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The polyphenol constituents of grape pomace

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Abstract

Chemical investigation of Chardonnay grape pomace has resulted in the isolation of 17 polyphenols which were identified by NMR spectroscopy as gallic acid, its 3- and 4-β-glucopyranosides, *trans*-caftaric acid, *cis*- and *trans*-coutaric acid, 2-hydroxy-5-(2-hydroxyethyl)phenyl-β-glucopyranoside, catechin, epicatechin, procyanidin B1, quercetin 3-glucoside, quercetin 3-glucuronide, kaempferol 3-glucoside, kaempferol 3-galactoside, eucryphin, astilbin and engeletin. Gallic acid 3-β-glucopyranoside, gallic acid 4-β-glucopyranoside and 2-hydroxy-5-(2-hydroxyethyl)phenyl-β-glucopyranoside are reported here as natural grape constituents for the first time.

1. Introduction

Grapes contain a large amount of polyphenols which include the phenolic acids (Reschke & Herrmann, 1981; Singleton, Timberlake, & Lea, 1978; Singleton, Zaya, & Trousdale, 1986), flavonoids (Cheynier & Rigaud, 1986; Trousdale & Singleton, 1983; Wulf & Nagel, 1980), anthocyanins (Bakker & Timberlake, 1985; Hebrero, Santos-Buegla, & Rivas-Gonzalo, 1988; Wulf & Nagel, 1978) and proanthocyanidins (Escribano-Bailon, Gutierrez-Fernandez, Rivas-Gonzalo, & Santos-Buelga, 1992; Escribano-Bailon, Guerra, Rivas-Gonzalo, & Santos-Buelga, 1995; Kantz & Singleton, 1990; Lea, Bridle, Timberlake, & Singleton, 1979; Ricardo da Silva, Rigaud, Cheynier, Cheminat, & Moutounet, 1991; Souquet, Cheynier, Broussaud, & Moutounet, 1996). Recently, there has been much interest in these phenolic compounds because of their health benefits, such as antioxidant activity (Kanner, Frankel, Granit, German, & Kinsella, 1994), acting as free radical scavengers (Ricardo da Silva, Darmon, Fernandez, & Mitjavila, 1991) and inhibition of lipoprotein oxidation (Kinsella, Frankel, German, & Kanner, 1993; Teissedre, Frankel, Waterhouse, Peleg, & German, 1996), and the oxidation of LDL has been suggested as a pivotal step in the development of heart disease (Steinberg, 1992). The French paradox (Renaud & De Lorgeril, 1992) has been attributed to their presence at high levels in red wines (Frankel, Kanner, German, Parks, & Kinsella, 1993; Frankel, Waterhouse, & Teissedre, 1995; Kinsella et al., 1993; Teissedre et al., 1996).

Grapes are cultivated largely for the wine industry, which generates huge amounts of grape pomace as an industrial waste. Some research work has been carried out to seek industrial uses for this waste, including use as animal feed (Larwence, 1991; Santiago, Ferrer, de Colmenares, & Paez, 1993), as nutritive ingredients (Ana, Croiter, Segal, & Sas, 1995; Igartuburu, Pando, Luis, & Gil-Serrano, 1997), in the production of citric acid (Hang, 1988) and the use of anthocyanins from grape skins as colorants (Francis, 1992). We have previously reported the identification of two novel biphenyl-linked biflavanoids in grape pomace (Foo, Lu, & Wong, 1998) and, in continuing this study, the isolation and identification of a range of polyphenols will be presented as part of our project to evaluate the pomace's potential as a source of nutritional polyphenols.

2. Materials and methods

2.1. Extraction

Chardonnay grape pomace was obtained from Dry River Vineyard, Martinborough, New Zealand. The grapes were bunch-pressed using a Willmes bladder machine so there was no soaking of the pomace in the juice. The pomace was stored in a cold room one day and then freeze-dried. The finely ground (sieve 1 mm) freeze-dried pomace (500 g) was extracted with 80% ethanol

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(3×1500 ml). The combined extract was concentrated in a rotary evaporator under vacuum and the resulting aqueous concentrate was defatted with hexane (3×1000 ml) and freeze-dried to afford 211 g (42%) solid extracts.

2.2. Fractionation and isolation

The ethanol extract (200 g), dissolved in 300 ml of distilled water, was applied to a HP-20 column (16×6 cm I.D.) and fractionated first with water then with various concentrations of aqueous methanol (from 0 to 80% in increments of 20%, 2×300 ml each) followed with 50% acetone (2×300 ml) and finally with 70% acetone (2×300 ml) to yield 14 fractions. Each fraction was analysed by HPLC and fractions of similar composition were combined to give five fractions: A, 92.2 g (fraction 1); B, 70.8 g (2–5); C, 3.6 g (6 and 7); D, 4.2 g (8–10) and E, 8.0 g (11–14).

Separation of fraction B on a Sephadex LH-20 column (60×4 cm I.D.) yielded five sub-fractions, which were then further separately treated on a MCI column (40×2 cm I.D.). Similar treatments of fractions C and D were performed separately on a Sephadex LH-20 column (45×2.5 cm I.D.). These resulted in the isolation of six phenolic acids and a phenolic alcohol from fraction B, three flavanols from fraction C and seven flavonoids from fraction D. Fractions were collected by an automatic fraction collector and monitored by HPLC. Fractions of pure compounds were combined and freeze-dried for structural elucidation by NMR spectroscopy.

