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Apple Peels, from Seven Cultivars, Have Lipase-Inhibitory Activity and Contain Numerous Ursenoic Acids As Identified by LC-ESI-QTOF-HRMS

Tony K. McGhie,* Sébastien Hudault, Rona C. M. Lunken, and John T. Christeller

The New Zealand Institute for Plant and Food Research Ltd., Private Bag 11600, Palmerston North 4442, New Zealand

ABSTRACT: Apple peel contains numerous phytochemicals, many of which show bioactivity. This study investigated the identity of triterpenoid compounds contained in ethanolic extracts of peel from seven apple cultivars. Using HPLC-ESI-QTOF-HRMS, accurate mass information was obtained for 43 compounds, and chemical identity was inferred from the calculated elemental composition, fragment masses, ms/ms, and a limited set of authentic standards. Compounds were identified as triterpene acids and tentatively identified as ursenoic (or oleanoic) acid derivatives containing hydroxyl, oxo, and coumaroyloxy groups. These apple skin extracts exhibited lipase-inhibitory activity, which may be linked to the ursenoic acid content. Furthermore, both triterpene content and lipase-inhibitory activity varied by cultivar.

KEYWORDS: *apple, Malus, peel, triterpenoid, ursenoic acid, lipase inhibition*

INTRODUCTION

Apples (*Malus* × *domestica* Borkh) are a globally distributed fruit that have been cultivated for human consumption for centuries. Current global apple production is approximately 70 million tonnes/year, making apple the third most-consumed fruit after banana and citrus (2008 data, FAOSTAT). When compared with other fruit crops, there is a very diverse range of apple cultivars available for human consumption. In addition to the plethora of skin colors and patterns, this diversity extends to other quality attributes such as taste, aroma, texture, and phytochemical composition.[1]

Apples contain relatively high concentrations of antioxidant polyphenolics,[2] and high apple consumption has been linked to improved health through the prevention of several chronic diseases.[3−7] In a recent review of apple and health, apple products were associated with beneficial effects for cancer, cardiovascular disease, pulmonary function, and age-related cognitive decline.[8] Apple fruits are composed of different tissue types (peel, cortex, core, and seed), and each tissue type contains a different composition of phytochemicals. For example, apple peel is particularly rich in polyphenols[2] and plays an important functional role for the protection of the fruit, as it is at the interface with the external environment. The apple peel is composed of several layers including an outer wax layer and underlying layers of epidermal cells. From a postharvest perspective, the integrity of the skin is important because it affects water loss during storage and preserves quality. Apple peel also protects against pests and diseases as both a physical and a chemical barrier. For example, endogenous proteinase inhibitors in plants are well-known to have insect-deterrent properties.[9,10] Recently, it was found that addition of apple leaf extract to the diet of the light brown apple moth (*Epiphyas postvittana* Walker; Tortricidae) changed the expression of gastric and pancreatic lipase genes.[11] This supports the hypothesis that plant compounds that have lipase-inhibitory activity may also have insect-deterrent properties.

The composition of apple peel has been studied to identify compounds that have high antioxidant capacity and to understand the origin and genesis of postharvest disorders.[12−15] Compounds that have been identified in the apple skin include organic acids, phenolic acids, flavonoids (flavonols, anthocyanins, and flavanols), triterpene acids, coumaryl fatty acid esters, and sesquiterpenes.[14−18]

One of these apple peel compounds is the triterpene, ursolic acid, which is thought to have a number of potential health benefits for humans.[19−25] The aim of this study was to investigate the triterpenic acid composition of apple peel from seven apple cultivars and determine if extracts of apple peel inhibit lipase activity.

MATERIALS AND METHODS

Chemicals and Reagents. LC-MS grade acetonitrile was from Fischer Scientific, methanol (ChromAR) was from Mallinckrodt Chemicals, and ethanol (95%) was from LabServ. Authentic standards of the triterpenes ursolic acid, oleanolic acid, asiatic acid, betulinic acid, and uvaol were purchased from Extrasynthese (Genay, France).

Source of Fruit and Extraction. Fruit (approximately 1 kg; 4−6 fruits) of seven apple cultivars planted in one- or two-tree plots at the Plant and Food Research site in Hawke's Bay, New Zealand (39° 39′ S, 176° 53′ E), were harvested at commercial ripeness and transported to Palmerston North. The cultivars assessed in this study were 'Braeburn', 'Granny Smith', 'Fuji', 'Scilate' (Envy), 'Cripps Pink' (Pink Lady), 'Sciros' (Pacific Rose), and an open pollinated seedling of 'Red Field' (O.P. 'Red Field'). Fruits were peeled, using a kitchen apple peeler (Cuisena), and the peels (1−2 mm thick) homogenized with an 8× volume of ethanol using a GLH homogenizer (Omni International). The mixtures were left to extract overnight at 4 °C and then filtered (no. 3 Whatman). The extract was concentrated to a syrup by rotary evaporation at 40 °C, which was then dissolved in methanol

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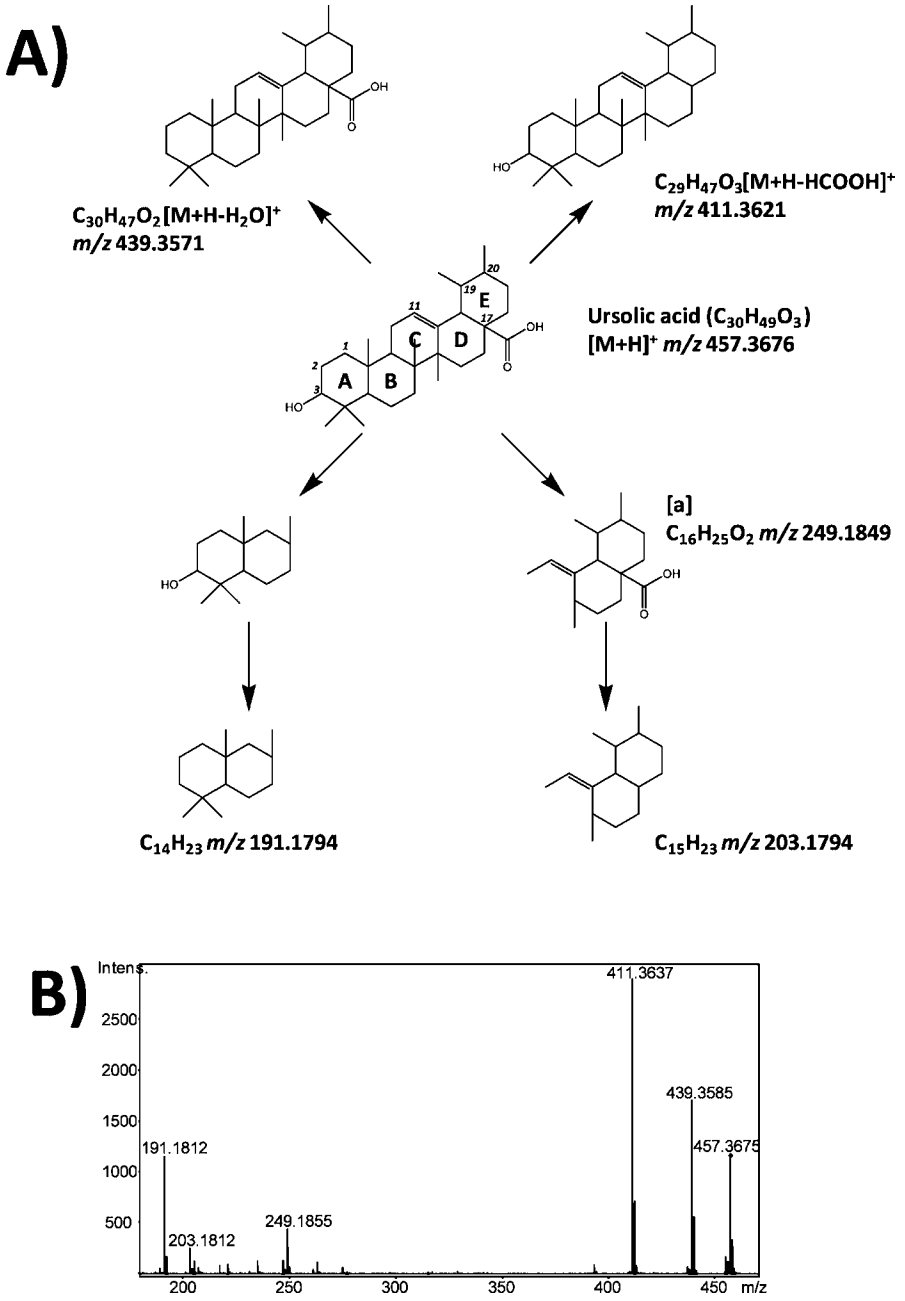

