Antioxidant Measurement and Applications

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Foreword

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Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

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Preface

Antioxidants play an important role in preventing oxidant damage both in vivo and in vitro. In the body, antioxidant enzymes, vitamins E and C, selenium, and dietary antioxidants neutralize free radicals and therefore control age-related and chronic diseases. In foods, antioxidants play a crucial role in preventing or delaying autoxidation and formation of off-flavors as well as loss of food quality and nutritional value. As the mechanisms involved in oxidation are different, there are also a number of mechanisms by which antioxidants render their effects. Therefore, to measure antioxidant activity, a variety of methods are available and so far no uniform procedure has been found to be applicable to all systems. There are also other complications related to matrix effect from other ingredients in the food as well as extraction methods for isolation and determination of antioxidants.

The aim of this book is to provide the latest findings and perspectives related to antioxidants (Chapters 1–3); methodologies and equipment for evaluation of antioxidant activity, including cell culture procedures (Chapters 4–11); as well as the presence and activity of antioxidants in selected plants and foods (Chapters 12–20). The health benefits of antioxidants from several sources are then discussed (Chapters 21–24) and finally, application of antioxidants in selected foods is presented (Chapters 25–27).

The book will serve as a useful reference for research workers and students in a variety of disciplines, including food science, nutrition, chemistry, and biochemistry as well as for health professionals. Researchers in the universities, industry, and government laboratories will find it of particular interest as it provides the most recent findings in this ever-growing area.

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We are indebted to all authors who contributed to this book and shared their vast knowledge in preparing a state-of-the-art information package for those interested in the area of food antioxidants. We are also grateful to Peggy-Ann Parsons for her hard work in formatting the manuscripts and in providing all the required assistance during preparation of this book.

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Chapter 1

Antioxidant Measurement and Applications: An Overview

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Antioxidants are added to fats, oils and fatty foods to prevent their oxidative deterioration. Antioxidants also protect the integrity of cellular structures and macromolecules from damage due to free radicals. Carotenoids and phenolic compounds are major dietary antioxidants. Because of the importance of antioxidant potential in foods and dietary supplements, it is necessary to have good and reliable methods for measuring antioxidant activity. Various *in vitro* and *in vivo* methods for assessing antioxidant activities are discussed.

Introduction

Autoxidation occurs widely in fats, oils and lipid-containing foods, and causes food quality deterioration with concomitant generation of loss of nutrients, unpleasant flavors, and even potentially toxic substances. Among the methods for preventing oxidation, addition of antioxidants is the most effective, convenient and economical one (*1*).

Antioxidants are also important to human health. Antioxidant protection from damage due to free radicals is vital for the integrity of cellular structures

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and macromolecules (*2,3*). As we age, the system which utilizes antioxidants for our defense and protection also declines, and can be aggravated by the presence of various oxidative stresses caused by pollution, exercise, smoke exposure and radiation. This defense system operates through a series complex networks between vitamins C and E, carotenoids, zinc, copper, selenium, and magnesium-dependent enzyme antioxidants as well as other phytonutrients, which together perform highly involved recycling and regeneration reactions to optimize free radical protection. Deficiencies in any of the mentioned necessary components could potentially lead to a severely compromised defense system (*4,5*). Owing to the incomplete efficiency of our endogeneous defense systems, dietary antioxidants are needed to overcome the oxidative damage (*5*).

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Dietary Antioxidants

Carotenoids and phenolic compounds are major dietary antioxidants. Both of these groups of compounds contain hundred of members (*6*).

Carotenoids are natural, fat-soluble pigments that provide bright coloration to plants and animals and act as antioxidants, which include the possibility of providing vitamin A activity. One defining characteristic of carotenoids is the chemical structure of what is often considered their backbone molecule, a 40-carbon polyene chain. The polyene backbone consists of a pattern of conjugated double bonds, which allows the carotenoids to take up excess energy from other molecules (*7*). This characteristic may be responsible for the antioxidant activity seen in biological carotenoids. In addition to scavenging free radicals, other health benefits related to this observed antioxidative activity include protection from sunburn and inhibition of the development of certain types of cancers (*8-10*).

β-Carotene is the most common carotenoid in food and the most potent of the provitamin A carotenoids. β-Carotene is believed to have antioxidant activity. It has been shown to exhibit radical- trapping behavior only at partial pressure of oxygen significantly less than that in normal air (*11*). Such low oxygen partial pressures are found in most tissues under physiological conditions. At higher oxygen pressure it loses the antioxidant activity and shows a pro-oxidant effect (*11*).

Lutein and its isomer, zeaxanthin, are yellow pigments that belong to the classes of non-provitamin A carotenoids. Unlike other carotenoids, hydroxyl groups are substituted on the ring structures at the end of the conjugated double bond chains of lutein and zeaxanthin. Lutein is naturally occurring and found predominantly in dark green, leafy vegetables such as spinach and kale. Zeaxanthin gives corn its yellow color. There is a growing body of evidence (including *in vivo*, *in vitro* and epidemiological studies) supporting the claim that

lutein and zeaxanthin contribute to health and delay age related macular degeneration of the eyes and, to a lesser extent, cancers and heart diseases (*12-14*). The evidence for the role of lutein and zeaxanthin in eye health is very strong because of their exclusive presence in the ocular tissues and the high numbers of epidemiological studies that have been conducted. With a high accumulation in the macula of the eye, the area of highest visual acuity, lutein and zeaxanthin are proposed to have the ability to filter out harmful blue light, while at the same time acting as antioxidants to quench potentially damaging reactive oxygen species (ROS; *15*).

Lycopene, a carotenoid found in tomatoes, watermelon, papaya, apricot, orange and pink grapefruit, exhibits antioxidant and anticancer activities (*16*). About 80% of lycopene is consumed through tomatoes and tomato-related products. Numerous studies have suggested reduced risk of prostate cancer from the consumption of processed tomato products (*17*). Although, these beneficial health effects of lycopene are thought to be due to its antioxidant properties, evidence is accumulating to suggest other mechanisms of action like hormone and immune system modulation (*18*). Lycopene is the most abundant carotenoid in human plasma, which may imply its elevated level of importance in the human body compared with other carotenoids, such as β-carotene and lutein (*19*).

Phenolic compounds occurring commonly in foods may be classified into simple phenols, hydroxybenzoic and hydrocinnamic acid derivatives, flavonoids, stibenes, lignans and hydrolysable as well as condensed tannins (*20*). Phenolics in foods may occur in the free, esterified, etherified and insoluble-bound forms.

The most abundant phenolic compounds in food are flavonoids. Flavonoids are present in edible fruits, leafy vegetables, roots, tuber bulbs, herbs, spices, legumes, tea, coffee, cocoa, chocolate and red wine. They can be classified into seven groups: flavones, flavanones, flavonols, flavanonols, isoflavones, flavanols (catechins) and anthocyanidins. In general, the leaves, flowers and fruits or the plant itself contain flavonoid glycosides, woody tissues contain aglycones, and seeds may contain both (*20*).

As a result of their ubiquity in plants, flavonoids are an integral part of the human diet. It is estimated that the average American's daily intake of the consumption of flavonols is close to 20-25 mg/day (*21*).

Almost all flavonoids possess several common biological and chemical properties: (a) antioxidant activity, (b) the ability to scavenge ROS, (c) the ability to scavenge electrophiles, (d) the ability to inhibit nitrosation, (e) the ability to chelate metals, (f) the potential to produce hydrogen peroxide in the presence of certain metals and (g) the capability to modulate certain cellular enzyme activities (*22*). It appears that diets rich in flavonoids may protect against cardiovascular diseases, neurodegenerative disorders and some forms of cancer.

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Antioxidant Measurement

The need to measure antioxidant activity is well documented; these are carried out for meaningful comparison of foods or commercial products and for provision of quality standards for regulatory issues and health claims (*23*). There are numerous methods for measureing antioxidant activity; these may be classified into two categories. The first category measures the ability of antioxidants in inhibiting oxidation in a model system by monitoring the associated changes using physical, chemical or instrumental means. Radical scavenging assays include methods based on hydrogen atom transfer (HAT) or electron transfer (ET) mechanisms. Oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant parameter (TRAP) and crocin bleaching assays are the major methods that measure HAT while Trolox equivalent antioxidant capacity (TEAC), ferric reducing antioxidant antioxidant power (FRAP) and 2,2-diphenyl-1-picrglhydrozyl (DPPH) assays represent ET-based methods. Extensive relevant reviews are provided in the literature (*23-25*) as well as in this volume (*26*).

It is interesting to note that DPPH radical is used to test antioxidant activity by its ability to abstract hydrogen atoms from polyphenols *(27)*. Another stable radical, tris(2,4,6-trichloro-3,5-dinitrophenyl)methyl radical, was developed as a good sensor to test the activity of polyphenols measuring their capacity to participate in electron transfer reactions (*27*).

Antioxidant activity of a compound can also be evaluated in different cell culture assay for the prevention of carcinogenesis. Because oxidative DNA damage is considered to be relevant in carcinogenic process, one can evaluate the possible anticarcinogenic effect of dietary antioxidants by determining their effect on 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-inducing ROS generation, H_2O_2 scavenging, H_2O_2-induced apoptosis, xanthine oxidase activity, and lipopolysaccharide (LPS)-inducing NO generation. Details are discussed in this volume (*28*).

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Bioavailability of Dietary Antioxidants

Bioavailability is the degree to which a drug, nutrient, dietary supplement or nutraceutical is available to the body. Bioavailability is influenced by how much of a substance is absorbed and circulated in the human body. Problems with bioavailability arise when trying to elucidate exactly what dose brings about the desired physiological response. Manufactures producing 500 mg vitamin C pills cannot claim that 500 mg of the vitamin are taken in and used by the body.

There are variations between different human subjects and their uptake of certain food-based chemicals. This means that two people taking the same dose could actually absorb different amounts of the same compound. One might only see the effect of 200 mg of a 500 mg pill while the other might see the effect of 100 mg. This disparity is due to variability in absorption, distribution, metabolism and excretion of the bioactives abbreviated as ADME. In addition, it is sometimes the case that the ingested chemical is not the final bioactive agent. Many molecules enter the digestive system in one form only to be broken down into smaller metabolites that interact through absorption. Science has yet to identify many of these breakdown reactions sufficiently to understand how these reactions affect the bioavailability of compounds.

Studies have shown that 11% of caffeic acid and trace amounts of chlorogenic acid, present in coffee, are found in urine indicating that they failed to be fully absorbed through the gut barrier (*29*). The exact fate of the remaining caffeic acid is unknown. This is the problem of bioavailability and it translates to nearly every aspect of nutraceuticals.

While it is difficult to say for sure how much of a compound will be taken in and used by an organism, there is some strong evidence showing how low some of the uptakes can be. In a study using chlorogenic acid, only 1.7% of it was recovered unchanged in the urine. In these cases, the colon could play a larger role in the metabolism of polyphenols (*30*).

Polyphenols reaching the colon can be broken into smaller metabolites by colonic microbiota, the bacteria found in the colon (*30*). These bacteria are able to break down phenols, allowing absorption of these smaller metabolites by the kidneys, liver and other organs. Later, these smaller metabolites may find their way into the urine. Without a clear understanding of their chemical nature, we cannot screen for them in the urine. The same study also concluded that a large part of the ingested polyphenols will probably never enter the peripheral circulation as smaller metabolites (*30*).

The problem of reduced antioxidant activity found in smaller metabolites of larger parent compounds increases the uncertainty of bioavailability studies. An organism is not likely to absorb an entire dose, and it is likely that the compound will be broken down into smaller, unidentified compounds. Further studies are warranted to identify these compounds. Considering these variables, it is very difficult to predict the total effect of an antioxidant on host cells.

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Chapter 2

Antioxidants and Cancer Therapy: To Take or Not to Take: That Is the Question?

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The development of chronic diseases such as cancer is attributed to oxidative damage by reactive oxygen species (ROS) to DNA. One way of reducing or minimizing the formation of ROS is by the addition of antioxidants in the diet. However, this strategy of supplementing cancer therapy with antioxidants is hotly debated because of the absence of clear data. Early studies indicated this could be detrimental in workers who smoke or are exposed to environmental carcinogens. Later studies showed ROS play a role in cell signaling and regulation which could lead to cell death or apoptosis. Thus supplementing with dietary antioxidants such as ascorbic acid would interfere with the formation of ROS and prevent apoptosis from occurring. Consequently, antioxidant supplements by cancer patients remains highly questionable.

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Reactive oxygen species (ROS) are normal metabolic by-products produced in the mitochondria (*1-3*). They are implicated in the genesis of atherosclerosis, diabetes, cancers as well as neurological disorders. Over 200 epidemiological studies point to a strong association between the low consumption of fruits and vegetables and the incidence of cancer (*4-5*). This is attributed to the presence of a wide array of antioxidants in fruits and vegetables capable of protecting normal cells from the damaging action of ROS. The latter includes free radicals such as superoxide, hydroxyl, peroxyl, alkoxyl, and hydroperoxyl radicals as well as nitric oxide and nitrogen dioxide. Consequently it would be safe to assume that the presence of antioxidants is essential for reducing oxidative damage and the development of cancers. For example, Thompson and co-workers (*6*) showed that increasing the consumption of fruits and vegetables in women with a family history of risk for breast cancer, significantly decreased urinary 8-hydroxy-guanosine (8 OhdG) and 8-isoprostane F-2α (8EPG), both markers of oxidative cellular damage (Table I).

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Table I. Overall Effect of the Vegetable and Fruit Intervention on Oxidative Indices*

Parameter	*Pre-Intervention*	*Post-intervention*	*Change (%)*
8-OHdG**	49.6 ± 12.4	21.4 ± 2.2	-52
8-EPG***	3.7 ± 0.3	2.4 ± 0.1	-33

Antioxidants

Antioxidants are organic compounds capable of neutralizing or detoxifying reactive oxygen or nitrogen species. These compounds can donate an electron to quench free radicals. In 2000, the National Academy of Sciences defined a dietary antioxidant as "*A dietary antioxidant in food significantly decreases the adverse effects of ROS, reactive nitrogen species, or both on normal physiological functions in humans* (*7*)." In addition there are a number of enzymes that are synthesized within the body to catabolize free radicals including superoxide dismutase, catalase, and glutathione peroxidase (*8*). A group of proteins which keeps metals such as iron and copper in the bound form also reduce the ability of these metal from catalyzing the production of free radicals. Vitamins E, C, A and β-carotene are generally classified as essential nutrients. Vitamin E is a fat-soluble vitamin associated with the cell membrane that works in conjunction with vitamin C, a water-soluble vitamin, to maintain its antioxidant potential. This is achieved by vitamin C regenerating vitamin E to its

reduced and active form. It is now recognized that many plant-derived compounds in the diet, such as flavonoids, carotenoids and steroids are also antioxidants (*9-11*).

Antioxidants and Cancer Treatment

β-Carotene and Vitamin E

While supplementation with antioxidants may be an appropriate strategy for the normal population, a number of studies suggest this could detrimental in individuals who smoke or are exposed to environmental carcinogens. For example, three randomized clinical trials showed that β-carotene, alone or in combination with vitamins A and E, increased the incidence of lung cancer and mortality in heavy smokers and in asbestos workers (*12-16*). A review of the β-carotene data by Paolini and co-workers (*17*) showed it was particularly harmful to give β-carotene as the sole supplement to smokers or individuals exposed to such environmental carcinogens as asbestos. Under these conditions β-carotene acts as a cocarcinogen by inducing higher levels of cytochrome P450 isoforms which activate procarcinogens or increases ROS levels. As a consequence these individuals would be exposed to a greater risk for cancer. Vitamin E, the most popular antioxidant taken as a supplement has also been evaluated for its health benefits. Several reviews have appeared that present conflicting views on its anticarcinogenic and cardioprotective effects (*18-25*).

Vitamin C

ROS are the major cause of cell damage but they also play an important physiological role in intracellular signaling and regulation (*26*). As a result ROS influences redox status and depending on their concentration can cause cell proliferation or arrest and cell death (apoptosis). Thus the production of ROS can play an important role in bringing about the death of cancer cells or apoptosis.Thus addition of antioxidants could inhibit apoptosis and prevent the destruction or elimination of precancerous and cancerous cells. Wenzel *et al.* (*27*) found ascorbic acid interfered with apoptosis of HT-29 human colon carcinoma cells induced by the drug camptothecin or the flavonoid flavone. The reduction of ROS by ascorbic acid inhibited disintegration of the plasma membrane of these cancer cells. Ascorbic acid reduced or prevented stimulation of caspase 3, downregulation of the mitochondrial antiapoptotic protein bcl-X1 as well as NK-kB mRNA levels. This study clearly showed that an increase in

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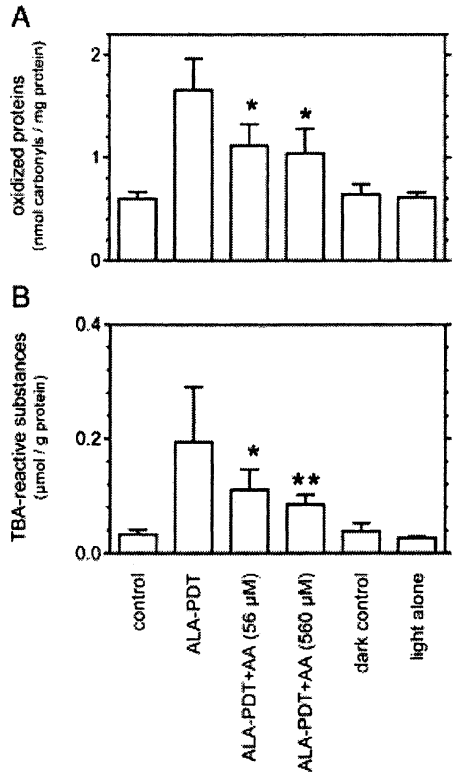

*Figure. 1. Effect of ascorbic acid (AA) on protein oxidation (**A**) and lipid Peroxidation (**B**). Both processes were significantly reduced by AA. Values were assessed 16 h after ALA-PDT treatment. Results represent means ± SD of at least six independent experiments. *p < 0.05, ** p<0.005 vs ALA-PDT (Reproduced with permission from reference 28. Copyright 2005 Elsevier.)*

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ascorbic acid intake should be avoided by cancer patients undergoing chemotherapy.

A recent study by Frank *et al.* (*28*) found ascorbic acid interfered with photodynamic therapy (PDT) of a rat DS-sarcoma cell line. Photodynamic therapy is a successful therapy for eliminating local malignant tumors by accumulating excessive amounts of ROS which brings about an early cure (*29-30*). One of the rapidly developing areas in PDT is 5-aminolevulinic acid (ALA)-mediated photosensitization. Using this technique, Frank *et al.* (*29*) showed

vitamin C or ascorbic acid (AA) reduced mitochondrial damage induced by 5-aminolevulinic acid-based photodynamic therapy (ALA-PDT). This was clearly evident by a reduction in lipid and protein oxidation as shown in Figure 1. Overall ascorbic acid countered the efficacy of ALA-PDT by increasing the cell survival rate by over twofold. Consequently patients undergoing ALA-PDT cancer therapy must avoid the use of ascorbic acid.

In contrast, a combination of ascorbic acid and vitamin K_3 was reported by Verrax and co-workers (*31*) to effectively kill cancer cells by a new type of cancer-cell death known as autoschizis. This process was independent of caspase 3 and characterized by oxidative stress, DNA fragmentation, cell-membrane damage, with progressive loss of organelle-free cytoplasm. Thus vitamin C and K3 could be considered coadjuvants for cancer therapy.

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Conclusions

Research on the relationship between antioxidants and human health remains a challenging field. While many phytochemicals with potential to serve as *in vivo* antioxidants have been identified, their effect on normal cells may differ from their effect on cancer cells. Clearly β-carotene acts as a promoter of carcinogenesis in patients who smoke or are exposed to environmental carcinogens. Ascorbic acid, another important antioxidant has been shown to interefere with therapies in which ROS is generated to destroy cancer cells. Based on the evidence to-date cancer patients should remain cautious and avoid supplementing their cancer therapy with antioxidants such as vitamin C. In the absence of safety and efficacy of supplemental antioxidants, a diet rich in vegetables, whole grains and adequate essential nutrients to maintain optimal growth and maintenance of vital bodily function remains the most prudent and recommended choice for the general population .

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Chapter 3

Antioxidants and Whole Food Phytochemicals for Cancer Prevention

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It is well established that regular consumption of fruits, vegetables, and whole grains is strongly associated with reduced risk of developing chronic diseases. It has been shown that fruit and vegetable phytochemical extracts exhibit strong antioxidant and antiproliferative activities. The additive and synergistic effects of phytochemicals in fruits and vegetables are responsible for these potent antioxidant and anticancer activities, and that the benefit of a diet rich in fruits and vegetables is attributed to the complex mixture of phytochemicals present in whole foods. This may explain why no single antioxidant can replace the combination of natural phytochemicals in fruit and vegetables to achieve the health benefits. The evidence suggests that antioxidants or bioactive compounds are best acquired through whole food consumption, not from expensive dietary supplements. Thus, it is recommended that consumers eat 5 to 10 servings of a wide variety of fruits and vegetables daily as an appropriate strategy for significantly reducing the risk of chronic diseases and to meet their nutrient requirements for optimum health.

Cancer and cardiovascular disease (CVD) are the top 2 causes of death in the United States and in most industrialized countries. Epidemiological studies have consistently shown that a high dietary intake of fruits and vegetables as well as whole grains is strongly associated with reduced risk of developing such chronic diseases (*1-3*). It is estimated that one-third of all cancer death in the United States could be avoided through appropriate dietary modifications (*3-5*). This suggests that change in dietary behavior, such as increasing consumption of fruits, vegetables, and whole grains, and related lifestyles is a practical strategy for significantly reducing the incidence of cancer.

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In 1982 the National Academy of Sciences of the United States included guidelines in their report on diet and cancer emphasizing the importance of fruits and vegetables (*6*). The value of adding citrus fruits, carotene-rich fruits and vegetables, and cruciferous vegetables to the diet for reducing the risk of cancer was specifically highlighted. In 1989, a report from the National Academy of Sciences on diet and health recommended consuming 5 or more servings of fruits and vegetables daily for reducing the risk of both cancer and heart disease (*7*). The Five-a-Day program was developed as a tool to increase public awareness of the health benefits of fruit and vegetable consumption and promote adequate intakes of known vitamins. Plant-based foods, such as fruits, vegetables, and whole grains, which contain significant amounts of bioactive phytochemicals (Figure 1) and have potent antioxidant activity (Figure 2), may provide desirable health benefits beyond basic nutrition to reduce the risk of chronic diseases (*8*). The beneficial effects associated with plant based food consumption are in part due to the existence of phytochemicals.

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Phytochemicals

The "phyto-" of the word phytochemicals is derived from the Greek word *phyto*, which means plant. Therefore, phytochemicals are plant chemicals and may be defined as bioactive nonnutrient plant compounds in fruits, vegetables, grains, and other plant foods that have been linked to reducing the risk of major chronic diseases. More than 5000 individual phytochemicals have been identified in fruits, vegetables, and grains, but a large percentage still remain unknown and need to be identified before we can fully understand the health benefits of phytochemicals in whole foods (*8*). However, more and more convincing evidence suggests that the benefits of phytochemicals in fruits, vegetables and whole grains may be even greater than is currently understood because the oxidative stress induced by free radicals is involved in the etiology of a wide range of chronic diseases (*9*). Because phytochemicals differ widely in composition and ratio from fruit to vegetable to grain (Figure 1), and often have complimentary mechanisms to one another, it is suggested that one consumes a wide variety of these plant-based foods.

Phytochemicals can be classified as carotenoids, phenolics, alkaloids, nitrogen-containing compounds, and organosulfur compounds (Figure 3). The most studied of the phytochemicals are the phenolics and carotenoids.

Phenolics

Phenolics are compounds possessing one or more aromatic rings with one or more hydroxyl groups and generally are categorized as phenolic acids, flavonoids, stilbenes, coumarins, and tannins (Figure 3). Phenolics are products of secondary metabolism in plants, providing essential functions in the

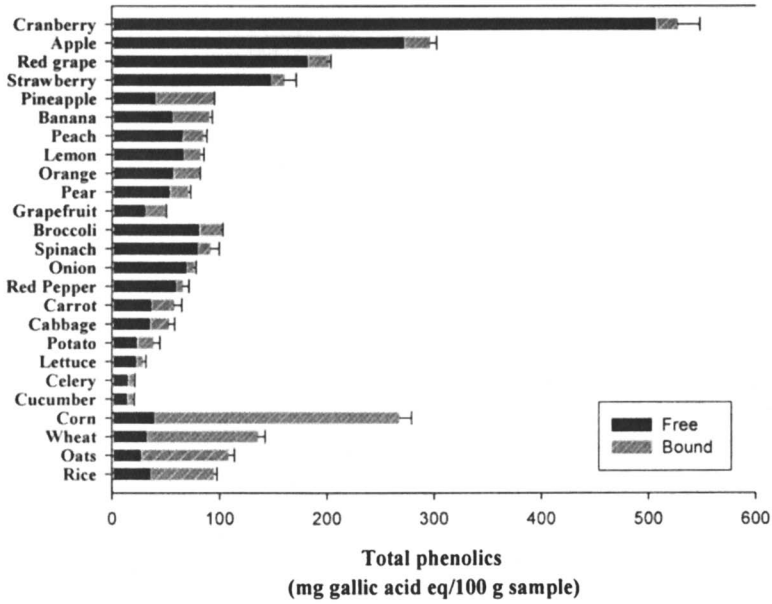

Figure 1. Total phenolic content of common fruits, vegetables and whole grains (adopted from references 11-13).

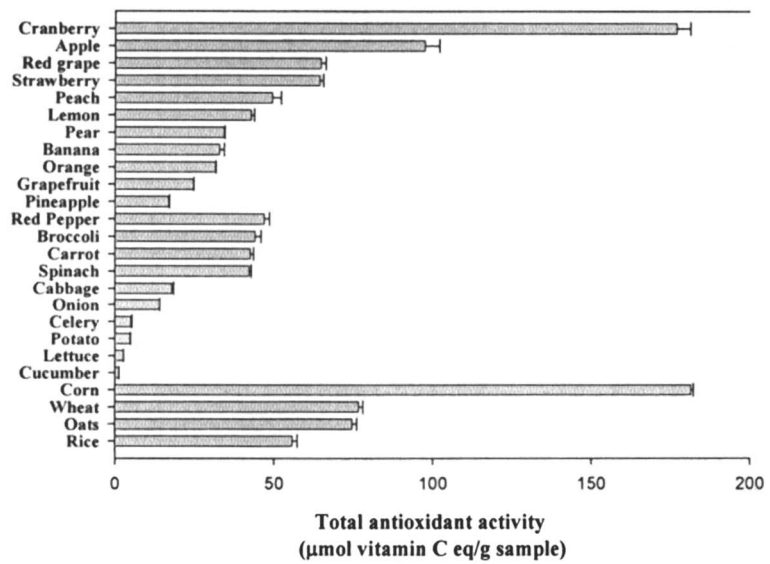

Figure 2. Total antioxidant activity of common fruits, vegetables and whole grains (adopted from references 11-13).

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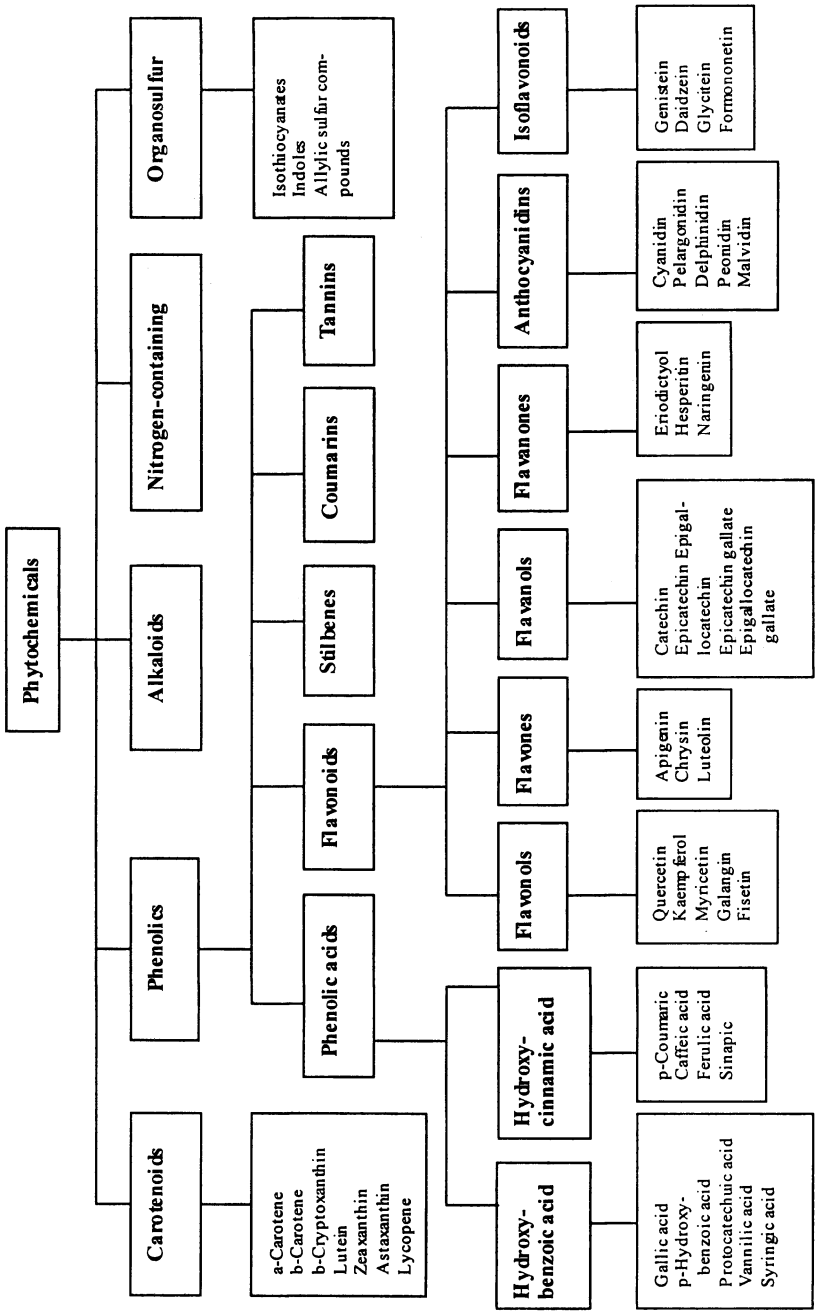

Figure 3. Classification of dietary phytochemicals

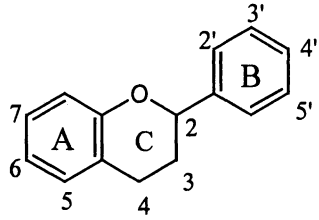

Figure 4. The generic structure of flavonoids

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reproduction and growth of the plants, acting as defense mechanisms against pathogens, parasites, and predators, as well as contributing to the color of plants. In addition to their roles in plants, phenolic compounds in our diet may provide health benefits associated with reduced risk of chronic diseases. Among the 11 common fruits consumed in the United States, cranberry has the highest total phenolic content, followed by apple, red grape, strawberry, pineapple, banana, peach, lemon, orange, pear, and grapefruit (*10*; Figure 1). Among the 10 common vegetables consumed in the United States, broccoli possesses the highest total phenolic content, followed by spinach, yellow onion, red pepper, carrot, cabbage, potato, lettuce, celery, and cucumber (*11*; Figure 1). Whole grains are also good sources of dietary phenolics on a per serving basis (*12*). Among the grains tested, corn had the highest content of phenolic compounds, followed by wheat, oats, and rice (Figure 1). It is estimated that flavonoids account for approximately two-thirds of the phenolics in our diet and the remaining one-third are from phenolic acids.

Flavonoids

Flavonoids are a group of phenolic compounds with antioxidant activity that have been identified in fruits, vegetables, and other plant foods and have been linked to reducing the risk of major chronic diseases. More than 4000 distinct flavonoids have been identified. They commonly have a generic structure consisting of 2 aromatic rings (A and B rings) linked by 3 carbons that are usually in an oxygenated heterocycle ring, or C ring (Figure 4). Differences in the generic structure of the heterocycle C ring classify them as flavonols, flavones, flavanols (catechins), flavanones, anthocyanidins, and isoflavonoids (Figure 3 and Figure 5). Flavonols (quercetin, kaempferol, and myricetin), flavones (luteolin and apigenin), flavanols [catechin, epicatechin, epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG)], flavanones (naringenin), anthocyanidins (cyanidin and malvidin), and isoflavonoids (genistein and daidzein) are common flavonoids in the diet (Figure 3 and Figure 6). Flavonoids are most frequently found in nature as conjugates in glycosylated or esterified forms but can occur as aglycones, especially as a result of the effects of food processing. Many different glycosides can be found

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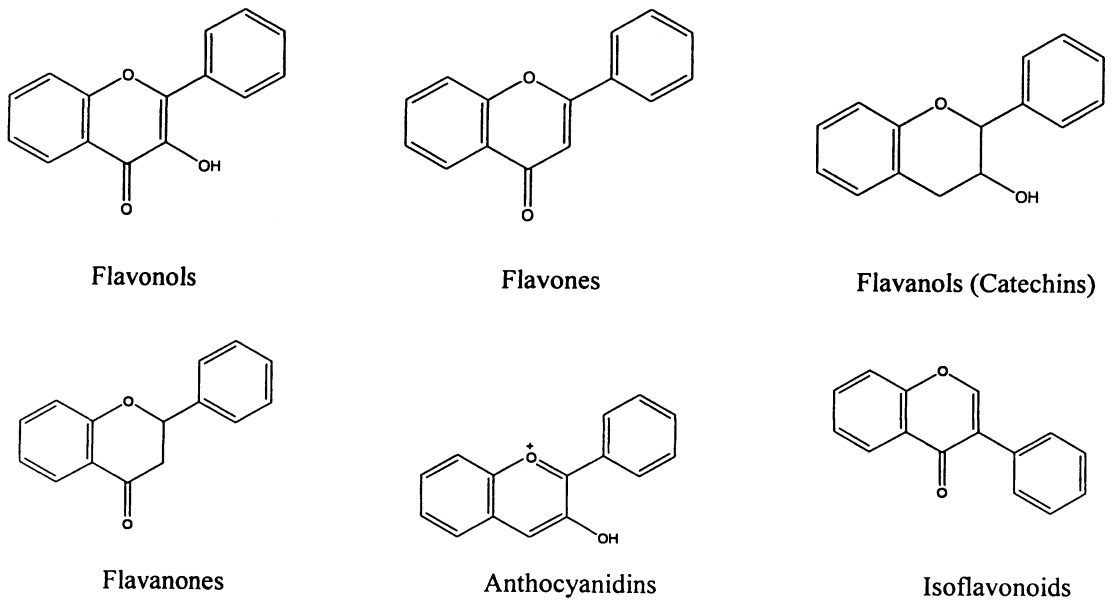

Figure 5. Structures of main classes of dietary flavonoids

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in nature as more than 80 different sugars have been discovered bound to flavonoids (*13*). Anthocyanidins give the red and blue colors in some fruits and vegetables.

Human intake of all flavonoids is estimated at a few hundred milligrams (*14*) to 650 mg/d (*15*). The total average intake of flavonols (quercetin, myricetin, and kaempferol) and flavones (luteolin and apigenin) was estimated as 23 mg/d, of which quercetin contributed ~70%, kaempferol 17%, myricetin 6%, luteolin 4%, and apigenin 3% (*16*).

Phenolic Acids

Phenolic acids, another major source of dietary phenolics, can be subdivided into two major groups, hydroxybenzoic acid and hydroxycinnamic acid derivatives (Figure 7). Hydroxybenzoic acid derivatives include *p*-hydroxybenzoic, protocatechuic, vannilic, syringic, and gallic acids. They are commonly present in the bound form and are typically a component of a complex structure like lignins and hydrolyzable tannins. They can also be found in the form of sugar derivatives and organic acids in plant foods.

Hydroxycinnamic acid derivatives include *p*-coumaric, caffeic, ferulic, and sinapic acids (Figure 7). They are mainly present in the bound form, linked to cell wall structural components such as cellulose, lignin, and proteins through ester bonds. Ferulic acids occur primarily in the seeds and leaves of plants, mainly covalently conjugated to mono- and disaccharides, plant cell wall polysaccharides, glycoproteins, polyamines, lignin, and insoluble carbohydrate biopolymers. Wheat bran is a good source of ferulic acids, which are esterified to hemicellulose of the cell walls. Free, soluble-conjugated, and bound ferulic acids in grains are present in the ratio of 0.1:1:100 (*12*). Food processing, such as thermal processing, pasteurization, fermentation, and freezing, contributes to the release of these bound phenolic acids (*17*).

Caffeic, ferulic, *p*-coumaric, protocatechuic, and vannilic acids are present in almost all plants. Chlorogenic acids and curcumin are also major derivatives of hydroxycinnamic acids present in plants. Chlorogenic acids are the ester of caffeic acids and are the substrate for enzymatic oxidation leading to browning, particularly in apples and potatoes. Curcumin is made of two ferulic acids linked by a methylene in a diketone structure and is the major yellow pigment of turmeric.

Carotenoids

Carotenoids are nature's most widespread pigments with yellow, orange and red colors, and have also received substantial attention because of both their provitamin and antioxidant roles. More than 600 different carotenoids have been identified in nature. They occur widely in plants, microorganisms, and animals. Carotenoids have a 40-carbon skeleton of isoprene units (Figure 8). The

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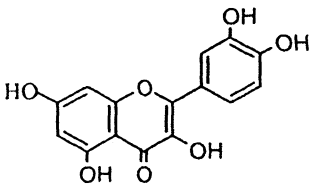
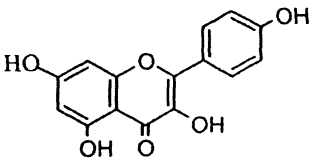
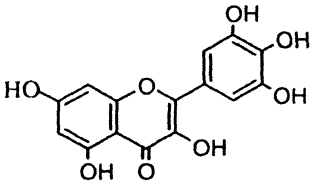


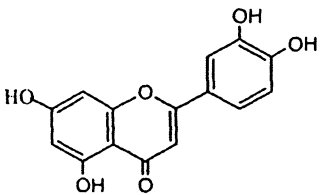
Quercetin



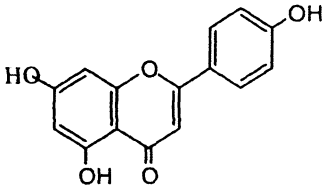
Kaempferol



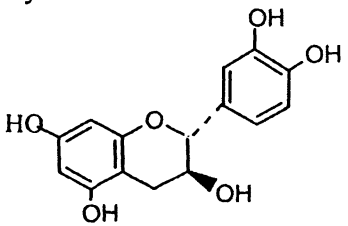
Myricetin



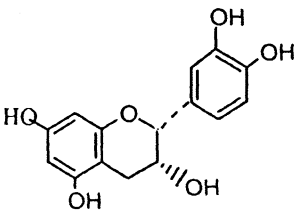
Luteolin



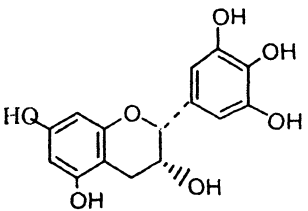
Apigenin



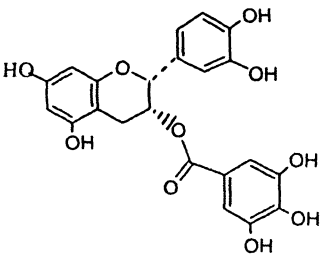
Catechin



Epicatechin



Epigallocatechin (EGC)



Epicatechin gallate (ECG)

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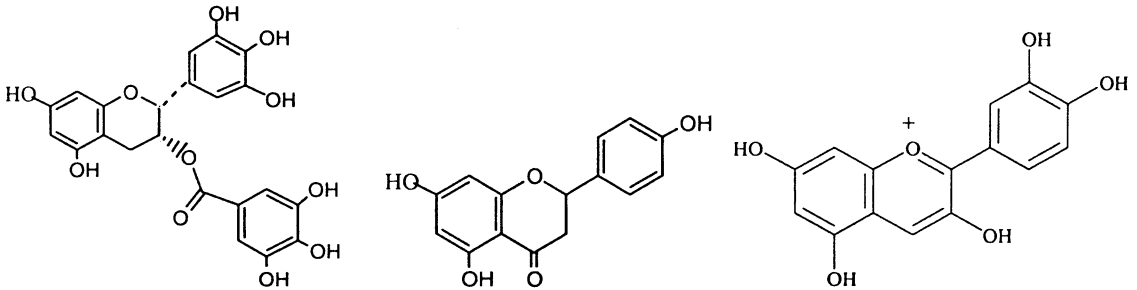
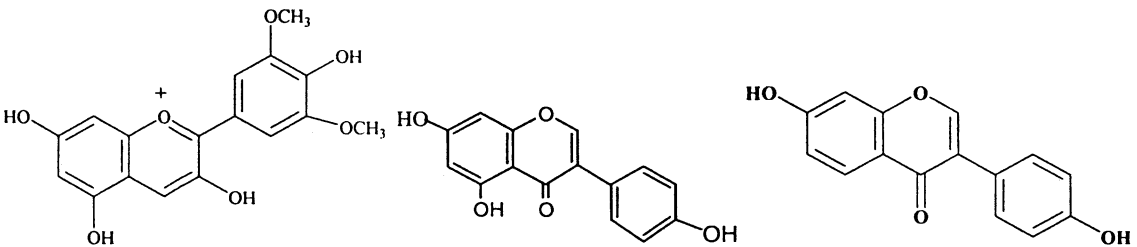


Epigallocatechin gallate (EGCG) Naringenin Cyanidin



Malvidin Genistein Daidzein

Figure 6. Chemical structures of common dietary flavonoids

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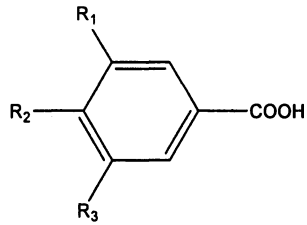
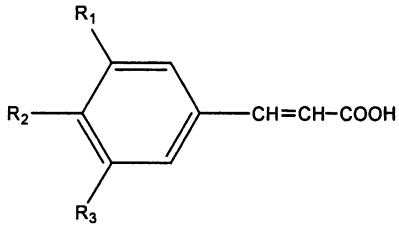
(a) Benzoic acid derivatives



Benzoic acid derivatives	Substitutions		
	R_1	R_2	R_3
Benzoic acid	H	H	H
p-Hydroxybenzoic acid	H	OH	H
Protocatechuic acid	H	OH	OH
Vannilic acid	CH_3O	OH	H
Syringic acid	CH_3O	OH	CH_3O
Gallic acid	OH	OH	OH

(b) Cinnamic acid derivatives



Cinnamic acid derivatives	Substitutions		
	R_1	R_2	R_3
Cinnamic acid	H	H	H
p-Coumaric acid	H	OH	H
Caffeic acid	OH	OH	H
Ferulic acid	CH_3O	OH	H
Sinapic acid	CH_3O	OH	CH_3O

Figure 7. Structures of common phenolic acids: (a) benzoic acid derivatives; (b) cinnamic acid derivatives.

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structure may be cyclized at one or both ends, have various hydrogenation levels, or possess oxygen-containing functional groups. Lycopene and β-carotene are examples of acyclized and cyclized carotenoids, respectively. Carotenoid compounds most commonly occur in nature in the all-*trans* form. The most characteristic feature of carotenoids is the long series of conjugated double bonds forming the central part of the molecule. This gives them their shape, chemical reactivity, and light-absorbing properties. β-Carotene, α-carotene, and β-cryptoxanthin are able to function as provitamin A. Zeaxanthin and lutein are the major carotenoids in the macular region (yellow spot) of the retina in humans.

Orange vegetables and fruits, including carrots, sweet potatoes, winter squash, pumpkin, papaya, mango, and cantaloupe, are rich sources of the carotenoid β-carotene. Tomatoes, watermelons, pink grapefruits, apricots, and pink guavas are the most common sources of lycopene; 85% of American lycopene intake comes from processed tomato products such as ketchup, tomato paste, and tomato soup.

Carotenoid pigments play important functions in photosynthesis and photoprotection in plant tissues. The photoprotection role of carotenoids originates from their ability to quench and inactivate reactive oxygen species such as singlet oxygen formed from exposure of light and air. This photoprotection role is also associated with its antioxidant activity in human health. Carotenoids can react with free radicals and become radicals themselves. Their reactivity depends on the length of the chain of conjugated double bonds and the characteristics of the end groups. Carotenoid radicals are stable by virtue of the delocalization of the unpaired electron over the conjugated polyene chain of the molecules. This delocalization also allows addition reactions to occur at many sites on the radical (*18*). Astaxanthin, zeaxanthin, and lutein are excellent lipid-soluble antioxidants that scavenge free radicals, especially in a lipid-soluble environment. Carotenoids at sufficient concentrations can prevent lipid oxidation and related oxidative stress.

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Role of phytochemicals in the prevention of cancer

Strong epidemiological evidence suggests that regular consumption of fruits and vegetables can reduce cancer risk. Block *et al.* (*19*) reviewed ~200 epidemiological studies that examined the relationship between intake of fruits and vegetables and cancer of the lung, colon, breast, cervix, esophagus, oral cavity, stomach, bladder, pancreas, and ovary. In 128 of 156 dietary studies, the consumption of fruits and vegetables was found to have a significant protective effect. The risk of cancer was 2-fold higher in persons with a low intake of fruits and vegetables than in those with a high intake. Significant protection was found in 24 of 25 studies for lung cancer. Fruit was significantly protective in cancer of the esophagus, oral cavity, and larynx. Fruit and vegetable intake was protective for cancer of the pancreas and stomach in 26 of 30 studies and for

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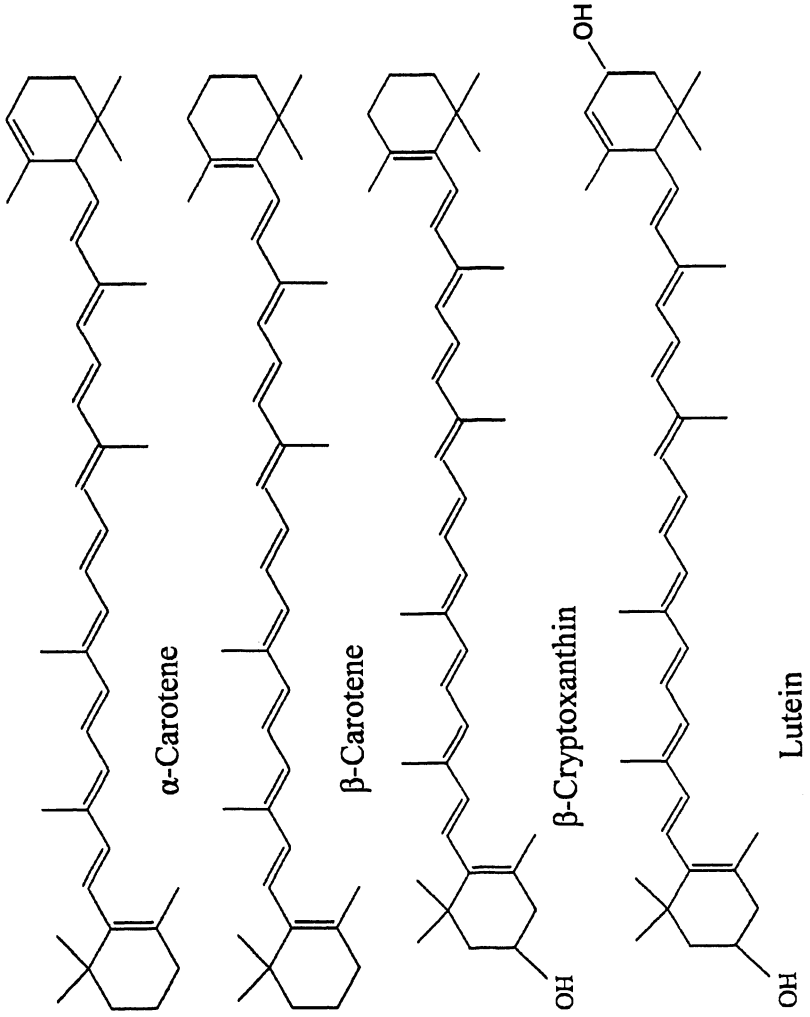

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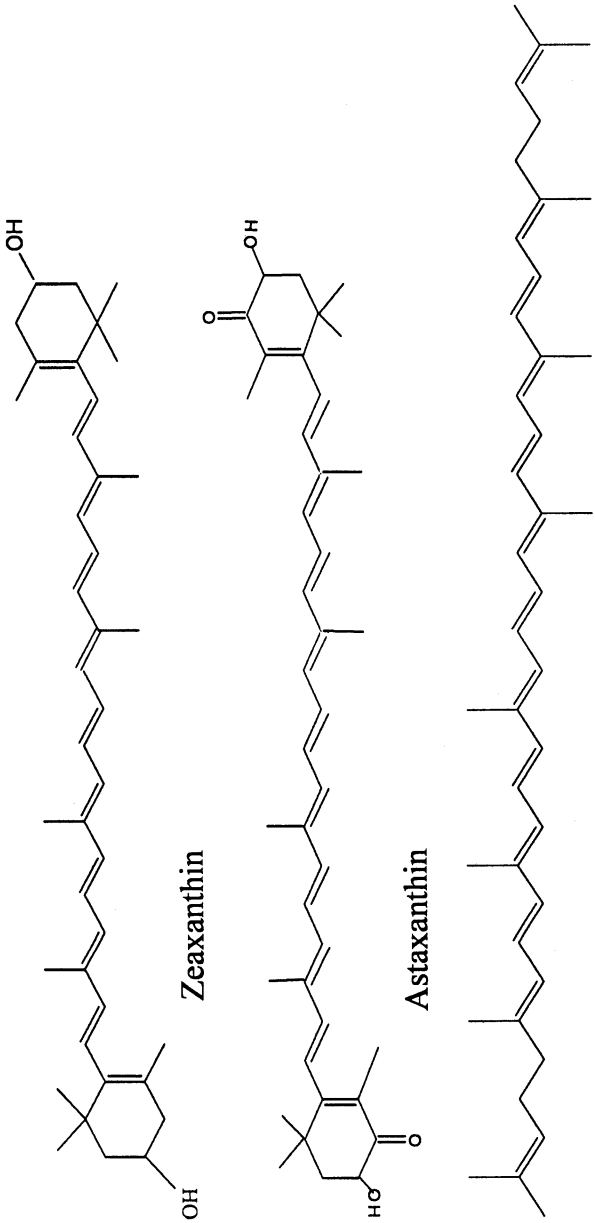

Figure 8. Chemical structures of common dietary carotenoids.

colorectal and bladder cancer in 23 of 38 studies. A prospective study involving 9959 men and women in Finland showed an inverse association between the intake of flavonoids and incidence of cancer at all sites combined (*20*). After a 24-y follow-up, the risk of lung cancer was reduced by 50% in the highest quartile of flavonol intake. Consumption of quercetin from onions and apples was found to be inversely associated with lung cancer risk (*21*). The effect of onions was particularly strong against squamous cell carcinoma. Boyle *et al.* (*22*) showed that increased plasma levels of quercetin after a meal of onions was accompanied by increased resistance to strand breakage by lymphocyte DNA and decreased levels of some oxidative metabolites in the urine.

Cells in humans and other organisms are constantly exposed to a variety of oxidizing agents, some of which are necessary for life. These agents may be present in air, food, and water or they may be produced by metabolic activity within cells. The key factor is to maintain a balance between oxidants and antioxidants to sustain optimal physiological conditions. Overproduction of oxidants can cause an imbalance leading to oxidative stress, especially in chronic bacterial, viral, and parasitic infections (*23*). Oxidative stress can cause oxidative damage to large biomolecules such as lipids, proteins, and DNA, resulting in an increased risk for cancer (*9, 23, 24*).

Carcinogenesis is a multistep process, and oxidative damage is linked to the formation of tumors through several mechanisms (*23, 24*). Oxidative stress induced by free radicals causes DNA damage, which, when left unrepaired, can lead to base mutation, single- and double-strand breaks, DNA cross-linking, and chromosomal breakage and rearrangement (*24*). This potentially cancer-inducing oxidative damage might be prevented or limited by dietary antioxidants found in fruit and vegetables.

Proliferation of normal healthy cells is tightly regulated by a myriad of cell cycle proteins. These proteins work together in complex pathways to ensure that the cell divides only when necessary and without error. Cells may respond to external mitogens, growth factors, or oxidative stress through the MAP (mitogen-activated protein) kinase signaling pathways (*25*). Oxidative stress on the cell membrane can stimulate signaling pathways that will protect the cell such as the stress-activated protein kinase/c-Jun NH_2-terminal kinase (SAPK/JNK) and p38 kinase pathways (*26*). For example, the oxidative stress will activate ASK1 (apoptosis-stimulating kinase 1), which in turn activates the SAPK/JNK kinases, MKK4 and 7 (*27*). These kinases phosphorylate and activate JNK which translocates into the nucleus where it can activate other regulatory proteins such as p53 and the transcription factor Elk-1. In the case of the stress response, p53 will respond by either arresting cell cycle or inducing apoptosis (*28*). In order to induce a G1-phase cell cycle arrest, p53 will stimulate expression of the cyclin-dependent kinase inhibitor (CKI) p21, whose role is to inhibit the Cyclin D1/CDK-4 complex.

Cells under oxidative stress are susceptible to DNA damage that may lead to mutations that alter expression or activity of key regulatory proteins. Such mutations can result in de-regulation of cell cycle and subsequent uncontrolled

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cell proliferation, otherwise known as cancer. These cells are then unable to properly respond to further oxidative stress and are highly susceptible to additional DNA damage. This cycle contributes to increasing genetic instability characteristic of tumor cells (*28*).

Phytochemicals appear to reverse the effects of such mutations by halting the uncontrolled proliferation of cancer cells *in vitro* through induction of cell cycle arrest or apoptosis. For example, the flavonol quercetin has been shown to cause a G2/M arrest and induce apoptosis in a dose-dependent manner in PC-3 androgen independent human prostate cancer cells (*29*). The cells were treated with 25 to 100 μM quercetin for 24 to 72 hours and the EC_{50} for growth inhibition was determined to be 50 μM at 24 h. Flow cytometric analysis revealed that quercetin treatment induced a G2/M accumulation. Effects of quercetin on the relevant cell cycle proteins were then analyzed. The CKI p21 was dramatically induced despite the absence of p53 (PC-3 cells contain a mutation in the p53 gene which renders it inactive). The quercetin was found to down-regulate expression of Cdc2/Cdk-1, which accounted for the increase in hypo-phosphorylated Rb while the amount of total Rb protein remained constant. Cyclin B1 was down-regulated while cyclin A was unaffected. Quercetin has effectively induced G1/S cell cycle arrest in other cell models such as colon and gastric cancers and leukemia, while it has caused G2/M arrest in others, including breast and laryngeals cancers and non-oncogenic fibroblasts (*30*).

Dietary phytochemicals can act to prevent cancer or interfere with its progression at virtually every stage of cancer development. Studies to date have demonstrated that phytochemicals in common fruit and vegetables can have complementary and overlapping mechanisms of action (Table I), including antioxidant activity and scavenging free radicals, regulation of gene expression in cell proliferation, cell differentiation, oncogenes, and tumor suppressor genes; induction of cell cycle arrest and apoptosis, modulation of enzyme activities in detoxification, oxidation, and reduction; stimulation of the immune system, regulation of hormone-dependent carcinogenesis, and antibacterial and antiviral effects (*10, 11, 29-34*).

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Health benefits of phytochemicals in whole foods–food synergy

Phytochemical extracts from fruits, vegetables, and whole grains were shown to have potent antioxidant activity (Figure 2), and the combination of phytochemicals from fruits and vegetables was proposed to be responsible for the potent antioxidant and anticancer activity of these foods (*10, 11, 35*). The total antioxidant activity of phytochemicals in 1 g apples with peel is equivalent to 83.3 μmol vitamin C equivalents; to put it another way, the antioxidant value of 100 g apples is equivalent to 1500 mg vitamin C (*35*). This is far higher than the total antioxidant activity of 0.057 mg vitamin C (the amount of vitamin C in

Table I. Proposed mechanisms by which dietary phytochemicals may prevent cancer

- Antioxidant activity
  - Scavenge free radicals and reduce oxidative stress
- Inhibition of cell proliferation
- Induction of cell differentiation
- Inhibition of oncogene expression
- Induction of tumor suppress gene expression
- Induction of cell cycle arrest
- Induction of apoptosis
- Inhibition of signal transduction pathways
- Enzyme Induction and enhancing detoxification
  - Phase II enzyme
  - Glutathione peroxidase (GPX)
  - Catalase
  - Superoxide dismutase (SOD)
- Enzyme Inhibition
  - Phase I enzyme (block activation of carcinogens)
  - Cyclooxygenase-2 (COX-2)
  - Inducible nitric oxide synthase (iNOS)
  - Xanthine oxide
- Enhancement of immune functions and surveillance
- Antiangiogenesis
- Inhibition of cell adhesion and invasion
- Inhibition of nitrosation and nitration
- Prevention of DNA binding
- Regulation of steroid hormone metabolism
- Regulation of estrogen metabolism
- Antibacterial and antiviral effects

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1 g apples with peel) that is equivalent to 0.32 μmol vitamin C equivalent. In other words, vitamin C in apples contributes less than 0.4% of its total antioxidant activity. Thus, most of the antioxidant activity comes from phytochemicals, not vitamin C. The natural combination of phytochemicals in fruits, vegetables, and whole grains is responsible for its potent antioxidant activity. Apple extracts also contain bioactive compounds that inhibit tumor cell growth *in vitro*. Phytochemicals in apples with peel (50 mg/mL on a wet basis) inhibit colon cancer cell proliferation by 43%. However, this was reduced to 29% when apple without peel was tested (*35*). Recently, we reported that whole apple extracts prevented mammary cancer in a rat model in a dose dependent manner at levels comparable to human consumption of 1, 3, and 6 apples a day (36). This study demonstrated that whole apple extracts effectively inhibited mammary cancer growth in the rat model, thus consumption of apples may be an effective strategy for cancer protection.

Phytochemicals and antioxidants in grains have not received as much attention as the phytochemicals in fruits and vegetables. When people talk about the sources of antioxidants, they always mention fruits and vegetables, not grains, because the antioxidant content in grains has been reported to be low in the literature. Antioxidant content and antioxidant activity of grains reported in the literature has been underestimated since unbound antioxidants are usually studied. We have reported that the major portion of phenolics in the grains is present in the bound form (85% in corn, 75% in oats and wheat, and 62% in rice) (*12, 37*). Therefore, it is clear that the total phytochemical contents of grains have been commonly underestimated in the literature, without determining the content of bound phenolics. Among the grains tested, corn had the highest content of phenolic compounds, followed by wheat, oats, and rice. Actually, whole grains are also good sources of dietary phenolics on a per serving basis (*12, 37*). In addition, bound phytochemicals cannot be digested by human enzymes and could survive stomach and small intestinal digestion to reach the colon, but would then be released in the colon by bacterial fermentation, and potentially play a protective role. This may partially explain the mechanism by which whole grain consumption acts to prevent colon cancer and other digestive cancers.

Different species and varieties of fruit, vegetables, and grains have different phytochemical profiles (*10-12, 37-40*). Therefore, consumers should obtain their phytochemicals from a wide variety of fruits, vegetables, and whole grains for optimal health benefits. In 2003, Temple and Gladwin (*41*) reviewed more than 200 cohort and case-control studies that provided risk ratios concerning intake of fruits and vegetables and risk of cancer. They concluded that cancer prevention is best achieved by consumption of a wide variety of fruits and vegetables, although one group of fruits and vegetables may dominate for a particular cancer. To improve their nutrition and health, consumers should be obtaining antioxidants from their diet and not from expensive dietary supplements, which do not contain the balanced combination of phytochemicals found in fruits and vegetables and other whole foods. More importantly,

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obtaining antioxidants from dietary intake by consuming a wide variety of foods is unlikely to result in consumption of toxic quantities because foods originating from plants contain many diverse types of phytochemicals in various quantities. Fruits and vegetables eaten in the recommended amounts (5–10 servings of fruit and vegetables per day) are safe. Furthermore, health benefits from the consumption of fruits and vegetables extend beyond lowering the risk of developing cancers and CVD: benefits also include preventive effects on other chronic diseases such as cataracts, age-related macular degeneration, central neurodegenerative diseases, and diabetes (*42*).

The additive and synergistic effects of phytochemicals in fruits and vegetables have been proposed to be responsible for their potent antioxidant and anticancer activities. The benefit of a diet rich in fruits and vegetables is attributed to the complex mixture of phytochemicals present in these and other whole foods (*10, 11, 35, 40*). This partially explains why no single antioxidant can replace the combination of natural phytochemicals in fruits and vegetables in achieving the observed health benefits. Thousands of phytochemicals are present in whole foods. These compounds differ in molecular size, polarity, and solubility, which may affect the bioavailability and distribution of each phytochemical in different macromolecules, subcellular organelles, cells, organs, and tissues. This balanced natural combination of phytochemicals present in fruit and vegetables cannot simply be mimicked by pills or tablets.

Research progress in antioxidant and bioactive compounds has boosted the dietary supplement and nutraceutical industries. The use of dietary supplements is growing, especially among baby-boomer consumers. However, many of these dietary supplements have been developed based on the research results derived from biochemical and chemical analyses and studies, *in vitro* cell culture studies, and *in vivo* animal experiments and not from human intervention studies. The health benefits of natural phytochemicals at the low levels present in fruits and vegetables does not mean that these compounds are more effective or safe when they are consumed at a higher dose, even in a pure dietary supplement form. Therefore, a thorough understanding of the efficacy and long-term safety of many dietary supplements needs further investigation.

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Conclusions

Dietary modification by increasing the consumption of a wide variety of fruits, vegetables, and whole grains daily is a practical strategy for consumers to optimize their health and reduce the risk of chronic diseases. Use of dietary supplements, nutraceuticals, and functional foods is increasing as industry is responding to consumers' demands. However, more information about the health benefits and possible risks of dietary supplements is needed to ensure their efficacy and safety. Phytochemical extracts from fruits and vegetables have strong antioxidant and antiproliferative activities, and the major part of total antioxidant activity is from the combination of phytochemicals. The additive

and synergistic effects of phytochemicals in fruits and vegetables are responsible for their potent antioxidant and anticancer activities. The benefit of a diet rich in fruits, vegetables, and whole grains is attributed to the complex mixture of phytochemicals present in these and other whole foods. This explains why no single antioxidant can replace the combination of natural phytochemicals in fruits and vegetables and achieve their health benefits. Therefore, the evidence suggests that antioxidants are best acquired through whole food consumption, not from expensive dietary supplements. Further research on the health benefits of antioxidants and phytochemicals in whole foods is warranted.

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Chapter 4

Measurement of Antioxidant Activity in Food and Biological Systems

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The importance of lipid oxidation and antioxidants in biological systems and foodstuff has been widely recognized. Oxidative changes involving free radicals in membrane lipids are thought to have destructive cellular effects *in vivo*; whereas oxidative processes in foods result in flavor and nutritional quality deterioration that may also affect their safety and wholesomeness. Antioxidants protect cells and foods against oxidative stress. Many methods have been developed for evaluating the activity of antioxidants; these may be classified into two categories. The first category measures the ability of antioxidants in inhibiting oxidation reaction in a model system by monitoring the associated changes using physical, chemical or instrumental means. Radical scavenging assays include methods based on hydrogen atom transfer (HAT) or electron transfer (ET) mechanisms. Oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant parameter (TRAP) and crocin bleaching assays are the major methods that measure HAT while Trolox equivalent antioxidant capacity (TEAC), ferric reducing antioxidant power (FRAP), and DPPH assays represent ET-based methods. An overview of relevant methods for evaluating antioxidant activity with emphasis on the chemistry, basic principles involved as well as advantages and disadvantages of each assay is provided.

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Lipid oxidation is a major cause of food quality deterioration. Concerns about lipid oxidation have been addressed in the food industry because the oxidation products influence the flavor and nutritional value of food products and some may render health-damaging effects to humans. Antioxidants have thus attracted much interest of the food scientists, medical and nutritional experts as well as the general public. The term antioxidants is defined as substances that when present in foods at low concentrations compared to that of an oxidizable substrate markedly delay or prevent the oxidation of the substrate (*1*). Antioxidants that fit in this definition include free radical scavengers, reducing agents, inactivators of peroxides and other reactive oxygen species (ROS), chelators of metals, and quenchers of secondary lipid oxidation products that produce rancid odors (*2*). These substances may occur naturally in foods, such as tocopherols, ascorbic acid and some phenolic compounds, or are synthesized and used as food additives such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and *tert*-butylhydroquinone (TBHQ). Addition of antioxidants has been carried out in many food systems in order to minimize or negate oxidative deterioration of lipid. Antioxidants have also been used in the health-related areas because of their ability to protect the body against damage caused by ROS as well as reactive nitrogen species (RNS) and those of chlorine (RCS) (*3*). Antioxidants can be broadly classified by their mechanism of action as primary antioxidants, which break the chain reaction of oxidation by hydrogen donation and generation of more stable radicals; and secondary antioxidants, which slow the oxidation rate by several mechanisms, including chelation of metal ions, regeneration of primary antioxidants, decomposition of hydroperoxides and scavenging of oxygen, among others (*4*). Primary antioxidants such as tocopherols and some phenolic compounds inhibit the chain reaction of oxidation (as shown below) by acting as hydrogen donors or free radical acceptors. The free radical mechanism of lipid oxidation includes three distinct steps of initiation, propagation and termination, as outlined.

The inhibition reaction is considered in competition with the propagation step of lipid oxidation and yields stable products that will not initiate new free radicals or bring about a rapid oxidation via a chain reaction (*5*).

Natural and/or synthetic antioxidants used in fat-containing foods inhibit lipid oxidation with a wide range of efficiencies, depending on their properties, concentrations and processing conditions, leading to varied antioxidant activities. Other terms such as antioxidant capacity, efficiency and potency have also been used to describe the power of an antioxidant to inhibit lipid oxidation.

Measurement of Antioxidant Activity

The need to measure antioxidant activity is well documented; these are carried out for meaningful comparison of foods or commercial products and for

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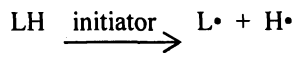
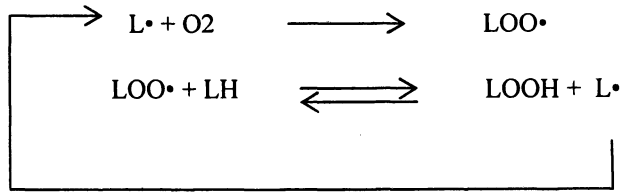
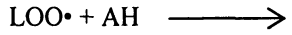
Initiation:



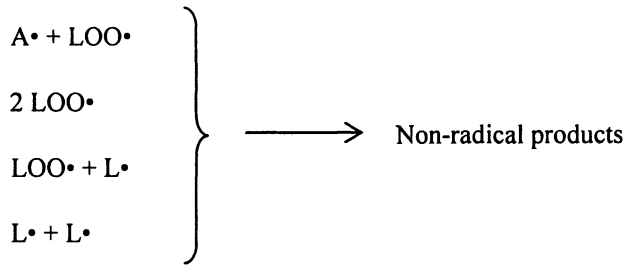
Propagation:



Inhibition:



Termination:



provision of quality standards for regulatory issues and health claims (*6*). There are numerous methods for measuring antioxidant activity, all of which include certain essential features such as a suitable substrate, an oxidation initiator, and an appropriate measure of end point (*7*). Therefore, these and other aspects should be taken into consideration when selecting a test for measuring antioxidant activity. Thus, model food systems may be used, some of which are carried out under accelerated oxidation conditions. Normally, most assessments of antioxidant activity are performed in oil, or other model systems, giving sensible prediction for the activity in oil or water-in-oil emulsions, whereas the results may be misleading for oil-in-water emulsions (*8*). It should also be ensured that the starting lipid does not contain high levels of oxidation products (*9*). Several oxidation products of lipids such as hexanal and 2,4-decadienal have been reported to exhibit pro-oxidative effect (*10*). Furthermore, stripping of oils may be necessary in such evaluations because the endogenous antioxidants in non-stripped oils are found to enhance the oxidative stability of test samples, thus giving rise to erroneous results about the efficiency of antioxidants under investigation (*11-13*). In addition to oils and fats, lipid substrates used for testing

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antioxidant activity may include fatty acids, fatty acid ethyl esters or triacylglycerols (*7*), and β-carotene (*14-16*). In some cases such as radical scavenging methods, no substrate is used. Most test procedures involve initiators to accelerate oxidation. The combination of increased temperature and oxygen supply, addition of metal catalysts, and exposure of the reactants to light can reduce the oxidative stability to a large extent (*7, 8*). Nevertheless, the elevated temperature may bring about changes in the oxidation mechanism thus causing difficulties in the prediction of antioxidant activity at low temperatures as compared with those at high temperatures (*7, 8*). Therefore, high oxidation temperatures (>60°C) are not recommended for testing antioxidant activity (*9*). After the substrate is oxidized under standard conditions, the oxidation is monitored by chemical, instrumental or sensory methods. An appropriate measure of end point is essential for assessing antioxidant activity. Analytical strategies for end point determination include measurement at a fixed time point, measurement of reaction rate, lag phase measurement, and integrated rate measurement (*7*). The resulting antioxidant activity may be expressed using a wide range of parameters (Table 1).

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Evaluation of Antioxidants in Inhibition of Lipid Oxidation in Model Systems

Methods proposed for testing antioxidant activity fall into two major categories: measuring of the current state of oxidation in model systems, and radical scavenging assays. A large group of tests for antioxidant activity measurements have been considered by assessing the current oxidative state of a model system in the presence and absence of an antioxidant. The extent that the oxidation process is inhibited by added antioxidant indicates its antioxidant activity. The process of lipid oxidation can be monitored through a number of chemical and physico-chemical procedures, including measuring the substrate, oxidant/initiator, intermediates or final products. Quantification of the loss of reactants, depletion of oxygen and formation of primary or secondary oxidation products may provide the most appropriate markers depending on the stage of oxidation (*17*). Methods for measuring lipid oxidation have been reviewed elsewhere (*18*).

Measurement of Oxygen Absorption

Consumption of oxygen during the initial stages of lipid oxidation results in an increase in the weight of the oil or a drop of headspace oxygen pressure (or concentration). Therefore, these parameters may be used for measuring

Table 1. Major methods of expressing results of antioxidant activity tests

Methods	Dimensions
Induction period	h (hour), d (day)
Time to reach a set level of oxidation (pre-induction period)	h, d
Rate of oxidation (pre-induction period)	mol kg^{-1} h^{-1}, gL^{-1} d^{-1}
Concentration of oxidation product after set time period	mg kg^{-1} (ppm, w/w)
Scale reading after set time period	Absorbance, conductivity, etc.
Concentration to produce equivalent effect to reference antioxidant (pre-induction period)	mol kg^{-1}, gL^{-1}
ORAC, oxygen radical absorbance capacity	μmol of Trolox equivalents
Total radical-trapping antioxidant parameter (TRAP)	μmol peroxyl radical deactivated L^{-1}
ABTS assay, phycoerythrin assay	TEAC (mM Trolox equivalent to 1 mM test substance)
Free stable radical quenching (DPPH)	Percentage inhibition; EC_{50}, concentration to decrease concentration of test free radical by 50%; T_{EC50}, time to decrease concentration of test free radical by 50%; AE, (1/ EC_{50}) T_{EC50}
FRAP assay	Absorbance of Fe^{2+} complex at 593 nm produced by antioxidant reduction of corresponding tripyridyltriazine Fe^{3+} complex
Metal chelating assay	Percentage of inhibition of ferrozine-Fe^{2+} complex formation

Adapted from reference (*7*).

antioxidant activity by comparing the results in the presence and absence of an antioxidant. The oxygen absorption is usually determined by means of monitoring weight gain or headspace oxygen uptake.

Heating an oil and periodically testing for weight gain is one of the oldest methods for evaluating its oxidative state (*7*). This method requires simple

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equipment and indicates directly oxygen absorption through mass change. Oil samples are weighed, and stored in an oven at a set temperature with no air circulation. To avoid the influence of mass change by volatiles, samples can be preheated in an inert atmosphere. Sample vials are then taken out of the oven at different time intervals, cooled to ambient temperature and reweighed; the weight gain is then recorded. The induction period can be obtained by plotting weight gain against storage time. As a physical method for measuring lipid oxidation, the weight gain method has several drawbacks such as discontinuous heating of the sample, which may give rise to nonreproducible results, and requiring long analysis time and intensive human participation (*19*). Nevertheless, this method offers advantages such as low instrumentation cost as well as a high capacity and processing speed of samples without limitation (*19*). Antolovich *et al.* (*7*) suggested that this technique may be extended to more sophisticated continuous monitoring of mass and energy changes as in thermogravimetry (TG)/differential scanning calorimetry (DSC). Nevertheless, this method is most suitable for evaluation of highly unsaturated oils, such as marine oils and vegetable oils containing a high content of polyunsaturated fatty acids as the oxidizable substrates.

In addition to the weight gain method, oxygen consumption can be measured directly by monitoring the drop of oxygen pressure. Using headspace oxygen method, an oil sample is placed in a closed vessel also containing a certain amount of oxygen at elevated temperatures, commonly around 100°C. The pressure reduction in the vessel which is due to the oxygen consumption is monitored continuously and recorded automatically. The induction period as the point of maximum change in rate of oxygen uptake can be calculated (*20*). Oxidograph is a commercial instrument that is available and measures the pressure change in the reaction vessel electronically by means of pressure transducers (*20*).

Oxygen consumption may also be measured electrochemically by detecting changes in oxygen concentration. The use of a semi-automatic polarographic method has been proposed as an improvement for evaluation of lipid oxidation by determination of oxygen consumption (*21*). This method is based on use of two oxygen meters with microcathode oxygen electrodes, coupled to a computerized data collection and a processing unit (*21*).

The headspace oxygen method is simple and reproducible and may be one of the best analytical tools for evaluation of lipid oxidation and antioxidant activity. However, it should be ensured that no protein is present in the model system since protein oxidation also proceeds with oxygen consumption (*22*).

Changes in the Reactants

Lipid oxidation may also be assessed quantitatively by measuring the loss of initial substrates. In foods containing fats or oils, unsaturated fatty acids are

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the main reactants whose composition changes significantly during oxidation. Changes in fatty acid composition provide an indirect measure of the extent of lipid oxidation (*22*). This method can also be applied in testing antioxidant activity as antioxidants exert a significant inhibitory effect on oxidation of unsaturated fatty acids, which can be detected by gas chromatography (GC). Fatty acid methyl esters (FAME) are commonly used for determination of fatty acid composition. Similar to the oxygen absorption method, it is recommended that highly unsaturated oils be used as the model system.

Measurement of Oxidation Products

Titration Method

Lipid oxidation involves continuous formation of hydroperoxides as primary oxidation products which may break down to a variety of non-volatile and volatile secondary products (*22, 23*). The rate of formation of hydroperoxides outweighs their rate of decomposition during initial stages of oxidation, and this becomes reversed at later stages. Therefore, the peroxide value (PV) is an indicator of the initial stages of oxidative change (*24*). Inhibition of formation and/or action of these unstable species by antioxidants can be used for assessing antioxidant activity (*7*).

A number of methods have been developed for determination of PV, among which the iodometric titration, ferric ion complex measurement spectrophotometry, and infrared spectroscopy are most frequently used (*25*). Iodometric titration assay, which is based on the oxidation of the iodide ion (I^-) by hydroperoxides (ROOH), is the basis of current standard methods for determination of PV. In this method, a saturated solution of potassium iodide is added to oil samples to react with hydroperoxides. The liberated iodine (I_2) is then titrated with a standardized solution of sodium thiosulfate and starch as an end point indicator (*19,26*). The PV is calculated and reported as milliequivalents of oxygen per kilogram of sample (meq/kg). The official determination is described by IUPAC (*27*). Chemical reactions involved are given below.

$$ROOH + 2H^+ + 2KI \longrightarrow I_2 + ROH + H_2O + 2K^+$$
$$I_2 + 2NaS_2O_3 \longrightarrow Na_2S_2O_6 + 2NaI$$

Although iodometric titration is the most common method for measurement of PV, it suffers from several disadvantages. The procedure is time consuming and labor intensive (*24*). This technique requires a large amount of sample and generates a significant amount of waste (*24, 28, 29*). Furthermore, possible absorption of iodine across unsaturated bonds and oxidation of iodide by

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dissolved oxygen are among potential drawbacks of this method (*7, 19*). Lack of sensitivity, possible interferences and difficulties in determining the titration end point are also among the main limitations (*23, 29*). To overcome these drawbacks, novel methods based on the same reaction have been developed. Thus, techniques such as colorimetric determination at 560 nm (*30*), potentiometric end point determination (*31*), and spectrophotometric determination of the I_3^- chromophore at 290 or 360 nm (*32, 33*) have been proposed. In addition, an electrochemical technique has been used as an alternative to the titration step in order to increase the sensitivity for determination of low PV by reduction of the released iodine at a platinum electrode maintained at a constant potential (*19*).

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Spectrophotometric Methods

Other chemical methods based on the oxidation of ferrous ion (Fe^{2+}) to ferric ion (Fe^{3+}) in an acidic medium and the formation of iron complexes have also been widely accepted for determination of PV. These methods spectrophotometrically measure the ability of lipid hydroperoxides to oxidize ferrous ions to ferric ions, which are complexed by either thiocyanate or xylenol orange (*29, 34*). Ferric thiocyanate is a red-violet complex that shows strong absorption at 500-510 nm (*23*). The method of determining PV by coloremetric detection of ferric thiocyanate is simple, reproducible and more sensitive than the standard iodometric assay. The ferrous oxidation of xylenol orange (FOX) assay uses dye xylenol orange to form a blue-purple complex with a maximum absorption at 550-600 nm (*23*). This method is rapid, inexpensive, and not sensitive to ambient oxygen or light (*35*). It can consistently quantify lower hydroperoxide levels, and good agreement between FOX and iodometric method has been found (*35*). The FOX method has been successfully adapted to a variety of applications. However, because many factors such as the amount of oil sample, solvent used and source of xylenol orange may affect the absorption coefficient, knowledge of the nature of hydroperoxides present in the oil sample and careful control of the conditions used are required for accurate measurements (*23*).

Conjugated Dienes and Trienes

Polyunsaturated fats and oils are generally methylene-interrupted in nature. Upon oxidation, conjugated dienes and trienes are formed and these give rise to absorption peaks at 234 and 268 nm in the ultraviolet (UV) region (*19*). An increase in UV absorption theoretically reflects the formation of primary oxidation products in fats and oils. Therefore, antioxidant activity can be assessed by lag phase measurements and percentage inhibition in the presence of

a certain antioxidant (*7*). Good correlations between conjugated dienes and peroxide value have been found (*36, 37*).

Ultraviolet detection of conjugated dienes is simple, fast, and requires no chemical reagents and only small amounts of samples are needed. However, this method has less specificity and sensitivity than PV measurement (*7, 8*). Furthermore, the result may be affected by the presence of compounds absorbing in the same region, such as carotenoids (*19*). To avoid these interferences, an alternate spectroscopic method measuring conjugable oxidation products (COPs) has been proposed. In this method, hydroperoxides and some decomposition products are converted to more conjugated chromophores by reduction and subsequent dehydration (Figure 1). The concentrations of the resultant conjugated trienes and tetraenes are determined from their respective absorption at 268 and 301 nm and expressed as COP values (*8, 19*).

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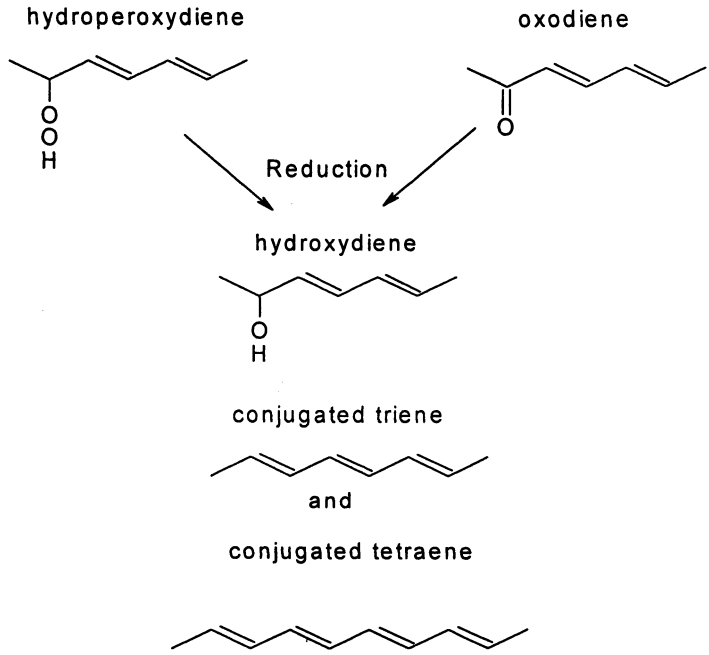

Figure 1. Chemical reaction steps in COP

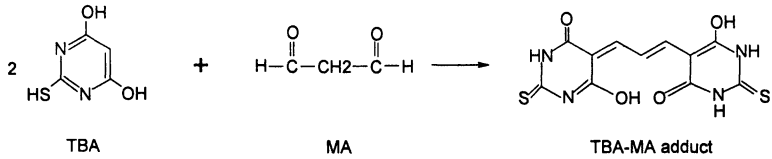

Figure 2. Reaction of 2-thiobarbituric acid (TBA) and malonaldehyde (MA)

Thiobarbituric Acid (TBA) Test

During lipid oxidation, malonaldehyde (MA), a minor component of fatty acids with 3 or more double bonds, is formed as a result of the degradation of polyunsaturated fatty acids. Thus, MA is usually used as an indicator of the lipid oxidation process, both for the early appearance as oxidation occurs and for the sensitivity of the analytical method (*38*). In this assay, MA reacts with 2-thiobarbituric acid (TBA) to form a pink MA-TBA complex that is measured spectrophotometrically at its absorption maximum at 530-535 nm (Figure 2) (*7, 39, 40*). The extent of oxidation is reported as the TBA value and is expressed as milligrams of MA equivalents per kilogram sample or as micromoles of MA equivalents per gram of sample. It must, however, be noted that alkenal and alkadienals also react with the TBA reagent and produce a pink color. Thus the term thiobarbituric acid reactive substances (TBARS) is now used instead of MA.

The TBA test is used frequently to assess the oxidative state of a variety of food systems, despite its limitations such as lack of specificity and sensitivity (*40*). As already noted, many other substances may react with the TBA reagent and contribute to absorption, causing an overestimation of the intensity of color complex (*40*). Interferences may come from additional absorption of other alkanals, 2-alkenals, 2,4-alkdienals, ketones, ketosteroids, acids, esters, proteins, sucrose, urea, pyridines, and pyrimidines, also referred to as TBARS (*39, 41*). For instance, the reaction of TBA with various aldehydes leads to the development of a yellow chromogen (aldehyde-TBA adduct) with an absorption maximum at 450 nm, which overlaps with the pink peak at 532 nm resulting in erroneously high TBA values in certain cases (*39, 42, 43*). Furthermore, the presence of impurities in the TBA reagent may produce TBA-MA-barbituric acid and MA-barbituric acid adducts that absorb at 513 and 490 nm, respectively, indicating that thiobarbituric acid should be purified before use (*39*). In addition, nitrite can interfere in the TBA test, while sulfanilamide could

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be added to samples to avoid the interference when residual nitrite is present (*44*). In order to improve the specificity and sensitivity of the TBA test, several modifications to the original TBA procedures have been proposed, including reduction of the heating temperature to stabilize the yellow color aldehyde-TBA complex (*45*), extraction of the MA prior to the formation of the chromogen (39), direct FTIR analysis of TBARS, and use of HPLC to separate the complex before measurement or to characterize the individual species of TBARS (*7, 39*). TBA test has been applied in antioxidant activity measurement. A reduction in absorbance by the addition of an antioxidant can be seen and quantified in terms of percentage inhibition of the oxidation.

p-Anisidine Value (p-AnV)

The *p*-anisidine value (*p*-AnV) is a measure of the content of aldehydes (principally 2-alkenals and 2,4-alkadienals) generated during the decomposition of hydroperoxides. It is based upon the color reaction of *p*-methoxyaniline (anisidine) and the aldehydic compounds (*46*). The reaction of *p*-anisidine reagent with aldehydes under acidic conditions affords yellowish products that absorb at 350 nm (Figure 3) (*8, 19*). The color is quantified and converted to *p*-AnV. The *p*-AnV is defined as the absorbance of a solution resulting from the reaction of 1 g of fat in isooctane solution (100 ml) with *p*-anisidine (0.25% in glacial acetic acid) (*8*). This test is more sensitive to unsaturated aldehydes than to saturated aldehydes because the colored products from unsaturated aldehydes absorb more strongly at this wavelength (*8*). However, it correlates well with the amount of total volatile substances (*46*).