2.3. HPLC analysis

A Hewlett Packard series 1100 equipped with a DAD detector was employed. The HPLC analysis of grape pomace extracts and their chromatographic fractions was performed on a LiChroCART® 125-4, LiChrospher® 100 RP-18 (5 μm) column held at 30°C using the following solvent gradient: solvent A (AcOH–H_2O, 2:98, v/v); solvent B (CH_3CN–AcOH–H_2O, 50:2:48, v/v); 0 to 20 min—8 to 24% B; 20 to 30 min—24 to 40% B and 30 to 45 min—40 to 100% B. The flow rate was set at 1 ml min^{-1} and detection of compounds was by UV absorption at 280 and 350 nm.

2.4. NMR Identification

The identification of purified phenolic compounds was made by NMR spectroscopy on a Bruker AC 300 instrument and chemical shifts (δ) were referenced to TMS (^1H) or solvent signal (^{13}C).

2.4.1. *Gallic acid* (**1**)

^1H NMR (300 MHz, D_2O): δ 7.10 (s, H-2, 6). ^{13}C NMR (75 MHz, D_2O): δ 110.52 (C-2, 6), 121.38 (C-1), 135.93 (C-4), 144.94 (C-3, 5), 170.76 (C-7).

2.4.2. *Gallic acid 4-β-glucopyranoside* (**2**)

^1H NMR (300 MHz, D_2O): δ 3.39–3.81 (m, sugar-H), 4.94 (d, *J* 7.5 Hz, H-1′), 6.99 (s, H-2, 6). ^{13}C NMR (75 MHz, D_2O): δ 60.68 (C-6′), 69.49 (C-4′), 73.78 (C-2′), 75.95 (C-5′), 76.75 (C-3′), 104.24 (C-1′), 109.66 (C-2, 6), 134.44 (C-1), 134.90 (C-4), 149.25 (C-3, 5), 174.67 (C-7). ESMS (negative mode): *m*/*z* (%) 331.5 ($[M–H]^-$, 100%), 169.6 (60%).

2.4.3. *Gallic acid 3-β-glucopyranoside* (**3**)

^1H NMR (300 MHz, D_2O): δ 3.38–4.10 (m, sugar-H), 5.09 (d, *J* 7.5 Hz, H-1′), 7.20 (d, *J* 1.9 Hz, H-2), 7.28 (d, *J* 1.9 Hz, H-6). ^{13}C NMR (75 MHz, D_2O): δ 60.90 (C-6′), 69.77 (C-4′), 73.31 (C-2′), 75.86 (C-5′), 76.60 (C-3′), 101.66 (C-1′), 109.94 (C-2), 112.54 (C-6), 128.42 (C-1), 134.48 (C-4), 144.60 (C-3), 145.21 (C-5), 174.77 (C-7).

2.4.4. Trans-*caftaric acid (caffeoyltartaric acid)* (**4**)

^1H NMR (300 MHz, D_2O): δ 4.82 (br s, H-3), 5.52 (br s, H-2), 6.43 (d, *J* 15.9 Hz, H-8′), 6.91 (d, *J* 8.2 Hz, H-5′), 7.09 (d, *J* 8.2 Hz, H-6′), 7.18 (br s, H-2′), 7.66 (d, *J* 15.9 Hz, H-7′). ^{13}C NMR (75 MHz, D_2O): δ 71.46 (C-3), 75.34 (C-2), 114.06 (C-8′), 115.39 (C-2′), 116.54 (C-5′), 123.23 (C-6′), 127.14 (C-1′), 145.11(C-3′), 147.38 (C-7′), 148.07 (C-4′), 168.35 (C-9′).

2.4.5. Trans *and* cis-*coutaric acids (coumaroyltartaric acids)*. Trans-*isomer* (**5**)

^1H NMR (300 MHz, D_2O): δ 4.91 (d, *J* 2.0 Hz, H-3), 5.61 (d, *J* 2.0 Hz, H-2), 6.48 (d, *J* 16.0 Hz, H-8′), 6.95 (d, *J* 8.6 Hz, H-3′, 5′), 7.57 (d, *J* 8.6 Hz, H-2′, 6′), 7.78 (d, *J* 16.0 Hz, H-7′). ^{13}C NMR (75 MHz, D_2O): δ 69.18 (C-3), 72.63 (C-2), 111.77 (C-8′), 114.71 (C-3′, 5′), 124.67 (C-1′), 129.43 (C-2′, 6′), 145.80 (C-7′), 157.89 (C-4′), 166.17 (C-9′), 169.20 (C-1), 172.05 (C-4).

2.4.6. Cis-*isomer* (**6**)

^1H NMR (300 MHz, D_2O): δ 4.89 (d, *J* 2.1 Hz, H-3), 5.54 (d, *J* 2.1 Hz, H-2), 5.99 (d, *J* 12.7 Hz, H-8′), 6.89 (d, *J* 8.7 Hz, H-3′, 5′), 7.07 (d, *J* 12.7 Hz, H-7′), 7.69 (d, *J* 8.7 Hz, H-2′, 6′). ^{13}C NMR (75 MHz, D_2O): δ 69.18 (C-3), 72.55 (C-2), 113.21 (C-8′), 113.75 (C-3′, 5′), 124.75 (C-1′), 131.44 (C-2′, 6′), 144.70 (C-7′), 156.80 (C-4′), 165.10 (C-9′), 169.20 (C-1), 172.05 (C-4).

