Comprehensive Analysis of Polyphenols in Fruits Consumed in Japan

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Polyphenols, a large group of natural antioxidants, are a versatile group of phytochemicals beneficial for disease prevention. In this study, we comprehensively analyzed polyphenols, catechins, procyanidins, simple polyphenols, anthocyanins and flavonoids, in fruits consumed in Japan by high performance liquid chromatography with photo-diode array and mass spectrometric detection to complete the database of food components.

Keywords: polyphenol, fruits, HPLC, PDA

Introduction

Polyphenols form a large versatile group of phytochemicals that are natural antioxidants found to be potentially beneficial for disease prevention. Epidemiologic studies have recently revealed the association of higher polyphenol intake from fruits and vegetables with decreased risk of cardiovascular diseases (Stoclet et al., 2004; Manach et al., 2005). Furthermore, the regular consumption of certain foods and beverages such as apples, berries, wine, coffee and tea may significantly influence the quantity of antioxidants in a diet (Scalbert and Williamson, 2000). Thus, it is important to determine the amounts and species of polyphenols in foods.

The most recent edition (fifth) of the “Standard Tables of Food Composition in Japan” edited by the Resources Council of the Science and Technology Agency of Japan published in 2000 serves as the database of food components in Japan. It lists the contents, such as proteins, lipids, carbohydrates, minerals and vitamins, of various foods consumed in Japan, but lacks data on polyphenols. For example, fruits are rich in polyphenols, but their polyphenol content is not given. Since the role of polyphenols in foods is gaining attention, we comprehensively analyzed the contents of polyphenols in fruits consumed in Japan to complete the database of food components.

There are over one million natural polyphenols, which generally occur as glycosides, and contain various sugar species with various binding forms (Wollenweber and Dietz, 1981). Aglycons of polyphenols can be generally classified into flavonoids and simple polyphenols. Flavonoids are a family of compounds with a C6-C3-C6 skeleton structure. Flavanols, flavonols and anthocyanins are included in this group. Most of them have been shown to possess antioxidant activity, which depends mainly on the number and position of hydroxyl groups in their structure (Rice-Evans et al., 1996). There are two subgroups of simple polyphenols: benzoic acids (protocatechuic acid, gallic acid, etc.) and cinnamic acids (coumaric acid, caffeic acid, etc.) (Sakakibara, 2003).

A high-performance liquid chromatography (HPLC) separation method with photo-diode array (PDA) detection has been proposed to determine and quantify polyphenols in fruits by several groups (Crozier et al., 1997; Merken and Beecher, 2000). Although PDA is a useful technique for characterizing the aglycons of flavonoids, information on the molecular weight of compounds is not obtained from this method. Therefore, in addition to PDA, mass spectrometric (MS) detection technique in HPLC analysis has been applied for the identification of polyphenol compounds (Stobiecki, 2000). In the present study, we also used HPLC-PDA and HPLC-MS analysis to identify and quantify the polyphenols.

Materials and Methods

Materials Catechins were purchased from Sigma (St. Louis, MO, USA), anthocyanins and flavonoids were from Funakoshi (Tokyo, Japan), and simple polyphenols were from Wako Pure Chemicals Industries (Osaka, Japan). These chemicals were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM and stored at −20°C in the dark for up to 3 months. Calibration curves of these polyphenols in solutions ranging from 1.0 to 1000 μM were made by HPLC with PDA.

Extraction of polyphenols from fruits Fresh fruit was obtained from local markets in Shizuoka City or directly...
from the producers. The edible portions were taken randomly from several individual samples and washed with tap water. After being chopped, they were homogenized in liquid nitrogen with a Nissei AM-8 homogenizer (Nihonseiki, Osaka, Japan). The homogenized powders were lyophilized for 48 h and stored at −20°C until use. Each sample was weighed after lyophilization and the water content was also obtained. The stored powders (50 mg) were extracted with 2 mL of 80% methanol containing 0.5% acetic acid, after adding 50 nmol of flavon in DMSO as an internal standard. The solution was allowed to stand in a sonicator for 1 min, and the supernatant was recovered by centrifugation at 1610 g for 10 min. After extraction three times, the extracts were concentrated with evaporator and dried with nitrogen gas. The residues were dissolved in 0.5 mL of 80% methanol or DMSO and filtered with a PTFE 0.45 μm membrane filter (Pall, East Hills, NY, USA) before HPLC analysis.

**Total polyphenol contents** Total polyphenol contents in the extracted powder from fruits were determined by the Folin-Ciocalteu colorimetric method (Kumazawa et al., 2002). Methanol extracts of the powders (1 mg/mL) were mixed with 1 mL of the Folin-Ciocalteu reagent (Kanto Chemicals, Tokyo) and 1 mL of 10% Na₂CO₃, and the absorbance was measured at 760 nm after 1 h incubation at room temperature. Total polyphenol contents were expressed as μmol/100 g gallic acid equivalents.

