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RAPID COMMUNICATION

# Identification and quantification of major polyphenols in apple pomace

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Apple pomace from juice processing was investigated as a potential source of fine chemicals. Major compounds isolated and identified include epicatechin, caffeic acid, phloretin-2′-glucoside (phloridzin), phloretin-2′-xyloglucoside, 3-hydroxyphloridzin, quercetin-3-arabinoside (avicularin), quercetin-3-xyloside (reynoutrin), quercetin-3-galactoside (hyperin), quercetin-3-glucoside (isoquercitrin) and quercetin-3-rhamnoside (quercitrin). The isolation of 3-hydroxyphloridzin as a natural component in apple was novel and its chemical structure, as well as those of the other co-occurring polyphenols, were established by $^{1}H$ and $^{13}C$ NMR spectroscopy and by acid hydrolysis. © 1997 Elsevier Science Ltd. All rights reserved

## INTRODUCTION

Apple polyphenols have been the subject of a number of investigations due mainly to their physiological and physical properties which cause problems during apple juice processing (Duncan & Dustman, 1936; Hulme, 1953; Siegelman, 1955; Sun & Francis, 1967; Schmidt & Neukom, 1969; Whiting & Coggins, 1975; Teuber & Herrmann, 1978; Wilson, 1981; Dick *et al.*, 1987; Oleszek *et al.*, 1988). The oxidation of these compounds by polyphenoloxidase (PPO) has been suggested to be the main cause of apple browning (Harel *et al.*, 1964; CoSeteng & Lee, 1987; Murata *et al.*, 1995). The rates of enzymatic browning as well as changes in their concentration during apple development and maturation and during storage have also been investigated (Mosel & Herrmann, 1974; Burda *et al.*, 1990; Lister *et al.*, 1994).

Polyphenols in apple pomace have not been investigated in any detail in the past. This study serves to bridge this gap by evaluating apple pomace as a potential source of polyphenols. There has recently been much interest in natural food polyphenols because of their potential to act as free-radical scavengers. Oxidative damage to cellular components such as lipids and cell membranes by free radicals and other reactive oxygen species is believed to be associated with the development of a range of degenerative diseases including cancer and atherosclerosis (Ames, 1983; Steinberg, 1988, 1992; Kinsella *et al.*, 1993).

## MATERIALS AND METHODS

### Extraction

Freeze-dried Gala apple pomace (200 g) from juice processing, supplied by Enza Processors (Hastings, New Zealand), was ground to powder (sieve 1 mm) and extracted at ambient temperature with 70% aqueous acetone (3×500 ml). The combined extract was concentrated on a rotary evaporator at 40°C under reduced pressure and the aqueous residue defatted with hexane (3×300 ml), then concentrated and freeze-dried to afford 45 g of solid.

### Chromatographic separation

Fractionation of the above freeze-dried extract was performed on a Sephadex LH-20 column (60 cm×4 cm ID) using aqueous methanol as solvent, the methanol content being increased from 0% to 100% in increments of 10%, then eluting with 1 litre of each solvent to yield 11 fractions. Fractions obtained from the 30–70% aqueous methanol containing the main bulk of the polyphenols were separately subjected to further chromatographic separation on Sephadex LH-20. Fractions were collected using a fraction collector and monitored by high-performance liquid chromatography (HPLC). Fractions of the same composition were combined and rechromatographed until pure compounds were isolated

and freeze-dried. In the case of 3-hydroxyphloridzin, a reverse-phase column (Mitsubishi Dianion HP-20) developed with aqueous methanol (30%) was used at the final purification step.

### HPLC analysis

HPLC analyses of extracts and chromatographic fractions were performed isocratically on a Pye Unicam Spherisorb ODS (5 μm) column (25 cm×4 mm ID) using 40% aqueous methanol containing 1% acetic acid. Samples or standard solutions prepared by dissolving 1 mg in 10 ml of 40% aqueous methanol were injected onto the column (injection volume 10 μl). The flow rate was set at 0·8 ml $min^{-1}$ and detection of compounds done by UV absorption set at 280 nm.

### Hydrolysis of phenolic glycosides

A small sample of purified phenolic glycosides (1–2 mg) was hydrolysed by dissolving it in 1 ml of 2 N HCl and then the solution was heated on a boiling water bath for 1 h. The hydrolysis products were compared with standard sugar samples on Whatman 3 mm chromatographic paper developed with *n*-butanol–pyridine–water (6:4:3, v/v). The sugar spots were visualised by spraying with a methanol solution of anilinium hydrogen phthalate and heating in an oven at 110°C for 10 min.

### Identification

Purified phenolic compounds were identified by NMR spectroscopy on a Bruker AC 300 instrument. Samples (10–20 mg) were dissolved in 0·4 ml of deuterated acetone, methanol or dimethylsulphoxide (DMSO) and chemical shifts were referenced to TMS ($^1$H) or solvent signal ($^{13}$C).

