leaves (Egger et al., 1976).

Homovanillyl alcohol, i.e. 2-(4-hydroxy-3-methoxyphenyl)ethanol (**21**), methyl vanillate (**22**), and propiovanillone (**23**), which were each present in a conjugated form in the Rhine Riesling C_{18} RP isolate, are known wine compounds. The first two were most recently observed in a Californian Riesling wine by Güntert et al. (1986), and ketone **23** has been identified in red wine by Etievant (1981).

Dihydroconiferyl alcohol, i.e. 3-(4-hydroxy-3-methoxyphenyl)propan-1-ol (**26**), has not been previously reported as a grape or wine product. Coniferyl alcohol itself, although not found in the DCCC fractions examined here, has been positively identified among the enzymically released products from Chardonnay C_{18} RP isolates during studies in our laboratories. Although coniferyl alcohol is widely distributed in plants (Harborne and Simmonds, 1964), it has not been previously reported in *Vitis* species. Zingerone (**24**), a pungent principle of ginger oleoresin (Connell, 1970), and its reduction product **25**, which occurred in conjugated forms in the Rhine Riesling DCCC fractions, have not previously been found in grapes or wines. The known flavor properties of zingerone (**24**) (Ohloff et al., 1985) add significance to the identification of a conjugate of this compound in grapes.

Several authors have ascribed the origins of those volatile phenols identified in wines as coming from oak during barrel storage and to microbiological action (Dubois, 1983; Etievant, 1981; Singleton and Noble, 1976). Additionally, Güntert et al. (1986) suggested that some volatile phenols could arise directly from the natural grape material, although their analytical data could not confirm this hypothesis. The results in Table I establish the presence of conjugates of several volatile phenols in unfermented grape juices. Further work is necessary to clarify the nature of the conjugation and the role of these compounds as precursors of phenolic flavorants in wine. If, as seems likely, the conjugation is glycosidic, then in the case of constituents **22–24** and the tentatively assigned dimethoxyphenol **27** the phenolic oxygen is derivatized and these compounds would not exhibit phenolic properties. For this reason such conjugates may have escaped detection in earlier studies on grape phenols.

Miscellaneous Compounds. Conjugated forms of 2-phenylethanol (**29**), benzyl alcohol (**28**), and 1-hexanol (**30**) were first recognized in Muscat grapes when C_{18} RP isolates from the fruit were acid hydrolyzed (Williams et al., 1982a). Subsequently, benzyl alcohol (**28**) and 2-phenylethanol (**29**) were shown to be glycosylated as β-rutinosides and 6-*O*-α-L-arabinofuranosyl-β-D-glucopyranosides in grapes (Williams et al., 1983). Of the volatiles released from the other conjugates collected in this group, neither cinnamyl alcohol (**31**) nor 3-phenylpropan-1-ol (**32**) have been reported as products of *V. vinifera* grapes. The conjugate of unknown **33**, which was found in Rhine Riesling DCCC fraction 63, is a glucosylated indole derivative. The structure of this apparently new grape constituent is under investigation.

CONCLUSION

This investigation was undertaken to determine the suitability of the DCCC technique for the analysis of minor polar constituents of grapes. In pursuit of this aim the work has led to the discovery of several new *V. vinifera* components and demonstrated that a range of important grape flavorants, including terpenoid and phenolic compounds, are present in nonvolatile conjugated forms. The work has thus opened a new avenue for research into flavor precursors of grapes and other fruits.

Because of the nature of the investigation it was not intended to identify all components present in the C_{18} RP isolates of the three juices studied. Accordingly, the absence of previously elucidated compounds from among those found in the juice samples does not imply that the particular compounds were not present in the juices. For example, several monoterpene glycosides have been earlier characterized in Muscat of Alexandria and Rhine Riesling C_{18} RP concentrates (Williams et al., 1982b). The report here, of linalool (**4**) as an unspecified glycoside in the Muscat juice and of geraniol (**8**), nerol (**9**), and α-terpineol (**6**) as conjugates in the Rhine Riesling sample, simply indicates that, for the purposes of this work, a detailed investigation of the conjugating moieties involved with these compounds was not made.

Also, the various C_{18} RP isolates were each subjected to different pretreatments prior to separation, and this factor obscures any relationships between composition and variety that might be drawn from the data in Table I. Although it was not the purpose of this study to correlate compositional differences between C_{18} RP isolates from the three cultivars, nevertheless it is evident that DCCC, together with the other techniques used, allows resolution and analysis of the numerous minor constituents of the

fruit. Such an analytical approach will facilitate research into varietally specific constituents of grapes.

ABBREVIATIONS USED

PVPP, polyvinylpolypyrrolidone; C_{18} RP, C_{18} reversed-phase; DCCC, droplet countercurrent chromatography.

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