Figure 1. (A) ESI-MS fragmentation of 3β-hydroxy-urs-12-en-28-oic acid (ursolic acid). (B) Mass spectrum obtained using direct infusion. MS conditions: MS/MS mode (m/z 457 CID 15 V), positive mode; capillary voltage, −4000 V; end plate offset, −500 V; source temperature, 180 °C; drying gas flow, 4 L/min.

(approximately 100 mL). The weights of apple peel and total volumes of the final extracts were recorded so that component concentrations and bioactivity could be accurately calculated. Extracts were stored at 1 °C until used for bioassay or LC-QTOF-MS analysis.

LC-QTOF-HRMS. For analysis by LC-MS, each ethanolic extract was further diluted with methanol. The LC-MS system was composed of a Dionex Ultimate 3000 Rapid Separation LC system and a micrOTOF QII mass spectrometer (Bruker Daltonics, Bremen, Germany) fitted with an electrospray source operating in positive mode. The LC system contained an SRD-3400 solvent rack/degasser, an HPR-3400RS binary pump, a WPS-3000RS thermostated autosampler, and a TCC-3000RS thermostated column compartment. The analytical column was a Zorbax SB-C18 2.1 × 100 mm, 1.8 μm (Agilent, Melbourne, Australia), maintained at 50 °C and operated in gradient mode. Solvents were A = 0.5% formic acid and B = 100% acetronitrile at a flow of 400 μL/min. The gradient was as follows: 70% A, 30% B, 0−0.5 min; linear gradient to 45% A, 55% B, 0.5−25 min; linear gradient to 2% A, 98% B, 25−45 min; composition held at 2% A, 98% B, 45−50 min; linear gradient to 70% A, 30% B, 50−50.2 min; return to initial conditions before another sample injection at 54 min. The injection volume for samples and standards was 2 μL. The micrOTOF QII source parameters were as follows: temperature, 200 °C; drying N_2 flow, 8 L/min; nebulizer N_2, 4.0 bar; end plate offset, −500 V; capillary voltage, −4000 V; mass range, 100−1500 Da, acquired at 2 scans/s. Postacquisition internal mass calibration used sodium formate clusters with the sodium formate delivered by a syringe pump at the start of each chromatographic analysis.

Compound Identification Criteria. High-resolution mass spectrometry (HRMS) was used to tentatively identify the compounds present in the apple peel extracts. The elemental compositions of the detected compounds were calculated from the accurate mass data, with additional confirmation provided by isotope ratio calculations

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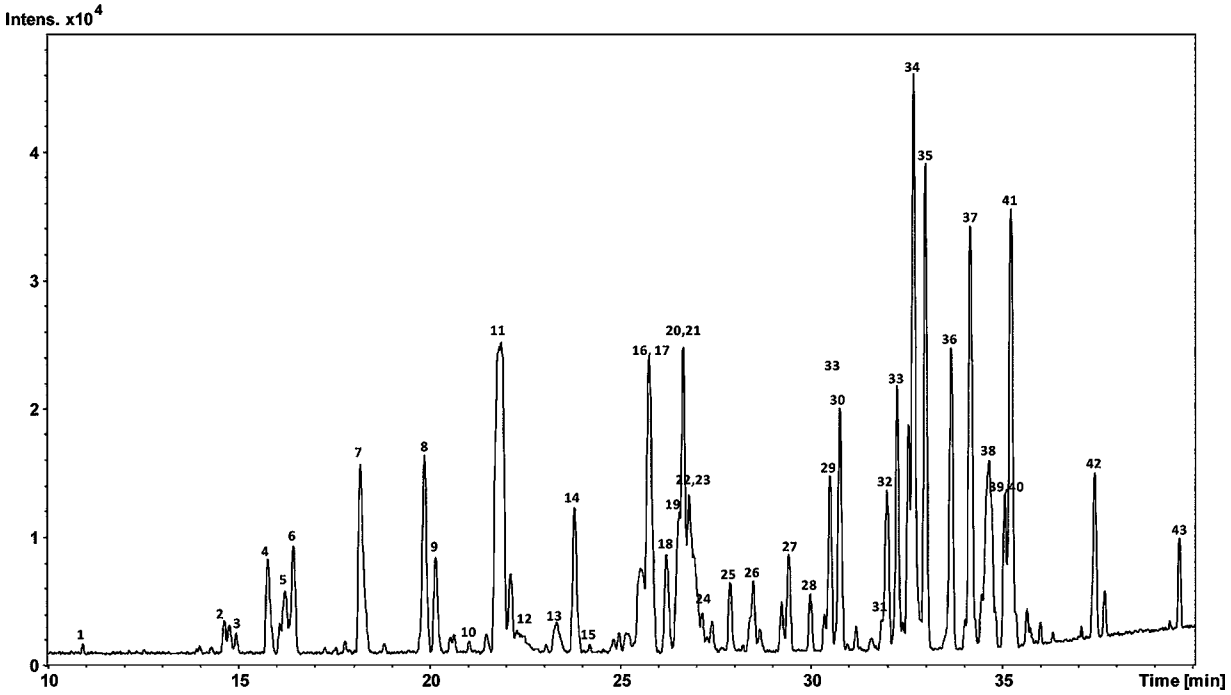

Figure 2. Typical reversed-phase separation of ursenoic acids in an ethanol extract of apple ('Pacific Rose') peel shown as a base peak chromatogram. Peak identities are given in Table 1. Analytical conditions are described in the text.