*Epicatechin* (1). $^1$H NMR (300 MHz, acetone-$d_6$): δ 2·71 (d, *J* 16·6 Hz, $H_\beta$-4), 2·85 (dd, *J* 16·6, 4·5 Hz, $H_\alpha$-4), 4·21 (m, H-3), 4·86 (s, H-2), 5·93 (d, *J* 2·2 Hz, H-6), 6·02 (d, *J* 2·2 Hz, H-8), 6·81 (m, H-5′, H-6′), 7·04 (s, H-2′). $^{13}$C NMR (75 MHz, acetone-$d_6$): δ 28·84 (C-4), 66·84 (C-3), 79·35 (C-2), 95·48 (C-8), 96·16 (C-6), 99·66 (C-10), 115·21 (C-2′), 115·48 (C-5′), 119·19 (C-6′), 132·00 (C-1′), 145·23 (C-3′), 145·36 (C-4′), 156·94 (C-5), 157·46 (C-7), 157·56 (C-9).

*Caffeic acid* (2). $^1$H NMR (300 MHz, DMSO-$d_6$): δ 6·22 (d, *J* 15·9 Hz, H-8), 6·81 (d, *J* 8·2 Hz, H-5), 7·02 (dd, *J* 8·2, 1·9 Hz, H-6), 7·08 (d, *J* 1·9 Hz, H-2), 7·47 (d, *J* 15·9 Hz, H-7). $^{13}$C NMR (75 MHz, DMSO-$d_6$): δ 114·66 (C-8), 115·23 (C-2), 115·82 (C-5), 121·21 (C-6), 125·77 (C-1), 144·60 (C-7), 145·62 (C-3), 148·19 (C-4), 168·00 (CO).

*Phloretin-2′-O-β-D-glucopyranoside* (*phloridzin*) (3). $^1$H NMR (300 MHz, DMSO-$d_6$): δ 2·79 (t, *J* 7·3 Hz, β-$CH_2$), 3·20-3·73 (m, sugar-H, α-$CH_2$), 4·58, 5·03, 5·13, 5·27 (4×sugar-OH), 4·93 (d, *J* 7·2 Hz, H-1″), 5·93 (d, *J* 2·1 Hz, H-5′), 6·13 (d, *J* 2·1 Hz, H-3′), 6·64 (d, *J* 8·4 Hz, H-3, H-5), 7·03 (d, *J* 8·4 Hz, H-2, H-6), 9·08 (OH). $^{13}$C NMR (75 MHz, DMSO-$d_6$): δ 28·98 (β-C), 44·93 (α-C), 60·53 (C-6″), 69·42 (C-4″), 73·17 (C-2″), 76·67 (C-3″), 77·24 (C-5″), 94·31 (C-3′), 96·80 (C-5′), 100·79 (C-1″), 105·14 (C-1′), 114·95 (C-3, C-5), 129·13 (C-2, C-6), 131·50 (C-1), 155·23 (C-4), 160·80 (C-2′), 164·44 (C-6′), 165·33 (C-4′), 204·67 (CO).

*Phloretin-2′-O-(β-D-xylopyranosyl-(1-6)-β-D-glucopyranoside)* (4). $^1$H NMR (300 MHz, DMSO-$d_6$): δ 2·78 (t, *J* 7·2 Hz, β-$CH_2$), 3·00 (m, H-2‴, $H_\alpha$-5‴), 3·15 (m, H-3‴), 3·28 (m, H-4‴), 3·30 (m, H-2″,3″,4″), 3·35 (m, α-$CH_2$), 3·55 (m, H-5″), 3·60 (m, $H_\alpha$-6″), 3·70 (m, $H_\beta$-5‴), 3·96 (d, *J* 11·0 Hz, $H_\beta$-6″), 4·18 (d, *J* 7·3 Hz, H-1‴), 4·89 (d, *J* 7·0 Hz, H-1″), 5·93 (d, *J* 1·9 Hz, H-5′), 6·20 (d, *J* 1·9 Hz, H-3′), 6·64 (d, *J* 8·4 Hz, H-3, H-5), 7·03 (d, *J* 8·4 Hz, H-2, H-6). $^{13}$C NMR (75 MHz, DMSO-$d_6$): δ 28·95 (β-C), 44·92 (α-C), 65·65 (C-5‴), 68·08 (C-6″), 69·37 (C-4″), 69·61 (C-4‴), 73·20 (C-2″), 73·44 (C-2‴), 76·02 (C-5″), 76·34 (C-3‴), 76·62 (C-3″), 94·56 (C-3′), 96·98 (C-5′), 101·05 (C-1″), 103·92 (C-1‴), 105·25 (C-1), 115·00 (C-3, C-5), 129·20 (C-2, C-6), 131·57 (C-1), 155·28 (C-4), 160·80 (C-2′), 164·40 (C-6′), 165·31 (C-4′), 204·73 (CO).