The *p*-AnV is a reliable indicator of oxidative rancidity in fats and oils and thus a good approach to measure inhibition of oxidation by antioxidants. A highly significant correlation between *p*-AnV and flavor scores and PV has been found (*47*). This method is used less frequently in North America, but is widely employed in Europe (*48*), particularly as a part of the Totox number (Totox value = 2 PV + *p*-AnV) determination. Nevertheless, it should be noted when using *p*-AnV as an index of oxidative stability that *p*-AnV is comparable only within the same oil type because initial AnV varies among oil sources (*49*). For instance, oils with high levels of polyunsaturated fatty acids might have higher AnV even when fresh (*50*).

Carbonyls

The carbonyl compounds, including aldehydes and ketones, are the secondary oxidation products generated from degradation of hydroperoxides, and are suggested to be the major contributors to off-flavors associated with the rancidity of many food products (*7*). The analysis of total carbonyl compounds,

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which is based on the absorbance of the carbonyl derivatives, provides another approach to measure the extent of lipid oxidation in fats and oils. In this method, the total carbonyl content is measured by a colorimetric 2,4-dinitrophenylhydrazone procedure. The carbonyl compounds formed during lipid oxidation are reacted with 2,4-dinitrophenylhydrazine (DNPH) followed by the reaction of the resulting hydrazones with alkali (Figure 4). The final colored products are then analyzed spectrophotometrically at a given wavelength (*19, 22*). Many variations of this method using an alternate solvent, reagent, wavelength, or workup have been reported.

The determination of total content of carbonyls has been used in different oxidative stability studies. However, when this method is used for evaluation of antioxidant activity, model system selected should be in absence of protein as carbonyls produced from protein oxidation may also give rise to higher values than those expected from lipid oxidation alone. Meanwhile, high temperature must be avoided because some short-chain carbonyls may be removed and hence affecting the total carbonyls.

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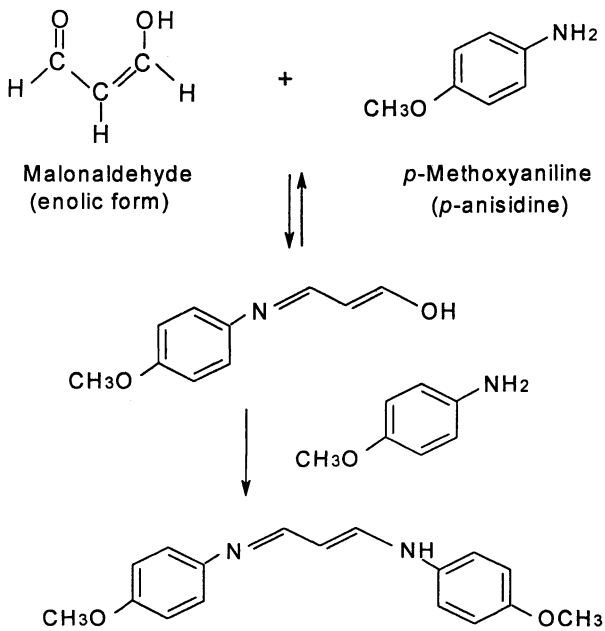

Figure 3. Possible reactions between p-*anisidine reagent and MA*

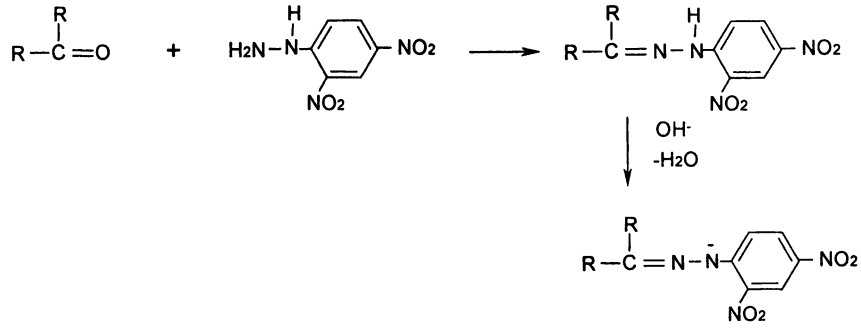

Figure 4. Reactions between carbonyls and 2,4-dinitrophenylhydrazine

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Infrared Spectroscopic Technique

It has been recognized that hydroperoxides, the primary products of lipid oxidation, can quantitatively be determined by IR spectroscopy via measurement of their characteristic O-H stretching absorption band (*51*). An absorption band at about 2.93 μm indicates the generation of hydroperoxides, whereas the replacement of a hydrogen atom on a double bond or polymerization accounts for the disappearance of a band at 3.20 μm.

A rapid Fourier transform infrared spectroscopy (FTIR) method based on the stoichiometric reaction of triphenylphosphine (TPP) with hydroperoxides has been developed and successfully applied to determination of PV (*52*). The hydroperoxides present in oil samples react stoichiometrically with TPP to produce triphenylphosphine oxide (TPPO), which has an intense absorption band at 542 cm^{-1} in the mid-IR spectrum (*23, 24*). The band intensity is measured and converted to peroxide value. The chemical reaction involved is given below.

$$ROOH + TPP \longrightarrow TPP{=}O + ROH$$

By using *tert*-butylhydroperoxide spiked oil standards and evaluation of the band formed at 542 cm^{-1} a linear calibration graph covering the range of 1-100 PV was obtained (*24*).

More recently, disposable polymer IR (PIR) cards have been used as sample holders where unsaturated oil samples oxidize at a fairly rapid rate (*53*). In the FTIR/PIR card method, warm air flows continuously over the sample allowing oxidation to be monitored at moderate temperatures. At periodic intervals, individual cards are removed and the FTIR spectra scanned (*53*). Another new FTIR approach uses flow injection analysis (FIA), which offers exact and highly reproducible timing of sample manipulation and reaction as well as a closed environment with oxygen and light being easily excluded (*24*).

The FTIR spectroscopy is a simple, rapid and highly precise method. It shows excellent correlation with the iodometric titration method and avoids the solvent and reagent disposal problems associated with the standard wet chemical method (*24, 52*). The FTIR method provides an automated, efficient and low-cost means of evaluating oxidation in oils undergoing thermal stress and has gained considerable interest for quality control in the industry (*23, 26, 54*). However, there is a need to characterize the spectral changes, assign wavelengths to more common molecular species produced and access potential spectral cross interferences (*26*). Recently, an improved FTIR attenuated total reflectance (FTR-ATR) method using the whole FTIR spectral data instead of particular wavenumbers has been proposed (*54*).

In addition to hydroperoxides, other oxidation products such as carbonyls can also be detected and quantified by IR spectroscopy. The formation of aldehydes, ketones or acids gives rise to an extra band at 5.72 μm. Furthermore, cis-, trans- isomerization and formation of conjugated dienes can be detected through the changes in the absorption band in the range of 10 to 11 μm (*19*). This method measures the current oxidative state of a model system and the inhibition of oxidation by an antioxidant can be obtained and used as an indicator of antioxidant activity.

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Chromatographic Methods

Oxidation products of fats and oils can also be measured by chromatographic techniques, mainly gas chromatography (GC) and high performance liquid chromatography (HPLC). These methods characterize specific oxidation products such as hydroperoxides and individual species of TBARS, while other assays measure their total amount. Various protocols have been employed for separation and detection of a selected compound depending on its physical or chemical properties. For example, GC, equipped with a headspace sampler, is frequently used in determining secondary volatile products formed during oxidation, such as propanal in oils high in ω3 fatty acids, and hexanal in oils high in ω6 fatty acids. GC has also been a useful technique for determination of hydrocarbon products from lipid oxidation such as ethane, propane and pentane. Volatilization is required in this method. HPLC, however, shows advantages over GC for hydroperoxide analysis. It operates at room temperature, thus decreases the risk of artifact formation, and no previous derivatization is required (*23*). A wide range of hydroperoxides can be analyzed using either normal or reverse phase HPLC.

Chromatographic methods require small amounts of sample, and interference from minor compounds other than the selected product can be easily excluded (*23*). Moreover, measurement of an individual compound offers the advantage of analyzing a single, well-defined end product for antioxidant activity studies (*7*).

Electrical Conductivity Method

During lipid oxidation, volatile organic acids, mainly formic and acetic acids, are produced as secondary volatile oxidation products at high temperatures, simultaneously with hydroperoxides (*26, 55*). In addition, other secondary products including alcohols and carbonyl compounds can be further oxidized to carboxylic acids (*26*). The formation of volatile acids can be measured by monitoring the change in electrical conductivity when effluent from oxidizing oils is passed through water (*8*). This method provides the oil stability index (OSI) value, which is defined as the point of maximal change of the rate of oxidation, attributed to the increase of conductivity by the formation of volatile organic acids during lipid oxidation (*56*). Two commercially available equipment, the Rancimat (Metrohm Ltd.) and the Oxidative Stability Instrument (Omnion Inc.) are employed for determining the OSI value. This method requires a somewhat higher level of oxidation (PV>100) to obtain measurable results than other methods in which hydroperoxides are the most important products formed and detected (*57*). Therefore, for some oils that are stable under normal conditions, the oxidation process is accelerated by exposing them to elevated temperatures in the presence of excess air or oxygen (*58, 59*). In the Rancimat assay, a flow of air is bubbled through a heated oil, usually at 100°C or above. For marine oils temperatures as low as 80°C are often used. Volatile compounds formed during accelerated oxidation are collected in distilled water, increasing the water conductivity. The change of conductivity is plotted automatically and the induction period of the oil or the time taken to reach a fixed level of conductivity is recorded (*26, 60*). The Rancimat assay enables continuous monitoring of the oxidation process. Excellent correlation between Rancimat and conjugated dienes has been found (*59*). Another apparatus, the Oxidative Stability Instrument, operates on the same principle as the Rancimat, and has the capacity of simultaneously analyzing up to 24 samples (*26*).

Although this method is useful for evaluation of lipid oxidation, it is not always recommended for measuring antioxidant activity, especially for evaluation of thermosensitive compounds. The high temperatures used do not allow reliable prediction of antioxidant effectiveness at lower temperatures. Volatile antioxidants may be swept out of the oil by the air flow under test conditions, and also the oils are severely deteriorated when end point is reached (*8*).

Formation of Free Radicals

The initial steps of lipid oxidation involve chain reactions of free radicals as important short-lived intermediates. Oxidation level of fats and oils can be measured directly by detecting the formation of radicals. Methods based on the

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detection of radicals or on the tendency for the formation of radicals provide a good indication of initiation of lipid oxidation (*61, 62*). Electron spin resonance (ESR), also referred to as electron paramagnetic resonance (EPR) spectroscopy, relies on the paramagnetic properties of the unpaired electrons in radicals and has been developed for assessing the formation of free radicals originating in the early stages of oxidation and the onset of primary oxidation (*62, 63*). The assay measures the absorption of microwave energy when a sample is placed in a varied magnetic field (*19*). Quantification of radical concentrations is complicated by comparison with stable paramagnetic compounds such as transition metals and nitroxyl radicals (*62*). However, the short life times and low steady state concentration of the highly reactive lipid-derived radicals make it difficult to detect these radicals at concentrations lower than the minimum detectable concentration of 10^{-9} M (*62*). To overcome this problem, various approaches have been used, including pulse radiolysis and UV photolysis, continuous flow systems and spin trapping, among which spin trapping has been the most widely employed procedure (*7*). Spin trapping technique allows the accumulation of detectable concentrations of longer-lived radicals by addition to samples of a spin trapping agent, which reacts with free radicals to form more stable spin-adducts, but often at the expense of the ability to identify the original radical (*7, 62, 63*). Nitroso compounds and nitrones are the most common spin traps, both leading to nitroxyl type spin-adducts such as α-phenyl-*tert*-butylnitrone (PBN)-adducts (Figure 5) (*62*).

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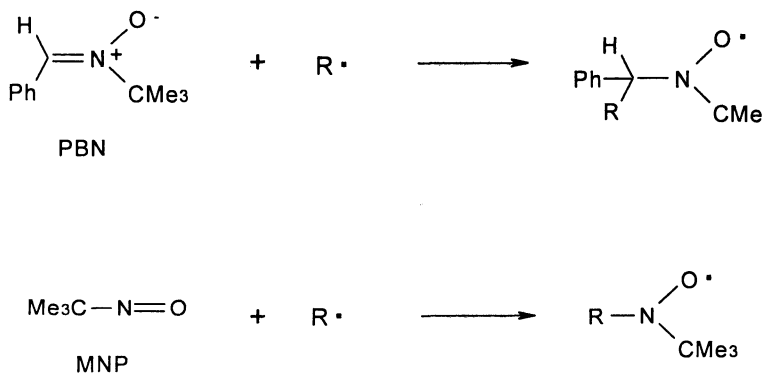

Figure 5. Formation of nitroxyl radical spin adducts

ESR spectroscopy is of great value for the study of the early stages of lipid oxidation and prediction of oxidative stability of fats and oils as well as evaluation of antioxidant activity. It has high sensitivity, and allows mild conditions by applying significantly low temperatures and requires little sample

preparation (*62-64*). Nevertheless, spin traps used in the ESR assay have been reported to exhibit widely differing trapping efficiencies for different radicals and show both prooxidant and antioxidant effects (*65, 66*), thus giving erroneous results of antioxidant activity.

Other Methods for Measuring Lipid Oxidation in Model Systems

Nuclear Magnetic Resonance (NMR) Spectroscopy

High-resolution ^{1}H NMR spectroscopy, in which hydrogen atoms (proton, ^{1}H) with various locations in the triacylglycerol (TAG) molecules are determined, has been used to evaluate oxidative deterioration of fats and oils (*19*). The principle of NMR is that hydrogen atoms in a strong magnetic field absorb energy, in the radiofrequency range, depending on their molecular environment, in which changes occur during the oxidation process (*19*). These changes may be monitored by NMR spectroscopy as a reflection of oxidation level of food lipids. The oil sample is dissolved in CDCl3 to avoid inference from the solvent, and its NMR spectrum recorded, with tetramethylsilane (TMS) as an internal standard (*19*). The spectrum shows several groups of signals, corresponding to the hydrogen atoms in different locations in the TAG molecules (Figure 6). The total number of each of these differently located protons can be calculated, from which ratios of aliphatic to olefinic protons (Rao) and aliphatic to diallylmethylene protons (Rad) may be obtained (*19*). Both ratios increase steadily during lipid oxidation, and this increment is inhibited by antioxidants. NMR method for evaluating lipid oxidation has been reviewed by Guillen *et al.* (*67*). NMR spectroscopy has been used by many researchers, and the changes in Rao and Rad measured by NMR correlated well with conjugated diene values and TBA values (*68*). In addition to ^{1}H NMR, ^{13}C NMR and ^{31}P NMR are also powerful tools to predict oxidative stability of oils (*69-71*). ^{13}C NMR enables direct observation of carbon atoms. The selectivity and dispersion of ^{13}C NMR spectra are very high (*70*). ^{13}C NMR assesses lipid oxidation by monitoring the changes of carbon chains in TAG molecules, revealing the specific sites that oxidative degradation occurs (*69*). However, because the abundance of the NMR active ^{13}C nucleus isotope is only 1.12% of ^{12}C, the sensitivity of ^{13}C NMR is usually much lower than that of ^{1}H NMR (*70*).

NMR spectroscopy is a rapid, non-destructive and reliable technique for assessing lipid oxidation. It simultaneously measures both the primary and the secondary oxidative changes in oils, and provides specific information on oxidative regions in the TAG molecules. Using NMR for antioxidant test, a reduction in oxidation of the model system relative to a control sample is used as an index of antioxidant activity.

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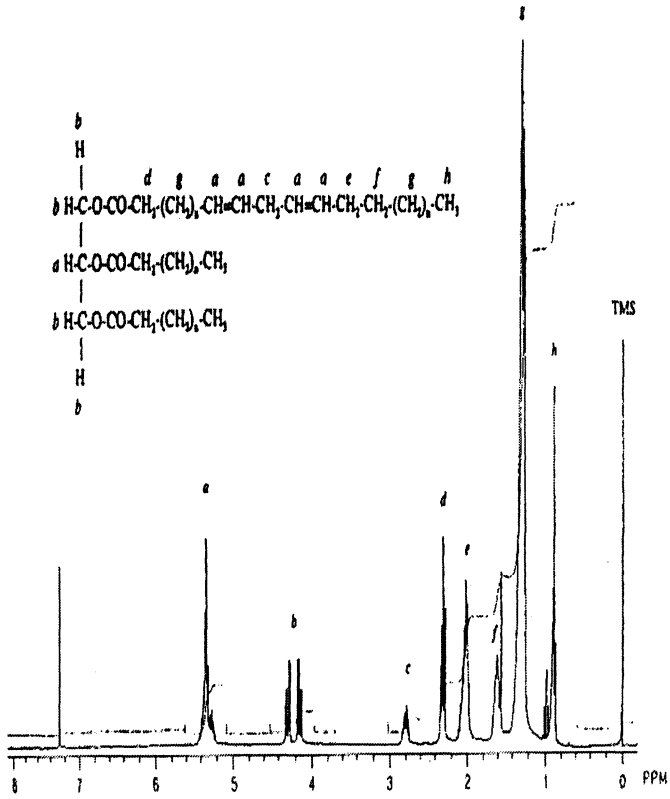

Figure 6. ^{1}H NMR spectrum of oxidized canola oil

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Differential Scanning Calorimetry (DSC)

During lipid oxidation, fats and oils reveal a number of thermally-induced transitions such as the transfer of oxygen molecules to unsaturated fatty acids (exothermic process) (*72*). Therefore, thermal analysis can be used in accelerated oxidation tests. The differential scanning calorimetry (DSC) technique, which is based on thermal release of oxidation reactions, has the potential as a nonchemical method for assessing oxidative stability of fats and oils, indicating the onset of advanced oxidation (termination) (*63*). It provides unique energy profile information, which specifically measures the temperature and heat flows associated with lipid oxidation as a function of time and temperature (*73*). The method uses isothermal or nonisothermal conditions and a flow of oxygen as purge gas, with a calorimeter measuring the heat flow into

(endothermic) or out of (exothermic) an oil sample undergoing oxidation changes (*63, 72*). The oxidation curves of the sample are obtained with different heating time, and a dramatic increase for the evolved heat can be observed with the appearance of a sharp exothermic curve during initiation of oxidation. The end point is taken at the time where a rapid exothermic reaction between oil and oxygen occurs, and induction period (IP) determined automatically by intersection of extrapolated baseline and tangent line (leading edge) of the exotherm (Figure 7) (*63, 72*). The DSC also measures oxidation onset temperature, the temperature at maximum reaction, and the ending temperature (*72*). The isothermal and nonisothermal DSC show good agreement, suggesting that both isothermal and nonisothermal DSC are suitable for oxidative stability studies of oils (*74*). Addition of antioxidants can improve the oxidative stability of the model system. This improvement can be determined by DSC method and used to indicate the antioxidant activity. The DSC technique has been reviewed by Tan *et al.* (*72, 75*).

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Radical Scavenging Assays

In addition to the methods discussed above, which measure the inhibition of lipid oxidation by an antioxidant in a model system, another group of assays directly measures hydrogen atom donation or electron transfer from the potential antioxidant to free radical molecules in simple "lipid free" systems (*17*). These assays require no lipid substrate and can be divided, based on the chemical reactions involved, into two categories: hydrogen atom transfer (HAT) reaction based assays and single electron transfer (ET) reaction based assays (*76*). Antioxidants can scavenge radicals by HAT and ET leading to the same end result, regardless of mechanism, although kinetics and potential for side reactions vary (*6*). Proton-coupled ET and HAT reactions may also occur in parallel, and in this case the dominating mechanism in a given system is dependent on antioxidant structure and properties, solubility and partition coefficient, and solvent system (*6*). These assays are available as commercial kits and is widely used for measurement of antioxidant activity. However, they have been criticized for not reflecting the situation in an oxidizing-food or an *in vivo* situation (*17*).

HAT-Based Methods

HAT-based methods measure the classical ability of an antioxidant to quench free radicals by hydrogen donation. These methods generally use a free radical generator that generates stable or short-lived radicals, an oxidizable molecular probe and an antioxidant (*76*). The added antioxidant competes with probes for the radicals and thus inhibits or retards the oxidation of the probes (*76*). Among a variety of HAT-based assays developed for measuring

antioxidant activity, oxygen radical absorbance capacity (ORAC) and total radical-trapping antioxidant parameter (TRAP) are used most extensively.

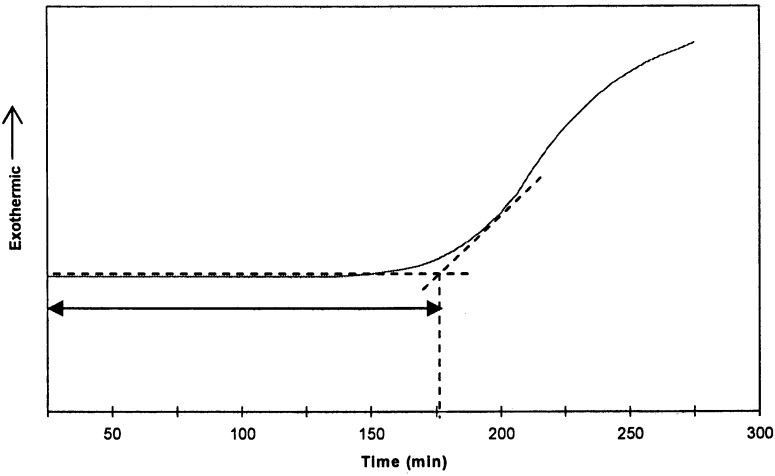

Figure 7. Determination of induction period (IP) by DSC

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Oxygen Radical Absorbance Capacity (ORAC) Assay

The ORAC assay measures the radical chain breaking ability of antioxidants by monitoring the inhibition of peroxyl radical induced oxidation by a certain antioxidant. In this assay, the peroxyl radical reacts with a fluorescent probe resulting in the loss of fluorescence, which is detected with a fluorometer. B-phycoerythrin (B-PE), a protein isolated from *Porphyridium cruentum*, was the fluorescent probe initially employed in early ORAC studies before it was found to suffer from several disadvantages. These include its large lot-to-lot variability in reactivity with peroxyl radicals as it is derived from red algae of various species, its interaction with polyphenols due to nonspecific protein binding, and its being photobleached under excitation light (*6, 76*). B-PE has therefore been replaced by a more stable and less reactive nonprotein probe fluorescein (FL:3'6'-dihydroxyspiro[isobenzofuran-1[3H],9'[9H]-xanthen]-3-one). The oxidation products of fluorescein with peroxyl radicals have been characterized, and the reaction mechanism has been verified as a classic HAT mechanism (*77*).

A group of azo compounds including the lipophilic AIBN (α,α-azobisisobutyronitrile) and AMVN (2,2'-azobis(2,4-dimethylnaleronitrile) and the hydrophilic AAPH (2,2' -azobis(2-amidinopropane) dihydrochloride) has been used as peroxyl radical generators (*17*). Added antioxidants can rapidly react with peroxyl radicals and inhibit the loss of fluorescence intensity, and this

inhibition is proportional to the antioxidant activity (*7*). A general pathway for the chemical reactions involved is given below (*6*):

Radical generation: $R—N=N—R \longrightarrow N_2 + 2ROO\bullet$

Probe oxidation:

$ROO\bullet$ + Fluorescence probe $O_2 \longrightarrow$
ROOH + oxidized probe (loss of fluorescence)

Inhibition:

$ROO\bullet + AH \longrightarrow ROOH + A\bullet$
$ROO\bullet + A\bullet \longrightarrow ROOA$

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A fluorescence decay curve (fluorescence intensity versus time) can be constructed (Figure 8). Instead of following the extension of the lag phase only, calculation of antioxidant activity employs a net integrated area under the fluorescence decay curves ($AUC = AUC_{sample}-AUC_{blank}$) that accounts for lag time, initial state, and total extent of inhibition (*6*). A series of Trolox solution with varying concentrations is used as antioxidant standard to obtain a standard curve (Trolox concentration versus AUC), and ORAC values are reported as Trolox equivalents.

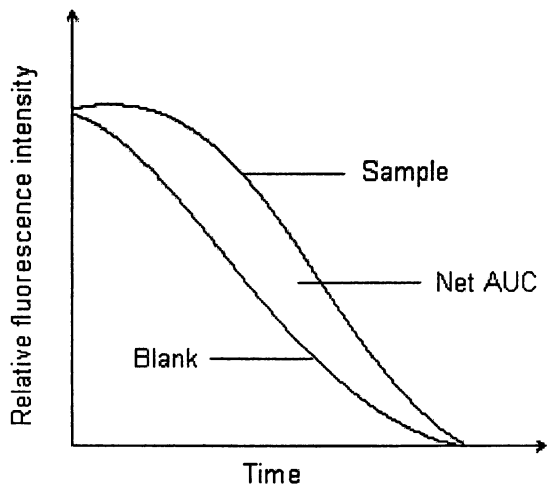

Figure 8. Kinetic curve of ORAC assay

Although it was originally developed for measurement of hydrophilic antioxidants, ORAC assay has recently been adapted to detect both lipophilic

and hydrophilic antioxidants by altering the radical source and solvent (*6*). The ORAC method is readily automated using a multichannel liquid handling system coupled with a fluorescence microplate reader (*6*). The detailed procedures of ORAC assay operating on a 48-well and a 96-well plate fluorescence reader are described by Hunag *et al.* (*78*) and Wu *et al.* (*79*), respectively. It should be noted, however, that the ORAC reaction is highly temperature sensitive and that small differences in temperature may decrease the reproducibility of the assay (6). Incubation of the reaction buffer at 37°C prior to the AAPH being dissolved is recommended to reduce the intra-assay variability (*6*).

Total Radical-trapping Antioxidant Parameter (TRAP) Assay

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Another radical scavenging assay that applies HAT mechanism is total radical-trapping antioxidant parameter (TRAP) assay, which has the same feature as ORAC assay. The TRAP method determines the ability of antioxidants to inhibit the reaction between peroxyl radicals and a target probe. Peroxyl radicals are generally provided from thermal decomposition of AAPH or ABAP (2,2'-azobis(2-amidinopropane) dihydrochloride). Various reaction probes have been used in this method, including oxygen uptake, fluorescence of R-phycoerythrin (R-PE) and absorbance of ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (*6*). Antioxidant activity is determined as the lag time of the kinetic curve in the presence of the antioxidant compared to corresponding lag time for trolox standard, and results are expressed as trolox equivalent (*6, 76*).

The TRAP assay was initially designed for measurement of *in vivo* antioxidant activity of certain nonenzymatic antioxidant compounds in serum or plasma, such as glutathione, ascorbic acid, α-tocopherol and β-carotene (*80*). The measurement of TRAP was based on the determination of the length of time that a subject's serum or plasma was able to resist artificially induced oxidation (*7*). This was approached by monitoring oxygen consumption in a thermostated oxygen electrode cell during oxidation of linoleate initiated by free radicals (*81*). The assay was later modified, due to the lack of stability of the oxygen electrode over the required period of time, using luminol-enhanced chemiluminescence (CL) as the end-point, which is enhanced by peroxyl radicals and extinguished by added antioxidants (*7*). More recently, TRAP assay using R-PE as a fluorescent probe has been widely adopted in assessing the activity of antioxidants in foods. Use of DCFH-DA (dichlorofluorescin diacetate) as the molecular probe has also been proposed (*82*).

The TRAP assay is automated, and serves as a useful tool for measuring antioxidant activity. However, the precision and reliability of this lag phase-based method has been questioned, owing to the fact that antioxidants also make contribution in inhibiting oxidation after the lag phase. This method has been reviewed by Ghiselli *et al.* (*83*).

Crocin Bleaching Assay

Carotenoids bleach under oxidation conditions and the decolorization can be prevented by antioxidants. Therefore, the activity of an antioxidant compound can also be determined by measuring the inhibition of carotenoids decolorization by the added antioxidant. Crocin, a naturally occurring carotenoid derivative, is used as the target more often than β-carotene, due to the fact that the decolorization of β-carotene at 470 nm can occur by multiple pathways, which brings difficulties in interpreting the results (*6*). Crocin bleaching assay has similar reaction pathways as another typical HAT-based method -- ORAC assay. Crocin bleaches through peroxyl radicals (e.g., AAPH) induced oxidation, and added antioxidants quench the free radicals by donation of hydrogen atoms and thus diminish the color loss of the crocin probe, which is recorded spectrophotometrically at 443 nm. Like the ORAC assay, crocin bleaching assay is temperature sensitive and close temperature control is critical in this assay.

Crocin bleaching assay requires no special instrumentation and can be readily adapted to high-throughput methodology such as microplates (*6*). It has been applied in analysis of plasma antioxidant activity (*84*). However, its application in food samples is limited, as many food pigments such as carotenoids also absorb light at the same wavelength. Being not commercially available, crocin is usually extracted from saffron, and hence it is subject to lot-to-lot variability, which imparts additional limitations to its industrial application in quantitative analysis of antioxidant activity (*6, 76*).

Photochemiluminescence (PCL) Assay

A photochemiluminescence (PCL) method based on the photochemical generation of free radicals and their chemiluminescent detection has recently been developed to quantify the antiradical property of antioxidant compounds. This method differs from other HAT-based radical scavenging assays because it does not require oxidizing reagent for production of radical species (*85*). This assay involves the photochemical generation of superoxide radical anion ($O_2^{\bullet-}$) and the sensitive detection with chemiluminescent. The reaction is induced by optical excitation of a photosensitizer (S), resulting in the generation of superoxide radical anion ($O_2^{\bullet-}$), as shown below (*85*):

$$S + h\upsilon + O_2 \longrightarrow [S^*O_2] \longrightarrow S^{\bullet+} + O_2^{\bullet-}$$

The free radicals are visualized with a chemiluminescence detection reagent, luminol, which also acts as the photosensitizer. Antioxidant activity is determined as the disappearance of these radicals caused by hydrogen donation from antioxidants. Two different protocols, ACW and ACL, are available as commercial kits to allow the measurement of antioxidant capacity of water- and lipid-soluble components, respectively.

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The PCL assay is known as a time- and cost-effective method for evaluation of the integral antioxidative capacity toward superoxide (*6*), and has been applied in measuring activity of food antioxidants, especially of phenolic compounds (*86, 87*). It does not require high temperature to generate free radicals and is reported to have a higher sensitivity (nanomolar range) than other radical scavenging assays such as ORAC and TRAP (*85*). Nevertheless, because PCL assay uses a novel radical source other than peroxyl radicals, further investigation will be necessary for a better understanding of superoxide radical in order to ascertain the correlation of PCL assay with other radical scavenging methods.

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ET-Based Methods

ET-based methods detect the ability of a potential antioxidant to transfer one electron to reduce any compound, including metals, carbonyls, and radicals (*88*). These assays involve an electron transfer reaction between the two components, antioxidants and oxidants (also the probe) (*76*):

probe (oxidant) + e (from antioxidant) $\longrightarrow$ reduced probe + oxidized antioxidant

This oxidation reaction is accompanied with color change of the probe and the extent of this color change is proportional to antioxidant concentration and thus reflects the reducing capacity of the antioxidant under investigation. However, because there is no oxygen radicals in these assays and the reaction does not mimic the radical scavenging mechanism of antioxidants in model systems, these methods are based on the assumption that antioxidant activity is equal to its reducing capacity (*89*).

Trolox Equivalent Antioxidant Capacity (TEAC) Assay

The TEAC assay was first reported by Miller and Rice-Evans (*90*), which measures the ability of antioxidants to scavenge a stable radical cation $ABTS^{\bullet+}$. In the original TEAC assay, metmyoglobin and hydrogen peroxide were used to generate ferrylmyoglobin radical, which is then reacted with ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) to produce $ABTS^{\bullet+}$, which is intensely colored and shows maximum absorption at 734 nm. The accumulation of $ABTS^{\bullet+}$ can be inhibited by the presence of antioxidants, to an extent dependent on the antioxidant activity (*7*). Modifications to the original assay have been proposed in many aspects, including the methods for $ABTS^{\bullet+}$ generation, wavelengths for monitoring the reaction, and the means of quantification, among others (*6*). In an improved version, $ABTS^{\bullet-}$, the oxidant, was generated by oxidation of $ABTS^{2-}$ using potassium persulfate, and the

radical anion $ABTS^{\bullet-}$ can be reduced by antioxidants to release the colorless product $ABTS^{2-}$ (*76*). The reaction scheme is as follows:

$$ABTS^{\bullet-} (\lambda_{max} = 734 \text{ nm}) \underset{K_2SO_5}{\overset{\text{antioxidants}}{\rightleftharpoons}} ABTS^{2-} \text{ (colorless)}$$

The percentage change of absorbance is recorded and results expressed as Trolox equivalents.

The TEAC assay is simple in operation and can be automated and adapted to microplates (*91*). It has been used to measure the total antioxidant activity in pure substances, body fluids and plant material as well. Although TEAC assay is classified as an ET-based method, HAT mechanism also applies; the radicals may be neutralized by either direct reduction via electron transfer or by radical quenching via hydrogen atom transfer, and the balance of these two mechanisms is generally determined by antioxidant structure and pH value (*6*). As an end-point assay, TEAC method requires reliable determination of end point of the reaction for quantitative evaluation of antioxidant activity, and the reaction rate is not reflected in the TEAC values (*6, 76*).

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2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay

DPPD is a stable chromogen radical which gives a deep purple color. It is commercially available and does not need to be generated prior to the assay. In the presence of antioxidants, DPPH radicals are reduced and the reduction reaction results in decolorization of the solution. The loss of color, which can be monitored spectrophotometrically at 517 nm, reflects the reducing ability of antioxidants toward DPPH radical. Two parameters EC_{50} and T_{EC50} are calculated and reported as the results for antioxidant activity. EC_{50} is defined as the concentration of the antioxidant necessary to decrease the initial DPPH concentration by 50%. T_{EC50} is defined as the time needed to reach the steady state with EC_{50}. More recently, a new parameter that combines both factors has been introduced and known as antiradical efficiency (AE). AE is calculated as:

$$AE = (1/ EC_{50}) T_{EC50}$$

The DPPH assay is a simple technique and requires only a UV spectrophotometer to perform. However, interference may occur when samples contain compounds that absorb at the same wavelength causing overlapped spectra of DPPH (*92*). The DPPH assay is believed to be mainly based on ET reaction, and HAT mechanism is a marginal reaction pathway in this assay (*6*).

Ferric Reducing Antioxidant Power (FRAP)

The FRAP assay is a typical ET-based method. It measures the reduction of ferric tripyridyltriazine (TPTZ) complex to the intensely blue colored ferrous complex by antioxidants in acidic medium. Antioxidant activity is determined as increase of absorbance at 593 nm, and results are expressed as micromolar Fe^{2+} equivalents or relative to an antioxidant standard (*7*). Differing from other ET-based methods, FRAP assay is carried out under acidic pH conditions (pH 3.6). This is to maintain iron solubility and more importantly drive electron transfer, and thus increase the redox potential, causing a shift in dominant reaction mechanism (*93*). Modified FRAP assays use ferric reagents other than ferric TPTZ to assess reducing power of antioxidants, among which potassium ferricyanide has been employed most frequently (*94*). Recently, a copper reduction assay which uses copper as the oxidant instead of ferric has been developed and used as a variant of FRAP assay (*95*).

The FRAP assay is simple, fast and cost-effective and does not require specialized equipment. It was originally used to measure reducing power in plasma but has been extended for assessing antioxidant activity in other biological fluids, foods, and plant extracts. However, potential problems for its application in foods exist, as many metal chelators in food extract could bind Fe^{3+} and form complexes that also react with antioxidants (*76*). Furthermore, Pulido *et al.* (*96*) reported that FRAP results might vary, depending on the analysis time. The time of reaction between antioxidants and Fe (III) TPTZ range from several minutes to several hours, as observed for some phenolic compounds (*96*). Therefore, a single-point absorption endpoint may not represent a complete reaction, since different antioxidants require different reaction times for detection (*6*). It has been argued that the FRAP assay utilizes ET mechanism, which has little relationship with radical quenching process (HAT mechanism) occurring in lipid systems, and has poor correlation with other antioxidant activity measurements. Other researchers have suggested that this assay could be used in combination with other methods in distinguishing dominant mechanisms for different antioxidants (*6*).

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Metal Chelation Capacity Test

Some antioxidants such as flavonoids are powerful metal chelators. These antioxidants can effectively deactivate prooxidant metal ions and thus prevent or retard metal ion-induced lipid oxidation. The antioxidant property of metal chelation is obtained when a complex is formed between the antioxidant and the metal, in such a way that metal ions cannot any longer act as initiator of lipid oxidation. Therefore, metal chelation capacity is also used as an indicator of antioxidant activity, usually in combination with other antioxidant assays.

Metal chelation capacity is determined by measuring the chelating effect of antioxidants for ferrous ion. Ferrous sulfate and ferrozine are the most commonly used reagents. A loss of absorbance at 485 nm (for ferrous sulfate) or 562 nm (for ferrozine) after the addition of antioxidants represents the formation of metal-antioxidant complex, and metal chelation capacity of the added antioxidant can be quantified spectrophotometrically. Metal chelation assay has been used in a number of antioxidant activity studies (*97-99*).

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Chapter 5

Hydrophilic and Lipophilic Antioxidant Capacity in Foods: Measurement and In Vivo Implications

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The measurement of both lipophilic and hydrophilic antioxidant capacity in food is possible using the oxygen radical absorbance capacity ($ORAC_{FL}$) assay. Data from these measurements provide a measure of antioxidant capacity using specific biologically relevant free radical sources, usually the peroxyl radical, but different radical sources can be utilized in order to obtain the relative response of different dietary antioxidants to different radical sources. The effects of food processing and of genetics, harvest maturity, and environmental growing conditions on antioxidant components can be evaluated. The *in vitro* measure of antioxidant capacity provides information on potential for dietary antioxidants to defend against free radical damage in the gastrointestinal tract; however, information on what the *in vivo* response will be following consumption of the dietary antioxidants may not directly correlate. It is clear that specific phytochemical components in foods are absorbed/metabolized quite differently; thus *in vivo* techniques are necessary to confirm that antioxidant status will be altered as a result from consumption of the specific source of dietary antioxidants.

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Several thousand polyphenolic compounds exist in plants and many of these have antioxidant capacity (AOC). Because of the difficulty of quantitating the individual antioxidant compounds, a method which provides a "sum" of the antioxidant components in plants and biological samples is useful. These types of assays are often referred to as "total" antioxidant capacity assays. However, there is in actuality no single assay which provides a "total" measurement of AOC. In thinking about antioxidant capacity methods, one has to first consider the oxidant source and the mechanism of reaction with potential antioxidants. Another component of any "total" measurement of AOC relates to hydrophilic and lipophilic antioxidants. It is possible to obtain a measure of "total" antioxidant capacity using a single radical or oxidant source if measures are taken to include both hydrophilic and lipophilic antioxidants. However, the protection observed against oxidant or radical damage may differ considerably depending upon the type of antioxidant phytochemical.

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Biologically Relevant Radical Species

Experimental evidence suggests that there are six major reactive oxygen species (ROS) causing oxidative damage in the human body. These species include superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), peroxyl radical ($ROO^{\bullet}$), hydroxyl radical ($HO^{\bullet}$), singlet oxygen ($^1O_2$), and peroxynitrite ($ONOO^-$). One of the most relevant radicals in biological regulation is superoxide (*1*). The superoxide anion is formed by the univalent reduction of triplet-state molecular oxygen. This process is mediated by enzymes such as NAD(P)H oxidases and xanthine oxidase or nonenzymatically by redox reactive compounds such as the semi-ubiquinone compounds of the mitochondrial electron transport chain. To counteract the assault of these ROS, living cells have a biological defense system composed of enzymatic antioxidants to convert ROS or reactive nitrogen species (RNS) to harmless species. For example, $O_2^{\bullet-}$ is converted to oxygen and hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) or reacts with nitric oxide ($NO^{\bullet}$) to form peroxynitrite. In biological tissues superoxide can also be converted nonenzymatically into the nonradical species hydrogen peroxide and singlet oxygen ($^1O_2$). Hydrogen peroxide can be converted to water and oxygen by catalase. In the presence of reduced transition metals (e.g., ferrous or cuprous ions), H_2O_2 can be converted into the highly reactive hydroxyl radical ($^{\bullet}OH$) (*1*).

In contrast, no enzymatic action is known to scavenge $ROO^{\bullet}$, $HO^{\bullet}$, $^1O_2$, and $ONOO^-$, so the burden of defense relies on a variety of non-enzymatic antioxidants such as vitamin C, vitamin E, and many phytochemicals that have the property of scavenging oxidants and free radicals. To comprehensively evaluate the oxidant-scavenging capacity of a food sample, assays have to be designed to include these ROS. However, so far the majority of assays are designed to measure a sample's capacity to react with one oxidant (either organic radical or redox active metal complex). The peroxyl radical has been the

most frequently used ROS in antioxidant capacity assays because it is the most relevant radical in lipid autoxidation and can be generated conveniently from azo compounds (*2,3*). The peroxyl radical has been used as a radical source in the oxygen radical absorbance capacity (ORAC) measurements (*4-6*).

Polarity of Antioxidants

An additional component to the assay of antioxidant capacity needs to be considered as antioxidants can be physically classified by their solubility into two groups: (1) hydrophilic antioxidants, such as vitamin C and the majority of polyphenolic compounds, and (2) lipophilic antioxidants, such as vitamin E and the carotenoids. Until fairly recently, there has not been much attention paid to the assay of both hydrophilic and lipophilic antioxidants (*4,7,8*), although both are extremely important sources of free radical protection within the cell. Any attempted measure of "total" antioxidant capacity needs to consider both lipophilic and hydrophilic antioxidant sources. This point is illustrated by studies of Faustino et al. (*9*) in which different phenolics displayed different antioxidant properties in protecting against LDL oxidation carried out with AAPH (2,2'-azo-bis(2-amidinopropane) dihydrochloride) or AMVN (2,2'-azo-bis(2,4-dimethylvaleronitrile)), as water or lipid soluble peroxyl radical generating systems, respectively. In an AAPH (hydrophilic) system, all polyphenolic compounds tested, except trans-resveratrol, displayed an antioxidant effect. LDL oxidation by AAPH was also inhibited by aliquots of a Merlot wine. No antioxidant effects were observed in an AMVN (lipophilic) environment except for a mild antioxidant effect by quercetin. Surprisingly, incubation of LDL with a Merlot wine strongly protected against oxidation by AMVN. Thus, red wines apparently contain unidentified antioxidants that provide protection against LDL oxidation within a lipid soluble environment (*9*).

Massaeli (*10*) found that Trolox, vitamin E, and vitamin C were more effective against free radicals generated in a water soluble medium than they were against free radicals generated in a lipid environment. Lipid solubility was an important factor for both the antioxidant and the free radical generating systems in determining the extent of lipid peroxidation in LDL. Antioxidant efficacy in one set of experimental conditions may not necessarily translate into a similar degree of protection in another set of conditions where lipophilicity is a variable (*10*). Because of these differences of function in different environments, there have been increased efforts to develop methods for measuring both lipophilic and hydrophilic antioxidant capacity.

In addition, in order to ascertain the role of dietary polyphenolics as antioxidants *in vivo* it is necessary to understand the chemical nature of the components that are absorbed into the circulation *in vivo* since during the absorption process many of these compounds can be metabolized to different forms that may change their polarity. Thus, understanding factors influencing processes such as de-glycosylation before absorption, conjugation in the small

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intestine and/or liver through glucuronidation, sulfation or methylation, among others, metabolism and degradation in the colon to smaller phenolic molecules are important. The chemical forms in which these compounds circulate *in vivo* will influence their polarity and, thus, their localization and bioactivities *in vivo.* The absorbed phenolic/polyphenolic components might function in the aqueous phase (like vitamin C) or in the lipophilic milieu (as vitamin E) *in vivo*. This will depend on their polarity, properties of uptake, how they are metabolized on absorption, and their resulting structural forms in the circulation (*11*). Thus the capability of assessing lipophilic and hydrophilic antioxidant capacity both in the food or dietary components and *in vivo* is important.

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Assay of Hydrophilic Antioxidant Capacity

Numerous methods have been developed and used for the assay of hydrophilic antioxidant capacity. These methods will not be reviewed here as some of the key methods being used have been reviewed recently (*2,3*). As has been pointed out in these reviews, it is very difficult to compare data obtained using different AOC methods, particularly when the reaction mechanisms involve different radical or oxidant sources. The relative ranking of AOC may be similar in some cases but will definitely differ in other cases. It all depends upon how the particular antioxidants in a given food or sample react with the radical source. Two highly relevant radical sources are the superoxide and peroxyl radicals. Considerable data published in the past few years has demonstrated the applicability of the $ORAC_{FL}$ assay in evaluating the antioxidant activity of diverse food samples (*4,5,8,12-22*), but only recently has there been much attention given to the use of the superoxide radical in the measurement of AOC of foods (*23*). A commercial instrument is available that uses a superoxide radical source, but very little published data is available currently.

The ORAC methodology has undergone some refinements since its first inception (*24,25*), primarily with the use of fluorescein as the fluorescent probe instead of β-phycoerythrin. Data obtained with the earlier version of ORAC using β-phycoerythrin (*26,27*) also cannot be directly compared to $ORAC_{FL}$ data, as the data from the latter are generally about 1.5-2 times greater. These differences produced by the different methods have been the major driving force for developing standardized methods for measurement of AOC (*2*).

Considerable work has now been done with the latest version of $ORAC_{FL}$ regarding measurement of both hydrophilic and lipophilic antioxidants. Wu et al. (*28*) tested four foods (strawberry, cashew, lettuce and avocado) for the effect of method of lipophilic extraction on the value for hydrophilic AOC. These four samples were directly extracted in an ASE® 200 Accelerated Solvent Extractor. Samples and sand were transferred to a 22 mL extraction cell and were initially extracted with hexane:dichloromethane (1:1, Hex/Dc) followed by

acetone/water/acetic acid (70:29.5:0.5; AWA), or directly extracted with AWA. Parameters for the ASE® 200 Accelerated Solvent Extractor were: static, 5 min; flush, 60%; purge, 60 sec; cycle, 3; temperature, 70 °C (Hex/Dc extraction), 80°C (AWA extraction); pressure, 1500 psi. Extracts from AWA were transferred to 25 mL volumetric flasks and diluted with AWA to 25 mL total volume. This solution was used to measure hydrophilic $ORAC_{FL}$ (H-$ORAC_{FL}$). Extracts using Hex/Dc were dried under nitrogen flow in a 30°C water bath and residues were reconstituted in 10 mL acetone. This sample solution was used to measure lipophilic $ORAC_{FL}$ (L-$ORAC_{FL}$). Their H-$ORAC_{FL}$ values are shown in Figure 1. From the results, it is clear that there was no significant difference between the $ORAC_{FL}$ values for AWA extracts without Hex/Dc extraction compared to that in AWA extracts with prior Hex/Dc extraction. Thus, there is a clear differentiation between the hydrophilic and lipophilic components with this extraction technique and also that there is not any contribution to the hydrophilic $ORAC_{FL}$ if the lipophilic components are not previously extracted.

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Assay of Lipophilic Antioxidant Capacity

The H-$ORAC_{FL}$ assay (*29*) is limited to hydrophilic antioxidants due to the aqueous environment of the assay. However, Kurilich et al. (*15*) extracted the lipophilic components from broccoli using hexane, and then dried the hexane extract and dissolved the lipophilic components with dimethyl sulfoxide (DMSO). Since DMSO acts as an antioxidant in the $ORAC_{FL}$ assay, DMSO was included in the blank. This is a critical point. If the solvent is not tested in the assay system, one might think that they are getting improved extraction, when in fact the extraction solvent is reacting to increase the background reading in the AOC measurement. Because of the low lipid content in broccoli, solubility in the aqueous system using DMSO was not a problem, but materials with much higher lipid content present problems without the use of something to solubilize the lipids. Huang et al. (*7*) expanded the current $ORAC_{FL}$ assay to include lipophilic antioxidants. Randomly methylated-cyclodextrin (RMCD) was introduced as a water solubility enhancer for lipophilic antioxidants. Seven percent RMCD (w/v) in a 50% acetone-H_2O mixture was found to sufficiently solubilize vitamin E compounds and other lipophilic phenolic antioxidants in 75 mM phosphate buffer (pH 7.4). This newly developed ORAC assay (L-$ORAC_{FL}$) was validated for linearity, precision, accuracy, and ruggedness. The L-$ORAC_{FL}$ assay was very reliable and robust. Using 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid as a standard (1.0), the L-$ORAC_{FL}$ values were as follows: α-tocopherol (0.5 ± 0.02), (+)-γ-tocopherol (0.74 ± 0.03), (+)-δ-tocopherol (1.36 ± 0.14), α-tocopherol acetate (0.00), tocotrienols (0.91 ± 0.04), 2,6-di-tert-butyl-4-methylphenol (0.16 ± 0.01), and γ-oryzanol (3.00 ± 0.26) (*7*). Naguib et al. (*30*) also measured vitamin E isomers using β-cyclodextrin in the ORAC assay. The mechanism for the reaction between the vitamin E and the

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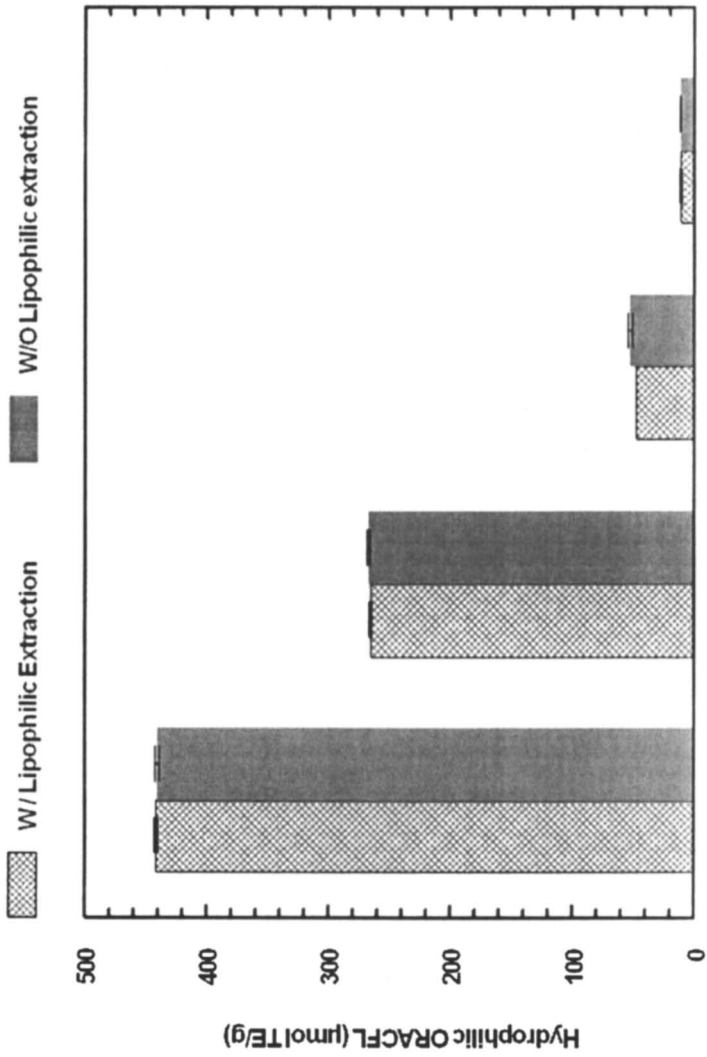

*Figure 1. Hydrophilic $ORAC_{FL}$ (H-$ORAC_{FL}$) following extraction of foods with hexane/dichloromethane (1:1, Hex/Dc) followed by Acetone:Water:Acetic Acid (70:29.5:0.5; AWA), or directly extracted with AWA without extraction with lipophilic solvents (Mean±SD, n=3) * (From: (28))*

peroxyl radical follows the hydrogen atom transfer mechanism, which is in agreement with the notion that vitamin E is a chain-breaking antioxidant (*7*).

Bangalore and coworkers (*31*) found that inclusion of β-cyclodextrin in the extraction media for lycopene improved the correlation between lycopene concentration and AOC as measured using the $ORAC_{FL}$ method. Lycopene was extracted according to a standard spectrophotometric assay procedure in the presence of β-cyclodextrin at concentrations of 0, 0.4, 0.8, and 1.6%, and the antioxidant activity of lycopene was measured with the $ORAC_{FL}$ assay. Lycopene concentration correlated poorly with $ORAC_{FL}$ (R^2 = 0.33) in the absence of β-cyclodextrin. Correlations improved with increasing levels of β-cyclodextrin (R^2 = 0.58 and 0.91 for 0.4 and 0.8% β-cyclodextrin, respectively). A very high β-cyclodextrin concentration (1.6%) decreased the correlation between $ORAC_{FL}$ and lycopene concentration. Inclusion of β-cyclodextrin in the ORAC assay thus was shown to expand the scope of the $ORAC_{FL}$ assay to include an additional fat-soluble antioxidant.

Wu et al. (*32*) have analyzed over 90 different foods and 500 individual samples with no apparent complications with very diverse types of foods. Data are presented in **Figure 2** on several diverse food samples with quite different values of L-$ORAC_{FL}$. However, difficulties can arise with the L-$ORAC_{FL}$ for the analysis of oil samples. Naturally occurring vegetable oils are prone to autoxidation due to their high content of polyunsaturated fatty acids. Protection of the oils from autoxidation becomes an important issue. The radical scavenging capacity can also be used as a measure of the nutritional value of the oils, which contain lipid soluble antioxidants such as vitamin E. However, there has not been a validated, high throughput assay to quantify radical scavenging AOC of oils.

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Hydrophilic and Lipophilic Antioxidant Capacity in Select Foods and Processing Effects

AOC of Foods: A number of factors, including genetics, and growing conditions (i.e. fertilization, moisture, pests and disease burdens, etc.), are known to affect the levels of what are considered 'plant secondary metabolites'. Many of these secondary metabolites have antioxidant capacity and may have important health consequences. The foods that our laboratory tested (See Figures 2 and 3) were sampled from 4 different locations in the U.S. and at two different time points during the year in an attempt to account for some of this variation that might exist in the U.S. market. For different foods, two sampling times (termed 'passes'), were planned to be 'in season' and 'out of season' for the fresh produce market in most U.S. markets. However, this differential sampling could not be maintained because of variation in production cycles within different localities within the U.S. Of the chosen samples, fruits and vegetables displayed significant variation in different sampling times, but nuts

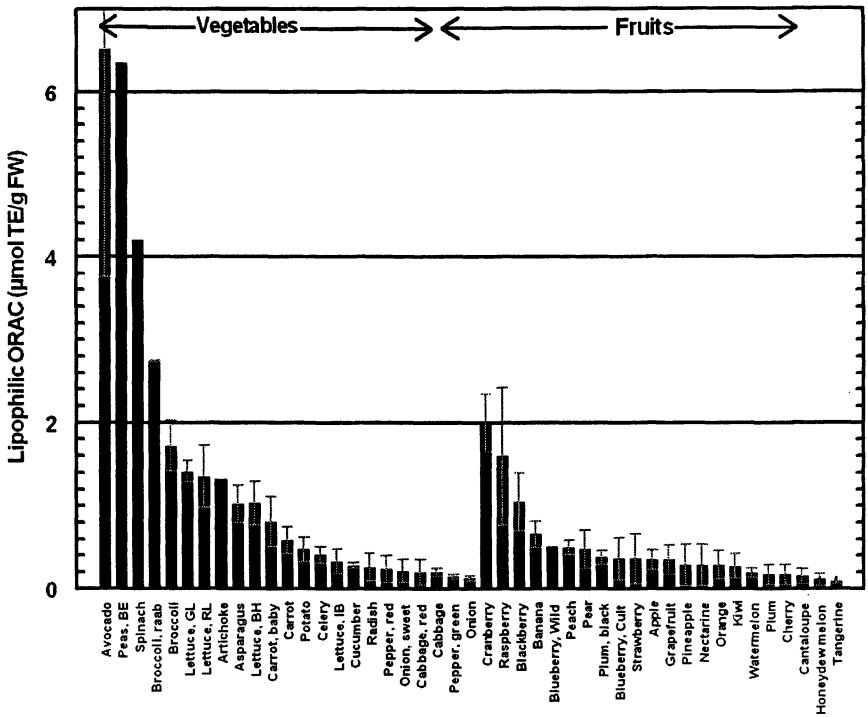

Figure 2. Lipophilic $ORAC_{FL}$ of selected fruits and vegetables (Adapted from: (32)). Data for most foods is the mean of 8 samples (4 sampling sites and 2 times).

and dried fruits did not. The second observation from these data was that the variation in L-$ORAC_{FL}$ and H-$ORAC_{FL}$ is quite different. L-$ORAC_{FL}$ is much more variable than H-$ORAC_{FL}$, perhaps due in part to the much lower concentrations. Furthermore, L-$ORAC_{FL}$ and H-$ORAC_{FL}$ were not always similarly affected by sampling time. Significant sampling period changes were observed for H-$ORAC_{FL}$ in six samples (honeydew, cantaloupe, watermelon, navel orange, grapefruit and iceberg lettuce) and for L-$ORAC_{FL}$ in thirteen samples (cantaloupe, kiwifruit, navel orange, strawberry, blueberry, raspberry, avocado, radish, celery, almond, pistachio, date and prune). These differences were as large as 3-fold in magnitude. Thus, it is clear that time of sampling is a factor that must be considered in developing an AOC database. These data also point to the importance of measuring both the hydrophilic and lipophilic AOC separately.

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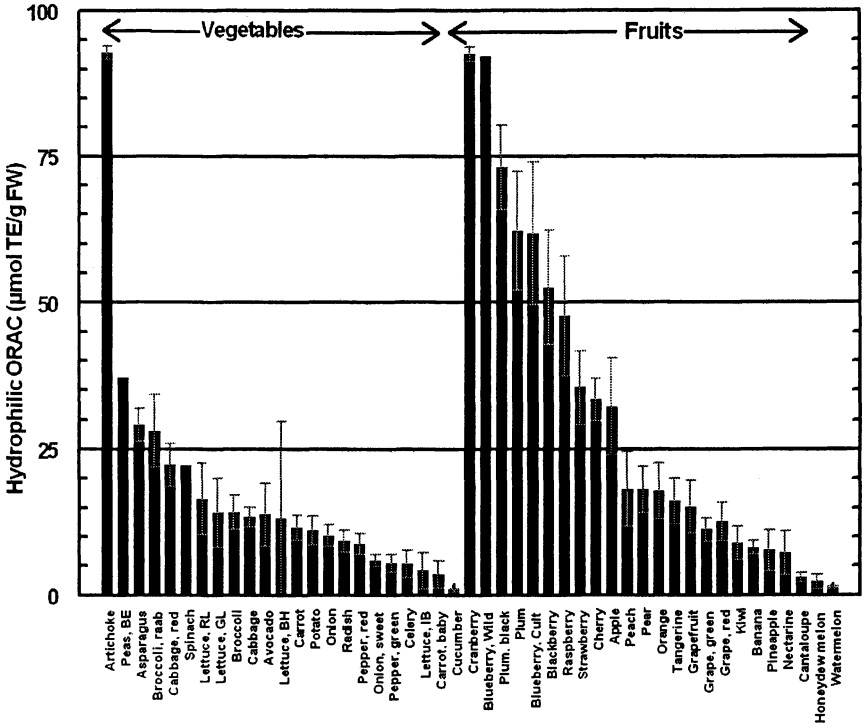

Figure 3. Hydrophilic $ORAC_{FL}$ of selected fruits and vegetables (Adapted from: (32)). Data for most foods is the mean of 8 samples (4 sampling sites and 2 times).

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Differences in lipophilic (Figure 2) and hydrophilic AOC (Figure 3) across foods vary by as much as 30- and 20-fold, respectively. Lipophilic AOC in all foods tested is much lower (by as much as 60-fold) than hydrophilic AOC (Figure 2 and 3). A sampling of the hydrophilic AOC of juices is presented in Figure 4. Lipophilic AOC was not measured because only the water soluble antioxidants will be captured during the juicing process. One can readily see the wide range of AOC for the different juices (Figure 4).

Effects of Processing on AOC Measurements: Processing, especially cooking of food, is another factor that can impact AOC (*33*). Some vegetables are commonly eaten in the cooked form (i.e., potatoes and asparagus), while others may be consumed in either raw or cooked forms (i.e., broccoli, carrots, and tomatoes). Thus, cooking is an important issue that needs to be considered in estimating the daily AOC intake. However, few studies have considered this

relative to antioxidants. Cooking is generally regarded as being destructive to antioxidant compounds (*34*). In our studies, both L-$ORAC_{FL}$ and H-$ORAC_{FL}$ of raw broccoli and carrots were significantly higher than that of their cooked forms (Figures 5 and 6). Baked russet potatoes showed a significantly increased H-$ORAC_{FL}$ (Figure 5) but a significantly decreased L-$ORAC_{FL}$ (Figure 6) compared to the raw forms. For tomatoes, both lipophilic and hydrophilic $ORAC_{FL}$ values of the cooked forms were significantly higher than those of their raw forms (Figures 5 and 6). Ascorbic acid and lycopene are the major hydrophilic and lipophilic antioxidants in tomatoes, and they are well correlated with hydrophilic and lipophilic antioxidant activities, respectively (*35*). A significant increase in both L-$ORAC_{FL}$ and H-$ORAC_{FL}$ in cooked tomatoes compared to raw was observed, which is in agreement with the results of Takeoka et al (*36*). Other studies have demonstrated an increased bioavailability of carotenoids from processed compared to raw tomatoes (*37*). Radical scavenging activity of peppers tended to decrease with cooking in boiled water, but were increased when heated in a microwave without water (*38*). Brett et al. (*39*) demonstrated that absorption of anthocyanins was greater in fresh fruit of black currants compared to syrup made from black currants. Cooking and processing can alter both lipophilic and hydrophilic antioxidants in both positive and negative ways, but in unpredictable ways at least based upon what we now know at this point.

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Hydrophilic and Lipophilic $Orac_{FL}$ Measurement and *In Vivo* Implications

The $ORAC_{FL}$ assay has also been utilized for assessment of antioxidant capacity of plasma and other body tissues and fluids (*4,40*) which is only one of many diverse assays that have been used to evaluate *in vivo* antioxidant status (*4,41,42*). Measurement of the area-under-the-curve of plasma hydrophilic and lipophilic AOC following a meal has been successfully utilized to study absorption/metabolism of antioxidant components in foods (*40*). The approach to the assay of both lipophilic and hydrophilic AOC in plasma is similar to that in foods in which the lipophilic components are extracted with hexane followed by the removal of protein using an ethanol/water/perchloric acid (PCA)(0.5 M) mixture. Addition of ethanol and water such that the final extraction solution contained 1 volume of plasma in 8 volumes total was determined to be important in order to get maximal extraction relative to a smaller total volume of extraction mix. A mixture of ethanol/plasma/H_2O/PCA of 2:1:1:4 (v/v/v/v) was found to be optimal based upon the combinations tested (*4*). Hexane extraction of plasma before analysis of hydrophilic $ORAC_{FL}$ did not alter the hydrophilic $ORAC_{FL}$ value obtained. This indicated that a good partitioning of the hydrophilic and lipophilic antioxidants into the two phases was obtained and that by adding the two values together, one could arrive at a measure of "Total" AOC of the plasma or serum sample using the peroxyl radical as the free radical source.

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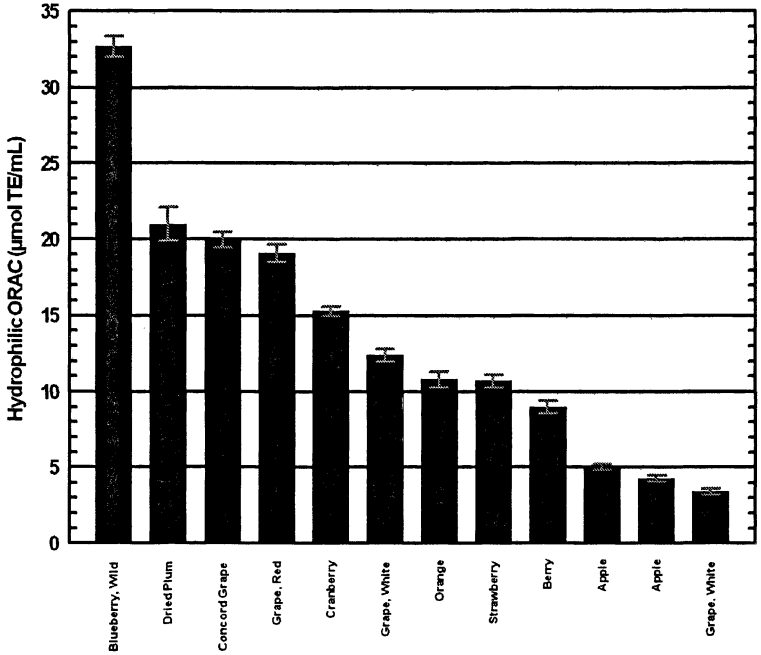

Figure 4. Hydrophilic $ORAC_{FL}$ of selected fruit juices (Adapted from: (4))

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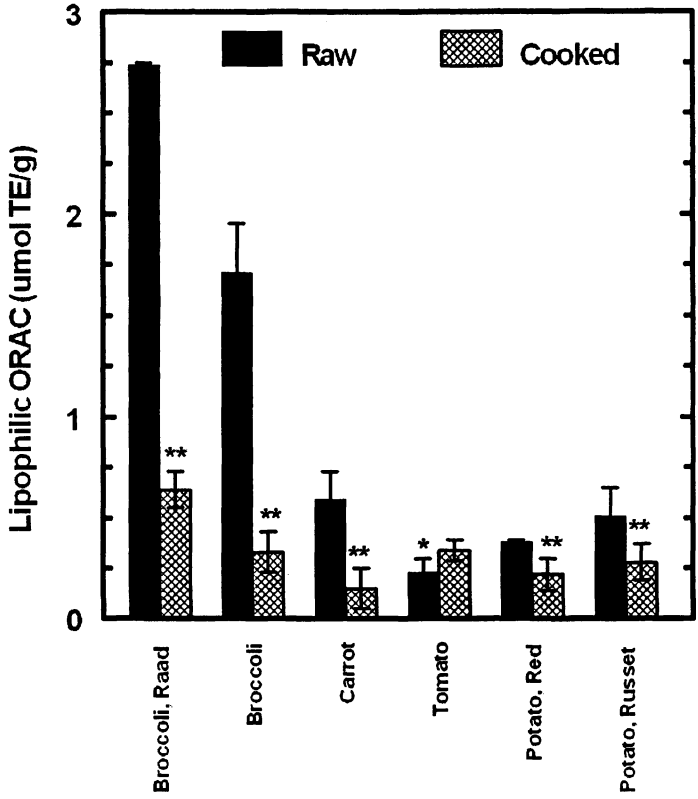

Figure 5. Effects of cooking/baking on lipophilic antioxidant capacity ($ORAC_{FL}$) of selected foods. Data adapted from (28,32). Four vegetables (russet potato, broccoli, carrot, and tomato) were cooked in boiling water in a stainless steel sauce pan for 3 to 4 min. Potatoes were baked for 50 minutes.

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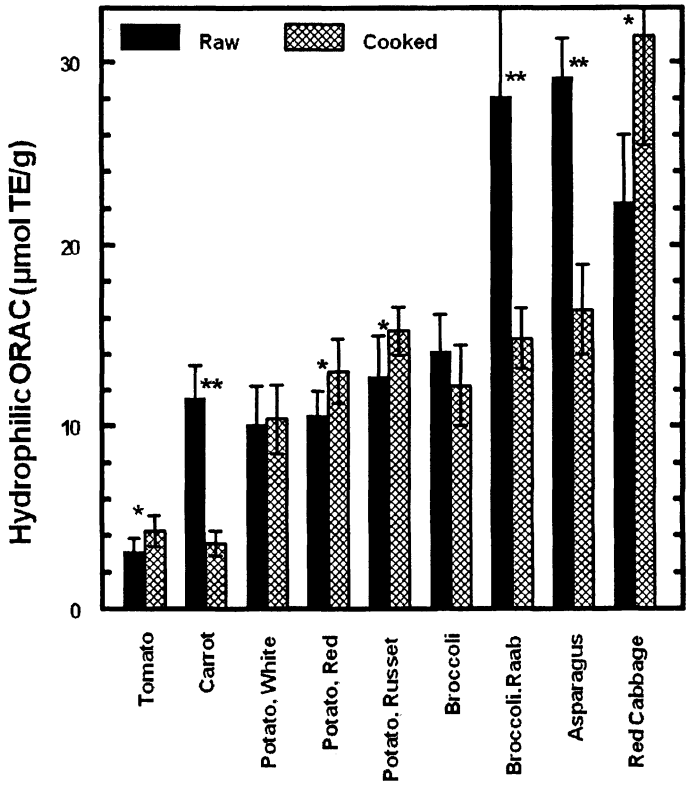

Figure 6. Effects of cooking/baking on hydrophilic antioxidant capacity ($ORAC_{FL}$) of selected foods. Data adapted from (28,32). Four vegetables (russet potato, broccoli, carrot, and tomato) were cooked in boiling water in a stainless steel sauce pan for 3 to 4 min. Potatoes were baked for 50 minutes.

Results from these studies clearly indicate that the antioxidant response *in vivo* can be altered by diet, but the response is dependent upon several factors such as 1) antioxidant capacity of the food, 2) the amount of dietary AOC consumed, 3) the type of phytochemicals and their content in the food, and 3) the amount of absorption and/or metabolism of the dietary antioxidants in the body. Although we have demonstrated that consumption of certain berries and fruits was associated with an increased plasma AOC in the postprandial state, one cannot necessarily translate that into a decreased risk of chronic degenerative disease (*40*).

Conclusions

What can be learned from the assay of lipophilic and hydrophilic antioxidant capacity in foods? (1) An assessment of the relative AOC of foods and dietary supplements can be obtained using specific biologically relevant free radical sources; (2) If different radical sources are tested, one can obtain the relative response of different foods and food antioxidants to different radical sources containing different antioxidants; (3) The effects of processing of foods on antioxidant components can be measured; (4) The effects of genetics, harvest maturity, and environmental growing conditions on antioxidant levels in foods can be determined; and (5) The potential for dietary antioxidant ingredients to provide a first line of defense against free radical damage in the gastrointestinal tract can be assessed.

However, *in vitro* antioxidant capacity assays do not provide information on what the *in vivo* response in terms of absorption/metabolism of dietary antioxidants might be. It is clear that specific phytochemical components in foods are absorbed/metabolized differently and thus the potential to provide *in vivo* antioxidant protection requires separate evaluation using *in vivo* techniques.

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Chapter 6

Cell Culture Models to Assess Bioactivity of Functional Foods and Dietary Supplements

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Antioxidant activity of pure compounds, foods, and dietary supplements has been extensively studied with the development of many new antioxidant and antioxidant activity assays in recent years. However, these assays, such as total phenolics, total flavonoids, and total antioxidant activity in vitro do not reflect the cellular physiological conditions and do not consider the bioavailability and metabolism issues. In addition, the mechanisms of action of antioxidants go beyond the antioxidant activity scavenging free radicals in disease prevention and health promotion. Animal models and human studies are expensive and not suitable for initial antioxidant screening of foods and dietary supplements. Therefore, there is a need for cell culture models to assess bioactivity of antioxidants. This article outlines potential cell culture models for initial antioxidant screening and antioxidant research.

Free radical-induced oxidative stress has been hypothesized to be a major factor in the development of several degenerative chronic diseases. Oxidative stress can cause damage to biomolecules such as lipids, proteins, and DNA, resulting in an increased risk of inflammatory diseases, cardiovascular disease (CVD), cancer, diabetes, Alzheimer's disease, cataracts, and age-related functional decline (*1-3*). To prevent or retard the oxidative stress induced by free radicals, sufficient amounts of antioxidants need to be consumed. Fruits, vegetables, and whole grains contain a wide variety of antioxidant compounds (phytochemicals), such as phenolics, flavonoids, and carotenoids (*4-9*), and these may help protect cellular systems from oxidative damage and also lower the risk of chronic diseases. The benefits of fruits and vegetables has been consistently supported by epidemiological studies reporting that regular

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consumption of fruits and vegetables as well as whole grains is associated with a reduced risk of developing chronic diseases such as cancer and cardiovascular disease (CVD) (*10-13*). Bioactive non-nutrient phytochemicals in fruits, vegetables, whole grains and other plant foods have been linked to reduced risk for major chronic diseases, including cancer and cardiovascular disease (*14*).

Antioxidant research has expanded dramatically since the mid-1990s with the development of several assays measuring the total antioxidant activity of pure compounds, foods, and dietary supplements (*15-18*). However, these total antioxidant activity assays in test tubes do not necessarily reflect the cellular physiological conditions and do not consider the bioavailability and metabolism issues. In addition, the mechanisms of action of antioxidants go beyond the antioxidant activity and scavenging free radicals in disease prevention and health promotion (*19*). Animal models and human studies are expensive and not suitable for initial antioxidant screening of foods and dietary supplements. Therefore, there is a need for cell culture models to support antioxidant research prior to animal studies and human clinical trials. This chapter outlines potential cell culture models for initial antioxidant screening.

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Potential Cell Culture Models For Cancer Research

Cells in humans and other organisms are constantly exposed to a variety of oxidizing agents, some of which are necessary for life. These agents may be present in air, food, and water or they may be produced by metabolic activity within cells. The key factor is to maintain a balance between oxidants and antioxidants to sustain optimal physiological conditions. Overproduction of oxidants can cause an imbalance leading to oxidative stress, especially in chronic bacterial, viral, and parasitic infections (*2*). Oxidative stress can cause oxidative damage to biomolecules such as lipids, proteins, and DNA, resulting in an increased risk for cancer.

Carcinogenesis is a multistep process, and oxidative damage is linked to the formation of tumors through several mechanisms (*2,3*). Oxidative stress induced by free radicals can cause DNA damage, which, when left unrepaired, can lead to base mutation, single- and double-strand breaks, DNA cross-linking, and chromosomal breakage and rearrangement (*3*). This potentially cancer-inducing oxidative damage might be prevented or limited by dietary antioxidants found in fruits, vegetables, and other plant foods. Studies to date have demonstrated that the mechanisms of action of antioxidants in the prevention of cancer go beyond the antioxidant activity scavenging free radicals. Antioxidants in fruits, vegetables, whole grains and other plant foods can have complementary and overlapping mechanisms of action (Table I), including antioxidant activity and scavenging free radicals, regulation of gene expression in cell proliferation, cell differentiation, oncogenes, and tumor suppressor genes, induction of cell cycle arrest and apoptosis, modulation of enzyme activities in detoxification,

Table I. Proposed Mechanisms of Action by Which Dietary Antioxidants May Prevent Cancer

- Antioxidant activity
  - Scavenge free radicals and reduce oxidative stress
- Inhibition of cell proliferation
- Induction of cell differentiation
- Inhibition of oncogene expression
- Induction of tumor suppress gene expression
- Induction of cell cycle arrest
- Induction of apoptosis
- Inhibition of signal transduction pathways
- Enzyme Induction and enhancing detoxification
  - Phase II enzyme
  - Glutathione peroxidase (GPX)
  - Catalase
  - Superoxide dismutase (SOD)
- Enzyme Inhibition
  - Phase I enzyme (block activation of carcinogens)
  - Cyclooxygenase-2 (COX-2)
  - Inducible nitric oxide synthase (iNOS)
  - Xanthine oxide
- Enhancement of immune functions and surveillance
- Antiangiogenesis
- Inhibition of cell adhesion and invasion
- Inhibition of nitrosation and nitration
- Prevention of DNA binding
- Regulation of steroid hormone metabolism
- Regulation of estrogen metabolism
- Antibacterial and antiviral effects

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oxidation, and reduction, stimulation of the immune system, regulation of hormone-dependent carcinogenisis, inhibition of arachidonic acid metabolism, and antibacterial and antiviral effects (*4, 5, 19-22*). Therefore, the potential cell culture models for cancer research should include this line of research (Table III). Obviously, no one cell culture system does it all.

Potential Cell Culture Models For CVDResearch

Several mechanisms for the prevention of atherosclerosis by dietary antioxidants in fruits and vegetables have been proposed (Table II). In the low-density lipoprotein (LDL) oxidation hypothesis (Figure 1), oxidized LDL cholesterol has been suggested as the atherogenic factor that contributes to CVD (*23, 24*). When circulating LDLs are present at high levels, they infiltrate the artery wall and increase intimal LDL, which can then be oxidized by free radicals. This oxidized LDL in the intima is more atherogenic than native LDL and serves as a chemotactic factor in the recruitment of circulating monocytes and macrophages. Oxidized LDL is typically taken up by macrophage scavenger receptors, thus inducing the formation of inflammatory cytokines and promoting cell proliferation, cholesterol ester accumulation, and foam cell formation (Figure 1). Gruel-like, lipid-laden foam cell accumulation in the blood vessel, forming fatty streak, would cause further endothelial injury and lead to atherosclerotic disease. Since oxidized LDL plays a key role in the initiation and progression of atherosclerosis, giving dietary supplements of antioxidants capable of preventing LDL oxidation has been an important therapeutic approach. Dietary antioxidants that are incorporated into LDL are themselves oxidized when the LDL is exposed to pro-oxidative conditions; this occurs before any extensive oxidation of the sterol or polyunsaturated fatty acids can occur (*25*). Therefore, dietary antioxidants might retard the progression of atherosclerotic lesions. In addition, phytochemicals have been shown to have roles in the reduction of platelet aggregation, modulation of cholesterol synthesis and absorption, and reduction of blood pressure. It was also reported that cranberry phytochemical extracts significantly induced expression of hepatic LDL receptors and increased intracellular uptake of cholesterol in HepG2 cells *in vitro* in a dose-dependent manner (*26*). This suggests that cranberry phtochemicals could enhance clearance of excessive plasma cholesterol in circulation.

C-reactive protein, a marker of systemic inflammation, has been reported to be a stronger predictor of CVD than is LDL cholesterol (*27, 28*), suggesting that inflammation is a critical factor in CVD. C-reactive protein is an acute phase reactant secreted by the liver in response to inflammatory cytokines (*28*). Inflammation not only promotes initiation and progression of atherosclerosis, but also causes acute thrombotic complications of atherosclerosis (*29*). Fruit and vegetable intake is found to be associated with lower plasma C-reactive

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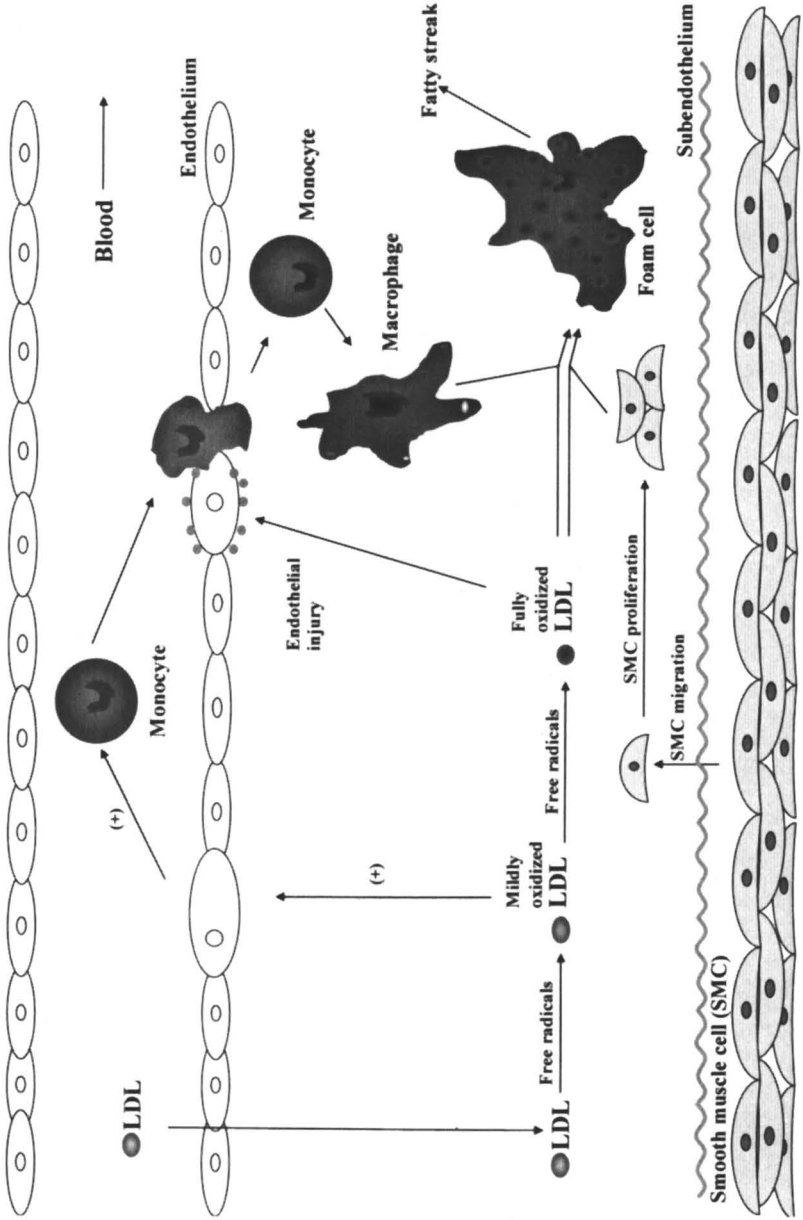

Figure 1. Proposed mechanism of LDL oxidation in fatty streak formation and atherosclerotic disease

protein concentrations (*30*), suggesting dietary phytochemicals can lower C-reactive protein. Therefore, the anti-inflammatory activity of phytochemicals may play an important role in prevention of CVD. Dietary antioxidants also have complementary and overlapping mechanisms of action in the prevention of CVD (Table II).

Bioavailability and Metabolism of Antioxidants

Bioavailability and metabolism are two important questions that need to be addressed when studying the biological effects of antioxidants (phytochemicals) in foods. The form of antioxidants found in foods is not necessarily the same as the form found in the blood or the targeted tissues after digestion, absorption and metabolism. In order to study the mechanisms of action of antioxidants in the prevention of chronic disease, two important questions must be asked. Firstly, are these antioxidants (phytochemicals) bioavailable? Secondly, are these original antioxidants or their metabolites the bioactive compounds? It is crucial to understand the bioavailability and metabolism of these compounds to gain knowledge of what compounds and at what level they actually reach target tissues. In some cases, the original phytochemicals may be excreted or metabolized and never actually reach target tissue, and the active compounds may not be the original antioxidant compounds found in foods rather than metabolites. To this date, many studies have not addressed the bioavailability and metabolism of phytochemicals from whole foods.

Examining the bioavailability of compounds from food sources can be challenging, because there are many factors that may influence bioavailability. Foods contain a wide variety of phytochemicals, and interactions with other chemicals in the food may affect bioavailability. Phytochemicals may be bound to different sugars (glycosides, xylosides, rhamnosides, galactosides) or to other compounds (fibers) that may affect the compound's bioavailability. Other factors, such as digestion, food processing, and stage of harvest may also affect phytochemical bioavailability. Although much progress has been made in understanding the bioavailability and further metabolism of pure compounds, more work is needed to further comprehend bioavailability of phytochemicals from complex food sources.

A good *in vitro* model would be beneficial in this area of study in evaluating bioavailability of phytochemicals from foods by offering a simple method to screen for factors that may affect intestinal absorption of phytochemicals, such as food matrix, food processing, digestion, and interactions with other foods. Human and animal models can be expensive and time consuming, while a cell culture model allows for rapid, inexpensive screenings. The Caco-2 cell culture model has the potential to be a good model to measure bioavailability of antioxidants, such as carotenoids and flavonids, from whole foods (*31-35*).

