Title

Identification/quantification of free and bound phenolic acids in peel and pulp of apples (Malus domestica) using high resolution mass spectrometry (HRMS)

Authors

Lee, Jihyun
Chan, Bronte Lee Shan
Mitchell, Alyson E

Publication Date

2017

DOI

10.1016/j.foodchem.2016.07.166

Peer reviewed

Food Chemistry 215 (2017) 301–310

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Identification/quantification of free and bound phenolic acids in peel and pulp of apples (*Malus domestica*) using high resolution mass spectrometry (HRMS)

Jihyun Lee [a], Bronte Lee Shan Chan [b], Alyson E. Mitchell [b,*]

[a] Department of Food Science and Technology, Chung-Ang University, Anseong 17546, South Korea
[b] Department of Food Science and Technology, University of California, Davis, One Shields Avenue, Davis, CA 95616, USA

ARTICLE INFO

Article history:
Received 31 March 2016
Received in revised form 16 July 2016
Accepted 28 July 2016
Available online 29 July 2016

Keywords:
Apple
Phenolic acid
High resolution mass spectroscopy
UHPLC-(ESI)QTOF MS/MS
QTOF
MS/MS
Peel
Pulp

ABSTRACT

Free and bound phenolic acids were measured in the pulp and peel of four varieties of apples using high resolution mass spectrometry. Twenty-five phenolic acids were identified and included: 8 hydroxybenzoic acids, 11 hydroxycinnamic acids, 5 hydroxyphenylacetic acids, and 1 hydoxyphenylpropanoic acid. Several phenolics are tentatively identified for the first time in apples and include: methyl gallate, ethyl gallate, hydroxy phenyl acetic acid, three phenylacetic acid isomers, 3-(4-hydroxyphenyl)propionic acid, and homoveratric acid. With exception of chlorogenic and caffeic acid, most phenolic acids were quantified for the first time in apples. Significant varietal differences ($p < 0.05$) were observed in both peel and pulp. The levels of total phenolic acids were higher in the pulp as compared to apple peel (dry weight) in all varieties. Coumaroylquinic, protocatechuic, 4-hydroxybenzoic, vanillic and t-ferulic acids were present in free forms. With exception of chlorogenic acid, all other phenolic acids were present only as bound forms.

1. Introduction

Phenolic acids are a diverse group of secondary metabolites widely distributed throughout the plant kingdom. Phenolic acids are interesting as a functional ingredient in foods because of their anticarcinogenic, antiviral, antibacterial and potent antioxidant activities (Khadem & Marles, 2010; Park et al., 2014). Phenolic acids (phenolcarboxylic acids) are composed of a phenol attached to an organic carboxylic acid function. There are two classes of phenolic acids which include hydroxybenzoic acids (C6–C1), derived from benzoic acid, and the hydroxycinnamic acids (C6–C3) which consist of a benzene ring coupled to a prop-2-enoic acid residue (—CH=CH—COOH). Both classes can be modified by hydroxylation (i.e. mono-, di-, or trihydric) and/or methoxylation of the aromatic ring (Khadem & Marles, 2010). The functional activity of phenolic acids is dependent upon the degree and arrangement of hydroxylation and methylation on the aromatic ring (Robards, Prenzler, Tucker, Swatsitang, & Glover, 1999). For example, the inhibition of LDL oxidation by methylated caffeic acid (ferulic acid) is lower as compared with caffeic acid (Meyer, Donovan, Pearson, Waterhouse, & Frankel, 1998). When caffeic acid is esterified to quinic acid (e.g., chlorogenic acid), *in vitro* antioxidant activity decreases whereas the dimer of caffeic acid demonstrates strong DPPH scavenging activity (Chen & Ho, 1997). Understanding the range and distribution of phenolic acids in a food is critical towards understanding the functionality of a food.

The phenolic acids most frequently consumed in the diet include the hydroxycinnamic acid derivatives such as *p*-coumaric acid, caffeic, ferulic and sinapic acids (El Gharras, 2009). In plants, only a small fraction exists as free esters. The majority are linked to structural components (cellulose, proteins, lignin), or small molecules (e.g. flavonoids, glucose, quinic, shikimic acid, lactic, malic and tartaric acid) or to other natural constituents (e.g., terpenes) through ester, ether, or acetal bonds (Bravo, 1998; Mattila & Hellström, 2007; Łata, Trampczynska, & Paczesna, 2009). Bound phenolic acids can be liberated using alkaline and acid hydrolysis (Kim, Tsao, Yang, & Cui, 2006; Mattila & Kumpulainen, 2002; Nardini et al., 2002). To date, few studies includes information on the composition of both free (nonhydrolysed) and bound (hydrolysed) phenolic acids in apples (Mattila & Kumpulainen, 2002; Soares, Kuskoski, Gonzaga, Lima, & Mancini Filho, 2008).

* Corresponding author.
E-mail address: aemitchell@ucdavis.edu (A.E. Mitchell).

http://dx.doi.org/10.1016/j.foodchem.2016.07.166
0308-8146/

In California, 130 million tons of apples are produced annually (USDA, 2011). About 60% of these apples are processed into canned, dried, or juice products; generating millions of pounds of processing co-product, largely composed of apple peel (USDA, 2011). Apple peels have the potential to be a valuable processing co-product however, there is little information available on the composition of phenolic acids in commercially important apple cultivars; and especially in the peels of different varieties of apple (Escarpa & González, 1998, 1999). Varietal differences in phenolic acids in apples have been reported for only the predominant phenolic acids (i.e. chlorogenic and caffeic acid) (Escarpa & González, 1998; Huber & Rupasinghe, 2009; Vrhovsek, Rigo, Tonon, & Mattivi, 2004; Łata et al., 2009). Levels of free 5′-caffeoyl quinic acid, p-coumaroylquinic acid and p-coumaric acid were evaluated in the combined pulp and peel of some apples (Vrhovsek et al., 2004) and free, esterified, and insoluble-bound phenolic acids were reported in the pomace (skin, core, seeds calyx and stem) of Gala and Fuji apples (Soares et al., 2008).

The goal of this study was to identify and quantify the composition of both free and total (free plus bound and hydrolysed) phenolic acids in the peel and pulp of four commercially important apple varieties (var. Fuji, Golden Delicious, Granny Smith and Pink Lady) grown in California. High-resolution mass spectrometry (HRMS) was used to aid in the identification of broader range of phenolic acids than previous studies. High resolution MS/MS offers the advantage of improved sensitivity and accurate mass measurements which facilitates compound identification/confirmation and differentiation between molecular formulas having the same nominal masses.

2. Materials and methods

2.1. Chemicals and reagents

Phenolic acid standards (3-(4-hydroxyphenyl) propionic acid, 4-hydroxy benzoic acid, caffeic acid, chlorogenic acid, *p*-coumaric acid, protocatechuic acid, salicylic acid, sinapic acid, *t*-cinnamic acid, *t*-ferulic acid, and vanillic acid) and carbohydrate standards (fructose, glucose, sucrose, and sorbitol) were purchased from Sigma-Aldrich (MO, USA). Acetic acid (85:15) and HPLC-grade methanol were obtained from Fisher Scientific (NJ, USA). Butylated hydroxyanisole (BHA) and LC/MS grade acetonitrile were obtained from Acros Organic (NJ, USA) and Burdick and Jackson (MI, USA), respectively.

2.2. Apple samples

Apples (cv. Fuji, Golden Delicious, Granny Smith, and Pink Lady) were obtained in Placerville, CA in January, 2014 in Apple Hill, Camino, CA. Apple trees were grown on Akin loam soil type and fertilized with calcium nitrate, urea and 15–15–15 formulation. Overhead watering was conducted every 14–21 days with micro sprinkler and drip. Apples were harvested at commercial maturity. At least 30 randomly selected apples of each variety were used to make a composite sample for each variety. The peels and pulp were separated using an apple peeler and by coring the apples. The thickness of peel was ~1 mm. Peels and pulp were freeze-dried using a VirTis 50-SRC lyophilizer (SP Scientific, Gardiner, NY, USA) and stored at −80 °C until analysis. The samples were ground before extraction and sieved to generate a uniform powder size.