2.4.7. *2-Hydroxy-5-(2-hydroxyethyl)phenyl-β-D-glucopyranoside* (**7**)

^1H NMR (300 MHz, D_2O): δ 2.73 (t, *J* 6.5 Hz, H-7), 3.5–3.6 (m, H-2′,3′,4′,5′), 3.70 (m, H-6′a), 3.75 (t, *J* 6.5 Hz, H-8), 3.90 (d, *J* 12.4 Hz, H-6′b), 5.1 (d, *J* 6.1 Hz, H-1′), 6.85 (d, *J* 8.7 Hz, H-4), 6.88 (d, *J* 8.4 Hz, H-3), 7.03 (s, H-6). ^{13}C NMR (75 MHz, D_2O): δ 37.95 (C-7), 61.29 (C-6′), 63.34 (C-8), 70.19 (C-4′), 73.63 (C-2′), 76.25 (C-3′), 76.88 (C-5′), 101.91 (C-1′), 117.15 (C-3), 117.98 (C-6), 125.04 (C-4), 132.55 (C-5), 144.53 (C-2), 145.15 (C-1). ESMS (negative mode): *m*/*z* (%) 315.2 ($[M–H]^-$, 80%), 212.3 (100%).

2.4.8. Catechin (**8**)

^{1}H NMR (300 MHz, acetone-d_6): δ 2.56 (dd, *J* 16.1, 8.2 Hz, H-4β), 2.93 (dd, *J* 16.1, 5.3 Hz, H-4α), 3.46 (s, OH-3), 4.04 (m, H-3), 4.59 (d, *J* 7.5 Hz, H-2), 5.90 (d, *J* 2.2 Hz, H-6), 6.04 (d, *J* 2.2 Hz, H-8), 6.77 (dd, *J* 8.2, 1.6 Hz, H-6′), 6.81 (d, *J* 8.1 Hz, H-5′), 6.91 (d, *J* 1.5 Hz, H-2′), 8.01 (br s, 4× OH). ^{13}C NMR (75 MHz, acetone-d_6): δ 28.66 (C-4), 68.33 (C-3), 82.56 (C-2), 95.45 (C-8), 96.17 (C-6), 100.60 (C-4a), 115.20 (C-2′), 115.33 (C-5′), 120.04 (C-6′), 132.09 (C-1′), 145.58 (C-3′), 145.64 (C-4′), 156.82 (C-8a), 157.14 (C-5), 157.63 (C-7).

2.4.9. Epicatechin (**9**)

^{1}H NMR (300 MHz, acetone-d_6): δ 2.72 (dd, *J* 16.7, 3.5 Hz, H-4β), 2.87 (dd, *J* 16.7, 4.5 Hz, H-4α), 4.24 (m, H-3), 4.88 (s, H-2), 5.96 (d, *J* 2.3 Hz, H-6), 6.06 (d, *J* 2.3 Hz, H-8), 6.83 (m, H-5′,6′), 7.05 (s, H-2′). ^{13}C NMR (75 MHz, acetone-d_6): δ 28.48 (C-4), 66.59 (C-3), 79.05 (C-2), 95.35 (C-8), 96.07 (C-6), 99.58 (C-4a), 115.05 (C-2′), 115.50 (C-5′), 119.05 (C-6′), 131.66 (C-1′), 144.93 (C-3′), 145.07 (C-4′), 156.62 (C-8a), 157.01 (C-5), 157.20 (C-7).

2.4.10. Procyanidin B1 (epicatechin-(4,8)-catechin) (**10**)

^{13}C NMR (75 MHz, acetone-d_6): Upper (epicatechin) unit: δ 36.76 (C-4), 72.58 (C-3), 76.74 (C-2), 95.54 (C-8), 96.11 (C-6), 100.95 (C-4a), 115.77 (C-5′), 116.06 (C-2′), 119.38 (C-6′), 132.37 (C-1′), 145.07 (C-3′), 145.30 (C-4′), 155.11 (C-8a), 155.63 (C-5), 157.40 (C-7); Lower (catechin) unit: δ 28.40 (C-4), 67.76 (C-3), 81.77 (C-2), 96.93 (C-6), 100.95 (C-4a), 107.49 (C-8), 114.93 (C-5′), 115.38 (C-2′), 119.38 (C-6′), 131.95 (C-1′), 145.07 (C-3′), 145.30 (C-4′), 155.11 (C-8a), 155.63 (C-5), 157.40 (C-7).

2.4.11. Quercetin 3-β-D-*glucopyranoside* (**11**)

^{1}H NMR (300 MHz, DMSO-d_6): δ 3.08–3.60 (m, sugar-H), 5.46 (d, *J* 7.5 Hz, H-1″), 6.20 (d, *J* 2.0 Hz, H-6), 6.40 (d, *J* 2.0 Hz, H-8), 6.84 (d, *J* 9.0 Hz, H-5′), 7.57 (m, H-2′, 6′). ^{13}C NMR (75 MHz, DMSO-d_6): δ 61.17 (C-6″), 70.13 (C-4″), 74.28 (C-2″), 76.70 (C-5″), 77.75 (C-3″), 93.67 (C-8), 98.82 (C-6), 101.07 (C-1″), 104.17 (C-10), 115.39 (C-2′), 116.39 (C-5′), 121.36 (C-6′), 121.78 (C-1′), 133.53 (C-3), 144.99 (C-3′), 148.64 (C-4′), 156.40 (C-2), 156.51 (C-9), 161.44 (C-5), 164.28 (C-7), 177.64 (C-4).

