# Preventive Factors in Food against Oxidative damage of DNA

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(Degree)
博士（学術）

(Date of Degree)
2002-03-31

(Date of Publication)
2008-11-27

(Resource Type)
doctoral thesis

(Report Number)
甲2587

(URL)
https://hdl.handle.net/20.500.14094/D1002587

# Preventive Factors in Food against Oxidative Damage of DNA

## DNA の酸化的損傷を予防する食品因子

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# Contents

# *Abbreviations*

| | | |
|---|---|---|
| AAPH | — | 2,2'-azo*bis*(2-amidinopropane)dihydrochloride |
| BHA | — | butylated hydroxyanisole |
| BHT | — | butylated hydroxytoluene |
| C | — | (+)-catechin |
| CG | — | (-)-catechin gallate |
| 2'-dG | — | 2'-deoxyguanosine |
| DMEM | — | Dulbecco's modified Eagle's medium |
| DMSO | — | dimethylsulfoxide |
| DPPH | — | 1,1-diphenyl-2-picrylhydrazyl |
| EC | — | (-)-epicatechin |
| ECD | — | electrochemical detector |
| ECG | — | (-)-epicatechin gallate |
| EDTA | — | ethylenediaminetetraacetate disodium |
| EGC | — | (-)-epigallocatechin |
| EGCG | — | (-)-epigallocatechin gallate |
| FT-IR | — | fourier transform infrared spectrometry |
| GC | — | (-)-gallocatechin |
| GCG | — | (-)-gallocatechin gallate |
| HPLC | — | high-performance liquid chromatography |
| LC/MS | — | high-performance liquid chromatography-mass spectrometer |
| MTT | — | microcultrue tetrazolium |
| NMR | — | nuclear magnetic resonance |
| 8-OHdG | — | 8-hydroxy-2'-deoxyguanosine |
| 8-OOHdG | — | 8-hydroperoxy-2'-deoxyguanosine |
| TBHQ | — | *tert*-butylhydroquinone |
| TLC | — | thin-layer chromatography |

# Chapter 1

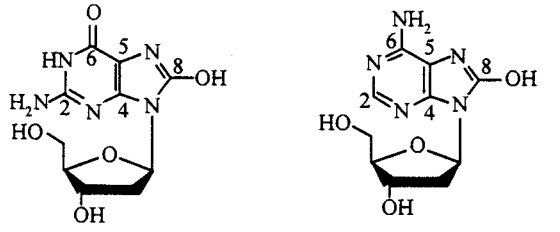
# General Introduction

# *General Introduction*

Including cancer, eleven degenerative diseases as shown in Table 1 are believed to be induced through oxidative genetic damage. The oxidative damage is caused by reactive oxygen species (Guyton and Kensler, 1993; Emerit, 1994; Marnett, 2000) and forms mainly 8-hydroxyls in the bases, 8-oxo-7,8-dihydrodeoxyguanosine (8-hydroxy-2'-deoxyguanosine, 8-OHdG) and/or 8-hydroxy-2'-deoxyadenosine (Fig.1) (Randerath, 1997; Boiteux and Radicella, 1999; Nomoto et al., 2000; Podmore et al., 2000). An accumulation of 8-OHdG has been found in several carcinoma cells and mutated genes (Yamamot et al. 1993; Kondo et al., 2000; Foksinski et al., 2000). Levels of 8-OHdG in human skeletal muscle, respiratory epithelium, and leukocytes are correlated with aging, exposure to urban pollution and smoking, respectively (Mecocci et al., 1999; Calderón-Garcidueñas et al., 1999; Lodovici et al., 2000). Urinary 8-OHdG is a biomarker of human oxidative stress accompanying cancer (Simic, 1992; Kasai, 1997; Halliwell, 1998; Zwart, 1999). An important issue in human health is the suppression of oxidative damage, which should associate with nutritional behavior. Thus, the better understanding is which

**Table 1.** Degenerative diseases directly caused by oxidative DNA damage

| | |
|---|---|
| cancer | diabetes mellitus |
| amyloidosis | hyperpiesia |
| arteriosclersis | pancreatitis |
| arthrosteitis | parkinson's facies |
| cirrhosis | senile cataract |
| dementia | |



8-hydroxy-2'-deoxyguanosine (8-OHdG)

8-hydroxy-2'-deoxyadenosine (8-OHdA)

**Fig. 1.** Structures of the oxidized DNA bases

food factors are sufficiently antioxidative to suppress the 8-OHdG formation.

Our daily edible plants contain a large number of phytochemicals without apparently nutritive value for human but with pronounced biologic activity (Wattenberg, 1990). They are polyphenols and occur ubiquitously in vegetables, fruits, and teas (Robards and Antolovich, 1997; Hollman and Katan, 1999). The term polyphenols includes various kinds of compounds that derive from the basic phenol-structures as Figs. 2, 3, and 4. They have some ability to scavenge reactive oxygen species and especially *ortho*-diol structure (catechol) shows strong activity (Rice-Evans et al., 1996; Nanjo et al., 1996). Food polyphenols, therefore, are expected to be able to prevent the degenerative diseases (Youdim and Joseph, 2001).

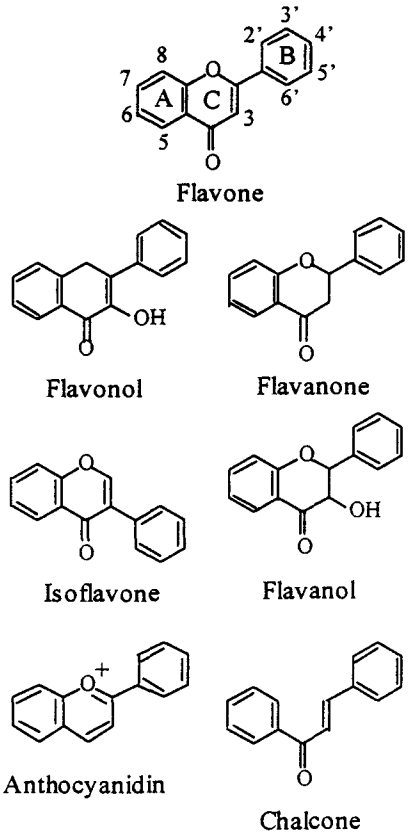



**Fig. 2.** Structures of the various classes of flavonoids

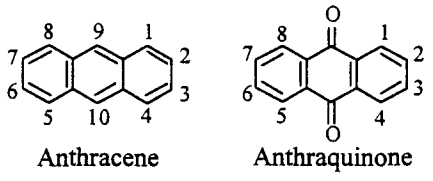



**Fig. 3.** Structures of Anthracene and Anthraquinone

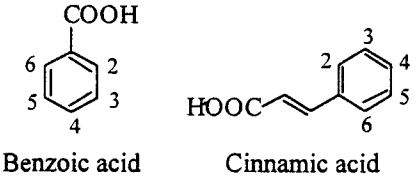



**Fig. 4.** Structures of the two types of phenolic acids

The first item requiring is which foods include the strong antioxidants to quench the reactive oxygen species sufficiently. The author has explored the strong antioxidants in the tropical fruits in Chapter 2. Since tropical fruits grow

under hot and intense sunlight that facilitates lipid peroxidation, the author thinks they should contain the strong antioxidants to shield against peroxidizing factors.

The second item requiring is an establishment of exact evaluation method for antioxidatively preventing diseases. To prevent genetic damage, antioxidants should trap the reactive oxygen species effectively enough easier than DNA. The putative reactive oxygen species that form 8-hydroxy derivatives are hydrogen peroxide (Floyd et al., 1998; Taylor et al., 1995; Abu-Shakra and Zeiger, 1997; Lloyd and Phillips, 1999), thiol radical (Goldman et al., 1999), and lipid peroxides (Kaneko and Tahara, 2000). However, they all require transition metals, UV rays or $\gamma$ rays to interconvert and generate oxidizing species. Thus, this Fenton's reaction complicates several subfactors such as hydrogen peroxide, iron(II), and ethylenediaminetetraacetate disodium salt (Nappi and Vass, 1997; Yokozawa et al., 1997). The various subfactors may result in errors, for example, interaction with metal ions and ray quenching (Sugihara et al., 1999; Puppo, 1992). A simple and convenient system is favor to evaluate which food factors can scavenge reactive oxygen species before the DNA bases are damaged. In Chapter 3, the author has established a novel evaluating method for the antioxidative potencies of food.

The third item requiring is an improvement for the analytical method to determine antioxidants in our daily foods. Considering the human, the quantitative data on the occurrence of antioxidants are strongly requesting. Recently, quantitative methods for flavonoids have been published based on HPLC with diode-array detection (Hertog et al. 1992a; Crozier et al, 1997a; Merken and Beecher, 2000) but have not been include the determination of other phenolics. Schieber et al. (2001) have reported an improved method for the simultaneous quantitation of both flavonoid glycosides and

phenolics but is lack in detection of flavonoid aglycon. The analytical method should be able to determine all antioxidants occurring in food in the species and the contents simultaneously. In Chapter 4, the author has developed and validated a complete method to determine almost all phenolic antioxidants in human diets.

The final item is the better understanding of bioavailability, which includes a physiological level of dietary antioxidants in the cells. It realizes for the first time to prevent diseases that the antioxidants in food would be absorbed in body and reach near DNA in the cells. In Chapter 5, the author has determined the plasma levels employing an animal model and further evaluated the preventing effect against oxidized DNA damages at the physiological levels of antioxidants using a cell line. Finally, the potentiality of maintaining the human health with daily diet is discussed.

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# Chapter 2

# Strong Antioxidants in Cavendish Banana

This chapter is based mainly on:

Large Amount of Dopamine: A Strong Antioxidant in Cavendish Banana

*Journal of Agricultural and Food Chemistry, 2000, 48(3): 844-848.*

# *Introduction*

The products of lipid peroxidation are part of our daily diet. The digestive system of the man possesses an ability to detoxify small amounts of these products (Kanazawa and Ashida, 1998a). However, those peroxides that cannot be detoxified are absorbed into the body (Kanazawa and Ashida, 1998b), where they can cause various degenerative diseases such as atherosclerosis (Nourooz-Zadeh et al., 1996), cancer (Chaudhary et al., 1994), diabetes mellitus (Novotny et al., 1994), Parkinson's disease (Yoritaka et al., 1996), and others (Kristal et al., 1996). It is important to our health to maintain the ability to detoxify in the digestive tract by eating antioxidative foods.

Plant foods, particularly vegetables, contain various antioxidants, tocopherols, ascorbic acid, carotenoids, and flavonoids. Strongly antioxidative phytochemicals also have been found; catechins in teas (Salah et al., 1995), sesaminols in sesame (Kang et al., 1998), chlorogenic and caffeic acids in coffee (Laranjinha et al., 1994), curcumin in herb turmeric (Toda et al., 1988), rosmanol, carnosol and carnosic acid in rosemary (Inatani et al., 1983; Aruoma et al., 1992), and so forth. The author is interested in fruit antioxidants. Wang et al. (1996) examined the antioxidative potency in 12 fruits and 5 fruit juices, and reported that strawberry had the highest activity followed by plum, orange, and etc. including banana. The author had also examined the antioxidative potency of several fruits, and found that tropical fruits had strong activity. For example, a banana water-extract suppressed the autoxidation of linoleic acid by 65-70% after a 5-day incubation in an emulsion system, as determined from the peroxide value and thiobarbituric acid reactivity. Tropical fruits grow under hot and

intense sunlight that facilitates lipid peroxidation. The author considered that bananas should contain antioxidants in pulp and peel to shield against peroxidizing factors. The objective of the present Chapter was an exploration of banana antioxidants.

The author first examined the peel, and identified dopamine as a strong water-soluble antioxidant, present at the level of one hundred mg per 100 g. Next, the author analyzed the pulp, and found it too contained dopamine at a few mg/100 g even in ripened bananas ready to eat.

# *Materials and Methods*

## *Materials*

The most popular banana on the world market, Musa *cavendishii*, imported from two different plantations in the Philippines, was obtained from a local market outlet before ripening treatment with ethylene gas. Linoleic acid purchased from Nacalai Tesque, INC. (Kyoto, Japan) was distilled under nitrogen in vacuo twice. Standard chemicals, dopamine and other catecholamines, amino acids, and tocopherols were used from Nacalai Tesque, INC. (Kyoto, Japan), Wako Pure Chemical Industries Ltd. (Osaka, Japan), and Ezai Co., Ltd. (Tokyo, Japan), respectively. Flavonoids and catechins were from Funakoshi Co., Ltd. (Tokyo, Japan). All other chemicals were commercially available in high grade.

### *Separation of banana antioxidants*

Banana peel (1050 g) was obtained from banana (3 kg) without fruit apex and stalk. The peel was allowed immediately to stand in boiling water for 20 sec to inactivate polyphenol oxidases (Jiménez and García-Carmona, 1999). It was minced with a Waring blender and centrifuged at 8000 × g. The supernatant in a cellulose tube (dialytic molecular size, less than 12,000-14,000) was dialyzed to 10 L of water overnight. The diffusate after being condensed with an evaporator under nitrogen was weighed (29.84 g by dry weight), and was subjected to the following chromatographic separations. The part of pulp was cut into one-cm pieces, and the 500 g was taken up randomly, and then treated similarly.

Products in the diffusate were purified while monitoring the antioxidative potency. An alumina (aluminum oxide 90, Merck & Co., INC., Darmstadt, Gemany) column (ϕ 3.5 × 30 cm, 0.05 M borate buffer (pH 8.6) for the immobile phase) was first used (Drell, 1970). The elution with water (fraction A-I), 0.2 N acetic acid (A-II), and 2 N HCl (A-III) gave 16.95, 10.38, and 2.46 g dry weight, respectively. The A-I was subjected to an ion-exchange chromatography (Dowex 50W × 4, 200-400 mesh, Na-form, ϕ 1.0 × 65 cm) (Dow Chemical Co., Midland, MI), and eluted with 0.2 M acetate buffer of pH 3.25 (I-a), the same buffer of pH 4.8 (I-n), and 0.02 N NaOH (I-b). The eluted matter weighed 9.00, 1.59, and 6.34 g dry weight, respectively. A-II was passed through a silica-gel column (Wakogel C-100, ϕ 2.0 × 40 cm) (Wako Pure Chemical Industries Ltd., Osaka, Japan). The elution with a mixed solvent of 1-butanol:acetic acid:water = 4:2:1 as the immobile phase gave a colorless fraction (II-S1, dry weight 0.34 g) and yellow fraction (II-S2, 8.85 g), and then a wash with 2 N HCl gave fraction II-S3 (1.17 g). II-S3 was finally subjected to purification of

antioxidant by cellulose (Avicel SF) thin-layer chromatography (TLC) (0.25 × 200 × 200 mm) twice, developing with 1-butanol:acetic acid:water = 4:2:1 and with ethanol:water = 63:37.

### *Evaluation of antioxidative potency*

The author employed an oxygen-absorption method using a Gilson differential respirometer (Model GRP 14) (Gilson France), because one can directly evaluate the antioxidative potency from the suppression of oxygen uptake during the peroxidation of polyunsaturated fatty acid (Kanazawa et al., 1973). Twenty micromoles of linoleic acid was dispersed with 20% Tween 40 in 2 mL of 0.1 M phosphate buffer (pH 7.0) containing a certain concentration of banana products. Shaking in a water bath at 37°C, the oxygen uptake was monitored and compared to that of the control without banana products.

Another evaluation method was used that employs a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. The banana antioxidant was added to 250 nmol DPPH in 50% ethanol in 0.05 M acetate buffer (pH 5.5), and immediately fading at 517 nm was monitored, comparing to the fading abilities of 130 nmol each of standard chemicals.

### *Instrumental analyses*

The purified antioxidant was elucidated as to chemical structure using infrared spectrometry (Shimadzu IR-408) (Shimadzu Co. Ltd.), nuclear magnetic resonance spectrometry (Bruker AC-250) (Bruker Analytik GMBH), and gas chromatography-mass spectrometry (JEOL DX 500) (JEOL Ltd.) with a column of 3% OV-1 using an

ionizing voltage of 20 eV.

### *High-performance liquid-chromatography (HPLC) for determining antioxidative phytochemicals*

The antioxidants in both banana peel and pulp were identified by HPLC (IRICA Σ871) (Irica Co. Ltd.) comparing with the respective standard chemicals. Levels of catecholamines were analyzed in the above A-II fraction, modifying the method of Causon et al. (1981): column, Wakosil-II 5C18AR (ϕ4.6 × 200 mm); mobile phase, 70 mM $KH_2PO_4$ containing 1 mM ethylenediaminetetraacetate disodium, 2 mM sodium 1-octanesulfonate, and 10% methanol; flow rate, 1.0 mL/min; column temperature, 35°C; and monitoring with an electrochemical detector (IRICA Σ875) (Irica Co. Ltd.) set at +600 mV. Flavonoid content also was determined for A-II, using a column SHISEIDO CAPCELL UG120 (ϕ4.6 × 250 mm) (SHISEIDO Co. Ltd.) maintained at 35°C, eluting with 45 mM potassium phosphate buffer (pH 3.3):methanol = 45:55, and monitoring with a photodiode array detector (Shimadzu SPD-M6A) (Shimadzu Co. Ltd.). Alternatively, banana peel or pulp was minced in ethanol containing 5% pyrogallol, saponified in 60% KOH for 3 min, and then extracted with diethyl ether or with methanol:dichloromethane = 1:2. The ether extract was passed through a Sep-Pak (Waters Florisil cartridges) (Nihon Waters K.K.) and submitted to tocopherol analysis on HPLC (Lumen and Fiad, 1982). Carotene contents were determined in methanol:dichloromethane extract (Miller et al., 1984). The determination of antioxidant levels was performed 3 times independently using 12 bananas each at the ripening stages.

# *Results*

## *Isolation of antioxidative ingredients in banana peel*

Fig. 1 shows the isolating procedures for antioxidants in banana peel. The antioxidative potency of isolated products was monitored from the suppressing effect on oxygen uptake of linoleic acid. Linoleic acid absorbs one mole of oxygen to form its hydroperoxides and another mole or a little more for decomposition of the hydroperoxides (Kanazawa et al., 1973). Finally, 20 μmol linoleic acid used here were

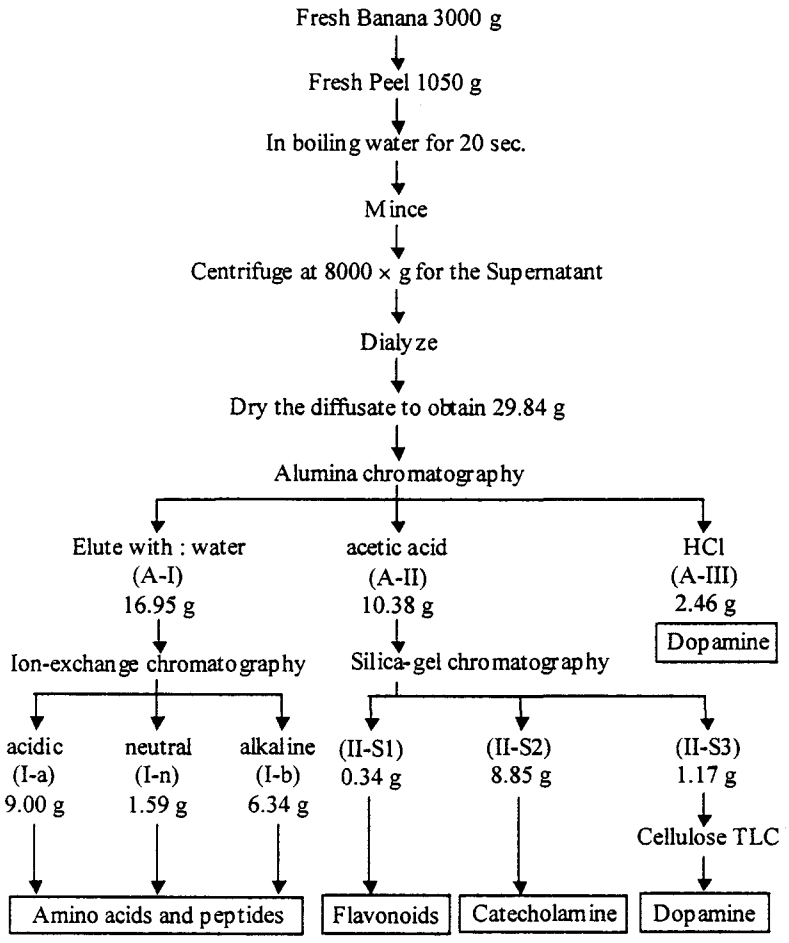


**Fig. 1.** Isolation of banana antioxidants. Details are given in the "MATERIALS AND METHODS". Figures next to the fraction names are the yield g as dry weight. The products in squares were identified here.

estimated to absorb around 50 μmol (1120 μL) of oxygen. Fig. 2 compares the oxygen uptake of linoleic acid with and without banana products. In a control without product, the uptake initiated after around 60 h-incubation, which is the induction period of autoxidation. Then, linoleic acid absorbed oxygen actively and the autoxidation almost finished at around of 230 h. In the linoleic acid-emulsion, 50 μg dry matter of the isolated fractions was added. The diffusate prolonged the induction period to around of 200 h and suppressed the oxygen uptake to 570 μL after 300 h (Fig. 2A).