2.3. Analysis of soluble solids content (SSC) in apple samples

Soluble solids content (SSC, °Brix) were measured with a refractometer (PR-32a, ATAGO, Tokyo, Japan) on apple juice.

2.4. Hydrolysis and extraction of phenolic acids

A sequential extraction was performed. The first extraction employed an acidified methanol extraction of the freeze dried apple powder to obtain the free phenolic acids (Mattila & Kumpulainen, 2002; Wojdyło, Oszmiański, & Laskowski, 2008). A second extraction was performed on the same material after alkaline and acid hydrolysis to obtain bound phenolic acids.

Briefly, the freeze-dried apple powder (0.4 g) was homogenized in 7 mL of methanol containing 2 g/L BHA and 10% acetic acid for 1 min and then ultrasonicated for 30 min with ice to prevent degradation of phenolic compounds. The sample was volumized to 10 mL with water. The supernatant (1 mL) was filtered through a 0.20 μm membrane filter and analyzed for free phenolic acids. After removing the 1 mL aliquot of the supernatant for free phenolic acid analysis, 5 mL of 10 M NaOH and 12 mL water (containing 1% ascorbic acid and 0.415% EDTA) were added to the remaining extract with the residual pellet. Nitrogen gas was bubbled into the extract, and the bottle was sealed. EDTA and ascorbic acid were added to prevent degradation of phenolic acids during alkaline hydrolysis (Mattila, Hellstrom, & Torronen, 2006; Nardini et al., 2002). The extract was stirred overnight at room temperature and then adjusted to pH 2 with hydrochloric acid. Liberated phenolic acids were extracted three times with 15 mL of a mixture of cold diethyl ether and ethyl acetate (1:1, v/v) by manually shaking and centrifuging at 2000 rpm. The organic extracts were combined. An acidic hydrolysis was then performed by adding 2.5 mL of concentrated HCl to the remaining pellet. The extract was incubated at 85 °C for 30 min, cooled, and adjusted to pH 2. A cold diethyl ether and ethyl acetate (1:1, v/v) extraction was performed again. The diethyl ether and ethyl acetate extracts from the alkaline hydrolysis and the acid hydrolyses were combined and evaporated to dryness (bound phenolic acids). The bound phenolic acid extract was re-dissolved into 2 mL of methanol and filtered through a 0.20 μm membrane filter. The extracts were stored at −20 °C until analyzed (within 48 h).

Prior to UHPLC-(ESI)QTOF MS/MS analysis, *t*-cinnamic acid (final concentration of 300 ng/mL) was added to each extract as an internal standard as it is not present in apples to correct signal suppression mediated by other sample components during analysis.

2.5. Method validation (detection limit, linearity, and recovery)

Detection limits were determined using a signal-to-noise ratio of 3:1. Linearity was established for each standard by evaluating a wide range of concentrations. Recovery was measured by adding known amounts of caffeic acid, chlorogenic acid, *p*-coumaric acid, and protocatechuic acid to freeze-dried apple powder of each variety at the beginning of the extraction process. The amount of standard added to each sample (45–180 μg caffeic acid, 5–20 μg chlorogenic acid, 45–180 μg p-coumaric acid, and 25–100 μg protocatechuic acid) was equal to, and four times the amount reported in apples. Recoveries were determined through all sample extraction and chemical hydrolysis steps.

2.6. UHPLC-(ESI)QTOF MS/MS analysis

Phenolic acid were analyzed on an Agilent 1290 Infinity ultra-high performance liquid chromatography (UHPLC) coupled to a 6530 accurate mass quadrupole time-of-flight mass spectrometer via an electrospray interface (Agilent Technologies, Santa Clara, CA, USA). The UHPLC was equipped with a binary pump with integrated vacuum degasser (G4220A), an autosampler (G4226A) with thermostat (G1330B), and thermostatted column compartment (G1316C). Phenolic acids were separated by a Poroshell C_{18} column

(2.1 × 100 mm, 2.7 μm, Agilent Technologies) with a C_{18} guard column (2.1 × 5 mm, 1.8 μm, Optimize Technologies, Inc., Oregon City, Oregon) maintained at 30 °C. The mobile phase consisted of a gradient of 0.1% acetic acid in water (A) and 0.1% acetic acid in acetonitrile (B) as follows: 5–10% B, 0–5 min; 10–12% B, 5–8 min; 12–15% B, 8–10 min; 15% B, 10–15 min; 15–55% B, 15–18 min; 55–90% B, 18–20 min. The column was re-equilibrated between injections for 4 min with initial mobile phase. The flow rate was 0.4 mL/min and the injection volume was 5 μL.

To identify all possible phenolic acids, total ion spectra were collected over a mass range of *m*/*z* 100–1000 in negative mode at an acquisition rate of 1.0 spectra/s. Negative ionization produced higher sensitivity as compared with positive ionization. The drying gas temperature and flow rate were 225 °C and 8.0 L/min, respectively. The sheath gas temperature and flow rate were 300 °C and 10.0 L/min, respectively. The nebulizer gas pressure, skimmer voltage, octopole RF, and fragmentor voltage were 45 psi, 65 V, 750 V, and 125 V, respectively. The capillary voltage was 2.5 kV. Continuous internal calibration was performed during analysis to achieve the desired mass accuracy of recorded ions with the ions of *m*/*z* of 119.0363 (proton abstracted purine) and 980.0164 (acetate adduct of protonated hexakis (1H, 1H, 3H-tetrafluoropropoxy)phosphazine or HP-921). An accurate mass database of all potential phenolic acids present in plants was built using a personal compound database library (PCDL) manager (Agilent Technologies). This database included phenolic acids from phenol explorer database (www.phenol-explorer.eu) and phenolic acids reported in the literature. The theoretical exact mass was calculated based on formula. This database was imported into MassHunter Qualitative Analysis Software (Agilent Technologies) and used to search potential phenolic acids in the MS data files of each sample obtained from QTOF in MS^1 mode. Phenolic acids were identified based on comparisons of their observed mass with calculated theoretical mass and isotope fidelity (i.e., abundance of the isotopes, and isotope spacing). The peaks in chromatograms corresponding to possible phenolic acids were further investigated using negative mode UHPLC-(ESI)QTOF MS/MS by applying optimum collision energy (15–20 V) for different analytes.

Quantification of each phenolic acid with authentic standards was performed in the QTOF in the MS^1 mode using protocatechuic acid, 4-hydroxybenzoic acid, vanillic acid, salicylic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, *t*-ferulic acid, sinapic acid, and 3-(4-hydroxyphenyl) propionic acid. Identified phenolic acids were quantified using peak areas extracted based on each *m*/*z* on the ESI/MS^1 mode. Absolute quantification was achieved for the 10 phenolic acids (i.e., protocatechuic acid, 4-hydroxybenzoic acid, vanillic acid, salicylic acid, chlorogenic acid, caffeic acid, p-coumaric acid, t-ferulic acid, sinapic acid, and 3-(4-hydroxyphenyl) propionic acid) with commercial standards. Relative quantification was performed on all other phenolic acids using commercial standards with close retention times to each phenolic acid. For example, relative quantification of methyl gallate and syringic acid was achieved using protocatechuic acid. Dicaffeoylquinic acid was quantified using 4-hydroxy benzoic acid. Ethyl gallate was quantified using chlorogenic acid. Hydroxy phenyl acetic acid, benzoic acid, phenylacetic acid (RT = 5.9), and rosmarinic acid (RT = 5.9) were quantified using vanillic acid. Three isomers of coumaroylquinic acid were quantified using 3-(4-hydroxyphenyl) propionic acid. Rosmarinic acid (RT = 9.3) was quantified using salicylic acid. Phenylacetic acid isomers (RT = 11.0 and 17.3) and homoveratric acid were relatively quantified using sinapic acid. Extracts that exceeded the concentration range of the standard curve were diluted before injection. The calibration curves were repeated several times during sample analyses. Blank extraction injections were run to evaluate background signal and cross contamination during sample analysis.