designated as mSigma (Bruker Daltronics) values. The accurate mass measured was within 5 mDa of the assigned elemental composition and mSigma values of <20 provided confirmation. Ursolic acid (3β-hydroxy-urs-12-en-28-oic acid) and derivatives have been previously reported in apple,[14,16,17,26] and ursolic acid was used as the base structure for the identification of triterpenic acids in the extracts. Thus, using accurate mass measurements, many of the detected compounds were assigned as structural analogues of ursolic acid that included additional hydroxyl, oxo, and coumaroyloxy groups. For example, an additional oxygen (plus m/z 15.9944) indicates an additional hydroxyl group, whereas an additional oxygen, less two hydrogen (plus m/z 13.9793), indicates an additional oxo group. Confirmation of the number of hydroxyl and oxo groups was provided by considering the number of losses of H_2O (m/z 18.0100) from the putative $(M + H)^+$. The presence of a carboxyl group was indicated by loss of HCOOH (m/z 46.0049) from the putative $[M + H]^+$. The pseudomolecular ion, $[M + H]^+$, was identified in each spectrum by consideration of the losses of H_2O, HCOOH, H_2O + HCOOH, and sometimes the presence of $[M + Na]^+$. The typical mass spectral fragmentation pathways exhibited by terpenoid acids are shown for ursolic acid, and these were obtained by direct infusion (Figure 1). This includes loss of H_2O (18.0100 Da) and HCOOH (46.0049 Da) and fragmentation by a characteristic retro-Diels–Alder reaction in ring C to yield [a].[27]

Lipase Inhibition Assay. Lipase enzymes were obtained from fourth-instar *Helicoverpa armigera* (Noctuidae, Lepidoptera) larvae from a colony maintained on lima bean diet[28] at Plant and Food Research (Mount Albert, Auckland, New Zeland). Following cold anesthesia, the midgut of each larva was removed under a dissecting stereomicroscope and carefully separated from potential contaminating tissues (silk glands, trachea, Malpighian tubules, fat body cells, and hemocytes) and then immediately stored at −20 °C until required. Samples of ∼1 g midgut were then ground in 8 mL of a 10 mM Tris buffer containing 20 mM NaCl, 2 mM sodium diethyldithiocarbamate, 2 mM sodium ascorbate, and 2 mM cysteine, at pH 7.5, and centrifuged, and the supernatant (in 0.5 mL aliquots) was freeze-dried and stored at −20 °C.[29]

The lipase inhibition assay was based on a previously widely used method.[30] A stock substrate solution was prepared with 10 g of glycerol, 600 mg of triolein (Sigma), and 100 mg of whey protein (mostly β-lactoglobulin, A. Hardacre, Department of Food Technology, Massey University, Palmerston North, New Zealand) as the emulsifier. Whey protein gave higher activities than a range of other emulsifying agents tested. The solution was mixed using an Ultra-Turrax (IKA-Werk, Staufen, Germany) for 15 s and could be stored at 4 °C for extended periods as a clear stable solution.

To assay lipase activity, a substrate mix was prepared daily using 250 μL of stock substrate solution, 800 μL of 50 mM HEPES buffer at pH 8.0, and 2 μL of [9, 10 ^{3}H(N)]-triolein (0.5 mCi/mL, Amersham, U.K.) and sonicated for 15 s/mL. This mix formed an opaque emulsion that was stable for several hours. The assay itself comprised 100 μL of substrate mix and 60 μL of enzyme preparation and was vortexed and incubated at 37 °C for 40 min. To test for the effect of apple peel extracts, 40 μL aliquots in dimethyl sulfoxide (DMSO) were added to the substrate emulsion and vortexed before addition of enzyme, that is, the "poisoned interface" method.[31] Control reactions contained 40 μL of DMSO. The reaction was stopped, and radioactive oleic acid was extracted using a mixture of 3 mL of heptane/chloroform/methanol (1.0:1.25:1.11) and 1 mL of aqueous 0.1 M sodium carbonate/0.1 M boric acid at pH 10.5. After vigorous vortexing for 15 s, the mixture was allowed to separate into two phases. One milliliter of the upper aqueous phase was counted in a scintillation counter. The assay was linear for up to 20% hydrolysis of the substrate. Under these conditions, all of the gastric lipase-like and all of the pancreatic lipase-like enzymes identified in *H. armigera* midgut should be active on the triacylglycerol substrate (F. Carrière, personal communication).

RESULTS AND DISCUSSION

The apple peel ethanolic extracts of the seven apple cultivars were a complex mixture of compounds. A typical example chromatogram for 'Sciros' is shown in Figure 2. Using DataAnalysis software (Bruker Daltonics, Bremen, Germany), approximately 150 components could be identified in these extracts within the 10–40 min retention time range. On the basis of a very limited range of authentic standards, many of these compounds appeared to be triterpene-like compounds, and tentative identifications were possible for 27 compounds based on the elemental formula calculated from accurate mass

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Table 1. Compounds Detected by LC-ESI-QTOF-MS in Apple Skins