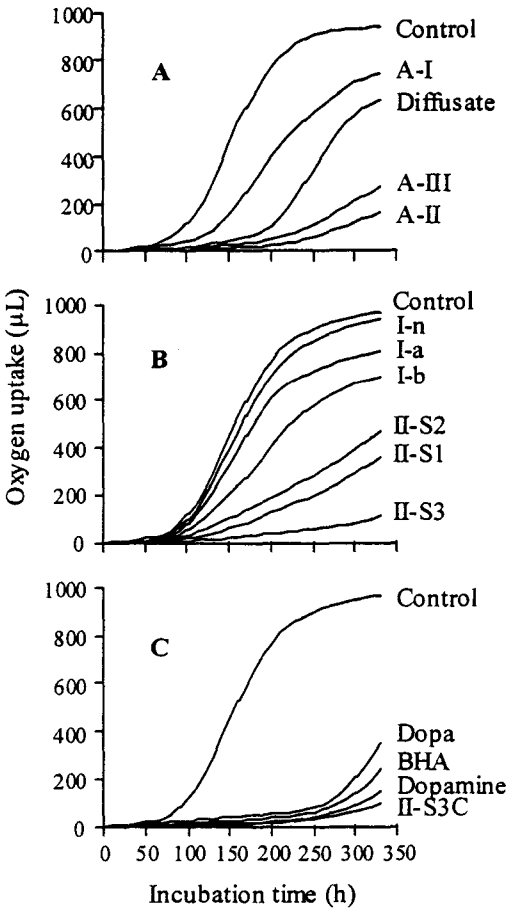


**Fig. 2.** Antioxidative potency of the isolated banana ingredients. A; the diffusate and isolated fractions obtained by the alumina column chromatography. B; the fractions on ion-exchange and silica-gel column chromatographies. C; a comparison of the antioxidative potency of 10 μg purified fraction II-S3C and 50 nmol each of standard chemicals; dopa, dopamine and BHA (butylated hydroxyanisole).

It had been reported by Udenfriend et al. (1959) that banana contained catecholamines. The strong antioxidative potency of banana diffusate might be attributed to the catecholamines. For the isolation of catecholamines, an alumina-column chromatography has been reported to be suitable (Drell, 1970). The author then employed the alumina chromatography and separated the diffusate into three fractions. Fraction A-I had weak antioxidative potency, whereas A-II and A-III were strong. A-I was further

separated through an ion-exchange column-chromatography but still gave no active fractions (Fig. 2B). In contrast, A-II passed through a silica-gel column gave a strong fraction, II-S3. Products in II-S3 were purified on cellulose TLC twice to II-S3C. The A-III fraction formed a dark-brown precipitate during a few h standing. These isolated fractions, purified products and precipitates were elucidated instrumentally as to chemical structure.

## *Identification of banana antioxidants*

Fraction A-I was positive for ninhydrin reagent and then analyzed by amino acid analyzer (HITACHI model 835) (Hitachi Ltd.) directly and after hydrolysis. It was composed mainly of peptides and free amino acids (data not shown). The most active I-b among the three A-I fractions included basic peptides and amino acids, particularly 1.77 mg free arginine in 6.34 g of I-b fraction (Fig. 1). The basic amino acids have been known to be more antioxidative than acidic and neutral amino acids (Matsushita and Ibuki, 1965). The activity of I-b was probably due to arginine. In I-n fraction (1.59 g), 0.30 mg free tyrosine and 0.44 mg phenylalanine were detected but Fig. 2B showed that I-n fraction was not antioxidative. This indicated that antioxidative potency of the neutral amino acids were weaker than arginine.

In the most antioxidative A-II fraction, the II-S3C purified from II-S3 showed a phenolic OH ($\nu_{max}$ 3300 $cm^{-1}$), amine ($\nu_{max}$ 1600 $cm^{-1}$), and aromatic ring ($\nu_{max}$ 1495 $cm^{-1}$) in an infrared spectrum in KBr. The mass spectrum after acetylation with trifluoracetic acid detected a molecular ion peak at *m/z* 441 ($M^+$) and fragment ion peaks; at *m/z* 372 (- $CF_3$), 329 (- $NHCOCF_3$) as a base ion peak, 315 (- $CH_2NHCOCF_3$), 301 (- $CH_2CH_2NHCOCF_3$), and 126 ($CH_2NHCOCF_3^+$) as the secondary peak. The

spectrum of $^{1}$H-NMR in dimethyl sulfoxide-$d_6$ was assigned as follows; at δ 2.66 (t, $J$ = 7.8) for H-1', δ 2.91 (t, $J$ = 7.8) for H-2', δ 6.48 (m) for H-4, δ 6.63 (d, $J$ = 7.9) for H-5, and δ 6.66 (d, $J$ = 8.0) for H-6. The $^{13}$C-NMR signals were as follows; at δ 32.7 for C-1', δ 41.0 for C-2', δ 116.3 for C-3, δ 116.5 for C-6, δ 120.2 for C-5, δ 128.6 for C-4, δ 144.3 for C-1, and δ 145.5 for C-2. Every analytical datum completely coincided with that of standard dopamine (Fig. 3). In addition, this II-S3C exhibited strong potency for antioxidant similar to the standard dopamine (Fig. 2C).

The II-S1 showed two large peaks on HPLC, having 3.9 and 4.1min as retention times. The mass spectra analyzing with a liquid chromatography/APCI-mass spectrometry (HITACHI M-1200H) (Hitachi Ltd.) at –50 V for ionizing voltage, showed ion peaks at *m/z* 577 (M-1-2H) and *m/z* 271 (M-1-rhamnoglucoside) for the former, and at *m/z* 609 (M-1) and *m/z* 301 (M-1-rutinoside) for the latter. The analytic data coincided with those of standard flavonoid glycosides, naringin and rutin, respectively. Flavonoids have been recognized to be potent antioxidants (Jovanovic et al., 1994; Cao et al., 1997). The activity of II-S1 was due to these flavonoids (Fig. 2B). II-S2 gave two major and several minor peaks on HPLC. The major peaks had 6.8 and 19.5 min of retention times and gave signals at *m/z* 168 (M-1) and *m/z* 152 (M-1), respectively, with a liquid chromatography/APCI-mass spectrometry. The information coincided with that of standard chemicals, and then they were identified as norepinephrine and dopamine, respectively.

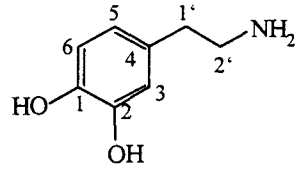


**Fig. 3.** Chemical structure of dopamine.

The precipitate from A-III had almost the same spectrum as dopamine in the

infrared analysis. This fraction was suggested to include a large amount of dopamine and to form oxidized products such as dopamine polymers during the preparation.

Then, because of the instability of dopamine, the antioxidant contents of banana were determined using fresh peel and pulp.

### *Antioxidant contents of banana at various ripening stages*

The peel contained antioxidative arginine, flavonoids and catecholamines, and probably others. The amounts of these compounds and other known antioxidative phytochemicals were determined in both peel and pulp at the various ripening stages (Table 1). The ripening stage was evaluated according to the common classification in the market. The classification defines 8 stages by color score; all green, light green, half green, half yellow, green chip, full yellow, star, and duffel, and by sugar content; around 1, 3, 5, 9, 14, 17, 19, and 19%, respectively. Stages 1-3 were just imported and had not received ripening treatment. Stages 4-8 were after the treatment with ethylene gas. Banana is usually served at a ripening stage of 6 or 7. At stages 1-3, it is not usually eaten because it is green and very hard. At 8 stage, it is overripe and muddy. Banana contained a large amount of dopamine in both peel and pulp. The amounts decreased a little with ripening and remained at levels between 80-560 and between 2.5-10 mg, per 100 g of peel and pulp, respectively, even at the edible stages. The variance was due to a difference of plantations in the Philippines. Interestingly, the metabolizing precursor dopa was less abundant than dopamine in peel and pulp. Naringin and rutin contents were a few 10 mg in the peel and negligible in pulp. The ascorbic acid content was constant at around 10 mg/100 g in both peel and pulp, regardless of the ripening stage. Carotenes and tocopherols were appreciable in peel

and less in pulp. Thus, the strong antioxidative potency of banana is attributable to dopamine, which is much more abundant than other antioxidants in this fruit.

**Table 1.** Levels of Antioxidative Phytochemicals in Banana at the Various Ripening Stage

| | Ripening stage[a] | | | |
|---|---|---|---|---|
| | 1-3 | 4-6 | 6-7 | 7-8 |
| | | In peel (mg/100g)[b] | | |
| Dopamine | 865-1940 | 185-705 | 80-560 | 235-930 |
| | (1290±420) | (430±210) | (380±160) | (500±270) |
| Dopa | 14-30 | 3.5-10 | 1.1-8.0 | 2.5-15 |
| Norepinephrine | 55-118 | 8.2-14 | ND[c] –24 | 38-43 |
| Naringin | 120-260 | 17-120 | 28-95 | 42-72 |
| Rutin | 16-23 | 11-14 | 11-16 | 15-17 |
| Ascorbic acid[d] | 6.8-8.2 | 6.6-8.7 | 5.8-8.0 | 3.2-7.9 |
| Carotenes[d] | 0.43-1.2 | 0.55-0.87 | 0.28-0.78 | 0.11-0.66 |
| Tocopherols[d] | 2.4-2.6 | 3.2-5.3 | 1.5-5.8 | 5.5-7.8 |
| | | In pulp (mg/100g)[b] | | |
| Dopamine | 4.7-10 | 6.1-15 | 2.5-10 | 0.72-6.1 |
| | (7.0±2.0) | (9.1±3.1) | (7.3±2.4) | (3.4±2.2) |
| Dopa | 1.3-1.9 | 1.3-1.9 | 1.0-1.5 | 0.95-1.4 |
| Norepinephrine | 0.80-1.7 | 0.84-2.1 | 0.82-1.6 | 0.62-1.5 |
| Naringin | ND[c] -65 | ND[c] | ND[c] -3.3 | ND[c] |
| Rutin | ND[c] -4.8 | ND[c] | ND[c] | ND[c] |
| Ascorbic acid[d] | 12-13 | 11-13 | 6.9-11 | 5.4-10 |
| Carotenes[d] | 0.09-0.12 | 0.06-0.12 | 0.03-0.12 | 0.02-0.05 |
| Tocopherols[d] | ND[c] | ND[c] -0.29 | ND[c] | ND[c] -0.45 |

[a] The ripening stage was determined as mentioned in the Materials and Methods
[b] The values are expressed as a range between the lowest and highest contents. In the lines for dopamine, values in parenthesis are the mean±SD.
[c] Not detectable.
[d] The ascorbic acid is the sum of reduced and oxidized forms. Carotenes are the sum of α- and β-carotenes, mainly α-carotene. Tocopherols are the sum of α-, γ- and δ-tocopherols, mainly α-tocopherol.

### *Strong antioxidative potency of dopamine*

The antioxidative potency of dopamine was compared with that of standard chemicals (Fig. 2C). Dopamine was stronger than dopa and the food additive BHA. In Fig. 4, another evaluation method was used with a DPPH radical. Dopamine scavenged the DPPH radical actively, more so than glutathione and another food-additive, BHT. The activity was also stronger than that of flavone luteolin and flavonol quercetin. Catechins have been found to be the strongest antioxidants in phytochemicals (Salah et al., 1995). Dopamine had a faster radical-scavenging rate than catechin, and was similar to gallocatechin gallate. Ascorbic acid is the strongest water-soluble antioxidant. Dopamine exhibited similar activity to ascorbic acid.

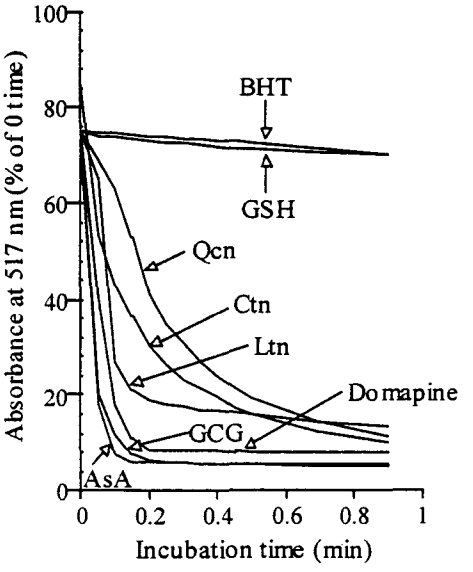


**Fig. 4.** Scavenging activity of dopamine against a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. Abbreviations: AsA, Ascorbic acid; GSH, glutathione reduced form; BHT, butylated hydroxytoluene; Ltn, flavone luteolin; Qcn, flavonol quercetin; Ctn, catechin; and GCG, gallocatechin gallate.

## *Discussion*

The present Chapter revealed that banana contained a strong antioxidant, dopamine, in large amounts. The antioxidative potency of dopamine was greater than that of BHA, BHT, flavonoids, glutathione, and catechin, and similar to that of the

strong antioxidants, gallocatechin gallate and ascorbic acid (Fig. 2C and 4). Bors et al. (1990) describes that the strong activity requires an *ortho*-dihydroxy (catechol) structure. Dopamine, one of the catecholamines, has this structure, and its amino residue facilitates the hydrophilic character. Thus, dopamine is a powerful water-soluble antioxidant like ascorbic acid. Edible banana pulp contained dopamine and ascorbic acid at high levels (Table 1). Banana is therefore one of the best sources of antioxidants.

The peel contained dopamine at the hundred-milligram level, and the antioxidative phytochemicals, flavanone glycoside naringin and flavonol glycoside rutin, at the ten-milligram level per 100 g at all ripening stages. Banana, a tropical plant, may protect itself from the oxidative stress caused by strong sunshine and high temperature by producing large amounts of antioxidants. A few milligrams of dopamine existed in the pulp at the stage that it is usually eaten. Dopamine has been found to protect against intestinal mucosal injury by modulating eicosanoid synthesis (MacNaughton and Wallace, 1989; Alanko et al., 1992). Banana may contribute to the anti-inflammation.

Dopamine is considered to be easily absorbed through a dopamine transporter and used in the body (Abi-Dargham et al., 1998). Although dopamine plays important roles as a nuerotransmitter and precursor for nor-epinephrine and epinephrine, an accumulation of the oxidized products of dopamine such as its quinone in brain with age can cause neurocells to undergo apoptosis, a process that is associated with Parkinson's disease (Luo et al., 1998; Cadet and Brannock, 1998). It is a remaining question whether a few milligrams of dopamine in pulp has a favorable or unfavorable effect on human health.

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# Chapter 3

# A Novel Method Using 8-Hydroperoxy-2'-deoxyguanosine Formation for Evaluating Antioxidative Potency to Protect DNA Damage

This chapter is based mainly on:

A Novel Method Using 8-Hydroperoxy-2'-deocyguanosine Formation for Evaluating Antioxidative Potency

*based on: Free Radical Research, in press*

# *Introduction*

Cancer and other degenerative diseases are believed to be induced by oxidative genetic damage. The oxidative damage is caused by reactive oxygen species (Gyuton and Kensler, 1993; Emerit, 1994; Marnett, 2000) and is mainly due to the formation of 8-hydroxyls, 8-oxo-7,8-dihydrodeoxyguanosine (8-hydroxy-2'-deoxyguanosine, 8-OHdG) and/or 8-hydroxy-2'-deoxyadenosine, in the bases (Randerath, 1997; Boiteux and Radicella, 1999; Nomoto et al., 2000; Podmore et al., 2000). An accumulation of 8-OHdG has been found in several carcinoma cells and mutated genes (Yamamoto to al., 1993; Kondo et al, 2000; Foksinski et al., 2000). Levels of 8-OHdG in human skeletal muscle, respiratory epithelium, and leukocytes are correlated with aging, exposure to urban pollution, and smoking, respectively (Mecocci et al., 1999; Calderón-Garcidueñas et al., 1999; Lodovici et al., 2000). Urinary 8-OHdG is a biomarker of human oxidative stress accompanying cancer (Simic, 1992; Kasai, 1997; Halliwell, 1998; Zwart, 1999).

Antioxidants can scavenge reactive oxygen species. The antioxidants in our diet may therefore prevent disease (Youdim and Joseph, 2001). A better understanding is needed of which dietary antioxidants can trap reactive oxygen species effectively enough to prevent genetic damage, i.e. easier than DNA. The putative reactive oxygen species that form 8-hydroxy derivatives are hydrogen peroxide (Floyd et al., 1988; Taylor et al., 1995; Abu-Shakra and Zeiger, 1997; Lloyd and Phillips, 1999), thiol radical (Goldman et al., 1999), and lipid peroxides (Kaneko and Tahara, 2000). However, they all require transition metals, UV rays or $\gamma$ rays to interconvert and generate oxidizing species, and several of them have to form hydrogen peroxide or lipid

peroxides. Thus, Fenton's reaction has been employed using 2'-deoxyguanosine (2'-dG) as a target (Kasai and Nishimura, 1984), when evaluates antioxidants. This system is composed of several subfactors such as hydrogen peroxide, iron(II), and ethylenediaminetetraacetate disodium salt (Nappi and Vass, 1997; Yokozawa et al., 1997). The various subfactors complicate the assay system and may result in errors during the evaluation, for example, several antioxidants interact with metal ions (Sugihara et al., 1999) and chelator (Puppo, 1992).

A simple system and a convenient method are better. In the present study, the author employed 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH) because it has been proved to produce stoichiometrically a molecular oxygen radical via an intermediary AAPH-peroxyl radical (Niki, 1990). The oxygen radical is present in normal cells at high steady-state concentrations (Simandan et al., 1998). Especially in mitochondria, side reactions of the electron transport chain with molecular oxygen directly generate its radical, superoxide anion (Cadenas and Davies, 2000; Atlante et al., 2000). Then, being 2'-dG as a target, it formed a novel product, 8-hydroperoxy-2'deoxyguanosine (8-OOHdG), quantitatively. The system could easily and accurately evaluate the potency of both water- and lipid-soluble antioxidants to suppress the formation by HPLC analysis.

# *Materials and Methods*

## *Chemicals*

AAPH, 2'-dG, 8-OHdG, and ascorbic acid were obtained from Wako Pure Chemical Ind., Ltd. (Tokyo, Japan). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was from Nacalai Tesque (Kyoto, Japan). Quercetin was from Extrasynthèse (Genay, France), epigallocatechin gallate (EGCG) was from Kurita Kogyo (Tokyo, Japan), and the other antioxidants were high-grade commercial products. These products were tested to be free from impurities by HPLC before use. Water was distilled twice and all other reagents used were of the highest grade available.

## *HPLC analysis for reaction products of 2'-dG and AAPH*

AAPH (25 mM) was mixed with of 2'-dG (0.5 mM) in water and incubated at 37°C with stirring under atmospheric pressure. The pH of the mixture was 7.0 and remained unchanged during the incubation. An aliquot (10 μl) was analyzed in a reverse-phased HPLC system equipped with UV (254 nm) (HITACHI L-7100 and L-7420) and electrochemical (+600 mV) (IRICA Σ 875; Kyoto, Japan) detectors. The conditions for HPLC were as follows: column, Capcell pak C18 UG120 (5 μm mesh and ϕ 4.6 × 250 mm) (Shiseido Co., Ltd., Tokyo, Japan), maintained at 35°C; mobile phase, a mixed solvent of 6.5% methanol and 93.5% 20 mM potassium phosphate buffer (pH 4.5) containing 0.1 mM ethylenediaminetetraacetate disodium salt; and flow rate, 1.0 ml/min.

### *Purification of the products*

To obtain the reaction products, another mixture of 250 mM AAPH and 2.5 mM 2'-dG was incubated for 6 h. This mixture (10 ml) was passed through an ion-exchange column (Dowex 1 × 4, 50-100 mesh, $OH^-$ form, ϕ 14 × 100 mm). The products were eluted with 0.1 N HCl after a wash with 50 ml of 0.1 N NaOH and 50 ml of water. The eluate was neutralized with 1 N NaOH and concentrated by an evaporator to around 5 ml. It was then subjected to HPLC with a Cosmosil Packed Column (preparative column, ϕ10 × 250 mm) (Nacalai Tesque, Inc., Kyoto, Japan) and eluted with water. The fraction showing absorption at 254 nm was collected and dried with a centrifugal concentrator (VC-96N, Taitec Co.). The dried products showed a single peak in the above analytical HPLC which coincided with one of the product peaks.