*3-Hydroxyphloretin-2′-O-β-D-glucopyranoside* (*3-hydroxyphloridzin*) (5). $^1$H NMR (300 MHz, DMSO-$d_6$): δ 2·68 (t, *J* 7·3 Hz, β-$CH_2$), 3·28 (m, α-$CH_2$), 3·17-3·66 (m, sugar-H), 4·56 (sugar-OH), 4·87 (d, *J* 7·2 Hz, H-1″), 4·99, 5·08, 5·21 (3×sugar-OH), 5·88 (d, *J* 2·1 Hz, H-5′), 6·08 (d, *J* 2·1 Hz, H-3′), 6·42 (dd, *J* 8·0, 2·0 Hz, H-6), 6·54 (d, *J* 8·0 Hz, H-5), 6·56 (d, *J* 2·0 Hz, H-2), 8·51, 8·58, 10·54, 13·48 (3, 4, 4′, 6′-OH). $^{13}$C NMR (75 MHz, DMSO-$d_6$): δ 29·30 (β-C), 45·05 (α-C), 60·75 (C-6″), 69·63 (C-4″), 73·32 (C-2″), 76·88 (C-3″), 77·43 (C-5″), 95·48 (C-3′), 96·98 (C-5′), 100·97 (C-1″), 105·37 (C-1′), 115·57 (C-2), 116·00 (C-5), 119·05 (C-6), 132·53 (C-1), 143·28 (C-3), 145·02 (C-4), 160·98 (C-2′), 164·56 (C-6′), 165·50 (C-4′), 204·92 (CO).

*Quercetin-3-O-β-D-galactopyranoside* (*hyperin*) (6). $^1$H NMR (300 MHz, methanol-$d_4$): δ 3·11-3·68 (m, sugar-H), 4·95 (d, *J* 6·7 Hz, H-1″), 6·02 (s, H-6), 6·21 (s, H-8), 6·71 (d, *J* 8·1 Hz, H-5′), 7·35 (d, *J* 8·1 Hz, H-6′), 7·59 (s, H-2′). $^{13}$C NMR (75 MHz, methanol-$d_4$): δ 61·87 (C-6″), 69·95 (C-4″), 73·20 (C-2″), 74·90 (C-3″), 77·06 (C-5″), 95·29 (C-8), 100·27 (C-6), 105·20 (C-1″), 105·81 (C-10), 116·52 (C-2′), 118·07 (C-5′), 123·03 (C-1′), 123·42 (C-6′), 135·82 (C-3), 145·73 (C-3′), 149·85 (C-4′), 158·43 (C-9), 159·07 (C-2), 162·60 (C-5), 165·75 (C-7), 179·63 (C-4).

*Quercetin-3-O-β-D-glucopyranoside* (*isoquercitrin*) (7). $^1$H NMR (300 MHz, methanol-$d_4$): δ 3·11-3·68 (m, glucose H), 4·85 (d, *J* 7·7 Hz, H-1″), 6·02 (s, H-6), 6·21 (s, H-8), 6·71 (d, *J* 8·1 Hz, H-5′), 7·35 (d, *J* 8·1 Hz, H-6′), 7·59 (s, H-2′). $^{13}$C NMR (75 MHz, methanol-$d_4$): δ 62·45 (C-6″), 71·10 (C-4″), 75·68 (C-2″), 77·85 (C-3″), 78·21 (C-5″), 95·29 (C-8), 100·27 (C-6), 104·22 (C-1″), 105·81 (C-10), 116·52 (C-2′), 117·87 (C-5′), 123·22 (C-1′), 123·62 (C-6′), 135·66 (C-3), 145·73 (C-3′), 149·85 (C-4′), 158·43 (C-9), 159·39 (C-2), 162·60 (C-5), 165·75 (C-7), 179·60 (C-4).

*Quercetin-3-O-β-D-xylopyranoside* (*reynoutrin*) (8). $^1$H NMR (300 MHz, methanol-$d_4$): δ 3·17-4·21 (m, sugar-H), 5·04 (d, *J* 7·1 Hz, H-1″), 6·06 (d, *J* 2·1 Hz, H-6), 6·25 (d, *J* 2·1 Hz, H-8), 6·74 (d, *J* 8·4 Hz, H-5′), 7·34 (d, *J* 2·1 Hz, H-2′), 7·46 (dd, *J* 8·4, 2·1 Hz, H-6′). $^{13}$C NMR (75 MHz, methanol-$d_4$): δ 67·3 (C-5″), 71·1 (C-3″), 75·4 (C-2″), 77·6 (C-4″), 94·9 (C-8), 100·0 (C-6), 104·8 (C-1″), 105·7 (C-10), 116·1 (C-2′), 117·4 (C-5′), 123·1 (C-1′), 123·2 (C-6′), 135·4 (C-3), 146·1 (C-3′), 149·9 (C-4′), 158·6 (C-9), 159·4 (C-2), 163·1 (C-5), 166·1 (C-7), 179·5 (C-4).

