ELSEVIER

PII: S0308-8146(97)00123-4

Food Chemistry, Vol. 61, No. 1/2, pp. 29–33, 1998

0308-8146/98 $19.00 + 0.00

Constitution of some chemical components of apple seed

Yinrong Lu & L. Yeap Foo

New Zealand Institute for Industrial Research and Development, Gracefield Road, PO Box 31310, Lower Hutt, New Zealand

(Received 18 April 1997; revised version received 27 May 1997; accepted 27 May 1997)

The hexane extract of apple seed was analyzed by GC–MS and found to consist mainly of fatty acids (80.9%) in its volatile fraction with linoleic acid as the most dominant one (51.2%), followed by palmitic, linolenic, stearic and oleic acids (10.5, 5.6, 4.3 and 4.1%, respectively). The seed pomace was further extracted with 70% aqueous acetone to yield two major compounds, [(6-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]benze-neacetonitrile (amygdalin) (1) and phloretin-2′-β-D-glucopyranoside (phloridzin) (2), which were identified by NMR spectroscopy. A number of minor polyphenols were also identified using HPLC/DAD as chlorogenic acid, *p*-coumarylquinic acid, 3-hydroxyphloridzin, phloretin-2′-xyloglucoside and quercetin glycosides.

INTRODUCTION

A large quantity of apple pomace from juice processing operations is produced worldwide. In New Zealand some 20 000 tonnes are produced annually and mostly used as stock feeds. As a result of its abundance, apple pomace has attracted numerous investigations primarily seeking better applications, but most of these have involved low value products (Kennedy, 1994). We recently investigated the polyphenol constituents contained in apple pomace (Lu and Foo, 1997) as such compounds have attracted a great deal of interest in potential health benefits due to their antioxidant activity (Okuda, 1993; Rice-Evans *et al.*, 1996). Seed makes up a significant part of apple pomace and previous studies on apple seed have mainly focused on the protein and fat contents as food and feed ingredients (Kamel, 1981). While there are extensive data on phenolic compounds in apples (skin and flesh) and apple products (Dick *et al.*, 1987; Oleszek *et al.*, 1988; Suarez Valles *et al.*, 1994), there is limited information on the chemical composition of apple seed (Durkee and Poapst, 1965). This work aims to fill the gap as part of our study on apple waste as a potential source of speciality chemicals.

MATERIALS AND METHODS

Extraction

Royal Gala apple seed was collected from apple pomace provided by Frucor (formerly Enza) Processors Ltd in Hastings, New Zealand. The finely milled (sieve 1 mm) seed (25 g) was firstly extracted with hexane (3×50 ml) and then with 70% aqueous acetone (3×50 ml) to yield the non-polar fraction as an oil (5.0 g) and the polar extract as a freeze-dried powder (1.6 g), respectively.

GC–MS analysis of hexane extract

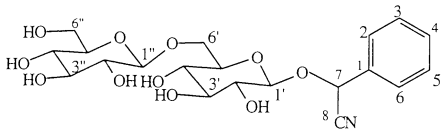
A drop of the hexane extract was dissolved in diethylether (1 ml) and methylated by addition of ethereal diazomethane solution (1 ml) freshly prepared from N-methyl-N-nitroso-*p*-toluenesulfonamide (Diazald®, Aldrich). The methylated sample (2 μl) was injected onto a HP Ultra-2 column (20 m×0.3 mm, I.D.) programmed from 80°C (2 min hold) to 250°C (20 min hold) at 10°C min^{-1} and installed in an HP 5890 GC instrument interfaced to a HP MSD 5970 spectrometer (m/z 40–400, EI at 70 eV). Helium was used as carrier gas. Free fatty acids were identified as the corresponding methyl esters.

Isolation and identification of major compounds from acetone extract

The freeze-dried acetone extract (1.5 g) dissolved in water (5 ml) was applied to a HP20 column (20×1.5 cm, I.D.) prepared in water. The column was eluted with water (100 ml) to remove sugars, followed by aqueous methanol (methanol content being increased from 10 to 70% in increments of 10%, each 50 ml). Chromatographic fractions in 20 ml lots were collected and monitored by UV. Fractions 1–6, and 11 and 12 were combined, respectively, to afford two compounds (1)

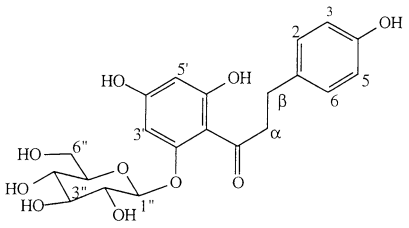
and (2). The former was *ca* 85% pure (estimated by ^{13}C NMR) while the purity of the latter was 95% (HPLC).