### *Elucidation of chemical structure*

The chemical structures of the dried products were elucidated using nuclear magnetic resonance (NMR) spectrometry (DPX-250, Bruker Analytik GmbH). Proton spectra were recorded at 250 MHz and $^{13}C$ at 62.5 MHz in $D_2O$ and 3-trimethylsilylpropionate-2,2,3,3, $D_4$. Mass spectra were obtained with an HPLC-mass spectrometer (LC/MS M-1200H, HITACHI) under atmospheric pressure, with chemical ionization and ionizing at +30 eV. Infrared spectra were measured with FT-IR spectrometer (FTIR-8600 PC, Shimadzu) in KBr powder by a diffusion reflectance method.

### *Antioxidant assay with 2'-dG and AAPH*

Antioxidants in 5 μl of dimethylsulfoxide (DMSO) were added at six different concentrations to the mixture of 0.5 mM 2'-dG and 25 mM AAPH. After 1 h incubation at 37°C, the amount of 8-OOHdG formed was determined with 10 μl of the mixture using the analytical HPLC system at UV 254 nm. When the chemicals constructed a dose-response curve, the $IC_{50}$ values were calculated by plotting the suppression of 8-OOHdG formation against the dose. The $IC_{50}$ value is the amount of antioxidant required for 50% suppression of 8-OOHdG formation. The assays were done independently in triplicate.

### *Another assay with DPPH*

The assay accuracy was compared to a convenient and widely used method employing the DPPH radical (Blois, 1958). Six different amounts of each antioxidant in ethanol were added to 250 nmol DPPH in 50% ethanol in 50 mM acetate buffer (pH 5.5) and the signal was monitored at 517 nm. The reduction in DPPH radical within 5 min was plotted against the respective concentrations of antioxidant. The $IC_{50}$ value was then calculated as the concentration of antioxidant required for scavenging 50% of DPPH radicals in the solution (125 nmol) within 5 min.

# *Results*

## *Reaction of AAPH and 2'-dG*

Fig. 1A is the HPLC profile obtained when the reaction mixture of AAPH and 2'-dG was incubated for 1 h and monitored at 254 nm. Other monitoring methods using different wavelengths and electrochemical detection which has been known to be sensitive to 8-OHdG (Sies, 1993) did not give additional peaks. Thus, the reaction of AAPH and 2'-dG gave four detectable products on HPLC.

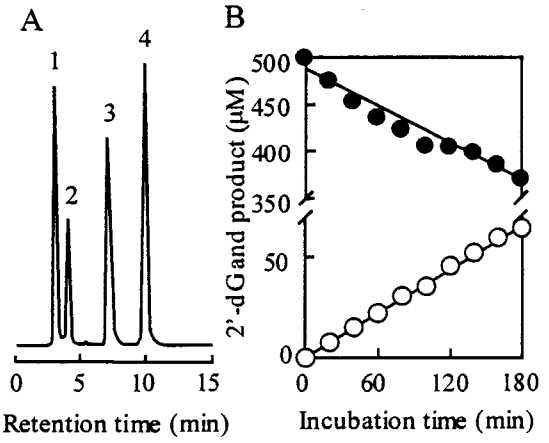



**Fig. 1.** HPLC for reaction products of AAPH and 2'-dG. (A) A profile of 1 h incubation of 25 mM AAPH with 0.5 mM 2'-dG at 37°C. The incubation mixture (10 μl) was analyzed by reverse-phased HPLC with monitoring at 254 nm. Peaks 1 - 4 were identified as AAPH, a reaction product, decomposed products of AAPH, and 2'-dG, respectively. (B) Incubation time-dependent formation of the product (○) and decrease of 2'-dG (●).

The first peak at a retention time of 3.3 min was the original AAPH while the third at 7.8 min was its decomposed product because the incubation of AAPH alone produced the same profile. The fourth at 10.1 min coincided with 2'-dG in retention time. A minor peak at 5.5 min was identified as guanine contaminating the reagent. Thus, the second peak at 4.3 min was the reaction product of AAPH and 2'-dG. The production involved time-dependent consumption of 2'-dG (Fig. 1B).

## *Identification of the product*

The characteristics of the purified product are summarized in Table 1 along with those of standard 2'-dG and 8-OHdG. The product gave a parent ion peak at *m/z* 300 suggesting an addition of 32 mass units ($O_2$) to 2'-dG. The UV spectrum indicated a purine skeleton as well as 2'-dG. Based on IR analysis, the bands were assigned to ν (O-O) and δ (OOH). Chemical determination of the peroxide value indicated one peroxide group per mole of the product. The value was negative in 2'-dG and 8-OHdG.

In NMR (Table 2), the product gave no signal for H-8 or 8-OHdG. The other signals were assigned to ribose protons from H-1' to H-5' (Lin et al., 1985), which shifted slightly to higher magnetic fields compared to those of 2'-dG, with a larger shift for H-1' than 8-OHdG. The shifts are a feature of substitution at the 8-position

**Table 1.** Spectrometric and Chemical Characters of the Reaction Product of AAPH and 2'-dG

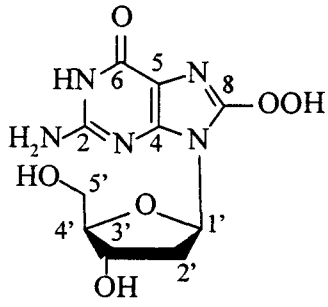
| Analysis | product | 2'-dG | 8-OHdG |
|---|---|---|---|
| LC/MS (*m/z*) | $(M+1)^+$, 300<br>(M+1) - ribose, 184 | $(M+1)^+$, 268<br>(M+1) - ribose, 152 | $(M+1)^+$, 284<br>(M+1) - ribose, 168 |
| UV ($H_2O$): $\lambda_{max}$ (ε)[a] | 254 (15,300) and 313 (1,670) | 253 (12,800) | 245 (12,300) and 293 (10,300) |
| ν (O-O)<br>δ (OOH) | 900 $cm^{-1}$<br>1125 $cm^{-1}$ | no signals | no signals |
| Peroxide value (mol/mol)[b] | 0.93 | negative | negative |

*a*, Molecular extinction coefficient of the product was calculated with the molecular weight obtained by LC/MS (all other values were from Kasai and Nishimura (1984)).

*b*, The value was determined according to the method of Sully (1954) and the ratio was calculated with the molecular weight obtained by LC/MS.

(Ikehara et al., 1972; Clup et al., 1989). In carbon spectra, C-8 shifted greatly to a lower magnetic field compared to the original 2'-dG, and C-2, C-4, and C-5 to higher fields as well as 8-OHdG (Uesugi and Ikehara, 1977). The shift of C-8 was greater than that in 8-OHdG. The proton and carbon spectra thus indicated a group other than

**Table 2.** Pertinent NMR Absorptions of the Product




| $^1H$ | chemical shifts (ppm), multiplicity, *J*=Hz | | |
|---|---|---|---|
| | product | 2'-dG | 8-OHdG |
| H-8 | no signal | 7.98, s | no signal |
| H-1' | 5.88, *t*, *J*=6.75, 6.50 | 6.30, t, *J*=6.80, 6.90 | 6.24, t, *J*=7.63, 6.70 |
| H-2' | 2.34, m; 2.63, m | 2.51, m; 2.80, m | 2.28, m; 3.07, m |
| H-3' | 4.07, m | 4.14, m | 4.09, m |
| H-4' | 4.49, m | 4.63, m | 4.65, m |
| H-5' | 3.73, m | 3.80, m | 3.83, m |
| $^{13}C$ | chemical shifts (ppm) | | |
| C-2 | 160.0 | 163.9 | 159.0 |
| C-4 | 149.8 | 153.7 | 150.3 |
| C-5 | 112.0 | 121.0 | 111.4 |
| C-6 | 172.4 | 171.1 | 172.9 |
| C-8 | 181.0 | 139.0 | 165.1 |
| C-1' | 84.8 | 87.3 | 85.1 |
| C-2' | 41.3 | 41.8 | 39.0 |
| C-3' | 74.2 | 74.6 | 74.6 |
| C-4' | 89.0 | 90.3 | 89.7 |
| C-5' | 64.7 | 64.9 | 65.0 |

OH substituted at the 8-position. The substitute was suggested to be a peroxide based on the data from mass and IR spectra and chemical determination.

A remaining question is whether the substitute is a hydroperoxide or endoperoxide such as 4,8 or 5,8. Then, the product was incubated in 0.17 N HCl at 37°C (Fig. 2). A 15-min incubation produced an appreciable amount of 8-OH-guanine and a small amount of 8-OHdG, and simultaneously the formation of 2'-deoxyribose was detected on thin layer chromatography with Merck Kieselgel 60 $F_{254}$ (Darmstadt, Germany) developed in methanol:ethyl acetate (5:2) with coloring by a reagent of diphenylamine:aniline:phosphoric acid (data not shown). The formation of 8-OHs indicated that the original product was 8-OOHdG as illustrated in Table 2.

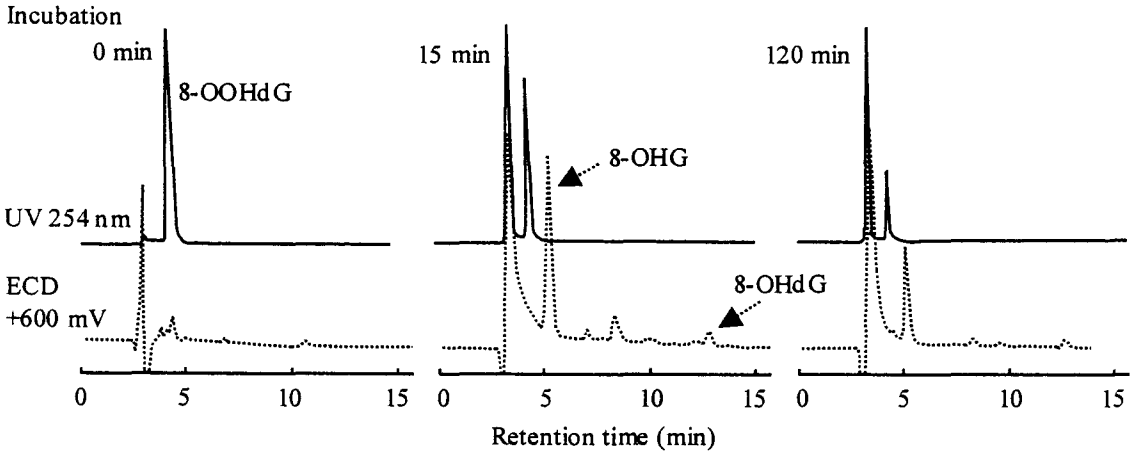



**Fig. 2.** Change of 8-OOHdG to hydroxyls. The purified 8-OOHdG was incubated in 0.17 N HCl at 37°C for 15 min, and then analyzed by HPLC as in Fig. 1, monitoring with UV 254 nm and electrochemically at +600 mV.

## *Quantitative formation of 8-OOHdG*

Fig. 3A is a calibration curve for 8-OOHdG obtained using the molecular extinction coefficient $\lambda_{254}$ ($\varepsilon$)=15,300 in Table 1. The determination limit was 3 pmol. The formation rates of 8-OOHdG depended on the incubation temperature (Fig. 3B) and every curve was almost linear with the incubation time. Further, the formation rates (8-OOHdG nmol/min) were linearly proportional to AAPH concentrations, indicating that the formation exhibited first order kinetics (Fig. 3C). These results show that the present system can be useful for antioxidant assay.

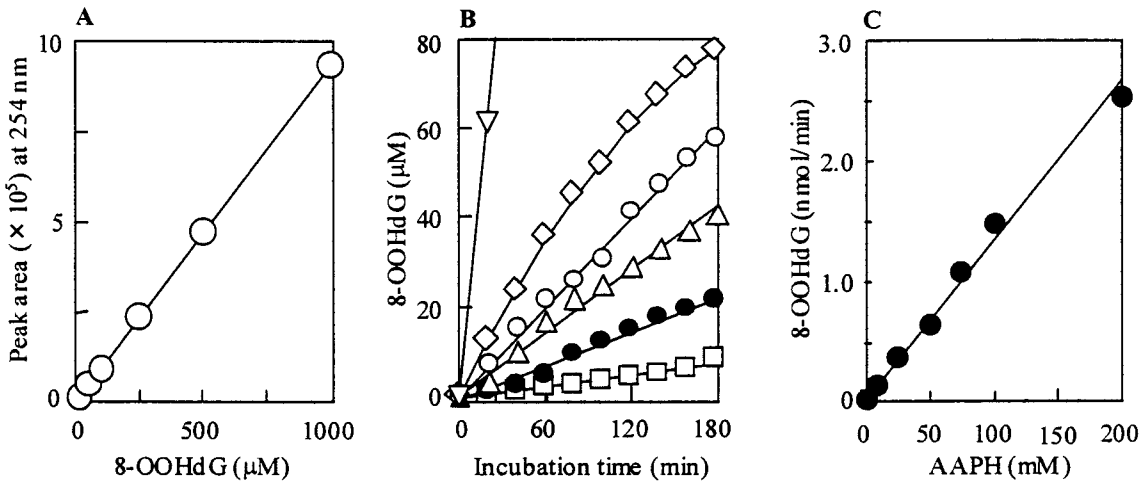



**Fig. 3.** Quantitative formation of 8-OOHdG under various conditions. (**A**) A calibration curve for 8-OOHdG with the molecular extinction coefficient of $\lambda_{254}$ ($\varepsilon$)=15,300 in Table 1. The determination limit was 3 pmol. (**B**) The formation of 8-OOHdG under various temperatures. The mixture of 0.5 mM 2'-dG and 25 mM AAPH was incubated at (□) 25, (●) 30, (△) 35, (○) 37, (◇) 40, and (▽) 50°C. (**C**) The formation of 8-OOHdG with various concentrations of AAPH. 2'-dG (0.5 mM) was incubated at 37°C with 1, 10, 25, 50, 75, 100, and 200 mM of AAPH. The amount of 8-OOHdG that formed in these incubation mixtures was determined by HPLC as in Fig. 1 every 10 min in 60 min.

## *Evaluation of antioxidative potency using the system of 8-OOHdG formation*

The author evaluated the antioxidative potency in 90 chemicals with the present system. They were dissolved in 5 μl of DMSO and added to the incubation mixture of 0.5 mM 2'-dG and 25 mM AAPH. The suppression ratios against the formation of 8-OOHdG without chemicals were determined as the antioxidative potency of the tested chemicals. The solvent DMSO had no effect on the 8-OOHdG formation.

Table 3 shows the antioxidative potencies of simple phenols, flavonoids, anthraquinones, other phytochemicals, food additives, and biological components, most of which occur in our diet. Almost all of them gave $IC_{50}$ values, meaning that they were antioxidative dependant on dose. Among the simple phenols, protocatechuic acid, catechol, caffeic acid, and chlorogenic acid showed the lowest values and *ortho*-dihydroxyl products were also recognized as potent antioxidants (Bros et al., 1990). In flavonoids, the antioxidative potencies almost coincided with their hydroxyl numbers, whereas no-hydroxyl flavone, flavanone, and chalcone were not effective and the one-hydroxyl flavonol was weaker than the polyhydroxyl flavonoids. Among anthraquinones, *ortho*-dihydroxyls showed relatively low $IC_{50}$ values but the others had higher values or were not effective. The biological components glutathione and ascorbic acid showed high $IC_{50}$ values, and the neurotransmitter dopamine and hormones showed relatively low values. Neither a quencher for singlet oxygen, β-carotene, nor a trapper for lipid-peroxyl radicals, α-tocopherol (Krinsky, 1989; Mascio et al., 1991), gave an $IC_{50}$ value. On the other hand, the DPPH method was not effective for scavenging radicals or for determining the $IC_{50}$ values of several simple phenols and flavonoids. For example, apigenin was evaluated as ineffective though it

is known to be an antioxidant (Cholbi et al., 1991; Yokozawa et al., 1998).

Most of the simple phenols and flavonoids tested here are lipid-soluble. Compared to the DPPH method, the proposed 8-OOHdG method was able to determine the activity of lipid-soluble chemicals. The present method was also useful for evaluating the potency of water-soluble antioxidants such as catechins, Trolox, glutathione, and ascorbic acid, while the DPPH method could not detect the activity of one biologically important antioxidant, glutathione.

**Table 3.** Evaluation of Antioxidative Potency with the Present System and DPPH Method

| Chemicals (position of substitute) | $IC_{50}$ value (μM) [a] against: | |
|---|---|---|
| | 8-OOHdG formation | DPPH radical |
| Simple Phenols | | |
| 1,2,4-benzenetriol | 15 | 19 |
| benzoic acid | ne | ne |
| caffeic acid | 6.2 | 26 |
| catechol | 7.6 | 24 |
| gallic acid | 16 | 10 |
| gallic acid n-butyl ester | 15 | 8.5 |
| hydroquinone | 23 | 25 |
| *ortho*-hydroxybenzoic acid | 8.9 | ne |
| *meta*-hydroxybenzoic acid | 8.9 | ne |
| *para*-hydroxybenzoic acid | 6.4 | ne |
| *ortho*-hydroxycinnamic acid | 12 | ne |
| *meta*-hydroxycinnamic acid | 11 | ne |

**Table 3** — continued

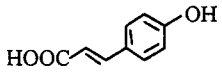
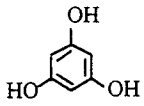
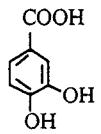
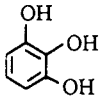
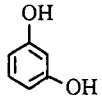
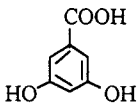
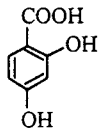
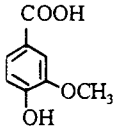
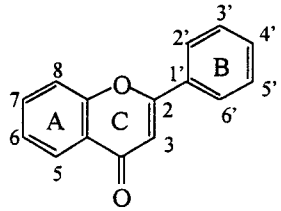
| Chemicals (position of substitute) | $IC_{50}$ value (μM) [a] against: 8-OOHdG formation | DPPH radical |
|---|---|---|
| *para*-hydroxycinnamic acid  | 8.4 | ne |
| phenol  | 10 | ne |
| phloroglucinol  | 23 | 90 |
| protocatechuic acid  | 6.9 | 22 |
| pyrogallol  | 20 | 12 |
| resorcinol  | 15 | ne |
| α-resorcylic acid  | 8.8 | ne |
| β-resorcylic acid  | 10 | ne |
| vanillic acid  | 12 | 170 |
| Flavonoids  | | |
| Flavones | | |
| flavone (none) | ne | ne |
| chrysin (5,7-OH) | 9.3 | ne |
| baicalein (5,6,7-OH) | 17 | 25 |
| apigenin (5,7,4'-OH) | 3.8 | ne |
| apigenin-6-*C*-glucoside (isovitexin) | 3.6 | ne |
| apigenin-7-*O*-glucoside (apigetrin) | 5.0 | ne |
| apigenin-8-*C*-glucoside (vitexin) | 3.6 | ne |
| luteolin (5,7,3',4'-OH) | 5.0 | 13 |
| luteolin-3',7-di-*O*-glucoside | 10 | 370 |
| luteolin-4'-*O*-glucoside | 4.1 | 460 |
| chrysoeriol (5,7,4'-OH, 3'-$OCH_3$) | 10 | ne |