*Quercetin-3-O-α-L-arabinofuranoside* (*avicularin*) (9). $^1$H NMR (300 MHz, DMSO-$d_6$): δ 3·17-3·75 (m, sugar-H), 4·16 (d, *J* 2·6 Hz, H-5″), 5·59 (d, *J* 1·0 Hz, H-1″), 6·20 (d, *J* 2·0 Hz, H-6), 6·41 (d, *J* 2·0 Hz, H-8), 6·86 (d, *J* 8·4 Hz, H-5′), 7·48 (d, *J* 2·2 Hz, H-2′), 7·55 (dd, *J* 8·5, 2·2 Hz, H-6′), 12·6 (OH-5). $^{13}$C NMR (75 MHz, DMSO-$d_6$): δ 60·66 (C-5″), 76·96 (C-3″), 82·12 (C-2″), 85·86 (C-4″), 93·57 (C-8), 98·70 (C-6), 103·93 (C-10), 107·85 (C-1″), 115·54 (C-2′, C-5′), 120·96 (C-1′), 121·71 (C-6′), 133·39 (C-3), 145·09 (C-3′), 148·48 (C-4′), 156·37 (C-9), 156·94 (C-2), 161·23 (C-5), 164·32 (C-7), 177·70 (C-4).

*Quercetin-3-O-α-L-rhamnopyranoside* (*quercitrin*) (10). $^1$H NMR (300 MHz, DMSO-$d_6$): δ 0·81 (d, *J* 6·8 Hz, H-6″), 3·14-3·64 (sugar-H), 3·97 (s, H-2″), 5·24 (d, *J* 1·2 Hz, H-1″), 6·19 (d, *J* 2·0 Hz, H-6), 6·38 (d, *J* 2·0 Hz, H-8), 6·86 (d, *J* 8·3 Hz, H-5′), 7·24 (dd, *J* 8·3, 2·1 Hz, H-6′), 7·29 (d, *J* 2·1 Hz, H-2′). $^{13}$C NMR (75 MHz, DMSO-$d_6$): δ 17·47 (C-6″), 70·04 (C-5″), 70·35 (C-3″), 70·56 (C-2″), 71·18 (C-4″), 93·61 (C-8), 98·67 (C-6), 101·82 (C-1″), 104·06 (C-10), 115·44 (C-2′), 115·64 (C-5′), 120·73 (C-1′), 121·09 (C-6′), 134·21 (C-3), 145·19 (C-3′), 148·42 (C-4′), 156·43 (C-9), 157·28 (C-2), 161·28 (C-5), 164·19 (C-7), 177·73 (C-4).

### Quantification

A 50 g sample of freeze-dried apple pomace was extracted with 70% aqueous acetone as described. The extract was concentrated to low volume on a rotary evaporator and the residue diluted to 100 ml with methanol. A 1 ml aliquot was pipetted out and diluted to 4 ml with 40% methanol. After filtration, a 10 μl sample was injected onto the HPLC column ($C_{18}$) (Spherisorb, 5 μm) operated isocratically with 40% aqueous methanol containing 1% acetic acid as eluent at a flow rate of 0·8 ml min$^{-1}$. Quantification of cinnamic acids, catechins and dihydrochalcone glycosides (detected at 280 nm) and quercetin glycosides (detected at 350 nm) was achieved by an external method using caffeic acid (Aldrich), epicatechin, phloridzin (both isolated from apple pomace) and quercetin-3-glucoside (isoquercitrin, isolated from grape pomace) as standards (all had purity greater than 95% determined by HPLC). Thus, the cinnamic acids were quantified as caffeic acid, catechins as epicatechin, dihydrochalcone glycosides as phloridzin and quercetin glycosides as isoquercitrin. The calibration curves were generated by repeated injections of a fixed volume (10 μl) of standard solutions covering a broad range of concentrations. Within the concentrations up to 200 mg litre$^{-1}$ the external standards gave satisfactory linearity with $R^2$ 0·996, 0·992, 0·995 and 0·986 for caffeic acid, epicatechin, phloridzin and quercetin-3-glucoside, respectively.

## RESULTS AND DISCUSSION

### Isolation and identification of major polyphenols

The HPLC chromatogram (Fig. 1) of apple pomace extract obtained under isocratic conditions (40% aqueous methanol containing 1% acetic acid as eluting solvent) showed that resolution of phenolic compounds accorded with structural class with first elution of polar phenolic acids and catechins (0–14 min), followed by dihydrochalcone glycosides (15–25 min) and lastly by quercetin glycosides (30–50 min). In addition, it gave successful resolution of most of the major phenolic