**HPLC-PDA analysis** The HPLC analysis of polyphenols was performed using a Jasco HPLC system (Tokyo) equipped with a PDA detector and a reversed phase column Capcell Pak C18 UG (5 μm; 250 × 4.6 mm i.d., 4.6 mm i.d., 5 μm; Shiseido, Tokyo, Japan). The flow rate was 1.0 mL/min, the injection volume was 10 μL, and the oven temperature was 30°C. The following solvents were used for analysis of catechins, procyanidins, simple polyphenols and anthocyanins: A, 0.1% trifluoroacetic acid (TFA) in water, and B, methanol. The gradient condition 1: 18% B (0–20 min), 18–22% B (20–50 min), 22% B (50–80 min), was applied for analysis of catechins, procyanidins and simple polyphenols. The gradient condition 2: 22–50% B (0–50 min), 50–70% B (50–80 min), was used for analysis of anthocyanins. For analysis of flavonoids, solvent A, 1% acetic acid in 10% methanol, and solvent B, 1% acetic acid in 70% methanol were used and the gradient condition 3: 0–30% B (0–15 min), 30–35% B (15–45 min), 35–40% B (45–65 min), 40–50% B (65–70 min), 50–100% B (70–85 min), 100% B (85–95 min), was applied.

**Quantification of polyphenols** Polyphenols were quantified under the analytical conditions described in HPLC-PDA. Each sample was injected in triplicate, and the standard calibration curves were constructed with the specific wavelengths of standard chemicals: 280 nm for catechins, procyanidins and simple polyphenols; 520 nm for anthocyanins and anthocyanidins; 350 nm for flavones and flavonols; and 290 nm for flavanones.

### Results and Discussion

Sakakibara et al. (2003) reported a method for simultaneously determining the polyphenols in foodstuffs. However, in the present study, we analyzed the polyphenols in 59 kinds (varieties) of fruits by different three HPLC conditions. Fruits used for the present experiment were purchased from a local supermarket or directly from the producers.

The detected polyphenol peaks from extracts of fruits were compared with respect to retention time with those of standard chemicals, and next the aglycons were identified by comparison with those spectra. When the detected polyphenol did not coincide with any of the standards, the food samples were subjected to hydrolysis or LC/MS analysis. Hydrolysis was performed by the method reported by Sakakibara et al. (2003).

Table 1 shows the water content (%), total polyphenol content (μmol/100 g) and polyphenol content (μmol/100 g fresh edible part) of each fruit. Sakakibara et al. (2003) examined using Japanese radish root to determine the recovery with extraction, and they obtained recoveries 68–92% for added flavonoids, and the analytical precision ranged from 1 to 9%. We also performed the recovery with extraction experiment, and obtained the similar results to those of Sakakibara et al. (data not shown). Sakakibara et al. (2003) used flavone as an internal standard, and they corrected the recovery rate with it. Thus, we also used flavone as an internal standard, and the same data analysis were carried out. The water content of each fruit was similar to that described in the Standard Table of Food Composition in Japan. The Folin-Ciocalteu method we used for determination of total polyphenol contents is interfered by reducing substances (Prior et al., 2005). For example, total polyphenol contents of acerola shows high values caused by the high ascorbic acid content (Hwang et al., 2001).

Catechins, procyanidins and simple polyphenols were analyzed using HPLC condition 1. We detected (+)-catechin in atemoyas, peach and plum, and (−)-epicatechin in apple, apricot and cherimoya. Although (+)- catechin and (−)-epicatechin have been reported to be in various fruits (Luo et al., 2002; Yilmaz and Toledo, 2004), these compounds were not detected in the present study. This is assumed to be because we analyzed only the edible parts of the fruit not the skin or seed, where these compounds are mostly located.

Large amounts of procyanidins B1, B2 and C1, oligomers of catechins, were detected in atemoyas. Cherimoyas also contained considerable amounts of procyanidins. White sapotes also had high contents of procyanidin B1 and B2, especially the latter. Procyanidins are reported to be detected in various fruits such as apples and grapes (Peng et al., 2001). Although procyanidins as well as catechins exist in the skins and seeds of fruits, we did not analyze polyphenols in the skins or seeds of these fruits. Thus, the contents of procyanidins we obtained were lower than the reported values. Most fruits containing large amounts of catechins and procyanidins belong to the rose family, such as example, apples, peaches and plums.

Of the simple polyphenols, chlorogenic acids were detected in many fruits especially marmelo. Other pears
<table>
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<tr>
<th>Food (scientific name)</th>
<th>Water content (w/w)</th>
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<th>Polyphenol content (μmol/100 g fresh edible part)</th>
<th>Polyphenols</th>
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Table 1-2. (Continued).

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<th>Food (scientific name)</th>
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<th>Polyphenol content (μmol/100 g fresh edible part)</th>
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<td>peonidins b 1.5—2.7</td>
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<td>Cherry (Prunus avium) Japan, sweet type</td>
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Table 1-3. (Continued).