no.	RT (min)	assigned compound name	elemental composition (M)	m/z (M + H)$^+$	difference (mDa)	mSigma[a]
1	10.95	dihydroxy-urs-12-en-28-oic acid	$C_{30}H_{48}O_4$	473.3622	0.4	28.6
2	14.64	unknown	$C_{30}H_{44}O_5$	485.3266	−0.4	8.0
3	14.94	unknown	$C_{18}H_{37}NO_3$	316.2842	−0.1	10.2
4	15.76	unknown	$C_{18}H_{37}NO_3$	316.2845	0.1	6.5
5	16.22	trihydroxy-urs-12-en-28-oic acid	$C_{30}H_{48}O_5$	489.3567	−1.9	12.0
6	16.45	trihydroxy-urs-12-en-28-oic acid	$C_{30}H_{48}O_5$	489.3558	1.6	24.4
7	18.18	unknown	$C_{18}H_{39}NO_3$	318.2999	0.4	6.1
8	19.86	unknown	$C_{30}H_{48}O_3$	457.3691	−1.4	4.8
9	20.17	tetrahydroxy-urs-12-en-28-oic acid	$C_{30}H_{48}O_6$	505.3537	−1.3	7.2
10	21.03	unknown	$C_{25}H_{39}N_3O_6$	478.2934	−2.2	7.5
11	21.86	3-oxo-1,19α-dihydroxy-urs-12-en-28-oic acid (annurcoic acid)	$C_{30}H_{46}O_5$	487.3433	−1.5	2.7
12	22.27	unknown	$C_{33}H_{46}NO_4$	520.3423	−0.2	36.3
13	23.30	unknown	$C_{33}H_{46}$ NO_4	520.3414	0.7	35.9
14	23.79	3-oxo-1α-hydroxy-urs-12-en-28-oic acid (pomonic acid)	$C_{30}H_{46}O_4$	471.3479	−1.0	5.0
15	24.19	3β-*p*-coumaroyloxy-dihydroxy-urs-12-en-28-oic acid	$C_{39}H_{54}O_7$	635.3964	−2.2	12.5
16	25.72	3β,19α-dihydroxy-urs-12-en-28-oic acid (pomolic acid)	$C_{30}H_{48}O_4$	473.3620	0.4	6.1
17	25.74	3-oxo-hydroxy-urs-12-en-28-oic acid	$C_{30}H_{46}O_4$	471.3483	−1.5	285.7
18	26.19	dihydroxy-urs-12-en-28-oic acid	$C_{30}H_{48}O_4$	473.3630	−0.4	23.4
19	26.51	dihydroxy-urs-12-en-28-oic acid	$C_{30}H_{48}O_4$	473.3631	−0.6	8.5
20	26.62	3β-*trans*-*p*-coumaroyloxy-2α,13β-dihydroxy-urs-12-en-28-oic acid	$C_{39}H_{54}O_7$	635.3970	−2.8	6.6
21	26.78	3β-*cis*-*p*-coumaroyloxy-2α,13β-dihydroxy-urs-12-en-28-oic acid	$C_{39}H_{54}O_7$	635.3976	−3.3	5.1
22	26.90	dihydroxy-urs-12-en-28-oic acid	$C_{30}H_{48}O_4$	473.3639	−1.4	7.8
23	27.00	3β-*trans*?-*p*-coumaroyloxy-2α,13β-dihydroxy-urs-12-en-28-oic acid	$C_{39}H_{54}O_7$	635.3955	−1.3	19.7
24	27.12	dihydroxy-urs-12-en-28-oic acid	$C_{30}H_{48}O_4$	473.3634	−0.9	4.2
25	27.85	unknown	$C_{30}H_{50}O$	427.3932	0.2	19.3
26	28.46	unknown	$C_{31}H_{50}O_6$	519.3681	−0.2	23.9
27	29.38	unknown	$C_{30}H_{50}O_2$	443.3886	−0.02	11.5
28	29.99	oxo-urs-12-en-28-oic acid	$C_{30}H_{46}O_3$	455.3538	−1.9	19.2
29	30.47	unknown (dihydroxy-urs-12-en-28-oic acid)	$C_{30}H_{48}O_4$	473.3657	−3.2	7.3
30	30.73	oxo-urs-12-en-28-oic acid	$C_{30}H_{46}O_3$	455.3542	−2.3	17.5
31	31.81	3-oxo-hydroxy-urs-12-en-28-oic acid	$C_{30}H_{46}O_4$	471.3500	−3.1	4.8
32	31.96	3-oxo-hydroxy-urs-12-en-28-oic acid	$C_{30}H_{46}O_4$	471.3502	−3.3	12.8
33	32.22	3β-?-*p*-coumaroyloxy-hydroxy-urs-12-en-28-oic acid	$C_{39}H_{54}O_6$	619.4049	−5.6	11.5
34	32.64	3β-*trans*-*p*-coumaroyloxy-2α-hydroxy-urs-12-en-28-oic acid	$C_{39}H_{54}O_6$	619.4049	−5.6	5.4
35	32.96	3β-*cis*-*p*-coumaroyloxy-2α-hydroxy-urs-12-en-28-oic acid	$C_{39}H_{54}O_6$	619.4048	−5.5	10.7
36	33.64	oxo-urs-12-en-28-oic acid	$C_{30}H_{46}O_3$	455.3548	−2.8	10.5
37	34.13	3β-hydroxy-lup-20(29)-en-28-oic acid (betulinic acid)	$C_{30}H_{46}O_2$	457.3676	−0.1	16.6
38	34.60	3β-hydroxy-urs-12-en-28-oic acid (ursolic acid)	$C_{30}H_{46}O_2$	457.3677	−0.1	24.1
39	34.99	unknown	$C_{29}H_{46}O_3$	443.3534	−1.4	2.1
40	35.04	unknown	$C_{30}H_{46}O_2$	457.3687	−1.1	17.8
41	35.18	unknown	$C_{30}H_{46}O_2$	439.3592	−2.2	5.2
42	37.32	3β-hydroxy-urs-12-en-28-ol (uvaol)	$C_{30}H_{50}O_2$	443.3900	−1.6	4.7
43	39.60	unknown	$C_{30}H_{51}O$	427.3934	0.0	6.4

[a]mSigma values are calculated from the isotope ratios expected for a given elemental composition, compared with that which is experimentally determined. Low values (<20) indicate a good fit and provide complementary evidence for assigning elemental composition.

data and previous literature reports (Table 1). Elemental compositions were calculated from accurate mass and isotope data for a further 16 compounds (Table 1).

To identify specific compounds, a series of exact ion chromatograms (EIC) were extracted from the MS data, and these are shown in Figure 3. Components identified from these chromatogram traces are discussed below.

Monohydroxy Ursenoic Acids m/z 457.3676; $[C_{30}H_{48}O_3 + H]^+$. Compound **38** had the same retention time as authentic ursolic acid (3β-hydroxy-urs-12-en-28-oic acid) and has previously been identified in apple peel.[14,16,17,26] Betulinic acid (3β-hydroxy-lupene-28-oic acid) has been previously identified in apple peel[16] and has the same elemental composition as ursolic acid. However, we were able to identify compound **37** as betulinic acid on the basis of the pseudomolecular ion at m/z 457.3691 and the same retention time as the authentic compound. The most intense ion in the mass spectra of both ursolic and betulinic acids was m/z 439.3571, because of the loss of H_2O from $[M + H]^+$. Two further triterpenes (**40** and **41**) produced spectra with m/z 439.3571 as the base ion and m/z 457.3691 as a minor ion; both were assigned the same elemental composition as ursolic and betulinic acids. These two compounds are probably structural isomers of betulinic and ursolic acid, and one may be oleanolic acid (3β-hydroxy-olean-12-en-28-oic acid), which has also been previously identified in apple peel.[16] We were not

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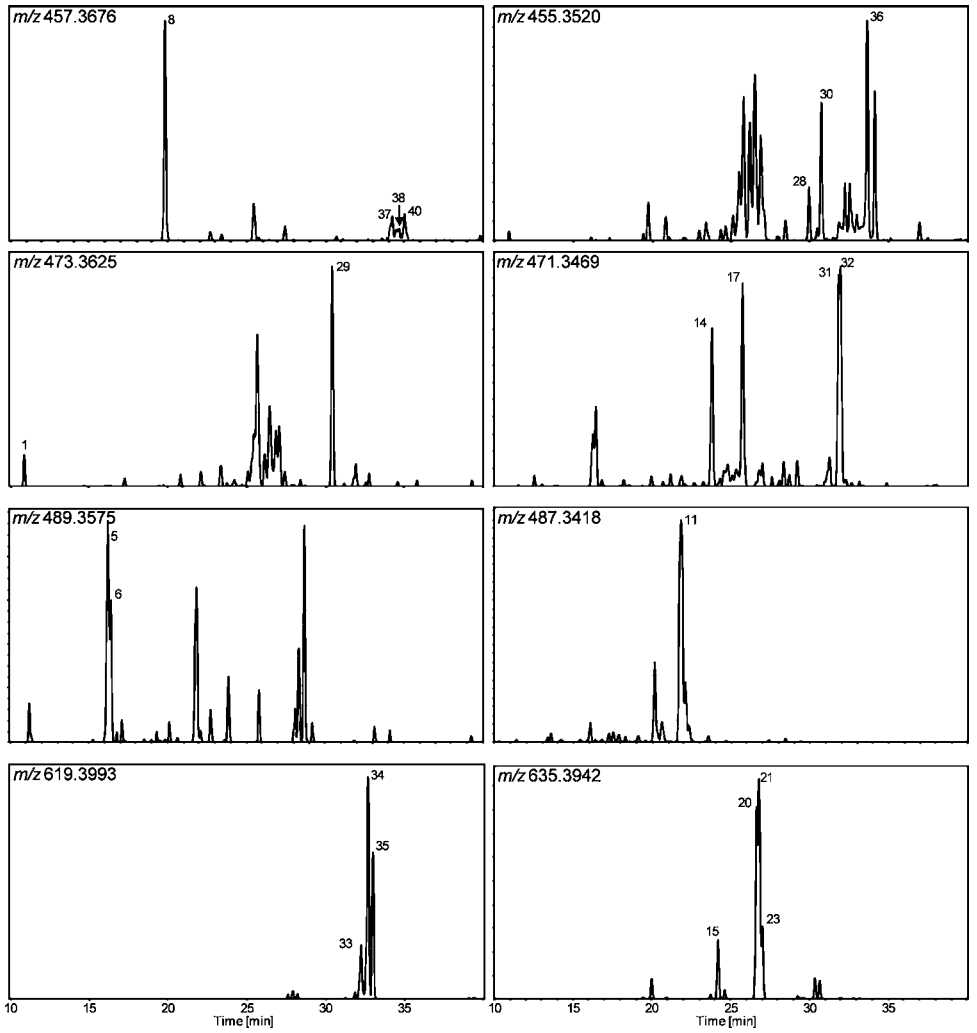