2.7. Statistical analysis

Statistical analysis was performed using IBM SPSS statistics software (v. 20.0, SPSS, Inc., Chicago, IL). Multivariate analysis of variance (MANOVA) was applied to evaluate the effect of varietal differences of apples and the effect of layers (peels or pulp) on phenolic acid levels, and variety × layer interaction. Significant differences of phenolic acid concentrations among apple varieties in the same layer (i.e., peel or pulp) were determined using an independent *t*-test at $p < 0.05$ or one-way ANOVA followed by the Duncan's multiple range test at $p < 0.05$. Significant differences of phenolic acid concentrations among layers (i.e., peel and pulp) in the same variety were determined using a paired *t*-test at $p < 0.05$.

3. Results and discussion

3.1. Soluble solids contents (SSC, °Brix) in apple pulp

The soluble solids content can be used as a fruit maturity index for determining apple harvest (Drake, Elfving, & Eisele, 2002). Herein, soluble solids were 14.82% for Fuji, Pink Lady, Golden Delicious and 14.04% for Granny Smith apples and demonstrate near equivalent maturity levels.

3.2. Method validation for phenolic acid quantification using UHPLC-(ESI)QTOF MS^1

Phenolic acids were quantified in extracts of apple peel and pulp using UHPLC-(ESI)QTOF MS^1. The limit of phenolic acid detection for the available standards ranged between 1 and 10 ng/mL with the linear dynamic ranges between 10–1000 and 30–1000 ng/mL (Table 1). The coefficient of determination for the standards (r^2) ranged between 0.9963 and 1.0000. Recoveries were determined using caffeic acid, chlorogenic acid, p-coumaric acid, and protocatechuic acid spiked at a high and low concentration and ranged between 87.8 and 110.2%.

3.3. Phenolic acid identification

A representative total ion chromatogram (TIC) of the free and bound phenolics in Fuji pulp is shown in Fig. 1a and b, respectively. Phenolic acids were identified by comparing the HRMS data obtained in single QTOF mode (MS^1) with their calculated masses and by examining their isotope fidelity (isotope abundance and isotope spacing). Peaks identified with a theoretical mass and observed *m*/*z* value of less than 5.2 ppm were further confirmed by obtaining QTOF MS/MS spectra and by comparison with authentic standards when available.

Table 1
Phenolic acid detection limit, linear range of quantification, and linearity.

Compound	Detection limit (ng/mL)	Linear range of quantification (ng/mL)	R^2
Protocatechuic acid	5	10–1000	0.9996
4-Hydroxy benzoic acid	10	30–1000	0.9997
Chlorogenic acid	5	30–1000	0.9990
Vanillic acid	5	10–1000	0.9996
Caffeic acid	1	30–1000	1.0000
3-(4-Hydroxyphenyl) propionic acid	5	30–1000	0.9996
p-Coumaric acid	1	10–1000	1.0000
Salicylic acid	10	30–1000	0.9993
t-Ferulic acid	1	10–1000	0.9994
Sinapic acid	5	30–1000	0.9963

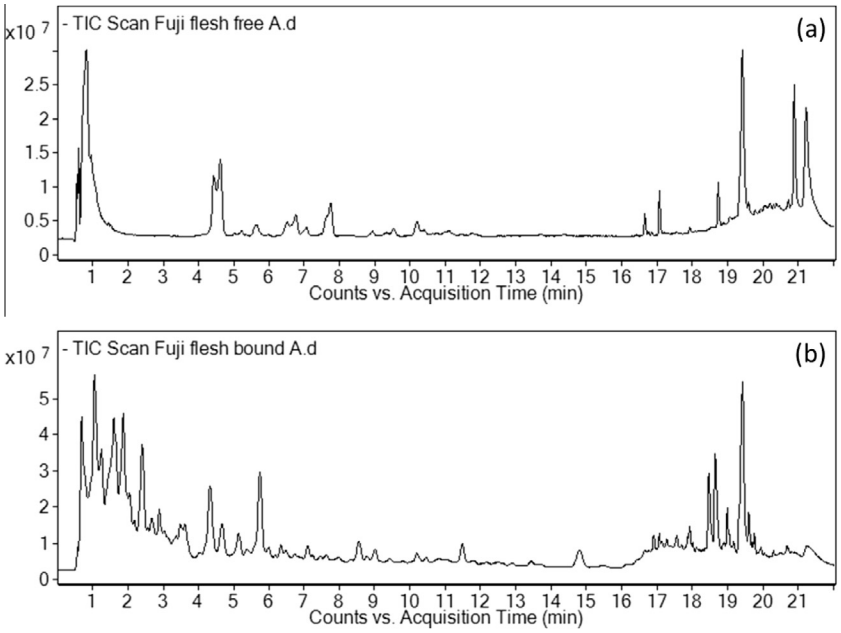

Fig. 1. A typical total ion chromatogram (TIC) of Fuji pulp (a) free phenolic acids (b) combined extracts of bound phenolic acids after alkaline and acid hydrolysis.

Twenty-five phenolic acids were characterized in apple peel and pulp of Fuji, Pink Lady, Golden Delicious and Granny Smith apples (Table 2 and Fig. 2). These included 8 hydroxybenzoic acid derivatives, 11 hydroxycinnamic acid derivatives, 5 hydroxyphenylacetic acid derivatives, and 1 hydoxyphenylpropanoic acid derivative. The identity of protocatechuic, 4-hydroxybenzoic, chlorogenic, vanillic, caffeic, 3-(4-hydroxyphenyl) propionic, *p*-coumaric, salicylic, *t*-ferulic, and sinapic acids were confirmed with authentic standards (Table 2). The remaining phenolic acids were tentatively identified based upon accurate mass and MS^2 spectra (Table 2). Methyl gallate, ethyl gallate, hydroxy phenyl acetic acid, three phenylacetic acid isomers, 3-(4-hydroxyphenyl)propionic acid, and homoveratric acid are tentatively identified in apples for the first time. Methyl gallate (Fig. 2**.1**) and ethyl gallate (Fig. 2**.6)** had pseudomolecular ions at *m*/*z* 183.0305 $[M-H]^-$ and *m*/*z* 197.0464 $[M-H]^-$, respectively and a base peak at *m*/*z* 125.0249 corresponding to the loss of either the methyl and CO_2 group for methyl gallate or ethyl moiety and CO_2 group for ethyl gallate.