^{1}H and ^{13}C NMR spectra of isolated compounds were recorded at ambient temperature on a Bruker AM 300. Chemical shifts (ppm) were referenced to TMS (^{1}H) or to the solvent signal (^{13}C).



amygdalin (1)

Compound (1) was identified as amygdalin. ^{1}H NMR (300 MHz, D_2O/CD_3COCD_3 2:1): δ 3.23-4.25 (12H, sugar-H), 4.53 (1H, *d*, *J* 7.7 Hz, H-1′), 4.60 (1H, obscured, H-1″), 5.91(1H, *s*, H-7), 7.51 (3H, *m*, H-3, H-4, H-5), 7.60 (2H, *d*, *J* 5.7 Hz, H-2, H-6). ^{13}C NMR (75 MHz, D_2O/CD_3COCD_3 2:1): δ 61.59 (C-6″), 69.13 (C-6′), 69.28 (C-7), 70.10 (C-4′), 70.49 (C-4″), 73.67 (C-2′), 74.05 (C-2″), 76.40 (C-3′, C-5′), 76.73 (C-3″, C-5″), 102.51 (C-1′), 103.67 (C-1″), 119.37 (C-8), 128.16 (C-2, C-6), 129.98 (C-3, C-5), 130.93 (C-4), 133.61 (C-1).



phloridzin (2)

Compound (2) was identified as phloridzin. ^{1}H NMR (300 MHz, CD_3COCD_3): δ 2.89 (2H, *t*, *J* 7.4 Hz, β-CH_2), 3.4-4.0 (8H, *m*, sugar-H, *a*-CH_2), 5.08 (1H, *d*, *J* 7.6 Hz, H-1″), 6.00 (1H, *d*, *J* 2.2 Hz, H-5′), 6.32 (1H, *d*, *J* 2.2 Hz, H-3′), 6.74 (2H, *d*, *J* 8.4 Hz, H-3, H-5), 7.10 (2H, *d*, *J* 8.4 Hz, H-2, H-6). ^{13}C NMR (75 MHz, CD_3COCD_3): δ 30.16 (β-CH_2), 46.29 (*a*-CH_2), 62.46 (C-6″), 71.04 (C-4″), 74.25 (C-2″), 77.90 (C-5″), 78.06 (C-3″), 95.42 (C-5′), 97.97 (C-3′), 101.95 (C-1″), 106.28 (C-1′), 115.90 (C-3, C-5), 130.17 (C-2, C-6), 133.19 (C-1), 156.28 (C-4), 162.08 (C-2′), 165.54 (C-6′), 167.09 (C-4′), 205.88 (C=O).

HPLC analysis of acetone extract

The HPLC analysis was performed on an HP instrument, series 1100 equipped with vacuum degasser, quaternary pump, autosampler, thermostatted column compartment and DAD detector, connected to Chemstation software. The column used was LiChrospher® 100 RP-18 (5 μm) (125×4 mm I.D.) equipped with a guard column of the same material and thermostatted at 30°C. Solvents used were solvent A (HOAc-H_2O, 2:98, v/v) and solvent B (CH_3CN-HOAc-H_2O, 20:2:78, v/v) starting with linear gradient from 20% B to 60% B in 40 min, increasing to 100% B in 20 min, then isocratically for 20 min. The flow rate was 0.5 ml min^{-1} and compounds were monitored by UV absorption at 280 nm.