Figure 3. Extracted ion chromatograms of specific compound types detected in the ethanolic extracts of apple peels. Mass m/z 457.3676 = hydroxy-urs-12-en-28-oic acid, m/z 455.3520 = oxo-urs-12-en-28-oic acid, m/z 473.3625 = dihydroxy-urs-12-en-28-oic acid, m/z 471.3469 = oxo-hydroxy-urs-12-en-28-oic acid, m/z 489.3575 = trihydroxy-urs-12-en-28-oic acid, m/z 487.3418 = oxo-dihydroxy-urs-12-en-28-oic acid, m/z 619.3993 = coumaroyloxy-hydroxy-urs-12-en-28-oic acid, and m/z 635.3942 = coumaroyloxy-dihydroxy-urs-12-en-28-oic acid.

able to identify compound **8**, which also has the same elemental composition as ursolic and betulinic acids, but eluted from the HPLC column almost 15 min earlier than these two acids. In contrast to ursolic and betulinic acids, the spectrum of **8** had m/z 457.3691 $[M + H]^+$ as the base ion instead of m/z 439.3571 $[M + H - H_2O]^+$ ion. The ion at $[M - 46]^+$ ($[M + H - HCOOH]^+$) was absent but was observed in the ISCID spectra, which suggests that a carboxylic group is present in compound **8**.

Oxo Ursenoic Acids m/z 455.3520; $[C_{30}H_{46}O_3 + H]^+$. The pseudomolecular ion of m/z 455.3520 corresponded to that of an oxo ursenoic acid. An EIC at m/z 455.3520 showed the presence of a number of compounds containing this mass (Figure 3). The mass spectra of compounds eluting between 25 and 28 min contained additional higher mass ions, suggesting these compounds have additional groups. For example, dihydroxy ursenoic acids are expected to produce an $[M + H - H_2O]^+$ ion, which will have the same exact mass as the $[M + H]^+$ ion of an oxo ursenoic acid, and these are discussed later. Compounds that produced spectra with ions at m/z 455.3546 and 437.3437, which correspond to $[M + H]^+$ and $[M + H - H_2O]^+$, respectively, were tentatively identified as oxo ursenoic acids, namely, compounds **28**, **30**, and **36** at 29.99, 30.73, and 33.64 min, respectively. Furthermore, the spectrum of compound **36** contained an ion at m/z 477.3349, which is consistent with $[M + Na]^+$, whereas the spectra of **28** and **30** did not. Therefore, compound **36** was tentatively identified as 3-oxo-urs-12-en-28-oic acid. The spectrum of compound **37** contained m/z 455.3534, consistent with an oxo ursenoic acid; however, the ions corresponding to $[M + H - H_2O]^+$ and $[M + Na]^+$ were absent. As compound **37** was confirmed to be betulinic acid, it may be that betulinic acid and a further oxo ursenoic acid coelute in the UHPLC system used in this study.

Dihydroxy Ursenoic Acids m/z 473.3625; $[C_{30}H_{48}O_4 + H]^+$. Six peaks were tentatively identified as probable dihydroxy ursenoic acids (compounds **1**, **16**, **18**, **19**, **22**, and **24**) from the EIC m/z 473.3625. Furthermore, their spectra mostly contained, in addition to $[M + H]^+$ pseudomolecular ions, ions corresponding to $[M + H - H_2O]^+$, $[M + H - 2H_2O]^+$, $[M + H - HCOOH]^+$, and $[M + H - H_2O - HCOOH]^+$ (Table 2). Both 2α,3β-dihydroxy-urs-12-en-28-oic (corosolic acid) [17,32] and 3β,19α,-dihydroxy-urs-12-en-28-oic (pomolic acid)[16,33] have been reported in apple peel. As pomolic acid was the only dihydroxy ursenoic acid isolated from apple by

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Table 2. Fragmentation Data for Each Compound

no.	RT (min)	compound name	composition	m/z (M + H)$^+$ (calcd)	− H_2O (18.0100)	− $(H_2O)_2$ (36.0206)	− $(H_2O)_3$ (54.0311)	− $COOH_2$ (46.0049)	− H_2O − HCOOH (64.0155)
1	10.95	dihydroxy-urs-12-en-28-oic acid	$C_{30}H_{48}O_4$	473.3625					409.3458
5	16.22	trihydroxy-urs-12-en-28-oic acid	$C_{30}H_{48}O_5$	489.3575	471.3465	453.3369	435.3257		
8	19.86	unknown	$C_{30}H_{48}O_3$	457.3676	439.3477			411.3611	
9	20.17	tetrahydroxy-urs-12-en-28-oic acid	$C_{30}H_{48}O_6$	505.3524	487.3426	469.3318			
11	21.86	3-oxo-1,19α-dihydroxy-urs-12-en-28-oic acid (annurcoic acid)	$C_{30}H_{46}O_5$	487.3418	469.3318	451.3216	433.3359 (sm)		
14	23.79	3-oxo-1α-hydroxy-urs-12-en-28-oic acid (pomonic acid)	$C_{30}H_{46}O_4$	471.3469	453.3372				
15	24.19	3β-*p*-coumaroyloxy-dihydroxy-urs-12-en-28-oic acid	$C_{39}H_{54}O_7$	635.3942					
16	25.72	3β,19α-dihydroxy-urs-12-en-28-oic acid (pomolic acid)	$C_{30}H_{48}O_4$	473.3625	455.3516	437.3423			409.3437
17	25.74	3-oxo-hydroxy-urs-12-en-28-oic acid	$C_{30}H_{46}O_4$	471.3469				425.3409	
18	26.19	dihydroxy-urs-12-en-28-oic acid	$C_{30}H_{48}O_4$	473.3625	455.3521	437.3423		427.3524	409.3461
19	26.54	dihydroxy-urs-12-en-28-oic acid	$C_{30}H_{48}O_4$	473.3625	455.3530	437.3422		427.3551	409.3469
20	26.62	3β-*trans*-*p*-coumaroyloxy-2α,13β-dihydroxy-urs-12-en-28-oic acid	$C_{39}H_{54}O_7$	635.3942					
21	26.78	3β-*cis*-*p*-coumaroyloxy-2α,13β-dihydroxy-urs-12-en-28-oic acid	$C_{39}H_{54}O_7$	635.3942					
22	26.90	dihydroxy-urs-12-en-28-oic acid	$C_{30}H_{48}O_4$	473.3625	455.3519	437.3421		427.3538	409.3425
24	27.12	dihydroxy-urs-12-en-28-oic acid	$C_{30}H_{48}O_4$	473.3625	455.3511			427.3555	
31	31.81	3-oxo-hydroxy-urs-12-en-28-oic acid	$C_{30}H_{46}O_4$	471.3469	453.3382	435.3277		425.3430	407.3326
32	31.96	3-oxo-hydroxy-urs-12-en-28-oic acid	$C_{30}H_{46}O_4$	471.3469	453.3390	435.3284		425.3436	407.3329
34	32.64	3β-*trans*-*p*-coumaroyloxy-2α-hydroxy-urs-12-en-28-oic acid	$C_{39}H_{54}O_6$	619.3993					
35	32.96	3β-*cis*-*p*-coumaroyloxy-2α-hydroxy-urs-12-en-28-oic acid	$C_{39}H_{54}O_6$	619.3993					
36	33.64	oxo-urs-12-en-28-oic acid	$C_{30}H_{46}O_3$	455.3520	437.3420				
38	34.60	3β-hydroxy-urs-12-en-28-oic acid (ursolic acid)	$C_{30}H_{48}O_2$	457.3676	439.3535				
42	37.32	3β-hydroxy-urs-12-en-28-ol (uvaol)	$C_{30}H_{50}O_2$	443.3884	425.3796	407.3662 (sm)			