2.4.12. Quercetin 3-β-D-*glucuropyranoside* (**12**)

^{1}H NMR (300 MHz, DMSO-d_6): δ 3.20–3.60 (m, sugar-H), 5.50 (d, *J* 7.3 Hz, H-1″), 6.21 (d, *J* 2.1 Hz, H-6), 6.41 (d, *J* 1.8 Hz, H-8), 6.84 (d, *J* 8.4 Hz, H-5′), 7.53 (d, *J* 2.1 Hz, H-2′), 7.60 (dd, *J* 8.4, 2.1 Hz, H-6′). ^{13}C NMR (75 MHz, DMSO-d_6): δ 71.78 (C-4″), 74.22 (C-2″), 76.31 (C-5″), 76.43 (C-3″), 94.04 (C-8), 99.20 (C-6), 101.51 (C-1″), 104.67 (C-10), 115.64 (C-2′), 116.46 (C-5′), 121.31 (C-6′), 122.15 (C-1′), 134.00 (C-3), 145.36 (C-3′), 149.06 (C-4′), 156.62 (C-2), 156.71 (C-9), 161.64 (C-5), 164.66 (C-7), 170.17 (C-6″), 177.61 (C-4).

2.4.13. Kaempferol 3-β-D-*glucopyranoside* (**13**)

^{1}H NMR (300 MHz, DMSO-d_6): δ 3.08–3.66 (m, sugar-H), 5.45 (d, *J* 7.2 Hz, H-1″), 6.19 (d, *J* 1.9 Hz, H-6), 6.42 (d, *J* 1.8 Hz, H-8), 6.88 (d, *J* 8.8 Hz, H-3′, 5′), 8.04 (d, *J* 8.9 Hz, H-2′, 6′), 12.60 (s, OH-5). ^{13}C NMR (75 MHz, DMSO-d_6): δ 60.81 (C-6″), 69.87 (C-4″), 74.18 (C-2″), 76.39 (C-5″), 77.45 (C-3″), 93.68 (C-8), 98.77 (C-6), 100.89 (C-1″), 103.84 (C-10), 115.07 (C-3′, 5′), 120.89 (C-1′), 130.84 (C-2′, 6′), 133.17 (C-3), 156.16 (C-2), 156.41 (C-9), 159.91 (C-4′), 161.19 (C-5), 164.52 (C-7), 177.38 (C-4).

2.4.14. Kaempferol 3-β-D-*galactopyranoside* (**14**)

^{1}H NMR (300 MHz, DMSO-d_6): δ 3.08–3.66 (sugar-H), 5.39 (d, *J* 7.6 Hz, H-1″), 6.19 (d, *J* 1.9 Hz, H-6), 6.42 (d, *J* 1.8 Hz, H-8), 6.86 (d, *J* 8.5 Hz, H-3′, 5′), 8.06 (d, *J* 8.7 Hz, H-2′, 6′), 12.60 (s, OH-5). ^{13}C NMR (75 MHz, DMSO-d_6): δ 60.15 (C-6″), 67.81 (C-4″), 71.17 (C-2″), 73.07 (C-3″), 75.74 (C-5″), 93.68 (C-8), 98.77 (C-6), 101.52 (C-1″), 103.84 (C-10), 115.07 (C-3′, 5′), 120.89 (C-1′), 130.84 (C-2′, 6′), 133.17 (C-3), 156.16 (C-2), 156.41 (C-9), 159.91 (C-4′), 161.19 (C-5), 164.52 (C-7), 177.38 (C-4).

2.4.15. Eucryphin (3,5,7-trihydroxychromone 3-α-L-rhamnopyranoside) (**15**)

^{1}H NMR (300 MHz, DMSO-d_6): δ 1.13 (d, *J* 6.2 Hz, H-6′), 3.58–3.68 (m, sugar-H), 3.89 (s, sugar-H), 5.22 (d, *J* 1.4 Hz, H-1′), 6.21 (d, *J* 2.0 Hz, H-6), 6.37 (d, *J* 2.0 Hz, H-8), 8.33 (s, H-2), 10.90 (s, OH-7), 12.42 (s, OH-5). ^{13}C NMR (75 MHz, DMSO-d_6): δ 18.09 (C-6′), 70.07 (C-5′), 70.11 (C-3′), 70.48 (C-2′), 71.79 (C-4′), 94.14 (C-8), 99.10 (C-6), 100.85 (C-1′), 105.08 (C-10), 138.34 (C-3), 148.30 (C-2), 157.60 (C-9), 161.80 (C-5), 164.58 (C-7), 177.53 (C-4). ESMS (negative mode): *m*/*z* (%) 339.4 ([M–H]$^-$, 15%), 127.5 (100%).

2.4.16. Astilbin (dihydroquercetin 3-α-L-*rhamnopyranoside)* (**16**)

^{1}H NMR (300 MHz, DMSO-d_6): δ 1.04 (d, *J* 5.9 Hz, H-6″), 3.09–3.91 (m, sugar-H), 4.04 (s, H-1″), 4.63 (d, *J* 9.8 Hz, H-3), 5.23 (d, *J* 9.8 Hz, H-2), 5.87 (d, *J* 2.0 Hz, H-6), 5.89 (d, *J* 2.0 Hz, H-8), 6.74 (s, H-5′, 6′), 6.88 (s, H-2′), 11.8 (OH-5). ^{13}C NMR (75 MHz, DMSO-d_6): δ 17.73 (C-6″), 68.95 (C-5″), 70.10 (C-3″), 70.39 (C-2″), 71.62 (C-4″), 75.62 (C-3), 81.48 (C-2), 95.11 (C-8), 96.06 (C-6), 100.02 (C-1″), 100.68 (C-10), 114.72 (C-2′), 115.31 (C-5′), 118.88 (C-6′), 126.97 (C-1′), 145.10 (C-3′), 145.90 (C-4′), 162.14 (C-9), 163.41 (C-5), 167.23 (C-7), 194.30 (C-4).

