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

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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).

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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