D'Abrosca et al.[33] and Cefarelli et al.,[16] compound **16** was tentatively identified as pomolic acid. The other four dihydroxy ursenoic acids detected are probably isomers with various configurations at the 2- and 3-positions, or dihydroxy oleanoic acids. The retention time (RT) of compound **1** was much lower than those of the other dihydroxy ursenoic acids, and although an ion with an accurate mass consistent with the elemental formula $C_{30}H_{48}O_4$ was present, no other confirmatory ions were observed. Compound **1** is only tentatively assigned as a dihydroxy ursenoic acid. Similarly, the spectrum of compound **29** contained an ion at m/z 473.3657, consistent with a formula of $C_{30}H_{48}O_4$, but the $[M + H - H_2O]^+$ ion indicative of hydroxylated ursenoic acid was absent. Consequentlyn the identity of compound **29** is unknown.

Oxohydroxy Ursenoic Acids m/z 471.3469; $[C_{30}H_{46}O_4 + H]^+$. Numerous peaks were observed in the EIC at m/z 471.3469 (Figure 3). The two peaks with retention times of about 16 min had spectra containing ions at m/z 489.3575 and are therefore probably trihydroxy ursenoic acids and are discussed later. From the EIC at m/z 471.3469, four peaks were tentatively identified as oxohydroxy ursenoic acids, as their spectra contained the $[M + H]^+$ pseudomolecular ions and, usually, ions equivalent to $[M + H - H_2O]^+$, $[M + H - 2H_2O]^+$, $[M + H - HCOOH]^+$, and $[M + H - H_2O - HCOOH]^+$ (Table 2). The spectra of compounds **14**, **31**, and **32** also contained ions at m/z 493.3282 $[M + Na]^+$, confirming the identity of the pseudomolecular ion. Compound **14** was tentatively identified as 3-oxo-1α-hydroxy-urs-12-en-28-oic and has been previously isolated and identified from apple peel.[16]

Trihydroxy Ursenoic Acids m/z 489.3575; $[C_{30}H_{48}O_5 + H]^+$ and Tetrahydroxy Ursenoic Acid m/z 505.3524; $[C_{30}H_{48}O_6 + H]^+$. The EICs at m/z 489.3575 and 505.3524 showed the presence of two trihydroxy ursenoic acids at 16.22 min (compound **5**) and 16.45 min (compound **6**) and one tetrahydroxy ursenoic acid at 20.17 min (compound **9**) (Table 1). The spectra of compounds **5** and **6** contained ions corresponding to $[M + H - H_2O]^+$, $[M + H - 2H_2O]^+$, $[M + H - 3H_2O]^+$, and $[M + H - 2H_2O - HCOOH]^+$, confirming that these compounds are acids containing multiple hydroxyl groups. The spectrum of compound **9** contained ions corresponding to $[M + H - H_2O]^+$, $[M + H - 2H_2O]^+$, $[M + H - HCOOH]^+$, and $[M + H - H_2O - HCOOH]^+$, confirming that compound **9** also contains multiple hydroxyl groups and a carboxyl group.

Oxodihydroxy Ursenoic Acids m/z 487.3418; $[C_{30}H_{46}O_5 + H]^+$. The EIC at m/z 487.3418 showed the presence of two compounds with RTs of 20.17 min (compound **9**) and 21.86 min (compound **11**). The spectrum of compound **11** contained the molecular ion $[M + H]^+$and ions corresponding to $[M + Na]^+$, $[M - H_2O + H]^+$, $[M - 2H_2O + H]^+$, $[M - HCOOH + H]^+$, and $[M - H_2O - HCOOH + H]^+$ (Table 2), confirming the pseudomolecular

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Table 3. Relative Amount of Each Compound Measured Normalized to Total Peak Area