The QTOF MS^2 spectra of protocatechuic, 4-hydroxy benzoic, hydroxy phenyl acetic, caffeic, 3-(4-hydroxyphenyl)propionic, *p*-coumaric, salicylic, and phenylacetic acids are characterized by a

Table 2
Phenolic acids identified in the peel and pulp of Fuji, Pink Lady, Golden Delicious and Granny Smith apples. Assignments are based on negative mode ESI and high resolution QTOF MS/MS data.[a]

Peak no.	t_R (min)	Compound	Predicted MS^1 *m*/*z*	Observed MS^1 *m*/*z*	Subclass
1	2.6	Methyl gallate	183.0299	183.0305	Hydroxybenzoic acid
2	2.6	Protocatechuic acid[b]	153.0193	153.0194	Hydroxybenzoic acid
3	3.0	Syringic acid	197.0455	197.0456	Hydroxybenzoic acid
4	3.5	Dicaffeoylquinic acid	515.1195	515.1195	Hydroxycinnamic acid
5	4.2	4-Hydroxy benzoic acid[b]	137.0244	137.0246	Hydroxybenzoic acid
6	4.7	Ethyl gallate	197.0455	197.0464	Hydroxybenzoic acid
7	4.7	Chlorogenic acid[b]	353.0878	353.0888	Hydroxycinnamic acid
8	5.1	Hydroxy phenyl acetic acid	151.0401	151.0402	Hydroxyphenylacetic acid
9	5.3	Vanillic acid[b]	167.0350	167.0355	Hydroxybenzoic acid
10	5.7	Benzoic acid	121.0295	121.0289	Hydroxybenzoic acid
11	5.9	Phenylacetic acid	135.0452	135.0459	Hydroxyphenylacetic acid
12	5.9	Caffeic acid[b]	179.0350	179.0352	Hydroxycinnamic acid
13	5.9	Rosmarinic acid	359.0772	359.0782	Hydroxycinnamic acid
14	6.1	Coumaroylquinic acid	337.0929	337.0930	Hydroxycinnamic acid
15	6.6	Coumaroylquinic acid	337.0929	337.0928	Hydroxycinnamic acid
16	7.1	Coumaroylquinic acid	337.0929	337.0930	Hydroxycinnamic acid
17	7.7	3-(4-Hydroxyphenyl)propionic acid[b]	165.0557	165.0565	Hydroxyphenylpropanoic acid
18	8.6	*p*-Coumaric acid[b]	163.0401	163.0400	Hydroxycinnamic acid
19	8.9	Salicylic acid[b]	137.0244	137.0247	Hydroxybenzoic acid
20	9.3	Rosmarinic acid	359.0772	359.0780	Hydroxycinnamic acid
21	10.3	*t*-Ferulic acid[b]	193.0506	193.0508	Hydroxycinnamic acid
22	10.8	Sinapic acid[b]	223.0612	223.0616	Hydroxycinnamic acid
23	11.0	Phenylacetic acid	135.0452	135.0450	Hydroxyphenylacetic acid
24	11.2	Homoveratric acid	195.0663	195.0664	Hydroxyphenylacetic acid
25	17.3	Phenylacetic acid	135.0452	135.0457	Hydroxyphenylacetic acid

[a] Deprotonated pseudomolecular ion $[M-H]^-$.
[b] Identified with commercial standards.

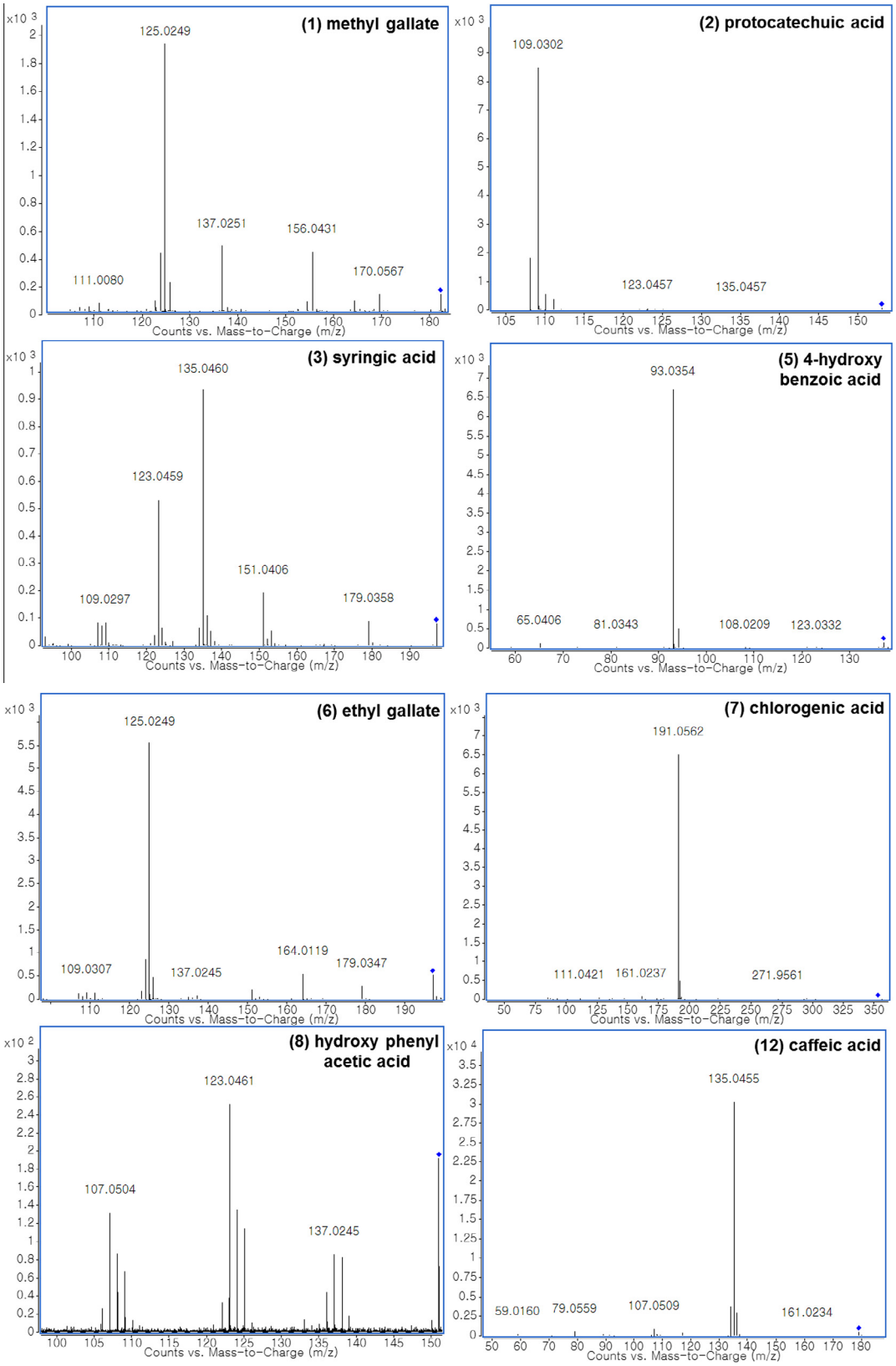

Fig. 2. UHPLC-(ESI)QTOF MS/MS spectra of chromatographic peaks obtained from apple extracts. A blue diamond indicates the pseudomolecular ion of the precursor ions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

strong fragment ion that corresponds to the loss of CO_2 from the carboxylic acid moiety (Fig. 2**.2**, **.5**, **.8**, **.12**, **.17**, **.18**, **.19** and **.25**). For protocatechuic, 4-hydroxybenoic, caffeic, p-coumaric, and salicylic acids the pseudomolecular ion was very weak. For syringic acid (Fig. 2**.3**) the base peak was observed at *m*/*z* 135.0460 corresponding to the loss of CO_2 and H_2O. Chlorogenic acid produced