RESULTS AND DISCUSSION

GC–MS analysis of hexane extract

The hexane extract of apple seed was methylated with diazomethane and its volatile fraction analyzed by gas chromatography-mass spectroscopy (GC–MS). Forty-six compounds were detected, most of which were fatty acids (as their methyl esters) (see Table 1). Linoleic acid was by far the most dominant fatty acid, followed by palmitic, linolenic, stearic, and oleic acids. Minor acids, found at levels between 0.2 and 4%, were eicosanoic acid, eicosenoic acid, docosanoic acid, hexanoic acid, octanoic acid and heptadecanoic acid. A number of fatty acids present below 0.2% were also detected and they are included in Table 1 as trace components. As shown in Table 1, some fatty acids were also detected as natural ethyl, butyl and hexyl esters, albeit in small amounts compared to the free acids. Linoleic acid, including its esters, made up 51.2% of the total volatile fraction of the hexane extract.

Squalene and nonacosane (3.4 and 3.6%, respectively) were also present and formed the two major components of the non-fatty acid fraction. Several compounds present at levels ranging from 0.2 to 2.6% were also detected in the GC–MS, but their identity was not established.

Isolation and identification of major compounds from acetone extract

The 70% aqueous acetone extract of the oil-free apple seed residue yielded a brown-coloured material which consisted predominantly of sugars, the ^{13}C NMR spectrum of which showed that it contained fructose, glucose and sucrose in a ratio of *ca* 2.5:2:1, respectively. These sugars were removed by chromatography on an HP20 column and the absorbed phenolics separated by elution with aqueous methanol. In this way, two compounds (1) and (2) were isolated and further characterized by ^{1}H and ^{13}C NMR spectroscopy.

The ^{1}H NMR spectrum of the more mobile compound (1) showed the presence of sugars as indicated by the multiple signals in the δ 3.2 to 4.3 region. This was corroborated by ^{13}C NMR which showed the presence of 12 signals assignable to two hexose molecules which were associated with the proton signals in the H,C-COSY spectrum. The downfield shift of the C-6 signal (δ 69.13) suggested involvement of the C-6 hydroxyl in

Table 1. Compounds detected in the methylated hexane extract of apple seed

Peak no	RT (min)	Compound (m/z)	%
1	4.03	benzaldehyde	t
2	4.76	unknown (45, 59, 72, 89)	0.42
3	6.52	methyl octanoate	t
4	6.77	unknown (41, 55, 69, 84)	0.40
5	7.43	2-dodecenal	t
6	8.11	methyl nonanoate	t
7	8.30	hexyl pentanoate	t
8	9.25	deca-2,4-dienal	0.49
9	9.60	isomer of peak 8	0.63
10	10.51	hexyl hexanoate	0.54
11	12.23	unknown (41, 91, 107)	1.74
12	12.83	unknown (41, 69)	0.49
13	12.98	unknown (41, 110, 152)	1.22
14	13.13	hexyl octanoate	0.49
15	14.26	unknown (43, 119)	0.77
16	14.41	unknown (43, 93, 162)	2.62
17	14.86	methyl myristate	t
18	15.33	unknown (43, 110)	0.99
19	15.99	methyl pentadecanoate	t
20	16.87	methyl palmitoleate	t
21	17.14	methyl palmitate	9.93
22	17.79	ethyl palmitate	0.56
23	18.13	methyl heptadecanoate	0.28
24	19.05	methyl linoleate	37.71
25	19.08	methyl linolenate	5.60
26	19.09	methyl oleate	4.12
27	19.20	methyl stearate	4.33
28	19.57	ethyl linoleate	4.31
29	19.61	ethyl oleate	t
30	19.81	ethyl stearate	t
31	20.19	methyl nonadecanoate	t
32	20.88	tricosene	4.29
33	21.15	methyl eicosenoate	1.05
34	21.47	methyl eicosanoate	2.18
35	21.74	an alkene	0.38
36	21.92	butyl linoleate	1.50
37	22.22	hexyl palmitate	0.61
38	23.01	methyl heneicosanoate	t
39	24.99	methyl docosanoate	0.72
40	25.59	hexyl linoleate	3.30
41	26.24	a benzyl ester	t
42	27.49	methyl tricosanoate	t
43	30.72	methyl tetracosanoate	t
44	31.88	unknown (91, 279)	1.13
45	34.96	squalene	3.40
46	38.52	nonacosane	3.59
		palmitic acid	10.49
		stearic acid	4.33
		oleic acid	4.12
		linoleic acid	51.15
		linolenic acid	5.60
		total fatty acids	80.91