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Table II. Proposed Mechanisms of Action by Which Dietary Antioxidants May Prevent CVD

- Antioxidant activity
  - Scavenge free radicals and reduce oxidative stress
  - Prevent LDL oxidation
- Induction of expression of hepatic LDL receptors
- Regulation of sterol regulatory element binding proteins ($SREBP_S$)
- Modulation of cholesterol synthesis
- Regulation of lipid profiles
- Inhibition of cholesterol absorption
- Regulation of prostanoid synthesis (PGE_2)
- Reduction of platelet aggregation
- Regulation of nitric oxide (NO•) production
- Lowering C-reaction protein (CRP)
- Regulation of blood pressure

Table III. Potential Cell Culture Models for Antioxidant Screening

Cell culture models	Biomarkers
Cancer	
Antiprolifertion	Inhibition of proliferation
Caco-2 colon cancer cells	
HepG2 liver cancer cells	
MCF-7 breast cancer cells	
Cell cycle arrest	G1 arrest, G1/S ratio
Apoptosis	Induction/inhibition of apoptosis
Antiangiogenesis	Inhibition of angiogenesis, MMP2
COX-2 inhibition	COX-2 expression, PGE_2
Quinone reductase	Induced Quinone reductase activity
Oxidative DNA damage	8-OH-dG
CVD	
Inhibition of cholesterol synthesis	Cholesterol, $SREBP_S$
Expression of hepatic LDL receptors	LDL receptors, Cellular LDL uptake
Bioavailability of antioxidant	
Flavonoid bioavailability	Cellular flavonoid uptake
Carotenoid bioavailability	Cellular carotenoid uptake
Metabolism of antioxidant	
Primary haptocytes	Metabolic compound(s)
Caco-2 colon cancer cells	Metabolic compound(s)
HepG2 liver cancer cells	Metabolic compound(s)

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Conclusions

Based on the proposed mechanisms of action by which dietary antioxidants prevent cancer and CVD (Table1 and Table 2), a number of potential cell assays are suggested for initial antioxidant screening and antioxidant research (Table 3). Future research is needed to develop cell-based antioxidant activity assays with consideration of bioavailability and metabolism of antioxidants. Mechanism-based cell culture models are valuable in future antioxidant research.

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Chapter 7

Antioxidant Activity of Phytopolyphenols: Assessment in Cell Culture Systems

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Reactive oxygen species (ROS), are generated during normal physiological processes. ROS are toxic and oxidize of various cell constituents such as DNA, lipids and proteins. The oxidation products so produced may cause damage to cellular machinery, ultimately leading to cell death. ROS have been implicated in a myriad of diseases such as various forms of cancer, atherosclerosis, ischemic reperfusion injury, neurodegenerative diseases, and chronic inflammatory diseases, such as rheumatoid and psoriatic arthritis. Tumor promoters, such as phorbol-12-myristate-13-acetate (PMA) enhance the generation of these ROS, through protein kinase C pathway, to activate NADPH oxidase and xanthine oxidase. Nitric oxide (NO) plays an important role in inflammation and in the multiple stages of carcinogenesis. The suppressive effect of polyphenols on ROS production, monitored by flow cytometry using dichlorodihydrofluorescein diacetate (DCFH-DA) and dihydroethidium (DHE), and NO generation are described.

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Reactive oxygen species (ROS) are broadly defined as oxygen containing chemical species having an unpaired electron or non-radical molecules and these include superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($HO^{\bullet}$) and hydrogen peroxide (H_2O_2). In recent years, considerable evidence has emerged implicating ROS as having an important role in the initiation of cellular injury which can lead to cancer development. ROS is not only formed by exogenous sources, but also as a result of normal aerobic metabolism, by neutrophils and eosinophils in their defense against microorganisms and during metabolism of steroids and arachidonic acid. Under normal physiological conditions a balance is maintained between endogenous oxidants and antioxidants, such as superoxide dismutase, catalase and glutathione peroxidase. When an imbalance occurs, created by the excessive generation of oxidants or a decrease of antioxidants, it leads to excessive generation of ROS (*1-3*).

ROS have been implicated in an ever increasing number of diseases and syndromes. It is known that ROS serve as messengers in cellular signaling transduction pathways, and that a moderate increase of certain ROS may promote cellular growth and proliferation and contribute to development of cancer and other diseases (*4,5*). These include various forms of cancers, atherosclerosis, ischemic reperfusion injury, neurodegenerative diseases and chronic inflammatory diseases, such as rheumatoid and psoriatic arthritis (*6-8*).

Acute and chronic inflammation induced by biological, chemical and physical factors is associated with increased risk of human cancer at various sites. Excessive ROS produced by a variety of inflammatory cells probably participates in the carcinogenic effects of inflammatory reactions. Current evidence indicates that these activated inflammatory cells, induce and activate several oxidant-generating enzymes such as NADPH oxidase, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (*9-12*). These enzymes produce high concentrations of diverse ROS and can cause gene mutation, DNA damage leading to increased mutation and altered functions of enzymes and proteins, thus contributing to the multistage carcinogenesis process thereby being a major factor in tumor promotion (*13-15*).

Tumorigenesis or carcinogenesis is a multistep process that begins with cellular transformation, progresses to hyperproliferation and culminates in the acquisition of invasive potential, angiogenic properties and establishment of metastatic lesions (*16*). This process can be activated by any one of the various environmental carcinogens, inflammatory agents, tumor promoters and ROS. As described earlier, excessive ROS react with and modify macromolecules resulting either in alterations of DNA structure such as DNA mutations, or in functional modifications of reactive proteins. The biological consequences of ROS are changes in signal transduction, gene expression, and post-transcriptional or post-translational modification that alter cell growth and differentiation and consequently cause carcinogenesis (*17,18*). In recent years

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several studies suggest that ROS also serve an important role as signaling molecules which regulate many genes, including MMPs (*19*).

In biological systems, ROS are constantly generated through a variety of pathways. These ROS can be a primary event in human disease progression or a secondary consequence of tissue injury. Free radicals formed by phagocytes are the first line of immune defense. However, that is accompanied by a dramatic increase in oxygen consumption with the attendant production of $O_2^{\cdot-}$, which is catalyzed by a membrane-bound NADPH oxidase system in the plasma membrane (*20*). Even though $O_2^{\cdot-}$ less reactive than $^{\cdot}OH$, it can react with nitric oxide ($NO^{\cdot}$) to yield an even more reactive species, peroxynitrite that is transformed by dismutates to H_2O_2 and attacks several biological targets (*21,22*). Lipid peroxidation products hydroperoxy radical ($^{\cdot}OOH$), peroxynitrite ($ONOO^-$) or hydroxyl radical ($^{\cdot}OH$), have also been implicated in several pathologic conditions including aging, hepatotoxicity, hemolysis, cancer, tumor promotion and inflammation (*23,24*).

Phytopolyphenols, as natural dietary phytochemicals, are widespread in fruits, vegetables, tea, medicinal herbs and other plants. Various phytopolyphenol have long been considered to exert protective effects against many diseases, in particular cardiovascular disease and cancer. The polyphenols might protect the body against cancer and heart disease through inhibition of oxidative damage due to their typical phenolic nature and hydroxyl groups and, therefore, act as potent metal chelators and free radical scavengers (*25*).

Phytopolyphenol actions *in vivo* or in food may be through inhibiting generation of ROS or increasing the level of endogenous antioxidants. They may also be up-regulated by increased expression of the genes encoding the antioxidant enzymes, such as superoxide dismutase (SOD), catalase or glutathione peroxidase (GPx). Further, phytopolyphenols could also inhibit the source of ROS such as NADPH oxidase (*26,27*) and xanthine oxidase (*28,29*).

Oxidative stress and inflammation have been reported as being closely associated with the tumor promotion stage of carcinogenesis. Nitric oxide (NO) is a short lived, highly reactive, free radical which is produced from L-arginine by nitric-oxide synthase. The overproduction of NO is thought to contribute significantly to the pathogenesis of inflammatory demyelinating diseases, such as multiple sclerosis, and neurodegeneration in certain diseases such as Alzheimer's and Parkinson's. Furthermore, NO and its oxidized forms have also been shown to be carcinogenic (*30,31*).

Materials and Methods

Cell Lines

The human gastric carcinoma cell line AGS, leukemia cell line HL-60 and mouse macrophage RAW264.7 (American Type Culture Collection [ATCC])

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were respectively cultured in Dulbecco's modified Eagle's F12, Roswell Park Memorial Institute 1640 and Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum. The human AGS gastric carcinoma cell lines (CCRC 60102) were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cell lines were grown at 37 °C in 5% carbon dioxide atmosphere in Dulbecco's modified Eagle's medium for RAW264.7 cells; DMEM/F12 for AGS cells; and RPMI for HL-60 cells, supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL, Grand Island, NY, 100 units/mL of penicillin, 100 μg/mL of streptomycin), and 2 mM l-glutamine (Gibco BRL).

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Measurement of Cellular GSH Content by Flow Cytometry

The level of intracellular GSH per cell was determined by flow cytometry after staining with chloromethylfluorescein-diacetate (CMF-DA) (*32*). CMF-DA, containing a mild thiol reactive chloromethyl reactive group, is colorless and nonfluorescent. This probe is primarily conjugated to the abundant tripeptide glutathione by glutathione S-transferase. Once inside the cell, cytosolic esterases cleave off their acetates and then the chloromethyl group reacts with intracellular thiols, transforming the probe into a cell-impermeant fluorescent dye-thioether adduct. In this experiment, AGS cells were treated with 60 μM acacetin for 0.5, 1 and 2 h, after which CMFDA (final concentration was 25 μM) was added into the medium. The cells and fluorescent dyes were incubated for 30 min at 37 °C. After 30 min, cells were washed with PBS, and the intracellular GSH levels were determined by flow cytometry.

$O_2^{\bullet-}$ Production Determination

To detect XO/xanthine-induced intracellular ROS accumulation, HL-60 cells were treated with various flavonoids (luteolin, apigenin, chrysin, diosmetin, acacetin, nobiletin and tangeretin) for 30 min prior to xanthine oxidase (9 U/L) / xanthine (50 μM) treatment for 30 min. The cells were washed once with PBS, and DHE (20 μM) was added into the medium for a further 30 min. The superoxide anion ($O_2^{\bullet-}$) production was monitored by flow cytometry.

H_2O_2-Induced Oxidative Cell Damage

HL-60 cells (2×10^5) were pretreated with test compounds for 30 min prior to the addition of 50 μM H_2O_2 into the medium for a further 8 h. The cells were then harvested, washed with PBS, resuspended in 200 μL of PBS, and fixed in

800 μL of iced 100% ethanol at -20 °C. After being left to stand overnight, the cell pellets were collected by centrifugation, resuspended in 1 mL of hypotonic buffer (0.5% Triton X-100 in PBS and 0.5 μg/mL RNase) and incubated at 37 °C for 30 min. Next, 1 mL of propidium iodide solution (50 μg/mL) was added and the mixture was allowed to stand on ice for 30 min. Fluorescence emitted from the propidium iodide-DNA complex was quantitated after excitation of the fluorescent dye by FAC Scan cytometry (Becton Dickinson, San Jose, CA).

DNA Extraction and Electrophoresis Analysis

HL-60 cells were treated, with or without flavonoids, in the presence of xanthine oxidase (9 U/L)/xanthine (50 μM) for 4 h. The cells were then harvested, washed with phosphate-buffered saline (PBS) and lysed with digestion buffer containing 0.5% sarcosyl, 0.5 mg/mL proteinase K, 50 mM tris(hydroxymethyl) aminomethane (pH 8.0) and 10 mM EDTA at 56 °C overnight after which they were treated with RNase A (0.5 μg/mL) for 3 h at 56 °C. The DNA was extracted by phenol/chloroform/isoamyl (25:24:1, v/v/v) before loading and was analyzed using 2% agarose gel electrophoresis. The agarose gels were run at 50 V for 120 min in Tris-borate/EDTA electrophoresis buffer (TBE). Approximately 20 μg of DNA was loaded into each well, visualized under UV light and photographed (*33*).

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Measurement of Intracellular ROS Accumulation

TPA is an inflammatory agent and potent tumor promoter which has been reporter to act through the generation of ROS. To investigate whether polyphenols could reduce TPA-induced ROS production, HL-60 cells were pretreated with test compounds for 30 min prior to TPA (100 ng/mL) treatment for a further 30 min. The intracellular ROS levels in HL-60 cells were detected by 2′,7′-Dichlorodihydrofluorescein diacetate (DCF-DA). DCF-DA is hydrolyzed by cellular esterases to firm DCF, thus, the fluorescence intensity is proportional to the amount of peroxide produced by the cells.

Nitrite Assay

Mouse macrophage RAW264.7 cells were treated with test compounds and LPS (100 ng/mL) for 24 h. The nitrite concentration in the medium was measured as an indicator of NO production according to the Griess reaction. One hundred microliters from each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1%

naphthylethylenediamine dihydrochloride in water) and absorbance of the mixture was read at 550 nm using an ELISA plate reader (Dynatech MR-7000; Dynatech Laboratories).

Results

Effect of Luteolin and Apigenin on H_2O_2 Production of TPA-stimulated HL-60 Cells

The effects of luteolin and apigenin on H_2O_2 production by TPA-stimulation HL-60 cells were analyzed by flow cytometry. The results showed that luteolin was a potent inhibitor of TPA-induced H_2O_2 production in HL-60 cells at 10 μM (Figure 1). The inhibitory potency was luteolin > apigenin at 10 μM (Figure 2). The effects of luteolin on H_2O_2 production in TPA-stimulated HL-60 cells are possibly due to involvement in the signal transduction of the PKC activation pathway that is stimulated by TPA.

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Effects of Flavonoids on H_2O_2 Levels in HL-60 Cells

The H_2O_2 levels was further compared in only flavonoid (10 μM) treated HL-60 cells using the fluorescent probe DCFH-DA and monitoring by flow cytometry. A decrease of intracellular peroxide levels by 10 μM tangeretin was detected for 0.5 h. The inhibitory potency was estimated in the following order: tangeretin > nobiletin > apipenin > luteolin > chrysin > diosmetin at 10 μM (Figure 3).

Effect of Flavonoids on H_2O_2-induced Apoptosis in HL-60 Cells

Hydrogen peroxide which has a permeable cell membrane and is a precursor of various free radicals, was chosen as an oxidant to induce apoptosis in our study. To further assess the antioxidant activity of flavonoids, we investigated whether exogenously administered H_2O_2 could induce apoptosis in HL-60 cells. As shown in Figure 4, luteolin (10 μM) proved to be an efficient protective agent for H_2O_2-induced apoptosis in HL-60 cells.

Inhibition of ROS Generation and Protection of HL-60 Cells from ROS Induced Apoptosis by Flavonoids

Xanthine oxidase (XO) is a complex enzyme. It causes gout and is responsible for oxidative damage to living tissues. XO is the major source of

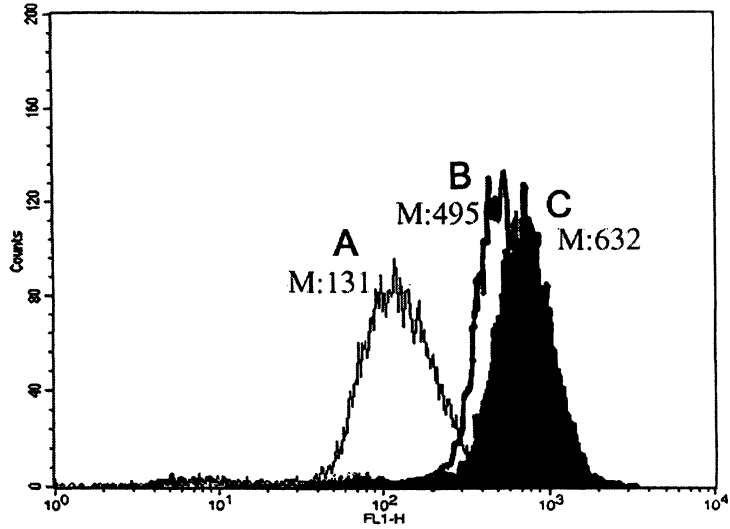

Figure 1. Effects of Luteolin on TPA-induced hydrogen peroxide (H_2O_2) generation in HL-60 cells. Cells were treated TPA only or treated with 10 μM luteolin prior to TPA (100 ng/mL) treatment.Tthe cells were then incubated with DCFH-DA (20 μM) for 30 min and analyzed by flow cytometry and presented as log fluorescence intensity. A, control, B, TPA 100 ng/mL + luteolin 10 μM, C, TPA 100 ng/L.

ROS candidacy, as strengthened by recent analyses of promoter regions of human, mouse, and rat enzymes, which suggest the presence of potential regulatory sites for cytokines known to stimulate generation of ROS. Flavonoids are known to inhibit xanthine oxidase activity and scavenge ROS. The flavonoid prevention of ROS generation induced by external xanthine/xanthine oxidase (X/XO) reaction was analyzed. The HL-60 cells were treated with xanthine and xanthine oxidase, and ROS generation and cell apoptosis were assayed by flow cytometry and DNA electrophoresis. The inhibitory ability of ROS generation by flavonoids is as follows: chrysin > nobiletin > diosmetin > luteolin > apigenin > tangeretin > acacetin at 10 μM (Figure 5). As shown in Figure 6, the DNA ladder was prevented when cells were treated with flavonoids. These results suggest that flavonoids significantly protected cells from ROS induced apoptosis in the X/XO reaction. These flavonoids were able to prevent cell damage induced X/XO reaction and could be potent inhibitor of xanthine oxidase.

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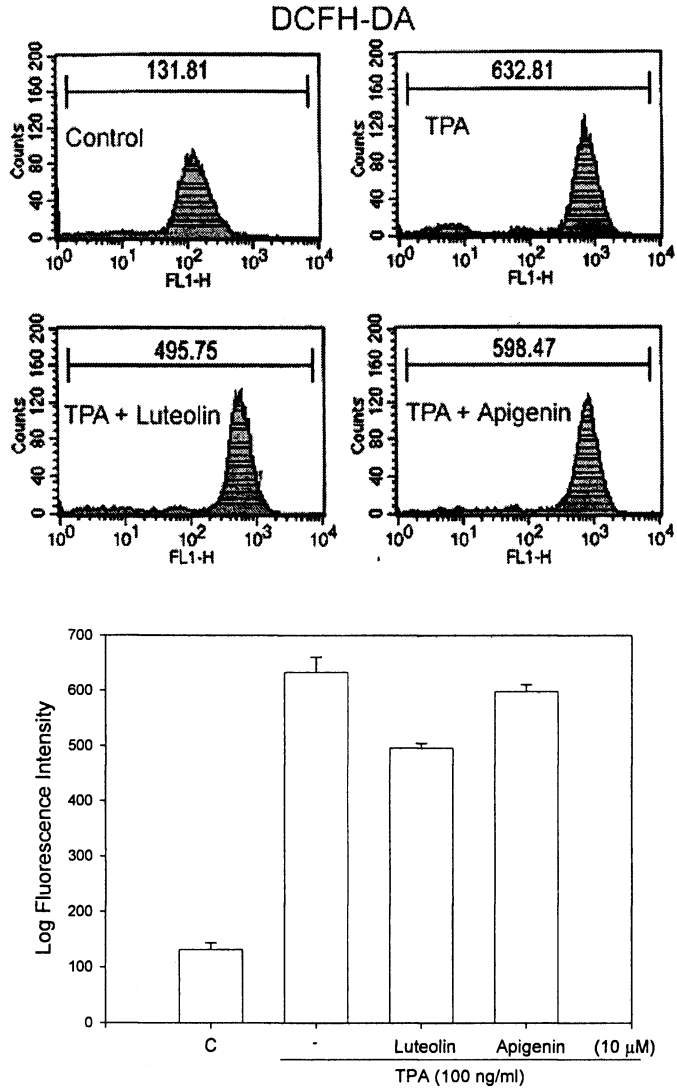

Figure 2. The effect of Luteolin and Apigenin on TPA-induced hydrogen peroxide (H_2O_2) production in HL-60 cells. Cells were treated TPA only or treated with 10 μM luteolin and apigenin prior to TPA (100 ng/mL) treatment. The cells were then incubated with DCFH-DA (20 μM) for 30 min and analyzed by flow cytometry and presented as log fluorescence intensity.

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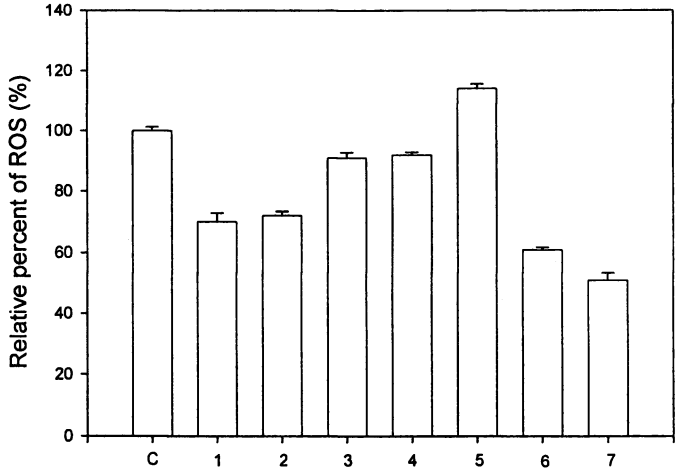

Figure 3. The effect of flavonoids on hydrogen peroxide (H_2O_2) levels in HL-60 cells. Cells were treated with 10 μM flavonids (1, apigenin; 2,luteolin; 3, chrysin; 4, diosmetin; 5, acacetin; 6, nobiletin; 7, tangeretin) 30 min and were then incubated with DCFH-DA (20 μM) for a further 30 min. Data are analyzed by flow cytometry and presented as log fluorescence intensity.

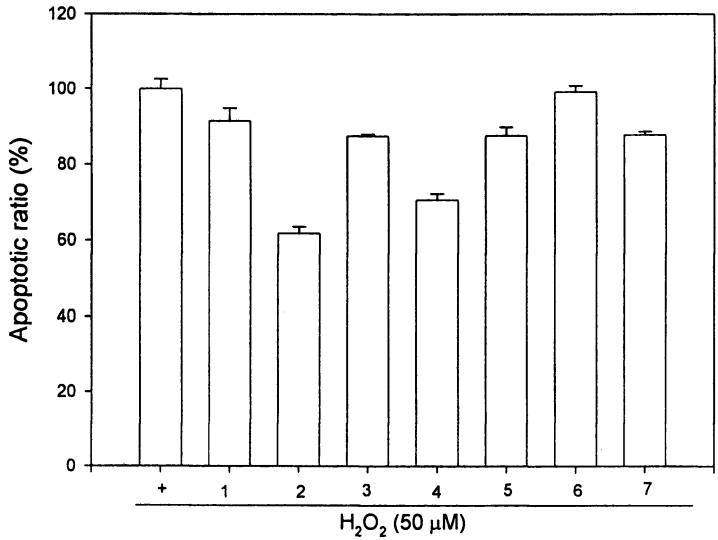

Figure 4. Effects of various flavonoids on H_2O_2-induced apoptosis in HL-60 cells. HL-60 cells were pretreated 10 μM flavonoids (1, apigenin; 2, luteolin; 3, chrysin; 4, diosmetin; 5, acacetin; 6, nobiletin; 7, tangeretin) for 30 min prior to treated with 50 μM H_2O_2 for 8 h. The apoptotic ratio (%) was determined by flow cytometry.

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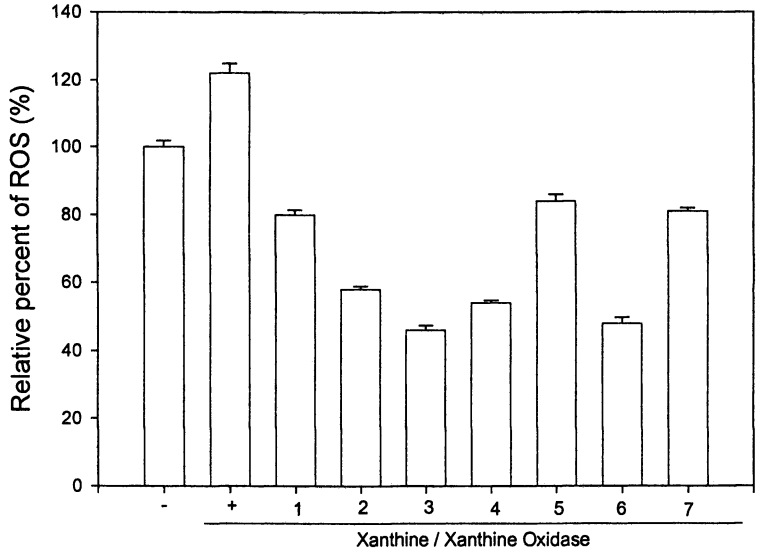

Figure 5. Effect of flavonoid on Relative percent of reactive oxygen species (ROS) in xanthine / xanthine oxidase-induced ROS production in HL-60 cells. Cells were treated with 10 μM flavonids (1, apigenin; 2, luteolin; 3, chrysin; 4, diosmetin; 5, acacetin; 6, nobiletin; 7, tangeretin) for 30 min, followed by incubated with xanthine/xanthine oxidase for a further 30 min. The cells were then incubated with DHE (20 μM) for 30 min and analyzed by flow cytometry and presented as log fluorescence intensity.

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Inhibition of NO Generation by Polyphenols in LPS-activated Macrophages

Nitric oxide plays an important role in inflammation and in the multiple stages of carcinogenesis. Of the polyphenols tested, theaflavin-3,3′-digallate (20 μM) inhibited LPS-stimulated NO generation the most strongly. As shown in Table I, the inhibitory potency was estimated to be in the following order: theaflavin-3,3′-digallate > acacetin > wogonin > penta-*O*-galloyl-β-D-glucose at 20 μM.

Discussion

Reactive oxygen species (ROS) are a family of active molecules including superoxide anion ($O_2^{\bullet -}$), peroxyl ($ROO^{\bullet}$), hydroxyl radical ($HO^{\bullet}$), and nitric oxide (NO), that are generated in cells by several pathways and involved in the

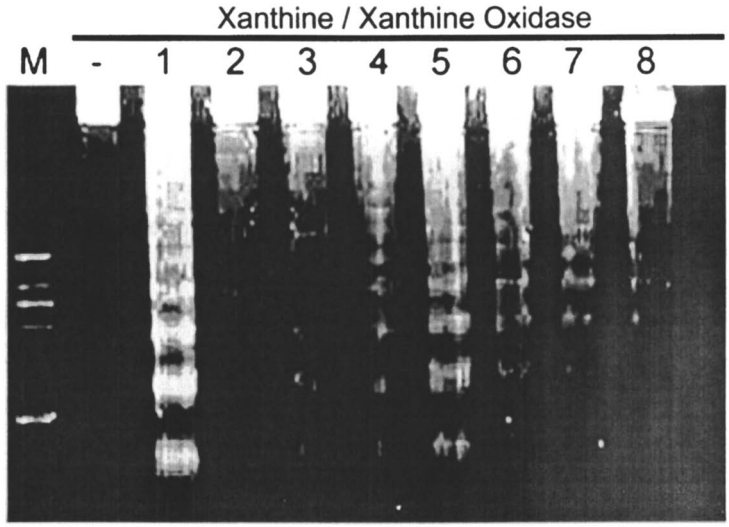

Figure 6. Flavonoids protected HL-60 cells from ROS damage in the xanthine oxidase/xanthine reaction. HL-60 cells treated with or without Flavonoids in the presence of xanthine oxidase/xanthine for 4h, and DNA fragmentation was shown. (1, apigenin; 2, luteolin; 3, chrysin; 4, diosmetin; 5, acacetin; 6, nobiletin; 7, tangeretin)

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modulation of biological cell functions. However, a large amount or sustained levels of ROS can result in the oxidation of biomolecules including lipid, protein, and DNA, resulting in cell damage leading to growth arrest, senescence, or death (*34*). Several studies have shown that differentiated HL-60 cells possess the phagocytic properties and the capability of generating ROS upon stimulation (*35*). Human carcinogenesis is known to progress through multiple stages of initiation, promotion, and progression. It is well known that generation of ROS is associated with the initiation and promotion of carcinogenesis.

Chemoprevention has had a potential impact on cancer incidence rates through the modulation of initiation and promotion stages (*36*). Epidemiological studies have increasingly demonstrated that the content of phytochemicals such as curcumin, tea polyphenols, and flavonoids occur ubiquitously in plant foods. They reduce the risk of cancer through their role in the metabolism of carcinogens, in hormonal binding, in regulation of gene expression, in antioxidant enzymatic activities, and in scavenging of free radicals (*37*).

The purpose of this study was to evaluate antioxidant activities of phytophenols in different cell culture assay for the prevention of carcinogenesis.

Table 1. Effects of Phytopolyphenols on LPS-induced Nitrite Production in RAW 264.7 Macrophage

Sample	*Test Compounds (μM)*		*Nitrite (μM)*	*Inhibition (%)*
control	-		4.6±0.6	-
LPS (100 ng/ml)	-		43.3±0.9	-
LPS	Wogonin	10	23.1±0.4	52
		20	17.1±0.6	68
LPS	Acacetin	10	14.6±0.3	74
		20	8.2±0.5	91
LPS	5GG	10	24.7±0.9	48
		20	18.3±0.6	65
LPS	TF-3	10	13.2±0.4	78
		20	6.8±0.2	94

The cells were treated with 100 ng/ml of LPS only or with different concentrations (10 and 10 μM) of various phytopolyphenols for 24 h. At the end of incubation time, 100 μl of the culture medium was collected for nitrite assay

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Because oxidative DNA damage is considered to be relevant in carcinogenic processes, we evaluated the possible anticarcinogenic effects of flavonoids by determining their effect on TPA-inducing ROS generation, H_2O_2 scavenging, H_2O_2-induced apoptosis, xanthine oxidase activity, and LPS-inducing NO generation. Assays of various antioxidant models produced different effects. Luteolin inhibited the H_2O_2 production of TPA-stimulated HL-60 cells (Figure 1 and 2). Tangeretin more potent removed the level of H_2O_2 in HL-60 cells (Figure 3).

TPA is known to induce H_2O_2 production by phagocytic cells and epidermal cells through increasing XO activity and by diminishing antioxidant enzyme activities (*38*). Recent studies show that, in double TPA treated mouse skin, ROS from leukocytes, including superoxide, plays an important role leading to chronic inflammation and hyperplasia. Superoxide generation inhibitors are effective in inhibiting this tumor promotion response. These findings might suggest that phytopolyphenols act at an earlier stage than has previously been suspected, by suppressing ROS production through inhibiting XO, rather than only scavenging the already formed ROS. This could partly explain some of the beneficial properties attributed to phytopolyphenols, such as antimutagenic and anticarcinogenic effects which are all mediated by ROS (*39*).

Acknowledgements

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Chapter 8

Lipid Oxidation, Antioxidants, and Spin Trapping

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Radicals are important intermediates in autoxidation of lipids. The short-lived radicals are often only encountered in steady-state concentrations that are too low to be detected directly by electron spin resonance (ESR) spectroscopy. The use of spin traps allow the indirect detection of lipid-derived radicals by formation of stable spin adducts that can accumulate in detectable concentrations. Spin trapping can be used for quantification of the tendency of radical formation in a lipid containing food sample by comparison with a standard curve of spin probes. Spin trapping may also under certain conditions be used for identification of radical intermediates in lipid oxidation reactions. Correct selection of spin traps is essential for a successful application of the method and recommendations are given for various food systems.

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Electron spin resonance (ESR) spectroscopy has proved to be useful for detection of radicals that are associated with oxidative processes in several different types of foods (*1,2*). However, the short lived nature of radicals that are involved in lipid oxidation makes it impossible to detect these radicals directly by ESR, although this technique is highly sensitive and can be used for detection of radicals down to a few nanomolar concentration nder favorable conditions. By using spin trapping technique, it is often possible to obtain indirect information about the presence of radicals in oxidative reactions, and this technique is finding increasing use in studies of the oxidative reactions that take place in foods (*2,3*). The spin trapping technique is based on forming stable paramagnetic spin adducts between so called spin traps and the reactive radicals (Figures 1 and 2). The accumulation of the stable spin adducts in concentrations above a few micromoles makes it possible to use ESR for their detection. The commonly available spin traps are either nitrones or nitroso compounds (Figure 1). The addition of radicals to nitrone or nitroso groups lead to the formation of nitroxyl radicals, which often are exceptionally stable (Figure 2).

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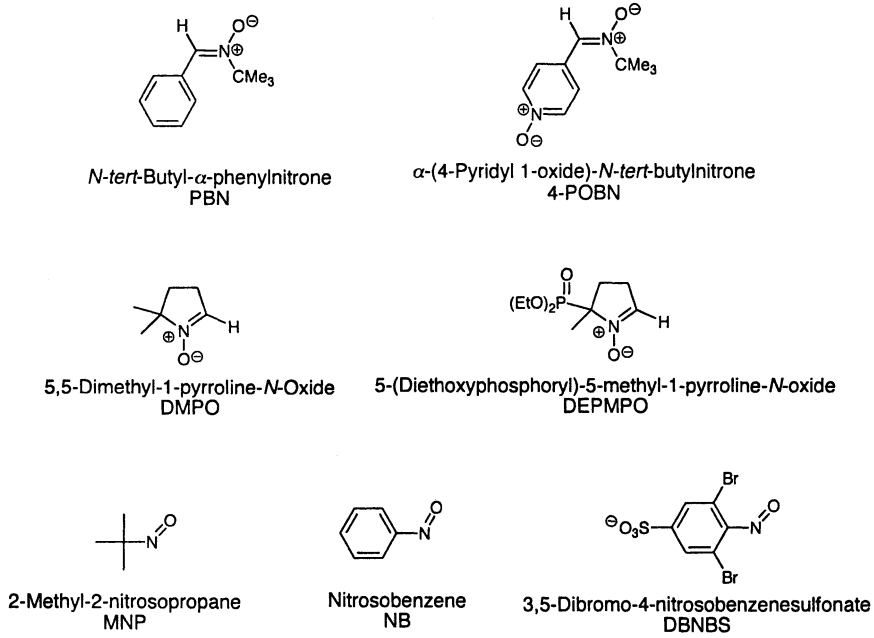

Figure 1. Structures of common spin traps.

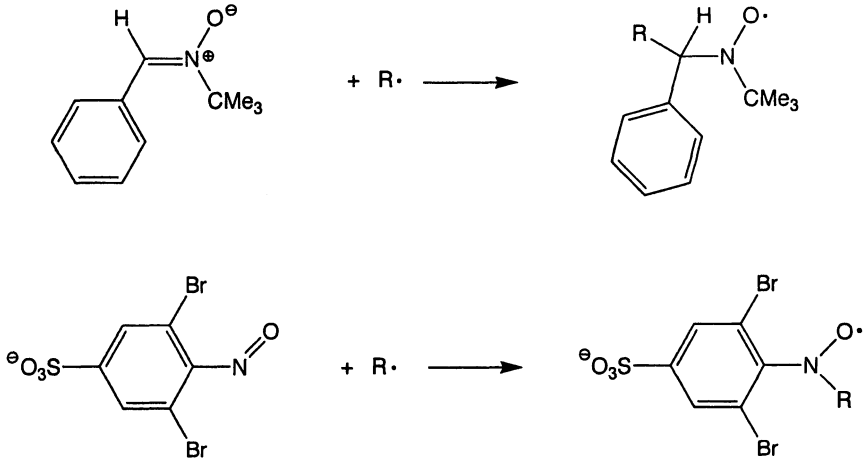

Figure 2. Formation of spin adducts by addition of radicals to spin traps.

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Spin trapping in food systems

The spin traps N-tert-butyl-α-phenylnitrone (PBN) and the more water-soluble α-(4-pyridyl 1-oxide)-N-tert-butyl nitrone (4-POBN) have often been used for detecting radicals during lipid oxidation, due to the good stability of the spin adducts. Unfortunately, the hyperfine coupling constants of the spin adducts of these two spin traps only show a small dependence of the added radicals, and the exact identifications of the trapped radicals are therefore problematic based only on the hyperfine coupling constants of spin adducts. The spin adducts are, however, often so stable that they can be separated by high performance high performance liquid chromatography (HPLC), detected by ESR and characterized by mass spectrometry (MS) (*4*). A single six-line ESR spectrum with hyperfine coupling constants a_N = 15.7 G and a_H = 2.8 G was observed when linoleic acid or arachidonic acid was oxidized by lipxoxygenase in the presence of 4-POBN (*4,5*). However, by using LC-ESR and LC-MS techniques a mixture of spin adducts formed by trapping of of linoleic alkyl radicals, epoxyallylic radicals, dihydroxyallylic radicals and alkyl radicals derived from β-scission of alkoxy radicals was identified. Similar six-line spectra were observed when 4-POBN was used in studies of lipid oxidation in meat systems (*6,7*). 4-POBN is normally to be recommended for water-containing systems like meat.

Oxidation of linoleic acid by lipoxygenase in the presence of 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) gave complex ESR spectra with overlapping signals from several different spin adducts (*8*). Computer simulations of the ESR spectra together with results from model experiments revealed that two diastereomeric DMPO alkoxyl radical spin adducts (with hyperfine coupling constants a_N = 14.08 G, $a_{H,\beta}$ = 10.33, $a_{H,\gamma}$ = 1.59 G and a_N = 14.15 G, $a_{H,\beta}$ = 11.33, $a_{H,\gamma}$ = 1.01 G respectively) accounted for 84 % of the ESR signal, while four radical adducts where present in smaller amounts (DMPO/·OH, a_N = 14.91 G and a_H = 14.79 G; DMPO/carbon centered radical adduct, a_N = 16.13 G and a_H = 23.32 G; DMPO/carbonyl radical adduct, a_N = 15.30 G and a_H = 18.85 G; oxidized DMPO (DMPOX), a_N = 7.24 G and a_H = 4.06 G). Similar results were obtained when arachidonic acid was oxidized under identical conditions.

PBN, although less water-soluble than 4-POBN, has been used for studies of oxidative reactions in beer (*9*). The presence of PBN during peroxidation of oils induced by heating gives a single six-line ESR spectrum with hyperfine coupling constants a_N = 14.8 G and a_H = 1.8 G (*10*). However, line broadening due to low rotational mobility of the spin adducts in viscous oils may cause the doublets to coalesce resulting in a three-line ESR signal (*11,12*). The use of deuterated PBN and the analysis by electron-nuclear double resonance (ENDOR) suggested that the ESR spectra were a mixture of spectra of PBN alkyl radical adducts, PBN peroxyl radical adducts and nitroxyl radicals arising from the decay of PBN peroxyl radical adducts (*13*).

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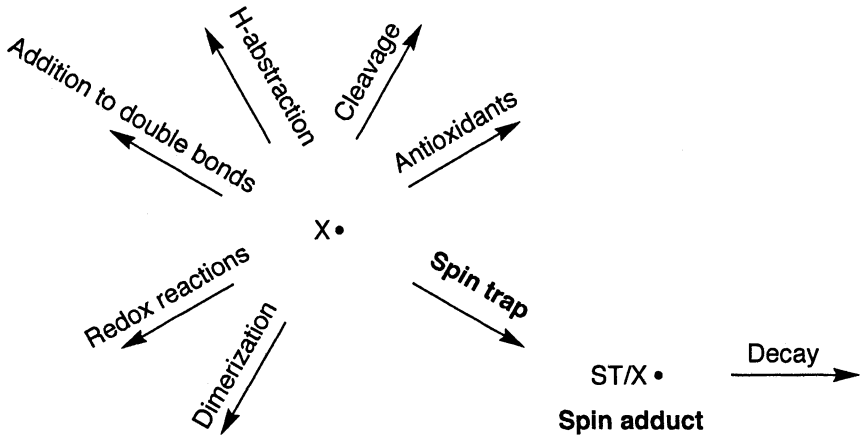

Figure 3. The formation of spin adducts always competes with other reaction pathways for the radicals X·

The high reactivity of radicals, which prevents the build up of detectable concentrations, is the main impetus for using the spin trapping technique for studying the chemistry of radicals. However, the high reactivity also means that radicals can have many different often unknown reaction pathway available in complex systems like foods, and it is therefore always important to take into consideration during the analysis and interpretation of results from spin trapping experiments that the formation of spin adducts is always the result of a delicate balance between several competing reactions (Figure 3). The specific properties of the spin traps should therefore be taken into account when experiments are designed and when the results of spin trapping experiments are evaluated and interpreted.

Most foods are heterogeneous systems containing both water and lipid phases, and the location of the spin traps will have a significant impact on the trapping of radicals. Most of the commonly used spin traps are hydrophilic which means that they will mainly be trapping radicals in the aqueous parts of complex foods (Table I). PBN is the only commonly available nitrone spin trap that has a partition coefficient in favor of distribution into lipid phases.

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Table I. Properties of Common Spin Traps

Spin trap	Partition coefficient [a]	Addition to spin traps	
	K_p	OH·	·CH(OH)CH$_3$
		k ($M^{-1}s^{-1}$)	k ($M^{-1}s^{-1}$)
DMPO	0.08 (*15*)	$3.3 \cdot 10^9$ (*17*)	$4.1 \cdot 10^7$ (*21*)
4-POBN	0.09 (*15*)	$3.5 \cdot 10^9$ (*18*)	$3.1 \cdot 10^7$ (*23*)
DBNBS	0.15 (*15*)	$4.0 \cdot 10^9$ (*19*)	$6.3 \cdot 10^7$ (*19*)
DEPMPO	0.16 (*16*)	$7.8 \cdot 10^9$ (*20*)	-
MNP	8.2 (*15*)	$2.5 \cdot 10^9$ (*21*)	$3.2 \cdot 10^8$ (*21*)
PBN	10.4 (15)	$6.1 \cdot 10^9$ (*22*)	$1.5 \cdot 10^6$ (*23*)
NB	73 (*15*)	-	-

[a] Partition coefficients of spin traps in n-octanol/phosphate buffer at pH 7 – 7.4.

The relative rate of spin trapping as compared to all other reaction pathways available for the radicals will also strongly influence the outcome of spin trapping experiments (*14*). In addition, kinetics of spin trapping should be considered when comparing the intensity of different spin adducts. The rate constant for reactions between different spin traps and hydroxyl radicals are close to being diffusion controlled, whereas the reactions with carbon-centered radicals are 100 – 1000 slower and show a greater variation between the spin traps as exemplified by the rate constants for addition of the 1-hydroxyethyl radical (Table I).

The success of detection of radicals by spin trapping depends not only on the ability of the spin traps to trap radicals, but also of the stability of the produced spin adducts. Spin adducts of oxygen centered radicals with nitroso spin traps are all unstable, and these spin traps are therefore mainly used for detection of carbon centered radicals. The hydroxyl radical adducts of PBN and 4-POBN have half lives less than 1-2 minutes, whereas spin adducts with carbon centered radicals are stable even at fairly high temperatures (*3,24*).

Spin trapping in edible oils

Addition of the spin traps PBN or DMPO to virgin olive oil have been shown to give rise to the formation of spin adducts (*25,26*). Irradiation with light or addition of iron or copper ions increased the yield of spin adducts.

The antioxidant efficiency of phenolic compounds towards lipid alkoxyl radicals have been studied by using the spin trap PBN to trap alkoxyl radicals (*27*). Lipid alkoxyl radicals were generated by adding Fe^{2+} to methyl linoleate, that had been enriched with lipid hydroperoxides by exposure to air for 3 days. The reaction of lipid hydroperoxides with Fe^{2+} produce alkoxyl radicals (reaction 1), that either initiate autoxidation chain reactions, or are trapped by antioxidants or by the spin trap. The level of produced spin adducts thus depend on the efficiency of the antioxidants.

$$LOOH + Fe^{2+} \rightarrow Fe^{3+} + LO\cdot + OH^- \quad (1)$$

A similar approach was used for examining the antioxidant efficiency of polar extracts from olive oil, where the spin trapping with PBN was carried out with low-density lipoproteins and lipid oxidation in the presence of olive oil extracts was initiated by addition of Cu^+ (*28*). Futhermore, studies of lipid oxidation in rapeseed oil encapsulated in a glassy food matrix have been performed by using the spin traps PBN and 4-POBN (*29*). The line shape of the ESR signals of the spin adducts made it possible to discern between radicals trapped in the encapsulated oils and radicals trapped in the glassy matrix.

Prediction of lipid stability

The formation of spin adducts in oil or lipid fractions isolated from foods that are mildly heated (40 – 70 °C) can be used for making early predictions of the oxidative stability of the lipids (*10,11,30*). Usually an induction period (or lag phase) for formation of spin adducts is observed, and the length of the induction period is a result of the competing effects of anti- and prooxidants.

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The induction periods for the onset of lipid oxidation in vegetable oils, which were determined by the spin trapping method with ESR detection, gave excellent linear correlations with induction periods determined by the Rancimat method and by differential scanning calorimetry (DSC) (Figure 4) (*30*). However, temperatures at 100 °C or above were needed by the latter two methods and the induction periods were between 2 and 20 h. The ESR method needed 60 °C and the induction periods were 6 h or less. The much milder conditions, which allow the presence of water in the samples, and the much shorter time for determination of the induction period make the ESR method favorable for determination of oxidative stability of not only pure oils but also for lipids in more complex food systems.

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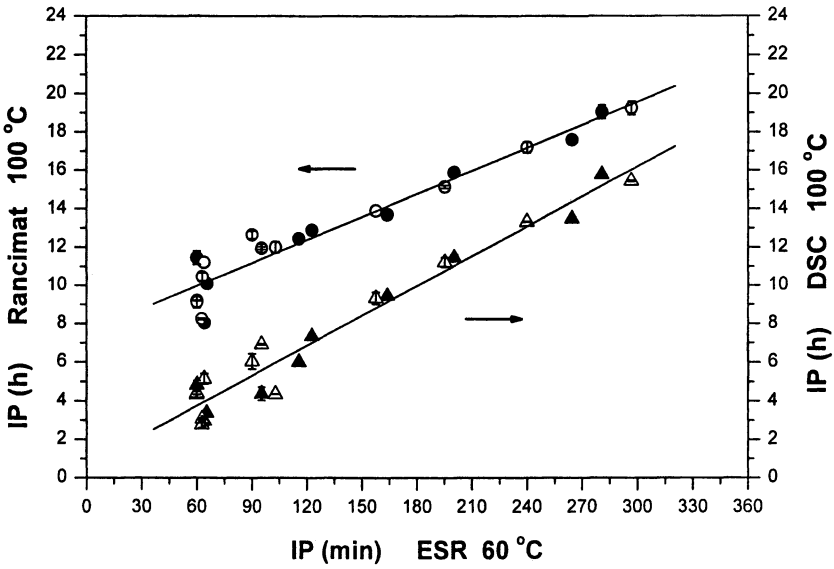
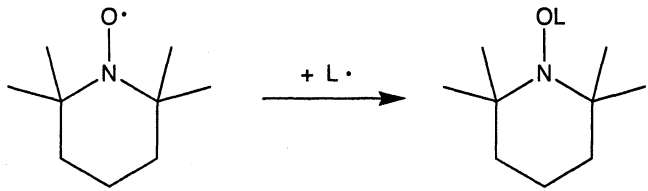


Figure 4. Linear correlations between induction periods (IP) for the onset of lipid oxidation in rapeseed oil and sunflower oil determined by ESR, the Rancimat method (circles) and DSC (triangles) (30).

Although PBN spin adducts were stable for days in oils stored at 40 °C, and thus were useful for studying the extent of the early stages of lipid oxidation, then the level of spin adducts leveled of after two weeks, indicating that the PBN spin adducts are not stable enough for studying lipid oxidation during long term storage experiments (*12*). Instead, it was suggested that lipid oxidation during extended storage of lipids could be studied by performing short duration spin

trapping experiments with samples that were collected at intervals during the storage, thus giving information about the tendency for radical formation at different times during the long term storage. A similar experimental approach was previously used for studying lipid oxidation in fish oil enriched mayonnaise, where samples stored for 2 and 4 weeks was added PBN and incubated at 37 °C for 24 h before the amount of spin adducts were determined (*31*). The concentration of PBN spin adducts in the mayonnaise samples could be quantified by standardization against a stable nitroxyl radical (*32*).



TEMPO

Figure 5. The stable nitroxyl radical TEMPO efficiently trap lipid radicals by forming non-radical adducts.

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Many spin adducts are stable, but the radical nature of these compounds also makes them efficient radical scavengers, thus producing non-paramagnetic molecules that are ESR invisible. Nitroxyl radicals such as the stable radical 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) couple with carbon centered radicals with rate constants up to 10^9 $M^{-1}s^{-1}$ forming non-radical adducts (Figure 5) (*33*). The nitroxyl group in TEMPO is structurally similar to the nitroxyl group in nitrone spin adducts, and the reactivity towards lipid radicals are expected to be similar for TEMPO and nitrone spin adducts. The trapping of two radicals by nitrone spin adducts leading to the formation of ESR-invisible compounds are expected to be favored under conditions with high levels of radicals (Figure 6). The amount of detectable spin adducts may thus decrease under highly oxidizing conditions.

The intensities of ESR signals of PBN spin adducts were found to decrease in salmon viscera oil after a few hours, while the signals of PBN spin adducts decreased within a few minutes in pure ethyl docosahexaenoate at 40 °C (*10*). On the other hand, PBN spin adducts were stable in cod liver oil up to 190 hours before the ESR signals began to decrease. The stable nitroxyl radical TEMPO decayed with nearly the same rate in the three lipid systems, which suggests that other mechanisms than trapping of two radicals might be responsible for the decay of the PBN spin adducts.

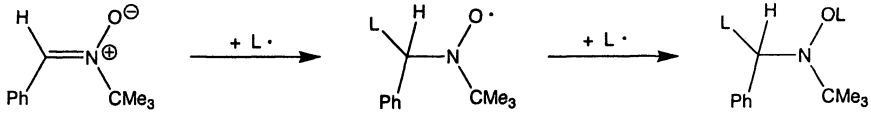

Figure 6. Trapping of two radicals per spin trap lead to formation of non-radical spin adducts which are ESR-invisible by trapping.

Spin traps as retarders of lipid oxidation

By trapping radicals the spin traps may act as antioxidants if they remove major amounts of the radicals that are formed during lipid oxidation. Lower levels of peroxides and thiobarbituric acid reactive substances (TBARS) were formed in rapeseed, sunflower and fish oils containing PBN than in controls without PBN during storage at 40 °C up to two months (*12*). The addition of PBN also protected tocopherols in the oils from oxidation during the storage. The inhibition of lipid oxidation by PBN decreased with the oxidative stability of the oils, which was explained by the ability of PBN to trap peroxyl radicals. The spin adducts between peroxyl radicals and PBN are known to be unstable, and break down to produce alkoxyl radicals, which are the actual radical species that are detected as stable spin adducts (*8*). The breakdown of the peroxyl radical spin adducts into alkoxyl radicals also suggest that PBN is not acting as a chain-breaking antioxidant, but more likely as a retarder of lipid oxidation, since the produced alkoxyl radicals will be able to initiate new chain of radical induced oxidation of unsaturated lipids. Barclay and Vinqvist (*34*) have shown that PBN only decrease the rate of oxygen consumption during lipid oxiddation, in contrast to chain breaking antioxidants that give an induction period where the oxygen consumption is inhibited.

Concluding remarks

The methods currently used for studying oxidation in foods, are not able to give any or only very crude information about the spatial distribution of oxidative processes, which is often caused by the need for samples on the gram-scale (*35*). The progression of oxidation between particles or different physical phases on a scale of millimeters or smaller have never been studied, despite the fact that most foods are heterogonous systems on this length scale. The spatial distribution of oxidation in foods is not only expected to vary between different particles and phases, but also vary with the distance from the outer surface of the

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food item, where atmospheric oxygen or irradiation with light may accelerate oxidation. A detailed knowledge of microscopic distribution and progress of oxidative reactions in foods will be useful for understanding the action of antioxidants and the role of emulgators during oxidative aging of foods, which can be used for better design of antioxidant defense in foods. The interaction between different antioxidants in membranes can also be followed and used for optimization of protection of heterogeneous food systems. Such work will depend on ESR imaging, a technique which can be based on L- and X-band ESR instruments. When this imaging technique is combined with oximetry (ESR probing of oxygen), a technique that is based on broadening of the line width by paramagnetic compounds, it should be possible to follow both formation of radicals and depletion of oxygen in localized structures in food systems.

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Chapter 9

Regulation of Antioxidant Response Element Pathways by Natural Chemopreventive Compounds

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Research indicates that protecting cells or tissues from carcinogens and carcinogenic metabolites through the induction of cellular defense enzymes, such as phase 2 detoxifying and antioxidant enzymes, is a method of chemoprevention that shows great promise. Antioxidant response element (ARE) is located in the promoter region of these defense genes and plays a key role in the induction of these enzymes. Many natural chemopreventive agents are known to induce ARE-mediated gene expression and regulate the upstream signalings involved in the ARE pathway.

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Cancer Chemoprevention

Carcinogenesis is typically classified into three stages of initiation, promotion, and progression (*1*). Various natural and synthetic chemicals, the so-called chemopreventive agents, can interfere with these stages. Chemoprevention is defined as a cancer-preventive approach that utilizes natural or synthetic pharmacological agents to impede, arrest, or reverse carcinogenesis at its early stages (*2*). Natural compounds found in foods and edible plants have gained much attention as potential chemopreventive agents due to their relatively low toxicity, low cost, and easy availability as well as their general identity as health foods (*3*). Therefore, the use of natural compounds as chemopreventive agents could be one of the most probable strategies for cancer chemoprevention.

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Induction of Cellular Defense Enzymes

One of the most important cancer chemopreventive properties of natural compounds could be their ability to induce cellular defense enzymes, such as phase 2 detoxifying and antioxidant enzymes, which can protect cells and tissues against various endogenous and exogenous carcinogens and carcinogenic metabolites. Examples of these defense enzymes are glutathione S-transferase (GST), NAD(P)H quinone oxidoreductase 1 (NQO1), γ-glutamylcysteine synthetase (γ-GCS) and heme oxygenase-1 (HO-1).

Nrf2/ARE Signaling Pathway

The induction of these defense genes is mediated, at least in part, by the antioxidant response element (ARE) in the promoter region of these genes (*4*).

Nuclear factor-erythroid 2-related factor 2 (Nrf2), a member of the Cap 'n' collar (CNC) family of basic region-leucine zipper (bZIP) proteins, plays a key role in ARE-dependent gene expression. Upon exposure of cells to its inducers, such as oxidative stress and certain chemopreventive agents, it dissociates from Keap 1, translocates from the cytosol to the nucleus, binds to AREs and transactivates the cellular defense enzymes (*5, 6*). Nrf2/ARE pathway is of great interest as a potential molecular target for cancer prevention. Several natural compounds including isothiocyanates, diallyl sulfides, indoles, terpenes, and phenolic compounds, such as tea catechins and curcuminoids, have been reported to modulate Nrf2/ARE signaling pathways (*7-14*).

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Naturally Occurring Potential Cancer Chemoprenventive Compounds Involved in Nrf2/ARE Pathways

Examples of natural chemopreventive compounds that have been studied include isothiocyanates, diallyl sulfides, tea catechins, curcuminoids, indoles and sesquiterpenes. The chemical structures of these compounds are illustrated in Figure 1.

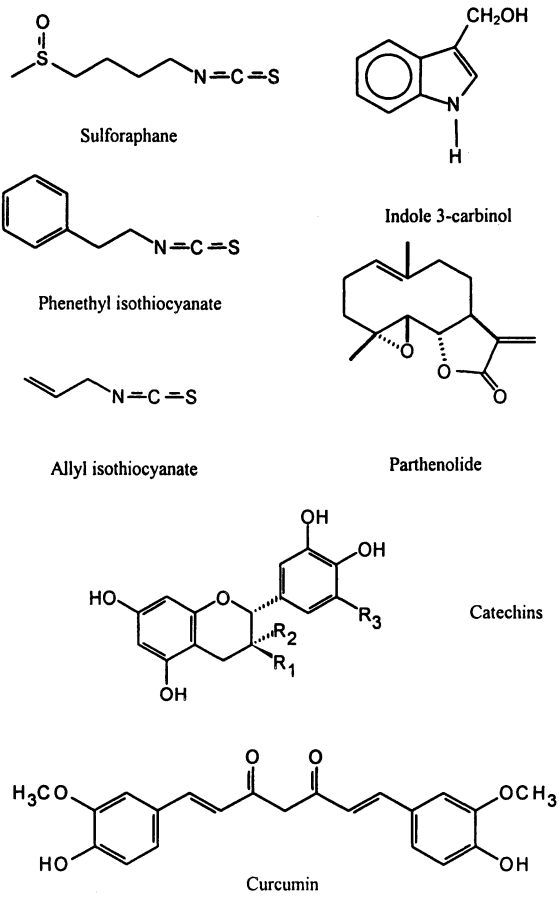

Figure 1. The Structures of natural chemopreventive compounds involved in Nrf2/ARE pathways.

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Isothiocyanates

Isothiocyanates are found mainly in cruciferous vegetables including broccoli, watercress, brussels sprouts, cabbage, and cauliflower (*15*). Glucosinolates are precursors of isothiocyanates in intact plant tissues. Upon physical stress followed by enzymatic reaction of plant-specific myrosinase or by intestinal microflora, they are converted to isothiocyanates such as sulforaphane, phenethyl isothiocyanate (PEITC), and allyl isothiocyanate (AITC) (*16*).

Sulforaphane could be one of the most potent inducers of cellular defense enzymes. It induces mitogen activated protein kinases (MAPKs), Nrf2, ARE reporter gene activity, and phase 2 detoxifying and antioxidant enzymes such as NQO1 and HO-1 (*9, 17, 13, 14*). One possible mechanism for this induction is that sulforaphane disrupts the cytoplasmic complex between Keap1 and Nrf2 by reacting with covalent bonds between the Nrf2-Keap1 complex, that results in the release of Nrf2 to the nucleus and the activation of ARE-dependent genes (*18*). Increased stability of Nrf2 protein also accounts for the induction of cellular defense enzymes by sulforaphane (*9, 14*).

PEITC is another promising isothiocyanate compound with cancer chemopreventive potential. It has been shown to dose-dependently activate ARE-reporter gene activity in a transiently transfected cell line model (*19*). In addition, co-transfection of Nrf2 and JNK1 together with PEITC treatment showed additional enhancement of the ARE-reporter activity. Overexpression of dominant-negative JNK1 suppressed Nrf2-induced ARE-reporter gene expression, suggesting a role of JNK1 in an upstream activation of Nrf2. Activation of JNKs by PEITC has also been implicated as an apoptotic mechanism induced by PEITC (*20*).

Induction of Nrf2 and antioxidant enzyme HO-1 was observed when HepG2 cells were treated with AITC but their potency was less than those of sulforaphane and PEITC (*9*).

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Diallyl Sulfides

The allium family, including garlic, onion, and chive, contains a series of potential chemopreventive agents such as diallyl sulfide (DAS), diallyl disulfide (DADS), and diallyl trisulfide (DATS). Induction of phase 2 detoxifying enzymes (GST, glutathione reductase, NQO1 and ferritin) has been reported *in vitro* and *in vivo* (*21-23*). A structure-activity relationship study with various organosulfides showed that DATS is a very potent stimulator of ARE activation as well as nuclear accumulation of Nrf2 (*8*). It can also strongly induce expression of phase 2 detoxifying and antioxidant enzymes such as NQO1, and HO-1 proteins. The third sulfur in the structure of the diallyl sulfides is proposed

to contribute to their bioactivities. In addition, allyl-containing sulfides were found to be more potent than propyl-containing sulfides. DATS also activated MAPKs such as ERK, JNK, and p38 (*8, 24*).

Phenolic Compounds

Phenolic compounds are found in almost all plants and a number of phenolic compounds have long been studied for their chemopreventive potential. However, their roles in cellular defense mechanisms, in particular their role in the Nrf2/ARE pathway, are not fully understood. Of phenolic compounds, those in green tea such as (–)-epicatechin (EC), (–)-epigallocatechin (EGC), (–)-epicatechin gallate (ECG), and (–)-epigallocatechin gallate (EGCG), are well known antioxidants and their beneficial properties in cardiovascular disease and cancer are well documented (*25, 26*). EGCG and ECG have been found to induce ARE-mediated gene expression as well as MAPKs in HepG2 cells (*27*). In this study, the induction of ARE reporter gene appears to be structurally related to the 3-gallate group.

Chlorogenic acid also increases the enzymatic activities of phase 2 enzymes such as GST and NQO1 through its stimulation of nuclear translocation of Nrf2 and subsequent induction of ARE in phase 2 genes (*28*). The PI-3 kinase pathway might be involved in the activation of Nrf2 translocation by chlorogenic acid.

Curcumin, found in tumeric, and caffeic acid phenethyl ester (CAPE) present in propolis of honeybee hives are also potential natural chemopreventive agents. Curcumin and CAPE stimulate the expression of Nrf2 as well as ARE-mediated phase 2 gene expression such as NQO1 and HO-1 in cell culture models (*29, 30*) Inactivation of the Nrf2-Keap1 complex or involvement of p38 by these compounds are suggested.

Other Compounds

Other natural compounds that possess chemopreventive properties through Nrf2/ARE pathway and subsequent induction of phase 2 detoxifying enzymes include terpenes and indole-3-carbinol (I3C). A sesquiterpene found in feverfew and parthenolide, stimulates ARE-reporter gene activity and potently induce expression of Nrf2 and HO-1 proteins in HepG2 cells (*9*). A recent study also indicates that parthenolide stimulates the binding activity of Nrf2 to ARE in HT22 cells (*31*). I3C is reported to retard the progression of aflatoxin B1-induced carcinogenesis in animals at both the initiation and promotion stages. Treatment with I3C has shown significant induction of GST Yc2, aflatoxin B1

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aldehyde reductase and quinone reductase (*32, 33*). In HepG2 cells, I3C was a weak inducer of ARE-reporter gene activity and Nrf2 protein expression but had no effect on HO-1 protein expression (*9*). An *in vivo* study using mice revealed that NQO and GST enzyme activities in the small intestine of mice increased about 2-fold after feeding with a mixture of coffee diterpenes, cafestol and kahweol palmitate (*11*).

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Chapter 10

Method Development for Monitoring Seal Blubber Oil Oxidation Based on Propanal and Malondialdehyde Formation

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Omega-3 polyunsaturated fatty acids (PUFA) in fish oils are extremely susceptible to oxidation and rapid degradation. Many studies have shown the health benefits of omega-3 fatty acids for human beings thus lead to the effort in improving the stability of these oils. Propanal, which is specific formed from the oxidation of omega-3 PUFA, could be used as an indicator of the extent of seal blubber oxidation. Solid-phase microextraction (SPME) is a solvent-free volatile extraction method and has been widely used in volatile analysis. However, short-chain aldehydes like propanal do not bind to the fiber. We have developed a rapid method to determine the extent of lipid oxidation of seal blubber oil based on propanal and malondialdehyde formation. *O*-(2,3,4,5,6-Pentafluorobenzyl) hydroxylamine hydrochloride (PFBHA) was used as derivatization agent to ensure the binding of short chain

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aldehydes to SPME fiber. Comparing with other traditional methods for measuring lipid oxidation, our method is convenient and reduces the amount of toxic solvent used. It targets the major end products produced by polyunsaturated omega-3 fatty acids oxidation, propanal and malondialdehyde.

Seal blubber oil is a good source of ω-3 fatty acids; it contains approximately 20% of eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) (*1*) (Table I). The source of long chain polyunsaturated fatty acids in marine oil is from microalgae (*2*). After consumed by the marine animals, those fatty acids, mainly EPA and DHA are accumulated in the food chain. Omega-3 fatty acids have aroused many interests because of its benefits on human health. The health benefits of omega-3 fatty acids have been thoroughly reviewed by many researchers (*3-6*) on cardiovascular disease (*7-9*), infant development (*10,11*), inflammatory and autoimmune disorders, neuronal and visual function.

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Table I: Major Fatty Acids from Seal Blubber Oil (*1*).

Fatty Acid	*Weight %*	*Fatty Acid*	*Weight %*
14:0	3.7	18:2ω6	1.5
14:1	1.1	20:1ω9	12.2
16:0	6.0	20:5ω3	6.4
16:1	18.0	22:1ω11	2.0
18:1ω9	20.8	22:5ω3	4.7
18:1ω11	5.2	22:6ω3	7.6

Omega-3 fatty acids are very susceptible to oxidation and generate an unpleasant odor due to their chemical structures. There are several commonly used indexes for monitoring lipid oxidation, such as measuring the conjugated dienes (*12*), peroxide value (*13, 14*), the oil stability index analysis method (OSI, AOCS official method Cd 12b-92) (*16-17*), gas chromatography method (*13, 18*), and 2-thiobarbituric acid (TBA) method.

Malondialdehyde is usually found in the oxidation products from PUFA containing 3 or more double bonds. The TBA method has been widely used to quantify the malondialdehyde because its simplicity, however, not only

malondialdehyde could react with thiobarbituric acid to form the chromogen, there are many other compounds, called thiobarbituric acid reactive substances (TBAR) that can react with thiobarbituric acid. The concept of TBA method is shown in Figure 1; thiobarbituric acid can react with the malondialdehyde in the oil sample and form the colored TBA chromagen (maximum UV absorbance at 532 nm). The methodology of this method has been reviewed thoroughly (*19-21*). Nonetheless, there are many drawbacks of TBA method, such as the use of toxic materials, 2-thiobarbituric acid, the reaction need to be conducted in hazardous solvent (1-butanol), time consuming procedures, and possible interference with many other aldehydes (*22*). Malondialdehyde can also be quantified by HPLC or GC analysis; however, further derivatization is required to generate the stable malondialdehyde derivatives (*20*).

Solid phase microextraction (SPME) is sample extraction technique, and it extracts the analytes without solvent. SPME was first invented by Arthur and

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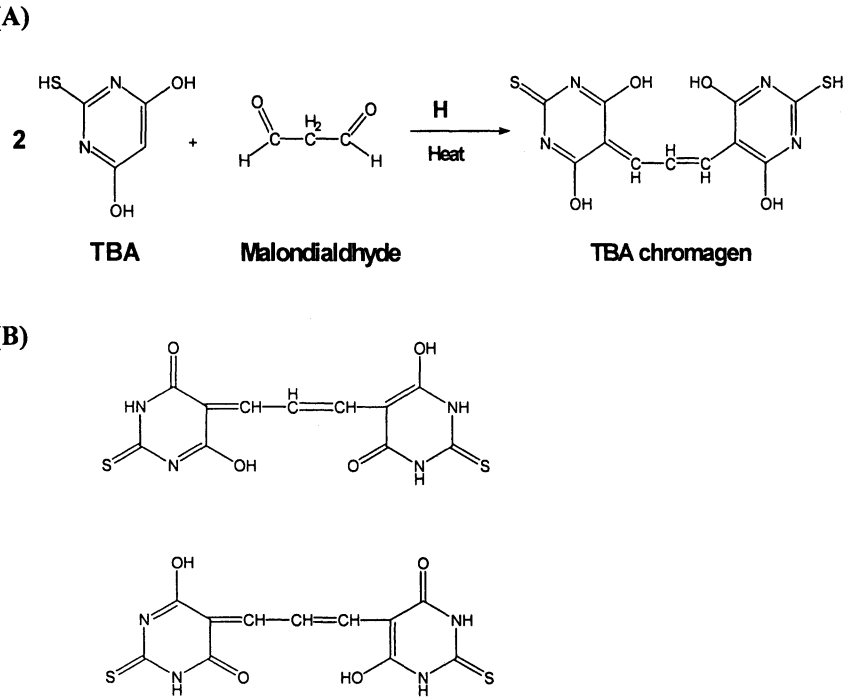

*Figure 1. **(A)** The formation pathway of TBA chromogen proposed by Sinnhuber et al. (23). **(B)** Two tautomeric TBA chromogen proposed by Nair and Turner (modified from 24).*

Pawliszyn in 1990 (*25*), after it became commercially available from Supelco in 1993; this technique was widely used because of its convenience, sensitivity, and omission of solvent. We applied this sample extraction technique to monitor the major oxidation end product from ω-3 fatty acids. The formation pathway of propanal and malondialdehyde is shown in Figures 2 and 3.

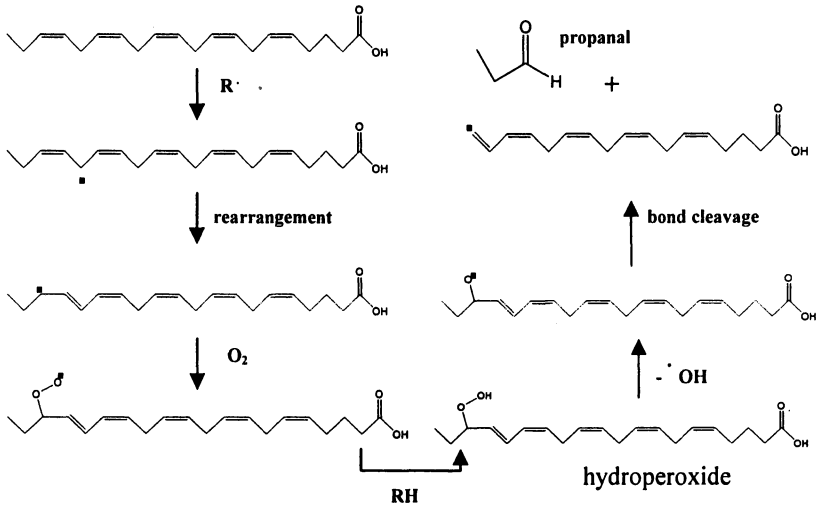

Figure 2. Formation of propanal from ω-3 eicosapentaenoic acid (EPA) oxidation.

The challenge in using SPME to sample propanal is that the binding affinity of short chain aldehydes to SPME fiber is low, and the derivatization reagent is required to ensure the binding of propanal to fiber. *O*-(2,3,4,5,6-Pentafluorobenzyl) hydroxylamine hydrochloride (PFBHA) is a derivatization reagent widely used for the analysis of carbonyl-containing compounds in variety of environmental and biological studies. It was first synthesized in 1975 for the analysis of keto steroids (*26*). Martos and Pawliszyn (*27*) have developed the on-fiber derivatization method to monitor formaldehyde in the environment using PFBHA as derivatization reagent. The theory of on-fiber derivatization is shown in Figure 4. The aromatic group on PFBHA has good binding affinity to the fiber while the hydroxylamine group can freely react with the approaching carbonyl group and form oximes on the fiber. Our objective was to develop a rapid method to monitor seal blubber oil oxidation based on propanal and malondialdehyde formation using on-fiber derivatization SPME.

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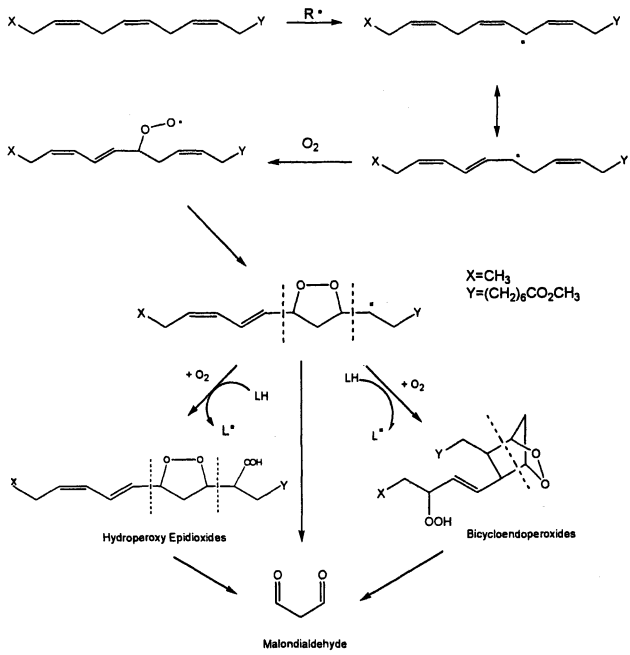

Figure 3. The conversion of methyl linolenate to malondialdehyde

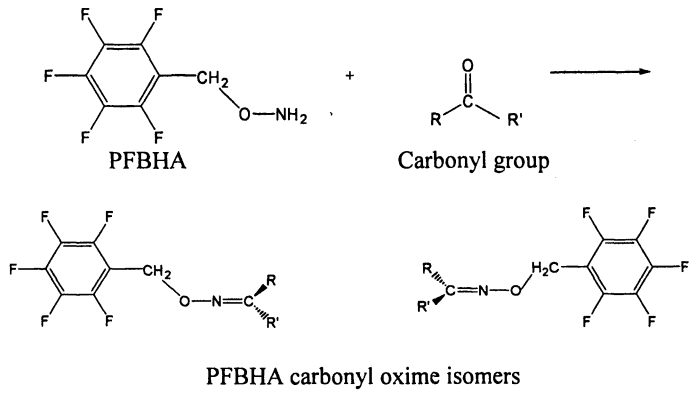

Figure 4. Reaction mechanism of PFBHA with carbonyl compounds (modified from 27).

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Materials and Methods

Propanal, corn oil, *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA), ether and 1,1,3,3-tetramethoxypropane were purchased from Sigma-Aldrich (St. Louis, MO). Malondialdehyde tetrabutylammonium salt was purchased from Fluka (Milwaukee, WI). Poly (dimethylsiloxane)/ divinylbenzene solid-phase microextraction (SPME) fiber, fiber holder, and crimp top amber glass vials (10 mL) were all purchased from Supelco (Bellefonte, PA). Crude seal blubber oil was rendered and subsequently subjected to refining, bleaching and deodorization.

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Sample Preparation

The oil (100 mL) was added into separatory funnel, and free fatty acids were then removed by mixing oil with suitable amount of sodium hydroxide solution. Rinse the oil with 80 mL x 3 distilled water. Dried the oil layer with anhydrous sodium sulfate. Seal blubber oil was further purified by passing through silica gel column. Vacuum was applied to accelerate the oil collecting process. The purified oil was transferred in an amber glass bottle, flushed the headspace with nitrogen, and stored the oil at -21 °C for further analysis.

Fish Oil Aging Study

Aging studies were conducted at 60 °C. Five milliliters purified seal oil were put into a 10 mL crimp top amber glass vial with a TFE starburst stirring head (diameter 9.5 mm). Sampling of propanal and malondialdehyde was conducted every 24 hours.

Loading SPME Fiber with PFBHA

One milliliter of PFBHA solution (17 mg/mL) was put into 4 mL amber Teflon-capped vials with a 1-cm stir bar. SPME fiber was inserted to the headspace of vial for 2 min to adsorb the volatile PFBHA, the solution was stirred at 600 rpm. The fiber was then inserted to the headspace of seal blubber oil containing vial. After 5 min, the fiber was removed and inserted to GC.

GC Conditions

An Agilent 6850 Gas Chromatography equipped with a flame ionization detector and a HP-1 methylsiloxane column (30.0 m x 250 μm x 0.25 μm) was

used. The inlet port and the detector were kept at 250 and 300 °C, respectively. The gas flow was controlled as follows: hydrogen flow at 30.0 mL/min and air flow at 300 mL/min. The oven temperature was hold at 45 °C for 1 min then increased to 200 °C at 10 °C/min and held at 200 °C for 8.5 min.

GC-MS Analysis of PFBHA Oximes

An Agilent 6890 Gas Chromatograph equipped with a Agilent 5973 Mass detector and HP-5 MS 5% phenylmethyl siloxane column (30.0 m x 250 μm x 0.25 μm) was used. The injection port was kept at 250 °C. The gas flow was controlled as follows: hydrogen flow at 30.0 mL/min and air flow at 300 ml/min. The oven temperature was hold at 45 °C for 1min then increased to 200 °C at 10 °C/min and held at 200 °C for 8.5 min. The mass detector was operated at the electronic ionization mode. The ionization voltage was held at 70 eV and ion temperature was at 280 °C.

Quantification of Propanal

Corn oil was used as a propanal free medium in our study. Different concentrations of propanal were added to 5 mL of corn oil and the SPME sampling procedure remained the same as describing above. The calibration curve was used to quantify the propanal concentration in seal blubber oil headspace.

Quantification of Malondialdehyde in Seal Blubber Oil

Malondialdehyde solution (10 mM) was prepared by dissolving Malondialdehyde tetrabutylammonium salt in 0.1 M pH 2.5 citrate buffer. 15 mL corn oil were used to extract the malondialdehyde from 15 mL 10 mM malondialdehyde solution. In order to test the efficiency for extraction of malondialdehyde from citrate buffer to corn oil, the standard curve was conducted to determine the amount of malondialdehyde remaining in the citrate buffer after oil extraction. Malondialdehyde solution (5 mL) (4, 6, 8, and 10 mM) was mix with 5 mL 30 mM PFBHA, extracted with ether (10 mL x 3) and then subjected for the GC analysis. Octanol was added as internal standard. The malondialdehyde containing oil was then used for conducting external standard curve in order to quantify the malondialdehyde existed in the seal oil blubber oil sample.

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Synthesis of Malondialdehyde Oximes

Tetrametoxypropane (160 μL) was added into 5 mL 1M HCl and heated at 95°C water bath for 3 min. The solution was slowly added into 5 mL PFBHA solution (34 mg/mL). The mixture was then shaken vigorously and put into ice bath. After centrifugation for 10 min, the precipitates were collected for LC-MS analysis.

LC-MS Analysis of Malondialdehyde Oximes

Analytical HPLC analyses were performed on a Hewlett-Pachard 1100 modular system equipped with an auto-sampler, a quaternary pump system, a photodiode array detector, and a HP Chemstation data system. A Luna C18 (2) analytical column (Phenomenex), 250 x 4.60 mm (5 μ particle size, 00G-4252-E0) was used. The mobile phase consists of 70% acetonitrile and 30% of 1% formic acid in water. The analyses were conducted at isocratic condition. Negative and positive ESI-mass spectra were measured on Agilent 1100 LC-MSD system (Agilent Technologies, Wilmington, DE) equipped with an electrospray source, Bruker Daltonics 4.0, and Data analysis 4.0 software. All the organic solvents are HPLC grade and obtained from Fisher Scientific Inc.

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Results and Discussion

SPME Sampling

A typical gas chromatogram of seal blubber oil incubated at 60 °C for 6 days is shown in Figure 5. Five aldehydes were identified based on the comparison of retention time and GC-MS spectrum with authentic compounds. Since there is no commercially available malondialdehyde, it was prepared by the hydrolysis of 1,1,3,3-tetrametoxypropane. Un-reacted PFBHA (peak 1) was also thermally desorbed from SPME fiber and the retention time is 8.7 min. Propyl-oxime isomers have retention time at 10.1 and 10.2 min, respectively. Propenyl-oxime isomers have retention time at 10.1 and 10.3 mins, which overlap with the propyl-oxime at 10.1 min; therefore, the quantification of propanal was based on the peak area at 10.2 min. Malondialdehyde has two carbonyl groups which can react with 2 molecule of PFBHA and formed 4 isomers (E, E-, E, Z-, Z, Z-, and Z, E-). The malondialdehyde oximes have retention time at 21.2 and 22.8 min in the ratio of 1:3.

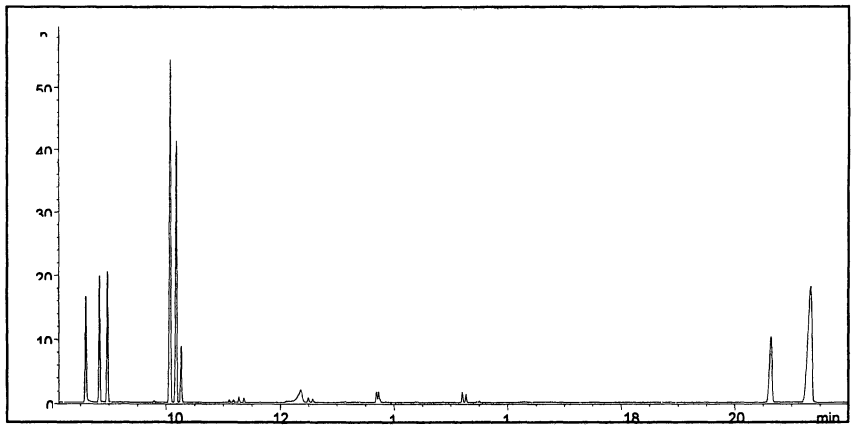

Figure 5. The representative gas chromatogram of seal blubber oil stored at 60°C for 6 days. Peaks are identified based on the comparison of retention time and MS spectrum with authentic compounds. 1. PFBHA, 2&3: acetaldehyde, 4: co-eluent of propanal and propenal, 5: propanal, 6: propenal, 7&8: malonadialdehyde

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LC-MS Analysis of Malondialdehyde-PFBHA Adduct

Malondialdehyde has two carbonyl group which can react with PFBHA to form oximes. Three major peaks were identified as malondialdehyde-PFBHA and PFBHA-malondialdehyde-PFBHA isomers with the molecular weight of 267 and 462 Da, respectively (Figure 6).

Quantification of Propanal

The external standard curve of propanal is shown in Figure 7A, and the linear regression is observed (R^2= 0.99). Propanal concentrations in the headspace of seal blubber oil containing vials were quantified by the external standard curve.

Rapid increase in propanal concentration was observed after 24 hours. After 6 days incubation at 60 °C, the propanal concentration reached about 1.6 ppm (Figure 7B). We've attempted to investigate the possibility of adding known amounts of different aldehydes into oil sample as internal standard, however, the competition of PFBHA between propanal and the additional aldehydes could lead to the misinterpretation of the data. (Data not shown). Thus, the external standard curve could give us a better quantification of propanal existed in the headspace of oil sample.

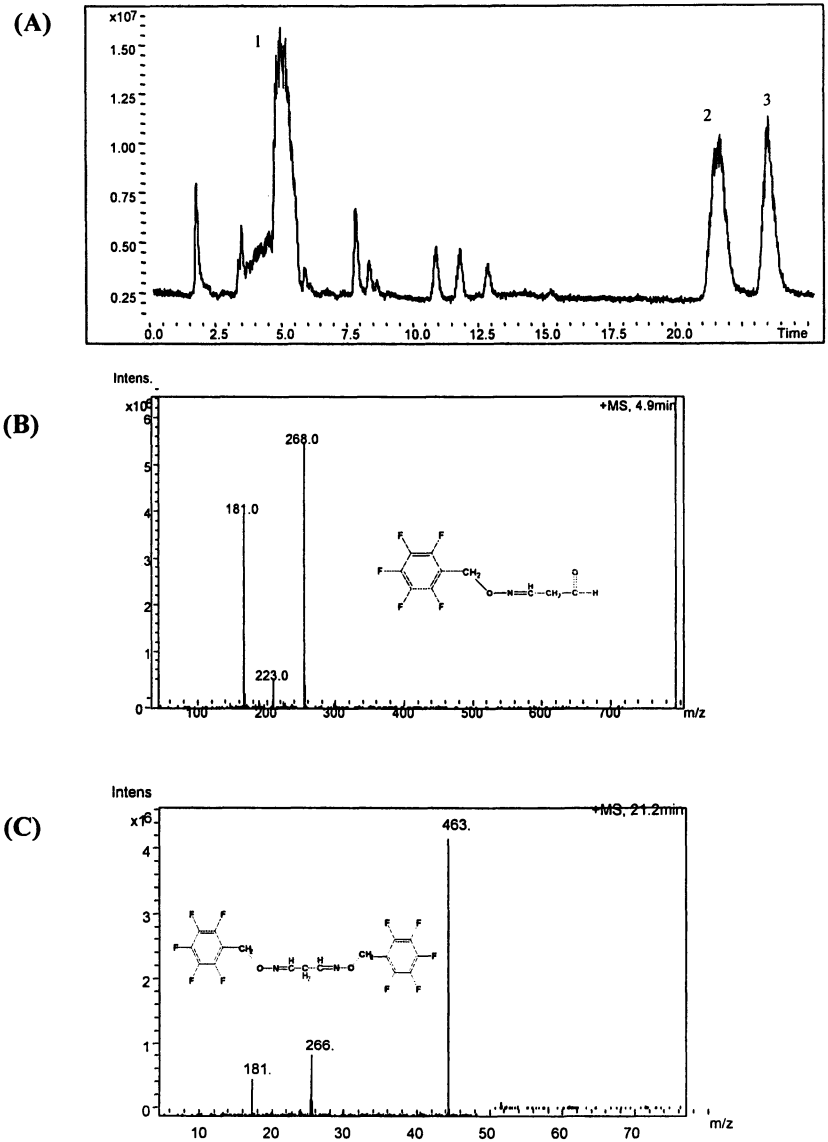

Figure 6. (A) LC chromatogram of malondialdehyde oximes. Peak 1: MA-PFBHA, peak 2 &3: PFBHA-MA-PFBHA. (B) LC-MS positive spectrum of MA-PFBHA (C) LC-MS positive spectrum of PFBHA-MA-PFBHA.