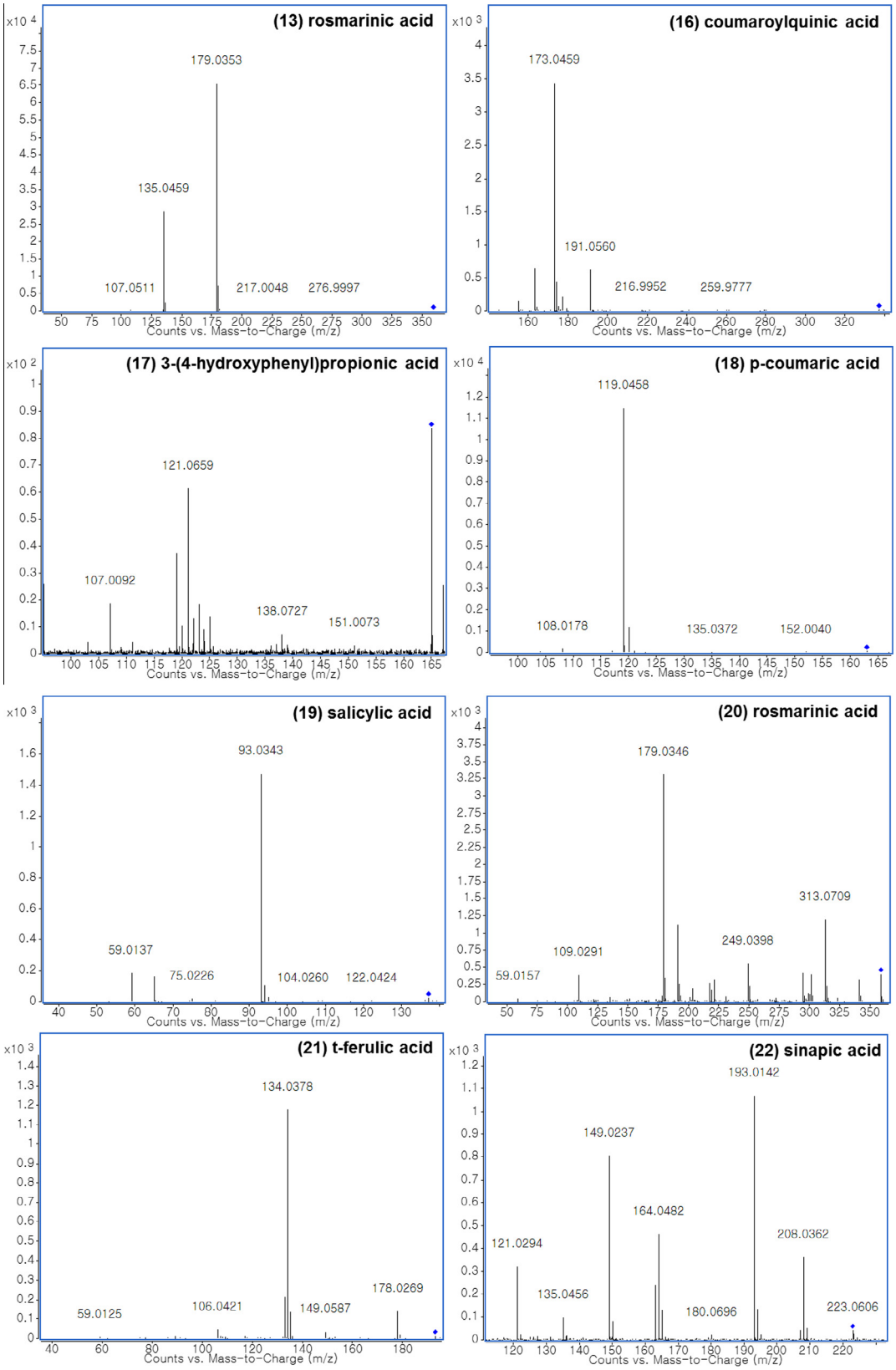

Fig. 2 (*continued*)

a weak pseudomolecular ion at m/z 353.0878 $[M-H]^-$ and a base peak ion at m/z 191.0562, corresponding to the loss of the caffeoyl moiety (Fig. 2.**7**). t-Ferulic acid produced a weak pseudomolecular ion and strong fragment ions, resulting from the loss of CH_3 moiety [m/z 178.0269] and CO_2 [m/z 134.0378] (Fig. 2.**21**). The two rosmarinic acid isomers demonstrated pseudomolecular ions at m/z

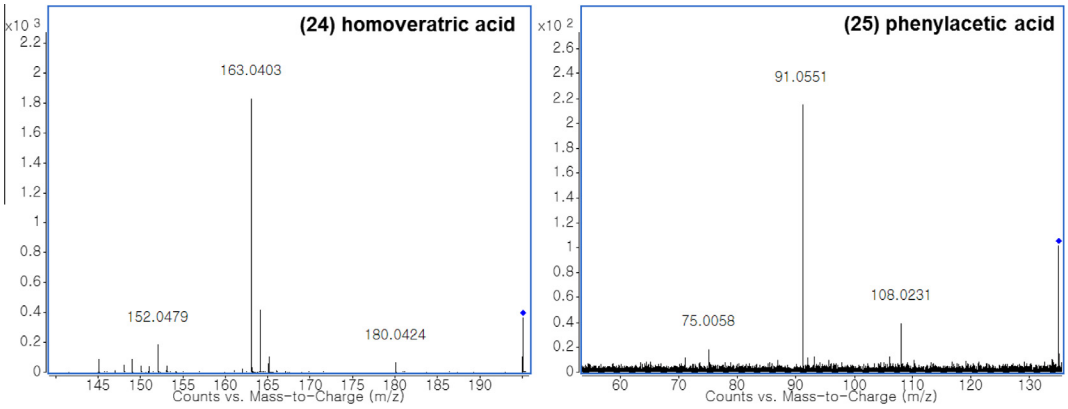

Fig. 2 (*continued*)

359.0772 $[M-H]^-$ and a predominate fragment ion of caffeic acid at *m*/*z* 179.0350 (Fig. 2**.13** and **.20**). Coumaroylquinic acid produced a distinct and strong fragment ion at *m*/*z* 173.0459, corresponding to the loss of coumaroyl and H_2O (Fig. 2**.16**). Sinapic acid demonstrated fragment ions at *m*/*z* 208.0362 and 193.0142, corresponding to the sequential loss of two CH_3 moieties (Fig. 2**.22**). Fragment ions were also observed for the sequential loss of CO2 at *m*/*z* 164.0482 and *m*/*z* 149.0237. Homoveratric acid (Fig. 2**.24**) displayed and intense fragment ion at *m*/*z* 163.0403, resulting from the loss of a methyl group and H_2O. No product ions were observed for dicaffeoylquinic acid, benzoic acid, the phenyl acetic acid isomers, and the coumaroylquinic acid isomers due to a low abundance of precursor ions.

3.4. Quantitative distribution of phenolic acids in apple peel and pulp

Absolute quantification of 10 phenolic acid compounds (protocatechuic acid, 4-hydroxybenzoic acid, vanillic acid, salicylic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, *t*-ferulic acid, sinapic acid, and 3-(4-hydroxyphenyl) propionic acid) with authentic standards and relative quantification of other phenolic acids in the peel and the pulp are presented in Table 3 The average total phenolic acid concentrations in apple peel and pulp are presented in Fig. 3.

Average phenolic acids levels of all apple varieties were significantly different ($p < 0.05$) in the pulp as compared to peel, on a dry weight basis, for all varieties compared (Fig. 3). In all varieties, levels of chlorogenic and caffeic acid predominate; with levels being significantly higher in the pulp as compared to the peel (Fig. 3). For example, levels of total chlorogenic acid ranged from 157.0–745.8 mg/kg DW in the pulp and 63.7–449.0 mg/kg DW in peel. Levels of caffeic acid ranged from 44.3–287.5 mg/kg DW in pulp and 1.4–125 mg/kg DW in peel. Chlorogenic and caffeic acid comprise between 59% and 68% of the total phenolic acids in peel and 75–83% in pulp of Fuji, Pink Lady and Golden Delicious apples. Levels were significantly lower in Granny Smith peel (29%) and pulp (39%). In an earlier study Escarpa and González (1998), reported a higher ratio of chlorogenic acid relative to caffeic acid in apple peel and pulp. Our results indicate that although chlorogenic acid was found in only the methanolic extracts, the bound forms could not be established as the ester bond could be hydrolyzed under the alkaline conditions used in this study. This result is comparable to results obtained by Mattila and Kumpulainen (2002) for Granny Smith apples (Mattila & Kumpulainen, 2002).