Free acids were identified as methyl esters in the methylated sample; t indicates that the level of the compound was less than 0.2%.

the 1→6 interglycosidic linkage. As the sugars commonly occurring in glycosidic form are all readily distinguishable from one another by ^{13}C NMR spectroscopy, comparison of the ^{13}C NMR data with those reported (Markham and Chari, 1982) led to the assignment of gentiobiose (two glucopyranoses linked by 1→6 bond). The ^{13}C NMR of compound (1) also showed the presence of an aromatic ring; the chemical shifts (δ 128.16 (×2), 129.98 (×2), 130.93 and 133.61) were consistent with a mono substituted benzene ring. A quarternary carbon observed at 119.37 ppm and an oxygenated carbon at 69.28 ppm in the ^{13}C NMR spectrum, together with a singlet at 5.91 ppm in the ^{1}H NMR spectrum, suggested the presence of a cyanohydrin group (Schwind *et al*., 1990). From this spectral evidence, the compound was assigned as [(6-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]benzene-acetonitrile, or amygdalin. Confirmation of the chemical structure of this compound was achieved by spectral comparison with published data for amygdalin (Ribeiro, 1990; Nahrstedt *et al*., 1990).

Amygdalin, a cyanogenic diglucoside, is widespread in the seeds of Rosaceae, principally in bitter almonds, but also in the stones of peaches and apricots (Nahrstedt, 1972; He and Li, 1988; Femenia *et al*., 1995). While its presence in apple seeds has also been reported, no details on its isolation or spectral data were available (Dziewanowska *et al*., 1979).

Compound (2) was identified as phloretin-2′-β-D-glucopyranoside (phloridzin). Its ^{1}H NMR spectrum showed, in the aliphatic region, two mutually coupled triplets (δ 2.9 and 3.5, J 7.4 Hz) which were attributable to the a and β methylene protons adjacent to a carbonyl group. In the aromatic region there were two mutually coupled doublets (δ 6.0 and 6.3, J 2.2 Hz) characteristic of the unsymmetrically substituted phloroglucinol ring, and an AA′BB′ type quartet (δ 6.7 and 7.4, J 8.4 Hz) for protons of the p-disubstituted phenyl ring. The presence of a sugar moiety was also apparent in the proton spectrum (δ 3.4–4.0) and the β-configuration of the sugar was indicated by the large coupling of the anomeric proton (δ 5.1, J 7.6 Hz). These data were consistent with the structure of phloridzin and further confirmed by spectroscopic and HPLC comparison with an authentic sample previously isolated from apple pomace (Lu and Foo, 1997). Phloridzin has been known as an apple constituent for a long time and generally accepted as a marker compound for apple products (Spanos and Wrolstad, 1992; Tomas-Barberan *et al*., 1993). Recently, its level in apple seed coat (0.2–5.2% DM) was reported (Jham, 1996), which is consistent with the present study. Phloridzin is clearly the most prominent polyphenol in apple seed, representing about 75% of the total polyphenols.

HPLC analysis of acetone extract

By UV detection the acetone extract showed one dominant peak (peak 14) in the HPLC chromatogram (see Fig. 1), accompanied by a number of smaller peaks. This major peak was identified as phloridzin, as described earlier. The identity of the minor polyphenols was achieved by HPLC and UV–VIS absorption comparison