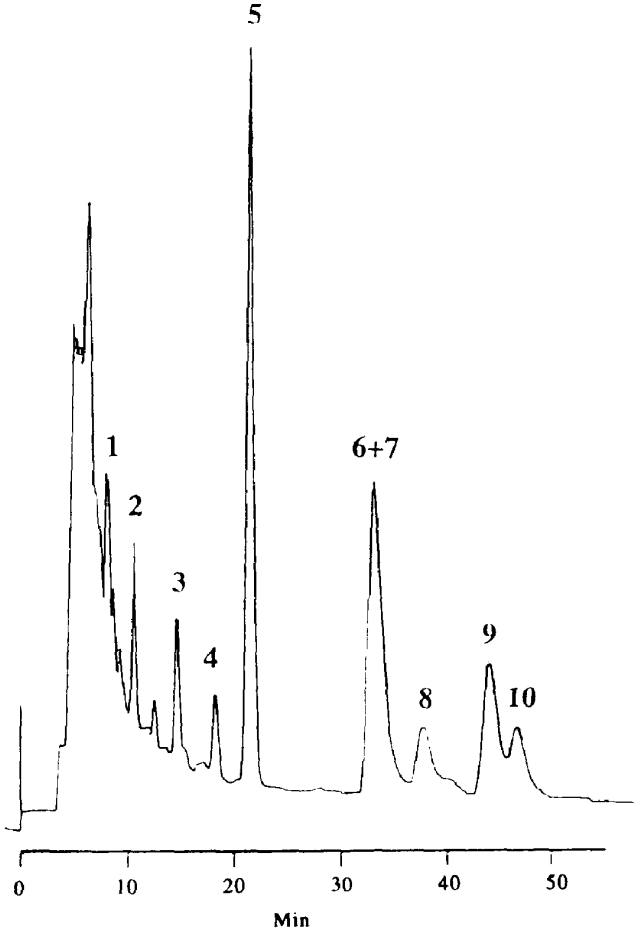



**Fig. 1.** HPLC chromatogram of 70% acetone extract of apple pomace. Peak identifications: **1**, epicatechin; **2**, caffeic acid; **3**, 3-hydroxyphloridzin; **4**, phloretin-2′-xyloglucoside; **5**, phloridzin; **6**, quercetin-3-galactoside; **7**, quercetin-3-glucoside; **8**, quercetin-3-xyloside; **9**, quercetin-3-arabinoside; **10**, quercetin-3-rhamnoside.

**Table 1. Main phenolics in each LH-20 chromatographic fraction**

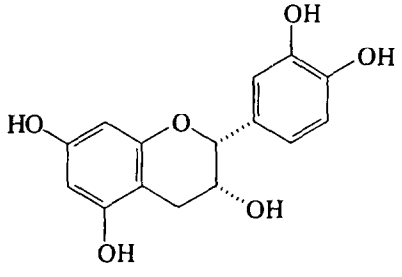
| Fraction no. | Eluent (% methanol) | Main phenolics |
|---|---|---|
| 0–2 | 0–20 | Sugars and polar phenolic acids (not yet identified) |
| 3 | 30 | Epicatechin |
| 4 | 40 | Phloretin-2′-xyloglucoside, phloridzin |
| 5 | 50 | Caffeic acid, phloridzin, 3-hydroxyphloridzin |
| 6 | 60 | Phloridzin, quercetin-3-galactoside/ glucoside, quercetin-3-rhamnoside |
| 7 | 70 | Quercetin-3-xyloside, quercetin-3-arabinoside |
| 8–10 | 80–100 | Procyanidins and oligomers |

compounds, with the exception of quercetin-3-galactoside (hyperin) and quercetin-3-glucoside (isoquercitrin).

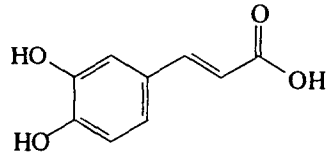
Individual phenolic compounds were isolated by repeated column chromatography on Sephadex LH-20. Initial fractionation of the extract was performed with aqueous methanol containing increasing methanol content (from 0% to 100%) to give 11 fractions (1 litre each). Fractions 3–7 eluted with 30–70% aqueous methanol were shown by HPLC to contain the major phenolics of interest (see Table 1). These were then separately subjected to further chromatographic separation on Sephadex LH-20 and on a reverse-phase matrix (Mitsubishi Dianion HP-20) until pure compounds, as indicated on HPLC, were obtained. In this manner all the four groups of compounds, namely catechins, cinnamic acids, dihydrochalcone glycosides and quercetin glycosides (for chemical structures see Scheme 1), were successfully isolated and identified by spectroscopic and chemical methods.

*Catechins*

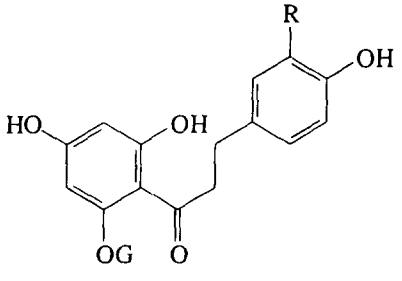
Compound (1) was readily isolated by re-chromatography of fraction 3 on a Sephadex LH-20 column. Its identity as epicatechin was apparent from the $^{13}C$ NMR spectrum which showed a methylene carbon in the upfield region (δ 28·84 ppm) and two oxygenated methine carbons in the heterocyclic region (δ 66·84 and 79·35 ppm) which were characteristic of the pyran C-ring of flavanols. The upfield position of the C-2 carbon (δ 79·35 ppm) was characteristic of the epicatechin