The levels of the hydroxycinnamic acids: coumaric, rosmarinic and t-ferulic were higher in the peel as compared to the pulp. All but t-ferulic in Fuji apple peel were present exclusively as bound forms. This result differs from Mattila and Kumpulainen (2002) for ferulic acid and may be explained by the unknown age/maturity/origin of the apples used Mattila and Kumpulainen (2002). Varietal differences in hydroxycinnamic acids were observed in both the peel and the pulp. For example, levels of rosmarinic acid were significantly lower in the peel and pulp of Granny Smith apples as compared with the other varieties. Varietal differences were also observed with the three coumaroylquinic isomers. For example, levels of the $t_R = 7.1$ isomer were significantly higher in the peel and pulp of Golden Delicious apples as compared to other varieties. This isomer was also present in higher concentrations in pulp, regardless of variety, as compared to the peel. All coumaroylquinic isomers were present exclusively in the free form. Sinapic acid was present in only the bound form and levels ranged between ND–3.9 mg/kg DW in the pulp and 0.9–1.3 mg/kg DW in peel. Varietal differences were more evident in the peel as compared to the pulp.

The levels of the hydroxybenzoic acids: syringic, 4-hydroxybenzoic and ethyl gallate were significantly higher ($p < 0.05$) in the pulp as compared to the peel whereas levels of protocatechuic, vanillic and benzoic acid were higher in the peel as compared to the pulp. With the exception of protocatechuic, 4-hydroxybenzoic, and vanillic acid all hydroxybenzoic acids were present in only the bound form. Protocatechuic acid was present as free and bound forms in only the peel of Granny Smith apples. This result is similar to Mattila and Kumpulainen (2002) who reported free and bound forms in Granny Smith apples (Mattila & Kumpulainen, 2002).

With exception of benzoic acid, our results comparing free and bound phenolics differ from those obtained by Soares et al. (2008) for Fuji apples. This is not unusual as Soares et al. (2008) investigated the composition of heat treated industrial apple pomace (Soares et al., 2008) whereas we investigated fresh apple peel and pulp individually. Varietal differences in hydroxybenzoic acids were present in both the peel and the pulp. For example, the levels of protocatechuic acid in the peel of Pink Lady apples are significantly higher than the other varieties examined. This may be significant as protocatechuic acid is reported as an effective antioxidant in both lipid and aqueous media by metal chelating and free radical scavenging (Li, Wang, Chen, & Chen, 2011). The levels of ethyl gallate were significantly higher (~30%) the peel of Fuji apples, and lower (~50%) in the pulp of Pink Lady apples. Levels of methyl gallate and salicylic acid varied little between the pulp and peel and variety.

The hydroxyphenylacetic acids are present as only bound forms and have less varietal variability. Levels of hydroxy phenyl acetic acid and homoveratic acid were higher in the peels of all varieties as compared to the pulp. No clear distribution of the two phenylacetic acid isomers (5.9 and 11.0 min) between pulp and peel

Table 3
Total and free phenolic acids in the peel and pulp of Fuji, Pink Lady, Golden Delicious and Granny Smith apples in mg/kg DW.[a]

Compound	Peel				Pulp			
	Fuji	Pink Lady	Golden Delicious	Granny Smith	Fuji	Pink Lady	Golden Delicious	Granny Smith
Hydroxycinnamic acid								
Dicaffeoylquinic acid	ND[b]	ND	ND	1.0 ± 0.2	ND	ND	ND	ND
				ND				
Chlorogenic acid[c]	449.0 ± 11.2d	428.8 ± 4.8c	335.2 ± 4.7b	63.7 ± 4.2a	745.8 ± 17.2c	569.1 ± 14.8b	781.8 ± 28.8c	157.0 ± 2.8a
	NA[d]	NA	NA	NA	NA	NA	NA	NA
Caffeic acid[c]	125.0 ± 3.6c	103.4 ± 16.6bc	80.9 ± 2.8b	1.4 ± 0.0a	277.4 ± 17.0c	184.6 ± 6.2b	287.5 ± 15.4c	44.3 ± 0.5a
	ND	ND	ND	ND	ND	ND	ND	ND
Rosmarinic acid (5.9 min)	34.1 ± 1.6c	27.0 ± 1.8b	23.5 ± 0.8b	3.2 ± 0.8a	33.3 ± 1.0d	23.7 ± 0.6b	31.2 ± 0.1c	4.5 ± 0.0a
	ND	ND	ND	ND	ND	ND	ND	ND
Coumaroylquinic acid (6.1 min)	16.5 ± 1.0b	1.9 ± 0.1a	34.3 ± 2.0c	4.0 ± 0.3a	3.2 ± 0.1c	1.0 ± 0.1a	6.0 ± 0.2d	1.8 ± 0.1b
	16.5 ± 1.0	1.9 ± 0.1	34.3 ± 2.0	4.0 ± 0.3	3.2 ± 0.1	1.0 ± 0.1	6.0 ± 0.2	1.8 ± 0.1
Coumaroylquinic acid (6.6 min)	6.9 ± 0.4c	2.7 ± 0.1b	0.1 ± 0.0a	0.1 ± 0.0a	7.8 ± 0.4c	2.9 ± 0.0b	3.0 ± 0.1b	0.1 ± 0.0a
	6.9 ± 0.4	2.7 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	7.8 ± 0.4	2.9 ± 0.0	3.0 ± 0.1	0.1 ± 0.0
Coumaroylquinic acid (7.1 min)	19.9 ± 1.3c	2.6 ± 0.1a	37.8 ± 0.9d	5.2 ± 0.4b	44.9 ± 1.3b	15.9 ± 0.0a	95.6 ± 0.4c	16.6 ± 0.1a
	19.9 ± 1.3	2.6 ± 0.1	(37.8 ± 0.9)	5.2 ± 0.4	44.9 ± 1.3	15.9 ± 0.0	95.6 ± 0.4	16.6 ± 0.1
p-Coumaric acid[c]	60.5 ± 2.3c	56.2 ± 5.0c	45.9 ± 3.2b	30.1 ± 0.4a	17.0 ± 0.8c	6.1 ± 0.2a	26.1 ± 0.9d	11.3 ± 0.8b
	ND	ND	ND	ND	ND	ND	ND	ND
Rosmarinic acid (9.3 min)	5.0 ± 0.5b	3.0 ± 0.4a	5.3 ± 0.1b	7.3 ± 0.4c	1.3 ± 0.1b	0.4 ± 0.0a	2.4 ± 0.1c	2.6 ± 0.4c
	ND	ND	ND	ND	ND	ND	ND	ND
t-Ferulic acid[c]	31.6 ± 0.7d	3.3 ± 0.0a	27.7 ± 0.7c	5.6 ± 0.1b	2.9 ± 0.2c	0.8 ± 0.1a	3.1 ± 0.1c	1.6 ± 0.0b
	0.3 ± 0.0	ND	ND	ND	ND	ND	ND	ND
Sinapic acid[c]	0.3 ± 0.0a	2.3 ± 0.0b	3.9 ± 0.4c	ND	1.1 ± 0.0b	0.9 ± 0.1a	1.3 ± 0.0c	1.1 ± 0.0b
	ND	ND	ND		ND	ND	ND	ND
Hydroxybenzoic acid								
Methyl gallate	5.9 ± 0.5	4.9 ± 0.7	5.1 ± 0.8	4.3 ± 0.5	6.3 ± 0.0b	6.3 ± 0.5b	4.5 ± 0.7a	3.9 ± 0.1a
	ND[b]	ND	ND	ND	ND	ND	ND	ND
Protocatechuic acid[c]	48.1 ± 2.5b	76.4 ± 4.9c	19.1 ± 3.2a	23.8 ± 0.6a	5.2 ± 1.5ab	3.6 ± 0.1a	9.6 ± 0.4c	6.3 ± 0.2b
	ND	ND	ND	(0.4 ± 0.0	ND	ND	ND	ND
Syringic acid	7.3 ± 1.2bc	5.8 ± 0.5b	1.6 ± 0.2a	9.4 ± 1.7c	29.8 ± 1.4bc	13.4 ± 5.4a	34.6 ± 3.4c	24.5 ± 1.0b
	ND	ND	ND	ND	ND	ND	ND	ND
4-Hydroxybenzoic acid[c]	4.2 ± 0.1b	6.6 ± 0.3c	2.2 ± 0.3a	3.8 ± 0.2b	14.8 ± 0.9b	10.2 ± 0.9a	17.7 ± 0.3c	17.2 ± 1.1c
	0.8 ± 0.0	ND	ND	0.9 ± 0.0	0.3 ± 0.0	ND	ND	ND
Ethyl gallate	34.3 ± 4.2b	1.3 ± 0.1a	0.1 ± 0.0a	0.1 ± 0.0a	92.2 ± 21.9ab	47.5 ± 27.0a	107.8 ± 20.5b	89.3 ± 7.2ab
	ND	ND	ND	ND	ND	ND	ND	ND
Vanillic acid[c]	2.2 ± 0.0c	1.8 ± 0.2bc	1.6 ± 0.0ab	1.4 ± 0.1a	ND	ND	0.7 ± 0.1	0.8 ± 0.0*
	1.0 ± 0.0	1.0 ± 0.1	0.8 ± 0.1	0.8 ± 0.1			ND	ND
Benzoic acid	8.7 ± 0.2ab	22.0 ± 1.6c	8.0 ± 0.6a	10.9 ± 0.0b	0.5 ± 0.0a	1.0 ± 0.0b	0.7 ± 0.3ab	1.1 ± 0.1b
	ND	ND	ND	ND	ND	ND	ND	ND
Salicylic acid[c]	ND	0.1 ± 0.0	0.4 ± 0.0*	ND	0.2 ± 0.0b	0.1 ± 0.0a	0.1 ± 0.0a	0.1 ± 0.0a
		ND	ND		ND	ND	ND	ND
Hydroxyphenylacetic acid								
Hydroxy phenyl acetic acid	26.8 ± 3.3	22.9 ± 1.2	37.3 ± 13.6	29.5 ± 6.0	3.9 ± 0.0c	1.7 ± 0.7a	3.2 ± 0.4c	2.8 ± 0.5ab
	ND	ND	ND	ND	ND	ND	ND	ND
Phenylacetic acid (5.9 min)	8.7 ± 0.2b	11.0 ± 0.5c	9.6 ± 0.3b	2.7 ± 0.3a	10.0 ± 0.3d	7.6 ± 0.4b	9.2 ± 0.2c	2.4 ± 0.0a
	ND	ND	ND	ND	ND	ND	ND	ND
Phenylacetic acid (11.0 min)	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	1.0 ± 0.0	0.3 ± 0.0a	0.3 ± 0.0a	0.3 ± 0.0a	1.6 ± 0.1b
	ND	ND	ND	ND	ND	ND	ND	ND
Homoveratric acid	3.5 ± 0.4	3.0 ± 0.2	2.5 ± 1.3	2.3 ± 0.6	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
	ND	ND	ND	ND	ND	ND	ND	ND
Phenylacetic acid (17.3 min)	1.4 ± 0.1ab	1.3 ± 0.0a	1.8 ± 0.1bc	1.8 ± 0.3c	6.2 ± 0.6ab	7.1 ± 0.5b	5.0 ± 0.6a	5.2 ± 0.4a
	ND	ND	ND	ND	ND	ND	ND	ND
Hydroxyphenylpropanoic acid								
3-(4-Hydroxyphenyl) propionic acid[c]	15.7 ± 1.9b	4.1 ± 0.0a	14.0 ± 1.4b	15.1 ± 0.7b	1.6 ± 0.1a	3.5 ± 0.2b	1.5 ± 0.3a	6.0 ± 0.1c
	ND	ND	ND	ND	ND	ND	ND	ND
Total	915.4 ± 13.4d	792.4 ± 27.2c	703.8 ± 3.4b	227.5 ± 3.1a	1305.7 ± 26.9c	907.9 ± 55.6b	1429.0 ± 74.4c	405.1 ± 7.2a
Free phenolic acid (%)	54.0	55.1	58.0	33.0	60.8	64.9	62.0	43.3