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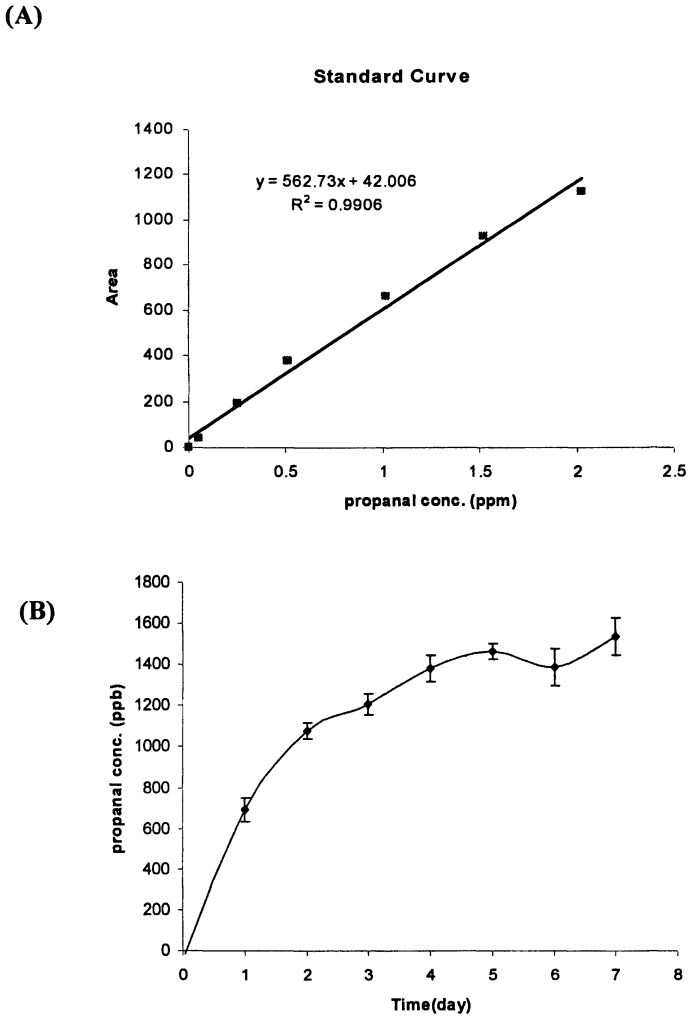
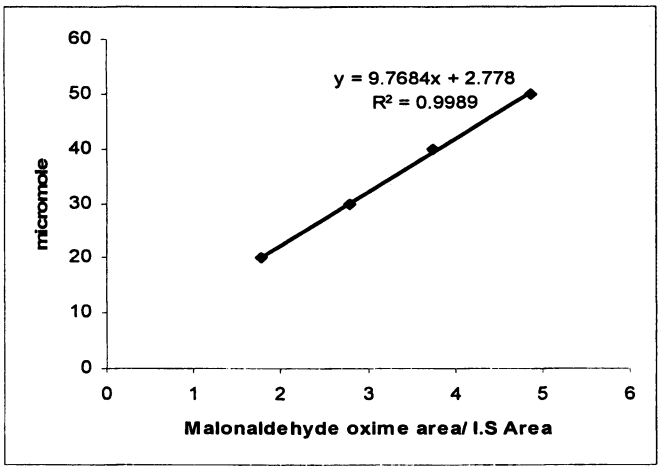


Figure 7. (A) Standard curve for propanal quantification. (B) Propanal formation of seal blubber oil incubated at 60 °C.



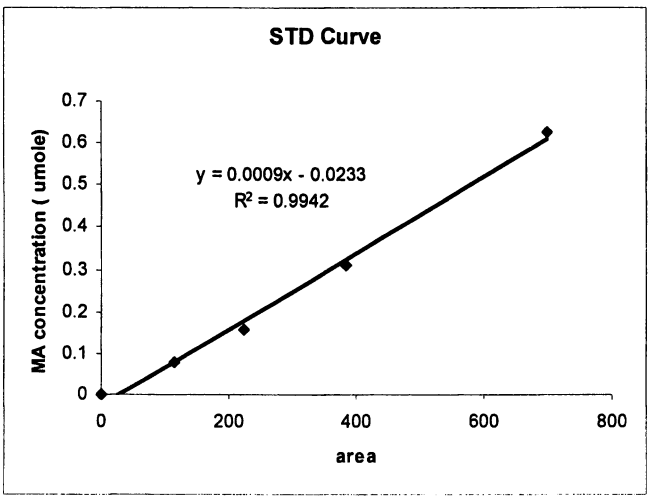


Figure 8. (A) Standard curve for determination of corn oil extraction efficiency. (B) Standard curve for the quantification of malondialdehyde.

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Quantification of Malondialdehyde

In order to generated the external standard curve to quantify the malondialdehyde in seal blubber oil, different amount of malondialdehyde need to be added into corn oil. However, malondialdehyde is a very unstable compound, it's not commercially available and usually generated from acid hydrolysis of its stable derivatives tetramethoxylpropane (TMP) (*28,29*), tetraethoxylpropane (TEP) (*30-32*) or dissolving tetrabutylammonium salt in water under acidic condition (*33*). Water is a polar solvent that would affect binding affinity of aldehydes to PFBHA. Since malondialdehydes generated by the methods described above were dissolved in water solution; further extraction step was needed to transfer standard malondialdehyde into corn oil.

The standard curve to quantify the efficiency for oil extraction is shown in Figure 8A. Malondialdehyde is a hydrophilic compound; only 2.34 out of 50 μmole of malondialdehyde was extracted into 15 mL corn oil. Figure 8B shows the external standard curve to quantify the malondialdehyde in seal blubber oil. Malondialdehyde formed rapidly after 1 day incubation at 60 °C and reached about 4 ppm in seal blubber oil headspace (Figure 9).

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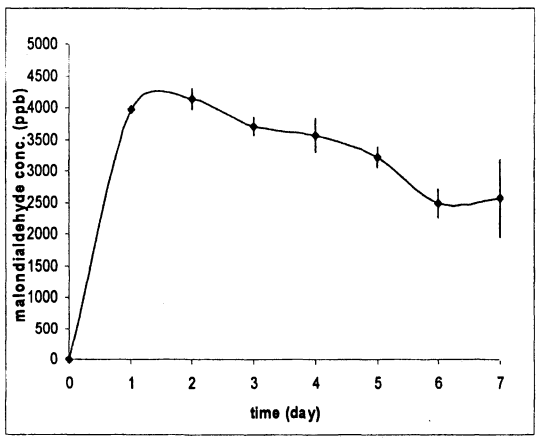

Figure 9. Formation of malondialdehyde from seal blubber oil oxidation.

Conclusions

A rapid method to monitor the oxidation of oil which rich in omega-3 fatty acids has been developed. It's very sensitive with the detection limit at ppb level. Comparing to the TBA method, this method is based on the measurement of major end products from omega-3 PUFA oxidation without the interference of TBARS generated during heating step with TBA method. It also saves time and substantially reduces the quantity of organic solvent used. Because of its high sensitivity, this method may be used to determine the effectiveness of antioxidants on lipid oxidation in food or other complex biological systems.

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Chapter 11

PHOTOCHEM® for Determination of Antioxidant Capacity of Plant Extracts

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The antioxidative capacities of water- (ACW) and lipid-soluble (ACL) compounds in selected plant extracts were investigated by a facile chemiluminescence assay using a PHOTOCHEM® device. Prepared extracts contained various classes of polyphenolics and included Cyclone canola hulls (*i.e.*, phenolic acids and condensed tannins), arugula greens (*i.e.*, quercetin and other flavonoids), bearberry-leaf (*i.e.*, arbutin, condensed and hydrolyzable tannins), as well as the leaf material (*i.e.*, a mixture of phenolics including condensed tannins) of east-coast Canadian wild blueberries. All extracts examined had demonstrated a capability to retard lipid oxidation in other model system studies, albeit to varying degrees. Using the PHOTOCHEM® device, the ethanolic extract from blueberry leaves exhibited the strongest antioxidative capacity at inhibiting the photo-induced chemiluminescence (PCL) of luminol; that is, 5.93 mmol ascorbic acid eq/g extract and 10.4 mmol Trolox eq/g extract for the ACW and ACL assays, respectively, while extracts from arugula greens were weakest with ACW data ranging from 0.06 to 0.16 mmol ascorbic acid eq/g extract. The crude

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In Antioxidant Measurement and Applications; Shahidi, F., el al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

bearberry-leaf extract also showed strong antioxidant activity; moreover, individual results for the ACW and ACL experiments correlated strongly to one another (r=0.955). The crude bearberry-leaf extract was fractionated on a Sephadex LH-20 column using 95% (v/v) ethanol and 50% (v/v) acetone as mobile phases. The ethanol fraction demonstrated a reduced antioxidative capacity at retarding PCL of luminol whereas the acetone fraction, which contained more tannin constituents, exhibited an enhanced capacity (ACW: 0.94 and 7.70 mmol ascorbic acid eq/g extract, respectively; ACL: 5.80 and 36.3 mmol Trolox eq/g extract, respectively). The simplicity and effectiveness of the PHOTOCHEM® device as a tool for measuring antioxidative capacity is discussed.

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Epidemiological studies have indicated the importance of consuming foodstuffs rich in phytonutrients, notably fruits and vegetables, to reduce the incidence of degenerative diseases like atherosclerosis and cancer (*1-5*). Although the mammalian body has certain defence mechanisms to combat and reduce oxidative damage, reactive oxygen species (ROS), in the form of superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^\bullet$), are natural by-products from our body's metabolism. They are dangerous to us when present in excess (possibly a result of oxidative stress), and can attack biological molecules such as lipids, proteins/enzymes, DNA and RNA, leading to cell or tissue injury associated with chronic diseases (*6-8*). In other words, free-radical oxidative stress can result in an increased risk of inflammatory diseases, cardiovascular disease (CVD), cancer, diabetes, Alzheimer's disease, cataracts and age-related functional decline (*9*).

Free radicals are transient species formed by homolytic cleavage of covalent bonds in molecules; this can be triggered by thermal, chemical, electrochemical and mechanical reactions, or radiation. They are very short-lived molecules and ions (*ca.* 10^{-3} to 10^{-10} s) having at least one unpaired electron in their electron shell, and are extremely reactive. The scavenging and dismutation of active oxygen radicals by enzymes like superoxide dismutase and catalase, as well as by low molecular-weight antioxidant compounds such as ascorbic acid and glutathione have been the subject of extensive studies from a pharmacological point of view (*10*). Endogenous antioxidants and antioxidant enzymes can often quench the reactivity of ROS before they do serious damage in the body. One of the most important free radicals resulting from metabolism is $O_2^{-\bullet}$. It has been implicated in initiating oxidation reactions associated with ageing (*11*), and plays an important role in the formation of other ROS like those described above (*12*).

Analytik Jena AG offers the PHOTOCHEM® device and standardized ready-to-use kits to measure the effectiveness of test compounds/preparations suspected of having antioxidant activity against $O_2^{-\bullet}$. Using a single system, the instrument can measure the antioxidative capacity of water- (ACW) and lipid-soluble (ACL) antioxidants. In the strictest sense, however, antiradical capacity is being measured. Differences exist between the ACW and ACL protocols and these will be discussed below after the general chemistry involved in the assay is outlined.

The approach employed by the PHOTOCHEM® device is based on methods developed by Popov and Lewin (*13-15*). The assay involves the optical excitation of a photosensitizing substance (*i.e.*, luminol) by UV irradiation and its reaction with molecular oxygen followed by luminometric detection. Though the complete reaction mechanism is not known (*13,16*), the operating principle is believed to be as follows:

- The superoxide anion radical ($O_2^{-\bullet}$) is generated by the reaction of dissolved oxygen with an intermediate product of photo-induced luminol (L*); no singlet oxygen $^1O_2$ is involved in the reaction.

$$\mathbf{L} + h\nu_1 \rightarrow \mathbf{L^*} + {}^3\mathbf{O_2} \rightarrow \mathbf{[L^*O_2]} \rightarrow \mathbf{L^{-\bullet}} + \mathbf{O_2^{-\bullet}}$$

  where **L** = luminol; $h\nu_1$ = UV radiation necessary to excite the luminol; **L*** = the photosensitizer in the triplet state; **[L*O$_2$]** = the intermediate product of excited luminol and oxygen; **L$^{-\bullet}$** = luminol radical; and **O$_2^{-\bullet}$** = superoxide anion radical.

- A portion of the $O_2^{-\bullet}$ radicals is quenched by antioxidants in the assay medium, if present, and the remaining ones are quantified by the luminometric detection reaction.

- The detection reaction is as follows:

$$\mathbf{L^{-\bullet}} + \mathbf{O_2^{-\bullet}} \rightarrow \mathbf{N_2} + \mathbf{AP^{*2-}} \rightarrow \mathbf{AP^{2-}} + h\nu_2$$

  where **L$^{-\bullet}$** = luminol radical; **O$_2^{-\bullet}$** = superoxide anion; **AP^{*2-}** = excited aminophthalate anion; **AP^{2-}** = aminophthalate anion; and $h\nu_2$ = blue luminescence.

Luminol plays a double role as both a photosensitizer and an oxygen radical detection reagent. During the assay, the test solution containing the photosensitizer and dissolved sample of interest (*i.e.*, plant extract or fraction) is exposed to UV light and then pumped to a measuring cell where the chemiluminescence is detected. The PHOTOCHEM® device is designed in such

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a way that during the assay, the test sample is continuously conveyed by pumps between the irradiated sample cell and the measuring cell; hence, a small volume of sample is always in the measuring cell (Figure 1). This allows the radicals generated in the sample liquid to be detected "time-resolved" by PCL (*17*).

The time course for PCL of luminol is such that there is a small lag phase before luminescence occurs and reaches a maximum. The presence of non-enzymatic antioxidants will quench the $O_2^{-\bullet}$ from the system and effectively attenuate the intensity of the PCL. The result is a lag phase before observing any chemiluminescence. It is only when the antioxidants are exhausted that interaction between the luminol anion radical (*i.e.*, $L^{-\bullet}$) and $O_2^{-\bullet}$ takes place, and chemiluminescence occurs. In other words, the presence of a water-soluble antioxidant leads to quantity-dependent temporal inhibition of PCL (*13*). An evaluating parameter of the curves for ACW is the duration of the lag-phase (L) of the PCL ...

$$L = L_i - L_o$$

where L_i is the lag phase in seconds of a sample with an antioxidant and L_o is the same for a blank sample. In the PHOTOCHEM® device, the duration of the lag phase is calculated by determining the first derivative and the turning point of the detected curve at its maximum. The intersection point of the slope straight line with the x-axis defines the lag time. For ACW, ascorbic acid in measuring ranges of 0-3 nmol is the typical calibration standard for hydrophilic antioxidant determinations.

The objective of this study was to examine the effectiveness of the PHOTOCHEM® device as a tool for measuring the antioxidative capacity of selected plant extracts. These included Cyclone canola hulls (*i.e.*, phenolic acids and condensed tannins), arugula greens (*i.e.*, quercetin and other flavonoids), bearberry-leaf (*i.e.*, arbutin, condensed and hydrolyzable tannins), as well as the leaf material (*i.e.*, a mixture of phenolics including condensed tannins) of east-coast Canadian wild blueberries.

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Materials and Methods

Preparation of Plant Extracts

Canola (*Brassica napus* cv. Cyclone) hulls

Canola hulls were prepared according to the procedure described by Sosulski and Zadernowski (*18*). Hulls were extracted with hexanes for 12 h using a Soxhlet apparatus and then dried by air at room temperature. Defatted

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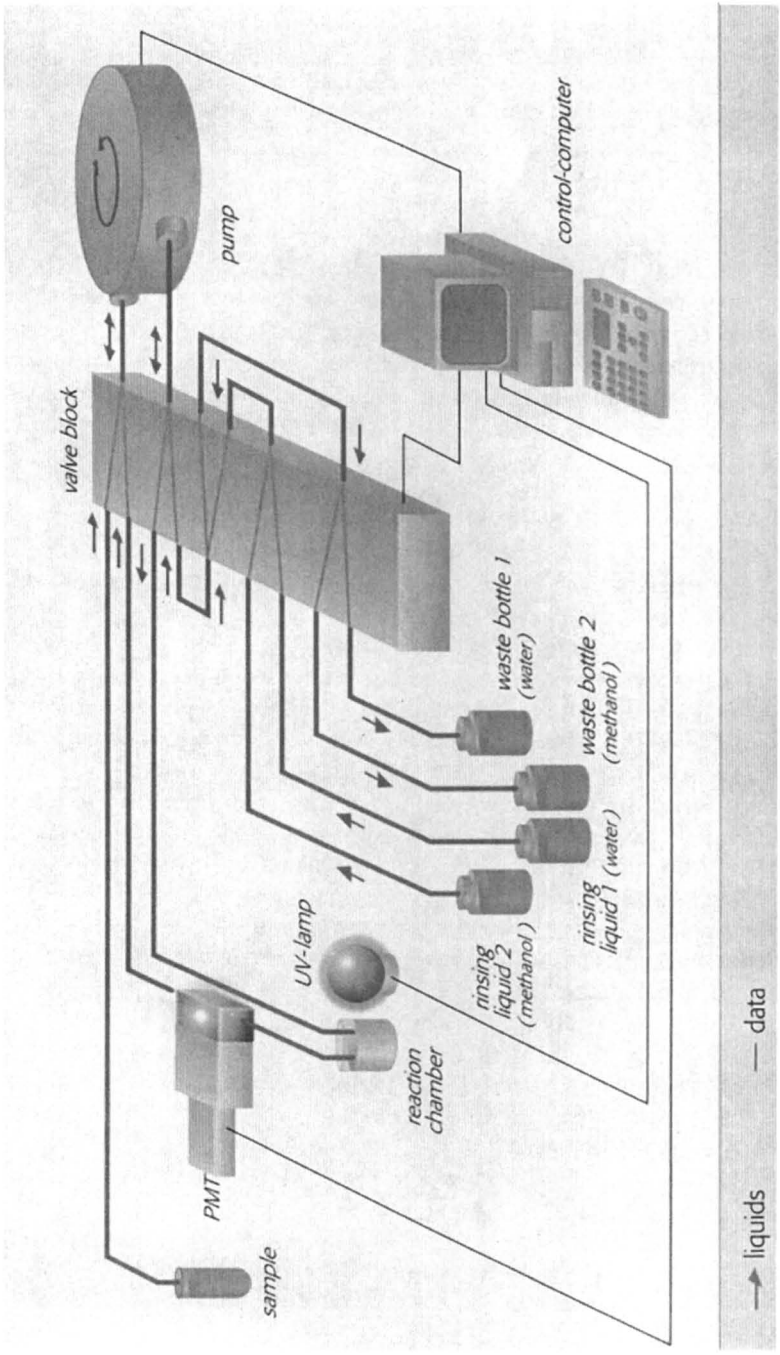

Figure 1. Functional design of Analytik Jena AG's PHOTOCHEM® device.

canola hulls were extracted twice at room temperature into 0, 30, 50, 70 and 80% (v/v) aqueous acetone or methanol using a Waring Blendor (Waring Products Division, Dynamics Corporation of America, Hartford, CT) for 2 min at maximum speed. Combined supernatants were evaporated to near dryness under vacuum at < 40°C using a Büchi Rotavapor/Water bath (Models EL 131 and 461, respectively, Brinkmann Instruments [Canada] Ltd., Mississauga, ON) and then lyophilized. Dried extracts were stored at 4°C in air until further analyzed.

Arugula (*Eruca vesicaria ssp. sativa* [P. Mill.] Thellung) greens

Authenticated arugula seeds were supplied by Mr. Larry Marshall of Marshall Farms Inc. (Shellbrook, SK). Seeds were planted in boxes of soil measuring an area of 25 cm × 25 cm. The plants were grown with sufficient watering under full spectrum fluorescent lighting with illumination from 7:00 am to 11:00 pm. To compensate for any variation in illumination from the light source, the boxes were randomly moved every second day. The arugula was grown for 36 days and then collected; harvested greens were lyophilized and then ground using a mortar and pestle. Crude extracts were prepared according to Hohl *et al.* (*19*).

Wild blueberry (*Vaccinium angustifolium*) leaves

Blueberry leaves, a by-product of mechanical harvesting, were collected from a wild blueberry farm located in Antigonish County, Nova Scotia, Canada. The leaves were separated from other debris, dried at room temperature and then stored in sealed polyethylene bags at –18°C. Crude polyphenolics were extracted from the leaf material with 95% (v/v) ethanol three times at 50°C for 30 min at a material-to-solvent ratio of 15:100 (w/v). The ethanolic extracts were combined and evaporated under vacuum at < 40°C using the rotavapor. Crude polyphenolics were also extracted from leaves using 70% (v/v) aqueous acetone in a similar fashion as outlined above; extracts were evaporated to near dryness at < 40°C using the rotavapor, and then lyophilized. Chlorophyll was removed from the leaf extracts as described by Pegg *et al.* (*20*).

Bearberry leaves (*Arctostaphylos uva ursi* [L.] Sprengel)

Dried bearberry leaves were ground in a coffee mill (Moulinex Corporation, Toronto, ON). Prepared material was transferred to dark-colored flasks, mixed with 95% (v/v) ethanol at a material-to-solvent ratio of 15:100 (m/v) and placed in a shaking Magni Whirl constant-temperature bath (Blue M Electric Company, Model MSG-1122A-1, Blue Island, IL) at 50°C for 30 min. Afterwards, the slurry was filtered through Whatman No. 1 filter paper and the residue was re-

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extracted twice more. Combined supernatants were evaporated to dryness under vacuum at < 40°C using the rotavapor. The crude preparation was then dechlorophyllized according to Pegg *et al.* (*20*) on a silicic acid column using hexanes and 95% (v/v) ethanol as mobile phases. Dried extracts were stored at 4°C in air until further analyzed.

Fractionation of the Crude Bearberry-Leaf Extract

Approximately 4 g of the crude dechlorophyllized bearberry-leaf extract were suspended in 20 mL of 95% (v/v) ethanol and then applied to a chromatographic column packed with Sephadex LH-20 (4.5 × 18 cm) and equilibrated with ethanol. The column was exhaustively washed with 95% (v/v) ethanol at a flow rate of 200 mL/h and then eluted with 50% (v/v) acetone at a flow rate of 150 mL/h. Eluates of ethanol represented the non-tannin fraction while those of 50% (v/v) acetone were the tannin fraction. The organic solvent of each fraction was removed under vacuum at < 40°C using the rotavapor. The tannin fraction was lyophilized to remove residual water.

ABTS Radical Anion ($ABTS^{\bullet-}$) Scavenging Activity

The scavenging effect of the bearberry-leaf extract and its two fractions on 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) radical anion ($ABTS^{\bullet-}$) was monitored by the method of van den Berg *et al.* (*21*) as modified by Kim *et al.* (*22*). The concentration of the $ABTS^{\bullet-}$ solution was adjusted to an absorbance of 0.50 at 734 nm. The stable $ABTS^{\bullet-}$ scavenging activity of the bearberry-leaf extract and its fractions was expressed as millimoles Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalent antioxidant capacity (TEAC) values per gram of extract or fraction. The TEAC value is reported as the slope of a line reflecting the amount of Trolox (expressed in *mM* equivalents per assay) as a function of the amount of phenolic extract or fraction added to the reaction mixture. The $ABTS^{\bullet-}$ stock solution was prepared fresh daily.

Chemiluminescence Assays

The inhibition of photochemiluminescence (PCL) of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) in both water and methanol by constituents of phenolic-containing plant extracts was monitored using the PHOTOCHEM® device coupled to a computer running PCLsoft® control and analysis software (Analytik Jena USA, The Woodlands, TX). PCL was brought about by the oxidation of luminol, and was delayed in the presence of antioxidants. The antioxidative capacity of water-soluble (ACW) and lipid (methanol)-soluble constituents (ACL) of extracts was determined as described by Popov and Lewin (*13*) and Popov and Lewin (*14*), respectively.

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For ACW studies, the luminol reagent and the ascorbic acid work solution (*i.e.*, AAWS; 1 nmol ascorbic acid/10 μL) were prepared on the day in which they were needed according to the ACW protocol supplied by Analytik Jena AG. To construct the ACW calibration curve, 1500 μL of deionized water less the volume of the AAWS (*i.e.*, ACW reagent 1, the diluent), 1000 μL of buffer (*i.e.*, ACW reagent 2), and a volume of the AAWS between 0 and 30 μL were added to a plastic sample tube. The contents were vortexed for 15 s. At this point, 25 μL of the luminol photosensitizer (*i.e.*, ACW reagent 3) were pipetted in, the contents again vortexed for 15 s and the tube then inserted into the PHOTOCHEM® sampling port. Presence of ascorbic acid (or any other antioxidant) retards luminescence for a period; hence, a lag time was noted before a PCL signal was measured. As greater concentrations of the AAWS were added, an extended lag time resulted. The duration of the lag, which is calculated by the computer software from the first derivative of the detector signal at its turning point and intersection with the *x*-axis, was plotted against the concentration of ascorbic acid added to the assay medium. For actual test samples, aliquots of an aqueous solution of prepared extracts were introduced to the reaction mixture instead of the AAWS. The concentration of the extract solution added was such that the generated luminescence fell within the limits of the standard curve (Figure 2). This was achieved by trial and error for each extract. The ACW was expressed as mmol ascorbic acid equivalents antioxidant activity per gram of extract and calculated as follows:

$$ACW = AA/TS$$

where AA is the quantity of ascorbic acid (mmol/assay), showing the same lag time as that of the test sample found on the calibration curve, and TS is the amount of extract tested (g).

For ACL studies, a Trolox (*i.e.*, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; a water-soluble analogue of vitamin E having potent antioxidant activity but is not a natural compound found in foods) working solution (*i.e.*, TWS; 1 nmol Trolox/10 μL methanol) was prepared instead of the AAWS. The calibration curve was constructed from 2300 μL of methanol less the volume of the TWS (*i.e.*, ACL reagent 1, the diluent), 1000 μL of buffer (*i.e.*, ACL reagent 2), a volume of the TWS between 0 and 30 μL, and 25 μL of the luminol photosensitizer (*i.e.*, ACL reagent 3) as described above. The kinetic light emission curve, which exhibits no lag phase in the ACL system, was monitored for 180 s. The areas under the curves were calculated using the PCLsoft® control and analysis software. As greater concentrations of the TWS were added to the assay medium, a marked reduction in the PCL signal and hence area calculated from the integral were observed. This inhibition was used as a parameter for quantification and related to the decrease in the integral of PCL

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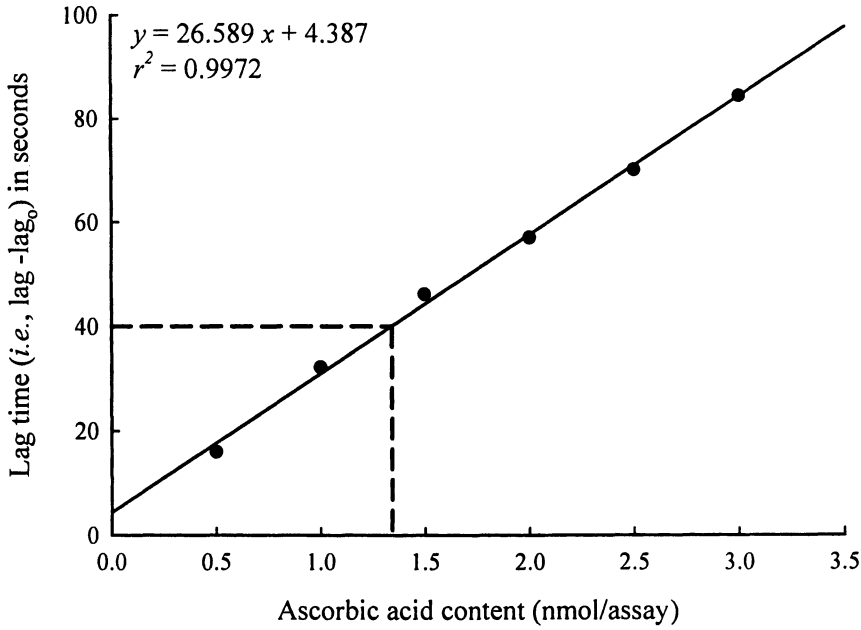

Figure 2. Calibration curve of ascorbic acid (AA) in the calculation of AA equivalents for ACW measurements. As an example, if the observed lag time of the test extract (0.64 μg in the assay medium) was 40 s, then this denotes a radical scavenging activity of 1.339 nmol AA, or 2.09 mmol AA eq/g extract.

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intensities caused by varying the concentrations of Trolox. The inhibition (I) of the signal was defined as follows:

$$I = (\int \text{Blank} - \int \text{TWS or Sample}) / \int \text{Blank}$$

The observed I was plotted against the concentration of Trolox added to the assay medium. For actual test samples, aliquots of a methanolic solution of prepared extracts were introduced to the reaction mixture instead of the TWS. The concentration of the extract solution added was such that the generated luminescence during the 180 s sampling interval fell within the limits of the standard curve. The ACL was expressed as mmol Trolox equivalents antioxidant activity per gram of extract and calculated as follows:

$$\text{ACL} = \text{TX}/\text{TS};$$

Where TX is the quantity of Trolox (mmol) displaying the same inhibitory effect as that of the test sample found on the calibration curve, and TS is the amount of extract tested (g).

Results and Discussion

The antioxidative capacity of selected plant extracts containing various phenolic constituents were compared against one another using the PHOTOCHEM® device and the ACW and ACL kits supplied by Analytik Jena AG. The PHOTOCHEM® device is the first system that can quantitate the antioxidative capacity of water- and lipid (*i.e.*, defined as methanol)-soluble substances. It combines very fast photochemical excitation of radical generation with highly sensitive luminometric detection. Due to the high sensitivity of luminol, only nanomolar concentrations of non-enzymatic antioxidant substances are required to delay the observance of luminol's PCL.

Performance of prepared extracts with the PHOTOCHEM® device

Extracts prepared from Cyclone canola hulls (*i.e.*, phenolic acids and condensed tannins), arugula greens (*i.e.*, quercetin and other flavonoids), bearberry-leaf (*i.e.*, arbutin, condensed and hydrolyzable tannins), and the leaf material (*i.e.*, a mixture of phenolics including condensed tannins) of east-coast Canadian wild blueberries were evaluated.

Canola hulls

Table I reports the antioxidative capacity of canola hull extracts as evaluated by the ACW and ACL protocols. Data indicate when a greater concentration of organic solvent was added to the extraction medium, more antioxidative constituents were recovered from the canola hulls. This is reflected in ACW and ACL data for both water- and lipid-soluble antioxidant compounds. For methanolic and acetonic extracts, ACW readings ranged from 0.15 to 0.85 and 0.29 to 0.94 mmol ascorbic acid eq/g extract, respectively. Counterpart ACL readings ranged from 0.27 to 0.73 and 0.43 to 1.73 mmol Trolox eq/g extract for the methanolic and acetonic preparations, respectively. In all cases but one, the antioxidative capacities of extracts for the ACL system were higher than those for the ACW system: the exception being when 80% (v/v) methanol was employed as the extraction medium. Compared with leguminous seeds (*i.e.*, white bean, pea, lentil, everlasting pea, faba bean and broad bean), whose antioxidative capacities as evaluated by the ACW system varied from 0.009 (white bean) to 0.48 (faba bean) mmol ascorbic acid eq/g extract and by the ACL system varied from 0.031 (white and broad beans) to 0.45 (lentil) mmol Trolox eq/g extract, the extracts from canola hulls exhibited a much stronger antioxidant activity (*23*).

Extraction of plant material with acetone is known to solubilize more condensed tannins (if present) than that of methanol. This is reflected in the total phenolics content of canola hull extracts reported by Naczk *et al.* (*24*): 70 and 80% acetonic extracts contained 94.3 and 104 compared to 36.5 and 40.4 mg sinapic acid eq/g extract for their methanolic extracts, respectively. The

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Table I. Water- and lipid-soluble antioxidative capacity of canola hull extracts[a]

Solvent system for canola hull extract	ACW mmol ascorbic acid eq /g extract	ACL mmol Trolox eq/g extract
Methanol		
30%	0.15 ± 0.010	0.27 ± 0.013
50%	0.38 ± 0.025	0.48 ± 0.018
70%	0.60 ± 0.037	0.69 ± 0.052
80%	0.85 ± 0.045	0.73 ± 0.029
Acetone		
30%	0.29 ± 0.031	0.43 ± 0.039
50%	0.53 ± 0.010	0.61 ± 0.032
70%	0.66 ± 0.034	1.47 ± 0.06
80%	0.94 ± 0.020	1.73 ± 0.13

[a]Sample measurements were repeated three times and are reported as the mean ± standard deviation.

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condensed tannins are oligomers and polymers of flavonoids, specifically flavan-3-ols such as (+)-catechin. The presence of hydroxyl moieties attached to the aromatic rings makes them capable of scavenging radicals, thereby functioning as antioxidants. It was presumed that ACW and ACL readings of acetonic extracts would be greater than their methanolic counterparts. This was so, but the results were not vastly different. This tends to suggest that either the condensed tannins were not being extracted from the canola hulls or if they were, they were not contributing significantly to the observed antioxidative capacity. Only when employing higher percentages of acetone (*i.e.*, 70 and 80%) do we see a significant increase in the antioxidant activity of the prepared extracts. This, however, was only observed for the ACL system. Lack of solubility of condensed tannins in water for the ACW system compared to that in methanol for the ACL system may be the reason for this observation.

Arugula greens

Arugula, also known as rocket, roquette, rugula and rucola, is a bitterish aromatic salad green with a peppery mustard flavor. Although it has long been

extremely popular with Italians, American palates often find its flavor too assertive. It is said, however, that a few seeds planted on an autumn day will provide spice for salads, sandwiches and egg dishes all winter long. The leaves are most tender when young, but as the plants age the flavor gets hotter and hotter. It is a rich source of iron as well as vitamins A and C (*25*); yet, not much is known about its flavonoid profile.

The extracts from arugula greens demonstrated poor antioxidant activity at delaying the onset of PCL in both the ACW and ACL systems. In fact, the arugula extracts exhibited the weakest activity of all those examined in this study and gave ACW data ranging from 0.06 to 0.16 mmol ascorbic acid eq/g extract. These values are in the same range as those for the leguminous seeds' extracts reported by Amarowicz and Raab (*23*).

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Bearberry-leaf extract

Bearberry is one of the most ubiquitous herbs on the Canadian Prairies. It comprises arbutin (5-15%), variable amounts of methylarbutin (up to 4%) and small quantities of the free aglycones. Other constituents include gallic acid, *p*-coumaric acid, syringic acid, galloylarbutin and up to 20% condensed and hydrolyzable tannins, as well as some flavonoids, notably glycosides of quercetin, kaempferol and myricetin (*26*). The antioxidant activity of the crude bearberry-leaf extract was assessed by a number of chemical assays including a beta-carotene-linoleic acid (linoleate) model system (*i.e.*, monitoring the coupled oxidation of beta-carotene and linoleic acid), reducing power, scavenging effect on $DPPH^{\bullet}$ free radical, liposome model system, scavenging capacity of hydroxyl radicals ($HO^{\bullet}$) by use of electron paramagnetic resonance (EPR) spectroscopy, and ability to curb lipid oxidation in meat model systems. Some results from these tests are reported by Amarowicz *et al.* (*27*). Amarowicz and Pegg (*28*) fractionated the crude bearberry-leaf extract and found that the tannin constituents play a significant role toward the antioxidant activity observed in model and food systems.

Preparation of a crude ethanolic extract from dried bearberry leaves, its dechlorophyllization and fractionation on a lipophilic Sephadex LH-20 column are depicted in Figure 3. The ethanol fraction comprises the phenolics which eluted from the column when 95% (v/v) was used as the mobile phase. Thin layer chromatography plates (*i.e.*, silica gel with fluorescent indicator and a mobile phase of chloroform/methanol/water, 65:35:10, v/v/v) with a ferric chloride spray and UV lamp detection were employed to indicate when no more relevant phenolics eluted from the column. Even though there are small quantities of tannin present, the constituents of the ethanol fraction are referred to as "non-tannins." Nearly 1000 mL of ethanol had passed through the Sephadex LH-20 column at which point the mobile phase was changed over to 50% (v/v) acetone. A similar approach as described above was employed when collecting the acetone fraction, except that vanillin spray was used instead of ferric chloride to indicate when all of the tannins had eluted from the column.

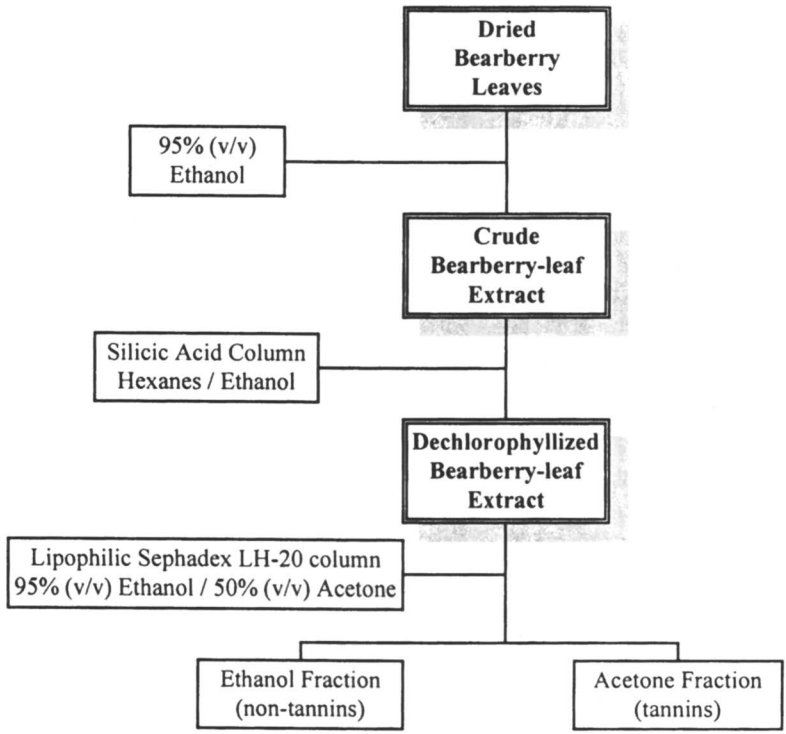

Figure 3. Flow diagram for the preparation of the crude bearberry-leaf extract and its ethanol and acetone fractions from a Sephadex LH-20 column.

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Although the constituents of the acetone fraction are not exclusively tannins, they do make up the bulk of the recovered product and therefore are referred to as "tannins." From a mass balance, the ethanol fraction (non-tannins) comprised 79.2% of the crude dechlorophyllzied bearberry-leaf extract and acetone fraction (tannins) consisted of 9.7% (*26*).

Water- (ACW) and lipid- (ACL) soluble antioxidative capacity data of the crude dechlorophyllized bearberry-leaf extract and its ethanol and acetone fractions, recovered from a Sephadex LH-20 column, are presented in Table II. The bearberry-leaf extract exhibited a strong antioxidative capacity at inhibiting PCL of luminol; that is, 2.50 mmol ascorbic acid eq/g extract and 10.1 mmol Trolox eq/g extract for the ACW and ACL assays, respectively. In total seven preparations of crude bearberry-leaf extracts were available for comparative purposes, but only data for the extract which was fractionated on the Sephadex LH-20 column are given in Table II. Moreover, when all individual results for ACW and ACL data from these preparates were examined, they strongly correlated to one another (r=0.955).

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In Antioxidant Measurement and Applications; Shahidi, F., el al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

Table II. Water- (ACW) and lipid- (ACL) soluble antioxidative capacity of bearberry-leaf extracts and its fractions[a]

Sample	ACW mmol ascorbic acid eq/g extract	ACL mmol Trolox eq/g extract	ABTS *mM* Trolox eq/g extract
Crude bearberry-leaf extract	2.50 ± 0.04	10.1 ± 0.17	2.89 ± 0.13
Bearberry-leaf fractions:			
Sephadex LH-20 / Ethanol	0.94 ± 0.02	5.80 ± 0.49	2.24 ± 0.07
Sephadex LH-20 / Acetone	7.70 ± 0.49	36.3 ± 4.0	3.94 ± 0.24

[a]Sample measurements were repeated three times and are reported as the mean ± standard deviation.

As aforementioned, the crude bearberry-leaf extract was fractionated on a Sephadex LH-20 column using 95% (v/v) ethanol and 50% (v/v) acetone as mobile phases. The ethanol fraction demonstrated a reduced antioxidative capacity at retarding the PCL of luminol whereas the acetone fraction, which contained more tannin constituents, exhibited an enhanced capacity (ACW: 0.94 and 7.70 mmol ascorbic acid eq/g extract, respectively; ACL: 5.80 and 36.3 mmol Trolox eq/g extract, respectively). Preliminary findings suggest that hydrolyzable tannins (*i.e.*, gallic or ellagic acid moieties linked to a central polyol, generally glucose) are important antioxidant constituents in the bearberry-leaf extract (*26*). Latté and Koldziej (*29*) reported that hydrolyzable tannins isolated from *Pelargonium reniforme* were 4 to 16 times more active than ascorbic acid at inhibiting PCL. Yet, the isolated gallotannins showed different trends in scavenging activity in a DPPH radical model. While gallic acid, possessing three hydroxyl groups, exhibited marked variation in radical scavenging capacity when tested against the DPPH radical [tentatively explained by the presence of carbonyl groups], no divergence was observed in the PCL measurements.

The ABTS radical ion assay, now commonly used for the estimation of antioxidant activities of plant phenolics, is based on the inhibition of the ABTS radical anion ($ABTS^{\bullet-}$) or the ABTS radical cation ($ABTS^{\bullet+}$). The assay of van den Berg *et al.* (*21*), used in this study, as modified by Kim *et al.* (*22*), is based on the inhibition of $ABTS^{\bullet-}$ preformed in the presence of 2,2'-azobis-(2-amidinopropane. As indicated in Table II, the mean TEAC value for the crude bearberry-leaf extract was 2.89 *mM* Trolox eq/g extract. As aforementioned, the crude preparation was fractionated on a Sephadex LH-20 column into non-tannin and tannin fractions using mobile phases of 95% (v/v) ethanol and 50% (v/v) acetone, respectively. It was expected that the TEAC value for the tannin fraction would be markedly greater than that of the non-tannin fraction, as was observed by the PHOTOCHEM® device for the ACW and ACL measurements, but this was not the case. The TEAC values for the ethanol and acetone fractions were 2.24 and 3.94 *mM* Trolox eq/g extract, respectively. Naczk *et al.* (*24*) reported that the TEAC values for 70 and 80% (v/v) acetonic extracts of canola hulls were ~ 2 to 3 times higher than those found for their methanolic counterparts. The difference in the TEAC values was possibly due to a higher level of both total phenolics and condensed tannins in the acetonic extracts compared to the methanolic preparations. When the acetonic and methanolic crude extracts of canola hulls were fractionated on a Sephadex LH-20 column into non-tannin and tannin fractions as described above, the antioxidant activity displayed by the tannin fraction was 4 and 8 times greater than that of the corresponding crude phenolic extract and non-tannin fraction, respectively. A similar finding was not observed for the fractions from the crude bearberry-leaf extract, however, this extract appears to be richer in hydrolyzable tannins as opposed to condensed ones, and this may account for the differences observed (*26*).

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Blueberry

The leaves of blueberry are by-products from mechanical harvesting of wild berries, and these are not commercially utilized. Naczk *et al.* (*30*) found that crude phenolic extracts from the leaves exhibited strong antioxidant activity when evaluated using a beta-carotene-linoleate model system, DPPH radical scavenging assay and by their inhibition of 2-thiobarbituric reactive substances formation in a meat model system. Using the PHOTOCHEM® device, the ethanolic blueberry-leaf extracts exhibited a strong antioxidative capacity at inhibiting PCL of luminol; that is, 5.93 mmol ascorbic acid eq/g extract and 10.4 mmol Trolox eq/g extract for the ACW and ACL assays, respectively. A similar story was found for the acetonic blueberry-leaf extracts; that is 1.66 mmol ascorbic acid eq/g extract and 5.72 mmol Trolox eq/g extract for the ACW and ACL assays, respectively. Again we see, the antioxidative capacities of extracts for the ACL system were higher than those for the ACW system. When scrutinizing the data, however, it is interesting to note that the ethanolic extract from blueberry leaves demonstrated stronger antioxidant capacity based on the ACW and ACL measurements than its acetonic extract. This is opposite to what was observed for the crude bearberry-leaf extract! Naczk *et al.* (*31*) reported that the total phenolics content and TEAC values for ethanolic extracts of blueberry leaves were greater than those for the acetonic counterparts. This was not only true for the crude preparations but also for the non-tannin and tannin fractions recovered by Sephadex LH-20 column chromatography. The differences in ACW and ACL measurements of the bearberry- and blueberry-leaf extracts are a good reminder that the makeup of the phenolic constituents and those which are solubilized by ethanol and acetone play a huge role in determining the antioxidant capacity of the resultant extracts.

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Overall effectiveness of the PHOTOCHEM® system

The PHOTOCHEM® device has been used to quantitate the capacity of water- and lipid-soluble antioxidants from a diverse list of foodstuffs and extracts prepared therefrom including fruits, vegetables, berries, teas, coffee, beer, legumes and oilseeds to name a few. The unit is marketed by Analytik Jena AG as a time- and cost-effective system for the determination of the integral antioxidative capacity toward superoxide ($O_2^{-\bullet}$) with ACW and ACL ready-to-use reagent kits. Because only one sample can be measured at a time, it is not, in its present configuration adaptable to a high-throughput assay system; a sample may require, at most, 3 min for analysis. Nevertheless, the conditions are standardized, so the results are comparable to other assays (*32*). One of the keys for success in garnering reproducible data is to have well-calibrated micropipettes and expert pipetting techniques by the researcher. Although this may sound elementary, it is an important consideration before

undertaking any ACW or ACL measurements. Moreover, ACW measurements tend to be more reproducible than ACL ones owing to the fact that pipetting methanol (*i.e.*, reagent 1; the diluent in the ACL kit) is not as precise as measuring the aqueous citrate buffer (*i.e.*, reagent 2 in the ACW kit).

It is important to remember the PHOTOCHEM® device only measures the antioxidative effectiveness of test samples against the PCL resulting from one free radical, namely $O_2^{-\bullet}$. Moreover, PCL measurements are collected at non-physiological pH values; hence, it is difficult to transfer the results of foodstuffs by this assay to the physiological environment of the human body. Naczk *et al.* (*24*) found that ACL values of extracts from canola hulls correlated with TEAC values, but did not correlate with ACW measurements. Prior *et al.* (*33*) reported that data from Dr. Luke Howard's lab clearly showed that there is little relationship between ORAC and PHOTOCHEM® data across a variety of foods. As they pointed out, this is not unexpected in that two completely different radical sources are being evaluated (*i.e.*, $ROO^{\bullet}$ and $O_2^{-\bullet}$). A better understanding of the potential importance of having data specific for the $O_2^{-\bullet}$ and how it might help in relating to potential *in vivo* effects is important. Nevertheless, results of these *in vitro* assays (*i.e.*, ACW and ACL) give an idea of the protective efficacy afforded by secondary plant metabolites.

Discussion in this paper suggests that antioxidative capacities depend not only on the chemical structure but also strongly on the model system employed. Accordingly, *in vivo* settings are essential to prove the therapeutic benefits of antioxidants in free radical diseases, though *in vitro* studies facilitate the primary evaluation (*29*). In this contribution a facile chemiluminescene assay using the PHOTOCHEM® device and standardized ready-to-use ACW and ACL kits from Analytik Jena AG has been described; this system should be considered as one of the best assays available for measuring the antioxidant capacity of water- and lipid-soluble antioxidants against $O_2^{-\bullet}$.

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Chapter 12

Antioxidants and Antioxidant Activities of Vegetables

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The health benefits of vegetables in preventing cancer, cardio- and cerebro-vascular diseases are mainly due to the antioxidants in vegetables. The major antioxidants in vegetables (phenolic acids, flavonoids, α-tocopherol, β-carotene, lutein, and ascorbic acid) are summarized in this review. The contents and composition of antioxidants of the mostly consumed vegetables in the USA vary greatly. Many methods have been used to measure the antioxidant activities of vegetables. Based on antioxidant activity values determined by oxygen radical absorbance capacity (ORAC), inhibition of oxidation of induced low-density lipoprotein (LDL), ferric reducing antioxidant power (FRAP), 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and total radical-trapping antioxidant parameter (TRAP), the rank of antioxidant activity of these vegetables from high to low is: garlic, asparagus, spinach, beet, bell pepper, mushroom, broccoli, cabbage, corn, onion, bean, carrot, cauliflower, sweet potato, tomato, potato, lettuce, squash, celery, and cucumber.

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Vegetables have anti-ageing and disease-prevention functions. These health benefits are mainly due to the antioxidants in these vegetables. Consumption of vegetables can lower incidence and mortality rates of cancer, cardio- and cerebro-vascular disease (*1*). More detailed disease-prevention function due to antioxidants can be obtained from certain chapters in this book. Several researches have shown that phenols in vegetables could be absorbed by human body (*2-4*). For example, quercetin in onions and kaempferol in broccoli after ingested by human were detected in the plasma and in the urine, respectively (*2,3*).

Based on per capita consumption, commonly consumed vegetables in the United States include asparagus, bean, beet, broccoli, cabbage, carrot, cauliflower, celery, corn, cucumber, garlic, lettuce, mushroom, onion, bell pepper, potato, squash, spinach, sweet potato, and tomato (*5,6*). The objectives of this review were to summarize the contents of phenolic acid, flavonoid, α-tocopherol, β-carotene, lutein, and ascorbic acid in vegetables and compare the antioxidant activity of these vegetables.

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Antioxidants in Vegetables

Extraction of Antioxidants

Antioxidants in plants can be extracted with solvents, such as methanol, acetone, ethanol, water, or their combination (*7*). The free phenolics are not linked to the large molecules in the plant cell wall and can be extracted using solvents with similar polarity. However, acid or alkali hydrolysis need to be used to extract the phenolics conjugated to the cell wall. For example, ferulic acid conjugates with other phenols or hemi-cellulose in the cell wall of asparagus. If extracted with methanol only, the conjugated ferulic acid cannot be dissolved in the extracting solution. Thus hydrolysis of the linking bond using sodium hydroxide can be used to extract the conjugated ferulic acid (*8*). The conjugated phenols in vegetables can also be extracted using acid hydrolysis (*6*). There was no positive correlation between the mole percent of conjugated phenols and the total phenol content of conjugated and free phenolics in vegetables (*6*).

Classes of Antioxidants

Simple Phenols and Phenolic Acids

Phenolic compounds have an aromatic ring with one or more hydroxyl substituents, or with functional derivatives (esters, methyl ethers and glycosides). Simple phenols include monophenols, such as cresol, and diphenols, such as hydroquinones. Phenolic acids include hydroxybenzoic acids and hydroxyl-cinnamic acids. Hydroxycinnamic acids (*p*-coumaric acid, caffeic acid,

chlorogenic and ferulic acid) may be more active antioxidants than the hydroxyl derivatives of benzoic acid (*p*-hydroxybenzoic, vanillic, and syringic acids) (*9*).

Flavonoids

The characteristic structure of a flavonoid is that of a diphenylpropane (C_6-C_3-C_6). The A and B phenyl rings and the central pyran C ring with variable oxidation level make up several types of flavonoids (*10*). The flavonoids include anthocyanidins, flavonols, flavones, isoflavones, catechins and flavanones (*11*). Flavonoids are important antioxidants that can donate hydrogens or combine with metal cations (*12*). In nature, flavonoids usually do not occur in a free state but as glycosides, and the third position of the C ring is the most common site to connect with the glycosyl group (*13*).

The number, arrangement and structural conjugation of the hydroxyl group in the phenol ring of flavonoids determines the availability of phenolic hydrogens and the stabilization of the resulting phenoxyl radicals (*14*). Flavonoids with high antioxidant activity have the following characteristic structures: (1) a 3',4'-dihydroxyl group in the B ring, (2) the 3-OH moiety in the C ring, (3) the C2-C3 double bond in the C ring conjugated with a 4-keto group causing electron delocalization from the B ring, and (4) both 3-OH group in C ring and 5-OH group in A ring combined with a 4-carbonyl group and C2-C3 double bond. Some flavonoids do not have the *o*-dihydroxyl structure in the B ring, and hydroxyl groups on the A-ring can compensate and become a major determinant of the antioxidant activity (*15*).

β-Carotene, α-Tocopherol, Lutein and Ascorbic acid

Ascorbic acid and carotenoids are major antioxidants in human food (*16*). Tocopherol is produced only by plants and mostly concentrated in the plant oils. Tocopherols can donate their hydrogen atoms to lipid free radicals, and neutralize the radical and form the tocopheroxyl radical. Lutein is a non-provitamin A carotenoid and a yellowish pigment. Vegetables and fruits are main sources of these compounds, making these foods essential to human health (*17*).

Antioxidant Content of Vegetables

There are several reviews available on the methods to determine the flavonoids and phenolics content in food (*12,18*). The phenolic acid and

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flavonoid content of some vegetables have been determined by several researchers (*19,20*). In most published work, only a limited number of the phenolic compounds of vegetables were determined. According to the available data, the content of flavonoids (Table I), phenolic acids (Table II), β-carotene, α-tocopherol, lutein and ascorbic acid (Table III) of some common vegetables are summarized.

The major vegetable sources of each antioxidant are listed in Table IV. Most flavonoids exist in food as glycosides and the content of aglycons forms of flavonoids are low in vegetables. The most abundant aglycons in vegetables are quercetin and kaempferol (*19*). In vegetables, quercetin glycosides predominate, but glycosides of kaempferol, luteolin and apigenin also exist (*21*). These glycosides are mainly distributed in leafy vegetables (cabbage, celery, lettuce, spinach and broccoli) (*19*). Some researches extracted the vegetables using acid or alkali hydrolysis to release the conjugated form of phenolics, thus some of the reported data in Tables I and II are total amounts of conjugated and free forms of phenolics. Except onion, broccoli, spinach, lettuce, bell pepper, tomato and bean, quercetin content of most vegetables are reported to be less than 7.0 μmol/100g (Table I). We can see from the data that flavones (luteolin and apigenin) are much less common in vegetables than flavonols (quercetin and kaempferol) (*22*). The main sources of luteolin are celery, broccoli and bell pepper, and the main source of apigenin is celery. Anthocyanins is also an important antioxidant occurring mostly in red celery, red potato, red cabbage and red leaf lettuce (*23*).

It has been reported that phenolic acids (chlorogenic, caffeic and ferulic acids) are widely distributed, especially in root vegetables (sweet potato, potato and carrot, etc.) (*19*). Compared to other phenolic acids, less data are available on the content of *p*-hydroxybenzoic acid and *p*-coumaric acid in vegetables (Table II). Either these two compounds are less abundant in vegetables or have not been extensively studied to determine their content. Cinnamic, caffeic and ferulic acids are more commonly found in vegetables compared to hydroxybenzoic acids, and these acids are rarely found in the free form (*22*). Caffeic and quinic acids combine to form chlorogenic acid, which has been found in many vegetables, such as celery, lettuce, tomato, carrot and asparagus. For vegetables, the major sources of caffeic acid include sweet potato, carrot and lettuce, and the major sources of ferulic acid are broccoli, corn and mushroom.

Ascorbic acid is widely present in vegetables in large amounts (Table III). The content of ascorbic acid for 8 of the 20 vegetables is more than 100 μmol/100g (Table III). The highest content of α-tocopherol, β-carotene, and lutein is 31.5 μmol/100g for celery, 12.1 μmol/100g (average value) for squash, and 13.9 μmol/100g (average value) for spinach, respectively (Table III). In many vegetables, ascorbic acid content is at least 10 times greater than the content of α-tocopherol, β-carotene, and lutein.

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Table I. Flavonoid Content (μmol/100g wet weight) of Some Common Vegetables.

	Myricetin	*Quercetin*	*Quercetin glycoside*	*Luteolin*	*Luteolin glycoside*	*Apigenin*	*Apigenin glycoside*	*Kaempferol*	*Kaempferol glycoside*
Asparagus	N.A.	N.A.	7.7-95.0(*19*) 46.9(*40*) (rutin) 73.8(*41*) (rutin)	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Bean	0.0-1.3(*42*)	5.3-6.6(*43*)	N.A.	0.3(*42*)	N.A.	N.A.	N.A.	0.8(*44*)	4.2-20.0(*19*)
Beet	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Broccoli	1.7(*42*)	9.9(*10*) 9.9-12.3(*43*) 1.8(*42*)	N.A.	13.3(*19*) 2.3(*42*)	0.6(*19*)	0.0(*42*)	N.A.	25.2(*10*) 21.0-25.2(*43*)	6.3(*19*)
Cabbage	4.4(*42*)	1.5(*43*) 0.4(*19*)	N.A.	0.0(*42*)	1.2(*19*)	0.0(*42*) 0.3-0.4(*45*)	N.A.	52.4(*20*)	1.6(*19*)
Carrot	0.0(*42*)	1.9(*42*)	N.A.	1.4(*42*)	N.A.	0.0(*42*)	N.A.	5.1(*42*)	N.A.
Cauliflower	0.0(*42*)	6.0(*42*)	N.A.	0.0(*42*)	N.A.	0.0(*42*)	N.A.	0.0(*42*)	N.A.
Celery	0.0(*42*)	0.0(*42*)	N.A.	2.3-14.0(*46*) 7.7(*21*) 1.8-7.0(*43*)	7.1-21.0(*19*)	6.3-70.7(*46*) 40.0(*21*) 5.3-16.0(*19*) 5.9-22.6(*43*)	18.0-51.0(*19*)	0.0(*42*)	N.A.

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Corn	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Cucumber	0.0(*45*)	0.003(*45*)	0.0(*47*) (rutin)	0.03(*45*)	N.A.	0.01(*45*)	N.A.	0.0(*45*)	N.A.
Garlic	80.7(*42*)	5.8(*42*)	N.A.	0.0(*42*)	N.A.	29.8(*42*)	N.A.	0.0(*42*)	N.A.
Lettuce	0.1(*45*)	3.6-302.0(*46*) 1.0-10.0(*10*) 4.6 - 26.2(*43*) 86.6(*20*)	1.7-4.8(*19*)	0.0(*45*)	N.A.	0.02(*45*)	N.A.	0.0(*45*)	N.A.
Mushroom	0.0(*42*)	0.0(*42*)	0.0-0.1(*48*) (rutin)	1.8-2.1(*48*)	N.A.	0.3-0.4(*48*)	N.A.	0.0(*42*)	N.A.
Onion	0.03(*45*)	94.0-161.0(*10*) 61.0-210.0(*46*) 93.0-162.0(*43*) 215.0(*20*)	N.A.	N.A.	N.A.	0.0-0.01(*45*)	N.A.	0.0(*49*) 3.3(*44*) 0.2-1.8(*45*)	N.A.
Pepper (Bell)	3.4(*42*)	9.3(*42*)	17.0-23.0(*19*)	2.5-4.9(*10*) 1.8-3.8(*43*)	13.0-(*19*) 37.0	6.3(*42*)	N.A.	0.0(*42*)	N.A.
Potato	0.003(*45*)	0.02(*45*)	N.A.	N.A.	N.A.	0.01(*45*)		0.2(*45*)	N.A.

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Table I. Continued.

	Myricetin	*Quercetin*	*Quercetin glycoside*	*Luteolin*	*Luteolin glycoside*	*Apigenin*	*Apigenin glycoside*	*Kaempferol*	*Kaempferol glycoside*
Spinach*	0.0(*42*)	90.1(*50*)	N.A.	0.0(*42*)	N.A.	0.0(*42*)	N.A.	0.0(*42*)	N.A.
	0.1(*45*)							0.2(*45*)	
Squash	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Sweet potato	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Tomato	1.4(*19*)	0.7-(*46*) 67.2	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	
		0.1(*19*)							
		0.7-(*43*) 20.9							

*Spinach contains patuletin glycoside 14.3-26.6, spinacetin glycoside 22.2-78.0, 5,3'-dihydroxy-3-methoxy-6,7-methylenedioxyflavone-4'-glucronide methyl ester 59.8-87.7, 5-hydroxy-3,3'-dimethoxy-6,7-methylenedioxyflavone-4'-glucronide methyl ester 19.8-27.1 (the unit of the data is μmol/100g wet weight) (*19*).
N.A.: no data available.
The numbers in parentheses in the table was the reference of the data.

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Table II. Phenolic Acid Content (μmol/100 g wet weight) of Some Common Vegetables

	Chlorogenic acid	Caffeic acid	Cinnamic acid	Ferulic acid	*p*-Hydroxybenzoic acid	*p*-Coumaric acid
Asparagus	9.7-24.0(*19*)	1.3-5.7(*19*)	1.7-16.0(*19*)	0.1(*51*)	N.A.	N.A.
Bean	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Beet	N.A.	N.A.	N.A.	0.3(*52*)	N.A.	N.A.
Broccoli	2.8(*19*)	9.4(*19*)	N.A.	12.9(*53*)	N.A.	N.A.
Cabbage	11.1(*19*)	7.1(*19*)	N.A.	N.A.	N.A.	N.A.
Carrot	0.6-1.4(*54*)	77.8(*55*)	0.5-0.6(*19*)	0.0(*47*)	N.A.	0.9(*55*)
	33.9(*55*)			0.6(*56*)		
Cauliflower	N.A.	N.A.	8.0(*19*)	N.A.	N.A.	N.A.
Celery	17.0-50.0(*19*)	N.A.	1.6-2.5(*19*)	N.A.	N.A.	N.A.
Corn		14.4(*57*)	2.0-27.0(*19*)	3.0-12.0(*19*)	4.1(*57*)	18.9(*57*)
Cucumber		N.A.	N.A.	0.0(*47*)	N.A.	N.A.
Garlic		N.A.	N.A.	N.A.	N.A.	N.A.
Onion		N.A.	N.A.	0.44(*58*)	N.A.	N.A.
Lettuce	3.0-82.0(*19*)	16.0-86.0(*19*)	N.A.	N.A.	N.A.	N.A.
	0.2(*20*)					
Mushroom	0.0-0.1(*48*)	0.-0.2(*48*)	0.1(*25*)	7.3(*25*)	0.0-0.5(*25*)	0.0-0.1(*48*)
		0.0(*25*)				

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Table II. Continued

	Chlorogenic acid	Caffeic acid	Cinnamic acid	Ferulic acid	*p*-Hydroxybenzoic acid	*p*-Coumaric acid
Pepper (Bell)		4.7-17.0(*19*)	N.A.	N.A.	N.A.	N.A.
Potato	1.9(*19*)	20(*55*)	18.0(*19*) 3.4(*55*)	0.2(*59*)	68.3(*59*)	0.6(*55*)
Squash		N.A.	N.A.	N.A.	N.A.	N.A.
Spinach	1.9-2.6(*19*)	N.A.	1.5-2.9(*19*)	0.5(*47*)	N.A.	N.A.
Sweet potato		0-407.4(*60*)	54.0(*19*)	N.A.	N.A.	N.A.
Tomato	17.9(*19*)	5.4(*19*) 13.3(*55*)	N.A.	1.5(*55*)	N.A.	5.8(*55*)

N.A.: no data available; The numbers in parentheses in the table was the reference of the data.

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Table III. The Contents of Tocopherol, Carotene, Lutein and Ascorbic acid (μmol/100 g wet weight) in Some Common Vegetables.

	α-Tocopherol		*β-Carotene*		*Lutein*		*Ascorbic acid*	
Asparagus	4.2	(*61*)	0.2	(*62*)	0.04	(*63*)	31.6-110.5	(*64*)
					0.8	(*62*)	86.8	(*65*)
Bean	0.5-2.2	(*66*)	0.3	(*67*)	0.9	(*67*)	99.4	(*68*)
			9.4	(*69*)	1.5	(*69*)		
Beet	N.A.		N.A.		N.A.		N.A.	
Broccoli	0.5-2.6	(*26*)	0.1-2.8	(*26*)	0.3-1.8	(*26*)	255.7	(*20*)
	3.8	(*70*)	1.7	(*70*)	5.8	(*69*)		
			3.3	(*69*)				
Cabbage	0.4	(*70*)	0.1	(*70*)	13.1	(*71*)	159.1-210.2	(*20*)
			10.7	(*71*)				
Carrot	3.7	(*72*)	10.2	(*73*)	0.9	(*73*)	0.0	(*47*)
			12.5	(*63*)	0.8	(*63*)		
Cauliflower	0.4	(*70*)	0.1	(*70*)	0.0	(*63*)	85.2	(*20*)
			0.0	(*63*)				
Celery	31.5	(*72*)	N.A.		N.A.		N.A.	
Corn	5.3	(*66*)	N.A.		N.A.		21.3	(*66*)
Cucumber	N.A.		N.A.		N.A.		0.0	(*47*)
							0.03	(*74*)
Garlic	2.2	(*75*)	N.A.		N.A.		0.0-272.2	(*76*)
	0.1-0.6	(*76*)						
Lettuce	0.1	(*77*)	4.4	(*69*)	7.3	(*69*)	N.A.	
Mushroom	0.0-0.4	(*78*)	0.0	(*78*)	N.A.		60.6-424.2	(*78*)

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Table III. Continued

	α-Tocopherol		*β-Carotene*		*Lutein*		*Ascorbic acid*	
Onion	N.A.		0.0	(*63*)	0.0	(*63*)	34.1	(*20*)
Pepper (Bell)	16.5	(*72*)	0.4-2.7	(*16*)	1.6	(*69*)	551.0-846.0	(*16*)
	0.3	(*77*)	0.6	(*69*)				
Potato	0.1	(*79*)	0.0	(*80*)	0.1	(*80*)	90.9-261.4	(*81*)
			0.0	(*63*)	0.2	(*63*)		
Spinach	3.7	(*82*)	6.4	(*69*)	11.2	(*69*)	352.3	(*83*)
	3.2	(*61*)	6.1	(*63*)	16.7	(*63*)		
Squash	0.1	(*84*)	3.9-43.8	(*85*)	8.3	(*85*)	N.A.	
			0.4	(*63*)	2.3	(*63*)		
Sweet potato	30.2	(*72*)	4.3	(*86*)	N.A.		N.A.	
Tomato	0.2-0.7	(*17*)	0.2-0.7	(*17*)	N.A.		125.0-272.7	(*17*)
							102.2	(*20*)

N.A.: no data available. The numbers in parentheses in the table was the reference of the data.

Table IV. Major Vegetable Sources of Specific Antioxidants.

Antioxidant	*Vegetables*
Myricetin	Garlic
Quercetin	Onion, spinach, lettuce, bean, tomato
Quercetin glycoside	Onion, asparagus, cabbage
Luteolin	Broccoli, celery
Luteolin glycoside	Celery
Apigenin	Garlic, celery
Apigenin glycoside	Celery
Kaempferol	Cabbage, broccoli
Kaempferol glycoside	Bean, broccoli
Chlorogenic acid	Celery, lettuce, tomato, carrot, asparagus
Caffeic acid	Sweet potato, carrot, lettuce
Cinnamic acid	Sweet potato, corn, potato
Ferulic acid	Broccoli, corn, mushroom
p-Hydroxybenzoic acid	Potato
p-Coumaric acid	Corn
α-Tocopherol	Celery, sweet potato, bell pepper
β-Carotene	Squash, Carrot
Lutein	Spinach, Cabbage, lettuce
Ascorbic acid	Bell pepper, spinach, broccoli, mushroom, cabbage, tomato

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The major antioxidants in 20 vegetables are summarized in Table V, which vary significantly from vegetable to vegetable. For example, the major antioxidants in asparagus are rutin and ascorbic acid, the major antioxidant in onion is quercetin glucoside, and the major antioxidants in celery are chlorogenic acid, apigenin and luteolin glycoside and tocopherol. These antioxidants in Table V are selected mainly according to their concentrations. In fact, both the antioxidant activity of each antioxidant and its concentration in vegetables decide its contribution to the total antioxidant activity of the vegetable. The selected categories of antioxidants in vegetables have different antioxidant activity. Determined by linoleic acid/β-carotene method, the ranking of antioxidant activity from high to low was: α-tocopherol, kaempferol, quercetin, kaempferol glycoside and myricetin (*24*). Determined by 2,2'-diphenyl-1-picrylhydrazyl (DPPH) method, the ranking of antioxidant activity from high to low was: kaempferol, quercetin, luteolin glycoside, myricetin, kaempferol glycoside, apigenin glycoside, and apigenin (*24*).

Compared to other vegetables, mushrooms contain a small amount or lack phenolic acids and flavonoids (*25*). Some vegetables have not been extensively

examined of their phenolic antioxidant contents. Little research has been done to identify the phenolic antioxidants in cucumber, beet, and squash.

The content of antioxidants in vegetables is affected by variety, cultivars, maturity and geographic origin. For example, the carotenoid, lutein and tocopherol contents of 22 broccoli genotypes varied significantly by 6-fold for lutein, 35-fold for β-carotene, and 5-fold for α-tocopherol, respectively (*26*).

Table V. Major Antioxidants with Significant Amounts in Some Common Vegetables.

Vegetable	*Major antioxidants*
Asparagus	Rutin, chlorogenic acid, ascorbic acid
Bean	Quercetin, kaempferol glycoside, ascorbic acid
Beet	Ferulic acid
Broccoli	Caffeic acid, ferulic acid, quercetin, kaempferol, ascorbic acid
Cabbage	Chlorogenic acid, caffeic acid, kaempferol, lutein, ascorbic acid
Carrot	Chlorogenic acid, caffeic acid, β-carotene
Cauliflower	Cinnamic acid, quercetin, ascorbic acid
Celery	Chlorogenic acid, apigenin, apigenin glycoside, luteolin glycoside, α-tocopherol
Corn	Caffeic acid, cinnamic acid, coumaric acid
Cucumber	Ascorbic acid
Garlic	Myricetin, apigenin, ascorbic acid
Lettuce	Chlorogenic acid, caffeic acid, quercetin
Mushroom	Ferulic acid, ascorbic acid
Onion	Quercetin glycoside
Pepper (Bell)	Caffeic acid, quercetin glycoside, luteolin glycoside, α-tocopherol, ascorbic acid
Potato	Caffeic acid, cinnamic acid, *p*-hydroxybenzoic acid, ascorbic acid
Squash	β-Carotene
Spinach	Quercetin, ascorbic acid, patuletin glycoside, spinacetin glycoside, 5,3′-dihydroxy-3-methoxy-6,7-methylenedioxyflavone-4′-glucronide methyl ester, 5-hydroxy-3,3′- dimethoxy-6,7-methylenedioxyflavone-4′-glucronide methyl ester
Sweet potato	Caffeic acid, cinnamic acid, α-tocopherol
Tomato	Chlorogenic acid, caffeic acid, quercetin, ascorbic acid

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Antioxidant Activity of Vegetables

Methods for Measuring Antioxidant Activities

More than 15 methods are available for determination of antioxidant activity of food. The merits and disadvantages of these methods have been fully discussed in several reviews (*27-30*). However, there are no officially standardized methods established yet for analyzing the antioxidant activity of food.

According to the chemical reaction measured, antioxidant assays can be classified into three classes: hydrogen atom transfer (HAT) based, electron transfer (ET) based reactions or other assays (*31*). Hydrogen atom transfer reactions include oxygen radical absorbance capacity (ORAC), total radical-trapping antioxidant parameter (TRAP), and inhibition of autoxidation of induced low-density lipoprotein (LDL). In most HAT-based methods, antioxidant and substrate compete for thermally generated peroxyl radicals through the decomposition of azo compounds. Electron transfer-based methods include 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ferric reducing antioxidant power (FRAP), DPPH and cupric reducing antioxidant capacity (CUPRAC) method. These methods measure the ability to reduce an antioxidant by the color change of the oxidant (*31*). Other assays include total oxidant scavenging capacity (TOSC), chemiluminescence and electrochemiluminescence, among others.

In the ORAC method, the peroxyl radical 2,2'-azobis-(2-amidinopropane) dihydrochloride (AAPH) reacts with a fluorescent compound (fluorescein) to form a nonfluorescent product, and the fluorescence is decreased over time. Antioxidant can scavenge the peroxyl radicals and retard the loss of the fluorescence, thus the area of time-fluorescence curve can be used to quantify the antioxidant activity of antioxidants (*32*). For the LDL oxidation method, LDL isolated from fresh blood samples is oxidized by Cu^{2+} or AAPH (*33*). The peroxidation of the lipid components in LDL can produce conjugated dienes which can be detected at 234 nm. Alternatively, lipid hydroperoxides can be determined by measuring peroxide values. Antioxidants can protect the LDL from oxidation. Thus the difference between blank assays and those with antioxidant is a measure of antioxidant potency. The DPPH method measures the free radical scavenging ability of antioxidants by monitoring the decrease of absorbance of DPPH free radical at 515 nm. ABTS method measures the reduction of absorbance of ABTS radical at 734 nm. The ABTS radical is produced by the reaction among ABTS, metmyoglobin, H_2O_2 and peroxidase, or between ABTS and persulfate. The ABTS method can determine both hydrophilic and lipophilic antioxidants in the same sample (*34*). The CUPRAC method utilizes the copper (II)-neocuproine [Cu (II)-Nc] reagent as the chromogenic oxidizing agent and the developed absorbance at 450 nm is measured after 30 minutes of incubation (*35*). For the chemiluminescence

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method, the chemiluminescent reaction between 9-benzylacridine and potassium superoxide can be measured using a luminometer. Antioxidants can compete with 9-benzylacridine to scavenge the superoxide radical anion. According to the reduced light signals, the antioxidant activity of the antioxidants can be quantified (*36*).

No single method can measure the total antioxidant activity of all the antioxidants in food (*27*). It is suggested that antioxidant activity of food should be evaluated using several methods to measure the oxidation products related to real food systems. To choose an antioxidant activity method, we need to consider the type of substrates, conditions of analysis, and concentrations of antioxidants found in the food under investigation.

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Analysis of Antioxidant Activities of Vegetables

Antioxidant activity of vegetables has been investigated using several methods, such as ORAC, FRAP, ABTS and LDL oxidation method. ORAC and FRAP methods were used to analyze the antioxidant activity of 13 vegetables (*37*). The data showed that the ORAC and FRAP values of vegetables gave different antioxidant activity trends. ORAC method is more relevant to the antioxidants' function in breaking the free radical chain compared to FRAP. The FRAP method can be affected by several factors, such as the interference due to compounds that absorb at the wavelength of analysis and reaction kinetics. ORAC method showed that green pepper, spinach, purple onion, broccoli, beet and cauliflower had greater peroxyl radicals scavenging ability than white cabbages, carrots, snap beans, white onions, pea, tomato and red pepper (*37*).

The antioxidant activities of 22 common vegetables were measured using ORAC assay with three different reactive species: a peroxyl radical generator, a hydroxyl radical generator, and Cu^{2+}(*5*). Garlic had the highest antioxidant activity against peroxyl radical followed by spinach, broccoli flowers, beets, red bell pepper, onion, corn, cauliflower, potato, sweet potato, cabbage, leaf lettuce, string bean, carrot, yellow squash, iceberg lettuce, celery and cucumber.

The antioxidant capacities of vegetables extracts were analyzed using the ABTS, FRAP and ORAC methods (*20*). The ABTS, FRAP and ORAC values for each extract were relatively similar. The relative antioxidant activities determined by ABTS were shown in the following order: cabbage > spinach > broccoli > onion > green cabbage > pea > cauliflower > tomato > lettuce.

The antioxidant activity of 23 vegetables after acid hydrolysis was evaluated by determining the amounts necessary to inhibit the oxidation of lower density lipoproteins (LDL) (*6*). The phenol antioxidant index, based on both the quantity and the quality of antioxidants present, was calculated using both the LDL method data and the total phenol concentration of the vegetable. Based on fresh weight, the order of the phenol antioxidant index was: kidney bean > pinto bean > garlic > yellow onion > red onion > asparagus > snap bean > beet > potato > broccoli > sweet potato > tomato > corn > bell pepper > carrot

> cauliflower > mushroom > spinach > celery > green squash > cabbage > head lettuce > cucumber.

Antioxidant activity of 43 vegetable extracts was examined by discoloration of β-carotene coupled with the oxidation of linoleic acid (*38*). The results showed that 13 vegetable extracts including asparagus belong to the high activity group, with 100 g of vegetable weight equals to more than 25 mg of BHA. Another 13 vegetable extracts (cucumber and others) belong to the low activity group with 100 g of vegetable weight equals to less than 5 mg of BHA. The activities of another 17 vegetables belong to the intermediate level of high activity group and low activity group.

The antioxidant activity of the common vegetables determined by several methods is summarized in Table VI. Based on the data in Table VI, a relative antioxidant activity index (%) was developed to facilitate the comparison of the antioxidant activity of vegetables determined using different methods. For the antioxidant activity analyzed by each method, the highest value of a vegetable was considered as 100%, and the values of other vegetables were expressed as a percentage of it. Then, for each vegetable, the mean value of the percentage from different methods was calculated as this vegetable's relative antioxidant activity index (%). The order of the antioxidant activity from high to low is garlic, asparagus, spinach, beet, bell pepper, mushroom, broccoli, cabbage, corn, onion, bean, carrot, cauliflower, sweet potato, tomato, potato, lettuce, squash, celery, and cucumber (Figure 1). To simplify the results, those with relative antioxidant activity index > 40% are classified as vegetables with high antioxidant activity: garlic, asparagus, spinach, beet, bell pepper, mushroom, and broccoli. Those with values from 40% to 15% are considered as vegetables with medium antioxidant activity: cabbage, corn, onion, bean, carrot, cauliflower, sweet potato, tomato, potato, lettuce, and squash. The rest with relative antioxidant activity index < 15 % are grouped as vegetables with low antioxidant activity: celery and cucumber.

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Future Research Direction

Antioxidants and antioxidant activity of vegetables have not been fully investigated. The future work in this area are suggested as the following: 1) identify the major antioxidants in uninvestigated vegetables and find out the contribution of the individual antioxidant to the total antioxidant activity of vegetables, 2) investigate the antioxidant activity of vegetables using *in vivo* experiments, 3) make full use of the vegetable byproducts, such as asparagus roots, which could be a good resource of antioxidants, 4) investigate the synergy mechanism effects of antioxidants in disease prevention. As the individual antioxidant studied in clinical trials do not appear to have consistent preventive effects, it is recommended that consumers eat 5 to 10 servings of a wide variety of vegetables daily to reduce the risk of chronic diseases and to meet their nutrient requirements for optimum health (*39*).

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Table VI. Antioxidant Activity of Selected Vegetables (μmol Trolox equivalent/g wet weight).

	**Total phenol antioxidant index × 0.001*	*ORAC (ROO•)*	*ORAC (OH•)*	*ORAC (Cu^{2+})*	*FRAP*	*ABTS*	*TRAP*
Asparagus	10.0(*6*)	30.2(*87*)	N.A.	N.A.	10.6**(*88*)	3.9(*88*)	9.7(*88*)
Bean	5.7-54.4(*6*)	2.4-2.9(*87*)	1.7(*5*)	0.2(*5*)	1.7(*37*)	1.3(*88*)	0.7(*88*)
		6.8(*37*)			2.3**(*88*)		
		2.0(*5*)					
Beet	5.3(*6*)	27.7(*87*)	3.1(*5*)	0.2(*5*)	13.2(*37*)	5.2(*88*)	2.7(*88*)
		8.4(*5*)			13.1**(*88*)		
		17.6(*37*)					
Broccoli	4.1(*6*)	15.9(*87*)	2.4(*5*)	1.6(*5*)	3.6(*37*)	6.5(*20*)	3.1(*88*)
		8.9(*5*)			8.3(*20*)	3.0(*88*)	
		11.2(*37*)			11.7**(*88*)		
		8.0-24.0(*26*)					
		13.4(*20*)					
Cabbage	1.6(*6*)	13.6(*87*)	1.5(*5*)	0.3(*5*)	6.9-18.7(*20*)	4.9-13.8(*20*)	2.6(*88*)
		3.0(*5*)			3.8(*37*)	1.6(*88*)	
		5.9(*37*)			7.0**(*88*)		
		11.8-21.2(*20*)					

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Carrot	2.3(*6*)	12.2(*87*) 2.1(*5*) 6.2(*37*)	0.8(*5*)	0.5(*5*)	13.3(*37*) 1.1**(*88*)	0.4(*88*)	0.7(*88*)
Cauliflower	2.2(*6*)	6.5(*87*) 3.8(*5*) 8.5(*37*) 4.3(*20*)	1.1(*5*)	0.2(*5*)	5.1(*37*) 2.6**(*20*) 4.3**(*88*)	3.0(*20*) 1.1(*88*)	1.6(*88*)
Celery	1.7(*6*)	5.7(*87*) 0.6(*5*)	0.3(*5*)	0.2(*5*)	1.2**(*88*)	0.5(*88*)	0.5(*88*)
Corn	2.8(*6*)	7.3(*87*) 4.0(*5*)	2.2(*5*)	1.0(*5*)	N.A.	N.A.	N.A.
Cucumber	1.1(*6*)	1.2(*87*) 0.5(*5*)	0.3(*5*)	0.3(*5*)	0.7** (*88*)	0.4(*88*)	0.0(*88*)
Garlic	31.5(*6*)	19.4(*5*)	1.1(*5*)	2.7(*5*)	N.A.	N.A.	N.A.
Lettuce	1.3(*6*)	4.5-15.5(*87*) 1.2-2.6(*5*) 3.2(*20*)	0.7(*5*) 1.4(*5*)	0.4(*5*) 0.1(*5*)	1.2**(*20*) 4.9**(*88*)	1.7(*20*) 1.3(*88*)	2.3(*88*)

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Table VI. Continued

	**Total phenol antioxidant index × 0.001*	*ORAC (ROO•)*	*ORAC (OH•)*	*ORAC (Cu^{2+})*	*FRAP*	*ABTS*	*TRAP*
Mushroom	2.0(*6*)		N.A.	N.A.	16.4**(*88*)	4.9(*88*)	6.3(*88*)
Onion	10.5-11.9(*6*)	4.5(*5*)	0.5(*5*)	0.6(*5*)	3.7**(*20*)	5.3(*20*)	2.4(*88*)
		10.3-11.5(*87*)			1.6-15.0(*37*)	1.8(*88*)	
		8.1-13.7(*37*)			5.3**(*88*)		
		9.9(*20*)					
Pepper (Bell)	2.7(*6*)	5.6-10.2(*87*)	0.6(*5*)	0.4(*5*)	11.6(*37*)	8.4(*88*)	5.5(*88*)
		6.1-9.7(*37*)			21.0**(*88*)		
		7.1(*5*)					
Potato	4.6(*6*)	3.1(*5*)	1.0(*5*)	0.5(*5*)	3.7**(*88*)	0.8(*88*)	0.9(*88*)
		10.6-13.2(*87*)					
Squash	1.6(*6*)	1.5(*5*)	1.1(*5*)	0.2(*5*)	N.A.	N.A.	N.A.
Spinach	2.0(*6*)	26.4(*87*)	2.8(*5*)	1.6(*5*)	4.0(*37*)	8.5(*88*)	5.8(*88*)
		12.6(*5*)			26.9**(*88*)		
		9.5(*37*)					

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Sweet potato	4.1(*6*)	9.0(*87*)	1.0(*5*)	0.3(*5*)	N.A.	N.A.	N.A
		3.0(*5*)					
Tomato	3.3(*6*)	3.4(*87*)	N.A.	N.A.	3.7(*37*)	2.5(*20*)	1.5(*88*)
		4.4(*37*)			3.4(*20*)	1.6(*88*)	
		4.2(*20*)			5.6**(*88*)		

* The phenol antioxidant index (PAOXI), a combined measure of quantity and quality of antioxidants, is calculated by dividing the total phenol concentration of the vegetable (μmol/kg) by the IC_{50} (μM). IC_{50} is the concentration of phenols in the extract to inhibit 50% of the oxidation of lower density lipoproteins (*6*); ** Data are expressed as μmol Fe equivalent/g wet weight.

N.A.: no data available.

The numbers in parentheses in the table was the reference of the data.