*2.4.17. Engeletin (dihydrokaempferol 3-α-L-rhamnopyranoside) (**17**)*

^{1}H NMR (300 MHz, DMSO-d_6): δ 1.05 (d, *J* 6.2 Hz, H-6″), 3.09–-3.94 (m, sugar-H), 3.98 (s, H-1″), 4.73 (d, *J* 10.3 Hz, H-3), 5.28 (d, *J* 10.3 Hz, H-2), 5.87 (d, *J* 1.9 Hz, H-6), 5.90 (d, *J* 1.9 Hz, H-8), 6.79 (d, *J* 8.5 Hz, H-3′, 5′), 7.33 (δ, *J* 8.5 Hz, H-2′, 6′). ^{13}C NMR (75 MHz, DMSO-d_6): d 17.52 (C-6″), 68.77 (C-5″), 69.95 (C-3″),

70.20 (C-2″), 71.42 (C-4″), 75.75 (C-3), 81.28 (C-2), 94.97 (C-8), 95.97 (C-6), 100.10 (C-1″), 100.61 (C-10), 114.96 (C-3′, 5′), 126.32 (C-1′), 128.82 (C-2′, 6′), 157.63 (C-4′), 161.99 (C-9), 163.21 (C-5), 167.28 (C-7), 194.32 (C-4).

3. Results and discussion

The ethanol extract of the grape pomace was dominated by flavonoids as evidenced from the HPLC chromatogram (Fig. 1). The extract was successfully fractionated by column chromatography on a Diaion HP-20 column with different methanol content as shown in Table 1 to give 17 phenolic compounds (see Scheme 1 for chemical structures) consisting of phenolic acids, flavan-3-ols, flavonoids and oligomeric procyanidins.

3.1. Phenolic acids

The phenolic acids were eluted with up to 40% methanol from the HP-20 column and were separated from the sugars by chromatography on Sephadex LH-20 to give compounds **1–6**. Compound **1** was identified as gallic acid from its only singlet (δ 7.10) in the ^{1}H NMR spectrum and seven carbon signals in the low field (δ 110.52×2, 121.38, 135.93, 144.94 ×2, 170.76) observed in the ^{13}C NMR spectrum, and further confirmed by comparison of its chromatographic behaviour and UV spectrum with those of an authentic sample.

The NMR spectra of compound **2** also showed the presence of a gallic acid moiety, but in addition there were six carbon signals in the upfield region which could be attributable to a sugar consistent with a glucopyranose unit with a β-configuration as deduced from the large coupling constant observed for the anomeric proton (δ 4.94, *J* 7.5 Hz). Based on the observed symmetry of the galloyl moiety and the fact that its HPLC retention time was different from that of 1-galloylglucoside isolated from evening primrose (Foo, Lu, & Lu, 1997), the glucose must therefore be attached to 4-OH of gallic acid. Compound **2**, therefore, was gallic acid 4-β-glucopyranoside. This assignment was also corroborated by spectral comparison with published data (Schuster, Winter, & Herrmann, 1986) and further supported by electrospray mass spectroscopy (ESMS) which showed a parent ion peak at *m*/*z* 331.

The sample of compound **3** was contaminated with compound **2**, but the NMR signals were readily established by subtracting those of compound **2** from the spectra. Compound **3** showed two *meta*-coupled doublets (δ 7.28 and 7.20, *J* 1.9 Hz) in the ^{1}H NMR spectrum while the ^{13}C NMR spectrum showed the presence of a carboxylic acid and six aromatic carbons of similar chemical shifts to gallic acid. In addition, both the spectra showed the presence of a β-glucopyranoside residue indicating that compound **3** was a regioisomer

Table 1
Composition of polyphenols in HP-20 chromatographic fraction

Fraction	Eluent	Main constituents
A	H_2O	Sugars
B	0–40% MeOH	Sugars and phenolic acids
C	40–60% MeOH	Flavan-3-ols
D	60–80% MeOH	Flavonoids
E	50–70% MeCOMe	Oligomeric procyanidins

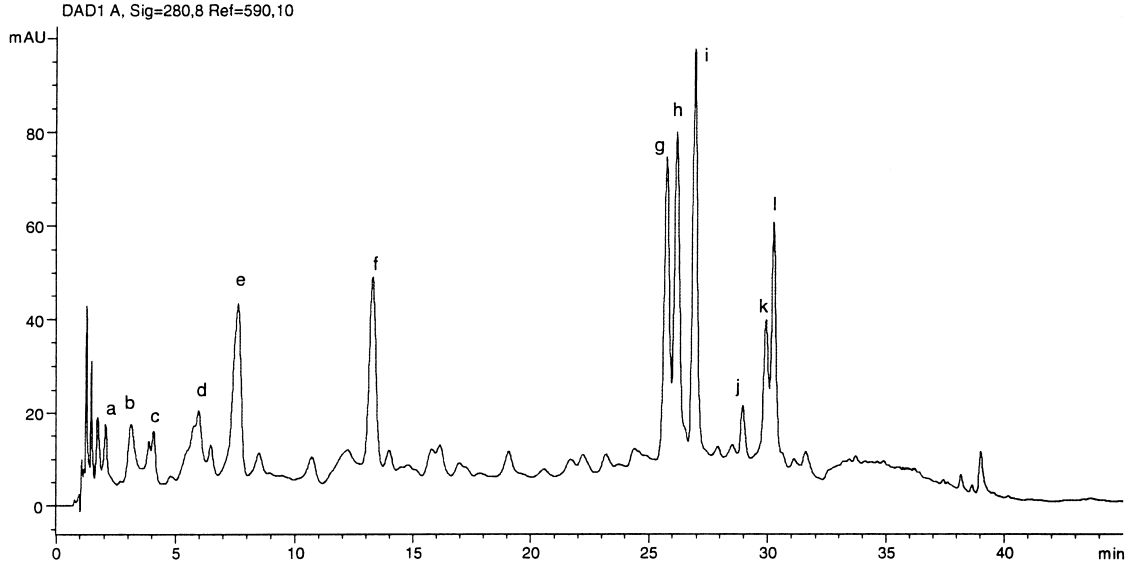
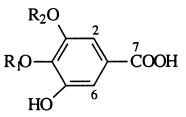
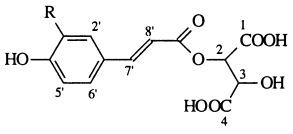