Total phenolic acids are the sum of free plus bound and hydrolysable phenolic acids.
[a] Mean values followed by different letters indicate significant varietal differences for the same layer at $p < 0.05$.
[b] ND stands for not detected.
[c] Absolute quantification using authentic standards.
[d] NA stands for not applicable as bound chlorogenic acid could be hydrolyzed under alkaline extraction conditions used in this study.
* Significant difference (*t*-test) in the concentrations of salicylic acid was noted at $p < 0.05$. A *t*-test was run for salicylic acid as only two varieties were available for comparison.

was observed whereas the 17.3 min was present in higher levels in the pulp as compared to the peel. The levels of 3-(4-hydroxyphenyl) propionic acid were higher in the peel as compare to the pulp in all varieties.

Comparisons of these data with other studies are difficult, as different methods are used for phenolic extractions and most studies do not include the measurement of the bound phenolic acids (Escarpa & González, 1998; Huber & Rupasinghe, 2009; Khanizadeh et al., 2008; Mattila & Kumpulainen, 2002; Sanoner, Guyot, Marnet, Molle, & Drilleau, 1999; Schieber, Keller, & Carle, 2001; Łata et al., 2009). Additionally, some studies report levels on a fresh weight basis (Escarpa & González, 1998; Khanizadeh

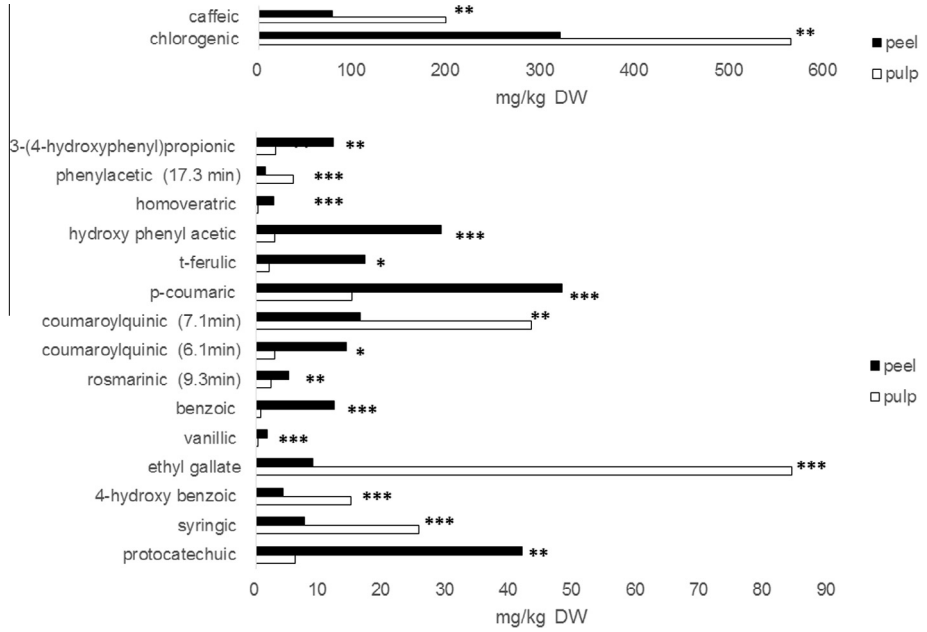

Fig. 3. Average total phenolic acids in apple peel and pulp of Fuji, Pink Lady, Golden Delicious, and Granny Smith apples in mg/kg apple DW. Significant difference between apple peel and pulp at $p < 0.05$, $p < 0.01$, and $p < 0.001$, are denoted by *, **, and *** respectively.

et al., 2008; Mattila & Kumpulainen, 2002; Sanoner et al., 1999; Łata et al., 2009) others on a dry weight basis (Huber & Rupasinghe, 2009; Schieber et al., 2001). Furthermore, extractions in alkaline conditions can promote the hydrolysis of caffeoylquinic acids such as chlorogenic to caffeic acid (Mattila & Kumpulainen, 2002). Herein, EDTA and ascorbic acid were added to extractions to prevent degradation of phenolic acids during alkaline hydrolysis (Mattila & Kumpulainen, 2002; Nardini et al., 2002). However, chlorogenic acid, can undergo hydrolysis in alkaline conditions even with the addition of EDTA and ascorbic acid (Nardini et al., 2002).