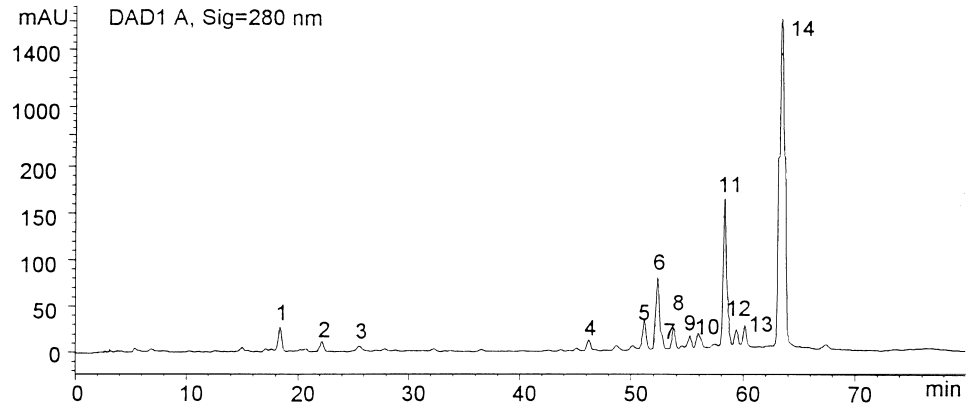


Fig. 1. HPLC chromatogram of 70% aqueous acetone extract of apple seeds. Peak identifications: 1, chlorogenic acid; 2, unknown; 3, *p*-coumarylquinic acid; 4, unknown; 5, unknown; 6, quercetin-3-galactoside; 7, quercetin-3-rutinoside; 8, quercetin-3-glucoside; 9, 3-hydroxyphloridzin; 10, quercetin-3-xyloside; 11, phloretin-2′-xyloglucoside; 12, quercetin-3-arabinoside; 13, quercetin-3-rhamnoside; 14, phloridzin.

with authentic samples obtained previously from apple skin pomace (Lu and Foo, 1997). These were chlorogenic acid (peak 1), *p*-coumarylquinic acid (peak 3), 3-hydroxyphloridzin (peak 9), phloretin-2′-xyloglucoside (peak 11) and a mixture of quercetin glycosides consisting of galactoside, rutinoside, glucoside, xyloside, arabinoside and rhamnoside (peaks 6, 7, 8, 10, 12 and 13, respectively). Chlorogenic acid, phloretin and quercetin glycosides are common constituents of apples (Dick *et al.*, 1987; Oleszek *et al.*, 1988), while *p*-coumarylquinic acid was first isolated from immature apple fruit as a minor component (Whiting and Coggins, 1975), and 3-hydroxyphloridzin was only recently identified as a natural apple constituent (Lu and Foo, 1997).

The UV absorption spectra of the three unidentified peaks, peak 2 (λ_{max} at 232 and 300 nm), peak 4 (λ_{max} at 232 and 284 nm) and peak 5 (λ_{max} at 230 and 280 nm) suggested they are of the dihydrochalcone or flavanone chemical constitution. The UV spectrum of peak 4 was similar to that of 3-hydroxyphloridzin (peak 9) and, because of its higher mobility in the HPLC, it was probably a disaccharide of 3-hydroxyphloretin, possibly 3-hydroxyphloretin-2′-xyloglucoside by analogy with phloretin-2′-xyloglucoside.

Phloridzin alone accounted for *ca* 75% of the total apple seed polyphenols, followed by phloretin-2′-xyloglucoside (*ca* 9%) and quercetin-3-galactoside (*ca* 6%). These values are in sharp contrast to the apple polyphenols where there was a more even distribution of various components with phloridzin comprising *ca* 20%. The range of polyphenols is very restricted in the seed while, in apple fruit, there was a wider range of compounds and in particular the presence of epicatechin and its oligomers (Burda *et al.*, 1990), which were absent in the seed.

CONCLUSION

Free fatty acids were the dominant GC-detectable components of the hexane extract in apple seed with linoleic acid (51.2%) as the principal acid. The polar fraction obtained by extraction with 70% aqueous acetone contained two major compounds, amygdalin and phloridzin, the latter making up to ca 75% of the total polyphenols. Minor components detected were chlorogenic acid, *p*-coumarylquinic acid, 3-hydroxyphloridzin, phloretin-2′-xyloglucoside and six quercetin glycosides (arabinoside, galactoside, glucoside, rhamnoside, rutinoside and xyloside). These results showed that apple seed is a good source of linoleic acid and phloridzin while flavonoids and procyanidins were absent or present at low levels.

ACKNOWLEDGEMENTS

The authors thank Dr Herbert Wong for the ^{1}H and ^{13}C NMR spectra and Mr John Marks and Ms Debbie Amoamo of Frucor Processors Ltd for the supply of apple pomace.

REFERENCES

Burda, S., Oleszek, W. and Lee, C. Y. (1990) Phenolic compounds and their changes in apples during maturation and cold storage. *Journal of Agricultural and Food Chemistry* **38**, 945–948.