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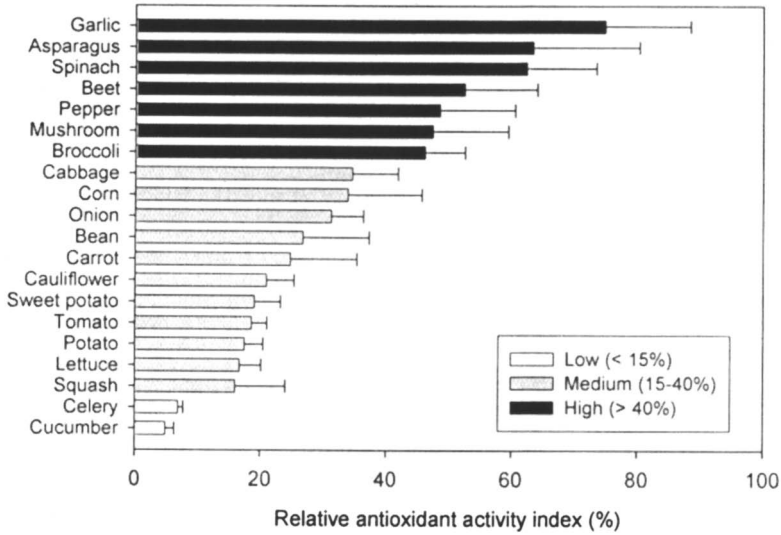

Figure 1. The relative antioxidant activity index (%) of the common vegetables (average ± standard error).

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Chapter 13

Antioxidant Capacity of Phenolic Extracts from Selected Food By-Products

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Potential use of blueberry leaves and canola hulls as a source of natural antioxidants was explored. Cyclone canola hulls and blueberry leaves were extracted with acetone (70%, v/v). Crude phenolic extracts were fractionated on Sephadex LH-20 column into two fractions; fraction A was rich in non-tannin phenolics while fraction B was rich in condensed tannins. The total content of phenolics in the extracts ranged from 44 to 517 mg catechin equivalents per gram of extract. Higher levels of condensed tannins were detected in blueberry leaves extracts than in canola hull extracts. The fraction B exhibited stronger free radical scavenging activity than both fraction A and crude phenolic extract.

The antioxidant activities of plant extracts have been associated with the presence of phenolic compounds such as phenolic acids, flavonoids and tannins (*1*). Canola and rapeseed hulls have been reported to contain up to 1000 mg of phenolic acids and from 89 to 1847 mg soluble condensed tannins per 100 g of sample (*2-4*). Therefore, the use of hulls, after dehulling, as a potential source of natural antioxidants would offer a commercial incentive for further exploitation. Amarowicz *et al.* (*5*) reported that crude extracts of condensed tannins isolated from high-tannin canola hulls displayed significantly greater scavenging effects for α,α-diphenyl-β-propyl hydrozyl (DPPH) radical than those from low-tannin rapeseed hulls. Subsequently, Amarowicz *et al.* (*6*) demonstrated that some fractions of non-tannin canola phenolics also exhibit a

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marked free radical scavenging activity. Later, Naczk *et al.* (*7*) reported that antioxidant activity of extracts of canola hulls in 80-100% (v/v) methanol and 70-80% (v/v) acetone in a β-carotene-linoleate model system was comparable to that exhibited by butylated hydroxyanisole (BHA). The existence of statistically significant linear correlation between the antioxidant activity of phenolic extracts from canola hulls, as measured by Trolox equivalent antioxidant capacity (TEAC) values, and total condensed tannin contents (as determined using the modified vanillin and proanthocyanidin assays) as well as the protein precipitation activity of phenolic extracts (as measured using the dye-labeled assay) has also been demonstrated (*8*).

Currently, there is an increasing interest in evaluation of plant leaves as a potential source of food–grade antioxidants. Wang and Lin (*9*) reported that phenolic extracts from strawberry, blackberry and raspberry exhibited significantly higher ORAC values than extracts from corresponding berries. Similarly, Ehlenfeldt and Prior (*10*) found that leaf extract from highbush blueberry displayed stronger antioxidant activity than corresponding extract from blueberry fruits. Recently, Naczk *et al.* (*11*), however, reported that the antioxidant activity of phenolic extract from wild blueberry leaf was only slightly higher than that exhibited by the corresponding fruit extract.

In this contribution we compare the antioxidant activity displayed by various phenolic extracts from canola hulls and blueberry leaves, both of which are processing by-products from well-known Canadian crops.

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Materials and Methods

Blueberry leaves (*Vaccinium angustifolium*), a by-product of mechanical harvesting of wild blueberries, were collected from a wild blueberry farm located in the Antigonish County of Nova Scotia, Canada, in July 2003. The leaves were separated from other debris by hand, dried at room temperature, and then stored in sealed polyethylene bags at -18 °C.

Cyclone canola hulls were prepared according to the procedure described by Sosulski and Zadernowski (*12*). Hulls were extracted with hexanes for 12 h using a Soxhlet apparatus and then air-dried at room temperature.

Blueberry leaves were extracted with 70% (v/v) acetone three times at 50 °C for 30 min at a solid-to-solvent ratio of 15:100 (w/v). The extracts were pooled, evaporated to near dryness under vacuum at 40 °C, lyophilized, and then the residue was weighed. The chlorophyll was removed from the lyophilized leaf extracts as described by Amarowicz *et al.* (*13*).

Cyclone canola hulls were extracted twice at room temperature into 70% (v/v) aqueous acetone using a Waring Blender (Waring Products Division, Dynamics Corporation of America, Hartford, CT) for 2 min at maximum speed at a solid-to-solvent ratio of 1:10 (w/v). The extracts were combined, evaporated to near dryness under vacuum at 40°C, lyophilized and then the residue was weighed.

The crude phenolic extracts of blueberry leaves and canola hulls were fractionated as described by Strumeyer and Malin (*14*). A sample (550 mg) of crude phenolic extract was suspended in 5 mL of 95% (v/v) ethanol and applied onto a chromatographic column (2.3 x 40 cm) packed with Sephadex LH-20. Sephadex LH-20 was equilibrated with 95% (v/v) ethanol for over 12 h and then the column was manually packed by elution with the same solvent. The column was exhaustively washed with 95% (v/v) ethanol at a flow rate of 60 mL/h and then eluted with 50% (v/v) acetone at a flow rate of 60 mL/h. The solvent was then evaporated from the ethanol eluate (referred to as fraction A) and acetone eluate (referred to as fraction B), under vacuum at <40 °C; each phenolic fraction was then lyophilized and the residue weighed.

The total content of phenolic compounds in the crude phenolic extracts and their fractions was estimated using the Folin-Denis reagent (*15*) and expressed as catechin equivalents per gram of extract. The content of soluble-condensed tannins in the extracts was measured using the modified vanillin assay and expressed as catechin equivalents per gram of extract (*3, 16*). The protein precipitating potential of phenolic extracts was assayed by the protein precipitation method of Hagerman and Butler (*17*) (at 1mg bovine serum albumin (BSA) per mL) and by the dye-labeled BSA assay of Asquith and Butler (*18*) (at 2 mg dye-labeled BSA per mL) as modified by Naczk *et al.* (*19*).

The scavenging effect of phenolic extracts from blueberry leaves on DPPH radical was monitored according to the method of Hatano *et al.* (*20*). An aliquot of methanolic solution (0.1 mL) containing 20-100 μg of phenolic extract of wild blueberry leaves was mixed with 2 mL of methanol and then added to a methanolic solution of DPPH (1 mM, 0.25 mL). The mixture was vortexed for 10 s, then left to stand at room temperature for 30 min, and its absorbance at 517 nm was recorded.

The scavenging effect of phenolic extracts from blueberry leaves on 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) radical anion was monitored using the method of van der Berg *et al.* (*21*) as modified by Kim *et al.* (*22*). The concentration of ABTS radical anion solution was adjusted to an absorbance of 0.50 at 734 nm. The ABTS radical anion scavenging activity of blueberry extracts was expressed in mM Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalent antioxidant capacity (TEAC) values per gram of extract. TEAC was expressed as a slope of a line reflecting the amount of Trolox equivalents per assay as a function of the amount of phenolic extract added to the reaction mixture. The ABTS radical ion solutions were freshly prepared each day.

All assays were conducted at room temperature (about 22 °C) using appropriate samples and blanks. Results presented in tables and figures are mean values of at least four determinations. The bars in the figures represent standard deviations from mean values.

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Results and Discussion

Aqueous acetone (70%, v/v) was used for the extraction of phenolics from wild blueberry leaves and canola hulls as this solvent system is commonly employed for the extraction of condensed tannins from plant materials (*1, 23, 24*). Blueberry leaves yielded an almost two-fold higher amount of crude phenolic extract than canola hulls (Table I). Crude phenolic extracts were fractionated according to the method of Strumeyer and Malin (*14*) into two fractions, namely fraction A rich in non-tannin phenolics, and fraction B containing predominantly condensed tannins. Fraction A constituted 53% and 90.1% of the total crude phenolic extracts of canola hulls and blueberry leaves, respectively. On the other hand, extracts of canola hulls yielded over 5-fold more of fraction B than the corresponding blueberry leaves extracts (Table I).

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Table I. The Yield (%) of Phenolic Extracts isolated from Blueberry Leaf and Canola Hull[1]

Phenolic Fraction	*Blueberry Leaf*	*Canola Hull*
Crude	14.2	7.7
Fraction A	12.8	4.1
Fraction B	1.1	3.2

[1] As percentage of fresh weight of sample.

The total phenolics content (TP) and the total soluble condensed tannins content (SCT) in the extracts were expressed as mg catechin equivalents per gram of extract to facilitate the comparison of the results. The proportion of TP and SCT in the extracts and their fractions is shown in Table II. The crude extract from blueberry leaves contained over 6-fold more TP than crude extract from canola hulls. Furthermore, blueberry leaves extracts contained 2-4 times more SCT than the corresponding extracts from canola hulls. SCT were predominantly found in fraction B from both blueberry leaves and canola hulls. Small quantities of SCT were also detected in fraction A. The SCT present in this fraction are probably composed of lower molecular weight SCT. Gu *et al.* (*25*) reported that only monomers, dimers and trimers may be eluted from Sephadex LH-20 column with aqueous alcohol.

Several assays have been proposed for the evaluation of protein-precipitating potentials of plant polyphenols (*1*). Of these methods, the dye-labeled BSA assay

Table II. Total Phenolic and Condensed Tannins Contents in Blueberry Leaf and Canola Hull Phenolic Extracts

Sample	*Phenolic Fraction*	*Total Phenolic*[1]	*Total Condensed Tannins*[1]
Blueberry Leaf	Crude	439±6	215±4
	Fraction A	247±3	39±8
	Fraction B	517±17	1095±10
Canola Hull	Crude	68±1.5	125±1
	Fraction A	44±0.6	10±1
	Fraction B	381±12	357±6

Results are means of six determinations ± standard deviation;
[1] Expressed in mg catechin equivalents per gram of extract.

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(DLPA) developed by Asquith and Butler (*18*) and the protein precipitation assay (PPA) developed by Hagerman and Butler (*17*) as modified by Naczk *et al.* (*19*) were selected for quantification of protein-precipitating potential of phenolic extracts isolated from canola hulls and blueberry leaves. The DPLA measures the amount of protein precipitated by polyphenols, while the PPA estimates the amount of phenolics precipitated as protein-phenolic complex.

The protein precipitating potential of tested phenolic extracts was expressed as the slope values of lines (Figure 1) depicting the amount of proteins or phenolics precipitated as a PP-protein complex with increasing quantities of total phenols added to a reaction mixture containing a known amount of protein (1 /mL for the PPA and 2 mg/mL for the DLPA). The numerical values of slopes for tested phenolic extracts are given in Table III. These values, obtained using the PPA, ranged from 0.82 to 7.9, while those obtained using the DLPA were between 9.5 and 104 for crude extracts of canola hulls and the fraction B of blueberry leaves, respectively. Fraction A, rich in non-tannin phenolics, did not exhibit any significant protein precipitating potential. On the other hand, phenolics present in fraction B were very effective protein precipitants. The observed differences between the numerical slope values for tested phenolic extracts may be due to the existing differences in their affinities for proteins. Similar differences in the slopes were reported by Naczk *et al.* (*19, 26*) for crude phenolic extracts isolated from low- and high-tannin hulls of canola and rapeseed (*Brassica* oilseeds), beach pea

Table III. Protein Precipitating Capacity[1] of Blueberry Leaf Canola Hull Phenolic Extracts

Sample	*Phenolic Fraction*	*PPA*[2]	*DPLA*[3]
Blueberry Leaf	Crude	1.5±0.02	22.5±1.6
	Fraction A	ND	ND
	Fraction B	7.9±0.2	104.0±11
Canola Hull	Crude	0.8±0.10	9.5±0.1
	Fraction A	ND	ND
	Fraction B	3.2±0.1	34.7±2.8

[1]Expressed as a slope values; [2]PPA, protein precipitation assay; [3]DPLA, dye-labeled protein assay; ND, not detected.

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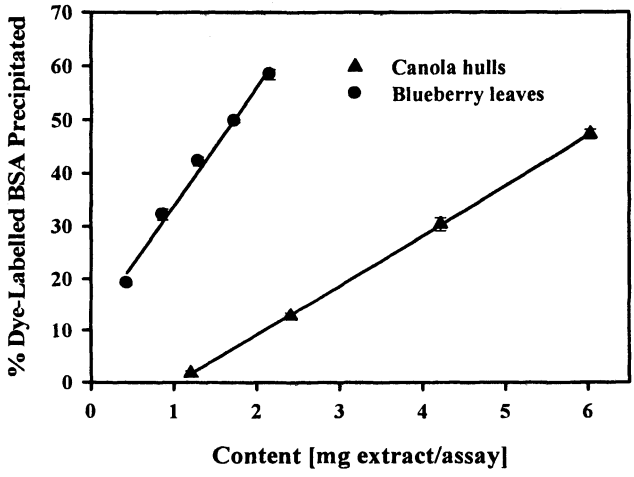

Figure 1. Titration curves of known amount of protein with increasing amounts of fraction B isolated from crude phenolic extract of canola hulls and blueberry leaves as determined by the dye-labeled protein assay of Asquith and Butler (18).

(*Lathyrus maritimus* L.), evening primrose seeds (*Oenothera biennis* L.), and faba beans (*Vicia faba*). According to Porter and Woodruffe (*27*) the ability of phenolics to precipitate proteins is influenced by their degree of polymerization. Furthermore, Asquith and Butler (*18*) have also noted that the precipitation of proteins was affected by the degree of SCT polymerization. The chemical structures, polymer chain lengths, and molecular weights of tested phenolics are still unknown and more detailed chemical analyses of these phenolics are still needed.

The DPPH radical and ABTS radical anion assays were selected for determination of radical scavenging potentials of phenolic extracts from blueberry leaves and canola hulls. The DPPH radical assay has been widely employed for estimation of antioxidant activity of plant phenolics (*6, 28, 29*). The tested extracts are composed of complex mixtures of phenolics with different chemical structures. Therefore, the amount of crude extract required for a 50% depletion of the DPPH radical present in the reaction mixture, $C_{50\%}$, was used as a measure for the radical scavenging activity; strong radical scavenging activity is associated with low $C_{50\%}$ values. The $C_{50\%}$ values for tested phenolic extracts were from 20 to 282 μg/assay (Figure 2). The extracts from blueberry leaves exhibited greater radical scavenging activities than corresponding canola hulls extracts. These differences may due to a higher level of both total phenolics and condensed tannins in blueberry leaves extracts than in canola hulls extracts (Table II). However, the $C_{50\%}$ values for canola hull extracts were in good agreement with those reported by Matthäus (*30*) for phenolic extracts from rapeseed residues and by Naczk *et al.* (*8*) for phenolic extracts from canola hull extracts. In addition, fraction B from both blueberry leaves and canola hulls displayed significantly stronger radical scavenging activity than crude phenolic extracts and their corresponding fraction A. This suggests that condensed tannins contribute greatly to the antioxidant capacity of the tested phenolic extracts.

The ABTS radical ion assay is based on the inhibition of the ABTS radical anion ($ABTS^{\bullet-}$) (*22*) or the ABTS radical cation ($ABTS^{\bullet+}$) (*31*). The assay used in this study was based on the inhibition of $ABTS^{\bullet-}$. The TEAC values given in Figure 4 are the slopes of lines depicting the relationship between the antioxidant activity of an extract, expressed as Trolox equivalents (TE)/assay, and the amount [in grams] of extract added to the reaction mixture (Figure 3). The TEAC values of crude phenolic extracts from canola hulls ranged from 0.39 for the fraction A of canola hulls extract to 6.42 for the fraction B of blueberry leaves extract (Figure 4). The TEAC values of phenolic extracts from blueberry leaves were up to six times higher than those found for canola hull extracts. Furthermore, free radical scavenging activity displayed by fraction B, rich in condensed tannins, was two and seven times higher than that of the corresponding crude phenolic extract and fraction A, predominant in non-tannin phenolics. In addition, Hagerman *et al.* (*32*) reported that tannins were 15-30

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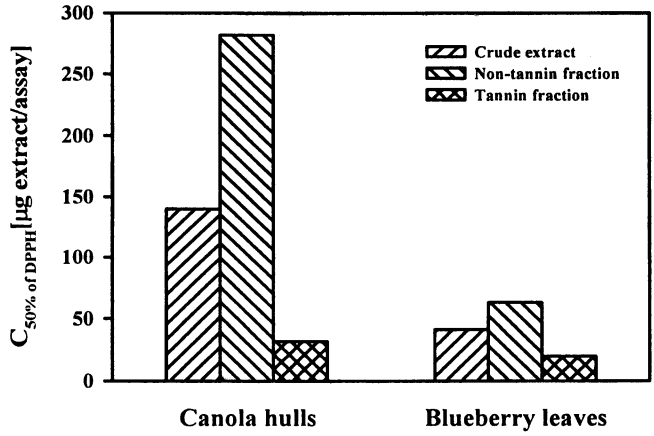

Figure 2. DPPH radical scavenging capacities of phenolics extracted from canola hulls and blueberry leaves.

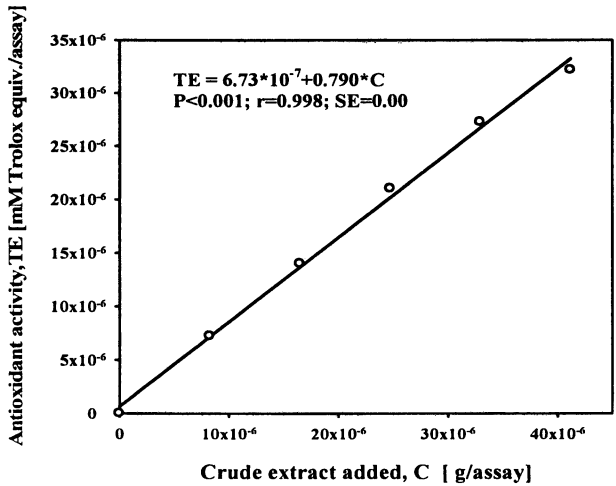

Figure 3. Determination of the TEAC value as a slope of the line depicting antioxidant activity of extract, expressed as Trolox equivalents per assay, as a function of the extract concentration in the reaction mixture. The data for the crude extract of phenolics from canola hulls are shown here. SE, standard error of estimate.

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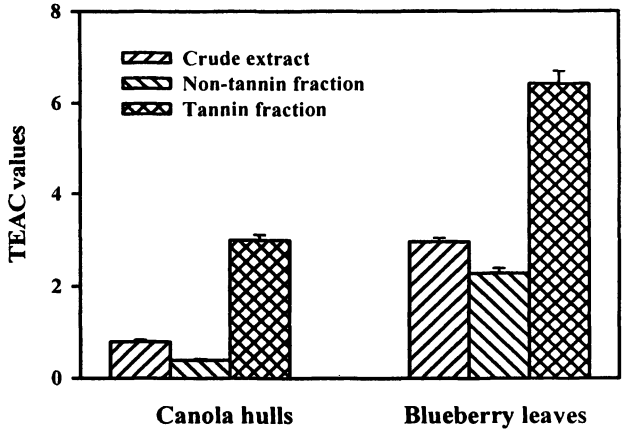

Figure 4. ABTS°⁻ scavenging capacities of phenolics extracted from canola hulls and blueberry leaves.

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times more potent scavengers of peroxyl radicals than simple phenolics. Subsequently, Riedl and Hagerman (*33*) demonstrated that procyanidins, even in the presence of proteins, were very effective free radical quenchers.

The phenolic extracts from blueberry leaves phenolics displayed stronger antioxidant capacities than those of canola hulls, possibly due to the existing differences in the total phenolics and tannins contents as well as the molecular composition of phenolics present in the extracts. Condensed tannin fraction of extracts (fraction B) contributed markedly to their antioxidant potential. More research is still needed to isolate the active phenolic components of the tested extracts.

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Chapter 14

Comparative Study on Total Polyphenol Content and Total Antioxidant Activity of Tea (*Camellia sinensis*)

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Total polyphenol content (TPC) and total antioxidant activity (TAA) of seventeen types of tea samples were evaluated and their relationship examined. TPC of tea was measured by the standard Folin-Ciocalteu method and a new enzymatic procedure involving the use of horseradish peroxidase and 4-aminoantipyrine, a chromagenic agent. TAA in tea samples was assessed using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging and ferric reducing antioxidant power (FRAP) assays. The highest content of polyphenol and strongest antioxidant activity was detected in green teas, followed by oolong teas and then black teas. The comparison of the above methods revealed the relationship between TPC and TAA of teas. Discrepancies in the results maybe explained by the different chemical principles upon which these assays are built. Moreover, use of a variery of standard compounds, along with a new enzymatic method, showed significant differences in the used TPC of teas, indicating need for further standardization of TPC and TAA methods.

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Tea is the most widely consumed beverage in the world. Regular intake of tea is associated with improved antioxidant status *in vivo* which may contribute to lowering the risk of coronary heart disease, stroke and certain types of cancer. There are three major categories of tea, non-fermented green tea, partially fermented oolong tea, and fully fermented black tea (*1*).

Considerable interest has been shown in the past decade regarding the health benefits of tea, particularly its polyphenolic components and its antioxidant activity (*2,3*). Catechins and theaflavins are two important groups of polyphenols present in tea (Figure 1). The health protective properties of tea are generally associated with its high polyphenol content. Future commercial exploitation of tea as a health beverage or nutraceutical will, therefore, require knowledge of the levels of tea polyphenols in different varieties of processed tea. However, tea polyphenol composition varies according to agronomic factors, leaf composition, variety, processing conditions, brewing conditions and degree of fermentation as well as storage method (*4,5,6*). Therefore, reliable compositional data on commonly consumed teas are also necessary for better understanding the health promoting effects of tea. Zhang *et al.* (1997), reported that jasmine tea GTP (green tea polyphenols) consist of 51.2% of EGCG (the most abundant catechin in tea), followed by 18.7 % EGC, 12.3% EC and 11.8% ECG *(7)*. Khokhar and Magnusdottir (*4*) reported the total polyphenol and catechins content in a selection of teas widely consumed in the United Kingdom.

Although Folin-Ciocalteu's method has been widely applied in the wine and tea industry, a more specific method for the determination of total polyphenol content in tea and wine was recently introduced by Stevanato *et al.* (*8*). The mechanism of this new enzymatic method is demonstrated in Figure 2. Horseradish peroxidase is oxidized in the presence of hydrogen peroxide. The oxidized form of this enzyme comes into contact with phenols, which results in the formation of phenoxyl radicals. These radicals further react with the aromatic amine group of 4-aminoantipyrine, giving rise to the final quinone-imine colored product *(9,10)*.

Methods for total antioxidant activity measurement from food matrix can be classified into two main categories. They are either based on a hydrogen atom transfer (HAT) reaction between a free radical and an oxidant or on single electron transfer (SET) reaction (*11*). DPPH (1,1-diphenyl-2-picrylhydrazyl) radical assay is one of the most commonly used HAT methods. Interaction of this radical with antioxidants results in a decrease in its absorbance intensity, thus providing a basis for the measurement of the antioxidant activity of investigational compounds. The radical scavenging action of antioxidants (phenol) (*12*) is indicated in Figure 3.

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The percent inhibition of DPPH radical by an antioxidant can be calculated with the following formula (*13*), where A_0 is the absorbance of the DPPH radical without antioxidant at t = 0 min while A_t is the absorbance of the DPPH radical with antioxidant at t = 30 min:

$$\% \text{ Inhibition} = [(A_0-A_t) / A_0] \times 100$$

The ferric reducing antioxidant power assay (FRAP) is one of the SET-based methods. As a result of the reaction, a single electron is transferred from the antioxidant to the oxidant. The change of the absorbance value of either the oxidant or the antioxidant, measured by spectrophotometer, is used to quantify the reducing power of the antioxidant. The principle of the FRAP method is demonstrated below.

$$Fe\ (TPTZ)_2(III) + ArOH \rightleftharpoons Fe\ (TPTZ)_2(II) + ArOH^{+\cdot}$$

Essentially, the FRAP method is based on the reduction of a ferric tripyridyltriazine $Fe(TPTZ)_2(III)$ complex to ferrous tripyridyltriazine $Fe(TPTZ)_2(II)$ by the antioxidant ArOH (*14*). Benzie and Szeto (*15*) compared total antioxidant capacity of 25 types of teas using a ferric reducing antioxidant power assay. The strongest antioxidant power was found in green tea (272-1144 μmol of antioxidant power/g of dried tea leaves), followed by oolong tea (233-532 μmol/g) and then black tea (132-654 μmol/g) (*15*). FRAP assay can be applied not only to investigate antioxidant efficiency of plasma or antioxidants in food matrix and beverages, but for investigation of pure dietary polyphenols as well *(16,17)*.

Little literature data exist on the characterization of total polyphenol content and evaluation of antioxidant activity in three categories of tea (green, oolong, black tea). Therefore, our systematic study on the total polyphenol, as well as antioxidant activity in green, oolong and black tea will build a valuable database for further investigation on tea and the tea beverage industry. In the present study we measure the total polyphenol content of 17 selected teas by applying Folin-Ciocalteu's method as well as a new enzymatic method. The total antioxidant activity of 17 teas is evaluated by DPPH and FRAP methods. The comparison of the data obtained from Folin-Ciocalteu's method and the enzymatic method suggests that the enzymatic method offers a greater specificity toward polyphenols and less interference. Correlation between total polyphenol content and total antioxidant activity of 17 teas are also elucidated.

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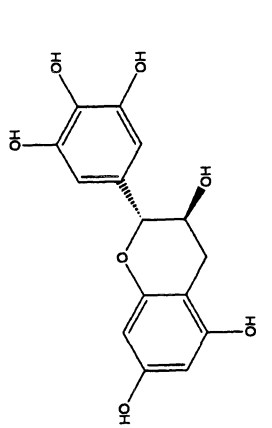

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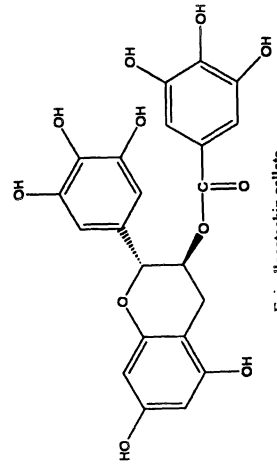
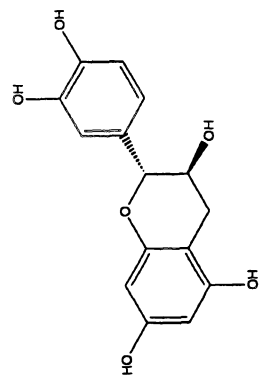
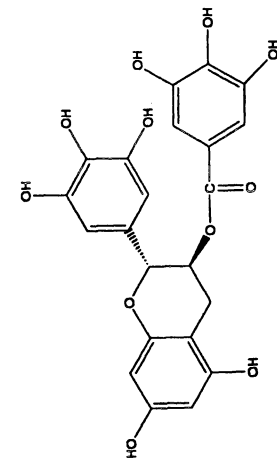


Epigallocatechin gallate



Epicatechin



Epigallocatechin gallate

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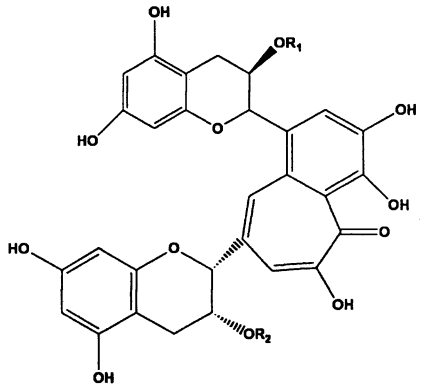

Theaflavin: R_1=R_2=H
Theaflavin-3-gallate: R_1=galloyl; R_2=H
Theaflavin-3'-gallate: R_1=H; R_2=galloyl
Theaflavin-3,3'-digallate: R_1=R_2=galloyl

Figure 1. Chemical structures of catechins and theaflavins in tea.

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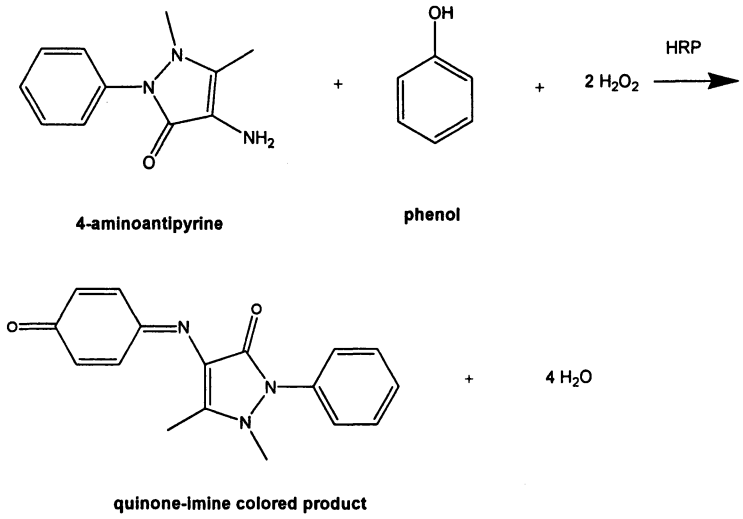

Figure 2. Mechanism of the new enzymatic method.

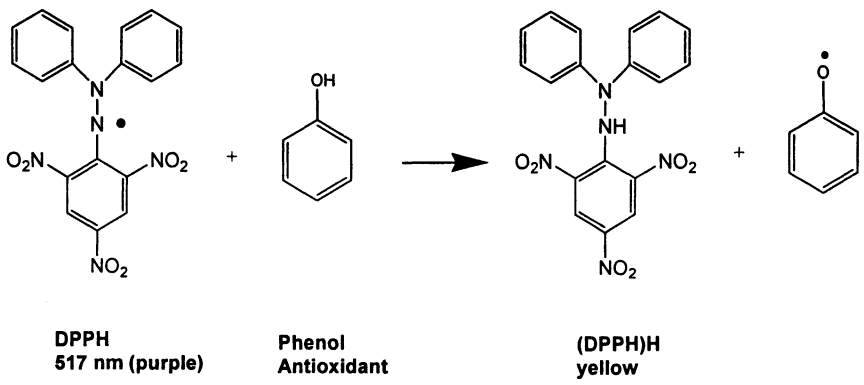

Figure 3. Radical scavenging mechanism of antioxidants (ArOH).

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Materials and Methods

Tea samples

A total of 17 commercial tea samples were collected from a local supermarket. They include 6 green teas, 5 oolong teas and 6 black teas. Their identities are listed in Table I.

Sample Preparation

In order to obtain comparable data, the following standard procedure were adopted. Dry tea leaves were ground into a fine powder. Approximately 107 g boiling distilled water was added to 1g sample of tea powder in a 300 mL beaker. The extraction was allowed to continue for 4 minutes, stirring continuously. After 4 min the tea powder was filtered through coffee filter paper into a tared beaker. The coffee filter paper containing the extracted tea powder was squeezed to express the remaining extract from the powder into the beaker. The beaker with the tea extract was placed on a balance and the net weight brought to 100 grams. The tea infusion was stored at -20 °C for further analysis.

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Table I. Tea Samples Tested

Category	*No.*	*Product identity*
Green Tea	1	Long-Jin
	2	Bi-Luo-Chun
	3	Mao-Jian
	4	Yunnan Green Tea
	5	Shanghai Pearl Tea
	6	High Mountain Japanese Green tea
Oolong Tea	7	Bai Hao Tea
	8	Oolong Tea
	9	Ti-Kuan-Yin
	10	Hou-Zi-Cai
	11	Pouchong
Black Tea	12	Qi-Men Black Tea
	13	Rose Black Tea
	14	Pu-Er Tea
	15	Black tea bag #1
	16	Black tea bag #2
	17	Black tea bag #3

Chemicals

Folin and Ciocalteu's phenol reagent, (+)-catechin, gallic acid, sodium carbonate, 4-aminoantipyrine, hydrogen peroxide, horseradish peroxidase (HRP) donor: hydrogen peroxide oxidoreductase, EC 1.11.1.7 type VI-A, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,4,6-tripyridyl-s-triazine (TPTZ), $FeCl_3 \cdot 6H_2O$ were all purchased from Sigma (St. Louis, MO).

Folin-Ciocalteu's Method

Total polyphenol content (TPC), determined by Folin-Ciocalteu reagent, was determined according to the procedure reported in the literature (www.teausa.com) with the following modifications. A saturated sodium carbonate solution was prepared by adding 50 g sodium carbonate to 200 mL distilled water in a 500 ml beaker. The solution was stirred and heated until the sodium carbonate was completely dissolved. The solution was stored in a refrigerator for about half an hour until crystals precipitated. Working standards (25 ppm, 50 ppm, 100 ppm, 150 ppm and 200 ppm gallic acid or catechin solution) were prepared freshly each time, at room temperature, before analysis. 0.5mL of 1:10 (v/v) diluted sample (water as blank, catechin or gallic acid as standard and tea infusion as sample), 4.5 mL distilled water, 0.2 mL Folin reagent and 0.5mL saturated sodium carbonate were added to each test tube and vortexed. Each of these additions had to be completed within 15 seconds and in the exact order described. Finally, 4.3 mL of distilled water were added to each and each tube was inverted to mix. The above procedure was repeated for all the samples. Samples were allowed to sit for at least 1 h, at room temperature to ensure completion of the reaction. A Milton Roy Spectronic 301 spectrophotometer was used to read the absorbance of each sample at 725 nm in a 1 cm cuvette. All measurements were carried out in triplicate. Final results were expressed as catechin equivalents (CE) or gallic acid equivalents (GE). Their values were determined by calibration curves using either catechin or gallic acid as the standard.

New Enzymatic Method

Tea infusion (100 μL) was added to a 0.1 M potassium phosphate buffer solution, pH 8.0, with 1 mL 9 mM 4-aminoantipyrine, 1 mL 31.3% 6 mM hydrogen peroxide and 0.9 mL 2.5 μM horseradish peroxidase and mixed in a 4 mL spectrophotometric cuvette. The absorbance of each tea sample was measured at 500 nm after 5 min of mixing. All measurements were performed in triplicate. Final results were expressed as either catechin or gallic acid equivalents, and their values were obtained utilizing catechin (50, 100, 150, 200

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μM) calibration curve and gallic acid (200, 400, 600, 800, 1000 μM) standard curve.

DPPH Assay

Ethanolic solution of DPPH (500 μM, 2 mL) was added to 2 mL of the test tea infusions. Each mixture was shaken vigorously by vortexing and kept in the dark for 30 min at room temperature using ethanol as a blank. Ethanolic solution of DPPH (500 μM, 2 mL) in 2 mL ethanol was used as the control. The decrease of the absorbance of DPPH radical was measured at 517 nm. All tests were performed in triplicate. The free radical scavenging activity of the tea samples was expressed as % decrease of absorbance of the DPPH radical.

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FRAP Assay

The total antioxidant activity of 17 tea samples was evaluated using a modified FRAP assay described by Benzie and Strain (1996). Briefly, FRAP reagent was prepared by mixing 25 mL of 300 mM, pH 3.6 acetate buffer, 2.5 mL of 10 mM TPTZ and 2.5 mL of 20 mM ferric chloride. These three solutions were added in the ratio of 25:2.5:2.5 (v/v/v). FRAP assay was performed by incubating 900 μL of freshly made FRAP reagent, 90 μL of distilled water and 30 μL of test sample or standard compound or water as blank, at room temperature for 4 min. Tea samples were allowed to melt and diluted 1:10 (v/v) prior to assay. Absorbance at 593 nm was measured relative to the water blank. The total antioxidant activity of samples was evaluated against a standard curve of gallic acid (50, 100, 200, 400, 600 μM). All final FRAP values are expressed as gallic acid equivalent (mg/L). Samples were analyzed in triplicate.

Results and Discussion

Folin-Ciocalteu's Method

Under the experimental conditions employed, two straight-line standard curves were obtained in the range of 25-200 μM catechin or gallic acid, characterized by a correlation coefficient of 0.9943 and 0.9953, respectively (Figure 4). Table II summarizes the total polyphenol content of 17 selected tea samples. The total polyphenolic compound content in the Folin-Ciocalteu's method ranged from 47 to 122 mg of gallic acid equivalent/g dry tea leaves or 68 to 170 mg of catechin equivalent / g dry tea leaves. Of the selected types of teas, Yunnan green tea showed the highest content of polyphenolic compounds

(112 mg of GE/g dry leaves or 170 mg of CE/g dry leaves) while Rose black tea had the lowest content of total polyphenols (47 mg of GE/g dry leaves or 68 mg of CE/g dry tea leaves). With the exception of Bi-luo-chun and Shanghai pearl tea, all tested green teas exhibited the highest concentration of polyphenols, followed by oolong teas and then black teas. Furthermore, no significant difference was observed in the CE value and GE value of all the teas. Gallic acid equivalent values were slightly lower than catechin equivalent values (Figure 5).

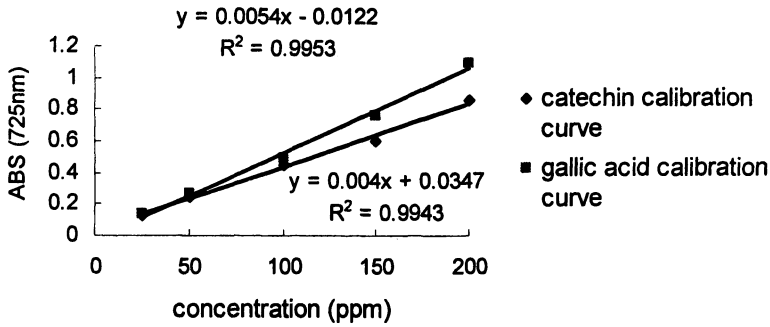

Figure 4. Calibration curves of gallic acid and catechin (Folin method).

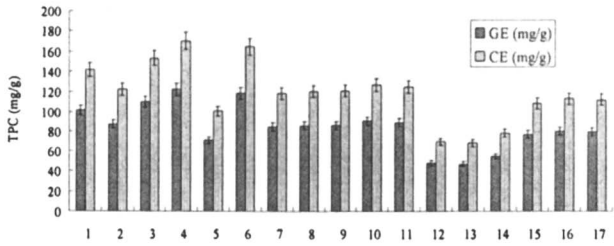

Figure 5. Comparison of two standards in Folin-Ciocalteu's method (1-6: green tea; 7-11: oolong tea; 12-17: black tea; n=3).

New Enzymatic Method

By using gallic acid and catechin as standards under the reported experimental conditions, two straight lines with correlation coefficient >0.994 were obtained (Figures 6 and 7). Catechin seems to be more sensitive in enzymatic method than gallic acid as shown by a higher concentration of gallic acid in order to reach the same range of absorbance as with catechin (Figures 6 and 7). Table III summarizes the total polyphenol content of 17 selected teas.

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The total polyphenolic compound content in the enzymatic method ranged from 153 to 394 mg of gallic acid equivalent/g dry tea leaves or 30 to 87 mg of catechin equivalent/g dry tea leaves. Of the selected types of teas, High Mountain Japanese green tea showed the highest content of polyphenolic compounds (391mg of GE/g dry leaves or 87 mg of CE/g dry leaves) while Pu-er tea had the lowest content of total polyphenols (153 mg of GE/g dry leaves or 30 mg of CE/g dry tea leaves). No clear trend of total polyphenol content was observed from the results (Table III and Figure 8). Moreover, a significant difference was found in the final results in terms of different standards. There was a big drop of the total polyphenol content expressed as CE as compared to GA (Figure 8).

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Table II. Total Polyphenol Content of 17 Teas by Folin-Ciocalteu's Method

Type of tea	*Total polyphenols[a] (mg of GE/g dry tea leaves)*	*Total polyphenols[a] (mg of CE/g dry tea leaves)*
Green tea		
Long-jin	101.43±1.19	141.65±1.61
Bi-luo-chun	87.26±1.75	122.53±2.37
Mao-jian	110.02±2.03	153.25±2.75
Yunnan green tea	122.47±1.88	170.07±2.53
Shanghai pearl tea	71.07±1.04	100.67±1.40
High mountain Japanese green tea	118.70±0.83	164.98±1.12
Oolong tea		
Bai Hao tea	84.56±1.57	118.87±2.12
Oolong tea	86.12±1.00	120.99±1.35
Ti-kuan-yin	86.34±0.60	121.28±0.81
Hou-zi-cai	90.79±0.41	127.29±1.55
Pouchong	89.34±1.38	125.33±1.87
Black tea		
Qimen black tea	48.21±0.36	69.81±0.49
Rose black tea	47.13±0.38	68.36±0.51
Pu-er tea	54.90±0.55	78.84±0.75
Black tea bag #1	77.04±0.16	108.74±0.21
Black tea bag #2	80.44±1.01	113.32±1.36
Back tea bag #3	79.66±0.39	112.27±0.53

[a] Results are expressed as mean±SD of three determinations.

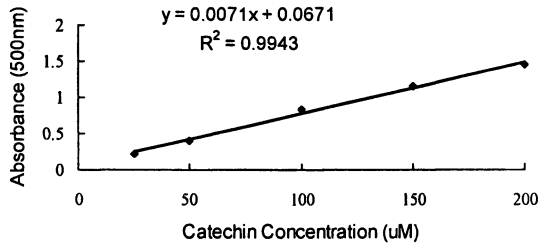

Figure 6. Standard curve of catechin (enzymatic method).

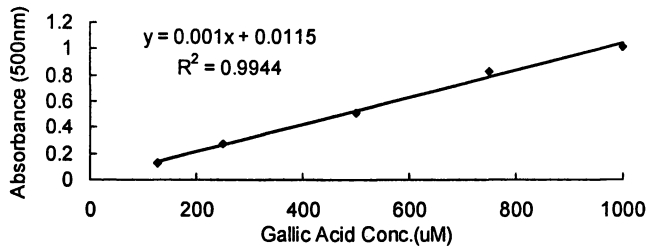

Figure 7. Standard curve of gallic acid (enzymatic method).

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Comparison between Folin and Enzymatic Method

Figure 9 shows the total polyphenol content for the tested teas in terms of GE as obtained from the Folin and enzymatic methods. The enzymatic method values were much higher than those of the Folin values. Using catechin as a standard, Figure 10 clearly show that the Folin values are much higher than the enzymatic values. This is consistent with those of Stevanato *et al.* (8). The different mechanisms behind these two methods may account for the discrepancy of the final total polyphenol content of the tea samples examined.

DPPH Assay

The inhibition percentage of 17 teas for DPPH radical ranged from 60 to 82% (Table IV). Of the selected types of teas, Yunnan green tea showed the highest inhibition (82%) with free radical induced oxidation while Pu-er tea possessed the lowest (60%). With the exception of Shanghai pearl tea, all tested green teas exhibited the highest inhibition, followed by oolong teas and then black teas (Table IV). No significant difference of the inhibition percentage was found among 17 teas (Figure 11).

Table III. Total Polyphenol Content of Selected Teas by Enzymatic Method

Type of tea	*Total polyphenols[a] (mg of GE/g dry tea leaves)*	*Total polyphenols[a] (mg of CE/ g dry tea leaves)*
Green tea		
Long-jin	291.16±5.33	63.16±1.28
Bi-luo-chun	250.84±5.68	53.47±1.37
Mao-jian	321.10±6.93	70.35±1.67
Yunnan green tea	391.02±13.31	87.16±3.20
Shanghai pearl tea	251.01±7.44	53.51±1.79
High mountain Japanese green tea	394.08±2.81	87.90±0.68
Oolong tea		
Bai Hao tea	278.23±6.80	60.05±1.63
Oolong tea	298.65±6.17	64.96±1.48
Ti-kuan-yin	312.43±3.90	68.27±0.94
Hou-zi-cai	312.77±4.13	68.35±0.99
Pouchong	302.73±8.45	65.94±2.03
Black tea		
Qimen black tea	157.45±3.57	31.02±0.86
Rose black tea	161.36±3.83	31.96±0.92
Pu-er tea	153.87±1.02	30.16±0.25
Black tea bag #1	300.86±5.38	65.49±1.29
Black tea bag #2	327.57±6.66	71.91±1.60
Black tea bag #3	309.19±0.78	67.49±0.19

[a] Results are expressed as mean ± SD of three determinations.

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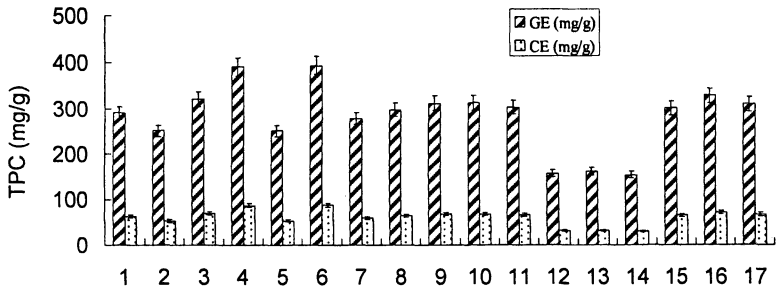

Figure 8. Comparison of two standards in enzymatic method (1-6: green tea; 7-11: oolong tea; 12-17: black tea; n=3).

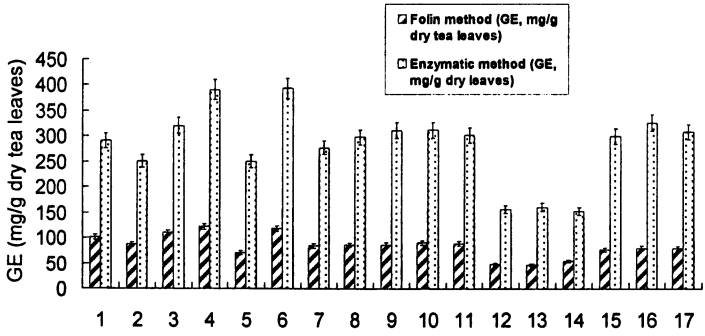

Figure 9. Comparison of Folin-Ciocalteu's and enzymatic method (1-6: green tea; 7-11: oolong tea; 12-17: black tea; n=3).

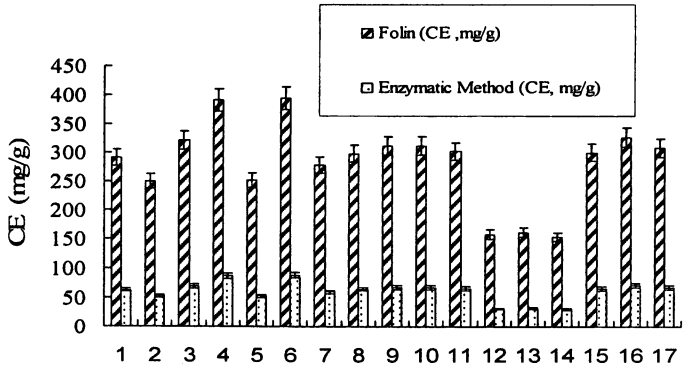

Figure 10. Comparison of Folin-Ciocalteu's and enzymatic method (1-6: green tea; 7-11: oolong tea; 12-17: black tea; n=3).

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Table IV. Inhibition Percentage of 17 Teas (DPPH Assay)

Type of tea	Inhibition %
Green tea	
Long-jin	79.33±0.27
Bi-luo-chun	75.39±0.41
Mao-jian	80.18±0.62
Yunnan green tea	82.06±0.69
Shanghai pearl tea	60.89±1.14
High mountain Japanese green tea	80.81±0.13
Oolong tea	
Bai Hao tea	69.40±0.84
Oolong tea	73.42±0.75
Ti-kuan-yin	75.70±0.27
Hou-zi-cai	76.15±0.61
Pouchong	75.30±0.40
Black tea	
Qimen black tea	71.15±0.51
Rose black tea	68.71±0.54
Pu-er tea	44.50±0.75
Black tea bag #1	66.47±0.46
Black tea bag #2	60.15±1.49
Black tea bag #3	63.41±0.51

Results are expressed as mean ± SD of three determinations.

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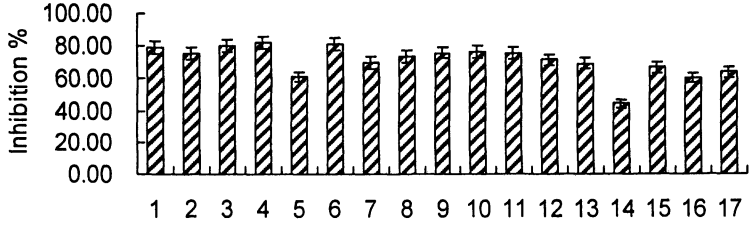

Figure 11. Inhibition percentage of 17 teas by DPPH assay (1-6: green tea; 7-11: oolong tea; 12-17: black tea; n=3).

FRAP (Ferric Reducing Antioxidant Power) Assay

Under the experimental conditions employed, a straight calibration curve was obtained in the range of 50-600 μM with gallic acid, characterized by a correlation coefficient of 0.9993 (Figure 12). Table V summarizes the ferric reducing antioxidant power of the selected tea samples. The ferric reducing antioxidant power value ranged from 118 to 482 ppm of gallic acid equivalent. Of the selected types of teas, Yunnan green tea showed the strongest antioxidant power (482 ppm of GA) while Rose black tea exhibited the weakest ferric reducing power (118 ppm of GA). With the exception of Bi-luo-chun and Shanghai pearl tea, all tested green teas exhibit the best antioxidant activities, followed by oolong teas and then black teas (Table V and Figure 13). Furthermore, a significant difference was found between the antioxidant power of green teas and black teas (Figure 13).

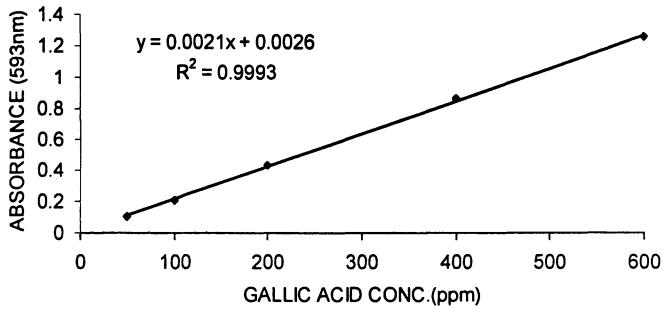

Figure 12. FRAP standard curve.

Comparative Study Among Methods

Regression analysis was used to correlate the results of the three methods. Figure 14 summarizes the results of total polyphenol content and total antioxidant activity measured by Folin, DPPH and FRAP assays. The highest correlation coefficient was exhibited between Folin-Ciocalteu's method and FRAP assay (R^2 = 0.8663), followed by that between DPPH free radical scavenging assay and FRAP assay (R^2 = 0.545); the correlation coefficient between Folin-Ciocalteu's method and DPPH assay was the lowest (R^2 = 0.4896). In addition, the correlations among the three methods were all positive which indicates that the higher the total polyphenol content of the sample, the better the antioxidant activity it possesses. Moreover, the comparison between

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Table V. FRAP Value of 17 Teas

Type of tea	GE (mg/L)
Green tea	
Long-jin	362.73±7.38
Bi-luo-chun	304.47±11.59
Mao-jian	414.79±5.31
Yunnan green tea	482.25±14.60
Shanghai pearl tea	240.35±6.76
High mountain Japanese green tea	470.98±21.74
Oolong tea	
Bai Hao tea	271.78±7.74
Oolong tea	332.25±9.29
Ti-kuan-yin	313.21±3.17
Hou-zi-cai	326.06±2.15
Pouchong	324.95±10.76
Black tea	
Qimen black tea	129.08±1.92
Rose black tea	118.13±2.40
Pu-er tea	176.22±3.51
Black tea bag #1	177.33±1.26
Black tea bag #2	163.52±4.69
Black tea bag #3	177.97±0.73

Results are expressed as mean ± SD of three determinations.

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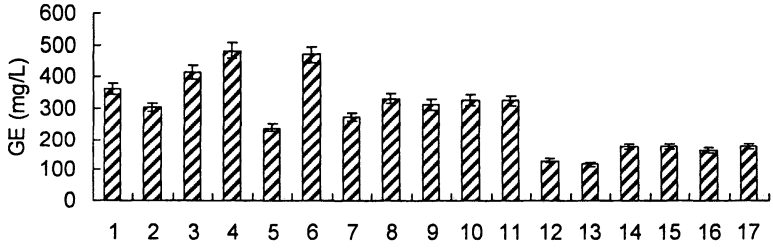

Figure 13. FRAP value expressed as gallic acid equivalent of 17 teas (1-6: green tea; 7-11: oolong tea; 12-17: black tea; n=3).

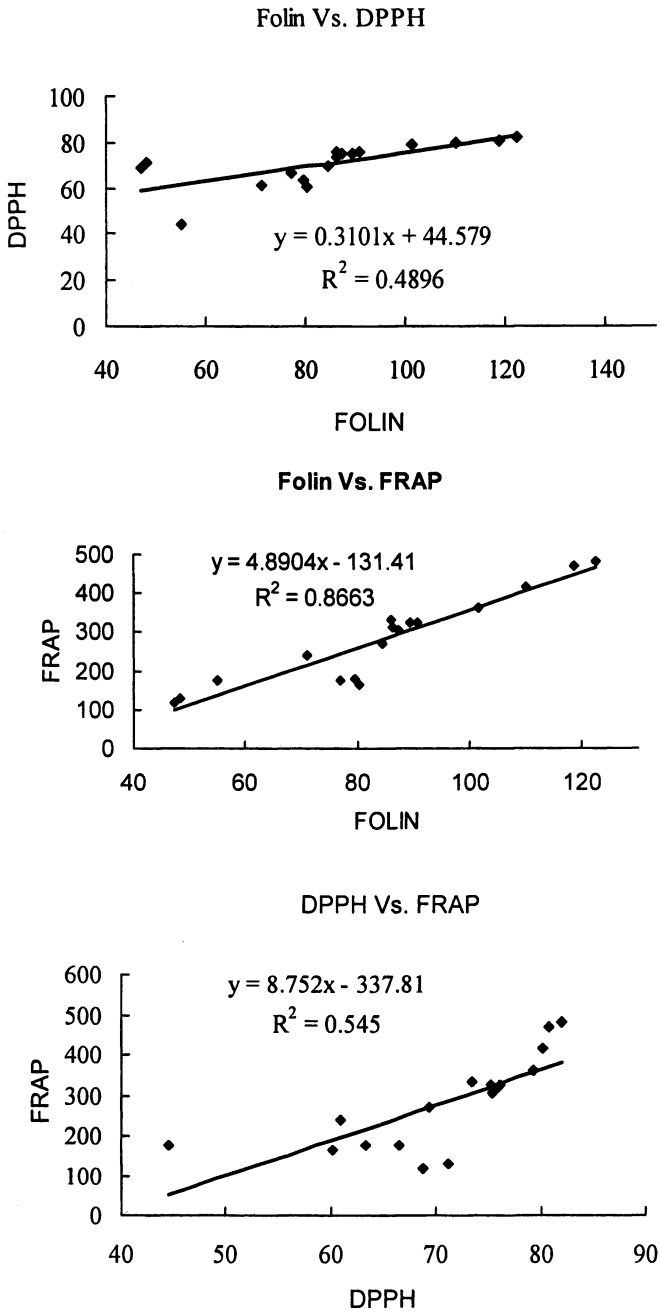

Figure 14. Correlation between three assays: Folin-Ciocalteu's assay; DPPH assay; FRAP assay.

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DPPH and FRAP tells us that the better the radical scavenging activity of the tea, the stronger the ferric reducing antioxidant power it has.

Conclusions

The highest content of polyphenol was detected in green tea, followed by oolong tea and then black tea. The degree of fermentation of tea leaves may account for the significant difference between green tea and black tea. The further the fermentation, the lower the total polyphenol content and total antioxidant activity of tea. The comparison of the total polyphenol content between Folin-Ciocalteu's method and the new enzymatic method indicates that the selection of the standard compound plays a very important role in the final results. Moreover, this study suggests that the new enzymatic method is a more specific and convenient assay than the Folin method to determine the total polyphenol content of tea.

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Chapter 15

Antioxidant and Anti-Cancer Activities of Green and Black Tea Polyphenols

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Tea (*Camellia sinensis*) has attracted public attention because of accumulating scientific evidence linking its consumption to health benefits. As a popular beverage, tea consumption may provide benefical biological activities, such as reducing the risk of mortality from cardiovascular disease (CVD) and delaying the onset of cancer. These biological activities are believed to arise from ability of tea polyphenols to effectively scavenge reactive oxygen species (ROS), as well as cancer chemopreventive effects associated with tea polyphenols and their molecular mechanisms. The biological activities of green and black tea polyphenols are summarized in this chapter.

Introduction

Consumer interest in health promoting foods has increased, and people believe that functional foods can help the prevention of certain diseases. Much scientific evidence demonstrating a positive correlation between health benefits and the consumption of certain foods has been accumulated, and scientists are performing systematic research to understand the chemistry and physiological effects of these foods. For example, cruciferous vegetables are rich in sulfur-containing phytochemicals known as glucosinolates which have been reported to

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exhibit anticancer activity (*1*). *Allium* vegetables are rich in organosulfur compounds and epidemiological evidence indicates that the consumption of these vegetables may decrease the risk of certain cancers (*2*). In addition, flavonoids are widely regarded as bioactive components in foods with health benefits. Fruits, vegetables and many food products derived from them are good dietary sources of flavonoids. For example, the flavonol quercetin can be found in onion, apple and cranberry, as well as many other vegetables and fruits. Anthocyanidins and their glycoside are pigments in red grapes and berries. Tea is rich in flavan-3-ols such as catechins.

Epidemiological studies provide strong evidence for the protective effects of dietary flavonoids against coronary heart disease and certain cancers (*3-5*). The principal hypothesis related to health benefits of flavonoids concerns the antioxidant properties of these compounds, specifically, their ability to scavenge reactive oxygen species (ROS). Flavonoids are polyphenolic compounds ubiquitous in plants. These compounds have a common three ring chemical structure: C_6-C_3-C_6. Various types of flavonoids exhibit different extents of oxidation in B and C ring, and these oxidation states have been used for their classification (*6*). The redox chemistry of flavonoids can provide useful information to understand the antioxidative mechanism of flavonoids (*7*). Due to their low reduction potential, flavonoids do not require much energy to donate an electron. Several elements of the chemical structure of these molecules have proven effective for radical scavenging activity (*7,8*). In addition, flavonoids prevent oxidative reactions by chelating free copper and iron through bidentate ligands. For example, quercetin has three potential metal chelating sites on the A, B and C-rings, of which the A-ring site has the highest affinity.

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Constituents of Green and Black Teas

Several types of tea products are commercially available, including green tea, oolong tea and black tea. Green tea is mostly consumed in China, Japan and the Middle East. Oolong tea, known as partially-fermented tea, is generally consumed in China and Taiwan. Black tea is manufactured through fermentation of the tea leaves. Fresh tea leaves contain four major tea catechins having unique biological activities. These compounds are (-)-epicatechin (EC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC) and (-)-epigallocatechin gallate (EGCG) (Figure 1). They account for about 30% of the dry weight of tea leaves (*9*). The other components in tea, such as quercetin, kaempferol and myricitin and their glycosides, account for about 3% of the dry weight of tea leaves (*7*).

To make green tea, tea leaves are steamed before it is dried to inactivate enzymes in the leaves. However, black tea manufacturing process requires additional fermentation process. The leaves are first subjected to a withering

process to reduce their moisture content and are then ruptured through a rolling process. This process initiates the enzymatic oxidation of polyphenols in the tea leaves by allowing polyphenol oxidase to diffuse into the cytoplasm. The rolled leaves are then allowed to ferment for between 40 minutes and 3 hours (*9*). During the fermentation process, important chemical changes occur due to the action of polyphenol oxidase (PPO). PPO is responsible for oxidizing the di-hydroxylated B-ring (catechol) and tri-hydroxylated B-ring (pyrogallol) of tea catechins to their *o*-quinones. Subsequent chemical reactions generate the various characteristic black tea pigments.

Roberts (*10*) reported that black tea contains polyphenolic pigments that are not found in unprocessed tea leaves. These pigments are designated as brown acidic pigments and yellow neutral pigments or thearubigins and theaflavins, respectively. Four major theaflavins have been identified from black tea, namely, theaflavin, theaflavin-3-gallate, theaflavin-3′-gallate, and theaflavin-3,3′-digallate (Figure 2), whereas thearubigins are a heterogeneous mixture of pigments. Theaflavins having orange or orange-red color and are formed from co-oxidation of selected pairs of tea catechins (Table 1).

Theaflavins account for 2-6% of the dry, solid weight of brewed black tea. The relative proportions of the theaflavins were found to be as follows: theaflavin, 18%; theaflavin-3-gallate, 18%; theaflavin-3′-gallate, 20%; theaflavin-3,3′-digallate, 40%; and theaflavic acids plus isotheaflavin, 4% (*9*). Several minor pigments, namely, theaflavate A, theaflavate B, isotheaflavin-3′-*O*-gallate, and neotheaflavin-3-*O*-gallate, have also been reported to be present in black tea (*11*, *12*). Compared to the amounts of theaflavins in black tea, epitheaflavic acid, theaflavic acid, and epitheaflavic acid gallate contents are quite low. Nonaka *et al.* (*13*) reported a new type of pigment named theaflagallin from chemical oxidation of tea catechins and gallic acid.

Thearubigins account for 10 to 20% of the dry weight of black tea. However, because of their solubility in hot water, they account for between 30 and 60% of the solids in black tea infusions (*9*). Thearubigins have a wide range of molecular weights, from 700 to 40000 Da, and they are regarded as polymeric compounds. Although Roberts (*14*) suggested thearubigins are further oxidation products of theaflavins and catechins, their mechanism of formation and chemical structure are still unclear.

Antioxidant Activiy of Tea Polyphenols

Tea consumption may provide health benefits, such as reducing the risk of mortality from cardiovascular disease (CVD) and delaying the onset of cancer (*15-18*). These biological activities are believed to arise from the antioxidant activity of these compounds, specifically their ability to effectively scavenge

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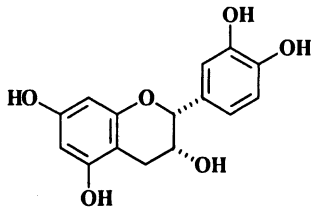
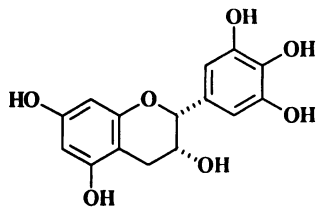
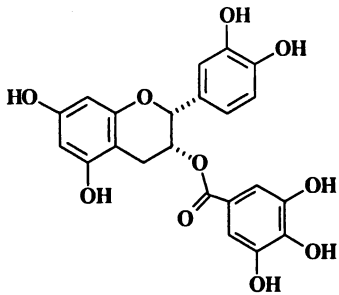


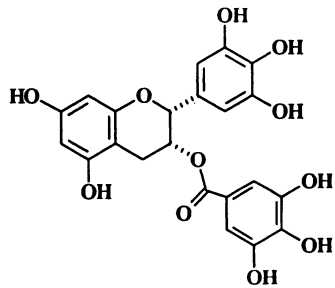
(-)-epicatechin (EC)



(-)-epigallocatechin (EGC)



(-)-epicatechin gallate (ECG)



(-)-epigallocatechin gallate (EGCG)

Figure 1. Structures of green tea polyphenols.

Table I. Parent Flavonols of Theaflavins

Theaflavins	*Parent flavonols*
Theaflavin	(-)-epicatechin and (-)-epigallocatechin
Theaflavin-3-gallate	(-)-epicatechin and (-)-epigallocatechin gallate
Theaflavin-3′-gallate	(-)-epicatechin gallate and (-)-epigallocatechin
Theaflavin-3,3′-digallate	(-)-epicatechin gallate and (-)-epigallocatechin gallate

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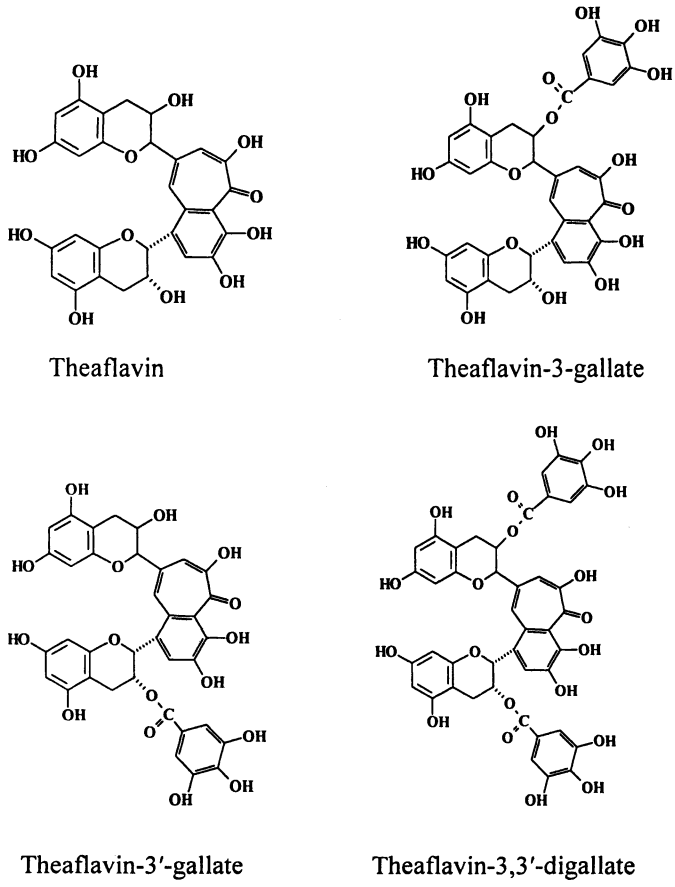

Figure 2. Structure of theaflavins in black tea.

reactive oxygen species (ROS). Although O_2 is essential for aerobic organisms to generate energy, it is also responsible for generating harmful components of ROS through metabolic pathways. Increased concentrations of ROS are associated with certain diseases, such as cardiovascular and cancer. Oxidative stress can also cause damage to important biomolecules, such as lipids, DNA, and proteins. ROS may play an important role in the development of atherosclerosis, which is characterized by a local thickening of a blood vessel through deposit of plaque on its inner walls. These deposits narrow the circulatory channels and partially block normal blood flow. Furthermore, fatty acids in low density lipoproteins (LDLs) are susceptible to oxidation in the presence of ROS (*19*). There is increasing evidence that ROS is involved in DNA damage. The hydroxyl radical generated from hydrogen peroxide increases the incidence of DNA strand breakage. The presence of 8-hydroxydeoxyguanosine (8-OHdG) in the DNA strand is regarded as a marker for oxidative stress (*20*). ROS can also directly damage proteins. For example, -SH groups in protein can be easily oxidized by ROS. Consequently important enzymatic functions can be compromised (*21*).

Antioxidants from diet, such as ascorbate and α-tocopherol, play an important role in scavenging ROS. In particular, α-tocopherol is the most important free radical scavenger for preventing lipid peroxidation. It is well known that synergy between α-tocopherol and ascorbate through redox cycles facilitates ROS scavenges. Moreover, we also have defensive enzyme systems in the body to remove harmful ROS. Superoxide dismutase (SOD) converts $O_2^{-\cdot}$ to H_2O_2. Hydrogen peroxide from dismuatation and metabolic pathways can be removed by catalase and peroxidase. Glutathione peroxidase also removes hydrogen peroxide by oxidation of glutathione (GSH).

A number of studies demonstrated the antioxidant capacity of green and black tea polyphenols using *in vitro* and *in vivo* methods. Guo *et al.* (*22*) investigated scavenging effects of tea catechins on superoxide anions ($O_2^{-\cdot}$), singlet oxygen ($^1O_2$), peroxyl radicals, and the DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals by ESR. The results indicated that galloylated catechins exhibited higher antioxidant activity than non-galloylated catechins, and the scavenging effects of catechins having pyrogallol in the B-ring were higher than those with catechol in the B-ring. Benzie and Szeto (*23*) reported that green tea has higher antioxidant capacity than fermented teas, such as oolong tea and black tea. They correlated antioxidant capacity, measured using the Ferric reducing antioxidant power (FRAP) assay, with total phenolic content in tea, and proposed that fermented tea may have lower antioxidant capacity because of the decreased content of tea catechins resulting from the fermentation process. However, many *in vitro* studies show that black tea has comparable antioxidant capacity to green tea. Researchers using the oxygen-radical absorbance capacity (ORAC) assay have shown that black tea has similar peroxyl radical scavenging capacity to green tea (*24*). Trolox equivalent antioxidant capacity (TEAC) values of green tea and black tea are comparable (*8*). It has also been demonstrated that

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black tea polyphenols have equivalent biological activities to green tea polyphenols. Black tea extracts have been shown to exhibit protection of human RBC against oxidative damage from lipid peroxidation (*25*). Sarkar and Bhaduri (*26*) reported that black tea extracts are more effective than green tea catechins in scavenging superoxide anion. Theaflavins have been regarded as one of the important biologically active components in black tea. Many studies show that theaflavins having more galloyl moieties have higher antioxidant activity (*27*). Theaflavins have been found effective in preventing Cu^{2+}-mediated LDL oxidation (*27*). O'Coinceanainn *et al.* (*28*) proposed theaflavin-3,3′-digallate has three potential chelating positions: two in galloyl moiety, and one in the benzotropolone moiety. Yoshida *et al.* (*29*) reported pretreatment with theaflavin-3,3′-digallate reduced cell-mediated LDL oxidation and inhibited superoxide anion production from macrophage, and that such inhibitory activity was higher for this compound than for the tea catechins EGC and EGCG. It is believed that the galloyl moiety in theaflavins may play an important role in such inhibitory activity.

Isolation and identification of the oxidation products of tea polyphenols can provide useful information to understand the mechanism giving rise to the antioxidant properties of these molecules. Several studies report a proposed mechanism for the reaction of tea catechins with radicals, and have proposed mechanisms for the antioxidant properties of these compounds (*30,31*). When tea catechins were reacted with peroxyl radical generated by thermolysis of the radical initiator 2,2′-azobis(2,4-dimethylvaleronitrile), the resulting seven-membered B-ring anhydride and symmetrical dimer were identified as the oxidation products of EGCG and EGC (*31*). These results indicate that the principal site for reaction is on the B-ring rather than the galloyl moiety.

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Anti-Cancer Activity of Tea Polyphenols

It has been reported that flavonoids affect phase I metabolism enzymes, such as the P-450s. For example, quercetin and apigenin have been shown to inhibit enzymes of the CYP1A family (*32*). CYP1A2 is regarded as a major enzyme in the bioactivation of chemical carcinogens. Flavonoids may induce enzymes of phase II metabolism, such as glutathione-*S*-transferase, quinone reductase, and UDP-glucuronyl transferase (*33*). Carcinogens can be detoxified through reaction with such phase II enzymes. Specifically, these enzyme systems convert the carcinogens to more polar compounds that are less toxic and can be easily removed from the body. Flavonoids can also prevent tumor cell proliferation by inactivation or down regulation of pro-oxidant enzymes and signal transduction enzymes. Pro-oxidant enzymes are induced or activated by various tumor promoters (*33*). It is believed that ROS act as a major catalyst of tumor promotion and progression.

A number of studies demonstrate that tea polyphenols are effective for prevention of cancer. Tea polyphenols have also been found to inhibit enzyme-mediated binding of carcinogens to DNA by inhibiting P-450 dependent enzyme systems in rat liver microsome (*18*). An *in vitro* cell culture experiment demonstrated polyphenols from green and black teas inhibited benz[α]pyrene adduct formation with DNA and induced phase II enzymes glutathione-*S*-transferase and quinone reductase (*34*). An *in vitro* study revealed that black tea as an aqueous extract effectively inhibited UV light-induced 8-OHdG formation (*35*). Feeding mice of decaffeinated black tea has been reported to inhibit the growth of papilloma induced by DMBA and promoted by TPA (*36*). Theaflavin from black tea polyphenols effectively inhibited cell proliferation of human A431 epidermoid carcinoma cells and mouse NIH3T3 fibroblast cells. Among the black tea polyphenols, theaflavin-3,3′-digallate showed higher inhibitory activity than EGCG (*37*). The antiproliferative activity of theaflavin-3,3′-digallate is proposed to arise from its ability to block the binding of growth factor to its receptor. Chen *et al.* (*38*) reported that black tea polyphenols effectively inhibited TPA tumor promotion in NIH3T3 cells. In particular, theaflavin-3,3′-digallate inhibited TPA-induced protein kinase C (PKC) and AP-1 binding activities. The black tea polyphenols theaflavin and theaflavin-monogallate and the oolong tea polyphenol theasinensin A have all exhibited strong inhibition of the growth of cancer cell line (*39*).

Chronic inflammation, an early event in carcinogenesis, is identified as a symptom of the onset of cancer. A large body of evidence has accumulated regarding the ability of tea polyphenols to prevent inflammation. Tea polyphenols may affect ornithine decarboxylase (ODC), cyclooxygenase (COX) and lipoxygenase (LOX) activity. Flavonoids, such as tea catechins, have anti-inflammatory activity via the inhibition of cyclooxygenase (COX)-2, and inducible nitric oxide synthase (iNOS). The COX enzymes convert arachidonic acid to prostanoids, which can cause various adverse physiological effects. COX-2 has been identified as an inducible isoform of cyclooxygenase which can produce large quantities of prostanoids involved in various inflammation-related diseases. Nitric oxide (NO) is important to the physiology of signaling functions, however, the increased generation of NO is implicated in certain joint disease states (*40*). Tea catechins inhibit the production of inflammatory mediators by down-regulating COX-2, and reduce NO by inhibiting the expression of iNOS (*41*). Black tea polyphenols have been found to effectively inhibit TPA induced epidermal edema and also to significantly inhibit epidermal ODC and COX activity (*42*). The effects of green tea and black tea polyphenols on COX and LOX-dependent arachidonic acid metabolism was demonstrated by Hong *et al.* (*43*). Lin *et al.* (*44*) demonstrated that theaflavin-3,3′-digallate and EGCG strongly inhibited inducible NO synthase in a murine macrophage cell line. The inhibition strength of the level of iNOS by tea polyphenols is in the

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following order: theaflavin-3,3′-digallate > EGCG > theaflavin-3-gallate and theaflavin-3′-gallate > theaflavin. Theaflavin-3,3′-digallate and EGCG inhibit the activation of transcription factor NF-*k*B and the transcription of iNOS in murine macrophages (*44*). Although chemical information about the chemistry of thearubigins as a major component of black teais limited, their biological activity is quite interesting. Maity *et al.* (*45*) reported that thearubigins significantly decreased degree of 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis, a model of inflammatory bowel disease in mice. Thearubigins have also been found to exhibit inhibitory activity against cell proliferation, TPA-induced PKC, and AP-1 binding activity (*37,38*), although such activities were lower than those of theaflavins.

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Conclusions

Many research groups have attempted to answer the following questions: What are the health-promoting bioactive components in foods? How do these compounds promote health in the body? As a popular beverage, tea (*Camellia sinensis*) has attracted public attention because of accumulating scientific evidence linking tea consumption with the health benefits discussed in this paper. Systematc rom systematic research to understand the chemistry and physiological effects of tea consumption reveals that, in particular, tea catechins and theaflavins are two of the most promising components responsible for significant biological activities, including antioxidant, anti-inflammatory and anti-cancer activities. In order to better understand these chemical and physiological mechanisms, we need further scientific evidence to support these perceived health benefits.

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Chapter 16

Peanut Skin Phenolics: Extraction, Identification, Antioxidant Activity, and Potential Applications

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Extraction and determination of total phenolics (TP) in peanut skins obtained from various processing methods was conducted. Total antioxidant activity (TAA) of peanut skin extracts was determined in order to identify and quantify different classes of phenolics in peanut skin. Peanut skins were removed by direct peeling, blanching, and roasting. TP and TAA of peanut skin extracts were determined using Folin-Ciocalteu and free radical scavenging methods, respectively. The composition of peanut skin extracts was determined by LC-MS. Peanut skin processing significantly affected total extractable phenolics and their composition. Roasting had limited effects on TP while blanching caused 89% loss of TP. TP in directly peeled, roasted, and blanched peanut skins were 130, 124, and 14.4 mg/g, respectively. TAA of all purified peanut skin extracts were higher than those of Trolox and Vitamin C at equivalent concentrations. LC-MS analysis of purified peanut skin extracts revealed the presence of catechins, A-type and B-type procyanidin dimers, trimers and tetramers. Total catechins, procyanidin dimers, trimers and tetramers in directly peeled peanut skin were 16.1, 111.3, 221.3 and 316.1 mg/100g, respectively, versus 8.8, 143.5, 157.5 and 203.9 mg/100 g, respectively, in roasted skin.

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Peanut is an important crop grown in the U.S. and worldwide. In 2005, peanut production in the United States was about 4.49 billion pounds (*1*). Georgia, Texas, Alabama, and North Carolina are the largest peanut producers in the United States. The edible part of peanuts consists of the kernel and protective skin. The peanut kernels are used to make peanut butter, roasted snack peanuts, peanut confections and peanut oil. The skin has a pink-red color with astringent taste, and is typically removed before peanut consumption. Peanut skin represents 3-7% of peanut kernel weight depending on the variety and size of the peanuts. Peanut skin, as a low cost peanut processing industry, contains 12% protein, 16% fat, 72% of carbohydrate, rich in polyphenols (*2*), and is mainly used as animal feed (*3,4*). The high polyphenol content of peanut skin may provide a potent source of antioxidants for use in foods and dietary supplements.

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Despite it high polyphenol content, limited studies have been carried out to evaluate the content and activity of peanut skin phenolics. For instance, six A-type procyanidin isomers were identified in peanut skin by Lou et al. (*5*) using ^{13}C NMR. These six compounds were found to inhibit the activity of hyaluronidase, an enzyme that responsible for the release of histamine which causes inflammation. In addition, resveratrol, a phytochemical found in grape seed and wine, was also found in peanut skin in much higher concentration than in peanut kernels (*6*). Previously published work indicated that the total phenolics of non-defatted peanut skin was about 90-125 mg/g dry skin, including phenolics acids, flavonoids and resveratrol (*7*). Catechins, B-type procyanidin dimmers, procyanidins trimers, tetramers and oligomers with higher degree of polymerization were also reported in peanut skin (*8*). Procyanidins are polyphenolic compounds generally found in pine bark (*9*), grape seeds and skin (*10-11*), as well as cocoa, cranberries and apples (*12*). Monomeric procyanidins are polyhydroxyflan-3-ol monomers, and are generally known as catechins. A-type procyanidins are formed through a 4→8 C-C bond and an interflavonoid C-O bond (*5, 13*). Procyanidins formed through 4→8 or 4→6 C-C bonds of flan-3-ol monomers are B-type procyanidins. Oligmeric procyanidins (OPCs) including dimmers, trimers and tetramers are also known as proanthocyanidins. These studies indicate that peanut skin could represent an inexpensive source of health promoting phenolics since peanut skin is a low cost by-product of the peanut industry.

Quantitative analysis of catechins and procyanidins in peanut skin has not been reported in the literature. Both identification and quantification of catechins and procyanidins in peanut skin are needed to determine their potential as functional ingredients in food, dietary supplement and non-food applications. In addition, the effects of processing methods commonly used to remove skin from peanut kernel on polyphenols retention and their antioxidant activity have not been reported in the literature. This study was conducted to determine the effects of three peanut processing methods on the composition of peanut; to identify/quantify peanut skin procyanidins compounds under each processing

and concentration process, and to evaluate the total antioxidant activity and free radical scavenging capacity of all peanut skin extracts.

Materials and Methods

Materials

Un-peeled raw peanuts were purchased from a local grocery store (Greensboro, NC). Standards including gallic acid (GA), catechin (C), epicatechin (EC), resveratrol, caffeic acid, *p*-coumaric acid, ferulic acid, ellagic acid and chlorogenic acid, and reagents 2,2'-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 2,2-Diphenyl-2-picrylhydrazyl (DPPH) free radical and peroxidase (EC 1.11.1.7) from horseradish were purchased from Sigma Chemical Co. (St. Louis, MO). Procyanidin B2 was purchased from INDOFINE Chemical Company (Hillsborough, NJ). Trolox, hydrogen peroxide (30%), methanol, ethanol, acetonitrile, trifluoroacetic acid and acetic acid were purchased from Fisher Scientific (Atlanta, GA).

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Removal of Peanut Skins

Peanuts skins were removed from kernels by three methods: direct peeling (DP), blanching (B) and roasting (R). In the direct peeling method, the skin was directly peeled by hands from raw peanut kernels, then freeze dried. For the blanching method, raw peanuts were boiled in water for 2 minutes, drained and their skin was hand-peeled then freeze dried. The roasting method consisted of heating peanuts at 175°C for 5 min, cooled to room temperature, then rubbing dry skin off. Further drying was not needed for the skin obtained by this method. Dry skins obtained from each method were separately blended into powder and stored at -20 °C in plastic bottles until used.

Extraction and Purification of Phenolics

Phenolics in peanut skin were extracted using 80% ethanol. Thirty milliliters of solvent were added in a centrifuge tube (50 ml capacity) containing 1.00 g of skin powder. The mixture was homogenized using a Polytron Homogenizer (Polytron PT-MR2100, Kinematica AG, Switzerland) for 2 min then centrifuged at 3000 g for 15 min at room temperature. The supernatant was collected in an amber bottle. The precipitate was subsequently extracted twice by the same procedure described above. The combined supernatant was filtered through a Whatman Anotop syringe filter (0.2 μm) and stored in a freezer until use. The extraction of each peanut skin was performed in triplicate.

The crude alcoholic extracts were evaporated under reduced pressure, and then purified by the method of Hara (*14*) to remove sugars, proteins and lipids. Phenolic acids and water soluble anthocyanins may have also been removed. The purified extracts were quantitatively re-dissolved in methanol and stored in amber glass vials in a freezer for subsequent analysis. The above extraction and purification process was completed under dim light to minimize light induced degradation/oxidation of phenolics, which are generally light sensitive.

Total Phenolics of Crude and Purified Peanut Skin Extracts

Concentrations of total phenolic in crude and purified extracts were determined by Folin-Ciocalteu method (*14*) using gallic acid as the standard. Results were expressed as mg/mL of gallic acid equivalent (GAE).

Free radical scavenging capacity and total antioxidant activity (TAA) of peanut skin extracts, total antioxidant activity (TAA) of crude and purified peanut skin extracts were determined by two free radical scavenging methods: $ABTS^{\bullet+}$ method (*16*) and $DPPH^{\bullet}$ method (*17*). Free radical scavenging capacity (FRSC) was calculated as the percentage of color reduction relative to control $FRSC\% = [A_{extract}/A_{control}] \times 100$ in comparison with that of Trolox and ascorbic acid used at the same concentration. TAA was calculated as Trolox equivalent antioxidant activity (TEAC), mM Trolox per mM of total phenolics.

LC-MS Identification and Quantification

The identification of the active compounds in peanut skin extracts was conducted using an Agilent 1100 LC/MS system (Agilent Technologies, Palo Alto, CA) equipped with HP ChemStation for data collection and analysis. The column used was a Nucleosil RP C_{18} (250 mm x 4.6 mm, and particle size of 5 μm, Alltec, Deerfield, IL). A binary mobile phase was used to elute injected samples. Mobile phases A and B were 0.1% formic acid in distilled water and 0.1% formic acid in 100% acetonitrile, respectively. The flow rate of mobile phase was 0.8 mL/min. The elution gradient was started with 88% of A and 12% of B. Mobile phase B increased to 15% in 15 minutes, then to 20% in 10 minutes at which it was maintained for 5 min. Afterward, mobile phase B was increased to 35% in 10 min, maintained at 35% for 5 min, and returned back to 12% in 5 min. The total run time was 50 min. Molecular weights of peaks resolved by LC were determined using Electron Spray Ionization (ESI) Mass Detector working at a negative mode (deprotonation) using both scan mode and selected ion monitoring. The mass range of m/z 5-2000 dalton was scanned at 1 dalton per second. Resolved peaks were detected by Waters 2487 Dual wavelength detector at wavelength 280 nm. The concentrations of catechin and epicatechin were expressed as (+)-catechin equivalents while procyanidin dimmers, trimers and tetramers were expressed as procyanidin B2 equivalent.