**Table 3** — continued

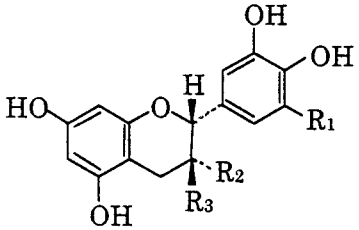
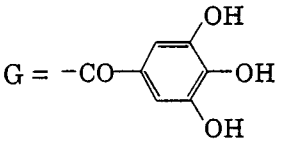
| Chemicals (position of substitute) | $IC_{50}$ value (μM)[a] against: 8-OOHdG formation | DPPH radical |
|---|---|---|
| Flavonols | | |
| flavonol (3-OH) | 41 | ne |
| galangin (3,5,7-OH) | 10 | 68 |
| datiscetin (3,5,7,2'-OH) | 6.9 | 58 |
| kaempferol (3,5,7,4'-OH) | 1.8 | 24 |
| kaempferol-3-*O*-rutinoside | 3.5 | ne |
| kaempferol-7-*O*-neohesperidoside | 4.3 | 29 |
| morin (3,5,7,2',4'-OH) | 4.1 | 26 |
| quercetin (3,5,7,3',4'-OH) | 3.8 | 11 |
| quercetin-3-*O*-glucoside (isoquercitrin) | 3.3 | 7.1 |
| quercetin-3-*O*-rhamnoside (quercitrin) | 1.3 | 18 |
| quercetin-3-*O*-rutinoside (rutin) | 3.6 | 15 |
| robinetin (3,7,3',4',5'-OH) | 8.3 | 7.4 |
| isorhamnetin (3,5,7,4'-OH, 3'-$OCH_3$) | 9.8 | 26 |
| quercetagetin (3,5,6,7,3',4',-OH) | 6.8 | 7.3 |
| myricetin (3,5,7,3',4',5'-OH) | 5.3 | 8.0 |
| myricetin-3-*O*-rhamnoside (myricitrin) | 4.6 | 6.7 |
| Flavanones (2-3 is saturated) | | |
| flavanone (none) | ne | ne |
| naringenin (5,7,4'-OH) | 3.9 | ne |
| hesperetin (5,7,3'-OH, 4'-$OCH_3$) | 4.8 | 260 |
| (+)-taxifolin (3,5,7,3',4'-OH) | 4.2 | 19 |
| Isoflavones (B-ring binds to 3 position) | | |
| daidzein (7,4'-OH) | 5.3 | ne |
| Catechins   | | |
| (+)-catechin ($R_1$=H, $R_2$=H, $R_3$=OH) | 7.4 | 16 |
| (-)-gallocatechin ($R_1$=OH, $R_2$=H, $R_3$=OH) | 9.3 | 12 |
| (-)-catechin gallate ($R_1$=H, $R_2$=H, $R_3$=OG) | 4.2 | 3.6 |
| (-)-gallocatechin gallate ($R_1$=OH, $R_2$=H, $R_3$=OG) | 5.1 | 3.2 |
| (-)-epicatechin ($R_1$=H, $R_2$=OH, $R_3$=H) | 3.7 | 4.9 |
| (-)-epigallocatechin ($R_1$=OH, $R_2$=OH, $R_3$=H) | 9.0 | 2.7 |
| (-)-epicatechin gallate ($R_1$=H, $R_2$=OG, $R_3$=H) | 5.3 | 3.4 |
| (-)-epigallocatechin gallate ($R_1$=OH, $R_2$=OG, $R_3$=H) | 5.8 | 1.6 |

**Table 3** — continued

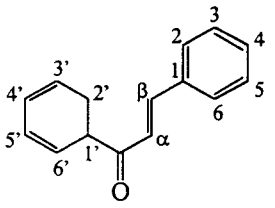
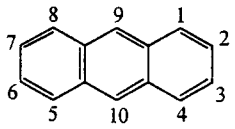
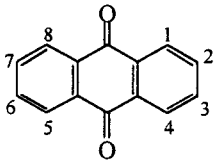
| Chemicals (position of substitute) | $IC_{50}$ value (μM) [a] against: 8-OOHdG formation | DPPH radical |
|---|---|---|
| Chalcones  | | |
| chalcone (none) | ne | ne |
| butein (3,4,2',4'-OH) | 5.4 | 17 |
| Anthracene and Anthraquinones   | | |
| Anthracenes | | |
| anthracene (none) | 93 | ne |
| 9(10H)-anthracenone (anthrone) | ne | 54 |
| 1,8-dihydroxy-9(10H)-anthracenone (anthralin) | 61 | 82 |
| Anthraquinones | | |
| anthraquinone (none) | ne | ne |
| alizarin (1,2-OH) | 14 | 42 |
| quinizarin (1,4-OH) | 58 | 130 |
| chrysazin (1,8-OH) | ne | ne |
| rhein (1,8-OH, 3-COOH) | ne | ne |
| chrysophanol (1,8-OH, 3-$CH_3$) | ne | ne |
| purpurin (1,2,4-OH) | 13 | 45 |
| emodin (1,3,8-OH, 6-$CH_3$) | 43 | ne |
| Other Phytochemicals and Food Additives | | |
| chlorogenic acid | 9.8 | 46 |
| curcumin | 10 | 37 |
| sesamol | 18 | 25 |
| 2,2,5,7,8-pentamethyl-6-chromanol (PMC) | 22 | 26 |
| Trolox | 23 | 34 |
| butylated hydroxyanisole (BHA) | 19 | 46 |
| butylated hydroxytoluene (BHT) | 52 | 69 |
| *tert*-butylhydroquinone (TBHQ) | 29 | 26 |

**Table 3** — continued

| Chemicals (position of substitute) | $IC_{50}$ value (μM) [a] against: | |
|---|---|---|
| | 8-OOHdG formation | DPPH radical |
| Biological Components | | |
| glutathione | 52 | ne |
| dopamine | 9.6 | 12 |
| adrenaline | 11 | 10 |
| noradrenaline | 6.5 | 12 |
| β-carotene | ne | ne |
| α-tocopherol | ne | 36 |

[a] Antioxidative potency was expressed as the $IC_{50}$ value; the concentrations required to suppress the formation of 8-OOHdG by 50% in the 1-h incubation of 0.5 mM 2'-dG with 25 mM AAPH or to scavenge 50% of DPPH radicals within 5-min incubation.

# *Discussion*

The present study found that the molecular oxygen radicals generated from AAPH formed a novel product, an 8-OOH derivative, on 2'-dG (Table 2). The formation of 8-OOHdG was linearly dependent on incubation time (Fig. 1 and 3) and so was useful for determining the antioxidative potency of various chemicals in our diet. This assay system is simple and easy taking 15 min for the HPLC following a 1-h incubation and is as convenient as the DPPH method. The DPPH method has been used widely, however, it employs an artificial DPPH radical making it difficult to evaluate the activity of antioxidants to prevent genetic damage. The method proposed here employs the molecular oxygen radical which is thought to be present in normal cells at high steady-state concentrations (Simandan et al., 1988) and especially in

mitochondria, side reactions of the electron transport chain with molecular oxygen directly generate its radical, superoxide anion (Cadenas and Davies, 2000). Also, the present method employs 2'-dG as the target and enables one to directly evaluate which antioxidants can inhibit the radical actions before the oxidative damage to 2'-dG. Additionally, this method could be used to evaluate both lipid- and water- soluble antioxidants (Table 3). This method assessed that β-carotene and α-tocopherol were not antioxidative to oxygen radical, and was correct because β-carotene had been recognized to be a quencher for singlet oxygen and α-tocopherol to be a scavenger for lipid-peroxyl radicals (Krinsky, 1989; Mascio et al., 1991). So, the 8-OOHdG method is considered to be more useful than the DPPH method.

Thus, the method proposed here based on the formation of 8-OOHdG is very useful for evaluating the effects of the dietary antioxidants and determining the potency at which they prevent degenerative diseases including cancer associated with oxidative genetic damage.

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# Chapter 4

# A Complete Analysis to Determine Phenolic Antioxidants in Vegetables, Fruits and Teas

This chapter is based mainly on:

A complete analysis to determine phenolic antioxidants in vegetables, fruits, and teas

*Journal of Chromatography A, in submitted*

# *Introduction*

Dietary polyphenols have the beneficial effects on prevention of degenerative diseases including cancer. Their actions can be classified into antioxidative potency and modulation of protein functions. The antioxidative potency can protect from oxidative genetic damages that associate directly with the degenerative diseases (Guyton et al., 1993; Emerit, 1994; Marnett, 2000). The intake of antioxidative polyphenols has been proved to reduce the incidences of cancer and life-style related diseases (Wattenburg, 1985; Mori et al., 1986; Verma et al., 1988, Wei et al., 1990; Tanaka et al., 1993; Yang et al., 2001; Han et al., 2001). Also, polyphenols can modulate the protein functions: they exhibit agonism and/or antagonism on several receptors such as arylhydrocarbon receptor (Ashida et al., 2000), estrogen receptor β (An et al., 2001), α-fetoprotein (Baker et al., 1998), brain $GABA_A$ receptor (Dekermendjian et al., 1999), adenosine receptor (Moro et al., 1998), epidermal growth factor (Agullo et al., 1997), and so on. They suppress or enhance gene expressions of several enzymes (Williamson, et al., 1996; Soriani et al., 1998; Wadsworth and Koop, 1999; Liang et al., 2001). They modulate secretion of cytokines that induce an arrest in cell cycle (Bhatia and Agarwall, 2001; Frey et al., 2001) and relate to allergy (Yamada et al., 1999), and inhibit the cytokine actions (Saliou et al., 1998; Xagorari et al., 2001). They regulate membrane transporters (Park, 1999; Revuelta and Hidalgo, 2000; Leslie et al., 2001) and further inhibit or stimulate various enzymes that mainly associating with tumorigenesis (Lepley and Pelling, 1997; Nagasaka and Nakamura, 1998; Yamaguchi and Sugimoto, 2000; Kobuchi et al., 1999; Kuppusamy and Das, 1994; Zhu and Liehr, 1996; Otake et al., 2000; Laughton et al., 1991; Gil et al., 1994;

Kitson and Kitson, 2000; Keung, 2001).

Polyphenols often occur as the glycoside forms in vegetables, fruits, and teas (Herrmann, 1976; Wollenweber and Dietz, 1981). The bio-activities, however, are based on their aglycone structures without sugar chains. The antioxidative potency is great in *ortho*-dihydroxyl (catechol) structure ones (Bros et al., 1990; Rice-Evans et al., 1996; Sakakibara et al., in press) and the modulating actions on proteins are contributed by their respective steric structures (Kanazawa et al., 1998; Ashida et al., 2000; Ferte et al., 1999; Casagrande and Darbon, 2001). Therefore, polyphenols in food should be determined in their aglycone structures.

Aglycones of polyphenols are classified into three classes, flavonoids, anthraquinones, and simple polyphenols (Fig. 1), although varieties of these polyphenols are beyond million species in plant kingdom. A large class, flavonoids, is constructed basically with A and C rings of benzo-1-pyran-4-quinone and B ring, and further classified to flavones (basic structure), flavonols (having hydroxyl on 3-position), isoflavones (B-ring binds to 3-position), flavanones (2-3 bond is saturated) including catechins (C-ring is 1-pyran), chalcones (C-ring is opened), and anthocyanidins (C-ring is 1-pyran, and 1-2 and 3-4 are unsaturated), which have a variety in substitute groups with hydroxyls and/or methoxyls. Anthraquinones are alizarin, rhein, emodin, and etc. (Sun et al., 2000), and rarely occur as glycoside forms. The other polyphenols are mainly constructed with a single ring and then generally called as simple polyphenols, which include three subclasses; cinnamic acids such as coumaric acid, ferulic acid, and caffeic acid; benzoic acids such as protocatechuic acid, gallic acid and vanillic acid; and others are sesamol and so on.

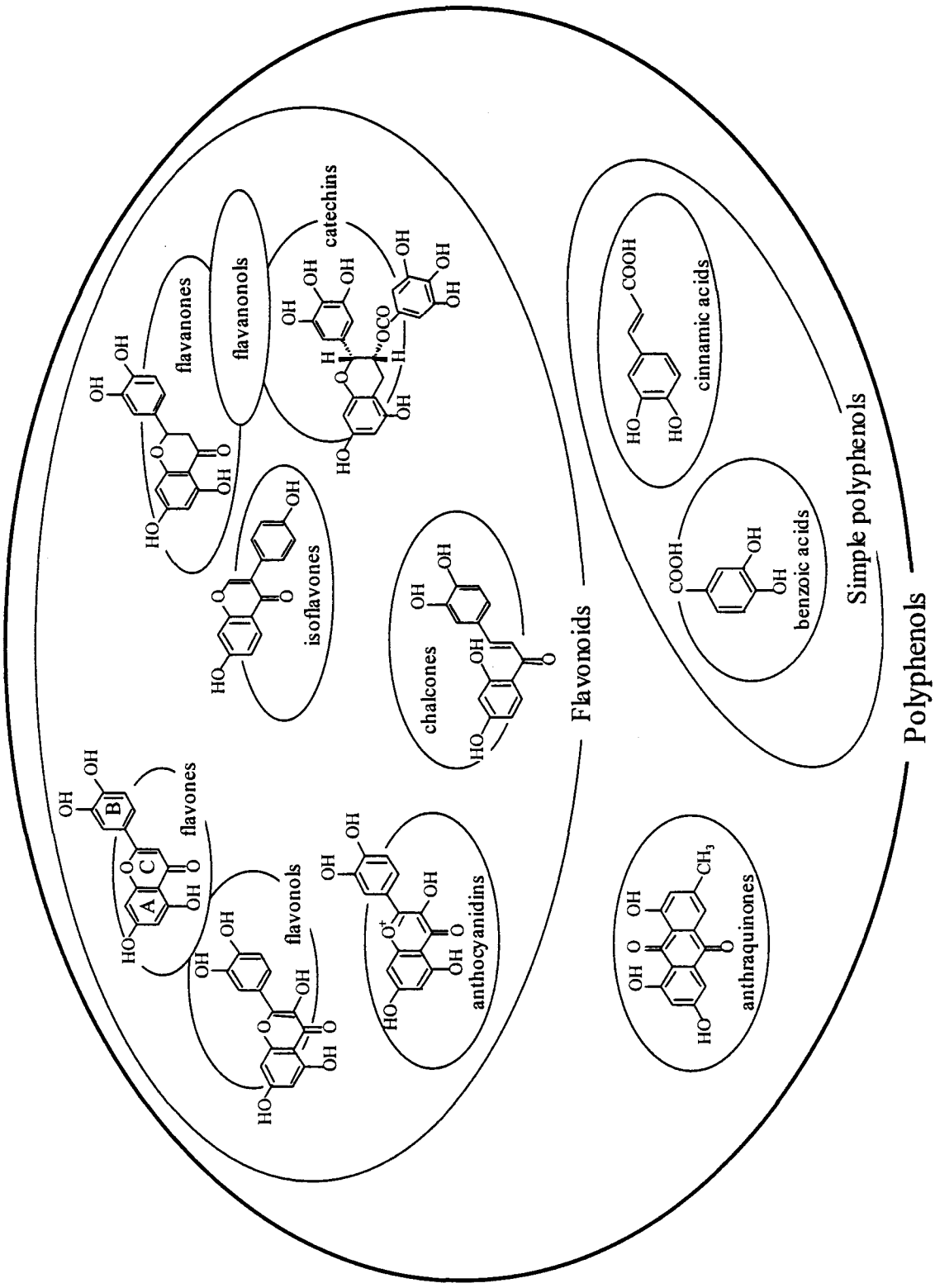



**Fig. 1.** Classification of polyphenols occurring in our vegetables, fruits and teas

Recently, several working groups have proposed the determination method for polyphenol aglycones employing HPLC with photo diode-array detector (Hertog, et al., 1992a; Crozier et al., 1997a; Haekkinen et al., 1998; Justesen et al., 1998; Merken and Beecher, 2000). Their method, however, have two demerits. They determined only flavonoid class but did not anthraquinones and simple polyphenols, and required a pretreatment of hydrolysis. The hydrolysis produces a loss of contents by decomposition and polymerization of polyphenols, for example, under the most optical condition the hydrolysis led to an underestimation of up to 50% of the true polyphenol level in food (Hertog et al., 1992a). Thus, the method is not suitable for the quantitative determination of dietary polyphenols. Schieber et al. (2001) have improved the HPLC method to detect simultaneously free polyphenols and its glycosides without hydrolysis. However, they could detect only one aglycone quercetin but not others.

The object of this chapter is the development of analytical method to determine both forms of free and glycoside in identifications of polyphenol classes and quantifications of the contents in food. The author established a novel determination method using HPLC and diode-array detector, with a simple extraction without hydrolysis. This method was able to accurately determine almost all of polyphenols in 47 kinds of vegetables, fruits, and teas.

# *Materials and Methods*

## *Standard chemicals*

The standard chemicals used here are summarized in Table 1. Among flavonoids, flavone and flavonol were purchased from Nacalai Tesque (Kyoto, Japan) and Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan), respectively. Catechins were obtained from Kurita Kogyo (Tokyo, Japan) and theaflavins were kindly furnished by Ito En, Ltd. (Tokyo, Japan). The other flavones, flavonols, flavanones, chalcones, and anthocyans were purchased from Extrasynthèse (Genay, France). Simple phenols were high-grade commercial products available. The standards were dissolved in dimethylsulfoxide (DMSO) at the concentration of 10 mM and kept at –20°C under dark for up to 3 months. Diluting to range from 1.0 to 1000 μM with DMSO, the calibration curves were made on a HPLC. Water was distilled twice and all other reagents were of the highest grade available.

## *Vegetables, fruits and teas*

Flesh vegetables and fruits were purchased from the local markets in Kobe City. The edible portion (100 g) were washed with tap water, chopped, and homogenized in liquid nitrogen with a homogenizer (Nihonseiki Kaisha Co., Ltd, Osaka, Japan). Teas, cacao, and coffee beans, which were obtained from local grocery stores in Kobe City, were powdered with a coffee mill. The homogenized powder was lyophilized at 0.2 Pa. for 48 h and stored at 4°C in a desiccator until use. The moisture contents were calculated with differences in weights before and after lyophilization.

## *Extraction of polyphenols*

The stored powders (50 mg) were extracted with 2 mL of 90% methanol containing 0.5% acetic acid, adding 50 nmoles of flavone in DMSO as an internal standard. Flavone was mostly used because vegetables, fruits, and teas have been found to rarely contain flavone. When food samples gave flavone peak on the HPLC, the internal standard was replaced with another internal standard such as flavonol or chalcone. The solution was sonicated by a Heart sonicater for 1 min, and the supernatant was recovered by a centrifugation at 3000 rpm for 10 min. The sonicating extraction was repeated three times, and the extracts were dried up with a centrifugal concentrator (VC-96N, Taitec Co., Saitama, Japan). The residues were dissolved in 0.5mL of DMSO and were filtered through a 0.2 μm membrane filter Millex-LG (Millipore Co., Bedford, America) before the HPLC analysis. The extraction was preformed independently tree times or more, until a variety of the recovery percentage calculated with internal standard was less than 5%.

## *HPLC analysis*

The HPLC system was employed a HITACHI HPLC series D-7000 (Tokyo, Japan), equipping with a Hitachi Model D-7000 chromatography data station software, a autosampler D-7200, a column oven D-7300, and a diode-array detection system D-7450. HPLC column was Capcell pak C18 UG120 (250 × 4.6 mm I.D., S-5, 5 μm, Shiseido Co., LTD. Tokyo, Japan) and jointed with guard column (10 × 4.0 mm I.D.). Both the main and guard columns were thermostatically maintained at 35°C and was 1.0 mL/min for the flow rate. For a mobile phase, gradient was programmed with A solution consisted of 50 mM sodium phosphate (pH 3.3) containing 10% methanol and

B solution of 70% methanol: 100% A as initial condition, 70% A for next 15 min, 65% A for another 30 min, 60% A for 20 min, 50% A for 5 min, 0% A for 15 min, and finally 0% A for 10 min. The injection volume for samples was suitable to be 10 μL. Spectra were recorded at wavelength ranged from 200 to 600 nm.

### *Identification and quantification of polyphenols on HPLC*

First, a HPLC library comprising by retention time and spectra of skeleton structures was constructed with standard chemicals in Table 1. When the food extract was analyzed on the HPLC, polyphenols were compared in retention time with that of standard chemicals in the library and then identified by a spectrum coincident with standard chemicals. Most of flavonoid glycosides disagreed in the retention time with standards though gave the similar spectrum to one of standards, because naturally occurring flavonoids have many varieties in glycoside forms. Then, food samples including these flavonoid glycosides were hydrolyzed as mentioned in next section and analyzed again on HPLC. A variety of flavonoid aglycones was limited and almost covered by the standards in Table 1. Thus, the parent aglycone was identified with retention time and spectrum comparing to those of standard aglycones, and determined in amount with absorbance of the related glycoside as mentioned below. Simple polyphenols have a typical spectrum as enrolled as cinnamic acids or benzoic acids. Hereby, comparison in the spectra between hydrolysis and non-hydrolysis could identify the respective parent structure. The identifications were confirmed further with a HPLC mass spectrometric analysis when required the more structural information.

In determining the amounts, the calibration curves were constructed with the specific wave lengths of standard chemicals: 250 nm for benzoic acids and isoflavones;

280 nm for flavanones, catechins, caffeine, ellagic acid, and internal standard flavone; 320 nm for cinnamic acids, flavones (except for flavone), and chalcones; 370 nm for flavonols; and 510 nm for anthocyans. With the corresponding standard calibration curves, the amounts of food polyphenols were calculated with integrated areas on HPLC. Among the glycosides of flavonoids, cinnamic acids, and benzoic acids, when they did not find in the library, the standard curves that made with glycoside of the same aglycone were used, for instance quercetin-3-*O*-rutinoside (rutin) for other quercetin glycosides and caffeic acid for cinnamic acid glycosides were used.

### *Hydrolysis treatment*

When the aglycone profiles were required on the HPLC, the stored food powders were submitted to hydrolysis treatment modifying the method of Hertog et al. (1992a). Fifty mg of powder were transferred in a test tube sealed with a rubber cap and mixed with 4 mL of 62.5% aqueous methanol containing 0.5 g/L *tert*-butylhydroquinone and 1 mL of 2 N HCl. After pin-holed on the rubber cap, the solution was heated at 90°C for 2 h and then extracted with two volumes of ethyl acetate. The extract was dried under nitrogen gas stream, dissolved in 0.5 mL of DMSO, filtered through a 0.2 μm filter, and analyzed with the HPLC.