Dick, A. J., Redden, P. R., DeMarco, A. C., Lidster, P. D. and Grindley, T. B. (1987) Flavonoid glycosides of Spartan apple peel. *Journal of Agricultural and Food Chemistry* **35**, 529–531.

Durkee, A. B. and Poapst, P. A. (1965) Phenolic constituents in core tissues and ripe seed of McIntosh apples. *Journal of Agricultural and Food Chemistry* **13**, 137–139.

Dziewanowska, K., Niedzwiedz, I. and Lewak, S. (1979) Hydrogen cyanide and cyanogenic compounds in seeds. III. Degradation of cyanogenic glucosides during apple seed stratification. *Physiologie Vegetale* **17**, 687–695.

Femenia, A., Rossello, C., Mulet, A. and Canellas, J. (1995) Chemical composition of bitter and sweet apricot kernels. *Journal of Agricultural and Food Chemistry* **43**, 356–361.

He, L. and Li, B. (1988) MicroHPLC determination of amygdalin in semen *pruni armeniacae* and semen *pruni persicae*. *Biomedical Chromatography* **2**, 271–273.

Jham, G. N. (1996) High-performance liquid chromatographic quantification of phloridzin in apple seed, leaf and callus. *Journal of Chromatography* **A 719**, 444–449.

Kamel, B. S. (1981) Nutritional properties of some agricultural seed byproducts. *Journal of Food Quality* **5**, 139–144.

Kennedy, M. J. (1994) Apple pomace and kiwifruit: processing options. *Australasian Biotechnology* **4**, 43–49.

Lu, Y. and Foo, L. Y. (1997) Identification and quantification of major polyphenols in apple pomace. *Food Chemistry* **59**, 187–194.

Markham, K. R. and Chari, V. M. (1982) Carbon-13 NMR spectroscopy of flavonoids. In *The Flavonoids: Advances in Research*, ed. K. R. Markham and T. J. Mabry, pp. 19–134. Chapman and Hall, New York.

Nahrstedt, A. (1972) Zur cyanogenese von *Prunus avium*. *Phytochemistry* **11**, 3121–3126.

Nahrstedt, A., Sattar, E. A. and El-Zalabani, S. M. H. (1990) Amygdalin acyl derivatives, cyanogenic glycosides from the seeds of *Merremia dissecta*. *Phytochemistry* **29**, 1179–1181.

Okuda, T. (1993) Natural polyphenols as antioxidants and their potential use in cancer prevention. In *Polyphenolic Phenomena*, ed. A. Scalbert, pp. 221–235. INRA Editions, Paris.

Oleszek, W., Lee, C. Y., Jaworski, A. W. and Price, K. B. (1988) Identification of some phenolic compounds in apples. *Journal of Agricultural and Food Chemistry* **36**, 430–432.

Ribeiro, A. A. (1990) ^{1}H and ^{13}C NMR analysis of D-amygdalin: oligosaccharide assignment and sequencing. *Magnetic Resonance in Chemistry* **28**, 765–773.

Rice-Evans, C. A., Miller, N. and Paganga, G. (1996) Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radicals in Biology Medicine* **20**, 933–956.

Schwind, P., Wray, V. and Nahrstedt, A. (1990) Structure elucidation of an acylated cyanogenic triglycoside, and further cyanogenic constituents from *Xeranthemum Cylindraceum*. *Phytochemistry* **29**, 1903–1911.

Spanos, G. A. and Wrolstad, R. E. (1992) Phenolics of apple, pear, and white grape juices and their changes with processing and storage—a review. *Journal of Agricultural and Food Chemistry* **40**, 1478–1487.

Suarez Valles, B., Santamaria Victorero, J., Mangas Alonso, J. J. and Blano Gomis, D. (1994) High-performance liquid chromatography of the neutral phenolic compounds of low molecular weight in apple juice. *Journal of Agricultural and Food Chemistry* **42**, 2732–2736.

Tomas-Barberan, F. A., Garcia-Viguera, C., Nieto, J. L., Ferreres, F. and Tomas-Lorente, F. (1993) Dihydrochalcones from apple juices and jams. *Food Chemistry* **46**, 33–36.

Whiting, G. C. and Coggins, R. A. (1975) 4-*p*-Coumarylquinic acid in apple fruits. *Phytochemistry* **14**, 593.