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Results and Discussions

Effect of processing on total phenolic content in crude peanut extracts. Direct peeling, blanching and roasting represent three most commonly used methods for peanut skin removal. Table I shows that total phenolics (TPs) were 131, 15 and 124 mg GAE /g dry skin for directly peeled, blanched, and roasted peanut skins, respectively. Total phenolics recovery obtained by the procedure used in this study was higher than that obtained by the procedure of Nepote *et al.* (118 mg/g dry skin) (*18*). With the exceptions of blanched peanut skin, the TPs of peanut skin tested in this study compared to that reported by Nepote *et al.* (150 mg per g defatted dry skin) (*2*) and significantly higher than the TPs of grape seeds. The latter was reported to be in the range of 5 to 8% of the dry weight of the seeds (*19*).

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Table I. Total phenolics in peanut skins as affected by skin removal methods and sequential extraction

	Total phenolics (mg GAE /g dry skin)*			
Skin removing method	1st extraction	2nd extraction	3rd extraction	Total**
Direct peeling	103.80 ± 1.80 (79.34%)	21.37 ± 2.45 (16.33%)	5.65 ± 0.11 (4.32%)	130.82 ± 4.16[a] (100%)
Peeling after blanching	8.77 ± 0.52 (58.22%)	4.62 ± 0.36 (30.66%)	1.68 ± 0.16 (11.15%)	15.07 ± 1.03[b] (100%)
Peeling after roasting	101.47 ± 0.87 (81.65%)	17.72 ± 0.70 (14.26%)	5.08 ± 0.53 (4.09%)	124.27 ± 2.10[a] (100%)

* Total phenolics expressed as mg gallic acid equivalent per g dry skin (GAE/g); value in parentheses represent % TP with respect to cumulative TP in 3 extractions.

** means with same letter superscripts are not significantly different at $p<0.05$.

Table I summarizes the total phenolics extracted from peanut skins following first, second and third extractions. Regardless of the peeling process employed, the amount of recovered phenolics was highest in the first extraction, followed by second and then third extraction. The data in Table I also show that the loss of TPs caused by roasting was very small (about 4.6% of TPs) compared to direct peeling but the loss caused by blanching was as high as 88.9%. Results of statistical analysis also indicate that blanching caused significant TP loss but roasting did not. Therefore, roasting, a common practice to remove skin in peanut industry would yield peanut skin with almost the same TP concentration and potency as the skin obtained by direct peeling. The stability of peanut skin phenolics under typical peanut processing conditions (e.g. roasting) provides a good indication of their potential as stable and low cost functional ingredients for the food and dietary supplement industries.

LC-MS identification and quantification of peanut skin polyphenols

Individual phenolic compounds in purified peanut skin extracts were identified and quantified by LC-MS. Directly peeled peanut skin extract was used as a control to evaluate the effects of blanching and short time roasting on the composition of the extract. Peaks identified were grouped according to the masses (m/z) of corresponding molecules (Figures 1 and 2). As shown in Figure1, directly peeled peanut skin extract contained two peaks with m/z = 289, six peaks with m/z = 575, eight peaks with m/z = 577, six peaks with m/z = 863, ten peaks with m/z = 865, two peaks with m/z=1149, and 7 peaks with m/z=1151. Based on the molecular structure and mass, these peaks were identified to be catechins (MW=289), A-type procyanidin dimers (MW=576), B-type procyanidin dimers (MW=578), A-type procyanidin trimers (MW=864), B-type procyanidin trimers (MW=866), A-type procyanidin tetramers (MW=1150) and B-type procyanidin tetramers (MW=1152), respectively. In addition to these major compounds, peaks with m/z=179, 353, 301, 227, and 389 were observed in the directly peeled peanut skin extract (Figure 2). These peaks represent caffeic acid (MW=180), chlorogenic acid (MW=354), ellagic acid (MW=302), resveratrol (MW=228), and resveratrol glycoside (MW=390). Other phenolic acids including ferulic acid and *p*-coumaric acid have been identified by HPLC (*7*). However, phenolic acids were present in relatively lower concentrations in the purified extract compared to procyanidins. They were possibly removed during the purification step.

Most peaks identified appeared at specific retention times, but some of the A-type and B-type procyanidin trimers had closer retention times causing peak overlap in total ion chromatograph (TIC). However, these peaks were resolved by MS and extracted by ChemStation based on the mass m/z. Overall, the results show that peanut skin contained two procyanidin monomers, eight B-type procyanidins dimers (MW=578), six A-type procyanidins dimers (MW=576), six A-type procyanidin trimers, ten B-type procyanidin trimers, four A-type procyanidin tetramers and 7 isomers of B-type procyanidin tetramer (Figure 1). These compounds were reported in peanut and peanut skin by Lazarus *et al.* (*8*). However, these authors did not report specific isomers. In addition, procyanidins with high degree of polymerization (e.g., pentamers, hexamers, heptamers and octamers, reported by Lazarus *et al.* (*8*), were not found in this study.

The procyanidin profiles of blanched and roasted peanut skin extracts were different from that of directly peeled peanut skin extract. The skin removal methods had a significant effect on the procyanidin contents of peanut skins (Table II). Procyanidin monomers and B-type dimers were present at high levels in directly peeled and roasted peanut skin extracts. Blanching leached 96.1% of procyanidin monomers, 88.5% dimers, 94.7% of trimers, 48.6% of B-type trimer, 93.5% of A-type tetramers and 73.9% B-type tetramers. This indicates that procyanidins in peanut skin are highly soluble in hot water. Changes caused by roasting (dry heat) were more complex. Compared to the extract of directly

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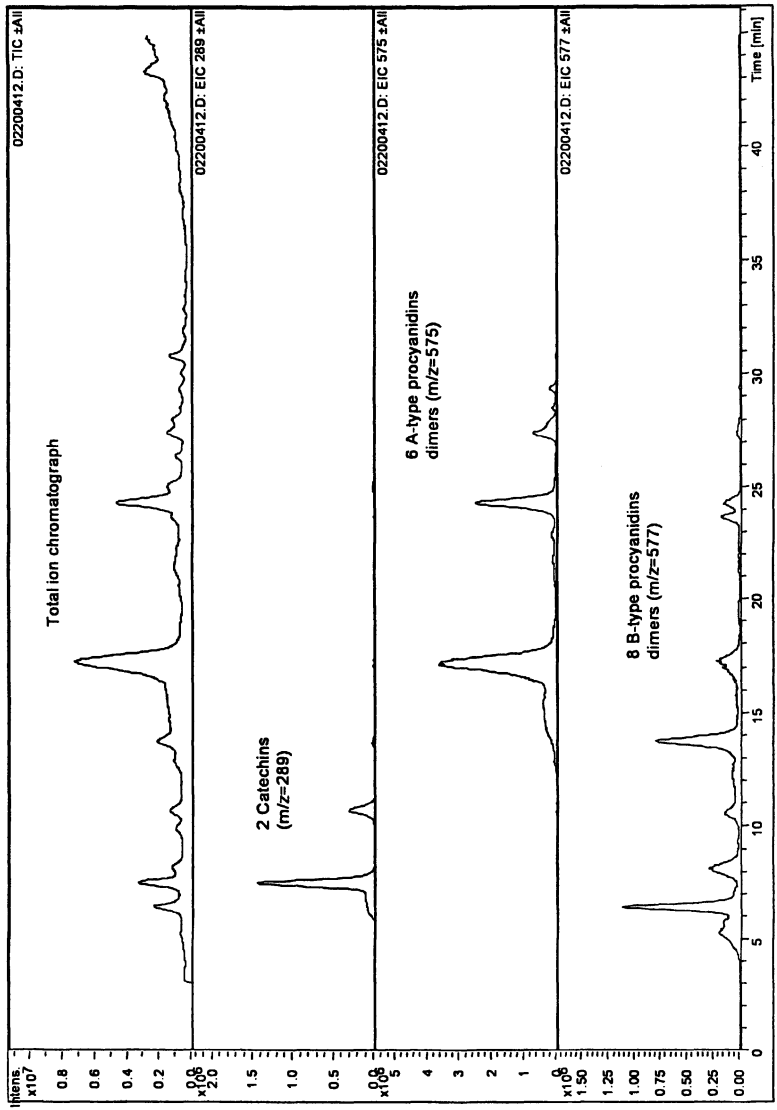

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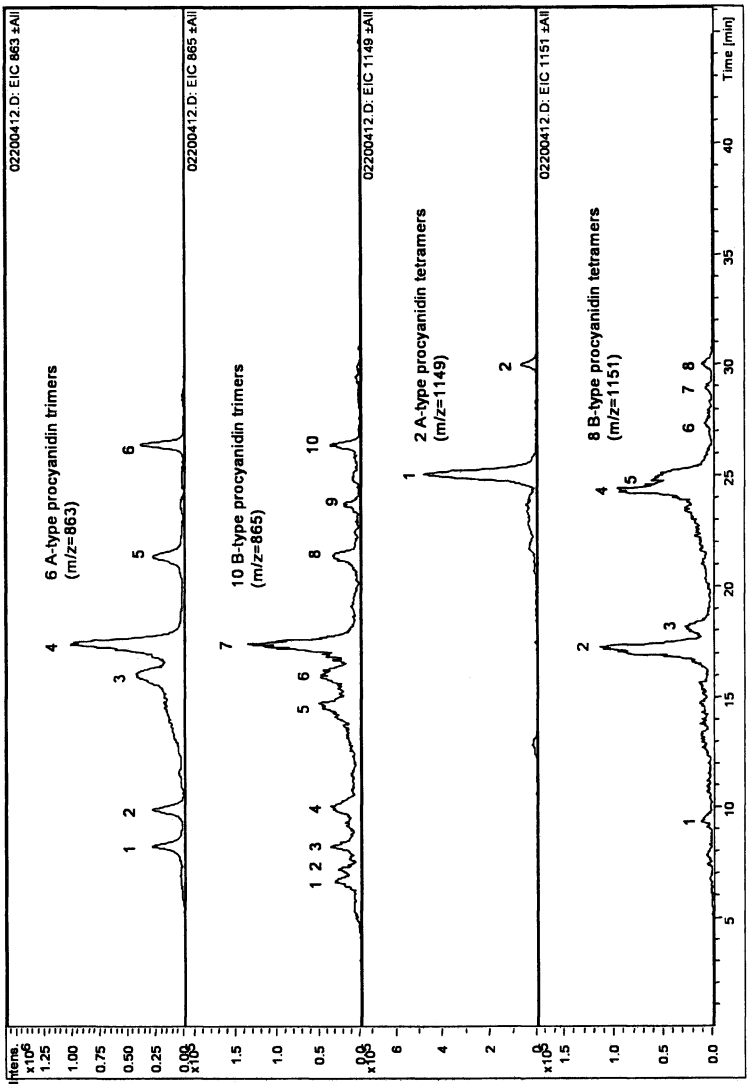

Figure 1. Major phenolic compounds in directly peeled peanut skin extract identified by LC-MS. (Peaks with numbers were confirmed by mass spectrometry.)

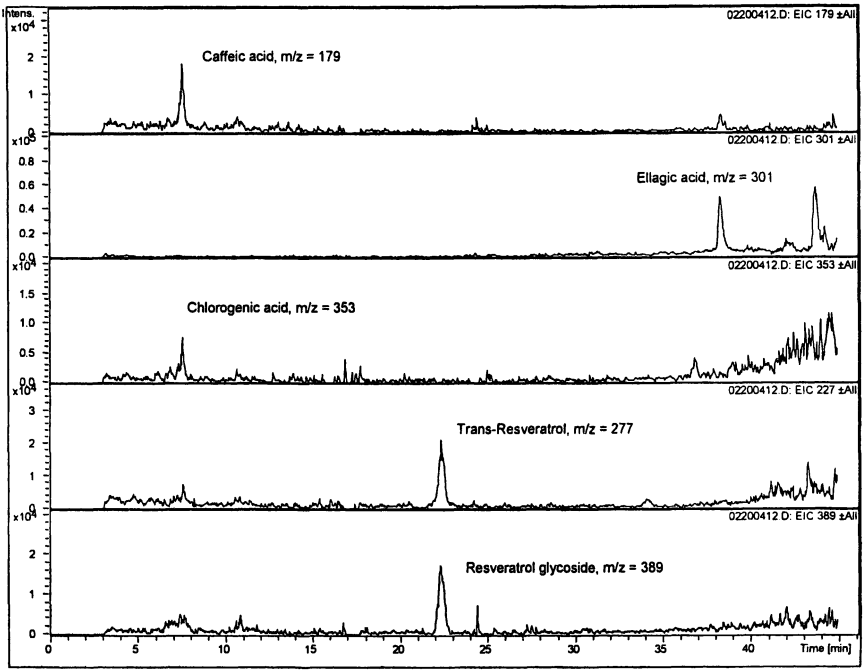

Figure 2. Minor phenolic compounds of directly peeled peanut skin extract identified by LC-MS (Peaks with numbers were confirmed by mass spectrometry).

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peeled peanut skin, the concentration of procyanidin monomers, B-type dimers, A-type trimers, and A-type tetramers decreased by 45.4, 14.7, 35.2, and 42.6%, respectively, while the concentration of A-type dimmers, B-type trimers and B-type tetramers increased by 41.0, 157.5 and 69.4%, respectively. The increase of A-type dimers might be the result of monomer polymerization or degradation of A-type trimers and tetramers through a mechanism that favors the formation of A-type dimers. The increase of B-type trimers and tetramers might be the result of polymerization involving monomers and B-type dimmers, which showed a parallel decrease in roasted skin.

The concentration of each type of procyanidin, the total catechins, procyanidin dimers, trimers and tetramers in processed peanut skins is given in Table II. Peanut skins exhibited a procyanidin dimmer content higher than that reported for 4 major varieties of grape seeds, namely Cabernet, Chardonnay, Merlot and Riesling grape seeds (54-126mg/100g), but slightly lower than values reported for Cabernet Franc, Gamay and Pinot noir grape seeds (169-375 mg/100g). Both directly peeled and roasted peanut skin contained a higher concentration of procyanidin trimers and tetramers than grape seeds. Peanut skin

Table II. Procyanidin contents of peanut skins removed by different processing methods

m/z	Compounds	*Concentration (mg/100g)		
		Directly peeled peanut skin	Blanched peanut Skin	Roasted peanut Skin
289	Procyanidin monomers	16.10 ± 1.29	0.62 ± 0.03 (-96.1%)	8.79 ± 0.32 (-454%)
575	A-type procyanidin dimers	90.20 ± 0.77	10.39 ± 0.33 (-88.5%)	127.19 ± 2.50 (+41.0%)
577	B-type procyanidin dimers	19.09 ± 0.99	1.90 ± 0.03 (-90.0%)	16.29 ± 0.45 (-14.7%)
863	A-type procyanidin trimers	214.04±7.60	11.39 ± 0.30 (-94.7%)	138.76 ± 2.04 (-35.2%)
865	B-type procyanidin trimers	7.29 ± 0.11	3.75 ± 0.11 (-48.6%)	18.77 ± 0.74 (+158%)
1149	A-type procyanidin tetramers	295.94 ± 5.31	17.92 ± 0.53 (-93.5%)	169.81 ± 0.20 (-42.6%)
1151	B-type procyanidin tetramers	20.13 ± 0.63	5.26 ± 0.11 (-14.87)	34.10 ± 0.49 (+ 69.4%)

* Concentration of monomers were expressed as (+)-catechin equivalent, dimmers, trimers and tetramers were expressed as procyanidin B2 equivalent.

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is the only source reported to contain A-type procyanidins, although catechin content of peanut skins (16.10 and 8.79 mg/100g for the directly peeled and roasted skin, respectively) was lower than that of grape seeds (*11*). This gives peanut skin a comparative advantage as a better source of potent antioxidants.

Free radical scavenging capacity and total antioxidant activity of crude and purified peanut skin polyphenols

The determination of $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging activity was carried out at concentrations of 0-5 mM and 0-200 μM, respectively. Free radical scavenging capacity of all extracts increased with increasing TP concentration and the best extracts exhibited maximum activity at 2.5 mM for $ABTS^{\bullet+}$ (98%) and 120 □M for $DPPH^{\bullet}$ (95%). These concentrations were used for subsequent comparisons shown in Figures 3 and 4. With the exception of blanched peanut skin, the free radical scavenging effects of crude peanut skin extracts were higher than those of Trolox and Vitamin C (Fig. 3 and 4). The crude extract of directly peeled peanut skin exhibited higher $ABTS^{\bullet+}$ scavenging capacity than

that of roasted peanut skin. There was no significant difference in DPPH• scavenging capacity for both directly peeled and roasted peanut skin crude extracts and none for scavenging capacities of the three purified peanut skin extracts. However, the purified extracts showed higher free radical scavenging effects than their respective crude counterparts, particularly for DPPH•. This suggests that the compounds responsible for the free radical scavenging effects in all purified peanut skin extracts were similar regardless the method of skin removal. The increase in % FRSC with the concentration of TP indicates that the antioxidant activity of peanut skin extracts is due to the presence of phenolics. Therefore, phenolics in peanut skin are more powerful than some well known antioxidants such as Trolox and Vitamin C and could have great commercial potential.

When the antioxidant activity of peanut skin extract was expressed as Trolox Equivalent Antioxidant Capacity (TEAC), it can be seen that the TEAC values of crude extracts measured by ABTS•+ and DPPH• scavenging methods were very close and significantly higher than those of Vitamin C except for blanched peanut skin crude extract (Table III). TEAC values of purified extracts measured by DPPH• method were significantly higher than those measured by

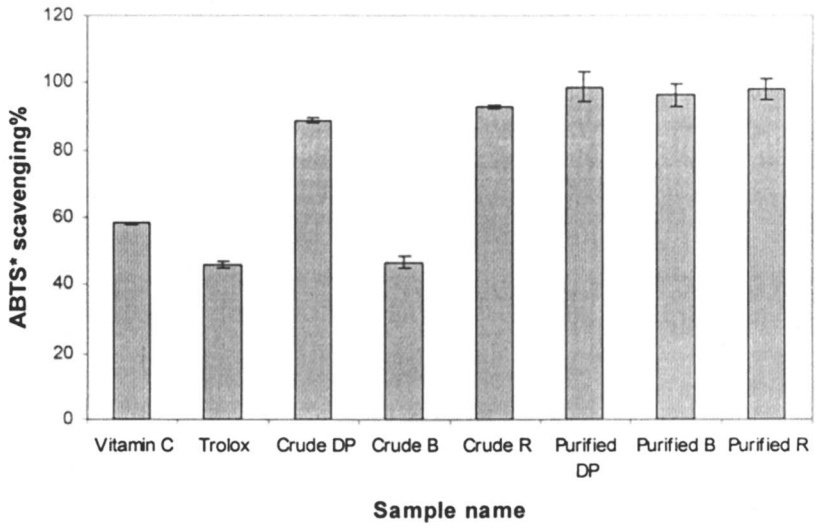

Figure 3. ABTS•+ scavenging capacity of peanut skin extracts as affected by skin removal methods and purification at 2.5 mM (crude - crude extract, pure - purified extract, DP-directly peeled skin, B-blanched skin and R-roasted skin) (Bars represent means and standard deviations of three replications.)*
**Concentration at which the most active extract reached maximum activity (>95%)*

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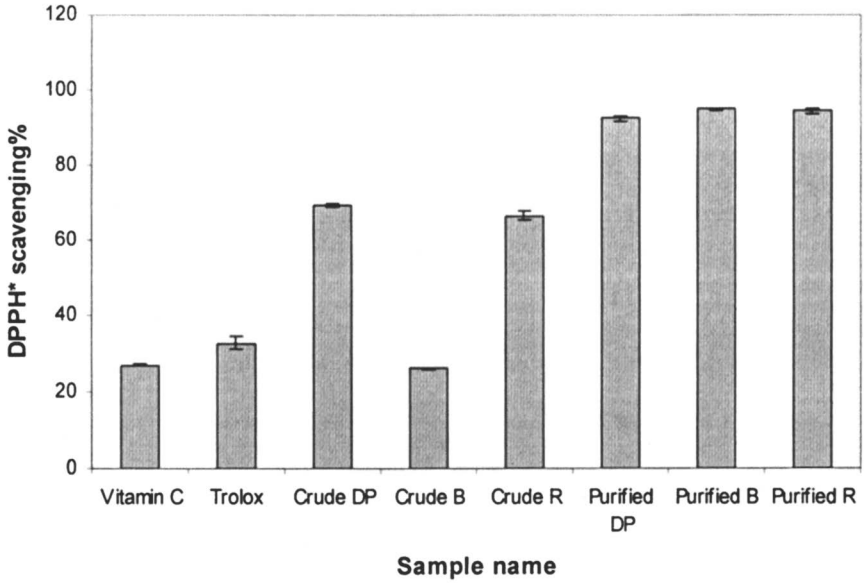

Figure 4. DPPH• scavenging capacity of crude and purified peanut skin extracts compared to Trolox and Vitamin C at 120 μM (crude - crude extract, pure - purified extract, DP-directly peeled skin, B-blanched skin and R-roasted skin, (Bars represent means and standard deviations of three replications.)*
** Concentration at which the most active extract reached maximum activity (>95%)*

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$ABTS^{\bullet+}$ method (Table III). Therefore, it is important to use the same method when comparing the antioxidant activities of different extracts/compounds. Regardless of the method of measurement, the measured antioxidant activity of peanut skin extracts was 2 to 4 time that of vitamin C or Trolox.

Statistical analysis shows that both skin removal methods and purification had significant effects on ABTS and DPPH free radical scavenging capacity at P = 0.05. A significant interaction ($p<0.0001$, $F=2053.99$) between skin removal methods and purification was observed. The interaction was caused by larger gain in TAA of blanched/purified peanut skin extract compared to the gains observed in purified extracts from directly peeled and roasted peanut skins. This suggests the presence of more non phenolic impurities in blanched peanut skin extract. These impurities may have interfered with the determination of TP and TAA of crude extract. Removal of interfering impurities brought the TAA of blanched skin extracts to levels similar to those observed in directly peeled and roasted skin extracts.

Table III. Antioxidant activities of peanut skin polyphenols determined by ABTS and DPPH free radical scavenging methods*

	Samples	TEAC for $ABTS^{\bullet+}$	TEAC for $DPPH^{\bullet}$
Reference	Trolox	1.01 ± 0.05	0.99 ± 0.09
	Vitamin C	1.25 ± 0.02	0.98 ± 0.02
Crude extracts	Directly peeled	2.26 ± 0.10	2.32 ± 0.18
	Roasted	2.09 ± 0.21	2.01 ± 0.02
	Blanched	1.25 ± 0.10	0.81 ± 0.07
Purified extracts	Directly peeled	2.43 ± 0.22	3.23 ± 0.61
	Roasted	2.67 ± 0.30	3.48 ± 0.52
	Blanched	2.51 ± 0.23	3.66 ± 0.63

* TAA expressed as Trolox Equivalent Antioxidant Capacity (mM Trolox per mM of total phenolics). TEACs were measured at linear range, and results were expressed as mean (of 3 replications) ± SD for all data in the table.

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It is important to note that antioxidant activity of peanut skin extracts measured by the chemical approach in this study can only give the relative ranking of extracts based on their reduction potential. It does not necessarily indicate that their biological/physiological effects are in the same order. More work is needed to test their antioxidant activity in biological systems, both *in vitro* and *in vivo*.

Health Benefit and Potential Applications of Peanut Skin

Peanut skins have been used as animal feed ingredients (*3*). Feeding lactating dairy cow with diet containing up to 16% peanut skin increased milk yield and milk fat, but decreased milk protein due to the reduced digestibility of protein-tannin complex (*4*). However, phenolic compounds identified in peanut skin are also found in green tea, fruits and grape seeds which have been extensively studied by many investigators for their health promoting effects and have been demonstrated to have multiple human health benefits, such as lower LDL level of serum/liver, inhibition of LDL oxidation thus preventing cardiovascular diseases, protection of DNA from free radical attack leading to lower risk of cancer and inhibition of histamine release thereby preventing inflammation. Catechins, procyanidins, anthocyanins and resveratrol in red wine and grapes have been attributed as the active compounds responsible for "the French Paradox", low mortality rate from cardiovascular diseases among people who drink red wine and have high intakes of saturated fatty acids (*20,21*).

Health benefits of catechins and procyanidins are well documented. Studies in animal models have shown that procyanidins from grape seeds inhibit

chemically induced lipid peroxidation, DNA fragmentation and subsequent apoptosis in a dose-dependent manner (*22,23*). Rats fed with diet enhanced with procyanidins had higher plasma antioxidant activity and their hearts were less susceptible to ischemia/perfusion damage induced by iron and copper ions (*24*). Human studies also show that diet rich in procyanidins decrease/inhibit lipid peroxidation of LDL cholesterol and increase free radical scavenging capacity (*25-26*). Procyanidins appear to have affinity for vascular tissue and they play a role in the protection of collagen and elastin, two critical proteins in connective tissue, by strongly inhibiting several enzymes involved in degradation of collagen, elastin and hyaluronic acid (*27*). Procyanidins were reported to selectively inhibit protein kinase C (*28*) and intensively promote hair growth by enhancing proliferation of mouse hair epithelial cells in vitro and activating hair follicle growth *in vivo* (*29*). Procyanidins were also found to slow the proliferation and decrease apoptosis of pancreas beta cells induced by hydrogen peroxide and promote proliferation of normal pancreas beta cells (*30*). Grape seed procyanidins are reported to have a photoreceptive action against UVB-induced erythrocytes damage in vitro by intercalating within the phospholipid bilayer, scavenging free radicals from UV exposure and sparing vitamin E at concentration as low as 0.1 μg/ml (*31*). Among phenolic acids, the antioxidant and anti-carcinogenic activities of ellagic acid have been studied most intensively (*32-34*). Chlorogenic acid is one of the major antioxidants in commercial long-life apple juice (*35*). Phenolic acids are important antioxidants of fruits and beverages because of their high concentrations. Peanut skin phenolics provide potent antioxidants for prevention of above mentioned diseases due to the high procyanidins content.

In addition to above therapeutical applications, a few studies have shown that extracts from red grape seeds, grape peel and peanut skin had antioxidative effect in food systems (*36-38*) to delay the onset of lipid oxidation and extend the shelf life of fat containing foods. Red and white wine phenolic extracts also showed potential antimicrobial activity against food pathogens such as *Staphylococcus aureus, Escherichia coli* and *Candida albicans* (*39*).

In conclusion, peanut skin represents a rich source of potent antioxidants, including procyanidins, phenolic acids and resveratrol. Potential commercial applications of peanut skin extract include use as dietary supplement (nutraceutical) to prevent chronic diseases such as cancer, cardiovascular diseases, hair loss, and inflammation, as functional food ingredient, as a natural antioxidant to prevent oxidation of oil/fat containing food products and a natural antimicrobial agent to inhibit the growth of food pathogens.

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Chapter 17

Antioxidant Activity in Relationship to Phenolic Content of Diverse Food Barley Genotypes

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The antioxidant activity and phenolic composition of 21 mostly hull-less food barley of diverse origin were determined. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) and superoxide radicals scavenging capacity assays were used to measure antioxidant activity. A photochemi- luminescence technique was used to measure water (ACW) and lipid (ACL) soluble substances. The DPPH radical scavenging capacities of the samples were significantly different ($p<0.05$), ranging between 13 and 27%. Significant differences existed in the ACW and ACL values. The total phenolic contents were determined using the Folin-Ciocalteu method; values ranged from 2,672 to 3,947 μg ferulic acid equivalents /g of barley. Tannin contents determined using the vanillin HCl method were significantly different and varied between 36 and 58 μg catechin equivalents /g of barley. Two hulless genotypes Peru 45, and Ex87xCI 19973 displayed consistently high values of the antioxidant parameters and can be used as antioxidant rich food ingredients. Peru 16, a genotype which had an intact hull that was removed prior to analysis also displayed good antioxidant properties. Due to the difficulty involved in processing, Peru 16 will have to be developed into a hull-less genotype through breeding before adoption for food use. Lack of correlation between tannin and total phenolic contents warrants the need for more detailed chemical analyses and evaluation of functional properties of phenolic compounds of diverse barley materials.

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Barley (*Hordeum vulgare* L.) is the fourth most important cereal in the world in terms of world production (*1*). It is a versatile cereal adapted to and produced over a wider range of environmental conditions than any other cereal. Barley is used mainly for brewing and as animal feed but there is a growing interest in it for human food uses primarily because of its reported health benefits (*2*). Barley contains significant amounts of phytochemicals such as phenolic compounds and lignans and is an excellent source of β-glucan soluble fiber. These components have biological activities that can reduce the risk of coronary heart disease, diabetes and certain cancers (*2*).

Although increasing, food use of barley is still relatively low in many western countries compared with other cereal grains. Breeding programs aimed at increasing the food use of barley are among other things targeting development of hulless barley cultivars (*2*). Hulless cultivars permit greater ease in milling and pearling with enhanced processing yields and have higher levels of β-glucan. The recent FDA amendment of the health claim on the relationship between oat β-glucan soluble fiber and reduced risk of coronary heart disease (CHD) adding barley as an additional eligible source of β-glucan soluble fiber (*3*) will likely have a significant effect on the use of barley as a food ingredient. Knowledge of the antioxidant properties of the barley cultivars being developed for food use would likely aid selection of those exhibiting high antioxidant potential. Breeding of such varieties may also further increase the food usage of whole grain barley.

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Phenolic compounds are secondary plant metabolites naturally present in cereals and other plants as minor non-nutritive components (*4*). Phenolic compounds are potent antioxidants, they inhibit lipid peroxidation by scavenging free radicals such as hydroxyl radicals ($HO^{\bullet}$) and peroxyl radials ($ROO^{\bullet}$) resulting in the formation of low energy phenolic radicals whose energy is not sufficient to promote lipid oxidation at biologically significant rates (*5*). Barley phenolics constitute 0.2 to 0.36% of grain by weight (*6*). These phenolics range from tyrosine, tyramine and its derivatives, phenolic acids and their esters and glycosides, and anthocyanins responsible for the color of barley tissue to lignans and substances related to lignin (*7*, *8*).

The phenolic composition of barley is well documented; however, literature on the associated antioxidant properties is limited. Tamagawa et al (*9-11*) reported the antioxidant activity of one class of phenolics, the proanthocyanidins separated from bran extracts. Goupy et al (*12*) determined the antioxidant composition and activity of barley and malt extracts and of phenolic compounds isolated from the extracts. They reported a positive correlation (r=0.89) between the antiradical power of the extracts against DPPH and the amount of total flavan-3-ols. Zielinski and Kozlowska (*13*) found the total antioxidant activity (TAA) based on the relative abilities of antioxidants in 80% methanolic extracts to scavenge the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), diammonium salt (ABTS) radical in comparison with Trolox to be correlated (r = 0.96) to the total phenolic content of barley, wheat, rye and oats. Among these cereals, barley had the highest TAA followed by the wheat c.v Henika then oat. Rye and wheat cv. Almari had the least TAA.

Research on the antioxidant properties of barley genotypes containing a colored aleurone; the location of most phenolics in barley grain (*14*), originating from several parts of the world and therefore having a huge genetic base is currently lacking. This study was carried out to evaluate the antioxidant activities, total phenolic contents and tannin contents of 21 food barley varieties from the world germplasm collection in order to develop new knowledge in the area of functional foods where phenolic compounds are likely to contribute potential health benefits as antioxidants. The nature of the relationships between antioxidant activities, total phenolic contents and tannin contents of the barley samples were also evaluated.

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Materials and Methods

Chemicals and Reagents

Folin-Ciocalteu reagent was purchased from BDH Inc., (Toronto, ON). Ferulic acid, catechin and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (Oakville, ON). Sodium carbonate, hydrochloric acid and methanol were procured from Fisher Scientific (Pittsburgh, PA). Antioxidant capacity for water (ACW) and lipid (ACL) soluble substances kits were supplied by Analytik Jena (The Woodlands, TX). All chemicals and solvents were of the analytical grade.

Sample Description and Preparation

Twenty-one barley cultivars of diverse origin (Table I) were grown and harvested at Brandon, Manitoba, Canada in 2002. The barley genotypes represented samples originating from Peru, Ethiopia, Siberia, Mongolia, Ecuador, Japan, and Canada. They were selected as they find food applications in their respective regions. Most genotypes were hulless, a few were mixed and only 4 had hulls (Table I).

Hulls were removed from all samples with hulls prior to grain milling. Dehulled and originally hulless whole grain barley samples were ground in a Retsch mill (F-Kurt Retsch, Haan, German) to pass through a 0.5 mm screen and stored in sealed plastic bags at –20 °C until analysis.

DPPH Radical Scavenging Activity

Extracts were obtained and analyzed using a modification of the method developed by Brand-Williams *et al.* (*15*) as described previously (*16*, *17*). Sample extracts (0.1 mL) were added to 60 μM of freshly prepared DPPH solution. Each sample extract (0.1 mL) was reacted with DPPH radical solution (3.9 mL) and absorbance measured at 515 nm using methanol as the blank. Absorbance was read at time t = 0 (when sample was added to DPPH solution)

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Table I. Sample description

Entry	*Plot*	*Description*	*Type of caryopsis*
Peru 3	1	Peruvian line from CYMMIT collection	hull
Peru 5	2	Peruvian line from CYMMIT collection	hulless
Peru 16	3	Peruvian line from CYMMIT collection	hull
Peru 35	4	Peruvian line from CYMMIT collection	hulless
Peru 45	5	Peruvian line from CYMMIT collection	hulless
EX116	7	Juton mutant line from World Collection	hull
EX127	8	Faust #2 mutant from World Collection	hulless
CI4325	9	Canadian Introduction entry from Ethiopia (landrace)	hull
EX82 X CI 9973	10	Genetic cross from Brandon Research Centre	hull + hulless (origin)
EX87 X CI 9973	11	Genetic cross from Brandon Research Centre	hulless
EX83	12	Mutant derived from Moncalm barley - BRC	hull
CI1370	13	Canadian Introduction entry from Ethiopia (landrace)	hull + hulless
CI4374	14	Canadian Introduction entry from Ethiopia (landrace)	hull + hulless (origin)
CI9977	15	Canadian Introduction entry from Mongolia (landrace)	hull + hulless
CI10151	16	Canadian Introduction entry from Siberia (landrace)	hulless
CI2318	17	Canadian Introduction entry from Ecuador (landrace)	hulless
CI1248	18	Canadian Introduction entry from Ecuador (landrace)	hulless
CI2230	19	Canadian Introduction entry from Ecuador (landrace)	hulless
CI4013	20	Canadian Introduction entry from Ethiopia (landrace)	hull + hulless
HOKUTO HADAKA	21	Japanese cultivar	hulless
SHIGA WASEH	22	Japanese cultivar	hulless

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and t = 30 min. The test was run twice on each replicate extract and antioxidant activity of barley grains were expressed as percentage of decoloration of DPPH radical calculated as follows: % decoloration of DPPH = $(1 - Abs_{t=0} / Abs_{t=30}) \times 100$.

Determination of ACL and ACW using Photochemiluminescence (PCL)

Ground samples (0.5 g) were extracted under nitrogen, at room temperature with 70% methanol (5 mL) for 1 h on a wrist action shaker (Burrel, Pittsburg, PA). After extraction, the samples were centrifuged at 3000 rpm for 10 min on a Sorvall table centrifuge (GLC-1, Newton, CT). Sample extracts were used for the determination of ACW and ACL in duplicates. The ACL and ACW of the extracts were determined using photochemiluminescence (PCL) methods developed by Popov and Lewin (*18*, *19*) and modified by Analytik Jena AG (Jena, Germany). The PCL assays were carried out in a photoluminometer Photochem® using ACW and ACL kits supplied by and following procedures described by the manufacturer (Analytik Jena, The Woodlands, TX). The ACW assay mixture was composed of 15 µL of an appropriate dilution of extract and the following quantities of ACW kit components: 1485 µL of reagent 1 (water), 1000 µL reagent 2 (buffer solution, pH 10.5), and 25 µL reagent 3 (photosensitizer). For determination of ACL, after an appropriate dilution the extract (20 µL) was added to components of the ACL kit: 2 280 µL reagent 1 (methanol), 200 µL reagent 2 (buffer solution) and 25 µL reagent 3 (photosensitizer). Results were obtained from a workstation employing PCL software and expressed as µg ascorbic acid equivalents (AAE) /g of barley for ACW and µg Trolox equivalents /g of barley for ACL.

Extraction of Samples for Determination of Total Phenolic and Tannin Content

Phenolics were extracted according to the method described by Gao and co-workers (*20*). Ground samples (200 mg) were extracted with 4 mL of acidified methanol (HCl/methanol/water, 1/80/10, v/v/v) at room temperature for two hours on a Barrel shaker (Pittsburgh, PA). The mixture was centrifuged for 10min at 3000 rpm on a Sorvall table centrifuge (GLC-1, Newton, CN) and the supernatant used for determination of total phenolic content and tannin content.

Determination of Total Phenolic Content

The Folin-Ciocalteu method (*21*) modified by Gao et al (*20*) as described previously (*16*, *17*, *22*) was used for determination of total phenolics. Acidified-methanol extracts (0.2 mL) were added to 1.5 mL of freshly diluted Folin-Ciocalteu reagent. The mixture was allowed to equilibrate then 1.5 mL of sodium carbonate (60 g /L) were added. The absorbance of the mixture was measured on a spectrophotometer at 725 nm with acidified methanol as the blank

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after incubation at room temperature for 90 min. Ferulic acid was used as the standard and results were expressed in μg ferulic acid equivalents (FAE) /g of barley. All analyses were performed in duplicate

Determination of Tannin Content

Tannin content was determined using the vanillin method (*23*). Fresh acidified methanol extracts (1 mL) were reacted with 5 mL of vanillin reagent (50:50 mixture of 1% vanillin in acetic acid / 8% HCl in acetic acid) for 20 min at room temperature, and absorbance read at 510 nm. Catechin was used as the standard and the results were expressed as μg catechin equivalents (CE) / g of barley.

Statistical Analysis

Results were based on duplicate analyses and are reported as mean ± standard deviation. Analysis of variance (ANOVA) was performed with the general linear model (GLM) of the SAS software package (release 8.2) (SAS Institute, Cary, North Carolina) Determination of significant differences among means was done by the least square difference (LSD) and significance declared at $p < 0.05$. Correlation analyses were performed with the PROC CORR procedure of the SAS software package using the Pearson correlation test.

Results and Discussion

DPPH Radical Scavenging Capacity

The reaction time for radical scavenging analysis was 30 min. A review compiled by Huang and co-workers (*24*) stated that previous works showed that antioxidant compounds activity slow down after 30 min. Thus, 30 min duration was chosen to observe the ability of antioxidant compounds to bind and scavenge the DPPH radical.

The DPPH radical scavenging capacity of the samples ranged from 13 to 27 % (Table II). The average value was 21.9 % and the least significant difference (LSD) between means was 1.67. Varieties with the uppermost mean percentage (> 25 %) had almost twice as much activity as those with the lowermost percentages (<14 %). Duh *et al.* (*25*) found scavenging activities of 35.2 ± 2.5% for 0.2 mg of unroasted freeze dried barley extracts. It should be noted that differences in results can be contributed by different extraction and analytical methods from each experiment. Whole wheat extracts obtained under similar conditions exhibited DPPH scavenging capacities of between 13.2 and 14.3 % (*16*) possibly indicating that whole barley grain might be a superior source of antioxidants than whole wheat grain. Antioxidant properties of grains are however susceptible to genetic and environmental variations which complicate such conclusions.

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Table II. DPPH radical scavenging capacities and antioxidant activities of water (ACW) and lipid (ACL) soluble substances of barley samples*

Genotype	*DPPH radical scavenging capacity (%)*[1]	*ACW (μg AAE /g)*[2]	*ACL (TE μg /g)*[3]
Peru 3	22 ± 1.4 ghij	1560 ± 193.0 bc	696 ± 16.3 cde
Peru 5	13.3 ± 0.0 o	1093 ± 81.3 defg	637 ± 14.1 de
Peru 16	24.7 ± 1.5 cd	1244 ± 270.1 cde	736 ± 137.2 cd
Peru 35	19.1 ± 0.9 lk	1373 ± 59.4 cde	795 ± 28.2 bc
Peru 45	22.1 ± 1.1 ghij	2030 ± 185.3 a	931 ± 110.3 a
EX116	22.8 ± 0.6 efgh	972 ± 8.5 efgh	580 ± 132.2 efg
EX127	17.3 ± 0.4 m	981 ± 108.2 efgh	485 ± 7.1 gh
CI4325	25.4 ± 1.4 bc	1215 ± 176.1 cdef	521 ± 21.2 fgh
EX82 X CI9973	24.2 ± 0.1 cde	1887 ± 291.3 ab	724 ± 60.1 cd
Ex 38	21.5 ± 0.2 hij	781 ± 48.8 fgh	582 ± 1.4 efg
CI1370	20.3 ± 0.1 jkl	642 ± 38.2 ghi	442 ± 46.0 ih
CI4374	26.9 ± 0.8 ab	697 ± 82.7 ghi	638 ± 7.1 def
CI9977	24.8 ± 1.1 cd	573 ± 82.7 hi	500 ± 67.9 gh
CI10151	23.6 ± 0.2 defg	602 ± 60.8 hi	735 ± 14.1 cd
CI2318	23.8 ± 0.4 cdef	763 ± 68.6 fgh	901 ± 10.6 ab
CI1248	15.4 ± 0.1 n	250 ± 29.7 i	362 ± 2.1 i
CI2230	21.4 ± 0.5 hij	753 ± 6.4 gh	742 ± 66.5 cd
CI4013	20.7 ± 0.9 ijk	970 ± 58.0 efgh	484 ± 26.9 gh
Hokuto Kadaka	18.7 ± 0.4 lm	587 ± 25.5 hi	421 ± 31.8 ih
Shiga Waseh	27.3 ± 1.1 a	608 + 20.5 hi	423 ± 8.4 ih
Mean	21.9 ± 3.6	1002.4 + 460.9	618.8 ± 159.2
LSD[4]	1.67	453.6	118

* Mean ± standard deviation (dry weight basis), values with the same letters are not significantly different

[1] DPPH radical scavenging capacity (% DPPH decoloration)

[2] Antioxidant activity of water soluble substances (ACW in μg Ascorbic acid equivalents (AAE) /g of barley)

[3] Antioxidant activity of lipid soluble substances (ACL in μg Trolox equivalents (TE) /g of barley)

[4] Least significant difference at $p<0.05$

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ACW and ACL of the Barley Samples

Pooled together, the samples had an average ACW of 1002 μg AAE /g. The genotype Peru 45 had the highest ACW (2030 μg AAE /g), while CI1248 had the lowest ACW (250 μg AAE /g). The average ACL value for the samples was 619 μg TE /g overall (Table II). Samples with the uppermost concentrations (> 750 μg TE /g) were Peru 45, CI4325 and Peru 35. Shiga Waseh, Hokuto Hadaka and CI1248 had the lowermost (< 450 μg TE /g) ACL values. In a similar manner with ACW values, the genotype Peru 45 had the highest ACL value (931 μg TE /g) and the genotype CI1248 having the lowest ACL value (362 μg TE /g).

The sample extracts had higher water than lipid soluble antioxidant activities. Lee *et al.* (*26*) reported a similar trend in soybeans. Determination of the antioxidant capacities of water and lipid soluble substances in various foods using the ORAC assay (*27*) also revealed a similar trend to the one we observed. Using a newly developed peroxyl radical scavenging capacity assay. Adom *et al.* (*28*) reported that hydrophilic antioxidant activity contributed more than 98% to total antioxidant activity of selected whole grain flours. Lipophilic and hydrophilic antioxidants are utilized differently in the body. Although their concentration is relatively low, lipophilic antioxidants can be absorbed through the lymphatic system and reach a higher level of bioavailability, whereas hydrophilic antioxidants can be absorbed in the small intestine and reportedly do not accumulate in the body (*29*).

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Total Phenolic Content

The mean total phenolic content for the genotypes was 3307 μg FAE /g. Genotypes with upper range total phenolic contents (>3500 μg FAE /g) were CI4374, CI2318, Peru 45, EX82 X CI9973, CI 10151 and EX87 X CI9973. Genotypes in the lower range had less than 3000 μg FAE /g.

Previous studies on total phenolic content of barley reported different values. A study of the antioxidant properties of barley and malt (*30*) reported total phenolic contents of 1.0 mg/g of dry matter barley on average. Bonoli *et al.* (*31*), on the other hand, reported total phenolic contents ranging from 0.13 to 0.68 mg gallic acid /g barley flour. These differences are likely a manifestation of the testing of different genotypes, differences in analytical methods particularly in the choice of extracting solvent and the use of different standards. Nevertheless, our study showed that phenolic contents of the barley genotypes tested varied significantly.

Tannin Content

The combined mean tannin content of the barley genotypes was 46 μg catechin equivalents (CE)/g of sample (Table III). Genotypes with higher tannin contents (>48 μg CE /g) were significantly higher ($p<0.05$) than all other samples. Lower range genotypes had less than 45 μg CE /g of sample. Peru 45 had the

Table III. Total phenolic contents and tannin contents of barley samples*

Genotype	*Total Phenolics[1] (μg/g)*	*Tannin[2] (μg/g)*
Peru 3	3062 ± 105.2 ghi	47.8 ± 0.8 cd
Peru 5	3248.4 ± 88.5 ef	38.6 ± 1.0 i
Peru 16	3062.7 ± 96.7 ghi	48 ± 0.6 c
Peru 35	3483 ± 57.1 c	45.3 ± 0.9 f
Peru 45	3840.7 ± 68.7 ab	58.1 ± 1.4 a
EX116	3212.8 ± 67.0 ef	52.3 ± 1.4 b
EX127	3007.6 ± 31.5 hi	43.8 ± 0.6 g
CI4325	3294.1 ± 68.0 de	52.2 ± 0.2 b
EX82 X CI9973	3703.7 ± 56.4 b	46.5 ± 0.7 def
EX87 X CI9973	3691.5 ± 207.4 b	46.9 ± 0.2 cde
EX38	3099 ± 84.4 fghi	45.8 ± 0.8 f
CI1370	3182.7 ± 80.8 efgh	47.5 ± 1.3 cd
CI4374	3947.3 ± 54.5 a	47.7 ± 0.2 c
CI9977	3439.5 ± 12.2 cd	38.4 ± 1.0 i
CI10151	3703.2 ± 100.8 b	43.7 ± 1.7 g
CI2318	3858.8 ± 169.5 ab	39.8 ± 0.3 h
CI1248	2672 ± 28.4 j	42.8 ± 0.3 g
CI2230	3348.6 ± 83.6 cde	45.9 ± 0.0 ef
CI4013	2972.4 ± 49.0 i	45.9 ± 0.4 ef
Hokuto Kadaka	2682.2 ± 88.5 j	36 + 0.3 j
Shiga Waseh	2943.1 ± 15.4 i	51.1 ± 0.7 b
Mean	3307.4 ± 367.2	45.9 ± 5.2
LSD[3]	185	1.47

* Mean ± standard deviation (Dry weight basis), values with the same letters are not significantly different

[1] Total phenolic content (μg Ferulic acid equivalents /g of barley)

[2] Tannin content (μg catechin equivalents /g of barley)

[3] Least significant difference at $p<0.05$

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highest total tannin content (58.1 μg CE /g). Research done by Yadav and co-workers (*32*) showed that hulled barley grains contained proanthocyanidins (tannins) ranging from 3.85 to 4.94 mg CE /g. Barley hull has a high concentration of tannins therefore determination of tannin content in hulled grain results in higher values than can be obtained in hulless genotypes. Results based on hulless or dehulled materials provide better information since the hull is removed during food processing of barley.

Correlations between the Measured Parameters

The ACW and ACL values were significantly correlated to each other, but not significantly correlated to the DPPH scavenging capacities (Table IV). The methods used to determine antioxidant activities (PCL and the DPPH assay) make use of organic radical producers. There are, however, fundaments differences in the reaction mechanisms involved. The DPPH method analyzes the ability to reduce the stable DPPH radical cation whereas the PCL assay measures delay in radical generation as well as the ability to scavenge the radical (*33*).

The total phenolic content of the barley samples was significantly correlated to their DPPH scavenging capacities and ACL, with a stronger correlation with the latter (Table IV). Methanol is the predominant solvent for extraction although the presence of water increases the polarity of the solvent used for the PCL assay. The hydrophilic components, enhanced under PCL assay conditions, could have contributed to a weak correlation between the DPPH and ACW results. The ACW-PCL assay displayed greater differences among genotypes giving a higher content (mean 919 μg/g AEAC) than the ACL-PCL test (mean 567 μg/g TEAC). The tannin content was significantly correlated ($p<0.05$) to the ACW; however, its correlation with DPPH free radical scavenging activity was insignificant. There was lack of correlation between total phenolic acid and tannin content. The method used for determining total phenolic content is not specific since phenolic compounds (phenolic acids, monomeric and polymeric flavanols) and easily reducing compounds such as ascorbic acid are simultaneously determined. The vanillin-HCl method takes advantage of the capacity of the flavanols to react with aromatic aldehydes (vanillin) in strongly acidic media and form a colored adduct. This reaction is more specific and takes place with both catechins (monomeric flavanol) and proanthocyanidins (polymeric flavanols).

The genotypes Peru 45 and Peru 16 (Peruvian lines from the CYMMIT collection) and EX87 X CI 9973 (a genetic cross from the Brandon research centre in Canada) appear to possess the most superior antioxidant properties as they displayed consistently high values of the antioxidant parameters measured. Peru 45 and EX87 X CI 9973 are hulless genotypes and hence can be easily adopted for food use. Peru 16 on the contrary has an intact hull and may have to be modified accordingly through breeding before it can be adopted for food use.

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Table IV. Correlation on different antioxidant assays on 21 barley samples*

	Total Phenolics	*DPPH*	*ACW*[1]	*ACL*[2]
DPPH	0.46361 0.0343			
ACW	0.39783 0.0741	0.11957 0.6057		
ACL	0.73973 0.0001	0.20954 0.3620	0.60637 0.0036	
Tannin content	0.21677 0.3452	0.42848 0.0526	0.50345 0.0200	0.24806 0.2783

* Cell contents: Pearson correlation coefficient
(values in bold are significant at $p<0.05$) and P-value (shown below)
[1] Antioxidant activity of water soluble compounds
[2] Antioxidant activity of lipid soluble compounds

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Conclusions

The results obtained clearly show that DPPH scavenging capacity, ACW, ACL, total phenolic content and tannin content vary significantly among the barley genotypes tested. This variation could be caused by either genetic or environmental factors (*17, 34*). In this study, the observed variation appears to be mainly genetic in nature because all the genotypes were grown in the same season, at one location. The knowledge generated from this study will likely contribute to the breeding of antioxidant rich barley varieties for food use. Testing of the genotypes across different growing environments to determine the environmental stability of their antioxidant properties as well as determination of other antioxidant parameters and compositional analysis will, however, be of interest.

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Chapter 18

Phytochemical Compositions and Free Radical Scavenging Capacities of Selected Cold-Pressed Edible Seed Oils

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Selected specialty cold-pressed edible seed oils including blueberry, raspberry, marionberry, boysenberry, roasted pumpkin, onion, milk thistle, and parsley, were examined for their phytochemical compositions including tocopherols, carotenoids, and total phenolic content (TPC). They were also investigated for their radical scavenging properties against DPPH radicals and for their oxygen radical absorbing capacity (ORAC). All of the tested cold-pressed edible seed oils differed in their tocopherol profiles, carotenoid compositions, TPC, and radical scavenging properties. The highest concentration of α-tocopherol was seen in the cold-pressed onion seed oil (663.1 mg/kg), but the highest δ- and γ-tocopherol contents were detected in boysenberry seed oil. Zeaxanthin was the primary carotenoid in all tested oils. The cold-pressed roasted pumpkin seed oil exhibited the highest β-carotene, lutein and cryptoxanthin contents at levels of 5981.2, 272.2, and 4916.7 μg/kg, respectively. ORAC values of the herb and spice seed oils were generally higher than those of the fruit seed oils. Parsley seed oil had an ORAC value of 537.0 TE μmol/g, and milk thistle oil had an ORAC value of 125.2 TE μmol/g. The ORAC values of the fruit seed oils ranged from 1.1 to 77.9 TE μmol/g.

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Introduction

With the evidence continually growing that dietary intake may greatly impact human health and the quality of life, many consumers are making dietary choices that may benefit their overall health and/or prevent disease. Previously obscure or non-existent cold-pressed seed oils are now commercially available from many plant sources including vegetable, herb, spice, and fruit seeds. The cold-pressing procedure is an oil extraction method that does not involve heat or an organic solvent to extract the seed oil. This may help to protect and retain valuable natural ingredients including vitamins and antioxidants. Vitamins and provitamins found in seed oils include tocopherols and carotenoids (*1*). Aside from β-carotene, the latter can also include other potentially beneficial chemicals such as lutein, cryptoxanthin, and zeaxanthin that may have other positive effects including antioxidant activity. Other important oil components include phenolic compounds that have also demonstrated antioxidant effects and have potential for health promotion and disease prevention against maladies such as cancer and atherosclerosis (*2,3*). Antioxidants are proposed to benefit human health by directly quenching dangerous free radicals and peroxides by the donation of a hydrogen atom or an electron, and the antioxidant may be converted to a harmless compound. Some antioxidants can also form chelating complexes with dangerous transition metals, and some may stimulate immune responses. New sources of natural dietary antioxidants are in high demand because of their improved safety and consumer acceptability.

Four cold-pressed seed oils including black caraway, carrot, cranberry, and hemp demonstrated significant radical scavenging ability against the peroxyl radical and $ABTS^{\bullet\pm}$, with the black caraway seed oil showing the best overall scavenging activity with an ORAC value of 220 μmole TE/g and an $ABTS^{\bullet\pm}$ scavenging capacity of 30.8 μmole TE/ g. TE represents the trolox equivalents. The cold-pressed seed oils also showed significant total phenolic contents and powerful chelating abilities. These results suggest the possible health benefits from the consumption of these oils (*4*). This report summarizes a wide range of different cold-pressed seed oils from vegetable, herb, spice, and fruit seeds that are now commercially available including cold-pressed extra virgin onion (*Allium cepa* L.), parsley (*Petroselinum crispum*), cardamom (*Elettaria cardamomum*), mullein (*Verbascum thapus*), roasted pumpkin (*Curcubita pepo*), milk thistle (*Silibum marianum*), and red raspberry (*Rubus Ideaus*), boysenberry (*Rubus* hybrid), marionberry (*Rubus* hybrid), blueberry (*Vaccinium corymbosum*), seed oils for tocopherol and carotenoid composition, oxygen radical absorbance capacity (ORAC), $DPPH^{\bullet}$ scavenging activity, and total phenolic content (TPC). The data obtained from these cold-pressed seed oils can

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be useful in evaluating their potential uses as food products for improving human health and nutrition.

Materials and Methods

Materials

Recently prepared, cold-pressed, unrefined onion, parsley, cardamom, mullein, roasted pumpkin, milk thistle, blueberry, red raspberry, and marionberry seed oils were supplied by Badger Oil Company (Spooner, WI), and extractions were prepared upon arrival. Fluorescein, 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), and gallic acid were purchased from Sigma-Aldrich (St. Louis, MO). 2,2′-azobis (2-aminopropane) dihydrochloride (AAPH) was obtained from Wako Chemicals USA (Richmond, VA), and β-cyclodextrin (RMCD) was purchased from Cyclolab R & D Ltd. (Budapest, Hungary). All other chemicals and solvents were of the highest commercial grade and used without further purification.

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Sample Preparation

Oil constituents were extracted using 100 % MeOH. One gram of oil was measured into a test tube followed by the addition of 3 mL of methanol. The test tube was vortexed for 30 seconds then centrifuged at 6000 rpm for 5 min. and the supernatant was collected. This procedure was repeated two more times. All three extractions were combined, and the final volume was brought to 10 mL with MeOH. The resulting antioxidant solutions were then kept in the dark under N_2 until further analyses including $DPPH^{\bullet}$ scavenging capacity, oxygen radical absorbance capacities (ORAC), and total phenolic contents (TPC).

Carotenoid Composition

Carotenoids (β-carotene, lutein, cryptoxanthin, and zeaxanthin) were measured following a previously described method (*1*). One milliliter of cold-pressed seed oil was dissolved in 160 mL of methanol/tetrahydrofuran (1:1, v/v) and analyzed for carotenoid profile using high performance liquid chromatography-diode array detector-electron spray ionization-tandem mass spectrometry (HPLC-DAD-ESI-MSMS) (*1,5,6*). An Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA) with a Zorbax SB C18 column 50mm ×

1.0 mm i.d. with a 3.5-μm particle size (Agilent Technologies, Palo Alto, CA), was used to perform the separations of the carotenoid compounds. Identification of the individual components was accomplished by comparing HPLC retention time and selected reactant monitoring (SRM) analyses of the sample peaks with that of the authorized pure individual commercial compounds. Quantifications for the carotenoid and tocopherol compounds were conducted using the total ion counts with an external standard.

Tocopherol Profile

The methanol/tetrahydrofuran solutions prepared for carotenoid composition were also used to quantify α-, δ-, and γ-tocopherol concentrations by a previously described method (*1*). An HPLC with a Zorbax SB C18 column 30 mm × 1.0 mm i.d with a 3.5-μm particle size (Agilent Technologies, Palo Alto, CA) was used to separate the tocopherols. The individual tocopherols were identified by peak retention time and selected reactant monitoring with those of the pure commercial compounds, quantification was determined using the total ion counts with external standards of the individual compounds.

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DPPH• Scavenging Activity

The DPPH• scavenging activity was determined using the procedure described by Yu (*2*). Seed oil extracts were mixed with a freshly prepared DPPH•-MeOH solution at concentrations of 6.7, 8, 10, 20 and 40 mg oil equivalents/ mL to start the radical-antioxidant reaction. The final concentration was 100 μM for DPPH• and the final reaction volume was 2.0 mL. The absorbance at 517 nm was measured against a blank of pure methanol at 0.67, 3, 6, 10, 20, 50, 80, and 1440 min of the reaction, and used to estimate the remaining radical. The absorbance at 517 nm after 10 minutes of reaction was used to compare the DPPH• scavenging capacities of individual oil extracts at 40 mg oil equivalents/mL. Dose and time dependencies of the seed oil extracts and DPPH• reactions were demonstrated by plotting the percent of DPPH• remaining against time for each level of the seed oil extract tested.

ORAC Assay

ORAC values were determined using a previously described protocol (*7*). The fluorescent probe was fluorescein (FL), and the complete assay mixture contained 0.067 μM FL, 60 mM of AAPH, 300 μL of oil extract or methanol

blank. Fluorescence measurements of the assay mixtures were recorded every minute and the area under the curve of fluorescence versus time was calculated and compared against a standard curve prepared with trolox.

Total Phenolic Contents (TPC)

TPC was determined using Folicin-Ciocalteu's (FC) reagent with gallic acid as the phenolic standard (*2*). The reaction mixture contained 250 μL of freshly prepared FC reagent, 0.75 mL of 20% Na_2CO_3, and 3 mL of pure H_2O to which 50 μL of oil extract or standard was added to start the reaction. Absorbance was measured at 765 nm after two hours of reaction at ambient temperature and used to calculate the TPC of oil extracts.

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Statistical Analysis

Data were reported as mean ± standard deviation. Analysis of variance and Tukey's post hoc test was conducted to identify differences among means. Statistical significance was declared at $P < 0.05$.

Results and Discussion

Seed oils and products containing seed oils are rich in fat soluble vitamins, provitamins, and antioxidants. Some of the vitamins/provitamins and antioxidants include tocopherols, carotenoids, and phenolic acids, which may provide health benefits by protecting cellular components against free radical damage. They may protect molecules such as fatty acids, phospholipids, and proteins, etc. by quenching free radicals before their attack. They may benefit overall human heath and reduce the risk of age-related diseases including cancer and atherosclerosis. Because of the health concerns related to synthetic antioxidants, there is an increasing demand for natural antioxidants because they are free of added chemicals and may provide additional health benefits in functional foods and supplements. Our current investigation examined the tocopherol and carotenoid compositions, radical scavenging activities, and total phenolic content (TPC) of cold-pressed onion, parsley, cardamom, mullein, roasted pumpkin, milk thistle, blueberry, red raspberry, marionberry and boysenberry seed oils to determine their potential use as health promoting ingredients. The oil extracts were tested for their radical scavenging activity against the stable $DPPH^{\bullet}$ and the peroxyl radical (ORAC). TPC were also

examined for these seed oils as TPC is possibly related to the total antioxidant capacity.

Carotenoids are well known for their health beneficial potentials. The cold-pressed seed oils were measured for their carotenoid composition including β-carotene, lutein, cryptoxanthin, and zeaxanthin. Zeaxanthin was the primary carotenoid in all the tested cold-pressed seed oils. The roasted pumpkin seed oil demonstrated significantly higher concentrations of all carotenoids compared to the rest (Table I). Boysenberry seed oil had the highest concentration among the berry seed oils in β-carotene, lutein, and zeaxanthin, however, blueberry seed oil had the highest cryptoxanthin concentration compared to the other berries. The cold-pressed parsley seed oil contained very high levels of carotenoids and was the second behind roasted pumpkin seed oil in total carotenoids. Cardamom seed oil contained very little total carotenoids compared to the others (Figure 1).

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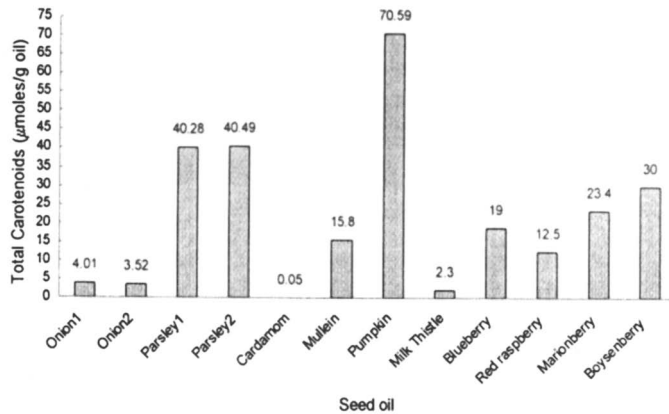

Figure 1. Total carotenoids. Total carotenoids in μmols/kg oil from β-carotene, lutein, cryptoxanthin, zeaxanthin. The vertical bars represent the SD (n = 3). Values marked by the same letter are not significantly different (P<0.05).

Alpha-, δ-, and γ-tocopherols, along with total tocopherols, were also determined for the cold-pressed seed oils. The onion seed oil had a significantly higher α-tocopherol concentration than all other seed oils (Table II). Boysenberry seed oil had the most γ-tocopherol, δ-tocopherol, and total tocopherols (Table II, Figure 2) compared to all others, and interestingly, the boysenberry and red raspberry seed oils, both from the genus, '*Rubus*,' had significantly more γ-tocopherol than the rest. These data suggested that these cold-pressed edible seed oils may contribute to the total dietary tocopherol intakes.

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Table I. Carotenoid Contents in the Cold-pressed Seed Oils*

	β-Carotene (μg/kg)	*Lutein (μg/kg)*	*Zeaxanthin (μg/kg)*	*Cryptoxanthin (μg/kg)*	*Total Carotenoids (μmoles/kg)*
Onion	nd	17.3f ± 0.3	1743.4f ± 13.5	507.8f ± 1.4	4.01
Parsley	783.8d ± 4.2	216.4b ± 2.4	20400.1b ± 128.4	1428.9d ± 19.1	40.28
Pumpkin	5957.6a ± 108.2	270.1a ± 1.1	28523.1a ± 508.4	4909.2a ± 8.1	70.59
Milk Thistle	nd	nd	1315.2g ± 0.6	nd	2.30
Blueberry	1352.3c ± 4.4	60.6e ± 0.0	7800d ± 600	1486.8c ± 1.8	19.0
Red Raspberry	82.2e ± 1.8	78.6d ± 0.5	5100e ± 300	1812.9b ± 11.6	12.5
Boysenberry	2405b ± 3.3	97.7c ± 1.0	13600c ± 300	717.6e ± 34.8	30.0

*Carotenoid content of each cold-pressed seed oils was reported as mean ± SD (n = 3). Different letters within columns represent significant difference ($P < 0.05$). nd: not detected.

Table II. Tocopherol Contents in the Cold-pressed Seed Oils*

	α-Tocopherol (mg/kg)	γ –Tocopherol (mg/kg)	δ-Tocopherol (mg/kg)	Total Tocopherols (μmol/kg)
Onion	681.9a±8.4	219.2c ± 4.6	28.6d ± 0.1	1973.8
Parsley	29.5e±0.3	2.8f ± 0.2	0.9h ± 0.0	77.6
Mullein	27.1f±0.2	213.3c ± 1.9	76.2c ± 0.2	759.4
Pumpkin	26.8f±0.9	216.3c ± 2.4	19.2e ± 0.0	625.6
Milk Thistle	156.3b±0.9	35.1d ± 0.4	7.0f ± 0.0	464.2
Blueberry	71.1d±1.1	33.6e ± 0.6	6.0g ± 0.0	260.6
Red Raspberry	150.9c±1.6	558.7b ± 7.7	178.9b ± 0.4	2135.4
Boysenberry	20.8g±0.1	688.6a ± 5.9	232.0a ± 2.1	2276.9

* Tocopherol contents were reported as mean ± SD (n = 3). Different letters within columns represent significant difference ($P < 0.05$).

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The cold pressed seed oil extracts were tested for their DPPH radical scavenging abilities and compared against each other under the same experimental conditions (Figure 3). Boysenberry seed oil extract was significantly superior to all of the other cold-pressed seed oil extracts, quenching more than 93 % of the DPPH$^{\bullet}$ in the reaction vessel in 10 min of reaction. Parsley seed oil extract was also very effective, quenching 86.6-90.8 % of the DPPH$^{\bullet}$. Milk thistle and roasted pumpkin seed oil extracts had the lowest DPPH$^{\bullet}$ scavenging activity; however all of the tested oil extracts significantly quenched DPPH radicals. The time and dose effects of the oil extracts-DPPH$^{\bullet}$ reactions were evaluated. All the oil extract-DPPH$^{\bullet}$ reactions were time and dose dependent. The time and dose effects for the parsley seed oil extract-DPPH$^{\bullet}$ reactions were demonstrated in Figure 4, whereas that for the blueberry seed oil-DPPH$^{\bullet}$ reactions were demonstrated in Figure 5.

It has been noted that radical system employed may alter the antioxidant activity estimation results, and two or more free radical systems have to be used for radical scavenging activity evaluation. The oxygen radical absorbance capacity (ORAC) test was used to determine the peroxyl radical scavenging activities of the cold-pressed edible seed oils. The strongest ORAC of 537 μmol TE/g was observed in the cold-pressed parsley seed oil (Figure 6). TE stands for trolox equivalents. Trolox is a water soluble vitamin E derivative and used as antioxidant standard. The ORAC value of the parsley seed oil was significantly higher than the ORAC values for cold-pressed black caraway (220 μmol TE/g oil), carrot (160 μmol TE/g oil), and hemp (28.2 μmol TE/g oil) seed oils (*4*). Cold-pressed boysenberry seed oil demonstrated the highest ORAC value among the berry seed oils, and was approximately 15 times higher than the cold-pressed blueberry seed oil (Figure 6).

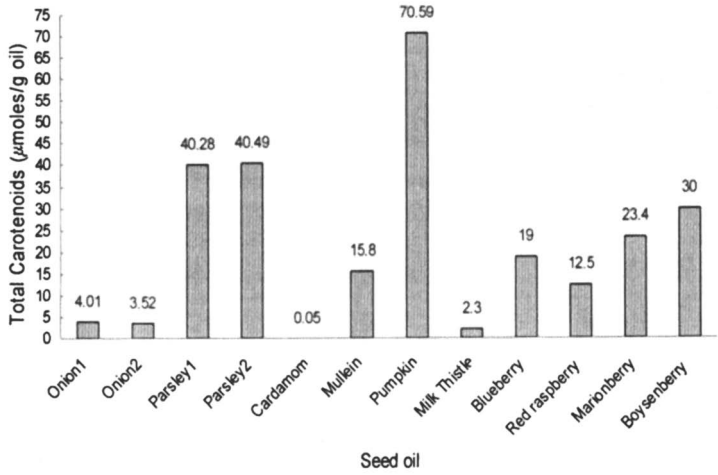

Figure 2. Total tocopherols. Total tocopherols in μmols / kg oil from α-tocopherol, δ-tocopherol, and γ-tocopherol. The vertical bars represent the SD (n = 3). Values marked by the same letter are not significantly different (P < 0.05).

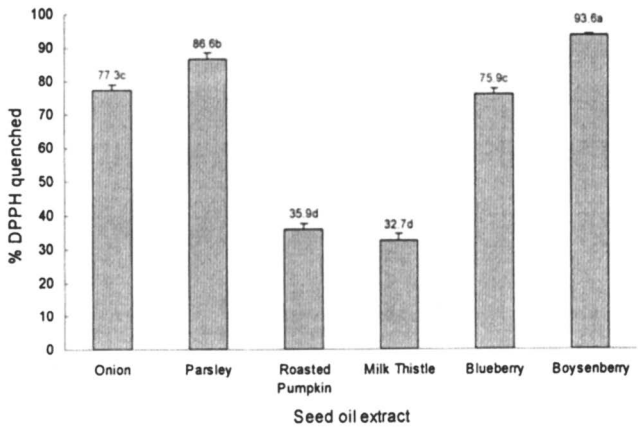

Figure 3. Dose and time effects on DPPH scavenging activity of cold-pressed parsley seed oil extract. Control, 6.7, 8, 10, 20, and 40 represent the final parsley seed oil extract concentrations of mg oil equivalent/mL in the reaction mixtures. The initial DPPH radical concentration was 100 μM in all reaction mixtures. All tests were conducted in triplicate

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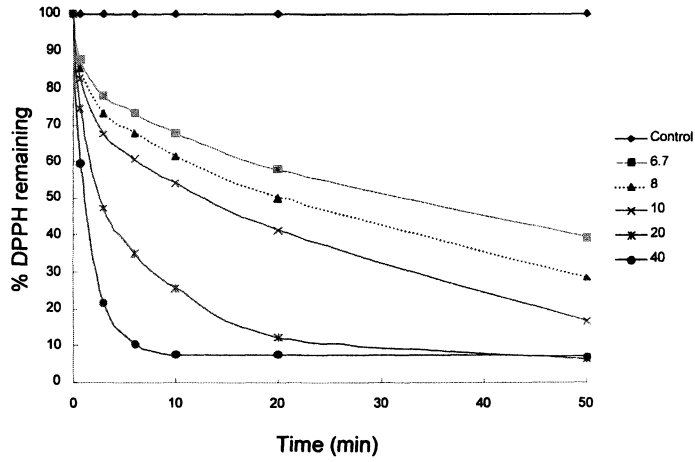

Figure 4. Dose and time effects on DPPH scavenging activity of cold-pressed parsley seed oil extract. Control, 6.7, 8, 10, 20, and 40 represent the final parsley seed oil extract concentrations of mg oil equivalent/mL in the reaction mixtures. The initial DPPH radical concentration was 100 μM in all reaction mixtures. All tests were conducted in triplicate.

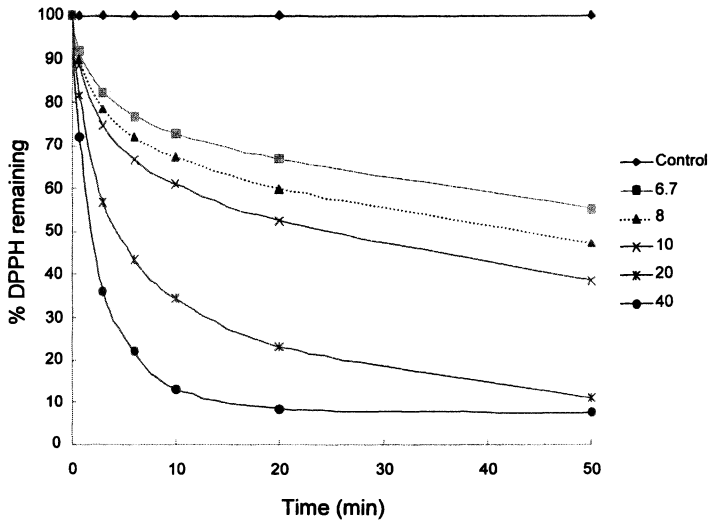

Figure 5. Dose and time effects on DPPH scavenging activity of blue berry seed oil extract. Control, 6.7, 8, 10, 20, and 40 represent the final parsley seed oil extract concentrations of mg oil equivalent/mL in the reaction mixtures. The initial DPPH radical concentration was 100 μM in all reaction mixtures. All tests were conducted in triplicate.

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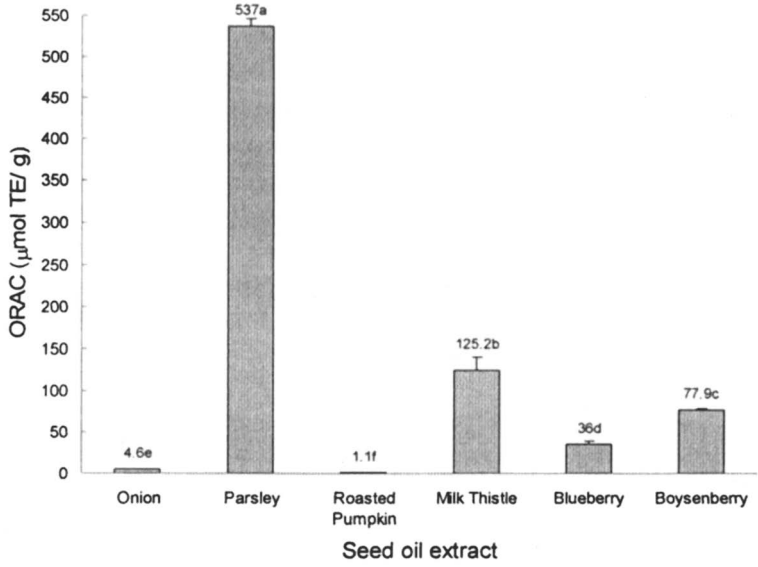

Figure 6. ORAC of selected cold-pressed edible seed oils. TE stands for trolox equivalents. Trolox is a water soluble vitamin E derivative and used as a common antioxidant standard. The vertical bars represent the SD (n = 3). Values marked by the same letter are not significantly different (P < 0.05).

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Total phenolic contents (TPC) of the oil extracts were also determined, since phenolic compounds have demonstrated antioxidative abilities and may contribute to the overall antioxidant capacity of the cold-pressed edible seed oils. The cold-pressed milk thistle seed oil demonstrated a very high TPC value of 3.1 mg GAE/g (Figure 7). However, this value was significantly lower then the TPC of the cold-pressed black caraway seed oil (3.5 GAE mg/g oil), but higher than the cold-pressed carrot, cranberry, and hemp seed oils (*4*). The cold-pressed roasted pumpkin seed oil had the lowest TPC (Figure 7).

In conclusion, all of the tested cold-pressed seed oils contained significant levels of tocopherols and carotenoids, although their contents may vary widely. Roasted pumpkin seed oil is an excellent source of carotenoids containing a very high level of total carotenoids. Onion seed oil contains a high concentration of α-tocopherol and may be used to significantly enhance dietary vitamin E intake at low doses while boysenberry seed oil had the most λ and total tocopherols. All of the tested seed oils have significant antioxidant activities, especially in parsley

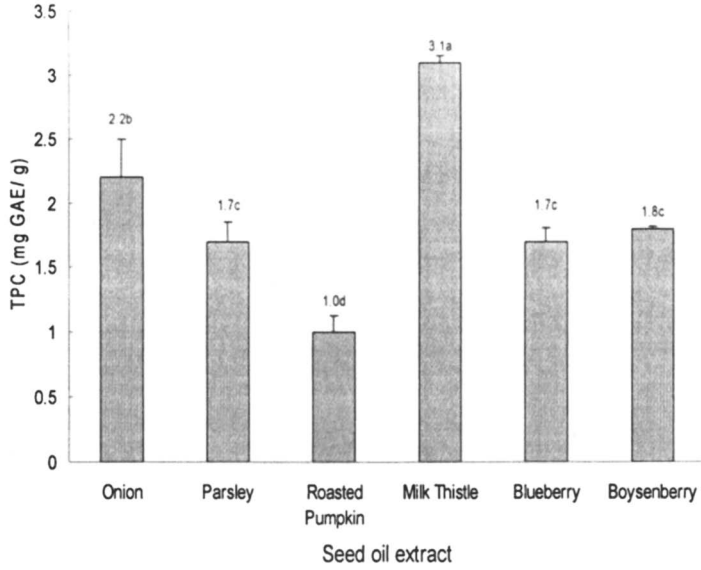

Figure 7. TPC of the selected cold-pressed edible seed oils. GAE stands for gallic acid equivalents. Gallic acid was used as the standard phenolic compound for TPC estimations. The vertical bars represent the SD (n = 3). Values marked by the same letter are not significantly different (P < 0.05).

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and boysenberry seed oils. The results indicate that these cold-pressed edible seed oils may serve as excellent dietary sources of phytochemicals and may be used as natural food ingredients to enhance the nutritional value of foods and benefit human health and well being.

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Chapter 19

Antioxidants from Edible Seaweeds

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There is a long history of seaweed consumption including *Phaeophyceae*, *Chlorophyceae* and *Rhodophyceae* taxonomies in Asia and the Pacifics, versus low to zero intakes in Europe and the Americas. This dietary difference between populations coincides with dramatic differences in diet-related chronic disease risks such as breast cancer in these groups. Animal model evidence points to a role for edible algae in antioxidant-mediated effects *in vivo*. As intertidal organisms, seaweeds require an endogenous antioxidant capacity to protect against oxidative stress from UV light and dessication during tidal fluctuations. The antioxidant capacity of algae include L-ascorbic acid, glutathione, carotenoids, tocopherols, chlorophyll derivatives, polyphenols such as the phlorotannins in brown kelp and mycosporine-like amino acids in red algae. This chapter reviews the evidence underlying the antioxidant mechanisms of these algal constituents and discusses various issues for their analysis.

Interest in naturally sourced antioxidant compounds continues to grow in the food, beverage and cosmetic industries not only as ingredients to take advantage of protective effects against lipid peroxidation in extending product shelf-life, but also as bioactive components of functional foods and nutraceuticals to reduce risk factors of diet-related chronic diseases such as breast, colon and prostate cancer or atherosclerosis. While well known sources of polyphenolic antioxidants in the lay and scientific literature include herbs and spices, various teas, fruits and vegetables, oilseeds and various alcoholic

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beverages, an underused source of antioxidant molecules in Europe and the Americas are the edible alga or seaweeds, sometimes referred to as sea vegetables. On the other hand, edible seaweeds from the *Protista* taxonomies *Phaeophyceae* (brown (B)), *Chlorophyceae* (green (G)) and *Rhodophyceae* (red (R)) are common in Pacific and Asian diets. Seaweeds are incorporated into these diets as sushi wrappings such as the red alga known in Japan as 'Nori' or Korea as 'Kim' (Laver, *Porphyra tenera* and *P. yezoensis*); seasonings and condiments such as the brown kelps in Japanese: 'Arame' (*Eisenia bicyclis*), 'Hijiki'(*Hijikia fusiformis*), 'Kombu' (*Laminaria digitata*), 'Wakame' (Sea Mustard, *Undaria pinnatifida*) or Chinese cuisines 'Hai dai' (*L .japonica*); green algae in Hawaiian cuisine 'Limu Pālahalaha' (*Ulva fasciata*); or snack foods and condiments such as the European/ North American red alga 'Dulse' (*Palmaria palmata*). Thus, while the bulk of research investigating the antioxidant activity and composition of edible seaweeds has taken place in China (*1,2*), Japan (*3-7*) and Korea (*8,9*), a few researchers are also active in this area in France (*10,11*) and Canada (*12-15*).

Much of the interest in the antioxidant capacity of algae has arisen from epidemiological evidence linking the habitual consumption of seaweed to reduced risk of particular chronic diseases in the Japanese and Chinese (including Korean, Mongolian and Hong Kong) populations (*16*). Japanese living in Japan typically consume between 0.4 to 29.2 g seaweed per day (approx. 10-25% of food intake) compared to the low to zero intake levels of migrant Japanese living in Hawaii as well as most Western populations (*17,18*). These population groups also have strikingly contrasted chronic disease incidences: the one-year prevalence cases per 100,000 for breast cancer in Japan and China were 42.2 and 13.1, respectively versus 125.9 and 106.2 in North America and Europe (*16*). Similarly, the one-year prevalence cases/100,000 statistics for prostate cancer in Japan and China were 10.4 and 0.7, respectively versus 117.2 and 53.1 in North America and Europe (*16*). Interestingly, one of the first references to a beneficial effect of seaweed on breast cancer was found in the ancient Egyptian 'Ebers Papyrus' dating back to approximately 1534 B.C. recommending that seaweed be administered as a treatment for breast cancer patients (*19*). Indeed there is evidence of antioxidant-mediated effects of dietary seaweeds *in vivo* such as increased antioxidant enzyme activity (GSH peroxidase) and reduced lipid peroxidation in hepatic tissue of rats treated with the mammary cancer carcinogen dimethylbenz[α]anthracene and fed on the brown kelp *L. religiosa* (*20*); as well as the inhibition of superoxide anion radical production by peritoneal activated leukocytes incubated with a sulfoglycolipid fraction from the red alga *Porphyridum creuntum* (*21*). Therefore, it is not surprising, that similar to terrestrial plants, seaweeds as intertidal organisms require an endogenous antioxidant capacity to protect plant cell membranes and organelle constituents against oxidative stresses associated with UV radiation (*22-24*) and dessication from tidal fluctuations (*25*). Indeed, algal antioxidants comprise enzymatic defenses (e.g. superoxide dismutase (SOD), glutathione reductase (GSSG-Red));

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aqueous and lipophilic molecules including L-ascorbic acid, glutathione (GSH), carotenoids, tocopherols and chlorophyll derivatives (e.g. chlorophyll a, pheophytin a) as well as secondary metabolites such as flavan-3-ols, phenolic acids, lignans, phlorotannins (phloroglucinol polymers) in brown kelp and mycosporine-like amino acids (e.g. mycosporine-glycine and shinorine) in red algae (Table 1). This chapter reviews the evidence and mechanisms underlying the antioxidant activities of edible brown, red and green seaweed constituents. It is noteworthy that antioxidant activity has been reported in both polar as well as lipophilic fractions isolated from these seaweeds; thus, this chapter will also address methodological considerations when analyzing these data where appropriate.

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Aqueous low-molecular-weight reducing agents – L-ascorbic acid and glutathione

Reducing activity, defined as the donation of electrons to another chemical; the supply of a hydrogen to another chemical; or the removal of oxygen from another chemical is key to maintaining the redox potential of intracellular systems, particularly in the presence of the potential for generation of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide radical anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical ($^{\cdot}OH$) during normal oxidative metabolism. Nonenzymatic aqueous low-molecular-weight reducing agents include ascorbic acid and glutathione (GSH, L-γ-glutamyl-L-cysteinyl-glycine) *in vivo* in human and animal tissues (*26*) as well as terrestrial and marine plants (*25,27,28*). A survey of the GSH contents of a variety of seaweeds harvested from the coast of Mie Prefecture, Japan, was recently carried out by Kakinuma and coworkers (*28*); GSH content was 0.1 200 mg/100 g dry wt in *Rhodophyceae*, 19-3082 mg/100 g dry wt in *Phaeophyceae* and 2-113 mg/100 g dry wt in *Chlorophyceae*. The one extremely low GSH content amongst the red algae was due to *Corallina pilulifera*, a species unusual for its highly calcified tissue, otherwise the range for red algae was 21-200 mg/100 g dry wt. Other workers investigating the red alga *Stictosiphonia arbuscula*, harvested in Brighton Beach, Otago, New Zealand, reported that ascorbate and GSH levels in fresh specimens were approx. 2.5 - 2.8 and 0.69 - 0.71 μmol/g wet wt., respectively (*25*). Fresh Dulse has been reported to contain 220-520 μg ascorbic acid/g wet wt. harvested from Spitsbergen, Norway and unknown locations in France and elsewhere (*24,27*). Fresh specimens of *Laminaria sp.* (B) harvested from Spitsbergen, Norway exhibited lower (170 μg /g wet wt) or only trace amounts of ascorbic acid. However, the air- and sun-drying, as well as storage of Dulse is known to reduce the vitamin C content measured in samples (*27*). The vitamin C content of Dulse harvested from an unknown location in Japan was approximately 2000 μg/g dry wt (*29*). Similarly, when McDermid and Stuercke (*30*) surveyed 22 species of algae harvested from various locations in the Hawaiian Islands; the vitamin C content of the oven-

dried samples (60°C) was minimal with detectable levels reported in only 4 samples: between 1300 and 3000 μg/g dry wt in the *Chlorophyceae*: *Enteromorpha flexuosa*, *Monostroma oxyspermum*, *Ulva fasciata* and 2000 μg/g dry wt in *Eucheuma denticulatum* (R). The vitamin C content of the brown alga *Ascophyllum nodosum* harvested from an unknown location was 550-1650 μg/g dry wt (*29*). The total content of combined reduced and oxidized ascorbate was not altered in dessicated *S. arbuscula*, but the concentration of reduced ascorbate did decline at 12 h and remained low at 48 h of dessication (*25*). Thus, aqueous low-molecular-weight reducing agents are likely most important to the oxidative status of algae prior to harvest, and are found in low concentrations in the dehydrated and processed products available to the consumer purchasing these products.