# *Results*

## *HPLC library with standard chemicals*

The HPLC library was constructed with 7 benzoic acids, 7 cinnamic acids, 55 flavonoids, 8 catechins, 4 theaflavins, 4 anthocyans, 4 chalcones, and 3 other phytochemicals, and their retention times on the present HPLC conditions and $\lambda_{max}$ obtained from the diode-array detector were summarized in Table 1. For every standard chemical, the calibration curve was made with the indicated wavelength. Since all chemicals constructed almost linear curves through zero point, the slopes of curves and the determination limits (pmol) were listed in Table 1. The most sensitive chemical was theaflavins-3,3'-digallate and was determined up to 10 pmol. The lowest one is (-)-epigallocatechin and the determination limit was 360 pmol.

**Table 1.** Analytical characteristics of the standard phenolic compounds

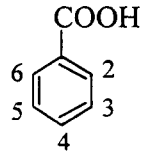
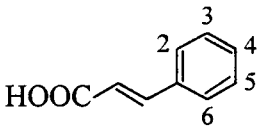
| Compounds | $\lambda_{max}$ (nm) | $t_R$[1] (min) | Calibration | | |
|---|---|---|---|---|---|
| | | | (nm)[2] | Slope[3] ($\times 10^{-4}$) | (pmol)[4] |
| Benzoic acids and Cinnamic acids | | | | | |
|  | |  | | | |
| Benzoic acids | | | | | |
| *o*-hydroxybenzoic acid (2-OH) | 273, 321 | 34.3 | 250 | 59.7 | 190 |
| *m*-hydroxybenzoic acid (3-OH) | 234, 293 | 16.5 | 250 | 19.7 | 103 |
| *p*-hydroxybenzoic acid (4-OH) | 211sh, 253 | 13.8 | 250 | 3.87 | 46 |
| protocatechuic acid (3,4- OH) | 257, 291 | 9.7 | 250 | 4.77 | 56 |
| *β*-resorcylic acid (2,4- OH) | 250, 291 | 10.9 | 250 | 8.48 | 60 |
| vanillic acid (4- OH, 3- $OCH_3$) | 259, 290 | 16.3 | 250 | 5.74 | 50 |
| gallic acid (3,4,5-OH) | 269 | 5.8 | 250 | 17.0 | 74 |
| Cinnamic acids | | | | | |
| *o*-coumaric acid (2-OH) | 213sh, 232sh, 276 | 21.9 | 320 | 4.36 | 38 |
| *m*-coumaric acid (3-OH) | 211sh, 231sh, 275, 320sh | 24.8 | 320 | 9.99 | 54 |

**Table 1.** — continued

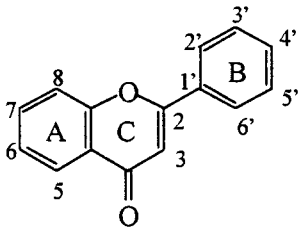
| Compounds | $\lambda_{max}$ (nm) | $t_R$[1] (min) | Calibration | | |
|---|---|---|---|---|---|
| | | | (nm)[2] | Slope[3] ($\times 10^{-4}$) | (pmol)[4] |
| *p*-coumaric acid (4-OH) | 213sh, 226sh, 291sh, 307 | 23.3 | 320 | 2.36 | 22 |
| caffeic acid (3,4-OH) | 215, 234sh, 291sh, 319 | 18.4 | 320 | 2.07 | 38 |
| chlorogenic acid (3,4-OH, glycosylation of the carboxylate group) | 217, 241sh, 294sh, 321 | 14.1 | 320 | 2.02 | 36 |
| ferulic acid (4-OH, 3-$OCH_3$) | 214, 239sh, 291sh, 325 | 25.8 | 320 | 2.09 | 33 |
| isoferulic acid (3-OH, 4-$OCH_3$) | 239sh, 291sh, 325 | 26.3 | 320 | 2.69 | 51 |
| Flavonoids |  | | | | |
| Flavones | | | | | |
| flavone (none) | 247, 295 | 88.8 | 280 | 2.53 | 32 |
| 7,4'-dihydroxyflavone | 230sh, 253sh, 307sh, 329 | 75.7 | 320 | 1.53 | 23 |
| 7,3',4'-trihydroxyflavone | 235sh, 307sh, 339 | 60.5 | 320 | | 41 |
| chrysin (5,7- OH) | 243sh, 267, 311 | 88.8 | 320 | 2.78 | 36 |
| gewkwanin (5,4'-OH, 7-$OCH_3$) | 265, 334 | 90.5 | 320 | 8.40 | 20 |
| baicalein (5,6,7- OH) | 274, 321 | 84.0 | 320 | 2.44 | 42 |
| baicalein-7-*O*-glucoside (baicalin) | 276, 315 | 51.0 | 320 | 1.93 | 64 |
| apigenin (5,7,4'-OH) | 265, 336 | 80.8 | 320 | 1.99 | 15 |
| apigenin-6-*C*-glucoside (isovitexin) | 269, 334 | 38.2 | 320 | 1.74 | 50 |
| apigenin-7-*O*-glucoside (apigetrin) | 265, 336 | 50.7 | 320 | 1.89 | 67 |
| apigenin-8-*C*-glucoside (vitexin) | 267, 334 | 31.7 | 320 | 2.18 | 44 |
| vitexin-2"-*O*-rhamnoside | 267, 336 | 35.2 | 320 | 2.24 | 62 |
| luteolin (5,7,3',4'-OH) | 252, 265sh, 347 | 78.9 | 320 | 3.03 | 26 |
| luteolin-6-*C*-glucoside (homoorientin) | 255sh, 267, 346 | 27.5 | 320 | 2.57 | 35 |
| luteolin-7-*O*-glucoside | 253, 265sh, 346 | 37.1 | 320 | 2.85 | 48 |
| luteolin-8-*C*-glucoside (orientin) | 254, 266sh, 346 | 26.2 | 320 | 2.76 | 42 |
| luteolin-3',7-di-*O*-glucoside | 239, 266, 338 | 31.6 | 320 | 2.63 | 74 |
| luteolin-4'-*O*-glucoside | 246, 265, 336 | 54.9 | 320 | 2.34 | 45 |
| diosmetin (5,7,3'-OH, 4'-$OCH_3$) | 250, 265, 344 | 84.0 | 320 | 2.84 | 32 |
| diosmetin-7-O-rhamnoside (diosmin) | 251, 265, 344 | 61.4 | 320 | 2.40 | 60 |
| chrysoeriol (5,7,4'-OH, 3'-$OCH_3$) | 249, 266, 344 | 83.8 | 320 | 2.51 | 22 |
| 5,7-dihydroxy-3',4',5'-trimethoxyflavone | 269, 329 | 88.3 | 320 | 2.25 | 33 |
| tangeletin (5,6,7,8,4'-$OCH_3$) | 269, 325 | 91.6 | 320 | 1.43 | 14 |
| gardenin A (5,6,7,8,3',4',5'-$OCH_3$) | 247, 295 | 88.6 | 320 | 2.49 | 30 |
| sinensetin (5,6,7,3',4'-$OCH_3$) | 267, 329 | 86.3 | 320 | 1.86 | 39 |
| Flavonols | | | | | |
| flavonol (3-OH) | 236, 305, 341 | 91.5 | 370 | 8.08 | 75 |
| galangin (3,5,7-OH) | 264, 307, 355 | 89.9 | 370 | 3.49 | 46 |
| datiscetin (3,5,7,2'-OH) | 257, 304, 346 | 83.1 | 370 | 6.10 | 112 |
| kaempferol (3,5,7,4'-OH) | 264, 363 | 82.3 | 370 | 1.90 | 20 |
| kaempferol-3-*O*-glucoside (astragalin) | 263, 344 | 55.6 | 370 | 4.42 | 41 |
| kaempferol-3-*O*-rutinoside | 263, 344 | 58.0 | 370 | 5.30 | 96 |
| kaempferol-7-*O*-neohespridoside | 246sh, 263, 318sh, 361 | 53.2 | 370 | 2.18 | 68 |

**Table 1.** — continued

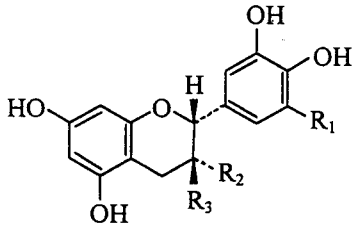
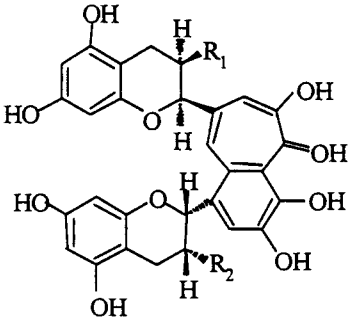
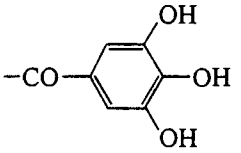
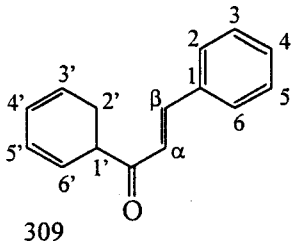
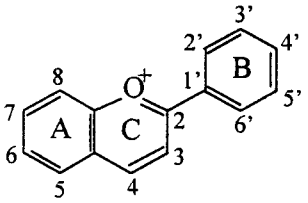
| Compounds | $\lambda_{max}$ (nm) | $t_R$[1] (min) | Calibration | | |
|---|---|---|---|---|---|
| | | | (nm)[2] | Slope[3] ($\times 10^{-4}$) | (pmol)[4] |
| morin (3,5,7,2',4'-OH) | 251, 261sh, 352 | 58.2 | 370 | 3.94 | 120 |
| quercetin (3,5,7,3',4'-OH) | 253, 297sh, 367 | 75.5 | 370 | 1.86 | 30 |
| quercetin-3-*O*-glucoside (isoquercitrin) | 253, 263sh, 294sh, 351 | 41.7 | 370 | 3.00 | 59 |
| quercetin-3-*O*-rutinoside (rutin) | 255, 265sh, 294sh, 352 | 40.6 | 370 | 3.06 | 55 |
| quercetin-3-O-rhamnoside (quercitrin) | 253, 263sh, 344 | 56.9 | 370 | 5.82 | 50 |
| robinetin (3,7,3',4',5'-OH) | 249, 317, 361 | 34.0 | 370 | 1.84 | 87 |
| isorhamnetin (3,5,7,4'-OH, 3'-$OCH_3$) | 253, 367 | 84.2 | 370 | 5.04 | 16 |
| tamarixetin (3,5,7,3'-OH, 4'-$OCH_3$) | 253, 268sh, 364 | 83.8 | 370 | 1.68 | 15 |
| quercetagetin (3,5,6,7,3',4',-OH) | 257, 273sh, 358 | 41.0 | 370 | 2.59 | 87 |
| myricetin (3,5,7,3',4',5'-OH) | 251, 300sh, 370 | 49.3 | 370 | 2.63 | 78 |
| myricetin-3-*O*-rhamnoside (myricitrin) | 256, 298sh, 349 | 36.4 | 370 | 3.77 | 84 |
| Flavanones (2-3 is saturated) | | | | | |
| naringenin (5,7,4'-OH) | 226sh, 288, 331sh | 75.2 | 280 | 2.63 | 38 |
| naringenin-7-*O*-rutinoside (naringin) | 224sh, 281, 332sh | 41.3 | 280 | 2.20 | 42 |
| eriodictyol (5,7,3',4'-OH) | 227sh, 286, 332sh | 54.4 | 280 | 2.10 | 40 |
| hesperetin (5,7,3'-OH, 4'-$OCH_3$) | 229sh, 286, 333sh | 79.4 | 280 | 2.28 | 20 |
| (+)-taxifolin (3,5,7,3',4'-OH) | 229sh, 287, 333sh | 26.7 | 280 | 2.33 | 30 |
| Isoflavones (B-ring binds to 3 position) | | | | | |
| daidzein (7,4'-OH) | 246, 300 | 64.1 | 250 | 1.61 | 52 |
| daidzein-7-*O*-glucoside (daidzin) | 248, 299 | 24.9 | 250 | 1.70 | 45 |
| daidzein-8-*C*-gulucoside (puerarin) | 248, 302 | 20.1 | 250 | 1.41 | 130 |
| genistein (5,7,4'-OH) | 259 | 79.8 | 250 | 1.34 | 15 |
| genistein-7-*O*-glucoside (genistin) | 259 | 31.9 | 250 | 1.32 | 34 |
| biochanin A (5,7-OH, 4'-$OCH_3$) | 259 | 88.8 | 250 | 1.34 | 120 |
| formononetin (7-OH, 4'-$OCH_3$) | 246, 302 | 85.1 | 250 | 1.63 | 21 |
| Catechins and Theaflavins |   G =  | | | | |
| Catechins | | | | | |
| (+)-catechin ($R_1$=H, $R_2$=H, $R_3$=OH) | 230sh, 278 | 13.6 | 280 | 11.3 | 180 |
| (-)-gallocatechin ($R_1$=OH, $R_2$=H, $R_3$=OH) | 268 | 8.1 | 280 | 140 | 290 |
| (-)-catechin gallate ($R_1$=H, $R_2$=H, $R_3$=OG) | 276 | 26.1 | 280 | 2.56 | 42 |
| (-)-gallocatechin gallate ($R_1$=OH, $R_2$=H,$R_3$=OG) | 273 | 19.7 | 280 | 3.62 | 68 |
| (-)-epicatechin ($R_1$=H, $R_2$=OH, $R_3$=H) | 229sh, 277 | 18.4 | 280 | 11.5 | 94 |
| (-)-epigallocatechin ($R_1$=OH, $R_2$=OH, $R_3$=H) | 229sh, 269 | 13.1 | 280 | 65.8 | 360 |
| (-)-epicatechin gallate ($R_1$=H, $R_2$=OG, $R_3$=H) | 276 | 22.9 | 280 | 2.12 | 30 |
| (-)-epigallocatechin gallate ($R_1$=OH, $R_2$=OG,$R_3$=H) | 273 | 17.0 | 280 | 3.24 | 80 |

**Table 1.** — continued

| Compounds | $\lambda_{max}$ (nm) | $t_R$[1] (min) | Calibration | | |
|---|---|---|---|---|---|
| | | | (nm)[2] | Slope[3] ($\times 10^{-4}$) | (pmol)[4] |
| Theaflavins | | | | | |
| theaflavin ($R_1$=OH, $R_2$=OH) | 267, 370, 446 | 80.8 | 280 | 1.92 | 18 |
| theaflavin-3-gallate ($R_1$=OG, $R_2$=OH) | 269, 370, 440 | 80.4 | 280 | 2.26 | 20 |
| theaflavin-3'-gallate ($R_1$=OH, $R_2$=OG) | 273, 370, 440 | 81.2 | 280 | 1.68 | 14 |
| theaflavin-3,3'-digallate ($R_1$=OG, $R_2$=OG) | 273, 370, 446 | 81.3 | 280 | 1.28 | 10 |
| Chalcones |  | | | | |
| chalcone (none) | 309 | 92.1 | 320 | 1.06 | 30 |
| isoliquiritigenin (4,2',4'-OH) | 298sh, 367 | 84.2 | 320 | 2.86 | 39 |
| butein (3,4,2',4'-OH) | 259, 305sh, 378 | 79.2 | 320 | 4.02 | 38 |
| phloretin (α–β bond is saturated, 4,2',4',6'-OH) | 225sh, 285 | 80.3 | 320 | 11.5 | 81 |
| Anthocianins |  | | | | |
| pelargonidin (3,5,7,4'-OH) | 267, 409, 503 | 47.7 | 510 | 9.03 | 30 |
| cyanidin (3,5,7,3',4'-OH) | 276, 503 | 36.3 | 510 | 13.3 | 39 |
| cyanidin-3-O-rutinoside | 278, 512 | 17.4 | 510 | 8.87 | 57 |
| delphinidin (3,5,7,3',4',5'-OH) | 271, 429, 521 | 28.2 | 510 | 9.94 | 27 |
| Others | | | | | |
| caffeine | 271 | 16.7 | 280 | 4.17 | 30 |
| sesamol | 230, 293 | 19.2 | 280 | 1.64 | 24 |
| ellagic acid | 251, 300sh, 358 | 43.1 | 250 | 0.84 | 12 |

[1]Retention times (min)
[2]Detection wavelengths correspond to the absorption maxim of the compounds.
[3]$y = ax$ where a is the slope, x is the peak area and y is the phenolic compound concentration (μM).
[4]Determination limits (pmol/10 μL injection) were calculated as amounts exhibiting ten times area of the largest noise peak

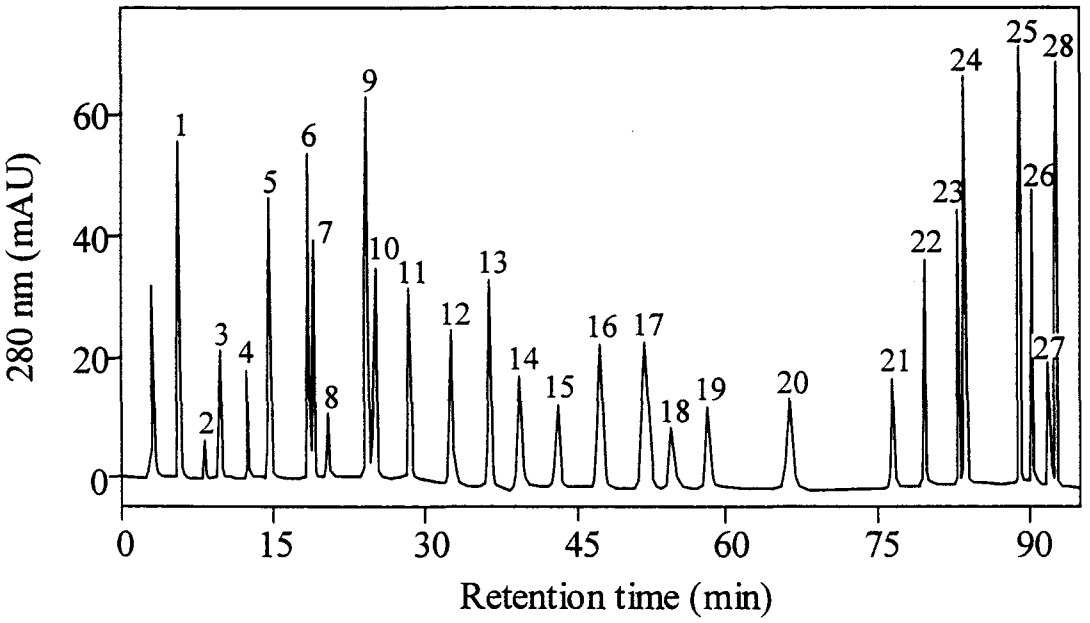



**Fig. 2.** Separation of a standard mixture of polyphenols by HPLC (280 nm).
1=gallic acid, 2=gallocatechin, 3=protocatechuic acid, 4=*β*-resorcylic acid, 5=chlorogenic acid, 6=caffeic acid, 7=epigallocatechin gallate, 8=sesamol, 9=*p*-coumaric acid, 10=Daidzein-7-*O*-glucoside, 11=catechin gallate, 12=luteolin-3',7'-*O*-diglucoside, 13=*o*-coumaric acid, 14=luteolin-7-*C*-glucoside, 15=quercetin-3-*O*-rutinoside, 16=hesperetin-7-*O*-rutinoside, 17=myricetin, 18=apigenin-7-*O*-glucoside, 19=kaempferol-3-*O*-glucoside, 20=daidzein, 21=quercetin, 22=luteolin, 23=kaempferol, 24=apigenin, 25=flavone, 26=galangin, 27=flavonol, 28=chalcone

In the present HPLC system, these chemicals were eluted as the respective single peaks when combined with others. For example, Fig. 2 shows a typical profile with 28 chemicals, which were mixed at the concentration of every 1 nmoles, picking up abundantly occurring ones in daily food (Robards and Antolovich, 1997; Herrmann, 1988). A good resolution was achieved for every chemical in the mixture. Subsequently, in the present HPLC system, simple polyphenols were eluted between 5.8-34.3 min at the retention time, catechins were between 8.1-26.1 min, glycosides of flavonoid were 17.1 and 58.8 min, anthocyans were 17.4-47.7 min, flavonoid aglycones were 34.0-91.5 min, chalcones were 79.2-92.0 min, and theaflavins were 80.4-81.3 min, and all chemicals were eluted in 95 min.