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<th>Food (scientific name)</th>
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<th>Polyphenol content (µmol/100 g fresh edible part)</th>
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<th>simple polyphenols</th>
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<th>Polyphenol content (μmol/100 g fresh edible part)</th>
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Table 1-6. (Continued).

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</table>
Total polyphenol contents were expressed as mmol/g gallic acid equivalents by the Folin-Ciocalteu method. Contents of these aglycons were determined by hydrolysis. Contents were determined using cyanidin-3-glucoside. Contents of these glycosides were determined using aglycons. Several analytical studies of anthocyanins in blueberries have been reported (Dugo et al., 2001; Faria et al., 2005). We identified each anthocyanin in blueberries using the HPLC conditions reported previously by comparison with standard anthocyanin compounds. Other red fruits such as acerolas, pomegranates and strawberries also have anthocyanins. Anthocyanidin peaks identified by hydrolysis were presented such as cyanidins and delphinidins. But anthocyanin peaks not in agreement with standard chemicals were quantified with cyanidin-3-glucoside and were presented as total anthocyanins. Anthocyanidin peaks obtained by hydrolysis of these unknown anthocyanins were similarly quantified with cyanidin and were presented as total anthocyanidins. Although red pitaya has a bright red color, no anthocyanins were detected. Color pigments of this fruits were expected to be betacyanins not anthocyanins (Wybraniec and Mizrahi, 2003). We could not quantitatively analyze the betacyanins because the standard chemicals were not available.

Many flavonoids have been isolated and identified from various plants including fruits. Flavonoids have been the subject of considerable scientific and therapeutic researches (Havsteen, 2002). We identified each flavonoid glycoside using three HPLC conditions. Most flavonoids occur in glycoside forms. To identify the aglycon of each flavonoid, we performed hydrolysis or the LC/MS/MS analysis. The flavonoid glycoside peaks not in agreement with standard chemicals but with aglycons identifiable by hydrolysis were quantified their aglycons and were presented such as quercetin glycosides. Consequently, as shown in Table 1, quercetin glycosides have been detected in various fruits. The flavanones narirutin and hesperidin were detected only in citrus fruits such as Satsuma mandarins and sudachi, while eriocitrin was detected in lemons and limes. These results were in agreement with those reported by Sakakibara et al. (2003).

In the present study, we analyzed polyphenols in fruits consumed in Japan. Although there have been many reports on polyphenols, there are few on the sample preparation and analysis under systematic conditions. The present findings may be helpful for understanding the physiological properties of polyphenols in fruits. In this study, however, several components reported previously were not detected, or the values obtained were different from the reported values. This is considered to be due to the difference between the quality or harvest season of fruits used in this study. Analysis of only the edible parts of fruits in this study may also be a reason. Perishable foods such as fruits and vegetables are different in their seasonal nature. Furthermore flavonoid content is known to be highly dependent on the cultivar and growing and processing conditions (Harnly et al., 2006). In Table 1, the results analyzed three times from different fruits (same kind) were also shown. Thus, it is considered that the results with the wide range are due to the difference of samples not inaccuracy of the analytical methods.

Table 1-7. (Continued).

<table>
<thead>
<tr>
<th>Food (scientific name)</th>
<th>Water content (%)</th>
<th>Total polyphenol content (μmol/100 g)</th>
<th>Polyphenol content (μmol/100 g fresh edible part)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>flavonoids</td>
<td>simple polyphenols</td>
</tr>
<tr>
<td>Watermelon (Citrullus vulgaris)</td>
<td>91.7</td>
<td>181</td>
<td></td>
</tr>
<tr>
<td>White sapote (Achras zapota)</td>
<td>81.8</td>
<td>980 procyanidin B2 87.1</td>
<td>caffeic acid 8.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>procyanidin C1 25.8</td>
<td></td>
</tr>
<tr>
<td>Yuzu (Citrus junos) fruit juice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>early ripening type</td>
<td>—</td>
<td>unexamined narirutin 44.6</td>
<td>cryptochlorogenic acid 1.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hesperidin 18.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>naringin 10.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>neohesperidin 4.8</td>
<td></td>
</tr>
<tr>
<td>normal ripening type</td>
<td>—</td>
<td>unexamined narirutin 29.7</td>
<td>cryptochlorogenic acid 0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hesperidin 14.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>naringin 7.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>neohesperidin 4.1</td>
<td></td>
</tr>
</tbody>
</table>

*Total polyphenol contents were expressed as mmol/100 g gallic acid equivalents by the Folin-Ciocalteu method.
*Contents of these aglycons were determined by hydrolysis.
*Contents were determined using cyanidin-3-glucoside.
*Contents were determined using cyanidin.
*Contents of these glycosides were determined using aglycons.
Some of the present data are available through the web page (http://www.nihn.go.jp/FFF/). A more complete database of functional food factors will be useful for further analyses of their effects on human health. Furthermore, the present findings should prove valuable for elucidating the roles that polyphenols in fruits may play in promoting health in Japan.

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References