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As a reflection of the potential bioactivity of the aqueous low-molecular-weight antioxidants discussed above, the reducing activity of water- and alcohol-soluble extracts from a variety of edible algae has been assessed using a variety of *in vitro* methodologies including the potassium ferricyanide assay of Yen and Chen (*31*), the hydroxyl radical scavenging deoxyribose assay of Halliwell and coworkers (*32*) as well as stable free radicals such as 1,1-diphenyl-2-picrylhydrazyl (DPPH•; *33*) and 2,2'-azino-bis (3-ethyl-benzthiazoline-6-sulfonic acid) radical cation ($ABTS^{\$+}$; *34*). For example, water extracts of 27 species of edible and non-edible seaweeds harvested in Shandong Province, PRC demonstrated hydroxyl radical scavenging activity in green, brown and red varieties, with the strongest activities in *Rhodomela teres* (R) and *Chorda filum* (B; *2*). Methanol extracts of these same seaweeds demonstrated relatively weak DPPH radical scavenging activities after 60 and 90 min incubation, with the strongest activities in *Phaeophyceae*: *Desmarestia viridis*, *Sargassum kjellmanianum* and *C. filum*. Similarly, a study conducted in Seoul, Korea with crude and fractionated methanol extracts of seaweeds indicated strong DPPH• scavenging activity in *U. pinnatifida* (B), *Enteromorpha sp.* (G), *P. yezoensis* (R) and *Dasya pedicellata* (R; *8*). Matsukawa and coworkers (5) reported that aqueous extracts of seaweeds harvested in Onagawa, Miyagi Prefecture, Japan, generally exhibited lower inhibition of DPPH radical compared to ethanolic extracts after 15 min incubation, regardless of seaweed variety. The DPPH• scavenging activity of the brown kelps was greater than that of the red or green algae studied, with the exception of *Porphya sp.* which exhibited strong inhibition of DPPH•. More recently, a 1-butanol soluble extract from Dulse (*P. palmata*) harvested in Grand Manan Island, New Brunswick, Canada, was reported to exhibit moderate $^{\bullet}OH$ and DPPH• scavenging activities compared to L-ascorbic acid and butylated hydroxyanisole (BHA) controls (*13*). It is noteworthy that the DPPH• scavenging activity of the Dulse extract was observed to take place over 105 min compared to 15 and 50 min for L-ascorbic acid and BHA, respectively. Thus, it is important to consider the assay incubation times when comparing DPPH• scavenging activities from different studies, as an insufficient incubation period may influence the results. Moreover, despite being widely used by researchers to evaluate the free radical scavenging

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Table 1. Antioxidant profile of edible seaweed varieties.

Seaweed class	Common name	Harvest location	Antioxidant compounds	References
Chlorophyceae				
Enteromorpha sp.	'Aonori'	Japan	Pheophytin a	53
Halimeda macroloba	'Hiroha-sabotengusa'	Okinawa Prefecture, Japan	Epigallocatechin, caffeic acid, Catechol, Hesperidin, Myricetin, Morin.	47
Halimeda opuntia	'Sabotengusa'	Okinawa Prefecture, Japan	Epigallocatechin, caffeic acid, Catechol, Hesperidin, Myricetin, Morin.	47
Phaeophyceae				
Ascophyllum nodosum		Pleubian, France	α-, δ-, γ-tocopherol	11
Ecklonia cava	"Kajime'	Jeju Island, Korea	Phlorotannins – phloroglucinol, eckol, dieckol, 6,6'-bieckol, phlorofucofuroeckol A.	9
Ecklonia kurome		Itoshima Peninsula, Japan	Phlorotannins – phloroglucinol, eckol, dieckol, 8,8'-bieckol, phlorofucofuroeckol A, unknown tetramer, other unknowns.	7

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Eisenia bicyclis	'Arame'	Tsuzumi island, Japan; Itoshima Peninsula, Japan	Phlorotannins – phloroglucinol, eckol, dieckol, 8,8'-bieckol, phlorofucofuroeckol A, unknown tetramer, other unknowns	53, 7
		Jogashima, Kanagawa Prefecture, Japan	Phospholipids – Phosphatidyl ethanolamine Phosphatidyl inositol	3
Fucus vesiculosus		Pleubian, France	α-, δ-, γ-tocopherol	11
Fucus serratus		Pleubian, France	α-, δ-, γ-tocopherol	11
Hijikia fusiformis	'Hijiki'	Store-bought, Tsukuba, Japan	Fucoxanthin, Phlorotannins	41
		Store-bought, Toronto, Canada	Mammalian lignans – Enterodiol, Enterolactone	12
Himanthalia elongata		Pleubian, France	α-, γ-tocopherol L-ascorbic acid	11, 10
Laminaria digitata	'Kombu'	Pleubian, France	α-, γ-tocopherol, L-ascorbic acid	11, 10

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Table 1. ***Continued.***

Seaweed class	Common name	Harvest location	Antioxidant compounds	References
Laminaria japonica	'Makonbu' 'Hai dai'	Qingdao, PRC	Chlorophyll a, L-ascorbic acid	61
Sargassum kjellmanianum		Taipingjiao, Qingdao, PRC	High and low MW Phlorotannins	1,2
Undaria pinnatifida (stipe)	'Wakame' 'Sea mustard'	Onagawa, Miyagi Prefecture, Japan	Fucoxanthin	56
Undaria pinnatifida (sporophyll)	'Mekabu'	Store-bought, Toronto, Canada	Mammalian lignans – Enterodiol, Enterolactone	12
Rhodophyceae				
Polysiphonia urceolata		Miyagi Prefecture, Japan	Bromophenols – 5-bromo-3,4-dihydroxybenzaldehyde 5-bromo-3,4-dihydroxybenzyl alcohol	56
Palmaria palmata	'Dulse'	Spitsbergen, Norway; Minami-Kayake, Hokkaido, Japan	MAAs – Mycosporine-glycine, Shinorine, Porphyra-334, Palythine, Asterina-330, Palythinol, Palythene, Usujirene.	22, 62
		Isle of Wight, UK	Lutein	40

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Porphyra tenera	'Laver' 'Nori' 'Kim'	Store-bought, Sendai, Japan	Phospholipids – Phosphatidyl choline Phosphatidyl ethanolamine	36
		Ota-ku, Tokyo, Japan	MAAs – Mycosporine-glycine, Shinorine, Porphyra-334	59
Porphyra yezoensis	'Susbinori'	Hyogo, Japan	MAAs – usujirene, β-carotene, Chlorophyll a, Pheophytin a, Amino acids - Alanine, Leucine, Phenylalanine	6

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activity of plant extracts, DPPH• does not solubilize easily, albeit, it does generate strongly colored solutions with methanol and ethanol as solvents (*13*). Re and coworkers (*34*) reported that the decolorization of the ABTS•$^{+}$ radical cation reflected the capacity of an antioxidant species to donate electrons or hydrogen atoms to inactivate this stable free radical. The ABTS•$^{+}$ radical cation scavenging activity of the Dulse extract and controls was very similar to the DPPH• results, reflecting the reaction stoichiometry of the number of electrons available to inactivate the free radical in the assay (*13*). For example, slowly reacting free radical scavengers such as butylated hydroxytoluene (BHT), or the closely related BHA, and the Dulse extract, are hypothesized to have a more complex reaction mechanism involving one or more secondary reactions in the quenching of the DPPH• (*35*) and ABTS•$^{+}$ radicals.

Essential considerations when determining the reducing activity of seaweeds are the topographic features of the geographic location and water depth of the harvest location of these intertidal species. Seaweeds are exposed to wide variations in photosynthetically available radiation (PAR; 400-700 nm) and more specifically, UVB (280-320 nm) and UVA (320-400 nm) radiation depending upon the season, water depth and turbidity as well as the presence of topographic features such as steep cliff faces (*14,22-25*). In fact, when Dulse was harvested from two locations known to vary in UV exposure, the sample from a sunny low-lying beach ('Passage' area of Grand Manan Island, NB) exhibited a 1.68 fold greater reducing activity than the corresponding sample from a shaded high-cliff area (Dark Harbour) on the opposite side of the island (*14*). Water depth appeared to influence the ability of *S. arbuscula* (R) to maintain GSH reserves upon dessication/dehydration, in that specimens from deeper water depths exhibited reductions in total GSH at 24 and 48 h of dessication, whereas specimens from shallow waters only experienced a decrease in GSH after 48 h (*25*). Thus, it is vital to collect water depth and topographic data about the harvest sites of seaweeds in order to compare data from different reports or even the same species from different locations.

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Lipophilic antioxidants – chlorophyll and its derivatives, carotenoids, tocopherols, and phospholipids

The importance of the presence of lipophilic antioxidants to the oxidative status of marine organisms such as algae is implicit to combat cell membrane damage against not only dessication during tidal fluctuations, but also photooxidative stress from UV radiation. This takes on particular importance given that the fatty acid profile of various edible seaweeds are rich in the long-chain polyunsaturated ω6 and ω3 fatty acids, including arachidonic acid (C20:4,ω6) and eicosapentaenoic acid (C20:5,ω3; 10,36-38). The fatty acid profiles of *Laminaria digitata* (B) and *Himenthalia elongata* (B) harvested from

Pleubian, France were enriched in C20:4, ω6 at 24.3 and 33.4%, respectively (10). Similarly, the kelps *L. ochroleuca* (B) and *H. elongata* (B) harvested from Bayona and Finisterre, Spain were richest in C20:4, ω6 at 14.20 and 10.69 % total fatty acids (*38*). Whereas *Rhodophyceae* such as commercial *P. tenera* (*36*), *Palmaria sp.* harvested from Rinlo, Spain (*38*) and *Porphyridium sp.* grown in culture (*37*) were rich in C20:5, ω3 at 40.8, 24.05 and 11.8 % total fatty acids, respectively. While chlorophylls are the major photosynthetic pigment in algae, carotenoids are also considered to be secondary photosynthetic pigments; thus, it is interesting to note that both classes of pigment also have antioxidant activity. Alpha- and β-carotene are well known as electron donors to free radicals and particularly as $^1O_2$ quenchers. Similarly, α-, γ- and δ-tocopherols are well known as electron or hydrogen donors as free radical scavengers as well as $^1O_2$ quenchers *in vitro* and *in vivo*. As photosynthetic pigments, tissue levels of chlorophyll a and carotenoids have been reported to vary seasonally depending upon UV irradiation exposure levels (*23,27*). When brown, red and green algae were harvested from Spitsbergen, Norway between early June (when the water was still covered by ice) and early August (well after sea-ice break-up), *L. saccharina* (B), the red alga Dulse and *Monostroma sp.* (G), as representative species, all exhibited greater levels of chlorophyll a (approx. 2.35 mg/ g wet wt) in early June compared to markedly lower levels (approx. 1.5 mg/g wet wt) in mid to late-July (*23*). Thus, algal tissues appear to concentrate chlorophyll a during periods of reduced UV radiation (i.e. ice cover, cloudiness) versus lesser pigmentation during periods of enhanced UV radiation (i.e. reduced turbidity, clear skies).

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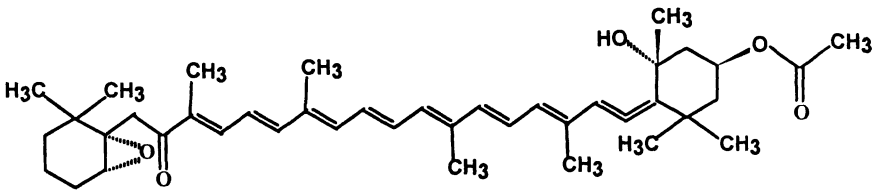
Carotenoids

In contrast to chlorophyll above, tissue levels of carotenoids (α- and β-carotene) in the red alga Dulse appear to peak during the Spring and Summer months (April to September) ranging between 110 and 420 μg/g dry wt in samples harvested from North Berwick, Scotland; Helgoland, Germany and unknown locations in Norway in comparison to the Winter (January) at 37 μg/ g dry wt (*27*). These data support a role for carotenoids as protective antioxidant molecules in algal tissues; indeed, carotenoids are known to provide photoprotection in plant tissues by preventing the photooxidation of chlorophylls since these pigments exist in close association with each other via weak non-covalent bonds (*39*). Tissue levels of β-carotene in algae harvested from different locations in the Hawaiian Islands varied widely depending on class, being 27-180, 60-97 and 15-430 IU/g dry wt in the *Chlorophyceae*, *Pheophyceae* and *Rhodophyceae*, respectively (*30*). Another factor which will impact the carotenoid levels reported in the literature include whether the data reflect total carotenoids, the separate identification of α- and β-carotene, or whether other carotenoids have also been determined, such as lutein, zeaxanthin or fucoxanthin (*27,40,41*). It is noteworthy that algal α- and β-carotene levels

can represent only approximately 1/3 to 1/8 of total carotenoid content in Dulse (*27*). For example, lutein has been reported to be a significant carotenoid in Dulse at 240 μg/g dry wt in a sample harvested from an unknown location in Norway (*27*) as well as that from Bembridge, Isle of Wight, UK (*40*). Moreover, all-*trans*-fucoxanthin, an orange colored carotenoid, has been reported to be a major carotenoid in *Hijikia fusiformis* (B) along with two *cis*-isomers of this powerful antioxidant (*41*).



Fucoxanthin

Fucoxanthin exhibits strong antioxidant activity against the DPPH radical. Interestingly, these results differ from a previous report wherein β-carotene, zeaxanthin and lutein did not scavenge the DPPH radical (*41*). Fucoxanthin has also been identified in the chloroform-soluble fraction of *Fucus vesiculus* (B) wherein it demonstrated antioxidant activity against lipid peroxidation, but did not exhibit any synergism with vitamin E (*11*). The structure of fucoxanthin differs from other carotenoids in that there is an unique double allenic carbon at C-7' near one of the two hydroxyl groups in its structure which may confer additional stability and resonance stabilization within the conjugated double bond structure of this molecule.

Tocopherols

Seaweed tissue levels of the tocopherols vary seasonally with typically low concentrations during Winter and Spring ranging between 22 and 35 μg/ g dry wt for the red alga Dulse and trace levels in *L. digitata* (B) compared to Summer and Fall levels of approximately 139 μg/g dry wt in Dulse and 2 % dry wt in *L. digitata*, similar to the phenomenon observed with the carotenoids above (*11,27*). The brown kelp *Ascophyllum nodosum* harvested from an unknown location was reported to contain 260-450 μg tocopherols/g dry wt (*29*). The tocopherol composition of various brown kelps (*L. digitata, H. elongata, F. vesiculus, F. serratus* and *A. nodosum*) has also been broken down into the α-, γ- and δ-isomers, with α-tocopherol making up the majority (*11*). Certainly, the tocopherol content of the organic solvent extracts of brown kelps was

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instrumental in the antioxidant effects observed against methyl linoleate oxidation when incubated with extracts of *L. digitata* harvested during the summer, which contained significant amounts of tocopherols, versus the winter when the alga contained only trace amounts of tocopherols (*11*).

Phospholipids

Despite the fact that the phospholipid (PL) fractions of algae are noted to be enriched in the long-chain polyunsaturated fatty acids, there is evidence of the antioxidant activity of these membrane lipids (*3,11,36,42*). Glycerophospholipids are characterized as lipids containing phosphoric acid as a diester. The phosphoric acid moiety of PL confers the ability to chelate transition metal ions, which may play a part in the antioxidant activity of these molecules. When Kaneda and Ando (*36*) investigated the antioxidant activity of the chloroform-soluble fraction of *P. tenera* (R), the PL fraction strongly inhibited the oxidation of the methyl esters of safflower oil fatty acids by extending the induction period on their own and also demonstrated synergistic effects when combined with butylated hydroxytoluene (BHT). Interestingly, the PL fraction of this red alga was enriched with C20:5, ω3 at 54.9 % fatty acids. When the PL fraction of *P. tenera* was separated into its component molecules, phosphatidylethanolamine and phosphatidylcholine predominated at 30.6 and 23.5 %, respectively, followed by phosphatidylserine (14.0%), lysolecithin (13.2%), sphingomyelin (9.9%) and phosphatidylinositol (4.5%). The chloroform-soluble fraction of *Eisenia bicyclis* (B) exhibited strong antioxidant activity against the oxidation of safflower oil fatty acid methyl esters at 37°C (*3*). Upon chromatographic separation of this extract, the PL fraction was shown to be responsible for the antioxidant activity; phosphatidylethanolamine and phosphatidylinositol were the main PLs. Other workers have reported that the antioxidant activity of PLs is due to synergism with other primary antioxidants such as the tocopherols (*11,42*). Indeed, crude organic solvent extracts of *L. digitata* (B) were not only effective against peroxide production in sunflower oil at 75°C on their own, but also exhibited synergistic effects in combination with vitamin E in extending the induction period during the oxidation of methyl linoleate at 60°C (*10*). Interestingly, despite the strong inhibition of methyl linoleate oxidation by organic solvent-soluble extracts of *F. vesiculus* (B), *F. serratus* (B) and *A. nodosum* (B) as well as their synergism with vitamin E, the purified phospholipid fractions of these kelps were not observed to be synergistic with vitamin E (*11*). The phospholipid fractions of these kelps were richest in phosphotidylinositol as opposed to phosphotidylethanolamine as observed in the algae above. Phosphatidylinositol lacks an amino-containing moiety necessary for phospholipid synergism with vitamin E as antioxidants (*11*). Thus, the synergism of phospholipids is specific to those which contain an

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amine group in their structure, i.e. phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine.

Chlorophyll derivatives

It is noteworthy that not only has the photosynthetic pigment chlorophyll a been identified as an antioxidant molecule in various algal extracts, but also that chlorophyll derivatives such as pheophytin a and pyropheophytin a have also been reported to be responsible for the antioxidant activity of organic solvent extracts of seaweeds. Chlorophylls contain a Mg^{2+} within the porphyrin structure (Figure 1) and are thus noted to provide a green-blue pigment to plant tissues. On the other hand, pheophytins are the Mg-free derivatives of chlorophylls which are most often formed during the heating of plant tissues in the presence of acid, leading to the loss of Mg^{2+} from the structure and an olive-brown coloration in tissues. Pyropheophytins are formed through the replacement of the C-10 carbomethoxy group of pheophytins by a hydrogen atom (Figure 1; *39*) leading to an olive colouration. Chlorophylls are classified as porphyrins or macrocyclic tetrapyrrole pigments: the four pyrrole rings are linked via methyne bridges forming a closed loop of conjugated double bonds. Thus, the conjugated system of double bonds can provide resonance stabilization to not only chlorophyll a, but also pheophytin and pyropheophytin a as potential antioxidant molecules reacting with free radicals. Moreover, chlorophylls are also noted to undergo a process called 'Allomerization' upon oxidation of the molecule with equimole O_2 uptake to the number of chlorophyll molecules present. Oxidation of chlorophylls occurs at the cyclopentanone ring forming either 10-hydroxychlorophylls or 10-methoxylactones (*39*). Studies evaluating the effect of chlorophyll and pheophytin on the stability of vegetable oil indicated that antioxidant activity was observed in chlorophyll containing oil samples stored at low temperatures in the dark (*42*). The antioxidant activities of pheophytin a and pyropheophytin a may also include indirect antioxidant activity through the chelation of transition metal ions due to the fact that in pheophytin, and thereby pyropheophytin, the two hydrogen atoms are easily displaced by Cu or Zn to form green coloured metallo complexes; moreover, Cu ions appear to be chelated more rapidly than those of Zn (*39*). Further work to elucidate the mechanism of antioxidant activity of chlorophyll a developed from initial results indicating that hexane-soluble extracts from *L. digitata* (B) and *H. elongata* (B) were protective against not only oxidation of methyl linoleate, but also exhibited synergism with vitamin E (*11*). These workers isolated two unidentified chlorophyll-related compounds from the hexane-soluble extract of *H. elongata* and chlorophyll a in *L. digitata*; chlorophyll a was previously reported to have synergism with vitamin E against lipid peroxidation. Le Tutour and coworkers (*11*) proposed that chlorophyll a can react with peroxyl radicals

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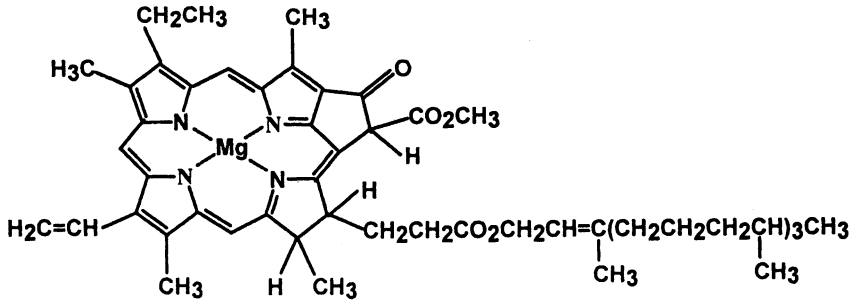
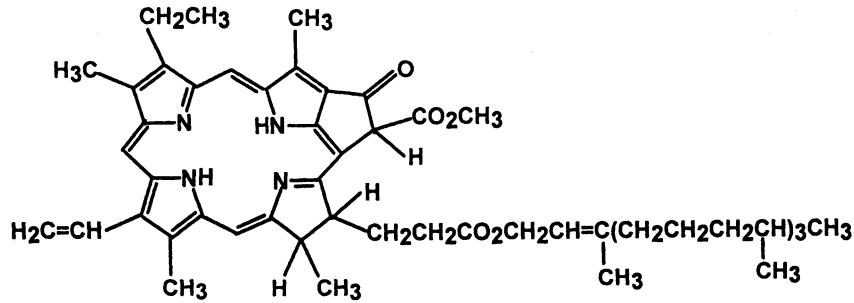
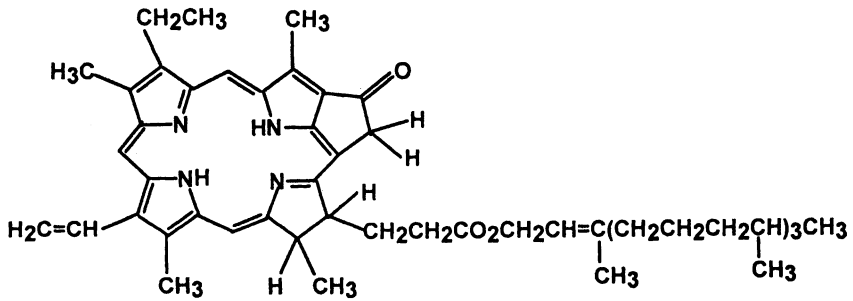


Chlorophyll a



Pheophytin a



Pyropheophytin a

Figure 1. Chlorophyll a and its derivatives.

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(ROO$^{\bullet}$) to form a complex with the π-cation radical of chlorophyll a bound to the negatively charged peroxyl radical:

$$ROO^{\bullet} + \text{chlorophyll a} \rightleftharpoons ROO^{-} + \text{chlorophyll a}^{\bullet+}$$

The complex can then react with another peroxyl radical to form inactive products

$$ROO^{\bullet} + \text{ROO–chlorophyll a}^{\bullet+} \longrightarrow \text{inactive products}$$

Moreover, the synergistic antioxidant effect of chlorophyll a in the presence of vitamin E was proposed to involve a non-reactive complex of an α-tocopheryl radical with chlorophyll a:

$$\alpha\text{-tocopheryl}^{\bullet} + \text{chlorophyll a} \longrightarrow \alpha\text{-tocopheryl–chlorophyll a}^{\bullet+}$$

which would then block further propagation of lipid peroxidation (*11*).

Polyphenolic antioxidants–flavonoids, phenolic acids, lignans, phlorotannins and bromophenols

As a broad class of compounds, polyphenols comprise the flavonoids including flavones, flavonols, flavanones, flavononols, chalcones and flavan-3-ols as well as lignans, lignins, tocopherols (discussed above), tannins and the related marine polyglucinol polymers (i.e. phlorotannins), plus phenolic acids. The flavonoids are synthesized via the Shikimic acid pathway from the aromatic amino acid precursors phenylalanine and tyrosine, giving rise to C_6-C_3 cinnamic acid derivatives (Figure 2). Cinnamic acid can then be metabolically converted to form flavonoids or various phenolic acids via *p*-coumaric acid and caffeic acid. Flavonoids are characterized by a C_6-C_3-C_6 skeleton as two aromatic rings bridged by a C_3 aliphatic chain often condensed into a pyran or furan ring. As well, flavonoids often occur as glycosides in plant tissues which are deglycosylated to the aglycones during digestion. The diversity of these compounds is also reflected in the antioxidant mechanisms involved such as ROS scavenging, free radical inactivation as well as transition metal ion chelation. It is well known that the position as well as the degree of hydroxylation influences the antioxidant activity of polyphenols. Thus, most monophenols are less effective than polyphenols; addition of a second hydroxyl group *ortho-* or *para-* to the first will increase the antioxidant activity (*42*). Interestingly, methoxy substitutions will also increase the antioxidant activity of a mono-phenol. However, methoxy substitution is noted to be less effective than hydroxylation at increasing antioxidant activity (*42*). In addition, cinnamic acid and its derivatives with the –CH=CHCOOH side chain are noted to be more

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efficient antioxidants than benzoic acid and its derivatives. Transition metal ion chelation can occur at the 3-hydroxyl, 4-keto group and/or the 5-hydroxyl, 4-keto group when the A ring is hydroxylated at the 5th position of flavonols (*43*). Generally, structures containing two or more of the following: -OH, -SH, -COOH, $-PO_3H_2$, C=O, $-NR_2$, -S- and -O- in a favorable structure-function configuration will exhibit transition metal ion chelation activity (*44*). Thus, the flavonol quercetin and its glycoside, rutin are noted to chelate transition metal ions (*45*).

Yoshie and coworkers (*46*) surveyed the flavan-3-ol profiles of Japanese green, brown and red algae, and found that catechin was present in only two *Chlorophyceae*: *Acetabularia ryukyuensis* (3330 μg/g dry wt) and *Tydemaniz expeditionis* (250 μg/g dry wt). It was also present as *Phaeophyceae* including *E. bicyclis* (1240 μg/g dry wt), albeit only trace amounts in *L. religiosa* and none in *U. pinnatifida* and *H. fusiformis*, the most commonly consumed kelps in Japanese cuisine; it was also present in most *Rhodophyceae* including *P. yezoensis* (36 μg/g dry wt). The catechin epimer, epicatechin (EC), was found in only five algae: *A. ryukyuensis* (500 μg/g dry wt), *E. bicyclis* (3860 μg/g dry wt), *Sargassum muticum* (B; 2620 μg/g dry wt), *Gelidium elegans* (R; 130 μg/g dry wt) and *Chondrococcus hornemannii* (R; 360 μg/g dry wt). The corresponding gallate esters were not widespread: catechin gallate was found in only *G. elegans* (70 μg/g dry wt) and epicatechin gallate (ECG) in *E. bicyclis* (290 μg/g dry wt) and *S. muticum* (120 μg/g dry wt). Epigallocatechin (EGC) was found in only one *Chlorophyceae*: *T. expeditionis* (350 μg/g dry wt); most *Phaeophyceae* including *E. bicyclis* (4770 μg/g dry wt), *H. fusiformis* (3770 μg/g dry wt), but none in *U. pinnatifida* nor *L. religiosa*; a few Rhodophyceae including *C. hornemannii* (16,000 μg/g dry wt), but not *P. yezoensis* (*46*). Epigallocatechin gallate (EGCG) was absent from all of the *Chlorophyceae* investigated and present in only three *Phaeophyceae*: *E. bicyclis* (280 μg/g dry wt), *Padina arborescens* (680 μg/g dry wt) and *P. minor* (490 μg/g dry wt); and in only three *Rhodophyceae*: *P. yezoensis* (32 μg/g dry wt), *Gracilaria texorii* (24 μg/g dry wt) and *Gracukarua asiatica* (18 μg/g dry wt). Taken together, these data suggest that the *Chlorophyceae* are relatively poor sources of the flavan-3-ols; as well, the flavan-3-ol gallate esters are not well represented amongst the Japanese edible algae.

Edible algae polyphenols with respect to their hydroxylated cinnamic acid derivatives, flavonols, flavanones and their glycosides have also been investigated (*47,48*). Analysis of two *Chlorophyceae* currently not used for food, revealed that *Halimeda macroloba* and *H. opuntia* were rich in EGC (28000 and 12700 μg/g dry wt, respectively), catechol (1880 and 384 μg/g dry wt), the flavonols myricetin (414 and 147 μg/g dry wt) and morin (429 and 234 μg/g dry wt; *47*). In addition, *H. macroloba* also contained caffeic acid (84.9 μg/g dry wt) and the flavanone glycoside hesperidin (hesperitin-7-rhamnoglucoside; 144 μg/g dry wt). While most Japanese edible green, brown and red algae were found to be rich in the flavonol morin, ranging between 257

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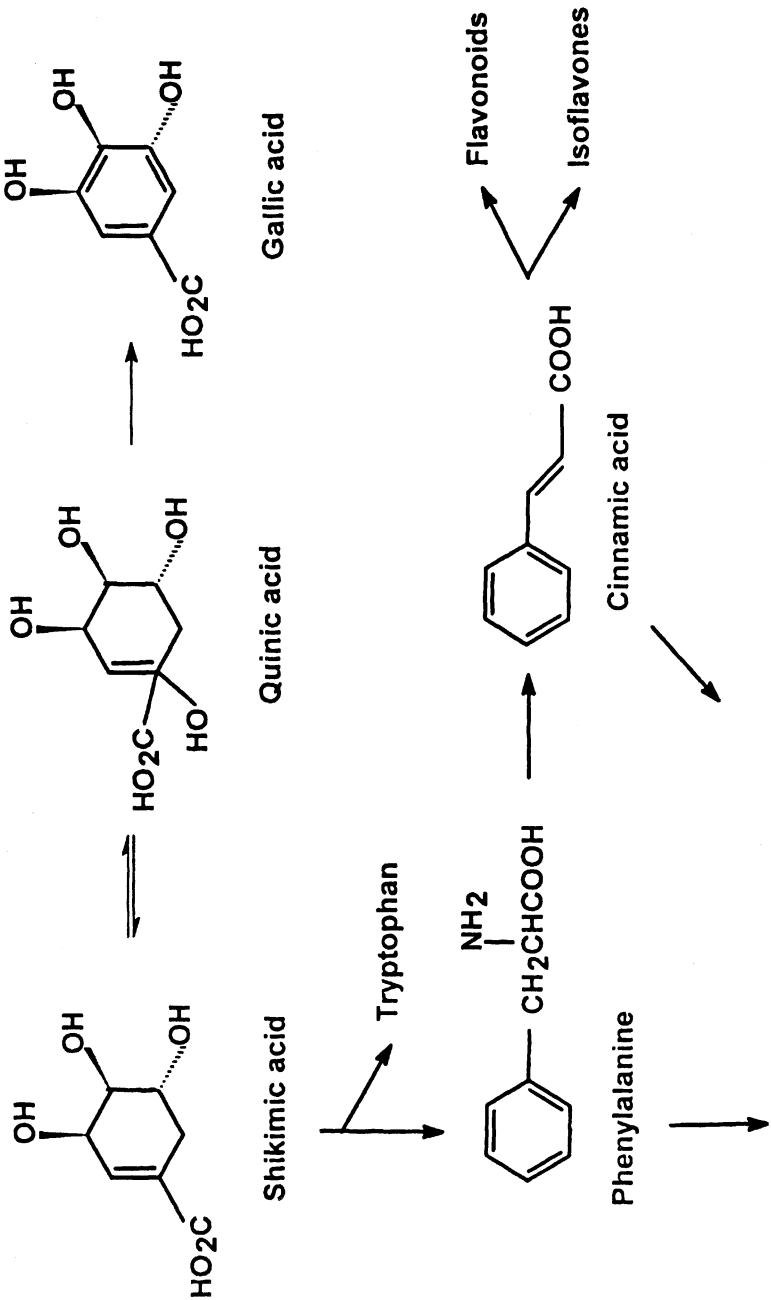

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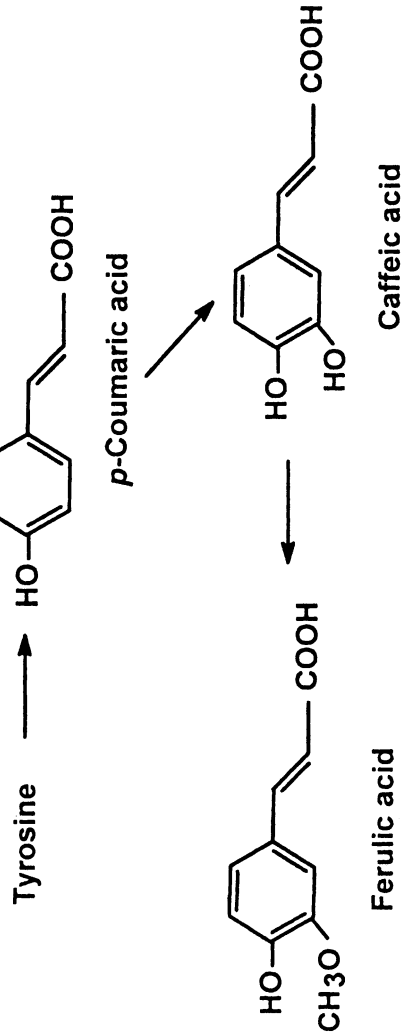

Figure 2. Shikamic acid pathway synthesis of phenolic acids.

and 2470 μg/g dry wt across the species, only two algae contained myricetin: *Tubinaria ornata* (B; 346 μg/g dry wt) and *Chondrus verruscosus* (R; 270 μg/g dry wt; *48*). Japanese green, brown and red algae were rich in catechol, ranging from a low of 241 μg/g dry wt in *L. religiosa* (B) to a high of 77,700 μg/g dry wt in *Caulerpa serrulata* (G). However, caffeic acid was measured in only one *Chlorophyceae*: *A. ryukyuensis* (317 μg/g dry wt); two *Phaeophyceae*: *U. pinnatifida* (53.6 μg/g dry wt) and *Ishige okamurae* (149 μg/g dry wt); and most *Rhodophyceae* including *P. yezoensis* (46.8 μg/g dry wt), *G. elegans* (132 μg/g dry wt) and *G. texorii* (168 μg/g dry wt). The flavonol glycoside rutin was particularly rich in most *Rhodophyceae*, including *P. yezoensis* (11400 μg/g dry wt), *G. elegans* (23,200 μg/g dry wt) and *G. texorii* (30,000 μg/g dry wt); but was measured in only three *Chlorophyceae*: *A. ryukyuensis* (26,900 μg/g dry wt), *Monostroma nitidum* (2700 μg/g dry wt) and *C. serrulata* (3370 μg/g dry wt) and three *Phaeophyceae*: *U. pinnatifida* (457 μg/g dry wt), *Ecklonia cava* (2730 μg/g dry wt) and *P. arborescens* (996 μg/g dry wt; 48). On the other hand, the flavonol glycoside quercitrin was absent from both green and red algae, and present in only two *Phaeophyceae*: *U. pinnatifida* (202 μg/g dry wt) and *P. arborescens* (466 μg/g dry wt). The flavanone glycoside hesperidin was particularly rich in most green, brown and red Japanese algae including *A. ryukyuensis* (G, 117,000 μg/g dry wt), *E. bicyclis* (B; 6930 μg/g dry wt) and *P.* yezoensis (R; 51300 μg/g dry wt; 48).

Lignans

Lignans are characterized as phenolic structures formed from two cinnamic acid residues, and thus contain a 2,3-dibenzylbutane nucleus. Amongst lignans, secoisolariciresinol diglucoside (SDG) has received much attention due to its potential role as an anticarcinogen and an antioxidant *in vitro* (*49*) and *in vivo* (*50*). The plant lignan SDG is metabolized through a series of hydrolytic, dehydroxylation and demethylation conversions by colonic microflora into the mammalian lignan enterodiol (ED; 2,3-*bis*(3-OH phenyl) methylbutane-1,4-diol), which is subsequently oxidized to form enterolactone (EL; *trans*-dihydro-3,4-*bis*(3-OH phenyl) CH_3-γ-butyrolactone).

While flaxseed is regarded as the richest dietary source of SDG, yielding 98.41 μg EL/g dry wt and 682.04 μg ED/g dry wt from an in vitro human fecal fermentation, commercially available Japanese seaweeds have also been reported to yield mammalian lignans following fermentation: *U. pinnatifida* with 1.84 μg EL/g dry wt and 10.83 μg ED/g dry wt; *H. fusiformis* with 2.97 μg EL/g dry wt and 4.32 μg ED/g dry wt (*12*). Thus, these brown kelps likely contain the plant lignan SDG and possibly the plant lignan matairesinol, which can be directly metabolized to yield enterolactone. SDG, ED and EL have been reported to be equally effective against non-site specific (in the presence of

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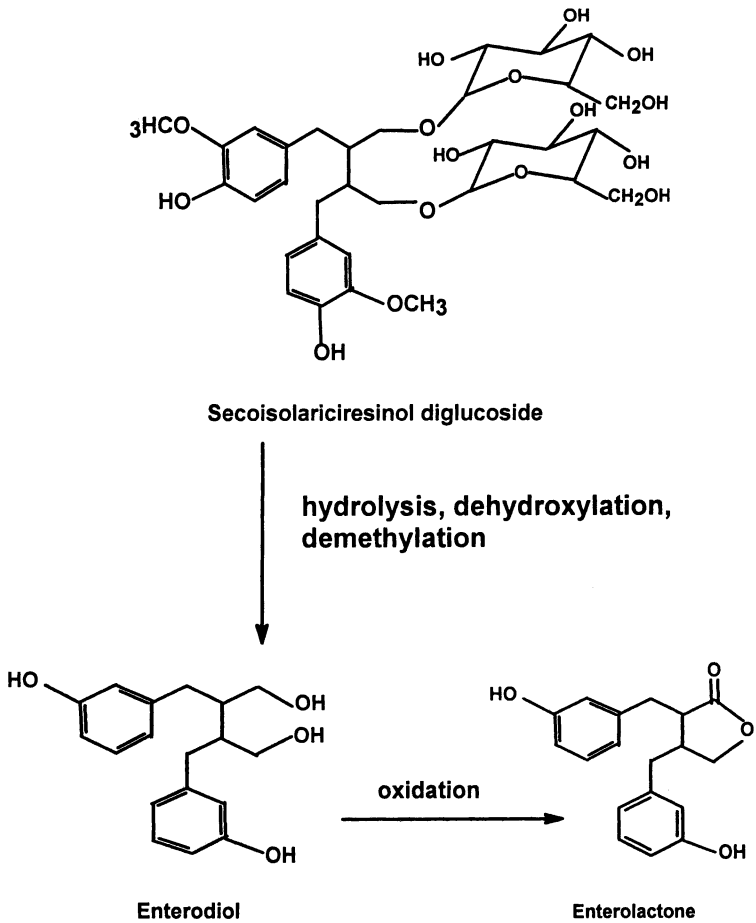

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EDTA) Fenton reaction-mediated plasmid DNA nicking *in vitro*; whereas, the mammalian lignans were more effective against hydroxyl radical scavenging and site-specific (in the absence of EDTA) Fenton reaction-mediated plasmid DNA nicking compared to SDG (*49*). Moreover, SDG, ED and EL were effective against lipid peroxidation of a linoleic acid emulsion system as free radical scavengers.

Phlorotannins

The brown to black coloration of kelps is associated with the phlorotannins and their oxidation products. Brown kelps synthesize these UV-inducible polyphenols which absorb in the UVB range (280-320 nm) and are inducible by UV-radiation below 400 nm (*51*). Moreover, kelp tissue levels of phlorotannins have been reported to increase during short-term exposure of *Macrocystis integrifolia* to UVA (320-400 nm) and UVB radiation (*51*). Some of these phlorotannins are also noted to be exuded into the surrounding seawater from damaged cells or tissues under UVA stress, thereby increasing water turbidity and reducing the impact of UV-radiation on the growing plants.

While the tannins of terrestrial plants, as gallo- or condensed tannins, are largely based on polymers of 4-8 linked flavan-3-ols, with some esterified to gallic acid; the related marine-derived compounds in the brown kelps, i.e. phlorotannins, are polymers of phloroglucinol (1,3,5-trihydroxybenzene) linked by C-C bonds or C-O-C aryl ether bonds (Figure 3). Thus, phloroglucinol and its oligomers and polymers may include those with masses exceeding 10,000 MW. Phlorotannins are unique to the *Phaeophyceae*, accounting for between 1 and 10% of the dry weight of these algae (*52*). For example, H. fusiformis and E. bicyclis have been reported to contain approximately 1.5% and 0.9% extractable phlorotannins, respectively (*41,53*). Up to 77 different phlorotannins have been detected in *Sargassum spinuligerum* (B; *54*). Similar to the terrestrial tannins, algal phlorotannins are also noted to precipitate proteins out of solution (*2*), have an astringent taste and chelate transition metal ions. Investigators separating high and low molecular weight fractions of phlorotannins as well as isolating and identifying phloroglucinol and its polymers in various brown kelp species, have also been able to demonstrate the efficacy of these kelp-derived polyphenols as free radical scavengers and inhibitors of lipid oxidation (*1,2,7,9,41,53*). A crude phlorotannin extract from *S. kjellmanianum* accounting for approximately 833.3 μg/g algal wet wt, exhibited dose-dependent inhibition of fish oil autooxidation with the high-molecular-weight fraction more efficacious than the low-molecular-weight compounds (*1*). *S. kjellmanianum* is not usually consumed as a food due to its bitterness, but does have a history of use as a tea in traditional Chinese medicine, thus the ability of a high molecular weight phlorotannin fraction and phloroglucinol to scavenge the DPPH radical with 27% and 21% inhibition of absorbance, respectively, may play a role in the therapeutic effects of this kelp (*2*). In *E. bicyclis* harvested from Tsuzumi Island, Fukuoka Prefecture, Japan, the phlorotannin profile consisted of: phloroglucinol

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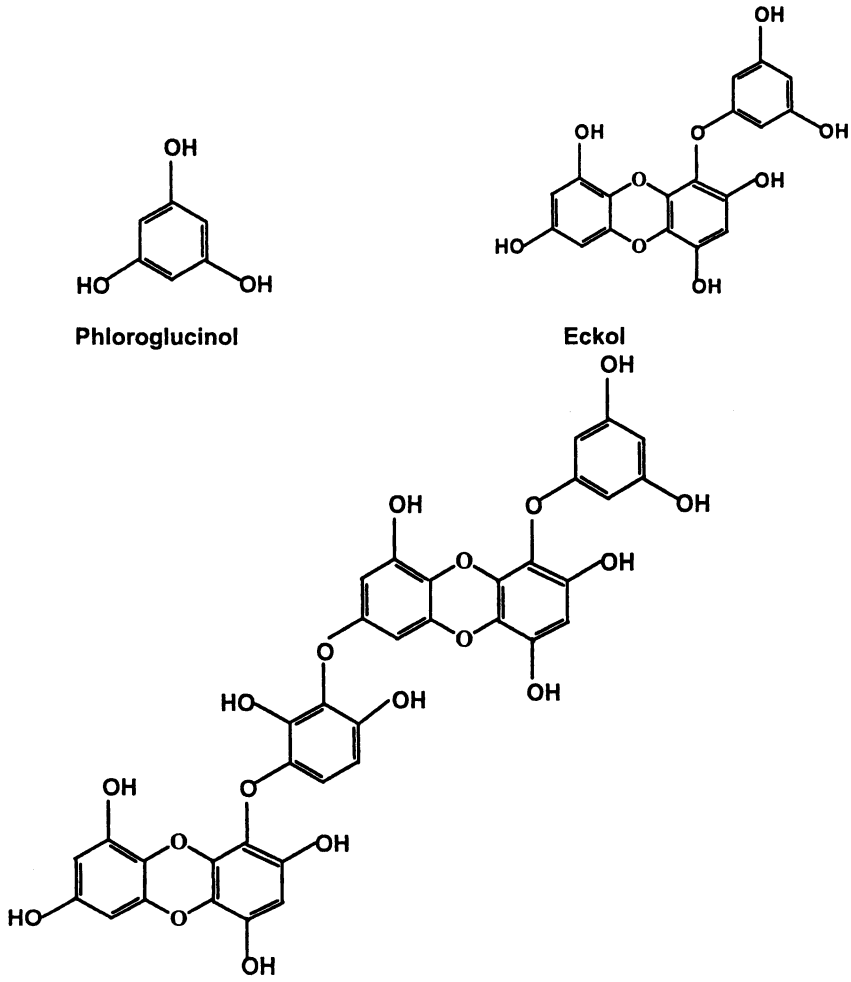
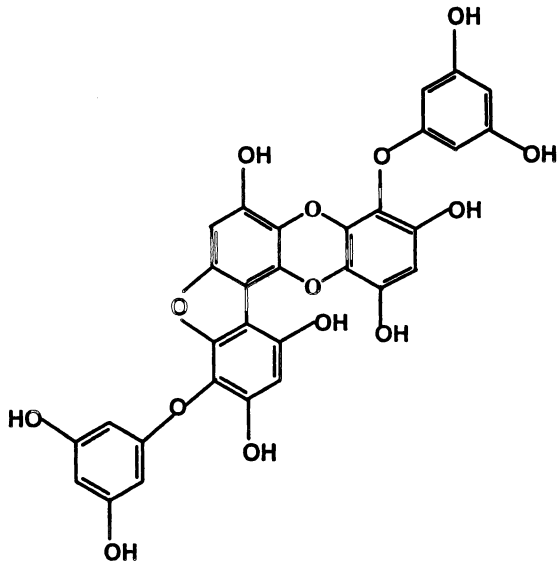
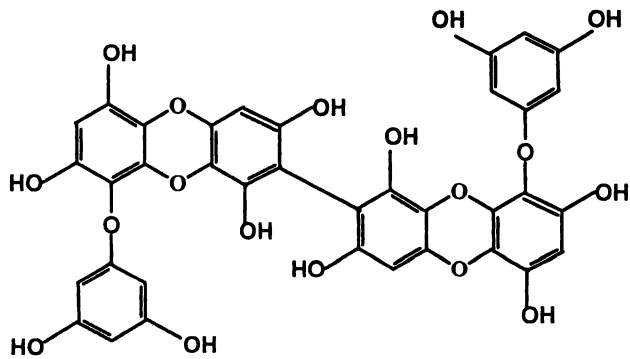


Figure 3. Phlorotannins from Phaeophyceae. *(Continued on next page.)*



Phlorofucofuroeckol A



8,8'-Bieckol

Figure 3. Continued.

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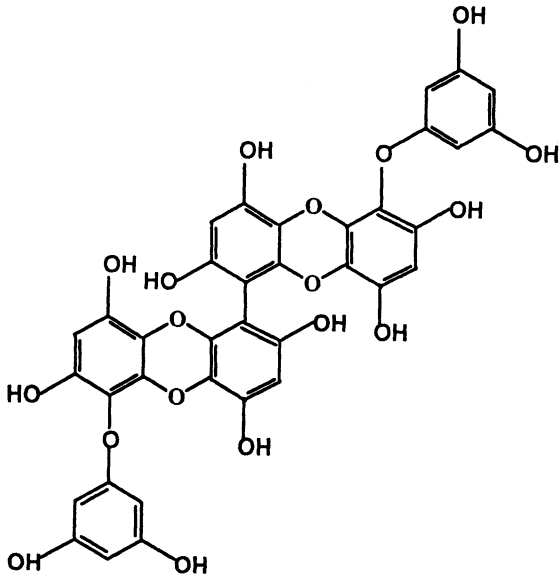

6,6'-Bieckol

Figure 3. Continued.

at 200 μg/g dry wt as well as eckol (trimer), phlorofucofuroeckol A (pentamer), dieckol (hexamer) and 8,8'-bieckol (hexamer) each at 1000-1500 μg/g dry wt (Figure 3; *53*). The phlorotannin profiles of *E. bicyclis* and *E. kurome* harvested from the Itoshima Peninsula, Fukuoka Prefecture, Japan, were reported to consist of phloroglucinol (0.7 and 2.2%, respectively), eckol (7.5 and 8.5%), an unknown tetramer of 478 molecular weight (3.4 and 0.6%), phlorofucofuroeckol A (21.6 and 27.6%), dieckol (21.9 and 23.6%), 8,8'-bieckol (24.0 and 6.8%) as well as an unidentified fraction (20.9 and 31.7%) indicating some differences in the phlorotannin profile in *E. bicyclis* which may relate to local growing conditions and geography (*7*). More recently, the phlorotannin profile of *E. cava* harvested from Jeju Island, Korea was reported to contain a different hexamer, 6,6'-bieckol (Figure 3; 59.2 μg/g dry wt) as well as phloroglucinol (1391.4 μg/g dry wt), eckol (124.3 μg/g dry wt), dieckol (3333.1 μg/g dry wt) and phlorofucofuroeckol A (60.4 μg/g dry wt; 9). The efficacy of individual phlorotannins in inhibiting oxidation of methyl α-linlenate at 0.05% (w/w) was greatest with the high-molecular-weight compounds *versus* phloroglucinol; however, the reverse was observed when concentrations were increased to 0.5% phlorotannins (*53*). On the other hand, individual phlorotannin efficacies to inhibit oxidation of a linoleic acid emulsion and scavenge DPPH radicals were dose-dependent and greater for high-molecular-weight species compared to phloroglucinol, demonstrating the strong antioxidant potential for oligomeric phlorotannins to donate electrons or hydrogen atoms from the multiple hydroxyl moieties of these compounds (*9*).

Jiménez-Escrig and coworkers (*55*) reported that extracts from *L. ochroleuca* (B) and *U. pinnatifida* (B) exhibited not only DPPH radical scavenging activity, but also ferric ion reducing activity, albeit, the reducing activity was lower than that of the red alga *Porphyra umbilicalis*. This latter evidence may relate to the low levels of free phloroglucinol reported in kelps compared to the higher molecular weight oligomers and polymers as discussed above (*52,53*). Moreover, the low reducing activities and total polyphenol contents of *Laminaria setchellii* (B), *Macrocystis integrifolia* (B) and *Nereocystis leutkeana* (B) 1-butanol soluble extracts reported by Yuan and coworkers (*15*) compared to those of the red alga *P. palmata*, likely reflected the oxidation and polymerization of phlorotannins in the kelps which form brown-colored high-molecular-weight compounds such as phycophaein (*52*). Thus, it is likely that the polyphenol contents of the 1-butanol soluble extracts from the three commercially available kelps; above were low due to the fact that during the isolation and purification of phlorotannins from kelps, Ragan and Glombitza (*52*) recommend that only fresh or frozen material be used, as opposed to air- or oven-dried algae. Edible kelps available for purchase by the consumer have usually been sun-dried prior to packaging for sale as health-food ingredients and condiments, thus air-oxidation has likely taken place in these products. This evidence suggests that the oxidation of phlorotannins, as well as the tendency of these pigments to bind to tissue protein, likely reduces the total polyphenols extractable from dried compared to fresh or frozen kelps. Thus, the processing

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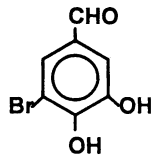
of kelp samples prior to phlorotannin extraction must be minimized to prevent alterations of the phlorotannin composition reported in the literature.

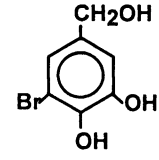
Bromophenols

Rhodophyceae such as the *Porphya sp.*, *Gelidium sp.* and *Pterocladia sp.* are noted to contain a variety of halogenated compounds including brominated mono- and dihydroxy C_6-C_1, C_6-C_2 and C_6-C_3 phenols comprising between 8 and 180 μg/kg, primarily 2,4,6-tribromophenol (*4,54,56*). *Phaeophyceae* such as the *Sargassum sp.* contain mono-, di- and tribromophenols with isoprenoid substituents comprising 4-84 μg/kg, primarily 2,4,6-tribromophenol and 2,4-dibromophenol (*54*). Fujimoto and Kaneda (*4*) reported that the antioxidant activity in the acetone-soluble fraction of the red alga *P. urceolata* was associated with the presence of four bromophenols, namely, 5-bromo- 3,4-dihydroxybenzaldehyde, 5-bromo-3,4-dihydroxybenzyl alcohol, 3,5-dibromo-4-hydroxybenzyl alcohol and 3-bromo-4-hydroxybenzyl alcohol.

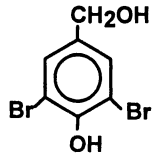
The most abundant bromophenol was found to be 5-bromo-3,4-dihydroxybenzaldehyde which was able to inhibit the oxidation of the methyl esters of safflower oil in a dose-dependent fashion by extending the induction period from 3.5 days to 11 days at a concentration of 200 ppm (*4*). Similarly, Fujimoto and coworkers (*56*) reported that the acetone-soluble fractions of both *P. urceolata* and *P. morrowii* exhibited strong antioxidant activity against lipid peroxidation of methyl esters of safflower oil at 45°C; however, the bromophenols were subsequently observed to be unstable when incubated at 97.8°C to determine effects against peroxide development in the AOM procedure. These workers also reported that 5- bromo-3,4-dihydroxybenzaldehyde comprised 75% of the total bromophenols in *P. urceolata* with substantial amounts of 5-bromo-3,4-dihydroxybenzyl alcohol present as well. Purified 5-bromo-3,4-dihydroxybenzaldehyde exhibited DPPH



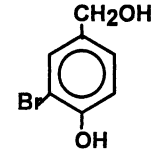
5-bromo-3,4-dihydroxybenzaldehyde



5-bromo-3,4-dihydroxybenzyl alco



3,5-dibromo-4-hydroxybenzyl alcohol



3-bromo-4-hydroxybenzyl alcohol

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radical inhibition (52%), similar to caffeic acid (45%) and greater than that of Trolox (26%) and BHT controls (13%), but less than that of gallic acid (82%; 2). Thus, these halogenated phenols are able to scavenge free radicals and inhibit lipid peroxidation likely through the donation of electrons or hydrogen atoms from their hydroxyl moieties.

Mycosporine-like amino acids

Mycosporine-like amino acids (MAAs) are characterized by a cyclohexenone or cyclohexenimine core conjugated with the nitrogen moiety of an amino acid (*57*). These aqueous or water-soluble secondary metabolites are noted to have absorbance maxima between 310 and 360 nm (*22*), and are thus, thought to function as UV-absorbing sunscreens in marine species including various red algae (*P. palmata*, *P. tenera*, *Devaleraea ramentacea*), corals (*Palythoa tuberculosa*) and small animals such as sea squirts (*Lissoclinum stellatus*; *22,23,58,59*). MAAs do not appear to exist in other algal species such as the *Phaeophyceae*, which synthesize the UV-protective phlorotannins as discussed above. Similar to the flavonoids and phenolic acids, MAAs are derived from the Shikimic acid pathway via 3-dehydroquinic acid and 4-deoxygadusol (4-DG; Figure 4; *57,60*). The resonance tautomers of 4-DG exist in equilibrium with each other prior to conjugation with an amino acid such as glycine or taurine to form the oxo-carbonyl MAAs mycosporine-glycine (Figure 4) or -taurine. Mycosporine-glycine can then undergo conversion to form one of several imino-MAAs such as shinorine or porphyra-334 (Figure 5; *57*). A protective UV-screening function for these compounds can be inferred from the fact that their absorbance maxima overlap not only with UVB irradiation (280-320 nm), but also the absorbance maxima for DNA. Moreover, 4-DG as a Shikimic acid pathway intermediate has been noted to possess strong antioxidant activity (*60*).

Takano and coworkers (*58*) isolated and determined the structure of a UV-absorbing MAA from the red alga *P. tenera*: porphyra-334. Eight MAAs were identified in Dulse (*P. palmata*) harvested from Spitsbergen, Norway: mycosporine-glycine, shinorine, porphyra-334, palythine, asterina-330, palythinol, palythene and usujirene (Figure 5; *22*). The highest amount of MAAs (2.74 mg/g dry wt) was isolated from Dulse samples collected from 1.5 m water depth; whereas samples collected from 3 m depth contained substantially less (0.68 mg/g dry wt). In the shallow water Dulse samples, palythine was the predominant MAA at approximately 1.4 mg/g dry wt compared to porphyra-334 and shinorine at 0.5 and 0.4 mg/g dry wt, respectively (*22*). On the other hand, in Dulse samples collected between 3 and 10 m depth, shinorine, prophyra-334 and palythine were present in very similar amounts of approximately 0.22 mg/g dry wt each. Further evidence for an UV-protective role for MAAs was demonstrated by the regional tissue distribution of these compounds in the Dulse plant; the young growing apical tips of this alga

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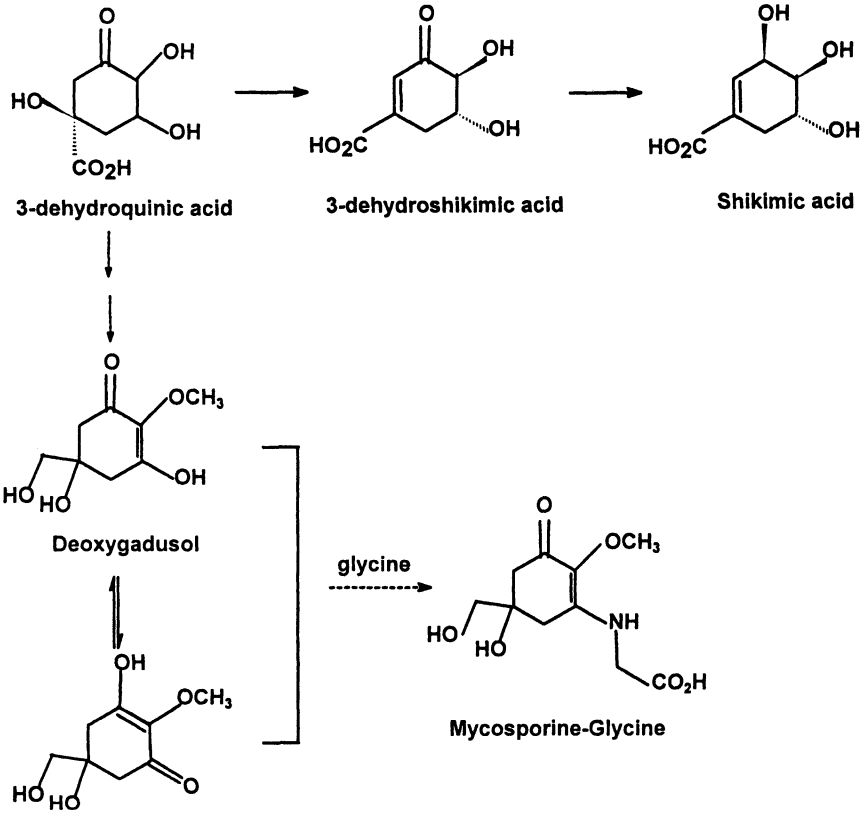

Figure 4. Shikamic acid pathway synthesis of MAAs.

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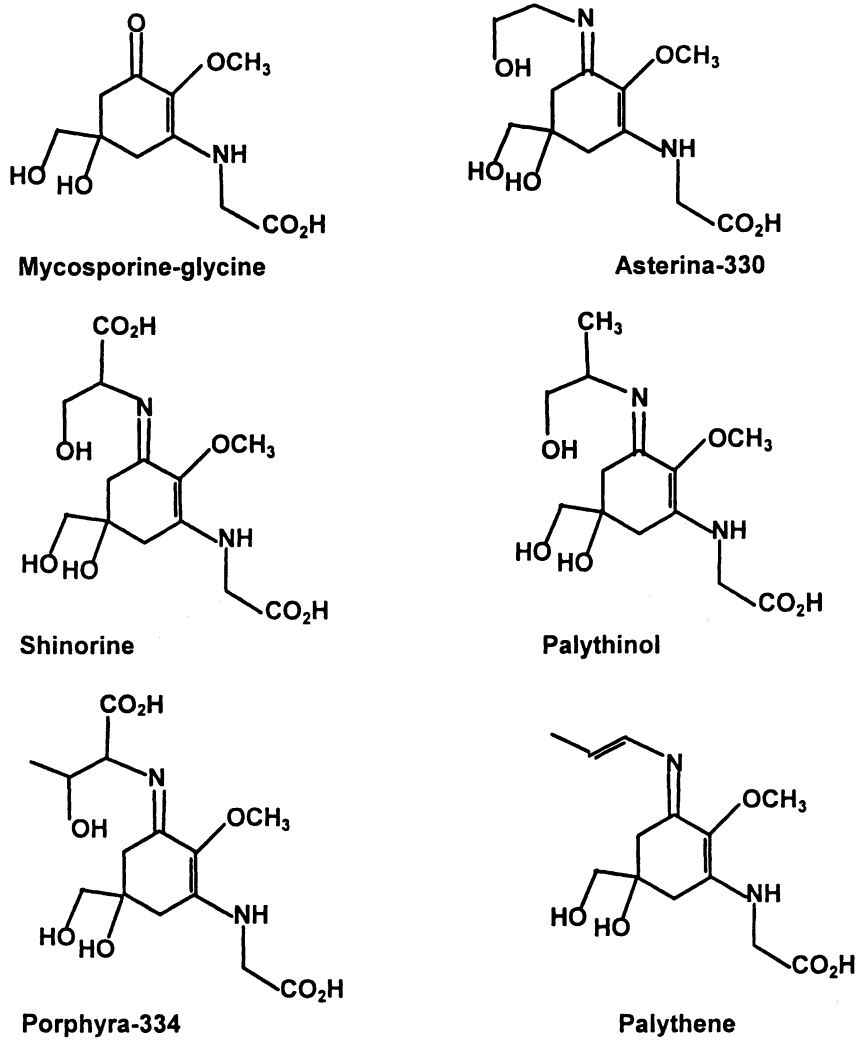

Figure 5. Mycosporine-like amino acids from Rhodophyceae

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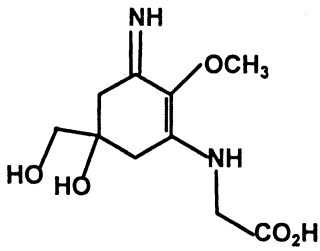
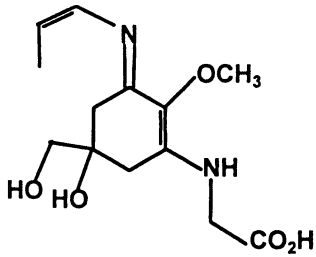


Palythine



Usujirene

Figure 5. Continued.

contained between 6 to 8 fold greater MAAs than the self-shaded older basal regions of the same plants (*22*). Similarly, when Aguilera and coworkers (23) examined the MAA contents of *P. palmata* and *D. ramentacea* samples during a period ranging from sea-ice cover to ice break-up in Spitsbergen, Norway, total MAA tissue concentrations ranged from lows of 0.55 and 0.75mg/g dry wt, respectively, under sea-ice cover in June, to highs of 1.0 and 1.3 mg/g dry wt, respectively, following ice break-up in July and August. The predominant MAA in *D. ramentacea* was palythine, whereas that for *P. palmata* was shinorine. While P. *palmata* contained the same eight MAAs as above, *D. ramentacea* samples contained all, except for usujirene (23).

Dunlap and Yamamoto (*59*) reported that UV-absorbing (334 nm) aqueous exract of *P. tenera* exhibited dose-dependent inhibition of peroxyl radical-initiated (2,2'-azo-bis(2-amidinopropane) dihydrochloride; AAPH) oxidation of a phosphatidylcholine emulsion system. Interestingly, when MAA-containing extracts were incubated with AAPH in the absence of lipid, UV-spectra and chromatographic results indicated that while mycosporine-glycine concentrations declined rapidly over time, shinorine, palythine, asterina-330, porphyra-334 and palythinol concentrations remained constant under these conditions. Thus, the imino-MAAs appear to have a greater one electron oxidation potential than the oxo-carbonyl MAAs against peroxyl radical oxidation (*59*). Thus, further investigations to characterize the antioxidant efficacy of individual MAAs against ROS and free radicals are needed to determine their potential efficacy *in vitro* and *in vivo*.

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Conclusions and Future Work

Edible and medicinal red, brown and green algae are rich sources of a great variety of polar as well as lipophilic antioxidant species. Many of these antioxidants also function as light-harvesting photosynthetic pigments (chlorophylls and their derivatives, carotenoids) and may also act as pro-oxidants depending on the test conditions. The current literature clearly demonstrates the potential for algae and their extracts to inhibit lipid peroxidation in model systems, scavenge free radicals or chelate transition metal ions. Moreover, there is growing evidence for chronic disease risk reduction in animal models fed on various edible seaweeds such as reduced mammary, intestinal or skin carcinogenesis. There is a need for information regarding the bioavailability and bioactivity of the unique algal antioxidants such as the phlorotannins and MAAs. It would be valuable to determine what role, if any, these unique antioxidant compounds play in the chronic disease risk reduction by edible algae observed in animal models which can pave the way for future clinical studies. With this evidence, edible algae may eventually have a place in Western diets as a functional food to promote consumption outside of traditional diets in Asia and the Pacific.

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Chapter 20

Extraction, Separation, Detection, and Antioxidant Activity of Apple Polyphenols

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There are mainly five major groups of polyphenolic compounds in apple, namely hydroxybenzoic/ hydroxycinnamic acids, flavan-3-ols, anthocyanidins, flavonols and dihydrochalcones. These are the principal contributors of the antioxidant activities of apple, among which flavan-3-ols such as epicatechin and its dimer procyanidin B2 are perhaps the most important. Polyphenols in apple are a highly complex mixture of aglycones, glycosides and polymers. The polyphenolic concentrations vary among different apple varieties and in different parts of the fruit. Certain varieties such as Red Delicious apple may contain significantly higher concentrations of polypohenols than others. Most of these compounds are in the peel as opposed to the flesh. Concentrations of polyphenols in apple also depend on other factors such as agronomic practice, environmental conditions and pre- and post- harvest handling, storage and processing. This chapter will therefore focus on the polyphenolic contents in apple, particularly, on the composition, characterization and factors affecting these phytochemicals and how they contribute to the antioxidant activities.

In recent years, increasing evidence suggests that consumption of diets high in fruits and vegetables leads to a lower risk of chronic diseases, particularly cancer and cardiovascular and heart diseases (*1-3*). Health regulatory agencies in many countries, including Canada, recommend 5-10 servings of fruits and vegetables per day (*4*). Although the exact active

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components in fruit and vegetables for these protective and preventative effects are not clearly known, many of these chronic diseases are related to oxidative stress caused by excess free radicals and reactive oxygen species (ROS), for which antioxidant phytochemicals in fruits and vegetables have been considered to play important roles. Phytochemicals such as polyphenols and some carotenoids may not be essential to human growth and development, however, they can possess significantly higher antioxidant capacity than essential antioxidants such as vitamins C and E, therefore contributing substantially higher proportion of the total antioxidant capacity of commonly consumed fruits and vegetables (*5-9*). Indeed, diets high in polyphenols have been associated with a lower risk of cancer, coronary heart disease and other chronic ailments (*10,11*).

Among all fruits and vegetables, apple has perhaps received the most attention from not only the researchers, but also the general public. The general perception that apple is good for health has intrigued scientists to search for the "magic" ingredient in apple, and how these phytochemicals help maintaining good health. Epidemiological studies have shown an inverse correlation between the consumption of apple and/or related products and many chronic diseases in humans. Most noticeably, diets containing a high apple content have been associated with lower risk of cardiovascular disease, lung dysfunctions, and cancers, particularly those of prostate, liver, colon and lung (*6,12-14*). Most of these biological effects of apple have been similarly linked to the high concentration of antioxidant phytochemicals present, particularly polyphenols, rather than vitamins C, E, or β-carotene (*6-9*). In apple, vitamin C only explains 0.4% of total antioxidant activity based on total oxyradical-scavenging capacity (TOSC) assay. Most of the activity is attributed to the polyphenolics in apple (*6,9,15*). Apples also ranked the second for total phenolic content and more importantly the highest portion of free phenolics when compared to other fruits (*15*).

These current researches have clearly shown that it is the polyphenols in apple that contribute the most to the antioxidant activity, which is one of the most important health benefit, among others, such as dietary fibres. This chapter, therefore will focus on the polyphenolic contents in apple, particularly, on the composition, analytical methods, characterization and factors affecting these phytochemicals during pre- and post- harvest handling and processing, and how they contribute to the antioxidant activities.

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Polyphenolic Profiles of Apple

Polyphenolic Compositions

Major groups of polyphenolic compounds in apple include benzoic acids, cinnamic acids, flavan-3-ols, anthocyanidins, flavonols and dihydrochalcones.

Benzoic and cinnamic acids are simple phenolic acids, and most of them are hydroxylated, thus hydroxybenzoic and hydroxycinnamic acids (Fig. 1). The other four groups of polyphenols, including the dihydrochalcones, are by and large categorized under flavonoids (Figures 2 and 3), despite the fact that chalcones do not contain a C-ring (Fig. 2). The polyphenolic profiles of apple are further complicated by varied degrees of polymerization of flavan-3-ols and glycosylation of other polyphenolics. The predominant sugars involved in glycosylation are galactose, glucose, rhamnose, arabinose and xylose; and the disaccharide, rutinose, has also been found in apple. The total polyphenolic concentrations of apple as determined by HPLC ranged from 177.4 to 933.6 μg/g fresh weight in the flesh and 1016.5 to 2350.4 μg/g fresh weight in the peel in a study on different Canadian apples (*17*).

Hydroxybenzoic acid content in apple is generally very low and often remains undetected as compared to other groups of phenolics. It is lower than 5% of the total phenolic contents, and found to vary significantly depending on cultivar and storage conditions (*18*). The total benzoic acid content was found to be between 40 and 80 μg/g fresh weight in the peel (*12*). Most recently, a new apple cultivar called Annurca grown in the southern region of Italy has been reported to contain several benzoic acid derivatives including protocatechuic, gentisic, vanillic, benzoic, and Salicylic acids (*19*) (Fig. 1). The concentrations of these benzoic acids were between 3 and 13 μg/g fresh weight (*19*).

The most frequently detected hydroxycinnamic acids in apple are chlorogenic acid with significantly lower concentrations of *p*-coumaric acid and caffeic acid (*16,20,21*). Chlorogenic acid is particularly found in the flesh of apple. It was nearly 90% of all polyphenolics detected in the flesh of Empire apple (*17*). Its concentration also varies in different cultivars and different parts of the apple fruit. The average concentration of chlorogenic acid in eight Ontario apples was 136 μg/g fresh weight in the peel, and 177 μg/g fresh weight in the flesh. The flesh of Northern Spy apple contained the highest chlorogenic acid concentration at 308 μg/g fresh weight (*17*). *p*-Coumaric acid was found to be present at approximately 1/10 of the chlorogenic acid in the same study.

The prevailing anthocyanin in apple has been found to be cyanidin glycosides with different sugar units attached at 3-position of the C-ring (Fig. 2). Cyanidin 3-galactoside is the most dominant form found in most apple, however, other glycosides such as cyanidin 3-rutinoside and 3-arabinoside have also been reported (*17,21*). Anthocyanins are only found in red apples, and they are not the dominant group among polyphenols in apple. The concentrations of cyanidin glycosides ranged from 42.9 to 208.2 μg/g fresh weight (*17*).

Flavonols in apple are nearly exclusively quercetin and its glycosidic derivatives. Schieber *et al.* (*22*) separated and detected six quercetin derivatives

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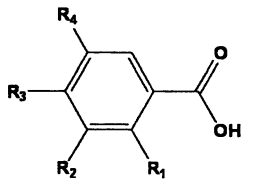
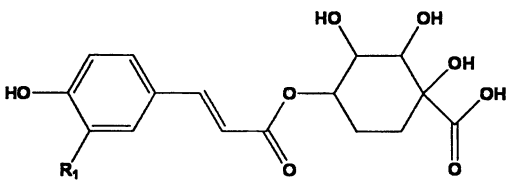
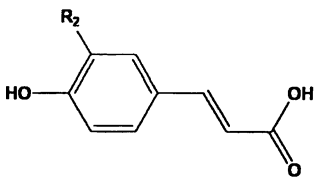


Hydroxybenzoic acid Derivatives	R_1	R_2	R_3	R_4
Benzoic acid	H	H	H	H
Salicillic acid	OH	H	H	H
Gentistic acid	OH	H	H	OH
Protocatechuic acid	H	OH	OH	H
Vanillic acid	H	OCH_3	OH	H





Hydroxycinnamic acid Derivatives	R_1	R_2
Chlorogenic acid	H	
p-Coumaroylquinic acid	OH	
Caffeic acid		OH
p-Coumaric acid		H

Figure 1. Typical phenolic acids found in apple (Modified from ref. 17 and 19).

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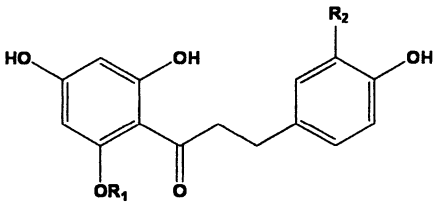
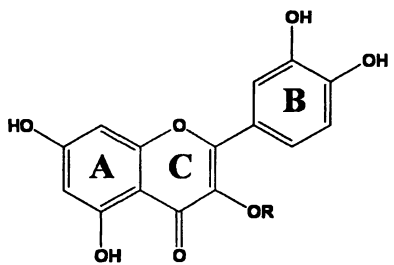
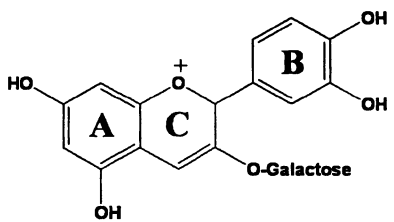


Dihydrochalcones	R_1	R_2
3-hydroxyphloretin-2'-xyloglucoside	Xylglu	OH
3-hydroxyphloretin-2'-glucoside	Glu	OH
phloretin-2'-xyloglucoside	Xylglu	H
phloretin-2'-glucoside (phloridzin)	Glu	H



Flavonols (Quercetin glycosides)

R = galactose, glucose, xylose, arabinose, rhamnose.



Anthocyanins

Cyanidin-3 galactose

Figure 2. Flavonols, dihydrochalcones and anthocyanins found in apple (Selected from ref. 17).

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including the aglycone and its five glycosides: 3-rutinoside (rutin), 3-galactoside, 3-glucoside, 3-xyloside, and 3-rhamnoside. In a recent study, the aglycone and its 3- rutinoside were not detected, however, a different glycoside, quercetin arabinoside was detected (*16*). It should be mentioned that not all quercetin glycosides are detected in a single study. For example, one of the quercetin 3-glycosides, rutin, was reported by Escarpa and Gonzalez (*23*) and Perez-Ilzarbe (*24*), but not in other similar studies (*17, 25, 26*). Most recently, Chinnici *et al.* (*26*) reported all the above 6 glycosides of quercetin in the Golden Delicious apple. Quercetin glycosides consisted of 18% of the total polyphenolic concentrations in the 8 apple varieties recently examined; ranging from 220.3 to 349.9 μg/g fresh weight in nearly exclusively the peels of apples. This makes the second largest polyphenolic group after the flavan-3-ols (*17*).

Phloretin 2'-glucoside (phlorizdin) and 2'-xyloglucoside are the dominant dihydrochalcones in apple, however, recently their hydroxylated derivatives, 3-hydroxyphloretin-2'-glucoside (*25*), and 3-hydroxyphloretin-2'-xyloglucoside have been identified in apple (*17*). The glycosylation pattern of phloretin glycosides were determined by collecting the HPLC (high performance liquid chromatography) fractions containing the individual peaks, and then analyzing the sugars and aglycone by HPLC-DAD (diode array detector) and LC-ESI-MS (electrospray ionization-mass spectrometry) (*17*). The majority of dihydrochalcones were found in the apple peel. The total dihydrochalcone content averaged 124 μg/g fresh weight in the peels of the 8 cultivars examined with that in the Red Delicious peel being at the highest concentration of 253 μg/g fresh weight (*17*).

Among all major polyphenolic groups in apple, the monomeric, dimeric and some times polymeric flavan-3-ols predominated the polyphenolic profiles of both apple peel (59.7%) and flesh (55.7%) (*17,28*) (Fig. 3). The two monomers, catechin and epicatechin, are stereoisomers, and in general, apples contain more epicatechin than catechin. The dimer procyanidin B1 is composed of one molecule of catechin and one molecule of epicatechin with C-C bond at 4, 8 positions, respectively. Procyanidin B2 is a dimer of two molecules of epicatechin connected similarly to procyanidin B1. Apples usually contain higher concentrations of procyanidin B2 than B1 (*17*). Procyanidins with higher degrees of polymerization have also been found in apple. Most recently, Shoji et al. (*29*) successfully separated and identified procyanidins from dimmers to octamers using normal phase HPLC, and ESI-MS and MALDI-TOF-MS (matrix-assisted laser desorption ionization-time of flight) (Fig. 3). The total procyanidin contents ranged from 151.3 to 1654.8 μg/g in the peel and 0 to 583.0 μg/g in the flesh; Red Delicious and Northern Spy had the highest amounts in the peel and flesh, respectively. Other tentatively identified, but extractable procyanidins may also be contributing to the total procyanidins (*17*).

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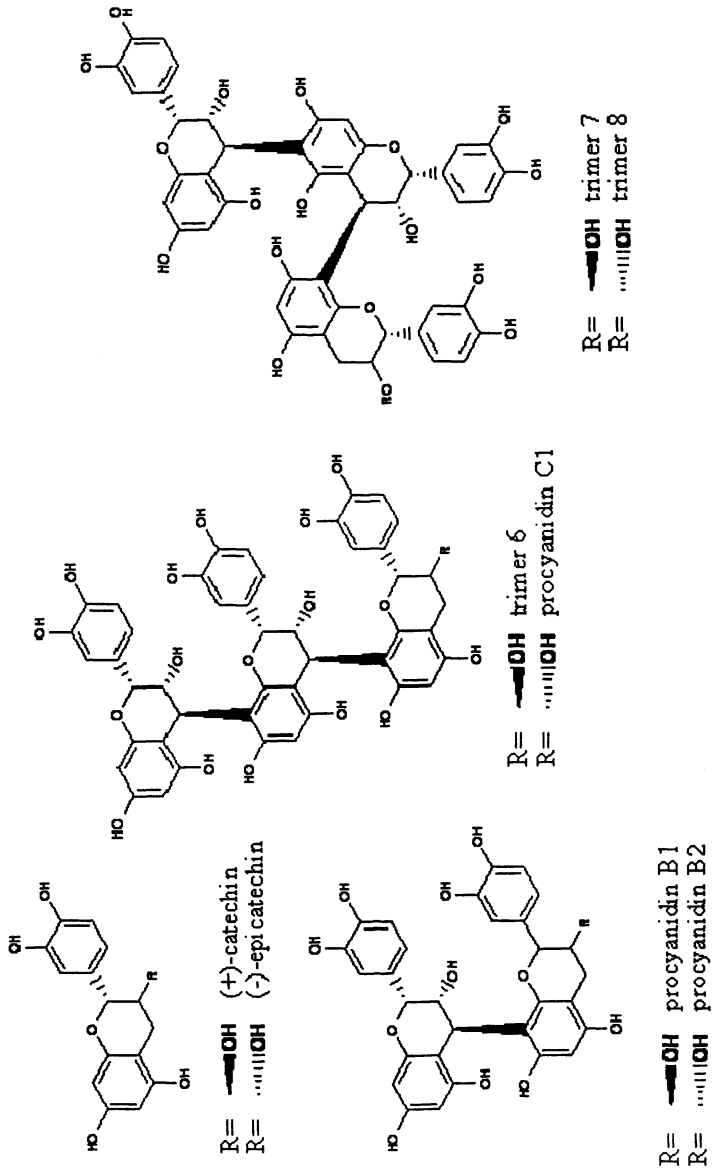

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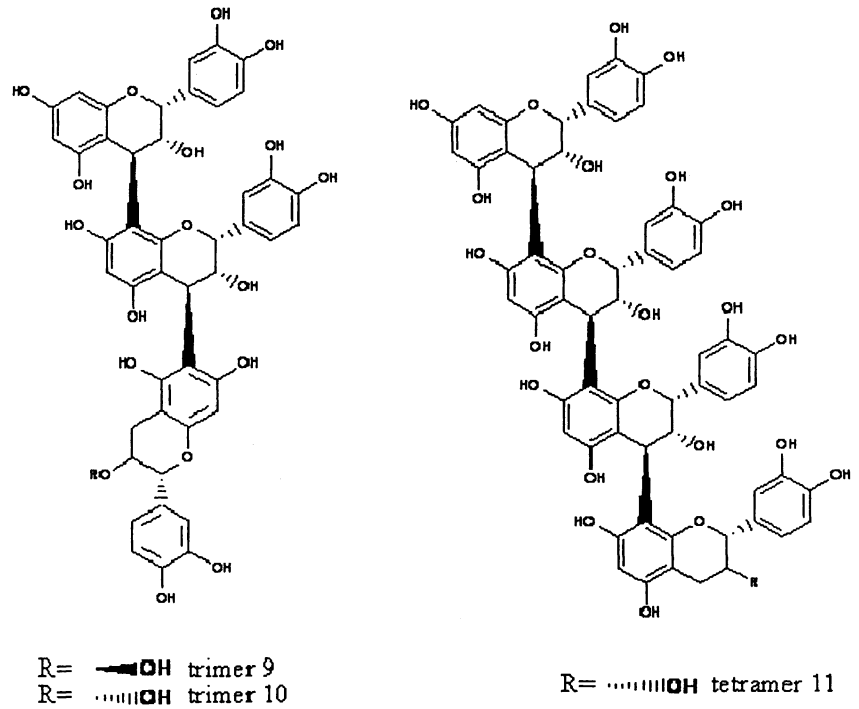

Figure 3. Typical monomeric and oligomeric flavan-3-ols found in apple (Modified from ref. 17 and 29).

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Extraction, Separation and Detection

Extraction

Polyphenols are relatively hydrophilic compounds that are extractable using water, polar organic solvents such as methanol, ethanol, acetonitrile and acetone, or a mixture of water and one such solvent. There is no specific extraction or analytical method solely developed for polyphenols in apple, however, methods for polyphenols in fruits and vegetables usually follow similar procedures. In terms of apple, there are basically two different procedures, one for fresh samples, and one for dried samples. Escarpa and Gonzalez (*23*) used methanol containing 1% BHT (2,6-di-*tert* -butyl-4-methylphenol) to extract polyphenols from fresh apple peel and pulp samples at room temperature and in the absence of light using an ultrasonic bath. The sample was extracted sequentially with 10 mL of solvent for 1 h, 10 mL for 30 min, and then 5 mL for 30 min. The three extracts were combined to a final volume of 25 mL before being analyzed by HPLC. Chinnici *et al.* (*27*) modified the method slightly. Samples were extracted three times with 95% methanol containing 300 mg/L sulfur dioxide as follows: 20 mL of solvent for 1 h, 10 mL for 1 h, and 10 mL for 30 min. The three extracts were combined and stored at -18 °C until analysis. If necessary, extractions was carried out to remove lipids, carotenoids, and chlorophylls (*30*).

Alonso-Salces (*31*) used a procedure for freeze dried apple samples. Apple peel was frozen in liquid nitrogen and freeze-dried. The dried peel was then crushed in closed vials to avoid hydration, obtaining a homogenous powder that was stored at room temperature in a desiccator until analysis. Freeze-dried apple peel powder (0.5 g) was extracted with 30 mL of methanol–water–acetic acid (30:69:1, v/v/v) in an ultrasonic bath over a 10 min period. Afterwards, the extract was freeze-dried once methanol had been evaporated at 40 °C in a rotary evaporator. The solution analysed was prepared by dissolving 50 mg of the extract in 1mL of methanol–water–acetic acid (30:69:1, v/v/v). This method has recently been modified by sonicating the freeze-dried apple tissue with 30 mL of the same solvent mixture in the presence of 2 g of ascorbic acid/L, for 10 min in an ultrasonic bath (*32*). Wu and Prior (*33*) also used similar mixture of solvents for the extraction of freeze-dried fruit samples including apples. The freeze-dried powders were extracted with methanol-water-acetic acid (85:15:0.5, v/v).