## *Analysis for food polyphenols*

In order to be accurate determination, first, recovery in the extraction process was examined. Radish root was used because it was known to contain little polyphenols (Eloesser and Herrmann, 1975). Fifty nmol each of 23 standard chemicals shown in Table 2 was added to the 50 mg powder of radish root, and then was extracted and analyzed on the HPLC as mentioned under Materials and Methods. Each recovery % of 23 chemicals was summarized in Table 2 when repeated independently three times. The recovery was in the range between 68 and 92%, and the variance was in 1 and 9%. These % of recovery and its variance were considered to be sufficient to determine food polyphenols quantitatively.

**Table 2.** Recoveries of the phenolic standards added to radish (root)*

| Standard added | Recovery (%) |
|---|---|
| Phenolic acids | |
| protocatechuic acid | 75 ± 2 |
| o-hydroxycinnamic acid | 78 ± 6 |
| p-hydroxycinnamic acid | 68 ± 1 |
| gallic acid | 87 ± 3 |
| chlorogenic acid | 70 ± 4 |
| caffeic acid | 77 ± 9 |
| Flavonoids | |
| apigenin | 78 ± 5 |
| apigenin-7-*O*-glucoside | 73 ± 3 |
| luteolin | 78 ± 7 |
| luteolin-7-*O*-ucoside | 80 ± 4 |
| kaempferol | 81 ± 2 |
| kaempfenol-3-*O*-ucoside | 81 ± 7 |
| quercetin | 83 ± 3 |
| quercetin-3-*O*-rutinoside | 77 ± 2 |
| myricetin | 73 ± 2 |
| myricetin-3-*O*-rhamnoside | 92 ± 8 |
| daidzein | 86 ± 3 |
| daidzein-7-*O*-glucoside | 74 ± 4 |
| naringenin | 79 ± 3 |
| naringenin-7-*O*-rutinoside | 77 ± 5 |
| flavone | 81 ± 5 |
| flavonol | 76 ± 3 |
| chalcone | 83 ± 3 |

*The amount of each standard added was 100 μM of final extract solution.

Next, the present method was applied to onion, because onion contains a large amount of quercetin glycosides and has been used as a model analysis sample by several workers (Hertog et al., 1992b; Crozier et al., 1997b; Justesen et al., 1998; Teyssier et al., 2001). Fig. 3 shows the HPLC and spectra of detectable three major

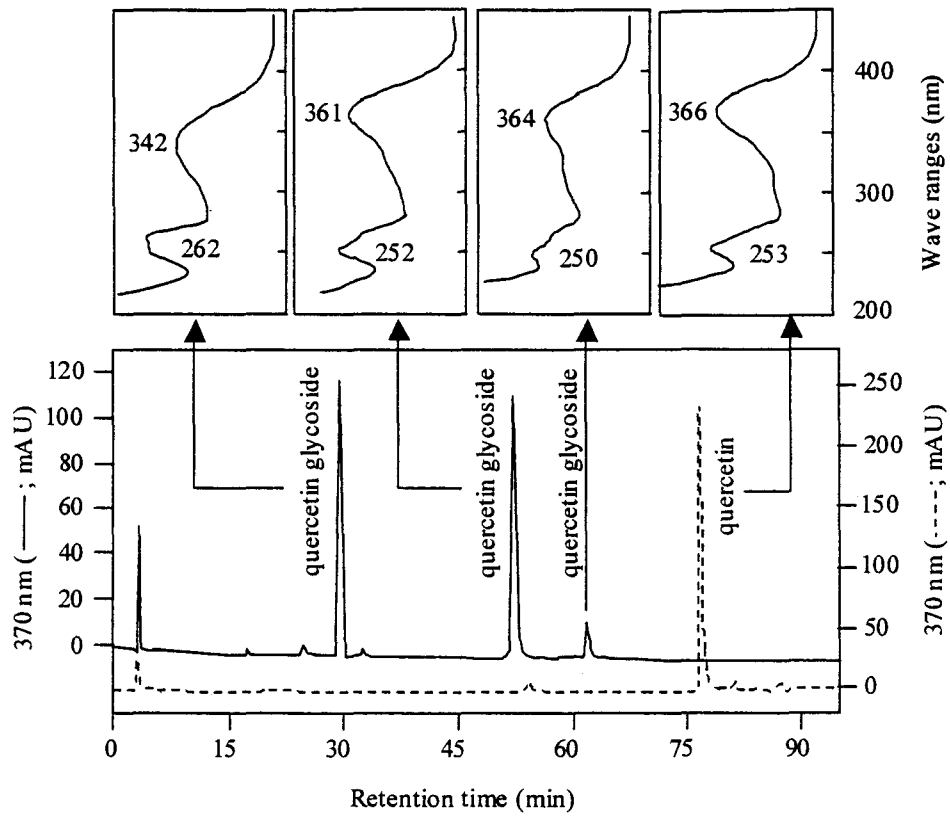



**Fig. 3.** HPLC-SPD analysis of onion extraction.
Chromatograms are shown extract before (line) and after (dotted line) hydrolysis as described in Materials and Methods. Diode-array detection was at 370 nm.

peaks. Every photodiode-array spectrum was very similar to that of quercetin aglycone. However, their retention times disagreed with those of quercetin glycosides in the library. Then, the onion powder was hydrolyzed and analyzed on the HPLC again. As shown with the dotted line in Fig. 3, the hydrolysis onion gave one single peak at 75.5 min of retention time, and this peak was exactly coincided with standard quercetin in the retention time and diode-array spectra. This means that all three products in onion were consisted of quercetin aglycone. As mentioned above, the bioactive moiety is aglycone that exhibits antioxidative potency and modulation of protein functions but is not sugars. Thus, the author considered that the polyphenol contents in food were better to determine by the aglycone amounts such as quercetin

glycosides. In the case of onion, they were determined with the calibration curve of the most abundant quercetin glycoside, rutin. The sum of amounts of three peaks in onion was 92-178 μmol/100 g fresh edible parts as aglycone quercetin (278-538 mg quercetin/kg). On the other hand, other working groups have reported in the amounts to be 284-486 mg/kg (Hertog et al., 1992b) and 185-634 mg/kg (Crozer et al., 1997b). The present result was in the ranges determined by others, though the sample onion was different in harvest seasons and lands from them. Further, the reproducibility in the present method was 3.6% in the variance when the extraction and analysis was repeated 6 times. The present analysis method was considered to be suitable for the determination of food polyphenols. In the following determinations, the hydrolysis was done when was required for the confirmation of aglycone structures.

Then, this method was applied to another sample, burdock. Burdock is consuming well in Japan but the polyphenol contents are little informed. The HPLC and spectra in Fig. 4 indicated that chlorogenic acid and ferulic acid were coincided in the retention times and spectra with the respective standards. All of the other three major peaks showed a spectrum to be typical for cinnamic acids. Thus, burdock contained only a class of simple polyphenols and no flavonoids. Calculating, chlorogenic acid was 4-178 μmol, ferulic acid 7-100, and the sum total of cinnamic acids was 15-532 μmol/100g flesh burdock and the variance of 6 determinations was 6.7%.

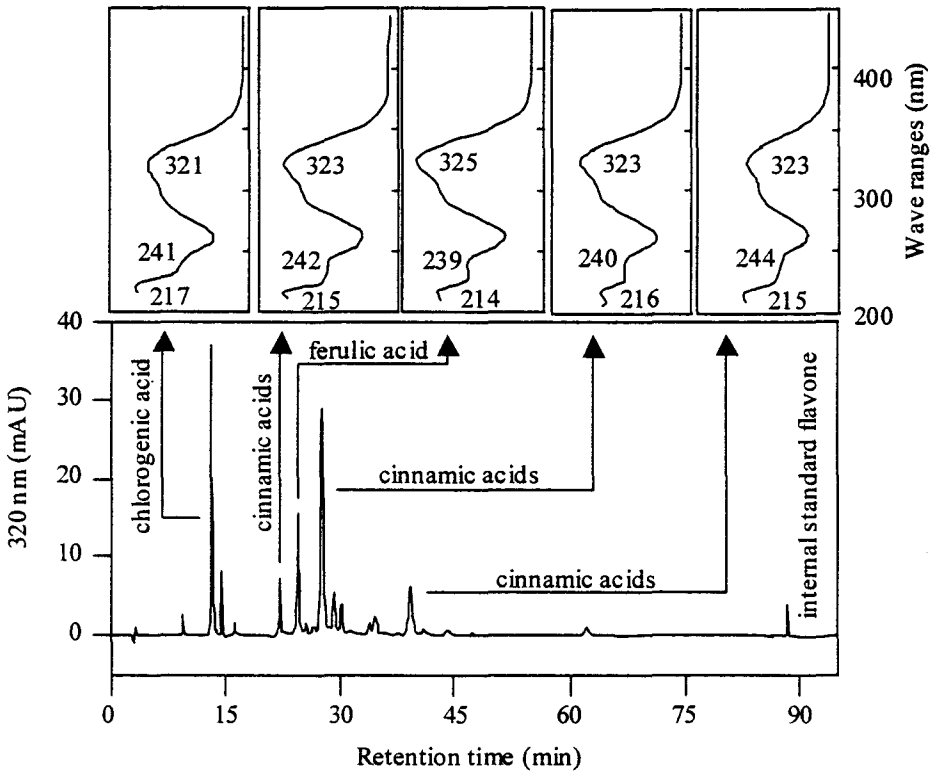



**Fig. 4.** HPLC-SPD analysis of edible burdock extraction.
Chromatogram is shown extract without hydrolysis as described in Materials and Methods. Diode-array detection was at 320 nm.

## *Determination of polyphenols in foods*

With the present analysis method, 35 vegetables, 9 fruits, and 3 teas were determined in the polyphenol contents and classes. Every food was analyzed independently three times with duplicate determination on HPLC and the ranges of variation are shown in the parenthesis (Tables 3-5).

Tables 3 and 4 are for vegetables and fruits, respectively. Flavonoids in them occurred as flavonoid glycosides and the major aglycones are quercetin and kaempferol as well as reported by Hertog et al. (1992b). Apigenin could only be detected in parsley (133 μmol/100 g fresh edible part for aglycone, 588 μmol/100 g for glycosides),

**Table 3.** Polyphenols contents in vegetables

| Product | μmol / 100 g fresh edible part[a] | | | |
|---|---|---|---|---|
| | Flavonoids | | Simple polyphenols and others | |
| Root | | | | |
| carrot *(Daucus carota* L.*)* | | u.d.[b] | cinnamic acids | 0.54 (0.5-0.6) |
| burdock *(Arctium lappa* L.*)* | | u.d.[b] | chlorogenic acid<br>ferulic acid<br>cinnamic acids | 63 (4-178)<br>42 (7-100)<br>197 (15-532) |
| radish *(Raphanus sativus* L.*)* | | u.d.[b] | cinnamic acids | 11 (5.8-13) |
| turnip *(Brassica campestris* L. var. *glabra Kitam.)* | | u.d.[b] | cinnamic acids | 8 |
| Tuber | | | | |
| potato *(Solanum tuberosum* L.*)* | | u.d.[b] | chlorogenic acid<br>cinnamic acids | 1.9<br>18 |
| sweet potato *(Ipomoea batatas* L.*)* | | u.d.[b] | cinnamic acids | 54 |
| chinese yam *(Dioscorea opposita* Thunb.*)* | | u.d.[b] | | u.d.[b] |
| Bulb | | | | |
| onion *(Allium cepa* L.*)* | quercetin glycoside<br>quercetin | 129 (92-178)<br>1.2 (u.d.-1.9) | | u.d.[b] |
| Leaf | | | | |
| celery *(Apium graveolens* L.*)* | apigenin<br>apigenin glycoside<br>luteolin glycoside<br>chrysoeriol glycoside | 5.3 (u.d.-16)<br>18 (u.d.-51)<br>7.1 (u.d.-20)<br>13 (u.d.-38) | chlorogenic acid<br>cinnamic acids | 17 (0.5-50)<br>1.6 (0.9-2.47) |
| chinese cabbage *(Brassica campestris* var. *perviridis* L.*)* | kaempferol glycoside | 91 (54-115) | caffeic acid<br>chlorogenic acid<br>cinnamic acids | 15 (8.6-22)<br>21 (13-28)<br>14 (1.9-26) |
| chinese chive *(Allium tuberosum* Rottle ex Spreng.*)* | kaempferol glycoside | 26 (16.-41) | ferulic acid<br>cinnamic acids | 4.3 (1.5-7.2)<br>19 (13-22) |
| garland chrysanthemum *(Chrysanthemum coronarium* L.*)* | | u.d.[b] | chlorogenic acid<br>cinnamic acids | 2.3 (1.7-3.5)<br>39 (28-59) |
| indian spinach *(Basella rubra* L.*)* | apigenin-6-*C*-glucoside<br>apigenin-8-*C*-glucoside | 13 (12-15)<br>267 (216-336) | caffeic acid | 4.2 (3.6-5.1) |
| lettuce *(Lactuca sativa* L.*)* | quercetin glycoside | 1.6 (0.3-4.8) | caffeic acid<br>chlorogenic acid | 25 (4.6-86)<br>3.2 (0.6-8.3) |
| nalta jute *(Corchorus olitorius* L.*)* | kaempferol glycoside<br>quercetin glycoside | 26<br>134 | cinnamic acids | 729 |
| pak choi *(Brassica campestris* var. *chinensis* L.*)* | kaempferol glycoside | 81 (53-133) | caffeic acid<br>chlorogenic acid<br>cinnamic acids | 8.6 (4.0-13)<br>21 (8.9-32)<br>23 (11-44) |

**Table 3.** — continued

| Product | µmol / 100 g fresh edible part[a] | | | |
|---|---|---|---|---|
| | Flavonoids | | Simple polyphenols and others | |
| parsley *(Petroselinum crispum Nym. ex A.W. Hill.)* | apigenin | 133 (8.6-331) | | u.d.[b] |
| | apigenin glycoside | 588 (275-873) | | |
| | kaempferol glycoside | 11 (7.8-17) | | |
| qing gin cai *(Brassica campestris* Var. *chinensis* L.*)* | kaempferol glycoside | 57 (24-101) | chlorogenic acid | 10 (5.4-19) |
| | | | cinnamic acids | 25.9 (15-36) |
| radish *(Raphanus sativus* L.*)* | quercetin glycoside | 233 | | u.d.[b] |
| | kaempferol glycoside | 27 | | |
| radish (maturity) *(Raphanus sativus* L.*)* | kaempferol glycoside | 76 (48-123) | cinnamic acids | 195 (168-229) |
| turnip *(Brassica campestris* L. var. *glavra.)* | kaempferol glycoside | 58 | cinnamic acids | 34 |
| water dropwort *(Oenanthe javanica* DC.*)* | quercetin glycoside | 16 | caffeic acid | 2.2 |
| | isorhamnetin glycoside | 65 | chlorogenic acid | 31 |
| | | | ferulic acid | 37 |
| | | | cinnamic acids | 3.7 |
| Leaf and spear | | | | |
| asparagus *(Asparagus officinalis* L.*)* | quercetin glycoside | 38 (7.8-95) | caffeic acid | 3.8 (1.3-5.7) |
| | | | chlorogenic acid | 18.9 (9.7-24) |
| | | | cinnamic acids | 8.9 (1.7-16) |
| Fruit | | | | |
| bell pepper (green) *(Capsicum grossum* L.*)* | quercetin glycoside | 25 (13-36) | | u.d.[b] |
| | luteolin glycoside | 20 (17-26) | | |
| bell pepper (maturity) *(Capsicum grossum* L.*)* | luteolin glycoside | 7.1 (6.3-14) | | u.d.[b] |
| bell pepper (small) *(Capsicum grossum* L.*)* | luteolin glycoside | 20 (15-27) | chlorogenic acid | 65 (34-106) |
| | | | cinnamic acids | 212 (93-280) |
| cacao *(Theobroma cacao* L.*)* | (+)-catechin | 305 | caffein | 1084 |
| | (-)-gallocatechin | 27000 | | |
| | (-)-epicatechin | 342 | | |
| | (-)-epigallocatechin | 512 | | |
| corn *(Zae mays* L.*)* | | u.d.[b] | benzoic acid | 72 (40-114) |
| | | | cinnamic acid | 11 (2-27) |
| | | | ferulic acid | 7 (3-12) |
| eggplant *(Solanum melongena* L.*)* | anthocyanins | 62 | chlorogenic acid | 229 |
| | | | cinnamic acids | 22 |
| okura *(Abelmoschus esculentus* L.*)* | quercetin glycoside | 82 (65-114) | | u.d.[b] |
| Bean | | | | |
| coffee bean *(Coffea* L.*)* | | u.d.[b] | caffeic acid | 166 |
| | | | chlorogenic acid | 698 |
| | | | cinnamic acids | 1350 |
| | | | caffein | 4032 |

**Table 3.** — continued

| Product | μmol / 100 g fresh edible part[a] | | | |
|---|---|---|---|---|
| | Flavonoids | | Simple polyphenols and others | |
| common bean *(Phaseolus vulgaris* L.*)* | kaempferol glycoside | 1.3 | | u.d.[b] |
| soybean *(Glicine max* L.*)* | genistein | 50 | | u.d.[b] |
| | daizein glycoside | 478 | | |
| | genistein glycoside | 346 | | |
| black soybean *(Glycine max* L.*)* | genistein | 70 | | u.d.[b] |
| | daizein glycoside | 263 | | |
| | genistein glycoside | 290 | | |
| | (-)-epicatechin | 129 | | |
| Bean peel | | | | |
| black soybean | (-)-epicatechin | 360 | protocatechuic acid | 30 |
| *(Glycine max* L.*)* | anthocyanins | 280 | | |
| Pea | | | | |
| garden pea *(Pisum sativum L.)* | quercetin glycoside | 63 | | u.d.[b] |

[a]mean (minimum – maximum)
[b]under detection limts

celery (5.3 for aglycone, 18 μmol/100 g for glycosides), indian spinach (280 μmol/100 g for glycosides), and lemon (8.5 μmol/100 g for glycosides); luteolin in only celery (7.1 μmol/100 g for glycosides) and three kinds of bell peppers (green, 20; mature, 7.1; and small, 20 μmol/100 g for glycosides); flavanones naringenin and hesperetin in only citrus fruits grapefruit (12 for naringenin and 258 μmol/100 g for naringenin glycosides), orange (167 for naringenin glycosides and 148 μmol/100 g hesperidin), and lemon (226 μmol/100 g for hesperetin glycosides); and anthocyanins in only eggplant (62 μmol/100 g), blueberry (324 μmol/100 g), and black soybean peel (280 μmol/100 g). Interestingly, the author detected *C*-glycoside flavonoid at a large amount in indian spinati. They are apigenin-6-*C*-glucoside (13 μmol/100 g) and apigenin-8-*C*-glucoside (267 μmol/100 g). Generally, flavonoids have been recognized to occur as

*O*-glycosides (Markham, 1982a). Further, the author found these *C*-glycosides were remained unchanged after the hydrolysis.