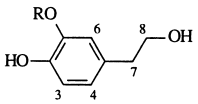
Fig. 1. HPLC chromatogram of ethanol extract of grape pomace. Peak identification: *a*, gallic acid and its β-glucopyranosides **(1–3)**; *b*, *trans*-caftaric acid **(4)** and 2-hydroxy-5-(2-hydroxyethyl)phenyl-β-glucopyranoside **(7)**; *c*, *trans*- and *cis*-coutaric acids **(5–6)**; *d*, procyanidin B1 **(10)**; *e*, catechin **(8)**; *f*, epicatechin **(9)**; *g*, astilbin **(16)**; *h*, quercetin 3-glucuronide **(12)**; *i*, quercetin 3-glucoside **(11)**; *j*, kaempferol 3-galactoside **(14)**; *k*, engeletin **(17)**; *l*, kaempferol 3-glucoside **(13)**.



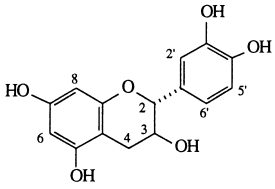
1. R_1=R_2=H, gallic acid
2. R_1=Glu, R_2=H, gallic acid 4-glucoside
3. R_1=H, R_2=Glu, gallic acid 3-glucoside



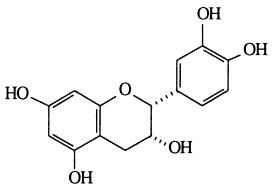
4. R=OH, *trans*-caftaric acid
5. R=H, *trans*-coutaric acid



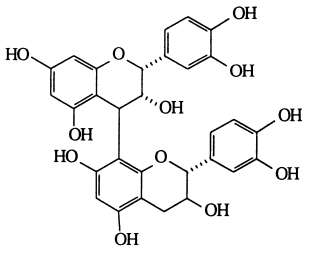
7. R=Glu, 2-hydroxy-5-(2-hydroxy-ethyl)phenylglucoside



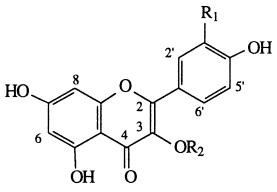
8. catechin



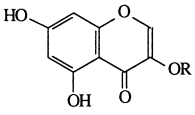
9. epicatechin



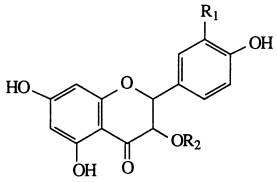
10. procyanidin B1



11. R_1=OH, R_2=Glu, quercetin 3-glucoside
12. R_1=OH, R_2=glucu, quercetin 3-glucuronide
13. R_1=H, R_2=Glu, kaempferol 3-glucoside
14. R_1=H, R_2=Gal, kaempferol 3-galactoside



15. R=Rha, eucryphin



16. R_1=OH, R_2=Rha, astilbin
17. R_1=H, R_2=Rha, engeletin

Scheme 1. Chemical structures of polyphenols from grape pomace.

having the sugar substituent in the 3-OH (or 5-OH) of gallic acid, namely gallic acid 3-β-glucopyranoside.

To our knowledge, this is the first time gallic acid 3-β-glucopyranoside and gallic acid 4-β-glucopyranoside have been reported in grape or grape products. Gallic acid 3-β-glucopyranoside was first isolated from rhubarb (Kashiwada, Nonaka, & Nishioka, 1986) and later found in tissue cultures of *Lobelia sessilifolia* (Yamanaka, Shimomura, Sasaki, Yoshihira, & Ishimaru, 1995) and *Quercus acutissima* (Tanaka, Shimomura, & Ishimaru, 1995), while its 4-β-glucopyranoside was more common and had been found in a large number of fruits and vegetables (Schuster & Herrmann, 1985; Schuster et al., 1986).

Compound **4** was the most abundant phenolic acid which showed nine strong carbon signals (δ 114.06, 115.39, 116.54, 123.23, 127.14, 145.11, 147.38, 148.07 and 168.35) consistent with a caffeoyl moiety and two weak signals at 71.46 and 75.34 ppm in its ^{13}C NMR spectrum. The presence of a *trans*-caffeoyl unit was also observed in the ^{1}H NMR spectrum which showed an ABX spin system at 6.91 (*J* 8.2 Hz), 7.09 (*J* 8.2 Hz) and 7.18 ppm and a pair of doublets at 6.43 and 7.66 ppm (*J* 15.9 Hz). Analysis using HMQC showed that the two weak carbon signals were correlated with the two broad singlet protons (δ 4.82 and 5.52), suggesting a tartaric acid moiety. The broad signals suggested there was some restriction to rotation about the sp^3 bond. These data suggested that compound **4** was *trans*-caftaric acid (caffeoyltartaric acid) and the ^{1}H NMR data were consistent with those published in the literature (Cheynier, Trousdale, Singleton, Salgues, & Wylde, 1986). The UV spectrum and the HPLC retention time were also consistent with those reported by Baranowski and Nagel (1981). *Trans*-caftaric acid has been reported in earlier studies to be the principal constituent in grapes (Singleton et al., 1986).