no.	compound name	average	'Braeburn'	'Granny Smith'	'Sciros'	O.P. 'Red Field'	'Cripps Pink'	'Fuji'	'Scilate'
1	dihydroxy-urs-12-en-28-oic acid	0.13	0.14	0.00	0.22	0.24	0.07	0.12	0.07
2	unknown	1.64	1.37	0.13	0.53	1.24	6.39	0.73	0.59
3	unknown	0.76	0.44	1.21	0.48	2.14	0.53	0.26	0.26
4	unknown	1.76	1.04	1.60	1.77	4.65	1.26	0.73	1.01
5	trihydroxy-urs-12-en-28-oic acid	2.48	2.03	1.88	0.63	0.49	4.10	3.16	5.34
6	trihydroxy-urs-12-en-28-oic acid	0.11	0.28	0.01	0.26	0.24	0.00	0.00	0.00
7	unknown	2.50	1.00	1.91	3.63	6.64	0.75	0.77	2.49
8	unknown	3.45	2.75	2.56	3.07	5.14	4.18	3.34	2.56
9	tetrahydroxy-urs-12-en-28-oic acid	1.26	5.28	0.08	1.16	1.68	0.38	0.20	0.23
10	unknown	0.93	1.25	0.88	0.35	1.84	0.74	0.78	0.64
11	3-oxo-1,19α-dihydroxy-urs-12-en-28-oic acid (annurcoic acid)	8.39	12.32	5.08	9.27	8.90	8.79	6.40	7.80
12	unknown	3.35	5.22	3.35	1.02	5.69	2.77	3.35	2.04
13	unknown	2.25	3.44	2.33	1.34	3.30	1.49	2.29	1.62
14	3-oxo-1α-hydroxy-urs-12-en-28-oic acid	2.14	4.91	1.25	2.02	1.98	2.21	1.21	1.55
15	3β-*p*-coumaroyloxy-dihydroxy-urs-12-en-28-oic acid	1.54	1.53	0.17	0.23	0.51	1.70	2.60	4.01
16	3β,19α-dihydroxy-urs-12-en-28-oic acid (pomolic acid)	1.86	1.73	1.66	1.96	1.78	1.69	1.62	2.65
17	3-oxo-hydroxy-urs-12-en-28-oic acid	3.69	5.38	4.98	5.45	2.74	2.58	2.21	3.20
18	dihydroxy-urs-12-en-28-oic acid	0.59	0.38	0.15	0.28	0.33	0.46	1.02	1.47
19	dihydroxy-urs-12-en-28-oic acid	1.23	1.38	1.31	1.23	1.04	1.25	1.01	1.51
20	3β-*trans*-*p*-coumaroyloxy-2α,13β-dihydroxy-urs-12-en-28-oic acid	3.06	2.88	2.03	4.28	1.55	3.09	4.10	3.10
21	3β-*cis*-*p*-coumaroyloxy-2α,13β-dihydroxy-urs-12-en-28-oic acid	1.84	1.75	0.65	2.94	2.07	1.89	2.09	0.94
22	dihydroxy-urs-12-en-28-oic acid	1.04	0.83	2.00	1.17	0.65	1.04	0.75	1.09
23	3β-*trans*?-*p*-coumaroyloxy-2α,13β-dihydroxy-urs-12-en-28-oic acid	0.34	0.40	0.00	0.73	0.56	0.57	0.00	0.00
24	dihydroxy-urs-12-en-28-oic acid	0.56	0.74	0.62	0.76	0.58	0.31	0.42	0.56
25	unknown	0.29	0.37	0.00	0.78	0.64	0.03	0.00	0.12
26	unknown								
27	unknown	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
28	oxo-urs-12-en-28-oic acid	0.59	0.90	0.61	0.81	0.51	0.37	0.49	0.51
29	unknown (dihydroxy-urs-12-en-28-oic acid)	1.91	3.13	1.73	2.38	1.86	1.39	1.59	1.35
30	oxo-urs-12-en-28-oic acid	1.96	3.21	2.04	3.39	1.33	1.16	1.29	1.49
31	3-oxo-hydroxy-urs-12-en-28-oic acid	2.56	8.63	0.00	0.00	2.21	2.01	3.28	1.79
32	3-oxo-hydroxy-urs-12-en-28-oic acid	2.04	0.00	1.93	2.97	2.57	1.94	2.15	2.42
33	3β-?-*p*-coumaroyloxy-hydroxy-urs-12-en-28-oic acid	2.00	1.08	1.94	2.83	1.21	1.49	2.94	2.31
34	3β-*trans*-*p*-coumaroyloxy-2α-hydroxy-urs-12-en-28-oic acid	6.34	3.67	4.94	8.94	4.18	5.29	8.95	7.65
35	3β-*cis*-*p*-coumaroyloxy-2α-hydroxy-urs-12-en-28-oic acid	4.35	1.33	7.18	5.76	2.32	3.55	6.05	4.46
36	oxo-urs-12-en-28-oic acid	2.83	3.39	4.29	4.20	2.25	1.63	2.06	2.50
37	3β-hydroxy-lup-20(29)-en-28-oic acid (betulinic acid)	6.03	3.80	9.62	5.85	4.92	6.76	5.33	6.85
38	3β-hydroxy-urs-12-en-28-oic acid (ursolic acid)	5.41	4.48	8.56	5.36	3.95	5.56	4.55	6.45
39	unknown	1.29	0.31	0.00	0.07	4.40	2.65	0.12	1.00
40	unknown	2.26	1.05	3.74	1.90	1.79	2.63	2.26	2.80
41	unknown	6.49	3.78	9.76	6.10	5.40	7.19	6.12	7.85
42	3β-hydroxy-urs-12-en-28-ol (uvaol)	5.06	1.53	6.42	2.53	3.23	5.70	11.70	3.12
43	unknown	1.70	0.86	1.39	1.36	1.25	2.40	1.97	2.60
	total ursenoic acids peak area		3,630,859	3,110,778	4,243,395	4,381,954	4,207,580	4,753,673	3,507,671
	% lipase inhibition		39	71	60	64	70	60	50

ion at m/z 487.3418 and elemental composition of $C_{30}H_{46}O_5$. Therefore, compound **11** was identified as 3-oxo-1α,19α-dihydroxy-urs-12-en-28-oic (annucroic acid), which is the only oxodihydroxy acid to have been previously reported in apple peel.[33]

Coumaroyloxyhydroxy Ursenoic Acids m/z 619.3993; $[C_{39}H_{54}O_6 + H]^+$. The EIC at m/z 619.3993 showed the presence of three compounds at 32.22 (compound **33**), 32.64 (compound **34**), and 32.96 min (compound **35**). For these three compounds, m/z 619.3993 is the base ion (Table 1), with

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less intense ions at m/z 641.3813 $[M + Na]^+$, 437.3414, and 409.3465. The ion at m/z 437.3414 corresponds to $C_{30}H_{45}O_2$, $[M + H - C_9H_7O_3 - H_2O]^+$, and the loss of the coumaroyloxy group and water. In a like manner, m/z 409.3465 corresponds to $C_{29}H_{45}O$, $[M + H - C_9H_7O_3 - HCOOH)]^+$, and the loss of the coumaroyloxy group and formic acid. Coumaroyloxy acid conjugates of ursolic acid have previously been reported in apple peel,[14,17] and in this study we observed two major and one minor compound that correspond to hydroxy ursenoic acid coumaryl conjugates. The two major compounds (**34** and **35**) are probably the cis and trans isomers, 3*β*-*cis*-*p*-coumaroyloxy-2*α*-hydroxy-urs-12-en-28-oic acid and 3*β*-*trans*-*p*-coumaroyloxy-2*α*-hydroxy-urs-12-en-28-oic acid, which have previously been identified in apple.[17] The minor hydroxy ursenoic acid coumaryl conjugate (compound **33**) has the same molecular weight and may have the hydroxy group located elsewhere in the molecule, such as on C19, where it is found for pomolic acid.

Coumaroyloxydihydroxy Ursenoic Acids *m/z* 635.3942; $[C_{39}H_{54}O_7 + H]^+$. The EIC at m/z 635.3942 showed the presence of nine compounds that are potentially coumaroyloxydihydroxy ursenoic acids; however, only four were considered further. These are compounds **15**, **20**, **21**, and **23** at 24.19, 26.62, 26.78, and 27.00 min, respectively. The spectra of these compounds contained a base ion at m/z 635.3942 (Table 1), and the ions m/z 657.3762 $[M + Na]^+$ (compounds **20**, **21**, and **23**) and m/z 453.3363. The ion m/z 453.3363 corresponds to $C_{30}H_{45}O_3$, $[M + H - C_9H_7O_3 - H_2O]^+$, the loss of the coumaroyloxy group and water, similar to that for coumaroyloxyhydroxy ursenoic acid above. He and Lui[14] previously identified cis and trans coumaroyloxydihydroxy ursenoic acids in apple, but appear to have mistakenly named the compounds 3*β*-*cis*-*p*-coumaroyloxy-2*α*,3*β*,13*β*-trihydroxy-urs-11-en-28-oic and 3*β*-*trans*-*p*-coumaroyloxy-2*α*,3*β*,13*β*-trihydroxy-urs-11-en-28-oic acid. Furthermore, data to support the C2 and C13 positions for the hydroxy groups were obtained by comparison with data for an authentic compound, 2*α*,3*β*,13*β*-trihydroxy-urs-11-en-28-oic acid. In contrast, several urs-12-en-28-oic acids (annurcoic acid, pomolic acid, and 2-oxopomolic acid) with hydroxyl groups at 2*α* and 19*α* have been identified, with robust NMR evidence for the positioning of the hydroxyl groups.[15,33] Therefore, two of the peaks identified here are likely to be 3*β*-*cis*-*p*-coumaroyloxy-2*α*,19*α*-dihydroxy-urs-12-en-28-oic and 3*β*-*trans*-*p*-coumaroyloxy-2*α*,19*α*-dihydroxy-urs-12-en-28-oic acid, and the other two are likely to be the corresponding oleanoic acid isomers.