Table 5 is for teas, and the major polyphenols were catechins in green, oolong, and black teas. The contents of epigallocatechin (EGC) and epigallocatechin gallate

**Table 4.** Polyphenol contents in fruits

| Product | μmol / 100 g fresh edible part[a] | | | |
|---|---|---|---|---|
| | Flavonoids | | Simple polyphenols and others | |
| Citrus | | | | |
| grapefruit | naringenin | 12 (4.6-27) | caffeic acid | 3.6 (2.1-5.8) |
| *(Citrus paradisi* Macf.*)* | naringenin glycoside | 258 (152-438) | cinnamic acids | 23.0 (15.6-27.0) |
| orange | hesperidin | 148 | | u.d.[b] |
| *(Citlus sinensis* L.*)* | naringenin glycoside | 167 | | |
| lemon | hesperetin glycoside | 226 (135-318) | caffeic acid | 1.8 (1.2-2.5) |
| *(Citrus limon* L.*)* | apigenin glycoside | 8.5 (5.0-12) | | |
| | quercetin glycoside | 3.8 (2.8-4.8) | | |
| | diosmetin glycoside | 48 (40-56) | | |
| Others | | | | |
| apple | quercetin glycoside | 8.0 | chlorogenic acid | 35 |
| *(Mauls pumila* Mill.*)* | | | cinnamic acids | 0.8 |
| blueberry | anthocyanins | 324 (168-471) | chlorogenic acid | 287 (273-325) |
| *(Vaccinium* L.*)* | quercetin glycoside | 7.7 (u.d.-21) | cinnamic acids | 13 (u.d.-21) |
| kiwi fruit | u.d.[b] | | cinnamic acids | 4.9 (4.6-5.3) |
| *(Actinidia chinensis* Planch.*)* | | | | |
| loquat | u.d.[b] | | caffeic acid | 16 |
| *(Eriobotrya japonica* Lindl.*)* | | | chlorogenic acid | 250 |
| | | | cinnamic acids | 33 |
| peach | quercetin glycoside | 3.4 (2.8-4.3) | chlorogenic acid | 14 (12-15) |
| *(prunus persica* L.*)* | catechins | 67 (29-93) | cinnamic acids | 16 (13-18) |
| strawberry | quercetin glycoside | 1.2 (1.0-1.6) | chlorogenic acid | 4.6 (3.7-5.8) |
| *(Fragaria × ananassa* Duch.*)* | | | | |

[a]mean (minimum – maximum)
[b]under detection limits

(EGCG) were the highest but their amounts differed markedly among three teas. The content of EGC of green tea, 17900 μmol/100 g fresh leaf, was 3.5 times greater than that of oolong tea and 18 times than black tea. In case of EGCG, the author found almost the same results. On the contrary, high amounts of theaflavins, which are reactive products between one molecule of a simple catechin and a gallocatechin during black tea fermentation (Owuor and Obanda, 1997), were contained in black tea but trace

**Table 5.** Concentrations of polyphenols in teas *(Camellia sinensis* L.*)*

| | μmol / 100 g fresh leaf | | |
|---|---|---|---|
| | green tea | oolong tea | black tea |
| Catechins | | | |
| . (+)-catechin | 280 | 210 | 160 |
| (-)-gallocatechin | 1460 | 1000 | u.d. |
| (-)-catechin gallate | u.d. | 50 | 120 |
| (-)-gallocatechin gallate | 380 | 300 | 270 |
| (-)-epicatechin | 5800 | 670 | 200 |
| (-)-epigallocatechin | 17900 | 4900 | 920 |
| (-)-epicatechin gallate | 2350 | 890 | 820 |
| (-)-epigallocatechin gallate | 14900 | 5400 | 1020 |
| Theaflavins | | | |
| theaflavin | u.d. | 27 | 310 |
| theaflavin-3-gallate | u.d. | 26 | 430 |
| theaflavin-3'-gallate or/and theaflavin-3,3'-digallate* | u.d. | 70 | 960 |
| Flavonols | | | |
| kaempferol-3-*O*-glucoside | 180 | 80 | 310 |
| kaempferol-3-*O*-rutinoside | u.d. | 60 | 250 |
| kaempferol glycoside | 260 | 40 | 120 |
| quercetin-3-*O*-rhamnoside | 120 | 140 | 630 |
| quercetin glycoside | 770 | 200 | u.d. |
| myricetin-3-*O*-rutinoside | 520 | 210 | 210 |
| isovitexin | 90 | u.d. | u.d. |
| Phenolic acid | | | |
| gallic acid | 250 | 1340 | 1800 |
| Others | | | |
| caffeine | 13500 | 11300 | 13900 |

*These theaflavins have an almost same spectrum and retention time in this method

for oolong tea and under detection limits for green tea. Three kinds of flavonol glycoside, kaempferol, quercetin, and myricetin, were also detected in the teas but were minor in the contents.

## *Discussion*

A quantitative determination in individual classes of polyphenols such as flavones, flavonols, catechins, and so on are strongly requested in vegetables, fruits, and teas been consumed commonly in Japan, whereas a large number of polyphenols are present in human diets. Various analytical methods published have determined the flavonoids as aglycone forms after hydrolyzing original glycosides to aglycones (Hertog et al. 1992a, Crozier et al, 1997a, Häkkinen et al, 1998, Justesen et al. 1998; Merken and Beecher, 2000). The hydrolysis under optimal reaction period and acid concentration for individual flavonoid glycosides are, however, difficult. Because time and acid concentration required for complete hydrolysis are dependent on the binding site of the sugar on the flavonoid nucleus (e.g. 7-*O*-glycosides > 4'-*O*-glycosides > 3-*O*-glycosides) and flavonoid *C*-glycoside were failing to produce an aglycone (Markham, 1982b). Furthermore, a simultaneous determination without hydrolysis of flavonoids and phenolic acids, e.g. chlorogenic acid and ferulic acid that has been reported as anticarcinogenic components on *in vivo* studies (Tanaka et al., 1993, Han et al., 2001), has been little published.

In this Chapter, a HPLC method based on diode-array detection is presented for the simultaneous identification and quantification of flavonoid aglycones and its

glycosides, viz. flavones, flavonols, flavanones, isoflavones, catechins, theaflavins, anthocyans, and chalcones and simple polyphenols, viz. benzoic acids and cinnamic acids in foods. In this elution system, 92 kinds of phenolic compounds were eluted within 95 min and displayed excellent resolution, and the present elution system allows the separation of 21 flavonoid glycosides used between retention times 17.1 for daizein-8-*O*-glucoside and 58.8 min for kaempferol-7-*O*-neohespridoside.

Using this extraction method, the recoveries of 23 kinds of additional phenolic compounds to the radish were good or reasonable (68-92%). The repeatability of this extraction method was good with coefficients of variation ranged from 3.6% for flavonoids (quercetin glycosides) and 6.7% for phenolic acids (cinnamic acids). As compared to values reported by Hortog et al. (1992a) and Häkkinen et al. (1998), the recoveries and repeatability in these results were almost same or higher.

An analysis method developed in the present study provides the precise amounts of flavonoids and phenolic acids within a single analysis. Furthermore, this system has been used for determination of phenolic compounds in 35 vegetables, 9 fruits, and 3 teas. Almost all flavonoids were existed as glycosides in foods analyzed and found in most foods, except for root and tuber vegetables, garland chrysanthemum, corn, coffee bean, kiwi fruit, and loquat. Flavonoid levels were highest in the leaves of most foods and lowest or under detection limits in foods that grown below the soil surface, with the exception of onion in which high levels of flavonoid quercetin glycosides were existed. These differences may be due to the differences of insolation because UV-B irradiation is known to induce the accumulation of flavonoids (Li et al., 1993; Lois, 1994).

Cinnamic and benzoic acids in simple polyphenol family widespread in plant foods, vegetables, fruits, and teas. High amounts of cinnamic acids were found in eggplant (229 μmol/100 g for chlorogenic acid), nalta jute (729 μmol/100 g for cinnamic acids), blueberry (287 μmol/100 g for chlorogenic acid and 13 μmol/100 g for cinnamic acids), loquat (16 for caffeic acid and 250 μmol/100 g for chlorogenic acid), and so on. On the other hand, benzoic acids were presented in corn (72 μmol/100 g for benzoic acids), black soybean peel (30 μmol/100 g for protocatechuic acid), and three kinds of teas as gallic acid between 250 for green tea and 1800 μmol/100 g for black tea. Interestingly, the major phenolic compounds existed in Sunflower Family, edible burdock, garland chrysanthemum, and lettuce, were cinnamic acids. These indicate that phenolic acids are major polyphenols in plant foods as well as flavonoid glycosides. Then, the author represented the typical polyphenols in daily plant foods in Table 6.

**Table 6.** Food sources of polyphenols

| Class of polyphenols | Specific example | Food source |
|---|---|---|
| Simple polyphenols | Chlorogenic, caffeic, ferulic and gallic acid | Widely distributed in food, especially root vegetables and teas |
| Flavones | Glycosides of apigenin and luteolin | Found mainly in leaf vegetables and citrus fruits, parsley, celery and lemon |
| Flavonols | Glycosides of quercetin, kaempferol and myricetin | Found in many leaf vegetables, fruits and teas |
| Isoflavones | | Soy beans |
| Flavanones | Glycosides of naringenin and hesperetin | Citrus fruits |
| Catechins | Epigallocatechin, epigallocatechin gallate and gallocatechin | Teas and cacao bean |
| Anthocyanins | Glycosides of anthocyanidin | Magenta colored vegetables and fruits such as eggplant and blueberry |
| Anthraquinones* | Emodin, chrysophanol and rhein | Medicinal plants, e.g. rhubarb |

*Sun et al. (2000)

Hollman and Katan (1999) reported that dietary flavonoids probably do not explain the cancer-protective effect of vegetables and fruits from their epidemiological study. However, phenolic acids were not taken account of a proportion of total dietary phenolic compounds intakes. These collective data presented in this study provide a more base for an epidemiological evaluation of possible anticarcinogenic effects of our daily diets, vegetables, fruits, and teas.

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# Chapter 5

# Bioavailability of Dietary Polyphenols to Prevent Oxidative Genetic Damage in a Cell

This chapter is based mainly on:

Bioavailability of dietary polyphenols to prevent oxidative genetic damage in a cell

*Free Radical Biology & Medicine, in preparation.*

# *Introduction*

Polyphenols ubiquitously widespread in plant foods, vegetables, fruits, and teas and their intakes closely correlate with incidences of degenerative diseases caused by genetic damages. Many epidemiological studies have been reported. In the Zutpen study, flavonoid intake was inversely associated with the incidences of coronary hear disease (Hertog et al., 1993; Hertog et al., 1995) and of stroke (Keli et al., 1996). In Finnish, the dietary flavonoids are closely inversed to the risk for lung cancer (Knekt et al., 1997). In an intervention study, the dietary antioxidants could suppress prostate, colon, spleen, and breast cancers by more than 50% (DeMarini, 1998). In Fukuoka, Japan, drinking of green tea was inversely associated with atherosclerosis (Sasazuki et al., 2000). On Japanese women, the daily intakes of flavonols and isoflavones were 16.7 and 47.2 mg, respectively, and were inversely correlated with serum and LDL cholesterol levels (Arai et al., 2000). In the Mediterranean bladder cancer, vegetables and fruits reduced an amount of DNA adducts in bladder leukocytes (Peluso et al., 2000). However, several reports described that the antioxidant intake did not correlate with the risk of diseases. In the same Zutphen study above, the dietary flavonoids did not reduce the risk of cancers (Hertog et al., 1994). High intake of flavonoids did not reduce the risk of bladder cancer (Garcia et al., 1999). In male smokers, intake of flavonoids was not associated with the risk of stroke (Hirvonen et al., 2000). In healthy subjects, the dietary quercetin from onion and black tea did not reduce the oxidative DNA damage in leukocytes (Beatty et al., 2000). With the supplementation study, rutin did not reduce 8-OHdG levels in urine (Boyle et al., 2000). In smoking healthy individuals, the consumption of black and green teas had no effect on

inflammation, haemostasis and endothelial markers (de Maat et al., 2000).

The discrepancy in epidemiological studies is probably to be due to lack in biochemical data. The polyphenols may have a specificity to absorb into body and further into the cells, depending on the chemical structures. Thus, the bioavailability is still unclear. In this chapter, the author investigated physiological levels of dietary antioxidants with rats, the incorporations of them into cells with HepG2 and then examined whether the antioxidants can exhibit the potency for the cellular DNA damage in order to understand the bioavailability for a goal of the cancer prevention.

# *Materials and Methods*

## *Chemicals*

Ascorbic acid, 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH), 2'-deoxyguanosine (2'-dG), and 8-hydroxy-2'-dG (8-OHdG) were purchased from Wako Pure Chemical Ind., Ltd. (Tokyo, Japan). Nuclease P1, alkaline phosphatase (from *Escherichia coli*), ribonuclease A, proteinase K, and β-glucuronidase/sulfatase (crude solution from *Helix pomatia*) were obtained from SIGMA-ALDRICH (Irvine, UK). Quercetin, rutin, kaempferol, and luteolin were from Extrasynthèse (Genay, France) and 8 kinds of catechins were from Kurita Kogyo (Tokyo, Japan). Commercial beverage, Green tea, was purchased from the local market. Water was distilled twice and all other reagents used were of the highest grade available.

### *Animals and diets*

Male Wistar rats (3 weeks old, Wistar/ST SPF, Shizuoka Lab. Anim. Agri., Shizuoka, Japan) were housed in temperature-controlled room (25 ± 1°C) and humidity (45-50%) with a 12 h light-dark cycle. The animals had free access to a commercial diet obtained from Japan SLC, Inc. (Shizuoka, Japan) and drinking green tea. The daily food intake (16.0 g/day) and fluid consumption (22.2 mL/day) were unaffected by the green tea supplementation. The rats were maintained in accordance with the Guidelines for Animal Experimentation of Kobe University.

### *Collection of plasma samples*

After 4 weeks of green tea supplementation, the blood were withdrew from rats by heart puncture using heparinized needles and syringes under anesthesia with pentobarbital. The plasma was immediately obtained from the collected blood by centrifugation at 800 *g* for 10 min at room temperature and stored at –80°C until analysis.

### *Determination of catechins in plasma*

Two aliquots of plasma (800 μL each) were acidified to pH 5.0 with 18.6 μL of 1.0% acetic acid and stirred. One of the aliquots was treated with 8 μL of $1 \times 10^5$ U/L β-glucuronidase/$7.5 \times 10^2$ U/L sulfatase for cleavage of all ester-bonds of glucuronides and incubated at 37°C for 30 min. Both aliquots of plasma sample were extracted with 2 volumes of ethyl acetate, evaporated to dryness under nitrogen gas, and dissolved in 200 μL of dimethylsulfoxide (DMSO) in an ultrasonic bath for 1 min. For HPLC analysis, the author injected 20 μL of these sample after filtration with a 0.2 μm

membrane filter Millex-LG (Millipore Co., Bedford, USA) into an Capcell pak C18 UG120 (250×4.6 mm I.D., S-5, 5 μm, Shiseido Co., LTD., Tokyo, Japan) protected by an guard column (10×4.0 mm I.D.) using acetritrile/100 mM sodium phosphate buffer (pH 2.5) containing 0.1 mM ethylenediaminetetraacetate disodium salt (EDTA), 14:86 (v/v) as mobile phase, at a flow rate of 1.0 mL/min. Both the main and guard columns were thermostatically maintained at 35°C. Catechins were detected by a electrochemical detector (ECD) (IRICA Σ 875, Kyoto, Japan) at +700 mV.

### *Determination of synergism of antioxidants*

Dietary antioxidants Ascorbic acid, quercetin and 8 kinds of catechins in DMSO were added to the mixture of 0.5 mM 2'-dG and 25 mM AAPH. The final concentrations of these antioxidants in reaction mixtures were 50, 5.0, and 1.5 μM, respectively. After incubation at 37°C for indicated time, the amount of 8-OOHdG formed was determined with 10 μL of the mixture using the analytical HPLC system at UV 254 nm. The detail conditions were described in Chapter 3. Simultaneously, the consumption of antioxidants was evaluated by HPLC. The mobile phases were a 100 mM sodium phosphate buffer (pH 2.5) containing 0.1 mM EDTA and 5 mM tetra-*normal*-butylammonium phosphate (flow rate, 0.7 ml/min) for ascorbic acid and 55% methanol in 50 mM sodium phosphate buffer (pH 3.3) containing 0.1 mM EDTA (flow rate, 1.0 ml/min) for quercetin. These were detected with an ECD at +600 mV. In case of catechins, the method mentioned above was used.

### *Cell culture*

Human hepatocellular carcinoma HepG2 cells was kindly supplied by Dr. Koji Ikura (Department of Applied biology, Faculty of Textile Science, Kyoto Institute of Technology, Kyoto, Japan) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (SIGMA-ALDRICH, Irvine, UK), 4 mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin. The cells were incubated at 37°C in a humidified atmosphere containing 5% $CO_2$.

### *Microculture tetrazolium (MTT) assay*

The non-toxic dosage level of AAPH was first established using MTT assay with some modifications (Alley et al., 1988). MTT assay results showed that AAPH at concentration by 25 mM exhibited little toxicity on the cells. Therefore, AAPH level of 25 mM was used as oxygen radical initiator in the following experiments.

### *Cell treatment*

When HepG2 cells in 100 mm dishes were about 90% confluent (3-4 days after seeding), they were pre-treated with 15 mL of DMEM containing 10 μM antioxidant in DMSO (0.1% final volume) or the same volume of DMSO. After 15 min incubation at 37°C, the medium was removed and the cell monolayer rapidly washed 3 times with DMEM. For intracellular evaluation of activity of the antioxidant added, the cells were incubated at 37°C for 9 h with 15 mL of Hanks' balanced salt solution buffered to pH 7.4 with 25 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphoneic acid) containing 25 mM AAPH. After incubation, the cells were rinsed 3 times with

ice-cold same buffer, collected in an Eppendorf tube, and centrifuged at 1000 *g*. The cell precipitates were kept at –80°C until further analysis.

### *DNA Isolation and 8-OHdG Analysis*

For 8-OHdG analysis, DNA isolation was carried out as previously described by Shiotani et al. (2001) with some modifications. Briefly, $2 \times 10^7$ cells were homogenized in TE buffer (10 mM Tris-HCl, pH 7.4, and 10 mM EDTA) containing 0.5% SDS. The homogenates were incubated with 500 μg/ml ribonuclease A at 50°C for 30 min and then with 500 μg/ml proteinase K at 50°C for 30 min. After addition of 0.5 M NaCl (at final concentration), DNA was precipitated in 50% ice-cold isopropanol. DNA precipitate was obtained by centrifugation at 10,000 *g* for 15 min and washed with 70% ethanol. The DNA samples were dissolved in 200 μl of 1mM EDTA and 15 μl of 0.5 M sodium acetate. Then, they were digested with nuclease $P_1$ (10 U, 30 min, 37°C) and were further incubated with alkaline phosphatase (3 U, 1h, 37°C) after addition of 80 μl of 0.4 M Tris-HCl (pH 7.4). After centrifugation (17,000 *g*, 10 min, 4°C), the reaction mixture was filtered through a 0.2 μm membrane filter before the HPLC analysis, and an aliquot (10 μL) was injected into the reverse-phased column Capcell pak C18 UG120 (5 μm mesh and 4.6 × 250 mm I.D.) (Shiseido Co., Ltd., Tokyo, Japan) connected to a Hitachi L-7100 pump (Tokyo, Japan) coupled to an UV detector (HITACHI L-7420) and an ECD (IRICA Σ 875; Kyoto, Japan). The solvent system used was a mixture of 6.5% methanol and 93.5% 20 mM potassium phosphate buffer (pH 4.5) containing 0.1 mM EDTA. The flow rate was 1.0 mL/min. The amounts of 8-OHdG and 2'-dG were calculated from peak height based on their corresponding standards, and the results expressed as the number of 8-OHdG/$10^5$ 2'-dG.

# Results

## *Catechins and its conjugates in plasma*

The amount of catechins in commercial green tea was determined (Table 1). The major catechin found in green tea is epigallocatechin (EGC, 64 μmol /100 mL) followed by gallocatechin gallate (GCG, 17 μmol/100 mL) and epigallocatechin gallate (EGCG, 15 μmol/100 mL). The total catechins content was 124 ± 11 μmol/100 mL. Rats used in this study ingested these green tea catechins 27.5 μmol/rat/day for 4 weeks. Catechin aglycone could only be detected as EGCG (34 nM) in rat plasma drank green tea (Table 2). C, EC, EGC, and EGCG were existed in plasma as conjugated forms 83, 93, 26, and 5 nM, respectively, and other catechins were under detection limits. The total catechins level in plasma was less than 1 μM.

