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Sinensetin, rutin, 3′-hydroxy-5, 6, 7, 4′-tetramethoxyflavone and rosmarinic acid contents and antioxidative effect of the skin of apple fruit

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ABSTRACT

A GC–MS method was developed for the separation and quantifiation of three flavones: sinensetin (SEN), rutin (RU) and 3′-hydroxy-5, 6, 7, 4′-tetramethoxyflavone (TMF) and rosmarinic acid (RA), a caffeic acid derivative, in the skin of apple fruit collected from different local markets of Bangladesh. The results showed significant variation in the amount of these markers in methanolic extracts of skin samples from different markets of Bangladesh, even though the values were almost identical for most of the cases. A variation in antioxidant activities, ranging from 62.82 to 92.34%, and variations in total phenolics, ranging from 6.69 to 10.20 mg caffeic acid/g dry weight of the methanol extracts, were observed. Antioxidative potency of the methanolic extracts was comparable to that of pure quercetin and the synthetic antioxidant butylated hydroxylanisole (BHA).

1. Introduction

Phenolics are a class of low molecular weight secondary metabolites found in most land plants. Phenolics (including flavonoids) protect plants against ultraviolet radiation, pathogens, and herbivores (Harborne & Willam, 2000). Although dietary intake varies considerably among geographic regions and cultures, the average daily consumption of flavonoids by humans is estimated to be 1 g (Bravo, 1998). Most of the protective effects of flavonoids in biological systems are ascribed to their antioxidant abilities, capacity to transfer electrons, quenching of free radicals and chelating abilities (Hirano et al., 2001), activate antioxidant enzymes, reduce alpha-tocopherol radicals and inhibit oxidases (Elliott, Scheiber, Thomas, & Pardini, 1992). Recently, there has been considerable interest in finding naturally occurring antioxidants to replace synthetic antioxidants in foods. Several studies have analyzed the antioxidant potential of a variety of herbs (Furuta, Nishiba, & Suda, 1997; Hertog, Hollman, & Katan, 1992). Among the different parts of plants studied, the leaves are reported to have highest antioxidant properties (Chung et al., 1999; Shahidi & Wanasundara, 1992; Venkatamuru, Patel, & Rao, 1983) and the most active principle among the phytochemicals is the phenolic fraction (Jung, Kim, & Kim, 1999; Nakasugi & Komai, 1998; Pietta, Simonetti, & Mauri, 1998; Shahidi & Wanasundara, 1992). The phenolics have in vivo antioxidant activities and have been used as natural antioxidants in food (Fuhrman, Lavy, & Aviram, 1995; Zloch, 1969).

The attractiveness of apples to consumers is determined both by their appearance and by attributes of firmness, taste, and health benefits. Flavonoids, secondary plant metabolites, contribute to both fruit colour and human health. Flavonoids are widely believed to possess antioxidative, antimicrobial, antimutagenic and anticarcinogenic properties (Formica & Regelson, 1995; Koes, Van Blokland, Quattrocchio, Van Tunen, & Mol, 1990; Robards & Antolovich, 1997; Shirley, 1996). Epidemiological studies have shown an inverse relationship between the intake of fruits, vegetables and beverages rich in flavonoids and the incidence of coronary heart disease, but the relationship with cancer is not clear (Hollman, 1997). Apple is one of the main sources for flavonoid intake in the European diet, after onion and tea (Hertog, Hollman, Katan, & Kromhout 1993).

The major flavonoid classes occurring in apple fruit are flavonols such as quercetin 3-glycosides, monomeric and oligomeric flavan-3-ols such as catechin, epicatechin and procyanidins, dihydrochalcones such as phloridzin, and in red-coloured cultivars, anthocyanins such as cyanidin 3-glycosides. Apple fruit also contains considerable amounts of hydroxycinnamic acid derivatives, which are mainly represented by chlorogenic acid (Lancaster, 1992; Nicolas, Richard-Forget, Goupy, Amiot, & Aubert, 1994). Flavonoids and chlorogenic acid also contribute to the quality aspects of apples. Their red colour is primarily due to the flavonoids

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cyanidin-3-galactoside located in the vacuoles of skin cells (Lancaster, Grant, Lister, & Taylor, 1994; Sun & Francis, 1967), and the browning occurring in processed apple such as juices and ciders is mainly due to oxidation of chlorogenic acid by oxidative enzymes (Nicolas et al., 1994).

The biosynthesis of flavonoids in apple, as in other plant tissues, includes precursors from both the shikimate and the acetate-malonate pathways via several enzymatic steps (Lancaster, 1992; Stafford, 1990; Van der Meer, Stuitje, & Mol, 1993). Flavonoids are generally present in plant tissues as glycosides. In apple, the predominant sugar involved in glycosylation is galactose. Other sugars involved are glucose, rhamnose, xylose, arabinose and the disaccharide rutinose. Contrary to other flavonoids, flavan-3-ols are generally found in the free rather than in the glycosylated forms. The different flavonoid classes are predominantly located in the skin (Guyot, Marnet, Laraba, Sanoner, & Drilleau, 1998; McRae, Lidster, De Marco, & Dick, 1990).