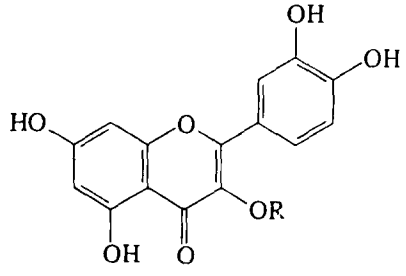



**1** epicatechin




**2** caffeic acid




| | | | | | |
|---|---|---|---|---|---|
| **3** | R=OH; G=Glu | 3-hydroxyphloridzin | **6** | R=Gal | quercetin-3-galactoside |
| **4** | R=H; G=Xyloglu | phloretin-2′-xylo-glucoside | **7** | R=Glu | quercetin-3-glucoside |
| | | | **8** | R=Xyl | quercetin-3-xyloside |
| **5** | R=H; G=Glu | phloridzin | **9** | R=Ara | quercetin-3-arabinoside |
| | | | **10** | R=Rha | quercetin-3-rhamnoside |

**Scheme 1.** Chemical structures of polyphenols from apple pomace.

chemical shift (Markham & Chari, 1982), and this was confirmed by the *cis* orientation of the C-2 and C-3 substituents on the heterocyclic C-ring as revealed by the small proton–proton couplings between the H-2 and H-3 protons. The presence of epicatechin was consistent with its reported presence in both the apple flesh and skin (CoSeteng & Lee, 1987; Burda *et al.*, 1990).

*Cinnamic acids*

Compound (2) was isolated as a minor constituent from fraction 5 by further treatment on Sephadex LH-20 column. The cinnamic acid constitution was evident from the $^{1}H$ and $^{13}C$ NMR spectra which showed the presence of an aromatic ring, an ethylene moiety as well as a carboxylic acid group. The *trans* configuration of the double bond was shown by the two doublets (δ 6·22 and 7·47 ppm) with a large coupling constant (*J* 16 Hz). The ABC type coupling pattern of the aromatic protons δ 6·81, 7·02 and 7·08 ppm) in the $^{1}H$ NMR spectrum was evidence of a 1,2,4-trisubstituted phenyl ring. This was corroborated by $^{13}C$ NMR data which were consistent with a catechol aromatic ring hence the compound was caffeic acid. The identity of caffeic acid was further confirmed by comparison with an authentic sample on HPLC.

The presence of caffeic acid in apple pomace which, to our knowledge, has not been reported as a natural apple constituent, could be a result of degradation of caffeoylquinic acids (chlorogenic acid and its isomers) which were tentatively identified as minor components. These cinnamic esters have been reported as natural apple constituents (Macheix *et al.*, 1990) and chlorogenic acid has been identified in apple as a major component (Hulme, 1953; Walker, 1963).

*Dihydrochalcone glycosides*

Compound (5) as a major phenolic in fraction 4 and fraction 5 was readily isolated from the Sephadex LH-20 column with aqueous methanol. Its $^{1}H$ NMR spectrum showed the presence of a sugar unit and two mutually coupled doublets (*J* 2 Hz) in the aromatic region indicative of an unsymmetrically substituted phloroglucinol ring. This proton coupling pattern was suggestive of the sugar being attached on the 2 position of the phloroglucinol ring. In addition, the presence of an AA′BB′-type quartet consistent with the protons of a *para* disubstituted phenyl ring and two mutually coupled triplets consistent with the α and β methylene protons to a carbonyl group indicated the compound (5) to be phloretin-2′-β-D-glucopyranoside (phloridzin). The β-anomeric configuration of the attached sugar was established by its coupling constant (*J* 7·2 Hz). Further confirmation of the compound identity was made by hydrolysis which yielded glucose and the aglycon phloretin.

Phloridzin has been known to occur in apple for a long time and its chemical structure fully characterised (Whiting & Coggins, 1975; Wilson, 1981; Dick *et al.*, 1987). Although phloridzin is the main phenolic compound in most cultivated apple, it has been also reported that in certain species it can be wholly or partly replaced by phloretin-4′-glucoside and 3-hydroxyphloretin-4′-glucoside (Williams, 1961). The presence of phloridzin has been regarded as characteristic of apple fruit and apple juice (Duggan, 1969) and has been employed in determining the authenticity of apple products (Spanos & Wrolstad, 1992).

Compound (3) which co-eluted with phloridzin from the Sephadex LH-20 column in fraction 5 was successfully resolved from phloridzin on a Mitsubishi Dianion HP-20 column. On acid hydrolysis it gave glucose. The $^{1}H$ NMR spectrum of the compound was similar to that of phloridzin with the pair of α and β methylene protons each appearing as triplets (the latter partially obscured by the sugar protons) together with a pair of doublets of the unsymmetrically substituted phloroglucinol ring. Instead of the four proton AA′BB′ quartet observed for phloridzin, the minor compound exhibited a three proton ABC pattern typical of 1,2,4-trisubstituted benzene. In the $^{13}C$ NMR spectrum two oxygenated aromatic carbons characteristic of the catechol ring were observed at 143·42 and 145·36 ppm. This observation unambiguously established the additional OH on the 3-position in the aromatic ring and established the structure as 3-hydroxyphloridzin. The $^{1}H$ NMR data were consistent with those reported for 3-hydroxyphloridzin (El-Naggar *et al.*, 1980) and the $^{13}C$ NMR data are given in Table 2 for the first time. The isolation of 3-hydroxyphloridzin from apple pomace established it to be a natural constituent of apple. It was first prepared by partial oxidation of phloridzin by apple enzymes in the presence of ascorbate (Hunter, 1975). It has also been isolated from the leaves of *Kalmia latifolia* (Ericaceae) (El-Naggar *et al.*, 1980) and manyspike tan oak (*Lithocarpus polystachyus*) (Yang *et al.*, 1991).