**Table 1.** Catechins in commercial green tea

| | Catechins (μmol/100 mL)* |
|---|---|
| catechin (C ) | 3.8 ± 1.1 |
| catechin gallate (CG) | 3.5 ± 0.1 |
| gallocatechin (GC) | 11 ± 1.0 |
| gallocatechin gallate (GCG) | 17 ± 1.8 |
| epicatechin (EC) | 8.0 ± 2.8 |
| epicatechin gallate (ECG) | 2.1 ± 0.5 |
| epigallocatechin (EGC) | 64 ± 15 |
| epigallocatechin gallate (EGCG) | 15 ± 2.4 |
| Total catechins | 124 ± 11 |

* Data are expressed as means ± SD (n=3)

**Table 2.** Cumulative absorption of catechins in plasma with green tea drinking rats

| | Catechins (nM)* | |
|---|---|---|
| | Aglycone | Conjugate |
| C | u.d.** | 83 ± 24 |
| EC | u.d.** | 93 ± 29 |
| ECG | u.d.** | 26 ± 2.1 |
| EGCG | 34 ± 22 | 5 ± 5 |

Other catechins were under detection limits.
*Data are expressed as means ± SD (n=5)
**under detection limits

## *Determination of synergism of antioxidants based on the formation of 8-OOHdG*

Dietary antioxidants ascorbic acid, quercetin, and catechins are major dietary antioxidants from vegetables, fruits, and teas. Then, they were tested for synergism using concentrations of 50, 1.5, and 5.0 μM, respectively, of these antioxidants (Fig. 1), which are close to their physiological levels (Levine et al., 1996; Mecocci et al., 2000; Conquer et al., 1998; de Vries et al., 1998; Nakagawa et al., 1997). While the formation of 8-hydroperoxy-2'-dG (8-OOHdG) that is oxidized product of 2'-dG as described in Chapter 3 immediately started on incubation with 2'-dG alone (0 min) (Fig. 1A), the addition of antioxidants obviously delayed the formation. The formation started at 60, 7.5, and 22.5 min into the incubation after the respective antioxidants ascorbic acid, quercetin, and EGCG were consumed completely (Fig. 1B-D). The presence of two antioxidants delayed the formation by the sum total of the delay caused

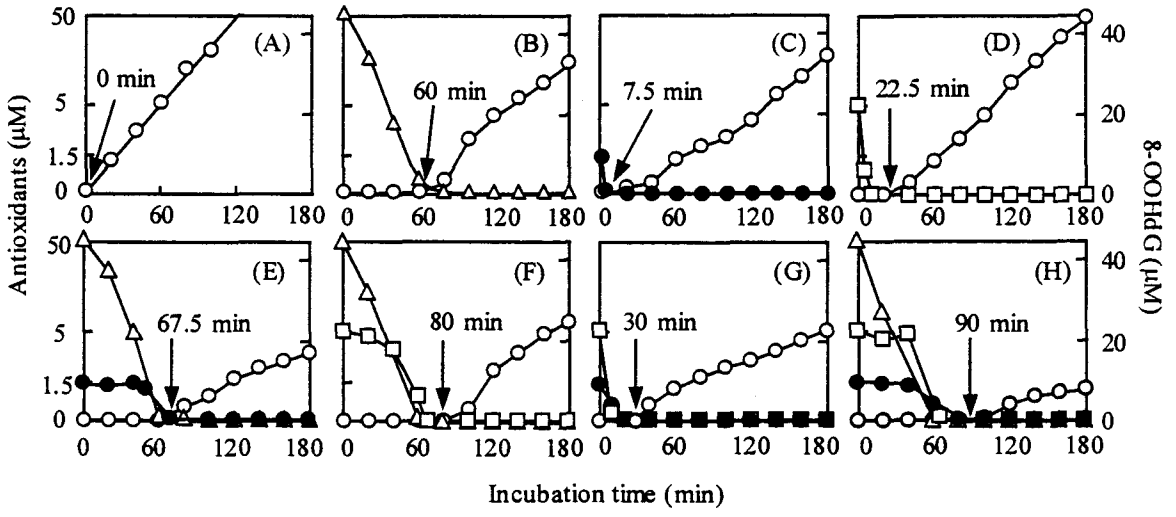


**Fig. 1.** Synergism assay for the antioxidants with 8-OOHdG formation. Ascorbic acid (50 μM), quercetin (1.5 μM) and/or EGCG (5.0 μM) were added to the incubation mixture of 0.5 mM 2'-dG and 25 mM AAPH at 37℃, and the amount of 8-OOHdG (○) that formed was determined by HPLC as in Materials and Methods. Simultaneously, the consumption of antioxidants was evaluated by HPLC: ascorbic acid (△), quercetin (●), and EGCG (□)

by each antioxidant alone (Fig. 1E-G). Then, simultaneous addition of these three antioxidants prolonged the formation by 90 min (Fig. 1H). When ascorbic acid and 8 types of catechin were coexisted in the oxidation of 2'-dG initiated by AAPH, the formation of 8-OOHdG was markedly suppressed. As shown in Fig. 2, ascorbic acid was consumed first linearly with time but catechins remained almost constant at the initial stage. Subsequently, gallocatechins, viz. EGCG, GCG, EGC, and GC that are the triol in the 3',4',5'-trihydroxy position in ring B were depleted in this order. Finally, other catechins, viz. ECG, CG, EC, and C, with diol in the 3' and 4' positions of B-ring were consumed. After all antioxidants were exhausted, 8-OOHdG formation proceeded rapidly. This delay (200 min) was almost same the sum total of the delay caused by each antioxidant used alone (Table 3). These results clearly show

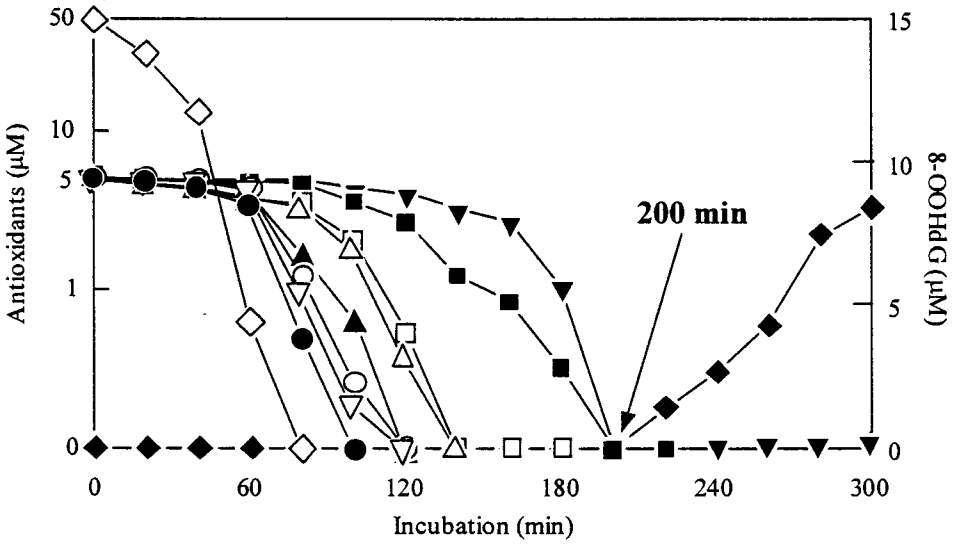


**Fig. 2.** Synergism assay for ascorbic acid and the 8 kinds of catechins with 8-OOHdG formation. Ascorbic acid (50 μM) and catechins (5.0 μM each) were added to the incubation mixture of 0.5 mM 2'-dG and 25 mM AAPH at 37℃, and the amount of 8-OOHdG (◆) that formed was determined by HPLC as in Materials and Methods. Simultaneously, the depletion of ascorbic acid and catechins was determined by HPLC: ascorbic acid (◇), EGCG (●), GCG (▽), EGC (○), GC (▲), ECG (△), CG (□), EC (■) and C (▼).

**Table 3.** Delay time of the 8-OOHdG formation by antioxidants

| Antioxidant | Delay time (min) |
|---|---|
| without antioxidants | 0 |
| ascorbic acid (50 μM) | 60 |
| catechins (5 μM) | |
| C | 15 |
| GC | 15 |
| CG | 17.5 |
| GCG | 20 |
| EC | 15 |
| EGC | 15 |
| ECG | 17.5 |
| EGCG | 22.5 |

that the oxidative damage to 2'-dG occurs only after all antioxidants have been consumed and that dietary antioxidants, such as quercetin and catechins, exhibit an additive effect together with biological antioxidant ascorbic acid on the genetic damage.

## *Formation of 8-OHdG in DNA of HepG2 cells exposed to AAPH*

Under the experimental conditions described in Materials and Methods, the formation of 8-OHdG in DNA bases from HepG2 cells exposed to oxygen radical initiator, AAPH was measured by HPLC with ECD. Fig. 3 illustrates a typical elution profile for 8-OHdG with ECD (+600 mV) and 2'-dG with UV (254 nm). The four most prominent peaks on UV detection were 2'-deoxycytidine (2'-dC, retention time 5.1 min), 2'-deoxyguanosine (2'-dG, 10.1 min), thymidin (2'-dT, 12.6 min), and 2'-deoxyadenosine (2'-dA, 21.0 min), respectively. At the sensitivity setting

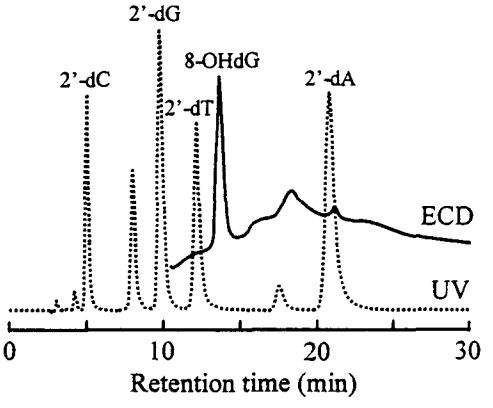


**Fig. 3.** Elution profile of ECD +600 mV (line) and UV 254 nm (dotted line). 8-OHdG and 2'-dG were quantitated as described in Materials and Methods.

used, 8-OHdG was mach sensitive to ECD in which detection limit was 0.1 pmol, unlike that of the UV absorbance signal for this compound. The time course of formation of 8-OHdG was shown in Fig. 4. In presence of 25 mM AAPH, its formation increased with an increase in the incubation time. AAPH level of 25 mM and incubation period of 9 h were used in the following experiment.

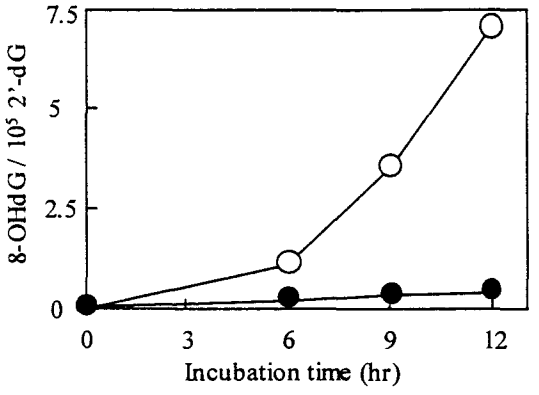


**Fig. 4.** Time-dependent formation of 8-OhdG in DNA by AAPH. HepG2 cells were reacted with 25 mM AAPH at 37°C for indicated time. After incubation, DNA was extracted and 8-OHdG level was determined as described in Materials and Methods. Results are expressed as 8-OHdG/$10^5$ 2'-dG in HepG2 cells incubated with (○) and without (●) AAPH.

### *Effects of antioxidants against intracellular oxygen radical*

HepG2 cells were pre-incubated with 10 μM antioxidants for 15 min before addition of AAPH. Every antioxidant at concentration of 10 μM exhibited no toxicity on the cells (data not shown). As shown in Table 4, antioxidants inhibit the oxygen radical induced 8-OHdG formation, for example 39% inhibition for quercetin, 58% for kaempferol, and 45% for EGCG.

**Table 4.** Percent (%) inhibition of 8-OHdG formation with dietary antioxidants

| | % inhibition |
|---|---|
| flavone | n.e.* |
| quercetin | 39 |
| kaempferol | 58 |
| luteolin | 45 |
| rutin | 22 |
| EC | 42 |
| EGCG | 45 |

*not effect

## *Discussions*

Dietary polyphenols, for example catechins and quercetin, are powerful antioxidants that can scavenge the reactive oxygen species before the DNA bases are damaged *in vitro* experiments (Chapter 3) and widely distributed in our diets, especially, green tea and onion, respectively (Chapter 4). On the other hand, cancer is believed to be induced by oxidative genetic damage caused by reactive oxygen species (Guyton et al, 1993; Emerit, 1994; Marnett, 2000). Further investigation needs to evaluate potentiality of dietary prevention our health from cancer. The author compared the antioxidative potencies of food factors based on 8-OOHdG formation (Chapter 3) with

its physiological levels. After consumption of 27.5 μmol green tea catechins/rat/day for 4 weeks, catechin aglycone could only be detected as EGCG (34 nM) in plasma. Conjugated derivatives of catechin were recovered in plasma between 5 nM for EGCG and 93 nM for EC. Total catechins level was less than 1 μM. On the contrary, their antioxidative potencies ($IC_{50}$ values for suppressing 8-OOHdG formation) ranged from 3.7 to 9.3 μM (Chapter 3). In flavonoids, their antioxidative potencies were around 5 μM or more but physiological levels are up to 1.5 μM in human (Paganga and Rice-Evans, 1997; Hollman et al., 1997; Conquer et al., 1998; de Vries et al., 1998). These results suggest that dietary antioxidant alone is insufficient to prevent the oxidative DNA damage. On the other hand, biological component, ascorbic acid has physiological levels of around 50 μM in human plasma (Lebine et al., 1996) and a few mM in cells (Mecocci et al., 2000). When the dietary phenolic antioxidants present together with ascorbic acid, they markedly delayed the start of genetic damage. This indicates the importance of the daily intakes of phenolic antioxidants from vegetables, fruits, and teas to facilitate the actions of ascorbic acid although individual physiological levels of dietary antioxidants are insignificant.

This chapter, furthermore, has demonstrated that oxygen radical generated from AAPH is capable of inducing 8-OHdG in DNA of HepG2 cells. 8-OHdG is the most major oxidative product of DNA bases (Chapter 1). The levels of 8-OHdG generated increases when the incubation time is increased. Dietary antioxidants, e.g. quercetin, kaempferol, and EGCG, prevented the formation of 8-OHdG, indicating that these antioxidants can be incorporated into cellular and scavenge the oxygen radical generating around DNA.

The author found in this chapter that vegetables, fruits, and teas consumption

can probably protect the oxidative DNA damage in our body and reduce risk of cancer.

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# Chapter 6

# Conclusion

# *Conclusion*

Degenerative diseases including cancer have been recognized to be induced by oxidative genetic damages. This means that antioxidants can probably prevent from the diseases. The antioxidants should be daily food factors but not medicines, because the degenerative diseases closely associate with the life style. Then, the author first explored the strong antioxidants in daily foods, focusing the target on tropical fruits. Since they are growing under such a condition, hot and intense sunlight, that facilitates lipid peroxidation, they should include several strong antioxidants to maintain the growths. Among the fruits, banana contained a strong antioxidant at large amount. It was identified to be dopamine. The antioxidative potency of dopamine was greater than that of known products such as BHA, BHT, flavonoids, glutathione, and catechin, further the content level was surprising, 80-560 mg per 100 g in peel and 2.5-10 mg in pulp (Chapter 2).

On the other hand, a better understanding of the antioxidant actions to prevent degenerative diseases is whether they can scavenge reactive oxygen species before the DNA bases are damaged. Little suitable method, however, has been available. Then, the author established a novel convenient method (Chapter 3). The author employed one of DNA bases 2'-deoxyguanosine as the target and 2,2'-azobis(2-amidinopropane)-dihydrochloride as the radical generator. This generator produces stoichiometrically superoxide anion that has been proved to occur in normal human cells. This system formed a novel product, and the author identified it 8-hydroperoxy-2'-deoxyguanosine (8-OOHdG). The formation of 8-OOHdG was linearly time and concentrations-dependent to follow the first order kinetics. Thus, this method was very suitable to

evaluate the antioxidants for the potency to be able to prevent genetic damages. This method could easily determine the $IC_{50}$ values to inhibit the genetic damages for 90 antioxidants such as simple phenols, flavonoids, anthraquiones, other phytochemicals, food additives, and biological components, most of which occur in our diet. Flavonoid catechins and quercetin are most powerful antioxidants that can scavenge the oxygen radical before the DNA bases are damaged, and their antioxidative potencies ($IC_{50}$ values for suppressing 8-OOHdG formation) are 3.7-9.3 and 3.3 μM, respectively. So, the author found that antioxidative potency to protect the oxidative DNA damage was great in *ortho*-dihydroxyl structures like chlorogenic acid, quercetin, rutin, luteolin, and catechins.

These antioxidants have been suggested to widespread in daily plant foods, and little exact analysis data have been published, especially in Japanese food. Thus, the analysis data are strongly requested, although the suitable analytical methods are absence. The author developed the accurate method and determined the contents identifying the species of antioxidants in over 50 foods in Chapter 4. Mostly, root vegetables included simple polyphenol species as the major antioxidants, leaf vegetables and fruits contained flavone and flavonol glycosides, orange family flavanone glycosides, and teas specifically included catechins.

A remaining question is whether these dietary antioxidants are available in humans. Epidemiologically, the intake of antioxidants closely correlates with the reduce of incidences in degenerative diseases but biochemically a little has been informed. The author investigated the bioavailability of antioxidants in physiological levels in the body and in the cells (Chapter 5). In rats, the physiological levels are less than 1 μM for total catechins and 1.5 μM for quercetin. This indicated that one dietary

antioxidant alone is insufficient to prevent the oxidative DNA damage because the concentration was not achieved near to $IC_{50}$ values. Contrary, catechins and quercetin markedly delayed the start of genetic damage when combined with a physiological concentration of ascorbic acid (50 μmol/L). This results strongly indicated that the importance of the daily intake of antioxidants from vegetables, fruits, and teas to facilitate the actions of ascorbic acid. Then, the author examined the cellular availability in incorporation into cell and further suppression of cellular genetic damages. When the cell was received the antioxidants and exposed to radical generator, quercetin and catechins markedly suppressed the formation of 8-OHdG in cellular DNA. Thus, the author concluded that the dietary antioxidants were partly absorbed into body and incorporated the cells, and then exhibited the antioxidative potency to prevent the DNA damage.

Another interesting issue is in which way 8-OOHdG formed in the cells interconverts to 8-OHdG. Fig. 2 in Chapter 3 shows the conversion occurs under acidic conditions, accompanying a cleavage of the base-ribose bond, and Fig. 3 in Chapter 5 shows that the major oxidative damage of intracellular DNA is 8-OHdG formation. On the other hand, the oxidative damage to DNA has been well recognized to accompany by strand breaks in the cells. As shown with Fig. 2 in Chapter 3, the interconversion of 8-OOHdG to 8-OHdG under acidic conditions accompanied the break of sugar moiety. Thus, it is possible that 8-OOHdG is a primary product of oxidative stress and subsequently 8-OH-guanine and strand breaks occur as detectable phenomena. This is going on as one of my future studied.

The author concludes that flavonoids and simple polyphenols in our diet contribute well to prevent from the degenerative diseases through the suppression of

DNA damages with the antioxidative potency. Consequently, consumptions of vegetables, fruits, and teas can antioxidatively reduce the risk of cancer in human, and especially, green tea is excellent resources for the antioxidants.

# *Acknowledgements*

I wish here to express my sincere gratitude to Dr. Kazuki Kanazawa, Professor of Kobe University, for his abundant guidance during the course of this study. He guided a stubborn student with extraordinary patience. Only his inspiring advice and cautious criticism enabled me to carry on this work and kept me from going astray.

I am deeply grateful to Dr. Gen-ichi Danno, Professor of Kobe University for his incessant enthusiastic encouragement. I am very grateful to Dr. Hitoshi Ashida, Associate Professor of Kobe University for his warm encouragement.

I would like to thank to Professor Dr. Taisaku Amakawa, Professor Dr. Takashi Ohno, and Professor Dr. Shin Hasegawa of Kobe University for their useful advice. I am very grateful to Professor Mitsuru Sasaki, Associate Professor Hideyoshi Miyake, and Assistant Professor Takeyuki Tanaka of Kobe University for their useful advice.

I am greatly indebted to the members in the Laboratory of Food and Nutritional Chemistry, Department of Agricultural Chemistry, Kobe University for useful advice and allowing use of their facilities.

I express my appreciation to Messrs. Takashi Hashimoto, Yutaka Hatanaka, Bunsyo Shiotani, Masakazu Kobayashi, Hiroaki Yanagitani, Satoshi Nakagawa, Yoshinori Honda, and Takatoshi Yamashita, and Ms. Kaori Furuse and Aya Yamanaka, for their help and friendship.

Last, but not least, I would like to express my hearty gratitude to my parents, without whose affection and assistance I have not continued this study.

# *List of Publications*

## *Papers*

1.
Kazuki Kanazawa and Hiroyuki Sakakibara
High Content of Dopamine, a strong Antioxidant, in Cavendish Banana
*Journal of Agricultural and Food Chemistry* (2000) **48,** 844-848.

2.
Mingzhou Sun, Hiroyuki Sakakibara, Hitoshi Ashida, Gen-ichi Danno, and Kazuki Kanazawa
Cytochrome P4501A-Inhibitory Action of Antimutagenic Anthraquinones in Medicinal Plants and the Structure-activity Relationship
*Bioscience Biotechnology and Biochemistry* (2000) **64,** 1373-1378.

3.
Mingzhou Sun, Hiroyuki Sakakibara, Hitoshi Ashida, Gen-ichi, Danno and Kazuki Kanazawa
Dietary Antioxidants Fail in Protection against Oxidative Genetic Damage in *In Vitro* Evaluation
*Bioscience Biotechnology and Biochemistry* (2000) **64,** 2395-2401.

4.
Hiroyuki Sakakibara, Hitoshi Ashida, and Kazuki Kanazawa
A Novel Method Using 8-Hydroperoxy-2'-deoxyguanosine Formation for Evaluating Antioxidative Potency
*Free Radical Research* (in press)

5.

Hiroyuki Sakakibara, Satoshi Nakagawa, Yoshinori Honda, Hitoshi Ashida and Kazuki Kanazawa

A complete analysis to determine phenolic antioxidants in vegetables, fruits and teas

*Journal of Chromatography A* (submitted)

6.

Hiroyuki Sakakibara, Hiroaki Yanagitani, Hitoshi Ashida and Kazuki Kanazawa

Bioavailability of dietary factors in prevention against oxidative genetic damage

*Free Radical Biology & Medicine,* (in preparation).

## *Patents*

1.

金沢和樹、榊原啓之

「遺伝子損傷マーカー」

平成 13 年 9 月 25 日提出（特願 2001-291793）