McRae et al. (1990) concluded that culture and growing conditions have limited effects on the polyphenol profiles of the cortex and peel of apple fruits but did not discuss effects on actual concentrations. There is considerable qualitative information on the developmental and environmental regulation of anthocyanin biosynthesis in apples (Lancaster, 1992; Saure, 1990), but quantitative information on the amplitude of variation and hence the potential for control is almost lacking. For other flavonoids even the variation in content due to parietal and environmental factors is poorly studied.

Plant health substances in contribute to fruit quality as perceived by consumers and more quantitative knowledge is needed for natural variation in order to increase or optimize their concentration in fruits.

The aim of this work was to compare the contents of SEN, RU, TMF and RA in the skin of apple fruit collected from different local markets of Bangladesh and to evaluate their antioxidative properties.

2. Materials and method

2.1. Chemicals and reagents

Standard samples of SEN, RU, TMF and RA were purchased from Indofine Chemical Co. (Hillsborough, NJ, USA). Solvents used for chromatography were methanol (GC grade) and water (GC grade), obtained from Merck (Darmstadt, Germany). Folin-Ciocalteu reagent, quercetin, butylated hydroxytoluene (BHT), Tween 20, beta-carotene (95%) and linoleic acid (99%) were purchased from Sigma chemical Co. (St. Louis, MO). All other chemicals were of analytical grade or GC grade.

2.2. Sample collection

There are different types of apples available in Bangladeshi markets all year round. Fuji is one of the most popular apple cultivars grown in Indian region. For our experiment we used the Fuji varieties. Fuji apples (*Malus domestica* Borkh) were collected from local markets of different districts of Bangladesh. The skin of Fuji apples is initially yellowish green, but turns red when exposed to sun radiation. Ripe apples without visual symptoms of damage are usually harvested from both the outer and inner part of the tree canopy, resulting in apples with various levels of flavonoids in the skin. The colour of the apple ranges from pale green to dark red. After harvesting, the apples are stored at 2 °C.

2.2.1. Polyphenol extraction

The Fuji apple was cut out as a plug of skin with a cork borer (17mm diameter) and immediately frozen in liquid nitrogen. An extraction procedure was established and optimized. Sonication followed by shaking the sample was more effective than using a Polytron homogeniser (PT 1200, Kinematica AG, Luzern, Switzerland). The skin (0.2 g and approx. 1 mm thick) was separated from the flesh while still frozen, cut into six pieces with a scalpel and rapidly transferred to a tube with 1 ml 0.01 M HCl in methanol. The sample was exposed to sonication for 30 min in an ultrasonic bath (CTU150, Coax Teknik, Lynge, Denmark) followed by 30 min shaking (1400 rpm) in a CM-9 Mixer (SARSTEDT, Numbrecht, Germany). The extract was transferred to another tube before re-extraction of the apple skin with the same procedure as described above. The sample was cut from the flesh that had been closest to the skin. The total extract (2 ml) from skin was filtered through a 0.45 μm Millex HA filter (Millipore, Molsheim, France) prior to GC–MS analysis. The entire procedure was carried out at 4 °C and shaded from incident light.

2.3. GC–MS analyses

2.3.1. Preparation of samples from different markets for GC–MS analyses

The methanol extract (1 ml) was diluted with 5 ml of methanol and the samples were filtered through 0.45 μm membrane filters (Molsheim, France) prior to GC–MS analysis.

2.3.2. Identification and quantification of markers in the skin samples by GC–MS

The GC–MS analysis of the methanolic crude extract of apple samples was performed using a Varian GC–MS (Model Varian CP 3800, USA) equipped with a VF-5 fused silica capillary column (30 m × 0.25 i. d. mm film thickness 0.25 μm, Varian, USA). For GC–MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium gas was used as a carrier gas at a constant flow rate of 1 ml/min. Injector and mass transfer line temperature were set at 250 and 300 °C, respectively. The oven temperature was programmed from 50 to 200 at 8 °C/min, and then held isothermal for 20 min and finally raised to 300 °C at 10 °C/min. Diluted samples (1/100 v/v, in methanol) of 0.2 μl were manually injected in the split less mode. Identification of compounds of the methanolic crude extract was based on GC retention time on VF-5 capillary column, computer matching of mass spectra with standards (Mainlab, Replib and Tutorial data of GC–MS systems). The following reference compounds were used as markers: RA, SEN, RU and TMF. The markers were accurately weighed and dissolved in methanol to produce a series of concentrations. Standard calibration curves were established by plotting the peak areas against different concentrations of the reference compounds (varying from 5.0 to 1000 ng on column for SEN, RA and TMF and 10 to 1000 ng for RU). The external standard method was used for quantification of the markers in the samples of apple extract from different places.

The system suitability of the method was evaluated by the intra- and inter-day precision and accuracy of replicates. The accuracy was evaluated through recovery studies by adding known amounts of the standard solution to the extract. Controls from all samples were prepared and analyzed. The recovery experiment was performed at three different standard concentrations.

2.4. Determination of total phenolic contents of methanol extracts

The concentrations of total phenols in extracts were determined using Folin–Ciocalteau reagent and external calibration with caffeic acid. Briefly, 0.2 ml of extract solution in a test tube and 0.2 ml of Folin–Ciocalteau reagent was added and the contents mixed thoroughly. After 4 min, 1 ml of 15% Na_2CO_3 was added, and then the mixture was allowed to stand for 2 h at room temper-