This study provides a comprehensive description of the composition of phenolic acids in the pulp and peels of 4 commercially important varieties of apples. For the first time, methyl gallate, ethyl gallate, hydroxy phenyl acetic acid, three phenylacetic acid isomers, 3-(4-hydroxyphenyl)propionic acid, and homoveratric acid were tentatively identified in apple samples. With exceptions of chlorogenic acid and caffeic acid, most phenolic acid compounds are quantified for the first time in apple samples.

Considerable varietal differences were observed. The major phenolic acids identified include chlorogenic acid, caffeic acid, *p*-coumaric acid, and protocatechuic acid in the peel, and chlorogenic acid and caffeic acid in pulp regardless of variety. Excluding chlorogenic acid and caffeic acid, the phenolic acid concentrations were higher in the peels of Fuji and Pink Lady apples as compared with their pulp. Protocatechuic, vanillic, *t*-ferulic, and 3-(4-hydroxyphenyl) propionic acid concentrations were significantly higher in the peel of all varieties examined as compared with the pulp. Homoveratric acid was found in only apple peel and not in pulp. This data indicates that apple peels are an excellent source of a range of phenolic acids and may be considered a valuable processing co-product.

Acknowledgements

The John E. Kinsella endowed chair in Food, Nutrition and Health provided financial support for this study. This research was supported by the Chung-Ang University Research Grants in 2015.

References

Bravo, L. (1998). Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition Reviews, 56*(11), 317–333.
Chen, J. H., & Ho, C.-T. (1997). Antioxidant activities of caffeic acid and its related hydroxycinnamic acid compounds. *Journal of Agricultural and Food Chemistry, 45*(7), 2374–2378.
Drake, S., Elfving, D., & Eisele, T. (2002). Harvest maturity and storage affect quality of Cripps Pink' (Pink Lady®) apples. *Hort Technology, 12*(3), 388–391.
El Gharras, H. (2009). Polyphenols: Food sources, properties and applications – A review. *International Journal of Food Science & Technology, 44*(12), 2512–2518.
Escarpa, A., & González, M. C. (1998). High-performance liquid chromatography with diode-array detection for the determination of phenolic compounds in peel and pulp from different apple varieties. *Journal of Chromatography A, 823*(1–2), 331–337.
Escarpa, A., & González, M. C. (1999). Fast separation of (poly)phenolic compounds from apples and pears by high-performance liquid chromatography with diode-array detection. *Journal of Chromatography A, 830*(2), 301–309.
Huber, G. M., & Rupasinghe, H. P. (2009). Phenolic profiles and antioxidant properties of apple skin extracts. *Journal of Food Science, 74*(9), C693–C700.
Khadem, S., & Marles, R. J. (2010). Monocyclic phenolic acids; hydroxy- and polyhydroxybenzoic acids: Occurrence and recent bioactivity studies. *Molecules, 15*(11), 7985–8005.
Khanizadeh, S., Tsao, R., Rekika, D., Yang, R., Charles, M. T., & Vasantha Rupasinghe, H. P. (2008). Polyphenol composition and total antioxidant capacity of selected apple genotypes for processing. *Journal of Food Composition and Analysis, 21*(5), 396–401.
Kim, K.-H., Tsao, R., Yang, R., & Cui, S. W. (2006). Phenolic acid profiles and antioxidant activities of wheat bran extracts and the effect of hydrolysis conditions. *Food Chemistry, 95*(3), 466–473.
Łata, B., Trampczynska, A., & Paczesna, J. (2009). Cultivar variation in apple peel and whole fruit phenolic composition. *Scientia Horticulturae, 121*(2), 176–181.
Li, X., Wang, X., Chen, D., & Chen, S. (2011). Antioxidant activity and mechanism of protocatechuic acid in vitro. *Functional Foods in Health and Disease, 1*(7), 232–244.
Mattila, P., & Hellström, J. (2007). Phenolic acids in potatoes, vegetables, and some of their products. *Journal of Food Composition and Analysis, 20*(3–4), 152–160.
Mattila, P., Hellstrom, J., & Torronen, R. (2006). Phenolic acids in berries, fruits, and beverages. *Journal of Agricultural and Food Chemistry, 54*(19), 7193–7199.
Mattila, P., & Kumpulainen, J. (2002). Determination of free and total phenolic acids in plant-derived foods by HPLC with diode-array detection. *Journal of Agricultural and Food Chemistry, 50*(13), 3660–3667.
Meyer, A. S., Donovan, J. L., Pearson, D. A., Waterhouse, A. L., & Frankel, E. N. (1998). Fruit hydroxycinnamic acids inhibit human low-density lipoprotein oxidation in vitro. *Journal of Agricultural and Food Chemistry, 46*(5), 1783–1787.
Nardini, M., Cirillo, E., Natella, F., Mencarelli, D., Comisso, A., & Scaccini, C. (2002). Detection of bound phenolic acids: Prevention by ascorbic acid and ethylenediaminetetraacetic acid of degradation of phenolic acids during alkaline hydrolysis. *Food Chemistry, 79*(1), 119–124.

Park, E. K., Ahn, S. R., Kim, D., Lee, E., Kwon, H. J., Kim, B. W., & Kim, T. H. (2014). Effects of unripe apple polyphenols on the expression of matrix metalloproteinase-1 and type-1 procollagen in ultraviolet irradiated human skin fibroblasts. *Journal of the Korean Society for Applied Biological Chemistry, 57*(4), 449–455.

Robards, K., Prenzler, P. D., Tucker, G., Swatsitang, P., & Glover, W. (1999). Phenolic compounds and their role in oxidative processes in fruits. *Food Chemistry, 66*(4), 401–436.

Sanoner, P., Guyot, S., Marnet, N., Molle, D., & Drilleau, J.-F. (1999). Polyphenol profiles of French cider apple varieties (*Malus domestica* sp.). *Journal of Agricultural and Food Chemistry, 47*(12), 4847–4853.

Schieber, A., Keller, P., & Carle, R. (2001). Determination of phenolic acids and flavonoids of apple and pear by high-performance liquid chromatography. *Journal of Chromatography A, 910*(2), 265–273.

Soares, M.C.R., Kuskoski, E.M., Gonzaga, L.V., Lima, A., & Mancini Filho, J. (2008). Composition of phenolic acids content in apple (Malus sp.) pomace. Semina: Ciências Agrárias, 29(2), 339–348.

United States Department of Agriculture (USDA) (2011). *Noncitrus fruits and nuts 2011 preliminary summary*. <http://www.nass.usda.gov/Statistics_by_State/California/Publications/Fruits_and_Nuts/201302frtrv.pdf> Accessed November 2015.

Vrhovsek, U., Rigo, A., Tonon, D., & Mattivi, F. (2004). Quantitation of polyphenols in different apple varieties. *Journal of Agricultural and Food Chemistry, 52*(21), 6532–6538.

Wojdyło, A., Oszmiański, J., & Laskowski, P. (2008). Polyphenolic compounds and antioxidant activity of new and old apple varieties. *Journal of Agricultural and Food Chemistry, 56*(15), 6520–6530.