In order to establish that 3-hydroxyphloridzin isolated from apple pomace was a natural apple constituent and not an enzymatic oxidation product of phloridzin, several varieties of apple available on the local market including Granny Smith, Red Delicious, Golden Delicious, Splendour, Braeburn, Fuji and Royal Gala were examined. 3-Hydroxyphloridzin was present at varying levels in all apple samples. Red Delicious in particular was found to contain as much 3-hydroxyphloridzin as phloretin-2′-xyloglucoside (about 14% of phloridzin level). However, quantitative comparison of the level of 3-hydroxyphloridzin in fresh hand-peeled Gala apple skin (0·04 g $kg^{-1}$ dry matter, DM) to that found in Gala apple pomace (0·27 g $kg^{-1}$ DM) suggested that the enzymic oxidation of phloridzin to 3-hydroxyphloridzin during juice processing could not be excluded.

Compound (4) as a minor phenolic in fraction 4, which eluted earlier than phloridzin from the Sephadex LH-20 column, was also isolated. Its $^{1}H$ NMR spectrum showed an AA′BB′ quartet characteristic of

**Table 2. $^{13}C$ NMR data (measured in DMSO-$d_6$) of phloretin, phloridzin, 3-hydroxyphloridzin and phloretin-2′-xyloglucoside**

| | Phloretin | Phloridzin | 3-Hydroxy-phloridzin | Phloretin-2′-xyloglucoside |
|---|---|---|---|---|
| C-1 | 131·63 | 131·50 | 132·53 | 131·57 |
| C-2 | 129·13 | 129·13 | 115·57 | 129·20 |
| C-3 | 115·05 | 114·95 | 143·28 | 115·00 |
| C-4 | 155·37 | 155·23 | 145·02 | 155·28 |
| C-5 | 115·05 | 114·95 | 116·00 | 115·00 |
| C-6 | 129·13 | 129·13 | 119·05 | 129·20 |
| C-β | 29·43 | 28·98 | 29·30 | 28·95 |
| C-α | 45·42 | 44·93 | 45·05 | 44·92 |
| CO | 204·21 | 204·67 | 204·92 | 204·73 |
| C-1′ | 103·73 | 105·14 | 105·37 | 105·25 |
| C-2′ | 164·19 | 160·80 | 160·98 | 160·80 |
| C-3′ | 94·66 | 94·31 | 94·48 | 94·56 |
| C-4′ | 164·58 | 165·33 | 165·50 | 165·31 |
| C-5′ | 94·66 | 96·80 | 96·98 | 96·98 |
| C-6′ | 164·19 | 164·44 | 164·56 | 164·40 |
| C-1″ | | 100·79 | 100·97 | 101·05 |
| C-2″ | | 73·17 | 73·32 | 73·20 |
| C-3″ | | 76·67 | 76·88 | 76·62 |
| C-4″ | | 69·42 | 69·63 | 69·37 |
| C-5″ | | 77·24 | 77·43 | 76·02 |
| C-6″ | | 60·53 | 60·75 | 68·08 |
| C-1‴ | | | | 103·92 |
| C-2‴ | | | | 73·44 |
| C-3‴ | | | | 76·34 |
| C-4‴ | | | | 69·61 |
| C-5‴ | | | | 65·65 |

the disubstituted phenyl protons and also a pair of doublets for the *meta* protons of the phloroglucinol ring and was hence similar to that of phloridzin. The $^{13}C$ NMR spectrum revealed the presence of two sugar molecules with a total of 11 carbon signals with the chemical shift at 68·08 ppm which was consistent with a 1–6 linkage between the two sugars. On acid hydrolysis it afforded phloretin and two sugars which were identified as glucose and xylose. Compound (4) was consistent with phloretin-2′-β-D-xylopyranosyl-(1–6)-β-D-glucopyranoside which was confirmed by comparison of its $^1H$ NMR data with those reported (Tomas-Barberan *et al.*, 1993). The $^{13}C$ NMR data of phloretin-2′-xyloglucoside has not been previously reported and are shown in Table 2. The disaccharide moiety in phloretin-2′-xyloglucoside was similar to those found in genkwanine-5-xyloglucoside (Garcia-Granados & Saenz de Buruaga, 1980) and vomifoliol-1-xyloglucoside (Schwab & Schreier, 1990).

*Quercetin glycosides*

The strong absorption at 350 nm of the fractions eluted after phloridzin on HPLC suggested that these compounds were flavonoids. Acid hydrolysis of these fractions (fraction 6 and fraction 7) afforded quercetin as the only aglycone and various sugars which included galactose (major), glucose, rhamnose (fraction 6), arabinose and xylose (fraction 7), thus indicating the fractions to consist primarily of quercetin glycosides.