Compounds **5** (major) and **6** (minor) were obtained as a mixture. Compound **5** showed two doublets (δ 4.91 and 5.61, *J* 2.0 Hz) in the ^{1}H NMR spectrum as well as two carboxylic acids (δ 169.20 and 172.05) and two aliphatic carbons (δ 72.63 and 69.18) in the ^{13}C NMR spectrum which were consistent with a tartaric acid

moiety. A *trans*-coumaroyl group characterized by an AB resonance system (δ 6.95 and 7.57, *J* 8.6 Hz) and a pair of proton doublets (δ 6.48 and 7.78, *J* 16.0 Hz) in the ^{1}H NMR spectrum were also observed. This was corroborated by its ^{13}C NMR spectrum which also revealed a carboxylic acid and eight aromatic/olefinic carbons of a coumaroyl moiety. Hence compound **5** was identified as *trans*-coutaric acid (coumaroyltartaric acid). As the minor signals of compound **6** showed similarity to *trans*-coutaric acid in both ^{1}H and ^{13}C NMR spectra, compound **6** was assigned to the *cis*-isomer which was supported by the smaller coupling constant of the two doublets (δ 5.99 and 7.07, *J* 12.7 Hz) observed for the olefinic protons.

The possibility that *cis*-coutaric acid was derived from its *trans*-isomer could not be ruled out as transformation of *trans*- to *cis*-coutaric acid could occur under UV light (Ong & Nagel, 1978; Singleton et al., 1978). The presence of a small peak before *trans*-caftaric acid in the HPLC chromatogram that had a UV spectrum similar to that of the *trans*-isomer could be the *cis*-caftaric acid. This was consistent with the report that the *cis*-cinnamic acids were eluted before the corresponding *trans* isomers under reverse-phase HPLC systems (Baranowski & Nagel, 1981). *Cis*-caftaric acid had been reported to occur in grapes at much lower level than the *trans*-isomer (Singleton et al., 1986).

3.2. Phenolic alcohols

The ^{13}C NMR spectrum of compound **7** (data see Materials and Methods section) displayed two methylene carbons (δ 37.95 and 63.34) established using DEPT, six sugar carbon signals (δ 61.29, 70.19, 73.63, 76.25, 76.88 and 101.91) consistent with a glucopyranoside residue and six aromatic carbons (δ 117.15, 117.98, 125.04, 132.55, 144.53 and 145.15) which were assigned to a 3,4-dihydroxyphenyl group. This catechol substitution pattern was corroborated by observation of an ABX system found in the ^{1}H NMR spectrum. The ^{1}H NMR spectrum also confirmed the presence of a–CH_2–CH_2– unit with two sets of triplets (δ 2.73 and 3.75, *J* 6.5 Hz) being observed. The location of the sugar was established using HQMC which showed that the phenolic carbon *meta* to the ethyl group was long range-coupled to the sugar anomeric proton (δ 5.1). Thus compound **7** was determined as 2-hydroxy-5-(2-hydroxyethyl)phenyl-β-glucopyranoside. This chemical constitution was supported by electrospray mass spectroscopy, which showed a parent peak at *m*/*z* 315 consistent with the expected $[M–H]^-$ and was confirmed by spectral comparison with literature data (Sugiyama & Kikuchi, 1992).

2-Hydroxy-5-(2-hydroxyethyl)phenyl-β-glucopyranoside was first isolated from *Osmanthus asiaticus* Nakai (Sugiyama & Kikuchi, 1992). Recently, its presence in the traditional Chinese drug Shengma (*Cimicifugae*) was reported (Li, Chen, & Xiao, 1994, 1995). In grapes and wines the 2-phenylethyl and benzyl monoglucosides and their oligosaccharides had previously been reported (Williams, Strauss, Wilson, & Massy-Westropp, 1983), but phenolic alcohol glucosides are not known grape constituents and 2-hydroxy-5-(2-hydroxyethyl) phenylglucoside is the first example of this class in grape.

3.3. Flavan-3-ols

Three flavan-3-ols (compounds **8**, **9** and **10**) were isolated from fraction C. Compounds **8** and **9** were identified as catechin and epicatechin by HPLC comparison with authentic samples and their identities were also apparent from both their ^{13}C NMR spectra which showed similar carbon signals for the phloroglucinol A-ring and catechol B-ring, but slight difference in signals for the pyran C-ring. Catechin (compound **8**) contrasted with epicatechin by the downfield signals for C-2 and C-3 (δ 82.56 and 68.33) compared to those (δ 79.05 and 66.59) for epicatechin. Furthermore, the large couplings observed for the H-2 to H-3 (δ 4.59 and 4.04, *J* 7.5 Hz) in the ^{1}H NMR spectrum indicated a *trans*-orientation in catechin, and the broad singlet (δ 4.88) for the corresponding H-2 proton in epicatechin suggested a *cis*-orientation between H-2 and H-3.