Compound **42** was identified as 3*β*-hydroxy-urs-12-en-28-ol (uvaol) on the basis of a mass of 443.3900 $[C_{30}H_{50}O_2 + H]$ and retention time identical to that of authentic uvaol.

Unknowns. The identities of 13 compounds (**2**–**4**, **10**, **12**, **13**, **25**–**27**, **39**–**41**, and **43**) remain unknown, even though robust elemental composition data could be calculated from the accurate mass data (Table 1).

The compound identifications indicated in Table 1 should be regarded as tentative unless supported by reference to an authentic standard. Accurate mass spectral data provided an indication of the type of group added to the base ursolic acid molecule. Although it is possible that these same modifications could be to a molecule of the same elemental composition, for example, oleanolic or betulinic acid, we have chosen to use ursolic acid as the basic structure because it is consistently known to be a triterpene component of apple peel.[14,16,17,26] However, it is feasible, but in our view unlikely, that the compounds tentatively identified have a base structure other than ursolic acid.

The mass spectrometric analysis presented here did not identify the locations of the additional hydroxyl, oxo, or coumaroyloxy groups within the molecule or the isomeric configuration of the groups (*α* vs *β*, and cis vs trans). Electron impact–mass spectrometry (EI-MS) of triterpenes is well-known, and the fragmentation includes a retro-Diels–Alder reaction in ring C that yields a fragment containing the D and E rings (Figure 1).[7] Identification of this fragment confirms the location of hydroxyl groups at position 19 in compounds such as annurcoic acid (compound **11**) and pomolic acid (compound **16**). Despite attempts using MS/MS and ISCID, this fragment could not be observed except when using direct infusion of the authentic ursolic acid, and so we could not confirm hydroxylation at C19. It may be that the loss of H_2O due to a ring hydroxylation is more facile than the retro-Diels–Alder reaction in ring C, making the ring D + E + hydroxylation unobservable.

The reversed phase chromatographic retention times generally supported the identifications made from the mass spectral data. In reversed phase HPLC, compounds with greater water solubility have shorter retention times. Increasing hydroxylation would be expected to increase water solubility and therefore shorten retention times. For example, the dihydroxy-urs-12-en-28-oic acids (compounds **18**, **19**, **22**, and **24**) have shorter retention times than hydroxy-urs-12-en-28-oic acid (compound **38**, ursolic acid). Similarly, the trihydroxy-urs-12-en-28-oic acid (compounds **5** and **6**) have shorter retention times than the dihydroxy-urs-12-en-28-oic acids. However, contrarily, the putative tetrahydroxy-urs-12-en-28-oic acid (compound **9**) has a longer retention time than the trihydroxy-urs-12-en-28-oic acids. Possibly, hydrogen bonding between adjacent hydroxyl groups negates the effect of additional hydroxyls on reversed-phase retention times.

Ursenoic Acid Variation by Cultivar. As authentic standards were not available for most of the compounds detected, absolute quantification is not possible. To assess the quantitative variation between cultivars, the TIC peak areas of each compound were determined, and then the relative amounts were expressed as percent total area of all 43 compounds (Table 3). The total peak areas for all 43 compounds varied from 3.11×10^6 ('Granny Smith') to 4.75×10^6 ('Fuji'). Although 'Granny Smith' is considered to have a waxy skin, it does not appear to contain the greatest amount of ursenoic acids, and possibly the relatively low concentrations are related to the increased susceptibility of 'Granny Smith' to superficial scald, as has been suggested by Rudell et al.[12,13]

In this study, the relative concentration of ursolic acid (compound **38**) was low compared with those of the other ursenoic acid components. This is in contrast to previous studies on apple epicuticular waxes, in which concentrations of ursolic acid may reach 30% of the total wax,[26,34] and other ursenoic acid components were not reported. In these previous studies, the wax layer was dissolved in solvent (chloroform and/or ethanol), whereas in this study, apple peel was extracted into aqueous ethanol by homogenization. These differences in the relative concentrations of the ursenoic acids between the studies may be a result of the extraction procedure or because the relative concentrations of ursenoic acids vary between the epicuticular waxes and the cell layers of apple peel.

Triterpene and Lipase Inhibition. Peel extracts from all of the apple cultivars exhibited lipase-inhibitory activity and ranged from the lowest for 'Braeburn' (39%) to the highest for 'Granny Smith' (70%). Ursolic acid was tested in this assay system and had substantial lipase-inhibitory activity (data not shown); however, the total ursenoic acid peak area was not correlated with lipase-inhibitory activity. A previous study reported that oligomeric procyanidins from apple inhibit porcine pancreatic lipase and that other polyphenols have much less inhibitory activity.[35] The data presented here indicate that apple peel extracts have lipase-inhibitory activity and contained substantial concentrations of ursenoic acids and probably polyphenols. Further research is required to identify conclusively the lipase-inhibitory active components in these extracts.

Several previous studies have identified triterpene compounds in apple skin; however, the only cultivars studied were 'Red Delicious'[14,17] and 'Annurca'.[15,16,33] These studies differed from the present, as compounds were isolated using multiple chromatographic steps and then chemically identified by a combination of MS and NMR. In contrast, we have used UHPLC-HRMS to provide a comprehensive analysis of the range of triterpenoic acids in seven apple cultivars. The results presented here show that apple peel contains a large number of ursenoic acids with different chemical structures and that the relative amounts of these compounds vary among apple cultivars. Previously, only a limited number of ursenoic acid derivatives had been identified in apple peel, but this study shows that for each compound class (oxo, hydroxyl, dihydroxy, trihydroxy, etc.) of the ursenoic acids, there are multiple compounds present, presumably with different isomeric chemical structures. Because ursolic acid and other terpenoic acids are known to have a variety of biological activities[19−25] and there is clearly significant variation in these compounds among apple cultivars, the breeding of novel apple fruit with improved health benefits and insect management attributes represents an opportunity for new product development. A better understanding of the components of apple peel will lead to an increasing understanding of physiological and postharvest disorders that are associated with apple peel.

AUTHOR INFORMATION

Corresponding Author

*E-mail: tony.mcghie@plantandfood.co.nz. Phone: +64 6 953 7684.

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ABBREVIATIONS USED

ESI, electrospray ionization; HPLC, high-performance liquid chromatography; HRMS, high-resolution mass spectrometry; ISCID, in-source collision-induced dissociation; LC, liquid chromatography; MS, mass spectrometry; QTOF, quadrupole time-of-flight; RT, retention time.

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