Further separation of fraction 6 on Sephadex LH-20 column afforded two fractions. The $^1H$ NMR spectra of both fractions showed evidence for the quercetin moiety, namely two singlets at approx. 6·0 and 6·2 ppm for the two *meta* protons on the A-ring and an ABC type protons of the B-ring. The $^{13}C$ NMR spectrum of the first fraction revealed two sets of sugar signals of unequal intensity assignable to galactose and glucose in the ratio of 65:35, respectively. The minor component (compound 7) was identified as quercetin-3-*O*-β-D-glucopyranoside by spectral comparison to an authentic sample and the major one (compound 6) therefore was quercetin-3-*O*-β-D-galactopyranoside. The β-anomeric configurations of both glycosides were derived from their coupling constants *J* 6·9 and 7·7 Hz for glucoside and galactoside, respectively (Markham & Geiger, 1993).

The $^{13}C$ NMR spectrum of fraction II (compound 10) showed an upfield signal at 17·5 ppm diagnostic of rhamnose. The small proton coupling constant (*J* 1·2 Hz) observed for the anomeric proton showed the rhamnose to have the α-configuration and hence compound (10) was quercetin-3-*O*-α-L-rhamnopyranoside.

Further chromatography of fraction 7 on Sephadex LH-20 column resulted in the isolation of the major compound quercetin-3-α-L-arabinofuranoside (9) and a minor compound quercetin-3-β-D-xylopyranoside (8). Both their chemical structures were deduced from their respective $^1H$ and $^{13}C$ NMR spectra and further confirmed by acid hydrolysis.

**Quantification of major polyphenols**

Concentrations of polyphenols in apple skin varied considerably from cultivar to cultivar (Burda *et al.*, 1990; Perez-Ilzarbe *et al.*, 1991); total quercetin glycosides for instance were found at 158 mg $kg^{-1}$ fresh weight (FW) in Reineta while in Granny Smith the level was 1425 mg $kg^{-1}$ FW (Perez-Ilzarbe *et al.*, 1991). An even higher level of quercetin glycosides in the skin of Granny Smith (4–7 g $kg^{-1}$ FW or 13–23 g $kg^{-1}$ DM) was reported (Lister *et al.*, 1994). Similar cultivar variation of other phenolics has also been observed for phloridzin (6–654 mg $kg^{-1}$ FW), epicatechin (85–670 mg $kg^{-1}$ FW), procyanidin $B_2$ (103–600 mg $kg^{-1}$ FW) and chlorogenic acid (0·9–204 mg $kg^{-1}$ FW) (Burda *et al.*, 1990; Perez-Ilzarbe *et al.*, 1991).

The major phenolics (see Table 3) in apple pomace were determined by HPLC using an isocratic system (40% methanol plus 1% acetic acid). The concentration of individual polyphenols did not appear to vary very significantly, with quercetin-3-galactoside and phloridzin being the most abundant compounds and phloretin-2′-xyloglucoside being the least abundant. The sum total of these phenolics was 7·24 g $kg^{-1}$ DM, of which more than a half were quercetin glycosides (4·46 g $kg^{-1}$ DM), followed by dihydrochalcone glycosides (1·86 g $kg^{-1}$), catechins (0·64 g $kg^{-1}$) and cinnamic acids (0·28 g $kg^{-1}$). These values were comparable to those in

**Table 3. Amount of major phenolics in apple pomace determined by HPLC**

| Peak | Compound | Amount (g $kg^{-1}$ DM) |
|---|---|---|
| 1 | Epicatechin | 0·64 |
| 2 | Caffeic acid | 0·28 |
| 3 | 3-Hydroxyphloridzin | 0·27 |
| 4 | Phloretin-2′-xyloglucoside | 0·17 |
| 5 | Phloridzin | 1·42 |
| 6 | Quercetin-3-galactoside | 1·61 |
| 7 | Quercetin-3-glucoside | 0·87 |
| 8 | Quercetin-3-xyloside | 0·53 |
| 9 | Quercetin-3-arabinoside | 0·98 |
| 10 | Quercetin-3-rhamnoside | 0·47 |
| | Total | 7·24 |

Granny Smith and Splendour (Lister *et al.*, 1994) and Golden Delicious (Mayr *et al.*, 1995). These results showed that apple pomace could serve as a potential source of polyphenols.

## CONCLUSION

Polyphenols extracted from apple pomace could be successfully separated by column chromatography on a Sephadex LH-20 column which resulted in the isolation of epicatechin, caffeic acid, three dihydrochalcone glycosides and five different quercetin glycosides. 3-Hydroxyphloridzin was characterised for the first time as a natural apple constituent. The total level of the polyphenols in the pomace was about 7·24 g $kg^{-1}$ DM, of which more than half consisted of quercetin glycosides (4·46 g $kg^{-1}$ DM). The result showed that apple pomace contained a high level of polyphenols which could be commercially exploited.

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