The ^{13}C NMR spectrum of compound **10** showed carbon signals for both catechin and epicatechin units, but its HPLC chromatogram gave only a single peak, indicating a procyanidin dimer consisting of epicatechin and catechin. The carbon signal at 76.74 ppm in the upfield region was characteristic for the C2 of epicatechin substituted on C-4 and that at 81.77 ppm was consistent with the C2 of catechin as the terminating unit (Foo & Karchesy, 1989). The position of the interflavanoid linkage was established as 4,8 by HPLC comparison with a procyanidin B1 authentic sample isolated from Douglas fir inner bark (Foo & Karchesy, 1989); hence compound **10** was procyanidin B1 (epicatechin-(4,8)-catechin).

3.4. Flavonoids

Seven compounds (**11** to **17**) were isolated from fraction D. Compound **11**, the most abundant polyphenol in the grape pomace extract, was determined as quercetin 3-glucoside from its NMR and also by comparison of its HPLC retention time and the UV absorption characteristic with that of an authentic sample isolated previously from apple pomace (Lu & Foo, 1997). The ^{1}H NMR spectrum of compound **11** showed two doublets (δ 6.20 and 6.40, *J* 2.0 Hz) consistent with the *meta* protons H-6 and H-8 on the phloroglucinol A-ring and an ABX system (δ 6.84, *J* 8.4 Hz; 7.53, *J* 2.1 Hz; 7.60, *J* 8.4 and 2.1 Hz) for the catechol B-ring protons, while the ^{13}C NMR spectrum was consistent with that of quercetin 3-glucoside reported by Markham and Chari

(1982). Compound **12** had similar UV and NMR spectra to those of quercetin 3-glucoside. The ^{13}C NMR spectrum showed, in addition to quercetin carbon signals, a carboxylic acid carbon (δ 170.17), a glycosidic carbon (δ 101.51) and four oxygenated carbons (δ 76.43, 76.31, 74.22 and 71.78). These data were typical for a sugar acid and consistent with glucuronic acid. Thus, compound **12** was assigned as quercetin 3-glucuronide.

Compounds 13 (major) and 14 (minor) were co-eluted as a mixture. The major compound exhibited a similar ^{13}C NMR spectrum to that of quercetin 3-glucoside but instead of the catechol B-ring, there was a *para*-substituted phenol ring (δ 115.07×2, 120.89, 130.84×2, 159.91). The latter was also confirmed by the ^{1}H NMR spectrum which contained an AB system (δ 6.86 and 8.06, *J* 8.6 Hz). These spectral characteristics suggested that compound 13 was kaempferol 3-glucoside. The minor compound **14** had carbon signals consistent with the presence of a kaempferol moiety; slight differences for the sugar carbon signals of compound **13** were due to the presence of a galactopyranoside instead. Thus the chemical structure of compound **14** was established as kaempferol 3-galactoside.

The ^{13}C NMR spectrum of compound **15** had similar carbon signals for the phloroglucinol A-ring and pyran C-ring, but none for the B-ring. The sugar moiety was identified as rhamnose from its characteristic high field methyl signal at 18.09 ppm in ^{13}C NMR spectrum and a doublet at 1.13 ppm (*J* 6.2 Hz) in the ^{1}H NMR spectrum. The very low field sharp singlet at 8.33 ppm was characteristic of the H-2 signal for isoflavonols. These spectral characteristics suggested compound **15** to be 3,5,7-trihydroxychromone 3-rhamnoside (eucryphin) which was confirmed by spectral comparison with published data (Tschesche, Delhvi, Sepulvedi, & Breitmaier, 1979). The structure was also supported by ESMS which showed a peak at *m/z* 339.4 consistent with the $[M–H]^{-1}$ ion.

The NMR spectra of compounds **16** and **17** showed that both contained a rhamnose residue with the characteristic doublet at 1 ppm (*J* 6 Hz) in the ^{1}H NMR spectra and carbon signal at 18 ppm in their ^{13}C NMR spectra. Compound **16** had similar carbon signals for the A- and B-rings as quercetin glycosides; the C-ring signals were different. The downshift of the C-4 signal (δ 194.30) and upshift of C-2 and C-3 (δ 81.48 and 75.62) suggested that the double bond was saturated and dihydroquercetin was the aglycone. This was evident also from the presence of two doublets (δ 4.63 and 5.23, *J* 9.8 Hz) for H-2 and H-3, respectively, in the ^{1}H NMR spectrum; hence compound **16** was dihydroquercetin 3-rhamnoside (astilbin). Similarly, by spectral comparison with those of astilbin and kaempferol 3-glycosides, compound **17**, because of the presence of an AB spin system in its ^{1}H NMR spectrum, was identified as dihydrokaempferol 3-rhamnoside (engeletin). The ^{1}H NMR data of both compounds **16** and **17** were consistent with those reported by Trousdale and Singleton (1983).

4. Conclusion

Grape pomace contained a variety of polyphenols which included phenolic acids (gallic acid, its 3- and 4-β-glucopyranosides, *trans*-caftaric acid, *cis*- and *trans*-coutaric acids), phenolic alcohol (2-hydroxy-5-(2-hydroxyethyl)phenyl-β-glucopyranoside), flavan-3-ols (catechin, epicatechin and procyanidin B1) and flavonoids (quercetin 3-glucoside and 3-glucuronide, kaempferol 3-glucoside and 3-galactoside, eucryphin, astilbin and engeletin). Gallic acid 3-β-glucopyranoside, gallic acid 4-β-glucopyranoside and 2-hydroxy-5-(2-hydroxyethyl)phenyl-β-glucopyranoside were all new grape constituents reported here for the first time.

These flavonoids including the oligomeric procyanidins made up approximately 4% of the dried grape pomace and presented a potential valuable source of natural polyphenols.

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