Procedures are also available for juice and pomace samples (*22*). Fresh sample of apple pomace may be extracted with 70% acetone for 1 h at ambient temperature. The aqueous extract is then evaporated to remove acetone, and then adjusted to pH 7, and extracted with ethyl acetate for flavan-3-ols and procyanidins, to pH 1.5 for all other polyphenols (also partitioned with ethyl acetate). Juice samples may be directly adjusted to pH 7 and 1.5, respectively, and extracted with ethyl acetate, dried over anhydrous sodium sulfate before

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analysis. Similar procedures have been reported for apple puree and concentrate (*34*).

In addition to the conventional solid-liquid extraction using large amounts of solvent, other methods have recently been developed. A new sample preparation technique, accelerated solvent extraction (ASE), or pressurized liquid extraction (PLE) has also been employed for extraction of apple polyphenols (*32,35*). PLE allows the volume of extraction solvent to be reduced, the analysis time to be shortened, and the handling necessary to produce more precise results to be decreased. The optimized conditions of the PLE method for the extraction of polyphenols from freeze-dried apple samples were use of methanol as solvent at 40 °C over a 5 min period at a pressure of 1000 psi and using two extraction cycles. The recovery rate for polyphenols by this method was between 86 and 95% (*35*).

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Separation and Detection

Conventional chromatographic techniques such as paper, packed column and thin-layer chromatography have been used for separation and purification of many polyphenols, however, lack of good separation efficiency and resolution as well as difficulties in detection, quantification and sensitivity has prevented their widespread use. These shortcomings of the conventional chromatography can not meet the increasing demand for profiling of the polyphenolics of fruits including apple. Gas chromatography meets these requirements, but its use is somewhat limited due to the non-volatility of polyphenols. This leaves HPLC the most popular and reliable system among all chromatographic separation techniques for separation of polyphenols, although other alternative separation techniques such as capillary liquid chromatography (CLC) exist (*36*). The versatility of HPLC is also aided by different separation modes and types of detection methods, among which diode array detection (DAD) coupled with mass spectrometry (MS) are most useful. For MS detection, several ionization techniques have been employed, however, electroscopy ionization (ESI) and atmospheric pressure chemical ionization (APCI) have most often been used in both positive and negative modes.

Measurement of food flavonoids by HPLC has recently been reviewed by Merken and Beecher (*37*). Methods of separation and detection of phenolic acids and flavonoids in apple are often similar to those for other fruits and vegetables; often the chromatographic conditions of the HPLC methods include the use of, almost exclusively, a reversed-phase (RP) C18 column; a DAD, and a binary solvent system containing acidified water (solvent A) and a polar organic solvent (solvent B). The separation normally requires 1 h at a flow rate of 1.0–1.5 mL/min. Solvent A usually includes aqueous acids or additives such as phosphate. Solvent B is normally pure or acidified methanol or acetonitrile (*38*). Recently, an RP monolithic column has been reported as a simple and rapid tool

for separation of polyphenols in apple (*26*). Other types of columns such as size exclusion chromatography (SEC) have also been investigated (*39*).

Some newer methods have been reported. Schieber (*22*) used a RP C18 column with a mobile phase consisting of 2% acetic acid in water (eluent A) and 0.5% acetic acid in 50:50 acetonitrile/water (v/v) (eluent B) and a gradient program, and separated 26 phenolic acids and flavonoids, although anthocyanins were not analyzed using this method. We developed a method using a binary mobile phase consisting of 6% (v/v) acetic acid in 2 mM sodium acetate buffer (solvent A, pH 2.55) and acetonitrile (solvent B), with a gradient program of 0% B to 15% B in 45 min, 15% B to 30% B in 15 min, 30% B to 50% B in 5 min, and 50% B to 100% B in 5 min, and a pump rate of 1.0 mL/min, and obtained a good separation of major polyphenols in apple peels and flesh (*17*). With the aid of DAD and ESI-MS, a total of 16 polyphenols from all major groups were positively identified, including a new dihydrochalcones, 3-hydroxyphloretin 2'-xyloglucoside (*17*). A solvent system involving the use of methanol by Alonso-Salces (*31*) also showed a remarkable separation of apple polyphenols. Their mobile phase consisted of: A: 0.5% (v/v) acetic acid in water, and B: methanol, and a gradient program: 0–10 min, 5%B isocratic; 10–50 min, linear gradient 5–15%B; 50–70 min, 15%B isocratic; 70–100 min, linear gradient 15–30%B; 100–125 min, linear gradient 30–60 %B; and a flow rate at 0.8 mL/min. This method also employed LC-DAD-APCI-MS and postcolumn addition of UV (ultraviolet) shift reagents, to assist the identification of two new hydroxyphloretin glycosides and quercetin in apple peel for the first time. The presence of isorhamnetin glycosides and not the isomeric glycosides of rhamnetin was also confirmed. Moreover, isorhamnetin-3-*O*-rhamnoglucoside was identified unambiguously by comparison with a standard (*31*). Although RP-HPLC has been the primary separation means for apple polyphenols, past studies illustrate the difficulty in separation of procyanidins, particularly determining the degree of polymerization of these antioxidants. For the separation and detection of procyanidins in apple, Shoji (*29*) developed a preparative normal phase HPLC method to separate procyanidins according to the degree of polymerization by using an Inertsil PREP-SIL (i.d. 30mm×250 mm) column (GL Science, Japan), which consists of porous spherical silica gel particles (10 □m) at the flow rate of 24 mL/min. The methods used a binary gradient with mobile phase containing hexane/acetone, (A1) 4:6 and (A2) 2:8 (v/v) in the hexane–acetone system. The fractionated procyanidins were then separated using a RP HPLC coupled with ESI-MS and matrix assisted laser desorption ionization of a flight mass spectrometry (MALDI-TOF-MS) detectors. A number of procyanidins from dimers to octamers were separated and identified (*29*) (Fig. 3).

Factors Affecting the Polyphenolic Profiles in Apple

Factors affecting the phytochemical profiles in fruits and vegetables have recently been reviewed (*40*). For apples, similar factors such as genetics,

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growing season, geographic locations, agronomic practices, plant/fruit parts, and pre- and post- harvest handling as well as storage and processing conditions are known to affect the polyphenol compositions.

Genetic Variation

Different cultivars of apple contain different levels of total polyphenolics and to some extent different profiles. A study in our laboratory on eight different Ontario apple cultivars showed that Red Delicious apple contained the highest content of total polyphenolics followed by Northern Spy, Ida Red, Cortland, McIntosh, Golden Delicious, Mutsu and Empire (*17*). Other studies have shown similar results (*41*). However, In terms of individual polyphenolic compounds, it has been found that flavan-3-ols and procyanidins predominate the total polyphenol profiles of apple (*17*). Certain polyphenols are specific to specific cultivars. Anthocyanins are only found in red-skinned apples.

Four apple cultivars grown in the Netherlands namely Jonagold, Golden Delicious, Cox's Orange and Elstar, were compared with regard to flavonol, catechins, phloridzin, and chlorogenic acid concentrations (*42*). Although concentrations of these major groups of polyphenols differed among the four cultivars, they did not follow the same trend. The total polyphenol content ranged from 265 to 467 mg/kg fresh weight, however, the highest amount was found in Jonagold apples followed by Golden Delicious, Elstar and Cox's Orange (*41*). Among the total phenolic contents of 9 apple cultivars studied, Braeburn and Empire had the highest and lowest total phenol content, respectively (*43*). The apple cultivars ranked in the following decreasing order of polyphenols: Braeburn > Red Delicious > Crisp Pink > Granny Smith > Royal Gala > Bramley > Golden Delicious > Fuji > Empire ranging from 360 to 535 mg GAE/100 g fresh weight. In this study, however, the apples were purchased form the UK market, and their original site of production was not clear. The concentrations reported were from apples with skin.

Vrhovsek (*44*) examined 41 apple samples, collected in Trentino, Italy, representing eight of the most widely cultivated varieties in Western Europe. Samples were extracted from fresh fruit with a mixture of acetone-water to achieve good extraction of polyphenols, including proanthocyanidin oligomers which were analyzed by normal-phase HPLC. Up to 20 compounds including catechin, epicatechin, B2 procyanidin, hydroxycinnamates, flavonols, anthocyanins, and dihydrochalcones were analyzed by reversed-phase HPLC and LC-MS. Total polyphenol content was independently measured with an optimized Folin-Ciocalteu (FC) assay. The mean content of total polyphenols was between 66.2 and 211.9 mg/100 g of fresh weight depending on the variety. With chromatographic analysis, it was possible to explain the whole amount of

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total polyphenols measured by the FC assay. Flava-3-nols (catechin and proanthocyanidins) are the major class of apple polyphenols (71-90%), followed by hydroxycinnamates (4-18%), flavonols (1-11%), dihydrochalcones (2-6%), and in red apple anthocyanins (1-3%). This is similar to that was found in apples from other countries (*17*). Fuji apples contain relatively lower total polyphenol content than others (*42,43*), however, it ranked the highest among the 13 apple varieties reported by Boyer and Liu (*45*). Further studies may be necessary to confirm the cause of this disparity.

Agronomic Practices (organically grown vs. conventionally grown apple)

Different agronomic practices also influence the polyphenolic contents of fruits and vegetables (*40*). Weibel (*45*) found that organically grown Golden Delicious apples had 19% higher total phenolic content than the conventionally grown apples. Some research have suggested that organically grown fruits and vegetables might contain more antioxidative phytochemicals, thus healthier to human, however, the increase is generally considered as a result of smaller fruit size. Information on the effect of agronomic practices on polyphenolic content and antioxidant activity of apple is generally lacking or the results are controversial. A recent report supported the concept that organic systems of cultivation do not generally provide increased phytochemical content and antioxidant activity, casting a question on whether organically grown produce provides greater health benefits (*47*). Another recent report showed that for individual polyphenolic compounds, the opposite might be true; in both peels and pulps, integrated production samples were found richer in polyphenols than organically grown apples. Among the 14 compounds identified, only phloridzin was present in higher amounts in organic peels (*48*).

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Environmental Factors

Growing season affects the total phenolic content and the concentration of individual polyphenolic compounds in many fruits and vegetable, however, a study on flavonoids concentration and antioxidant activity in three apple cultivars showed no significant differences among apples harvested in three different years (*42*). Geographic location affects the polyphenolic contents as well, however such influence seemed to be cultivar dependent. There were significant differences in polyphenolic concentrations in fruit from different regions of New Zealand for some cultivars but not for others (*48*). Positions within the same apple tree may also affect the concentration of polyphenols of the fruit, particularly the skin. Awad et al. (*50*) found that fruit from the top of the canopy contained the highest percentage of blush and the highest level of

cyanidin 3-galactoside (anthocyanin) and quercetin 3-glycosides, followed by fruits from the outside of the canopy, and then those from the canopy interior. Within the fruit, the most consistent finding is perhaps the different polyphenols in the peel and the flesh of apples. Several studies have shown that the total polyphenolic content in the peel can be several folds higher than that in the flesh (*17,41*). Certain polyphenolic compounds were only found in the peal whereas others were only or predominantly found in the flesh (*17,41*).

Pre- and Postharvest Handling, And Storage And Processing Conditions

Preharvest handling of apple, particularly harvesting timing relates to the fruit maturity. Apples at different maturation stages contain different polyphenolic profiles. Quercetin glycosides, phloridzin, catechins and chlorogenic acid concentrations in Jonagold and Elstar were found to be at the highest level early in the season and decreased to a steady level during maturation and ripening (*51*). Napolitano et al. (*20*) found that storage time significantly affected individual and total phenolic concentrations among different apples. However, long-term storage, both at refrigerator temperature and under controlled atmospheric conditions did not influence flavonoid concentration or antioxidant activity (*42*). Phenolics in apple did not change significantly during cold storage for up to 9 months (*18,52*). Lattanzio et al. (*53*) investigated the effect of microbial infections during low temperature storage of apple and found that the concentration of major polyphenolics and polyphenol peroxidase (PPO) activity in infected apple tissues were significantly higher than in healthy tissues. More recently, Tarozzi et al. (*47*) investigated the influence of commercial cold-storage period on total phenolics, and found that cold storage rapidly decreased these compounds in skin but not in pulp. The flavonoids in apple were also significantly affected by certain plant growth regulators during pre- and post-harvest handling. An ethylene-releasing synthetic plant hormone Ethephon was found to greatly increase anthocyanin accumulation, but not that of other flavonoids and chlorogenic acid. Other growth regulators were found to retard anthocyanin accumulation, but not on the synthesis of other flavonoids (*54*). When production of plant hormone in apple was inhibited by compounds like 1-methylcyclopropene (1-MCP), the total flavonoid concentration in Red Delicious apple was 5% greater in fruit treated with 1-MCP, whereas chlorogenic acid levels were 24% lower. All compounds analyzed increased in concentration during fruit harvest; however, the anthocyanins generally declined after storage, while chlorogenic acid levels increased (*21*).

Processing apples into juice also affected the polyphenolic content. Raw juice obtained from Jonagold apples by pulping and straight pressing or after pulp enzyming, showed reduction of catechin and chlorogenic acid by 97% and 50%, respectively. Most of the polyphenols remained in the pomace rather than

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being transferred into the juice (*55*). This effect was found in all three apple cultivars (Elstar, Golden Delicious, and Jonagold) tested by these authors. The same researchers also found that it is possible to improve flavonoid content in juice by applying an alcoholic extraction either on the pulp or on the pomace. The levels of flavonoids and chlorogenic acid in enriched juice were between 1.4 (chlorogenic acid) and 9 (quercetin glycosides) times higher than in conventional apple juice (*56*). Different polyphenolic compounds were affected differently by storage temperature and oxygen concentration (*56*). The most thermally sensitive compounds were the various quercetin glycosides and epicatechin, whereas phloridzin and chlorogenic acid were more stable. The quercetin glycosides showed differences in their stability: quercetin galactoside>quercetin rhamnoside > quercetin glucoside/rutinoside > quercetin arabinoside. The effect of the presence of oxygen on the degradation rates was clear for only quercetin and to a lesser extent for epicatechin (*57*).

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Antioxidant Activities of Apple and Apple Polyphenols

Evaluation of Antioxidant Activity of Phytochemicals

It is highly difficult to measure the antioxidant capacity *in situ* of human body, therefore the antioxidant activity of phytochemicals from fruits and vegetables are often measured *in vitro* with different model systems. Over the years, many *in vitro* models have been developed, however, caution has to be exercised in the interpretation of results from different systems. Currently, there is no one single system that is perfect. All model systems are based on different mechanisms, therefore often give considerably varied results. On the other hand, the active form of antioxidants *in vivo* may not necessarily be in the same form originally present in fruits and vegetables, as there are a host of factors affecting the metabolism and bioavailability of these antioxidants. There is no perfect system to learn about the "true" antioxidant power or capacity of a single antioxidant or a complex medium of antioxidant phytochemicals (*58,59*). In the past several years, we have used and modified a few different *in vitro* systems, namely, the ferric reducing antioxidant power (FRAP) assay, the β-carotene–linoleic acid model system (β-CLAMS), the oxygen radical absorption capacity (ORAC) and the photochemiluminescence (PCL) method, and found that these systems gave consistent and reproducible results (*15,60,61*). For fruit extracts, results from FRAP and β-CLAMS often correlated positively and the activities were also proportional to the total phenolic content (*15,60*) (Fig. 4). More discussions on the separation and evaluation of phytochemical antioxidants can be found in Tsao and Deng (*38*). However, it was recently decided that the ORAC method be used as the first standard method for evaluation of antioxidant activity (*62*). The ORAC method is simple and can be used to measure both lipophilic and hydrophilic antioxidants, and it measures the antioxidant activity

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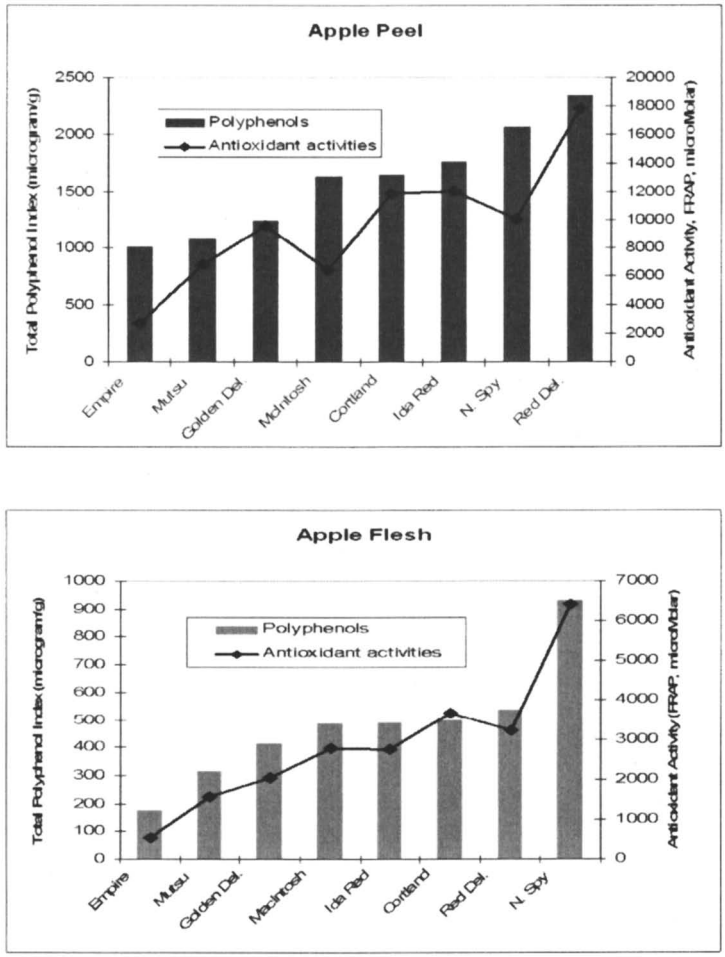

Figure 4. Total polyphenolic concentrations analyzed by HPLC and antioxidant activities measured by FRAP of eight apple varieties.

against one of the most important free radicals in biological systems, the hydroxyl radical (*63*). Recently, the antioxidant activities have also been measured *in vitro* by using cell line models (*47*). However, interpretation of results obtained from these model systems also has to be dealt with caution.

Phytochemicals Contributing To The Antioxidant Activities Of Apple

The health benefits of apple have been well documented (*45*). Consumption of apple has been associated with a lower risk of cancer, particularly cancer of the prostate, liver, colon and lung (*6,13,14,64*) and cardiovascular diseases (*12,65*). These chronic diseases are considered to be caused by oxidative processes; especially those involving excess free radicals and ROS. Thus compounds in apple that have antioxidative properties have been the focus of many studies. According to Eberhardt et al. (*6*), vitamin C in apples contributed less than 0.4% of total antioxidant activity. The complex mixture of phytochemicals, particularly polyphenols, were suggested to provide protective health benefits, and possibly through a combination of additive and/or synergistic effects. There are five major groups of polyphenolic compounds in apple, including hydroxybenzoic/hydroxycinnamic acids, flavan-3-ols/procyanidins, anthocyanidins, flavonols and dihydrochalcones (*17*), and the predominant portion of phenolics are in the free rather than in the bound state, suggesting that consumption of fresh apple can attain most of the health benefits such as antioxidant activity and anti-proliferation effects (*16*).

In general, a stronger antioxidant activity was found in apple cultivars or parts (peel vs. flesh) with higher phenolic content (*15,41*) (Fig. 4). Apple had the second highest total phenolic content and antioxidant activity measured by TOSC assay, and the third in antiproliferation activity using HepG2 human liver-cancer cells (*16*). However, it remains unclear as to which group(s) or individual compounds found among major apple phenolics contribute the most to its antioxidant activity. Most published papers have attributed the total phenolic content to the total antioxidant activity of apple (*41*). However, different phenolic acids, flavonols, anthocyanins, flavan-3-ols and chalcones have varying degrees of antioxidant activity, therefore contribute differently to the total antioxidant activity (*15,48*). Chinnici et al. (*48*) found that in apple peels, the top contributors to the antioxidant activity were flavonols, flavan-3-ols and procyanidins, which accounted for approximately 90% of the total calculated activity whereas in pulps, the total antioxidant capacity was primarily derived from flavan-3-ols (monomers and polymers) together with hydroxycinnamates. Among single classes of compounds, procyanidins (in peels and pulps) and flavonols (in peels) were statistically correlated to the total radical scavenging activity. Similarly, Lee et al. (*66*) also found that the antioxidant activity of different phenolic groups were in the order of quercetin> epicatechin > procyanidin B2 > phloretin > chlorogenic acid. Based on these

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data, these authors concluded that the estimated contribution of major phenolics to the total antioxidant capacity of fresh apples followed the same order (*66*). The antioxidant activities of apple phenolics are dependent on the methods used because multiple action mechanisms have been found for phenolic phytochemicals. At least two methods with different mode of actions are recommended to be used in one study in order to accurately evaluate the antioxidant activity (*67*). Single method such as radical scavenging activity alone thus can lead to different conclusions. Using several different methods in the evaluation of the antioxidant activity of apple, we have found that both of the monomeric and dimeric flavan-3-ols, particularly epicatechin and procyanidin B2, contributed the most to the total antioxidant activity of eight different apple varieties (*15*) (Fig. 5). Results from measuring the peroxyl radical trapping efficiency of apples by Vanzani et al. (*68*) also agreed that procyanidins were the major contributors to the total antioxidant activity of apple.

Factors that affect the phenolic contents in apple also affect the antioxidant activities in a similar manner. The effect of processing on polyphenolic contents in juice also leads to differences in observed antioxidant activities. Raw juice obtained from Jonagold apples by pulping and straight pressing or after pulp enzyming had an antioxidant activity that was only 10 and 3%, respectively, of the activity of the fresh apples (*55*); newer processing technology improved the recovery of polyphenols and hence the total antioxidant activity of apple juice. In enriched juice the antioxidant activity was 5 times higher than that in conventional apple juice, with 52% of the antioxidant activity of the originating fruits present (*56*). Storage conditions also affect the antioxidant activity of apple juice. van der Sluis et al. (*57*) found that polyphenol-enriched apple juice had decreases of 20-40% in the antioxidant activity during 4 days of storage at 80 °C. However, polyphenolic antioxidants and antioxidant activity of enriched apple juice were quite stable at ambient or refrigerated storage conditions up to half a year.

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Conclusions

Apples top the most consumed fruits in North America by their antioxidant activities. The dietary intake of apple can potentially contribute to the health benefits associated with prevention of many chronic diseases such as cancer and cardiovascular diseases. The major contributors to such effects are not the vitamins in apple, but rather different groups of phytochemicals present. In apple, phenolic acids and flavonoids were among the principal antioxidant phytochemicals; most importantly flavan-3-ols such as epicatechin and its dimer procyanidin B2. Polyphenols in apple are a highly complex mixture of different groups of phytochemcials; many of them are present in minute amounts. This makes their separation and detection a difficult task. Among the many methods

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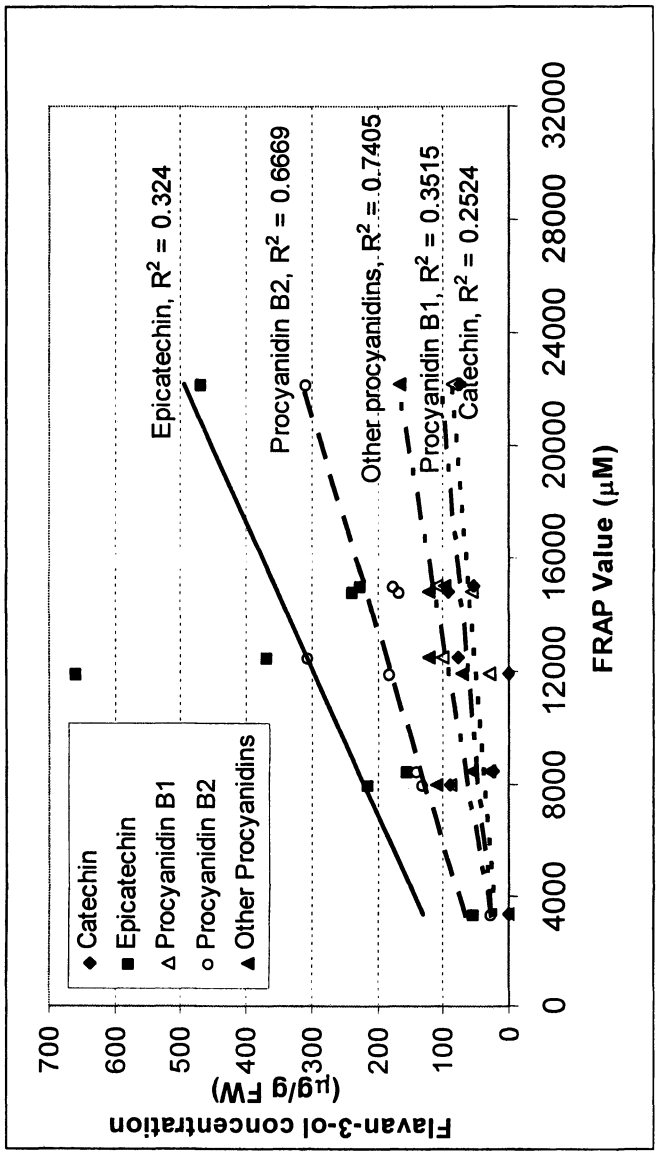

Figure 5. Epicatechin and procyanidin B2 contributed the most to the total antioxidant activity of apple.

developed, LC-DAD-MS will continue to play an important role in the separation, quantification and characterization of polyphenols in apple. The content of polyphenolics varies among different apple varieties and in different parts of the fruit. Certain varieties such as Red Delicious apple may contain significantly higher concentrations of polypohenols than others. Most of these compounds are in the peel as opposed to the flesh. Concentrations of polyphenols in apple are also affected by factors such as agronomic practice, environmental conditions and pre- and post- harvest handling, storage and processing.

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Chapter 21

Impact of Berry Phytochemicals on Human Health: Effects beyond Antioxidation

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It is now well accepted that a phytochemical rich diet contributes towards reducing the risk of oxidative-stress mediated diseases such as certain types of cancers, heart and neurodegenerative diseases. Phytochemical rich foods include fruits, vegetables, whole grains, spices, and certain beverages and other food products such as tea, wine, beer, chocolate, cocoa, etc. Among fruits, berries contain high levels of a diversity of phytochemicals known as phenolics including flavonoids (anthocyanins, flavonols and flavanols), proanthocyanidins, ellagitannins and gallotannins, stilbenoids and phenolic acids. Popularly consumed berries include blackberries, black raspberries, blueberries, cranberries, raspberries and strawberries. Although berry phenolics are potent *in vitro* antioxidants, they exert *in vivo* biological activities beyond antioxidation and can have complementary and overlapping mechanisms of action. For example, berry phenolics can impart preventive benefits through the regulation of enzymes important in metabolizing xenobiotics and carcinogens, by modulating nuclear receptors, gene expression and sub-cellular signaling of proliferation and apoptosis, and by acting indirectly through antioxidant actions that protect DNA from damage. This overview examines the impact of consumption of natural berry bioactive compounds on human health and disease prevention.

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Dietary phytochemicals having a phenolic structure (i.e. aromatic ring bearing hydroxyl, -OH, group) are the most abundant antioxidants in the human diet and are present in fruits, vegetables, whole grains, spices and some beverages such as tea and wine. Dietary antioxidants are of significant importance in human nutrition because the oxidative stress induced by free radicals is involved in the etiology of a wide range of chronic human illnesses (*1,2*). Oxidative stress can cause oxidative damage to large biomolecules such as lipids, proteins, and DNA, resulting in an increased risk for inflammatory diseases, cardiovascular disease (CVD), some cancers, diabetes, Alzheimer's disease, cataracts, and age-related functional decline. Hence antioxidants may help to protect cellular systems from oxidative damage thereby also lowering the risk of certain chronic human diseases.

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Antioxidant research has grown exponentially over the past decade with the development of numerous laboratory or *in vitro* assays which measure the total antioxidant capacity of pure compounds, foods, and dietary supplements (*3,4*). However, these *in vitro* assays do not necessarily reflect the cellular physiological conditions and do not consider the bioavailability, metabolism and other 'in the living body', or *in vivo*, issues. In addition, emerging scientific evidence has shown that the mechanisms of action of many phytochemicals go beyond the antioxidant activity of scavenging of free radicals thereby impacting disease prevention and health promotion in numerous other ways. Phytochemicals can have complementary and overlapping mechanisms of action and perform functions ranging from phytoprotectants in the plant to *in vivo* antioxidant activity- including scavenging reactive oxygen species-acting as antagonists toward oxidative enzymes such as cyclooxygenases, and influencing the expression of multiple genes (*5,6*). Ways in which phytochemicals impart preventive benefits beyond their antioxidative potential also include: the regulation of enzymes important in metabolizing xenobiotics and carcinogens; modulation of nuclear receptors and sub-cellular signaling of proliferation, cell cycle arrest and apoptosis, cell differentiation, oncogenes, and tumor suppressor genes; stimulation of the immune system; regulation of hormone-dependent carcinogenesis; inhibition of arachidonic acid metabolism; reduction of proliferation and protection of DNA from damage; antibacterial and antiviral effects (*5,6*).

Among fruits, berries contain a wide diversity and high levels of phytochemicals including flavonoids (anthocyanins, flavonols and flavanols), condensed tannins (proanthocyanidins), hydrolysable tannins (ellagitannins and gallotannins), stilbenoids and phenolic acids. Berries have been an important part of human diet for centuries. Popularly consumed berries in the USA include blackberries, black raspberries, blueberries, cranberries, raspberries and strawberries. However, berries are not only widely consumed as fresh fruits but

also in processed forms as beverages, snacks, yogurts, preserves, jellies and jams etc. In addition, extracts of berries are consumed in dietary supplements as active botanical ingredients for their potential health benefits. Although these natural berry antioxidants have been shown to have potent *in vitro* antioxidant biological activities, scientific evidence has revealed that their *in vivo* biological effects extend beyond antioxidation. This overview examines the impact of consumption of natural berry phytochemicals on human health and disease prevention.

Chemistry of Berry Phytochemicals

The structural diversity of berry phenolics imparts unique chemical and biological properties which affect the absorption, distribution, metabolism, bioavailability and excretion of these compounds in humans. Berry phenolics differ structurally based on varying types and oxidation levels of their heterocycle ring, their substitution patterns of hydroxylation, the existence of stereoisomers, their glycosylation by sugars and/or acylation by organic and phenolic acids, and by conjugation with themselves to form polymers. Figure 1 shows examples of chemical structures of the general classes of berry phenolics.

The main class of berry phenolics includes the most abundant group, the flavonoids. Berry flavonoids have a basic skeletal structure of C6-C3-C6 and, based on their degree of oxidation and substitution in the 3-position, can be further sub-divided into: flavonols (e.g. quercetin, kaempferol), flavanols (e.g. catechin, epicatechin), and anthocyanidins (e.g. cyanidin, pelargonidin). Anthocyanins, the glycosides of anthocyanidins, are the pigments responsible for the attractive red, blue and purple colors of berry fruits. Berry phenolics are also found in polymeric forms known as tannins and are categorized into condensed tannins (proanthocyanidins) and hydrolysable tannins (ellagitannins and gallotannins). Other classes of berry phenolics include stilbenoids, e.g. resveratrol and its analogs (*7*), that are found in a variety of other dietary sources including red grapes (and hence red wine) and muscadine grapes. Berries also contain phenolic acids, which include derivatives of hydroxybenzoic acid (e.g. gallic acid) and cinnamic acid (e.g. caffeic acid). Although these phenolic acids occur naturally in their free forms in berry fruits, they can be considered as structural moieties in polyphenol compounds i.e. 'embedded within a polyphenolic structure.' Hence phenolic acids can be released from the metabolism of polyphenols and their polymers *in vivo* and then conjugated (glucuronidated, sulfated, methylated etc.) in the liver before excretion, contributing immensely to bioactivity in the human body.

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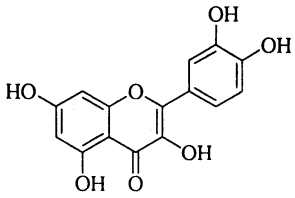
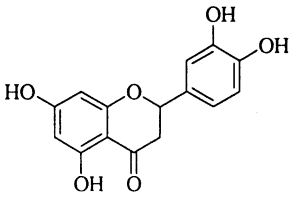
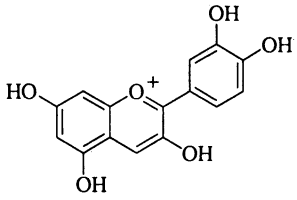


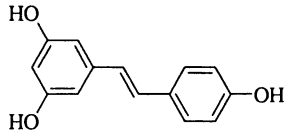
a) Flavonol (e.g. quercetin)



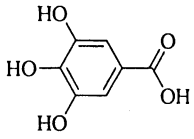
b) Flavanol (e.g. catechin)



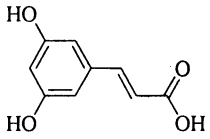
c) Anthocyanidin (e.g. cyanidin)



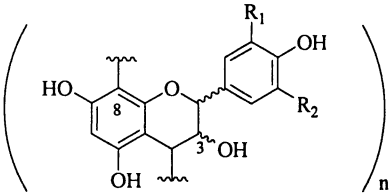
d) Stilbenoid (e.g. resveratrol)



e) Phenolic acid (e.g. gallic acid)

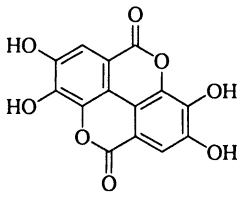


e) Phenolic acid (e.g. caffeic acid)



R_1, R_2 = H, propelargonidins
R_1= H, R_2= OH, procyanidins
R_1, R_2 = OH, prodelphinidins

f) Proanthocyanidins



g) Ellagitannin (e.g. ellagic acid)

Figure 1. Examples of chemical structures of some berry phenolics: a) flavonol (e.g. quercetin); b) flavanol (e.g. catechin); c) anthocyanidin (e.g. cyanidin); d) stilbene (e.g. resveratrol); e) phenolic acid e.g. gallic acid (a hydroxybenzoic acid) and caffeic acid (a hydroxycinnamic acid); f) condensed tannins (proanthocyanidins) and g) hydrolysable tannins (e.g. ellagitannins).

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Biology of Berry Phytochemicals

Antioxidation is by far the most widely reported bioactivity attributed to berry fruits due to the fact that they contain not only high levels, but also a wide diversity of antioxidant phenolics. Although epidemiological data suggest an inverse relationship between fruit and vegetable consumption and oxidative stress mediated diseases, whether the protection can be related to particular fruits or vegetables or whether it is related to overall intake of antioxidants is not clear (*8*). However, as previously mentioned, antioxidants exert their *in vivo* biological activities that go beyond their *in vitro* antioxidant activities. The antioxidant properties of berry phenolics as well as their bioactivities beyond antioxidation are outlined below.

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Antioxidation

In vitro

Numerous studies have been published on the antioxidant capacities of berries in hydrophilic and lipophilic assays (*4, 9-14*). However, comparisons and correlation of antioxidative potential of foods are difficult considering the wide variety of assays based on different mechanisms being used by different laboratories (*3,4,15*). In addition, difficulty with extraction of food matrices also further complicates matters. Nevertheless, there are few reports of correlation between antioxidant assays. For example, the oxygen radical absorbance capacity (ORAC) and ferric-reducing antioxidant power (FRAP), both aqueous-based antioxidant assays, have been reported to correlate well with each other and with a lipid emulsion-based methyl linoleate (MeLo) assay (*16,17*).

The scavenging property of berry phenolics of superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH), and singlet oxygen ($^1O_2$) has been shown (*18*). Berry phenolics have also been shown to inhibit lipid peroxidation (*9*) as well as protein and lipid oxidation in liposomes (*10*). Berry phenolics are highly active radical scavengers in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) test and effective antioxidants in emulsion and human low density lipoprotein (LDL) (*19-22*). Berry phenolics have also been shown to have high antioxidant activity in the ORAC assay (*23-24*).

In vivo

As outlined in the previous section, there is considerable evidence for the *in vitro* antioxidative potential of berry phenolics. However there are fewer reports of human *in vivo* plasma antioxidant status being enhanced by consumption of

berries. Most of the human studies examining biomarkers of oxidative stress include phytochemical rich diets consisting of high fruit and vegetable intake. Oxidative stress biomarkers include evaluation of plasma antioxidant status or total antioxidant capacity namely, by FRAP, ORAC and Trolox Equivalent Antioxidant Capacity (TEAC) assays; enzymatic antioxidant defenses (superoxide dismutase, catalase, glutathione reductase, and glutathione *S*-transferase enzymes), and lipid oxidation (isoprostanes and malon-dialdehyde).

Mazza *et al.* (*25*) reported an increase in serum antioxidant capacity ($ORAC_{acetone}$, $P < 0.01$) in 5 healthy human subjects after consumption of freeze-dried blueberry powder and concluded that food sources with high *in vitro* antioxidant properties can be associated with a diet-induced increase in *ex vivo* serum antioxidant status. Netzel *et al.* reported that various berry juice antioxidants are absorbed and are active as antioxidants *in vivo* in plasma of 6 healthy volunteers (*26*). However, in healthy volunteers with adequate vitamin intakes, 6-wk diets differing markedly in the amounts of vegetables, berries, and apples did not differ in their effects on lipid peroxidation, lipoprotein metabolism, thrombosis or inflammation (*27,28*). Moller *et al.* (*29*) showed that consumption of large amounts of antioxidants in the form of blackcurrant juice in 57 healthy adequately nourished humans did not decrease oxidative DNA damage. Nevertheless, in an older population (men of average age 60 yr), the effects of berry consumption on antioxidant potential and diene conjugation in LDL particles, albeit small, were demonstrable (*30*).

In conclusion, there are inherent difficulties in designing human studies that aim to measure biomarkers of oxidative stress to clarify the role of dietary antioxidants in disease prevention. In studies where subjects are under higher oxidative stress, for example, older target populations (> 60 yr) or smokers, some beneficial effects are observed. It should be noted that although measurement of plasma antioxidant status is a useful biomonitoring tool in supplementation and health related studies of redox balance, an understanding of the physiologic mechanisms of control of the body's redox defenses is an important issue that must be addressed in human studies (*31*).

Anticancer

The inhibition of the growth of human tumor cell lines is important in cancer therapy and there have been numerous reports attributed to the antiproliferative effects of berry extracts (*32-35*). For example, Seeram *et al.* reported that cranberry extract and its purified phenolics including its

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proanthocyanidins, anthocyanins, and other flavonoids inhibited the proliferation of human oral, colon and prostate tumor cells *in vitro* (*32*).

Apoptosis or programmed cell death is also a major mechanism of cancer suppression. Generally, the growth rate of pre-neoplastic or neoplastic cells outpace that of normal cells because of malfunctioning or dysregulation of their cell-growth and cell-death machineries. Therefore, induction of apoptosis or cell-cycle arrest can be an excellent approach to inhibit the promotion and progression of carcinogenesis and to remove genetically damaged, pre-initiated or neoplastic cells from the body. Berry extracts have been shown to have pro-apoptotic effects in human cancer cells (*36-37*). Among the berry phenolics, its anthocyanins have been shown to be major contributors towards the induction of apoptosis. For example, strawberry phenolics and especially its anthoycanins were shown to inhibit oxidative-stress induced apoptosis in PC-12 rat pheochromocytoma cells (*37*).

Angiogenesis, another major mechanism involved in cancer, is the process by which new blood vessels grow. Hence tumor cell growth can be affected through anti-angiogenic activity by reducing the supply of oxygen and nutrients. Berries have been shown to be potent chemopreventive agents via inhibition of angiogenesis (*38-40*). Atalay *et al.* (*40*) not only demonstrated that edible berry extracts potently suppressed *in vitro* angiogenesis but showed *in vivo* angiogenic effects in mice.

Also involved in cancer are sub-cellular signaling effects which have been demonstrated in numerous studies by berry extracts. Berries have been shown to target the down-regulation of activating protein (AP-1) and transcription factor NF-κB, blocking MAPK signaling, stopping the production of Tumor Necrosis Factor (TNF-α) and suppressing cancer cell proliferation and transformation (*41-43*).

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Effects on Neurodegenerative Diseases

Scientific evidence suggests that it may be possible to overcome genetic predispositions to neurodegenerative diseases, such as Alzheimer's disease, through a phytochemical rich diet (*8*). Blueberry supplementation were shown to enhance signaling and prevent behavioral deficits in a mice model of Alzheimer's disease (*44*). The berry phenolics are thought to offer protective effects through enhancement of memory-associated neuronal signaling (e.g. extracellular signal-regulated kinase) and alterations in neutral sphingomyelin-specific phospholipase C activity (*44*). Strawberry and blueberry supplementation to senescent rodents have also been shown to reverse the deleterious effects of aging on motor behavior and neuronal signaling (*45-46*).

Cardiovascular Health

Diets rich in fruits and vegetables have been of interest because of their potential health benefits in preventing chronic diseases such as cardiovascular disease. A high fruit, berry and vegetable intake was shown to be associated with reduced risk of mortality in middle-aged Finnish men (*47*). Rietbrock *et al.* (*48*) showed the efficacy and safety of a standardized berry extract in 88 patients with congestive heart failure with regard to exercise tolerance, dyspnea and quality of life.

Antiadhesion

Berries, such as cranberries and blueberries, have been reported to have antiadhesion effects (*49-51*). Among berries, probably the best known antiadhesion effect is that of cranberries on urinary tract infections which is attributed to specific structural types of proanthocyanidins present in the fruit (*49*). Recently, berries have also been implicated in the management of *Heliobacter pylori* infections (*52-54*). It is interesting to note that *H. pylori* has not only been linked to peptic ulcer but recently has also been both epidemiologically and pathogenetically linked to cardiovascular diseases including coronary atherosclerosis, acute ischemic stroke, and thrombosis (*55-56*).

Future Research: Nutritional Genomics

The post-genomics era has brought with it ever increasing demands to observe and characterize variations within biological systems. This variation has been studied at the genomic (gene function), proteomic (protein regulation) and the metabolomic (small molecular weight metabolite) levels. Nutritional genomics includes nutrigenomics, which explores the effects of nutrients on the genome, proteome and metabolome; and nutrigenetics, which explores the effects of genetic variation on the interaction between diet and disease (*57,58*).

Hence future studies on the metabolomics of berry phenolics are necessary and should focus on the *in vivo* bioactivities of their metabolites to evaluate the impact of consumption of berry bioactives on human health. Whether bioactivity of berry phytochemicals is made stronger by the interactions of the many substances within a particular fruit, as well as in combination with phytochemicals from other fruits and vegetables, should be investigated. In addition, future research should focus on studying gene-nutrient interactions, and health outcomes to achieve individual dietary intervention strategies aimed at preventing disease, improving quality of life and achieving healthy aging in humans.

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Conclusions

Berry phenolics constitute a substantial portion of natural phytochemicals in our diet and they have bioactivities that go beyond their antioxidative properties. Hence the inclusion of berries- such as blackberries, black raspberries, blueberries, cranberries, raspberries and strawberries- in the diet may have a positive impact on the prevention and progression of chronic human illnesses including heart disease, inflammation, neurodegenerative diseases and certain types of cancers.

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Chapter 22

Health Benefits of Edible Berry Anthocyanins: Novel Antioxidant and Anti-Angiogenic Properties

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Edible berries provide for some of the richest sources of anthocyanins and antioxidants. Numerous studies have substantiated why consumption of anthocyanin-rich fruits and vegetables might retard or prevent the onset of chronic diseases. The therapeutic and pharmacologic potential of berry anthocyanins demonstrate significant chemoprotective, anti-platelet aggregation, anti-angiogenic and anti-cancer properties. A significant number of studies have discovered that berry anthocyanins are novel cardioprotectant, beneficial in reducing age-associated oxidative stress, improving neuronal and cognitive brain function as well as ocular health, and protecting genomic integrity. Anthocyanin derivatives exhibit a broad spectrum of pharmacologic, therapeutic and medicinal properties including antioxidative, anti-bacterial, anti-viral, anti-hypertensive and anti-angiogenic effects. Furthermore, anthocyanins enhance immune system and detoxification, and modulate cholesterol biosynthesis. Edible berry anthocyanins have been shown to inhibit cellular transformation and this study demonstrates the potent inhibitory effect on inducible VEGF expression. Overall, anthocyanin-rich fruits and vegetables and their constituents favorably regulate a number of mechanistic pathways to promote human health and to prevent diseases.

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Anthocyanins are known to exhibit some of the strongest therapeutic effects of any phytochemical, and have been used as a viable therapy for disease prevention and health promotion. During the past two decades a growing number of studies have investigated the diverse medicinal and protective abilities of anthocyanins present in various fruits and vegetables. *In vitro*, *in vivo* and human clinical studies have shown that berries possess many biological functions that confer potent health benefits including cardiovascular and neurological health, blood sugar support, anti-aging, anti-inflammatory and anti-cancer properties, urinary tract health, healthy vision and dermal health.

In the United States, daily intake of anthocyanins in humans has been estimated to be 180–215 mg/day, which is much higher than the intake (23 mg/day) of other flavonoids (*1,2*). Of all the antioxidants, anthocyanins have shown their ability to penetrate into cell membranes, enhance and demonstrate therapeutic benefits. Main therapeutic benefits attributable to anthocyanins include antioxidant cytoprotection and maintaining genomic DNA integrity. Numerous studies have linked anthocyanin activity to an increase in peripheral circulation, improved vision, enhanced immune systems, and wound healing. Anthocyanins also serve as anti-inflammatory, antibacterial and anti-mutagenic agents, and provide cardioprotection by maintaining vascular permeability.

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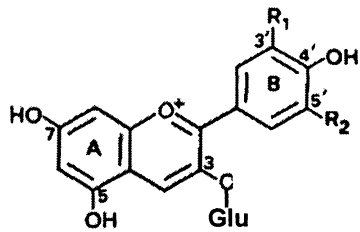
Chemistry and Bioavailability of Berry Anthocyanins

Anthocyanins, a sugar or acid form of anthocyanidins, are powerful natural antioxidants of the flavonoid family. Anthocyanins represent a large group of water-soluble plant pigments of the 2-phenylbenzophyrylium (flavylium) structure (Figure 1) (*1*). The anthocyanin class consists of approximately 200 or more compounds chemically combined with a sugar moiety such as glucose, rhamnose, galactose, xylose and arabinose (*3*). The positively charged oxygen atom in the anthocyanin molecule makes it a more potent hydrogen-donating antioxidant compared to other flavonoids (*4*). Berry anthocyanins are one of the most important group of plant pigments visible to the human eye. Anthocyanins differ from other natural flavonoids in the range of blue to red colors imparted by the anthocyanins and by their ability to form resonance structures dependent largely upon the changes in pH (*5,6*). At pH 1-3 the flavylium cation is red colored, at pH 5 the colorless carbinol pseudo base (pb) is generated, and at pH 7-8 the blue purple quinoidal base (qb) is formed (*7,8*).

Consumption of natural polyphenolic-enriched foods including strawberries, spinach or red wine has been shown to increase the serum antioxidant capacity in humans (*10*). The serum total antioxidant capacity was assessed following consumption of strawberries (240 g), spinach (294 g), red wine (300 ml) or vitamin C (1250 mg) in 80 elderly women using different assay techniques including oxygen radical absorbance capacity (ORAC) assay, Trolox equivalent antioxidant capacity (TEAC) assay and ferric reducing antioxidant potential (FRAP) assay. The results showed that the total antioxidant capacity of serum determined as ORAC, TEAC and FRAP, using the area under the curve,



$R_1 = H$	$R_2 = H$	: Pelargonidin
$R_1 = OH$	$R_2 = H$	: Cyanidin
$R_1 = OH$	$R_2 = OH$	: Delphinidin
$R_1 = OCH_3$	$R_2 = H$	: Peonidin
$R_1 = OCH_3$	$R_2 = OH$	: Petunidin
$R_1 = OCH_3$	$R_2 = OCH_3$	: Malvidin

Figure 1. Chemical Structure of Anthocyanins.

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increased significantly by 7-25% during the 4-h period following consumption of red wine, strawberries, vitamin C or spinach. The total antioxidant capacity of urine showed that ORAC increased ($p<0.05$) by 9.6, 27.5, and 44.9% for strawberries, spinach and vitamin C, respectively, during the 24-h period following these treatments. The plasma vitamin C level after a strawberry drink, and the serum urate level after the strawberry and spinach treatments, also increased significantly. However, the increased vitamin C and urate levels could not fully account for the increased total antioxidant capacity in serum the following consumption of strawberries, spinach or red wine (*9*).

Cao and Prior (1999) demonstrated the direct evidence of bioavailability of anthocyanins in humans by combining an octadecylsilane (ODS) solid-phase extraction procedure for plasma sample preparation and an HPLC system for anthocyanin separation and detection. One 35 year old male subject consumed 25 g elderberry extract containing 1.5 g of total anthocyanins after fasting overnight. Two main peaks at 520 nm were revealed in the plasma samples collected 30 min or 60 min after the consumption of an elderberry extract. The elution times (71.3 min and 71.9 min, respectively) and spectra of these two peaks in the plasma samples were the same as those of the two anthocyanins detected in the elderberry extract, indicating that anthocyanins can be absorbed in their glycosidic forms in humans (*10*).

Pedersen *et al.* (*11*) assessed whether consumption of 500 ml of blueberry juice or cranberry juice by healthy female subjects can increase plasma phenolic content and antioxidant capacity. After an overnight fast, nine volunteers consumed 500 ml of blueberry juice, cranberry juice or a sucrose solution (control). Each volunteer participated on three occasions one week apart, consuming one of the beverages each time. Blood and urine samples were

obtained 4 h after consuming the juices. Consumption of cranberry juice resulted in a significant reduction of plasma potassium nitrosodisulfonate and Fe (III)-2,4, 6-Tri (2-pyridyl)-s-triazine, measurements of antioxidant capacity attaining a maximum after 60-120 min. This corresponded to a 30% increase in vitamin C and a small but significant increase in total phenols in plasma. Blueberry juice exhibited no such effects (*11*).

Antioxidant Potential of Berry Anthocyanins

Anthocyanins have been found to serve as a potent antioxidants compared to traditional antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoulene (BHT) and α-tocopherol (vitamin E). Extracts of edible berries are effective scavengers of peroxyl radicals and a non-competitive inhibitor of lipid peroxidation. This natural agent has been shown to prevent autoxidation of lipids as well as lipid peroxidation in biological systems (*12*). In a recent clinical study conducted by Marniemi *et al.* (*13*) the long-term and short-term effects of increased intake of deep-frozen berries (bilberries, lingonberries or black currants) on antioxidant potential and lipid peroxidation were evaluated. Healthy 60-year-old men were randomized to berry, supplement and control groups (20 men in each group). The berry group consumed a 100 g portion of deep-frozen berries (bilberries, lingonberries or black currants) daily for 8 weeks in addition to their normal diet. Other groups ingested daily 100 mg of α-tocopherol and 500 mg of ascorbic acid (supplement group) or 500 mg of calcium gluconate (control group). Serum ascorbate concentrations increased significantly in both the berry and the supplement group. Serum α-tocopherol levels and the antioxidant potential (TRAP) in low density lipoprotein (LDL) increased in the supplement group only. In the berry group, slightly lowered LDL diene conjugation ($p = 0.074$) and slightly increased total serum TRAP ($p = 0.084$) values were observed. No changes were found in these measures in the supplement or the control group. In a short-term experiment, LDL TRAP showed a small increase (about 10%, $p = 0.039$) during 5 h after the intake of 240 g berries (*13*).

The potential antioxidant properties of blueberry polyphenolics were investigated *in vitro* and *in vivo* using red blood cell (RBC) resistance to reactive oxygen species (ROS) as the model (*14*). *In vitro* incubation with anthocyanins or hydroxycinnamic acids (HCA) (0.5 and 0.05 mg/ml) was found to significantly enhance RBC resistance to H_2O_2 (100 μM)-induced ROS production. This protection was also observed *in vivo* following oral supplementation in rats at 100 mg/ml. However, only anthocyanins were found to afford protection at a significant level at 6- and 24-h post-supplementation. This protection was not consistent with the measured plasma levels of anthocyanins. Indeed, plasma polyphenolic concentrations were highest after 1 h, declining considerably after 6 h and not detected after 24 h. The difference in absorption between anthocyanins and HCA is likely to have contributed to the

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observed difference in their abilities to afford protection to RBC. This protection represents a positive role following dietary consumption of polyphenolics from blueberries against ROS formation within RBC *in vivo* (*14*).

The comparative antioxidant efficacy of six individual berry extracts (wild blueberry, wild bilberry, cranberry, elderberry, raspberry seed and strawberry) was assessed with a novel combination of these six berry abstracts, OptiBerry, which was developed based on oxygen radical absorbance capacity (ORAC) assay, cellular uptake and cytotoxicity (LDH leakage) assays (*15*). OptiBerry demonstrated comparable antioxidant activity to wild blueberry and wild bilberry extracts, and significantly superior antioxidant activity compared to all other berry extracts tested (*16*). In a more recent study, we demonstrated the whole body antioxidant efficacy of OptiBerry using a novel EPR methodology (Figure 2) and substantiated the validity of this model using *in vivo* antioxidant assays (*17*). In this study, anesthetized mice were injected with a nitroxyl radical through tail vein, placed in a custom-built 750MHz EPR spectrometer (32 mW microwave power, 0.7G modulation amplitude, 100 KHz modulation frequency, 80ms time constant and 35 G scan range). Images were taken every 4 minutes. Antioxidants reduce the nitroxyl radical to hydroxyamine, and thus the signal decays with time. A higher intensity signal from an area indicates lower reduction capability. Three groups of animals were used: (a) Control group: a high signal intensity is observed in the peritoneum at 4 and 8 min, and in the bladder at 12 and 16 min; (b) Hyperbaric oxygen (HBO) group: in treatment with HBO at 2 atm for 120 min, a higher signal intensity was observed at all time points as compared to the control group, and the nitroxyl radical stays in the body for a longer time; and (c) Treatment with OptiBerry (OB) fed diet for two weeks and hyperbaric oxygen at 2 atm for 120 min (Figure 2). OB administration reduced HBO-induced oxidative stress as demonstrated by the signal intensity obtained. The radical was retained in the OB-treated group for a much shorter time period than the other two groups *(17)*.

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Anthocyanins and Cardioprotection

Cardiovascular disease is a major killer of older men and women in the United States and around the world. Epidemiologic data support the association between high intake of anthocyanin-rich fruits and vegetables and low risk of cardiovascular disease (*18-20*). Anthocyanins have been shown to reduce platelet aggregation *in vitro*, improve endothelial function and offer vascular protection comparing favorably with other cardioprotective drugs.

Capillary strength is a key component in cardiovascular health, while damaged capillaries contribute to electrolyte imbalances and lead to edema and other dysfunctions. Hypertension, atherosclerosis and diabetes reduce the flexibility of arterial walls, which contributes to poor blood flow, plaque formation and thicken capillary walls due to collagen and glycoprotein deposits. The thickened capillaries are less flexible and more susceptible to blockage,

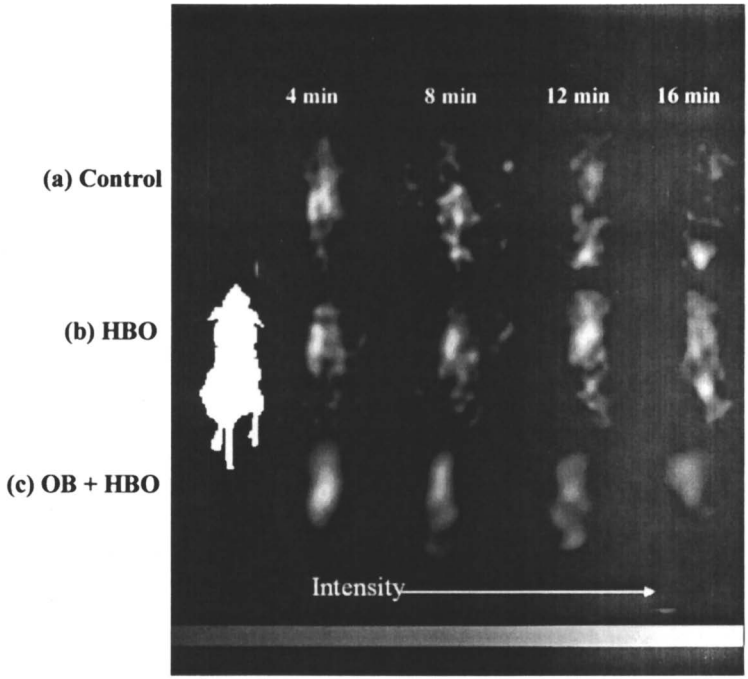

Figure 2. Comparison of redox status in mice. Data was obtained from a custom-built 750MHz EPR spectrometer. Three groups were used: (a) Control group; (b) Hyperbaric oxygen (HBO) group; and (c) OptiBerry (OB) treatment group. Animals were administered OB-enriched diet for two weeks and hyperbaric oxygen at 2 atm for120 min.

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leading to atherosclerosis. Rat aorta smooth muscle cells incorporated less radiolabeled amino acids when cultured with *Vaccinium myrtillus* anthocyanidins suggesting one mechanism by which bilberry maintains normal capillary structure (*5*). Furthermore, anthocyanidins decrease the proliferation of the intima, the extracellular matrix production, the calcium and lipid deposition in the aorta, and the DNA and lipid contents (*21*).

Bilberry extract has been shown *in vitro* to enhance relaxation of calf aortas that had been exposed to adrenalin. The dose response was nearly linear as the extract concentration was increased from 25-100 μg/mL. It has been proposed that relaxation of the blood vessels is due to the inhibition of catechol-O-methyltransferases. Anthocyanins and ascorbic acid inhibit contractile responses of calf aortas in the presence of histamine and angiotensin II. Hamsters treated with 36% bilberry anthocyanidins (10 mg/10 g body weight) for 2 or 4 weeks exhibited better capillary perfusion and fewer sticking leukocytes in ischemia-induced capillaries (*22*).

Antioxidants have shown the promise to improve endothelial function, which plays an important role in the initiation and development of vascular disease. Youdim *et al.* (2000) examined the ability of endothelial cells (EC) to incorporate anthocyanins, and evaluated the potential benefits of anthocyanins against various oxidative stressors. Elderberry extract containing four anthocyanins were incorporated into the plasma membrane and cytosol of EC following 4 h of incubation at 1 mg/ml. The elderberry enriched EC conferred significant protective effects against diverse oxidative stressors including hydrogen peroxide H_2O_2, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) and $FeSO_4$/ascorbic acid. Thus anthocyanins may have important implications on preserving EC function and preventing the initiation of EC changes associated with vascular diseases (*23*).

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Anthocyanins, Diabetes and Metabolic Syndrome

The leaves and fruits of *Vaccinium myrtillus* are used as a traditional therapy to treat many conditions resulting from diabetes. Flavonoids inhibit aldose reductase, an enzyme that converts sugars to sugar alcohols and is implicated with diabetic complications such as neuropathy, retinopathy and heart disease.

Active constituents of *Vaccinium myrtillus L.* (blueberry) leaves have been shown to be potentially useful for treatment of dyslipidaemiae associated with impaired triacylglycerol-rich lipoprotein clearance. In a study conducted by Cignarella *et al.* (*24*) an aqueous alcohol extract of blueberry leaves produced a 26% reduction in plasma glucose levels in streptozotocin-diabetic rats. Plasma triacylglycerol decreased by 39% in proportion with the amount of blueberry leaf extract given to rats (1.2 or 3.0 g/kg body weight) fed a hyperlipidemic diet (*24*).

Parameters of retinal oxidative stress, protein kinase C activity, and nitric oxide remained elevated for at least one year of hyperglycemia, and these abnormalities were normalized by multi-antioxidant therapy. Long-term administration of antioxidants may inhibit the development of the early stages of diabetic retinopathy, and the mechanism by which these actions occur, warrants further investigation (*25*).

Neuroprotection, Enhanced Brain Function and Mental Clarity

Brain functions such as balance, coordination, short-term memory and information retrieval can be impaired with advancing age. Recent research has indicated that increased vulnerability to oxidative stress may be the major factor involved in central nervous system (CNS) functional declines in aging and age-related neurodegenerative diseases, and that berry anthocyanins may ameliorate or prevent these declines. Diets supplemented with either spinach, strawberries or blueberries, nutritional sources of antioxidants, have been shown to prevent

and/or reverse age-related declines in cerebellar noradrenergic receptor function (*26,27*). Studies examined whether long-term feeding of Fischer 344 rats, beginning when the rats were six months of age and continuing for eight months, with diets supplemented with a fruit or vegetable extract identified as being high in antioxidant activity, could prevent the age-related induction of receptor-mediated signal transduction deficits that might have a behavioral component. A number of relevant parameters including oxotremorine-enhanced striatal dopamine release (OX-K^+-ERDA), cerebellar beta receptor augmentation of gamma-aminobutyric acid (GABA) response, striatal synaptosomal $^{45}Ca^{2+}$ clearance, carbachol-stimulated GTPase activity and Morris water maze performance. The rats were given control diets or those supplemented with strawberry extracts (SE), 9.5 gm/kg dried aqueous extract (DAE), spinach (SPN 6.4 g/kg DAE), or vitamin E (500 IU/kg). Results indicated that SPN-fed rats demonstrated the greatest retardation of age-effects on all parameters except GTPase activity, on which SE had the greatest effect, whereas SE and vitamin E showed significant but equal protection against these age-induced deficits on the other parameters. For example, OX-K^+-ERDA enhancement was four times greater in the SPN group than in controls. Thus, phytochemicals present in antioxidant-rich foods such as spinach may be beneficial in retarding functional age-related CNS and cognitive behavioral deficits and, perhaps, may have some benefit in neurodegenerative disease (*28*). Treatment of rats with anthocyanosides of *Vaccinium myrtillus* before the induction of hypertension has also been shown to keep the blood-brain barrier permeability normal which tends to become impaired with aging, showing a decrease in vascular density, increased permeability and other abnormalities *(20)*.

Saija *et al.* (*29*) demonstrated that dietary antioxidants can protect the brain from oxygen-induced damage and prevent decreases in nerve growth factor in the basal forebrain and mediated T3 transport at the capillary level. After eight weeks on the anthocyanin diet, the rats were subjected to 48 h of 100% oxygen-induced damage similar to that found in aged rats. Rats given intraperitoneal injections of bilberry anthocyanins (200 mg/kg/day) for 5 days had significantly more T3 in their brains than rats given only the solvent (26% alcohol). The specific portions of the brain responsible for better memory, vision and control of sensory input contained more T3 in the bilberry treated animals (*29*).

Spinach and strawberry supplementation primarily increased striatal muscarinic receptor sensitivity, and this appeared to be reflected in the reversal of cognitive behavioral deficits, where all of the diets, including the blueberry-supplemented diet, decreased the latency to find the platform and distance to the platform. Striatum has been demonstrated to be involved in mediating cognitive performance. Thus, dietary enhancement of striatal muscarinic receptor sensitivity in the striatum ultimately may be expressed as improved cognitive performance (*30*).

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Beneficial Role of Anthocyanins in Advancing-Age

Oxidative stress is particularly vulnerable with advancing age; therefore antioxidant-rich phytochemicals or red wine may be beneficial in reducing or retarding the functional central nervous system deficits seen in advancing-age or oxidative insult. Researchers determined whether increased oxidative stress protection via nutritional increases in antioxidant levels can be achieved in rats using diets supplemented with vitamin E (500 IU/kg), strawberry extracts (9.4 g/kg dried aqueous extract, DAE), spinach (6.7 g/kg DAE), or blueberry extracts (10 g/kg DEA for six weeks)], and whether these antioxidants can protect against exposure to 100% oxygen (a model of accelerated neuronal aging). These diets were effective in preventing oxidative stress-induced decrements in several parameters. Thus, antioxidant-rich phytochemicals may be beneficial in reducing or retarding the functional nervous system damages observed in advancing-age or oxidative stress (*31*).

Among the most effective agents that antagonized cellular vulnerability to oxidative stress was the combination of polyphenolics found in fruits (e.g. blueberry extract) with high antioxidant activity. Subsequent experiments using dietary supplementation with fruit (strawberry) or vegetable (spinach) extracts have shown that such extracts are also effective in forestalling and reversing the deleterious effects of behavioral aging in F344 rats. Thus, fruit and vegetable-derived polyphenolic antioxidants can demonstrate significant benefits in neuronal aging disorders and behavior (*32*).

It is often assumed that antioxidant nutrients contribute to the protection afforded by fruits, vegetables and red wine against diseases of aging. The consumption of strawberries, spinach or red wine, which is rich in antioxidant phenolic compounds, can increase the serum antioxidant capacity in humans (*9*).

Another study by Joseph *et al. (30)* showed that supplements (strawberry, spinach, or blueberry at 14.8, 9.1, or 18.6 g of dried aqueous extract per kg of diet, respectively) fed for eight weeks to 19-month old Fischer 344 rats were also effective in reversing age-related deficits in several neuronal and behavioral parameters including: oxotremorine enhancement of K^+-evoked release of dopamine from striatal slices, carbachol-stimulated GTPase activity, striatal Ca^{45} buffering in striatal synaptosomes, motor behavior performance on the rod walking and accelerated tasks, and Morris water maze performance. These findings suggest that antioxidant-rich foods may be beneficial in reversing the course of neuronal and behavioral aging *(30)*.

Urinary Tract Health

Cranberry (*V. macrocarpon* Ait.) juice has long enjoyed a folk reputation as a treatment for urinary tract infections (UTI). Although the low pH of the fruit is was at first believed to be the antimicrobial agent, fructose and high-molecular-

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weight phenolic compounds have been found to prevent the adhesion of *Escherichia coli* cells *in vitro*. These compounds have also been identified in blueberry juice. Fructose and polyphenols prevent mannose-resistant adhesions on certain P-fimbriated *E. coli* isolates from attaching to epithelial tissues in the urinary tract. Purified cranberry proanthocyanidins were reported to possess anti-adherence properties in an *in vitro* assay. Blueberry juice demonstrated a similar activity. Cranberry products provide women and their physicians with a helpful and effective new tool to manage recurrent urinary infections and a means to substantially reduce the amount of antibiotics used in prophylaxis against UTI (*33*).

Healthy Vision

Bilberry anthocyanins have been used for nearly a century to improve ocular health, and shown to be effective in the prevention and treatment of certain eye disorders such as cataracts, age-related macular degeneration, and other ocular dysfunctions. Bilberry extract is believed to improve eyesight, particularly night vision by enhanced generation of retinal pigments, increasing circulation within the capillaries of the retina, decreasing macular degeneration and diabetic retinopathy, improving or preventing glaucoma and cataracts, and relieve eye strain. Since carotenoids with vitamin A activity are found in *Vaccinium* species, some of the benefits pertaining to vision are attributable to these compounds. Anthocyanosides are potent antioxidants with a particular affinity for the eye and vascular tissues. In a randomized, double-blind, placebo-controlled trial, fifty outpatients, 21 men and 29 women (mean age 67 years, range 48-81) suffering from mild senile cortical cataract (62 eyes) underwent therapy with Vitamin E plus *Vaccinium myrtilluis a*nthocyanosides (1, 2 tabs b.i.d.) for 4 months. This combination was able to stop lens opacity progress in 97% of the eyes without any side effects or adverse-drug reactions (*7*). In a recent report of 50 patients with senile cataracts, a combination of bilberry extract standardized to contain 25% anthocyanosides (180 mg twice daily) and vitamin E in the form of dl-tocopheryl acetate (100 mg twice daily) administered for four months stopped the progression of cataracts in 96% of the subjects treated (n=25) compared to 76% in the control group (n=25) (*34*).

Research has shown that excess exposure to light, particularly bright daylight, high-contrast lights and even computer screens deplete rhodopsin, result in poor night vision. Anthocyanins have been implicated to improve visual functions and have effects on the regeneration of rhodopsin. Black currant fruit anthocyanins have a major effect on the regeneration of rhodopsin. Using frog rod outer segment membranes, cyanidin 3-glycosides, glucoside and rutinoside were found to stimulate the regeneration (*35*). In a double-blind, placebo-controlled study, subjects given oral doses of bilberry anthocyanins adapted to the light within 6.5 min, compared with 9 min for the control group (*8*).

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Anthocyanosides have also been shown to be effective therapy for diabetic or hypertensive vascular retinopathy. In a double-blind study, fourteen diabetic or/and hypertensive outpatients with vascular retinopathy underwent therapy with *Vaccinium myrtillus* anthocyanosides (160 mg b.i.d.) or placebo (n=20) for one month. At the end of the month, patients placebo-treated received the active drug for one additional month. Ophthalmoscopic and fluoroangiographic findings recovered before and after treatments showed an improvement ranging from 77 to 90% of anthocyanoside-treated patients (*36*). Ocular circulation and neovascularization has also been shown to play a significant role in diseased conditions of the eye, in particular diabetic retinopathy, retrolental fibroplasia and neovascular glaucoma (*37,38*). Berry anthocyanins and its effects on angiogenesis is discussed in another section.

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Anti-cancer and Anti-angiogenic Potential of Anthocyanins

Studies have demonstrated potent anti-cancer properties of blueberry, strawberry, raspberry, bilberry and cranberry *in vitro* and *in vivo* models (*39,40*). Blueberries have been shown to have an inhibitory effect on the growth of prostate cancer cells. Schmidt *et al.* (*41*) demonstrated that wild and cultivated blueberry fruits have inhibitory effects on the proliferation of LNCaP, an androgen-sensitive prostate cancer cell line, and DU145, a more aggressive androgen insensitive prostate cancer cell line. Wild blueberry proanthocyanidin fraction (20 μg/ml) was added to LNCaP media, in which growth was inhibited to 11% of control with an IC_{50} of 13.3 μg/ml. Two similar proanthocyanidin-rich fractions from cultivated blueberries at the same concentration inhibited LNCaP growth to 57 and 26% of control with an IC_{50} of 22.7 and 5.8 μg/ml, respectively. In DU145 cells, the only fraction that significantly reduced growth compared to control was from cultivated blueberries with an IC_{50} value of 74.4 μg/ml, indicating only minor inhibitory activity. These results have also determined that the differences in cell growth inhibition of LNCaP and DU145 cell lines by blueberry fractions rich in proanthocyanidins indicate that blueberry proanthocyanidins have an effect primarily on androgen-dependant growth of prostate cancer cells (*41*).

Black raspberries have been shown to prevent esophageal cancer and keep precancerous growths from becoming malignant. Esophageal cancer is the fifth leading cause of cancer death in the world with only 8% to 12% of individuals surviving 5 years after being diagnosed with the disease. In a recent study, rats injected with a cancer-causing compound were less likely to develop cancer of the esophagus when black raspberries comprised 5% to 10% of their daily diet. Researchers injected rats with NMBA, an inducer of esophageal cancer. Rats that consumed the greatest amount of black raspberries both 2 weeks before and up to 30 weeks after NMBA injections had 49% fewer tumors than rats whose diets did not include black raspberries. Additionally, tumors that developed in

raspberries-fed rats were found to decrease in size after 15 weeks. After 25 weeks, rats fed diets of 5% to 10% black raspberries exhibited a decrease in the number of esophageal tumors by 43% to 62% (*42*). Other studies have demonstrated that freeze dried strawberries also have potential inhibitory effects against esophageal cancer (*43*).

Angiogenesis is a key event that feeds tumor growth and cancer metastases, and therefore, anti-angiogenic approaches to prevent and treat cancer represent a priority area in investigative tumor biology. Six individual berry extracts including wild blueberry, bilberry, cranberry, elderberry, raspberry seed and strawberry, and OptiBerry, a novel combination of these six edible berry extracts, possess dramatic anti-angiogenic properties as demonstrated by VEGF inhibition and Matrigel assay (*16*). Surprisingly, grapeseed proanthocyanidin extract, with comparable ORAC, or α-tocopherol did not influence inducible VEGF expression suggesting that the observed effect of berry extracts was not dependent on their antioxidant property alone. However, pure flavonoids such as ferrulic acid, catechin and rutin shared the ability to suppress VEGF expression and exhibit anti-angiogenic properties (*16*). Thus, results demonstrate that anthocyanin structural moiety may be responsible for the anti-angiogenic effect.

Matrigel assay, a highly reliable approach to study *in vitro* angiogenesis, induces endothelial cells to differentiate as shown by morphological changes and by the reduction in proliferation, which offers a convenient model to study biochemical changes associated with angiogenesis. In a study conducted by Atalay *et al.* (*44*), human dermal microvascular endothelial cells (5,000 cells/well), harvested in the presence and absence of OptiBerry to determine its anti-angiogenic potential, were added on top of the solidified matrix solution and maintained in an incubator at 37°C overnight. OptiBerry exhibited significant anti-angiogenic potential in this model (*16*).

Hemangiomas are the most common infancy tumors, which occur in approximately 1:100 normal newborns. Approximately 5% of hemangiomas cause serious tissue damage, while 1-2% of all hemangiomas are life threatening. Proliferating hemangiomas are highly angiogenic and thus represent a powerful model to study *in vivo* angiogenesis.

Pretreatment of the endothelioma (EOMA) cells, derived from the spontaneously arising hemangioma, with OptiBerry significantly inhibited TNFα-induced basal MCP-1 and NF-κβ transcription. Furthermore, in an *in vivo* model, eight week old 129P3/J mice (Jackson Laboratories, Bar Harbor, ME) were treated with 100 μl of EOMA cell suspension (5 x 106 cells) with or without OptiBerry pretreatment. OptiBerry treated group did test positive for the presence of hemangioma, the average mass of such tumor growth was below 50% as compared to the untreated control group. Histological analysis demonstrated markedly decreased infiltration of macrophages in hemangioma of treated mice compared to controls (*15,44*).

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Conclusion

There are numerous reasons why consumption of anthocyanin-rich fruits and vegetables might retard or prevent the onset of chronic diseases. Edible anthocyanin and anthocyanidin phytopharmaceuticals exhibit complementary, pharmacologic and diverse overlapping mechanisms of action, including antioxidative, antibacterial, antiviral, induction of detoxification enzymes, stimulation of the immune system, reduction of platelet aggregation, modulation of cholesterol synthesis, anti-hypertensive and anti-angiogenic effects. Edible berry anthocyanins have been shown to inhibit cellular transformation and this study exhibited the potent inhibitory effect on inducible VEGF expression (*15*). It is worthwhile to mention that a number of novel antioxidants didn't exhibit anti-angiogenic effect like berry anthocyanins (*15*). Thus, antioxidant property alone may not account for the total observed anti-angiogenic effect. OptiBerry, a novel combination of six berry anthocyanins, significantly inhibited basal MCP-1 and inducible NF-κβ transcriptions, and that EOMA cells pretreated with OptiBerry showed a diminished ability to form hemangioma (*15,44*). Histological analysis exhibited markedly decreased infiltration of macrophages in hemangioma of treated mice compared to the control animals (*15,44*). Thus, berry anthocyanins may exert novel chemoprevention, as well as antioxidant and anti-angiogenic properties by several mechanistic pathways.

These broad spectrum beneficial and mechanistic effects have been examined primarily *in vivo* models, experimental dietary studies in humans have also demonstrated the capacity of anthocyanin-rich fruit and vegetables and their constituents to modulate some potential disease-preventive mechanisms.

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Chapter 23

Lutein: Separation, Antioxidant Activity, and Potential Health Benefits

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Lutein is an oxygen-containing carotenoid found in many food plants. Although it is not a vitamin A precursor, many health beneficial effects have been associated with high dietary intake of this phytochemical. Lutein, along with its isomer zeaxanthin, may be the most important xanthophylls for human, as they have been found to play important roles in protecting human from many chronic diseases. In particular, lutein has been found in recent years to protect against age-related macular degeneration (AMD), a leading cause of irreversible vision loss in the elderly population. Lutein is also found to enhance immune function, to prevent cancer, coronary heart disease, and to protect skin from damages caused by ultraviolet light. Our recent study also showed a protective role of lutein against mutagens. Lutein is a strong antioxidant, which may help explain its above physiological functions. However, currently accumulated knowledge on lutein requires further studies on several aspects including the mechanisms of its various bioactivities in the biological system, and how it is absorbed and transported to the site of action. All these require good separation, quantification and detection methods for low concentrations of lutein. Samples containing trace amount of lutein in biological tissues resulting from animal and human clinical trials are particularly challenging. High performance liquid chromatography (HPLC) couple with photodiode array and mass spectrometric

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detector (LC-DAD-MS) will continue to play an important role in quantification and identification of lutein. Preparative high-speed counter-current chromatography may be a good tool for high quality lutein standard for the various bioassays and trials. This chapter is therefore to briefly review the chemistry and biochemistry of lutein, with an emphasis on its occurrence, distribution, separation and bioactivities, and discuss about its possible adverse effect.

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Carotenoids are a group of important phytochemicals that contribute to human health. However, most of the reported studies have so far focused primarily on the provitamin A carotenoids, such as α- and β-carotene, and β-cryptoxanthin because of the perception that provitamin A activity may have been a contributing factor in their observed health benefits in epidemiological studies (*1,2*). Lutein is an oxygen-containing carotenoid known collectively as xanthophylls. Many plants, particularly the dark green leafy vegetables such as spinach and kale, contain lutein. Certain medicinal plants such as. marigold flower (*Tagetes erecta* L.), may contain an even higher concentration of lutein (*3*). Lutein is not a vitamin A precursor, however, many health benefits have been associated with high dietary intake of this phytochemical. Lutein, along with its isomer zeaxanthin, may be the most important xanthophylls for humans as they play important roles in protection from many chronic diseases. In particular, lutein has been found to protect against age-related macular degeneration (AMD), a leading cause of irreversible vision loss in the elderly population in the Western world. It is estimated that 1.6% of the population in the 50-65-year-old age group is affected, rising to 30% in the over-75-year-old age group. As the proportion of the elderly in our population increases, the public health impact of AMD will become even more severe (*4-6*). In addition to lowering the AMD risk, lutein is also found to enhance immune function, suppress mammary tumor growth and enhance lymphocyte proliferation (*7,8*). Lutein protects skin from damages caused by ultraviolet light, and also prevents cardiovascular hardening caused by ageing, coronary heart disease and cancer (*9-11*). Many of these diseases are considered to be related to the oxidative stress from excess free radicals, thus the effect of lutein in disease risk reduction may have arisen from its strong antioxidant activity found in recent studies (*12,13*). The antioxidant activity of lutein was confirmed by Khachik *et al,* (*14*) by identifying its oxidation products in the macular region of the human and monkey retinas. Lutein has also been found to be more bioavailable from food sources than is β-carotene (*1*). Lutein and zeaxanthin were detected in human plasma and breast milk (*15,16*), and more significantly, in the macular pigment of the human retina where they play most critical protective roles (*17-20*). While both lutein and zeaxanthin are found to be important for human health,

lutein is considered more valuable because its concentration, both in the human body and in most diets, is generally higher than zeaxanthin (*2*).

Many lutein-containing functional foods and nutraceutical products have been developed in recent years to help needy populations acquire sufficient lutein intake through supplementation. Lutein is also among the 10 phytochemicals recommended by the FDA as GRAS (generally recognized as safe) nutritional supplements (http://www.cfsan.fda.gov/~rdb/opa-g110.html). However, caution must be exercised when lutein is purified and given in high doses to human subjects as supplements. Certain carotenoids such as β-carotene have been found to cause negative effect at high dosages, especially among smokers (*21,22*). In addition, some biological functions of lutein are not consistent; many effects for purified lutein at higher dosages are not clear (*6*). The objective of this chapter is to briefly review the chemistry and biochemistry of lutein, with an emphasis on its occurrence, distribution, separation and bioactivities, and discuss its possible adverse effects.

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Occurrence and biological function of lutein in plants

Carotenoids are very common both in the plant and the animal kingdoms though they are always of plant origin. There have been more than 600 carotenoids identified to date, mostly from plants and algae (*23*). Lutein, as many other carotenoids, is an integral component of higher plant photosystems, and it is the most abundant among all carotenoid compounds in photosynthetic plant tissues (*24*). Lutein acts as a photosensitizer for the photosynthesis process, and protects plants and algae from damage caused by ultraviolet radiation and oxygen.

In addition to photosynthetic plants and algae, lutein also occurs in some non-photosynthetic bacteria, yeasts, and molds, where it may carry out a protective function against damage by light and oxygen. Lutein occurs in high concentrations in green leafy vegetables, particularly kale and spinach. Fruits such as mango, papaya, oranges, kiwi fruit, melons and peaches are also known to contain lutein, although their concentrations may not be as high as in some vegetables (*25,26*). Cereal grains including corn and wheat are known to have high lutein content, particularly ancient grains (*27*). Major dietary sources of lutein can be found in vegetables like squash, pumpkin, potato, peas, lima beans, green beans and broccoli (Table 1) (*2,28*).

The total xanthophyll content of marigold flower can be as high as 2.5% on a dry weight basis, of which 88-92% are from lutein and zeaxanthin (*29*). Lutein and zeaxanthin are responsible for the intense yellow/orange coloration of the crown petals of marigold flower. Lutein is the overwhelmingly predominant xanthophyll (72.3-87.3%), whereas zeaxanthin is approximately 4.0-16.4% of the total carotenoids content; lutein and zeaxanthin occur mainly in ester forms of saturated fatty acids, for example, ca. 95% of the lutein occurs

Table 1. Lutein and zeaxanthin concentrations in common of foods [a]

Food Source	*Lutein/zeaxanthin (μg/ 100g)*	*Food Source*	*Lutein/zeaxanthin (μg/ 100g)*
Kale, raw	40,000	Green beans	600
Kale, cooked	16,000	Okra	400
Spinach, raw	11,000	Iceberg lettuce	350
Collards, cooked	8,100	Cabbage	300
Spinach, cooked	7,000	Carrots	300
Broccoli	2,500	Tangerine	250
Romaine lettuce	2,500	Celery	200
Squash, Zucchini	2,100	Orange	200
Sweet corn	1,800	Tomato	150
Brussels sprouts	1,500	Green peppers	75
Peas	1,400	Egg	55
Persimmon	800	Wheat [b]	50

[a] Data were extracted from (*8*).
[b] Average of the three wheat varieties reported by (*2*).

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as esters with fatty acids (*30,31*). The majority of lutein esters in marigold are diester, and dimyristate and dipalmitate were reported as the major diesters of lutein (*3*).

Stability and factors affecting lutein in foods

Many factors affect the level of lutein in plants and foods. Concentration of lutein varies significantly among different vegetables, fruits and grains, although factors such as geographic location and growing season may also affect the concentration of lutein in the crops (*25*). Genetic variation of phytochemicals such as lutein can be used for breading functional fruits and vegetables with elevated concentrations (*32*). A more interesting source of lutein can be the industrial waste or by-products from the food processing industries. A recent study examined peels of squashes, a major component in the pomace generated in the canning industry, and found them to contain high concentrations of free lutein as opposed to the esters in marigold (*28*).

Processing is another important factor affecting carotenoid's concentration. Lutein is relatively unstable under ambient conditions due to its sensitivity to light and oxygen. Heating of pure lutein might be different from lutein-rich food, as processing conditions such as high temperature experienced during heating/baking increase the release of carotenoids including lutein in certain foods (*28*), however, cooking in other cases have been found to reduce lutein contents (*25*).

Separation, purification and detection of lutein

Lutein does not exist in plants or dietary sources alone, but often co-exists with many other carotenoids and phytochemicals. This makes separation and detection of lutein a highly difficult task. In addition, lutein is often found to occur as mono- or di- ester forms with different saturated fatty acids in the plants. Although monoesters are readily separated from diesters, it requires extra effort in separating highly structurally similar monoesters or diesters from each other in their respective categories. Occurrence of stereo- and regioisomers of lutein esters makes the separation even more arduous (*3*).

The highly conjugated double bond system in the molecule of lutein makes it readily attacked by light and oxygen. Thus, lutein or its derivatives are labile under ambient conditions, hence special care has to be taken during sample collection, storage and preparation (extraction, concentration and purification) procedures. Exposure to light and oxygen can also lead to isomerization to different geometric isomers. Differences in plant matrix, plant part, complexity of other phytochemicals, food type and composition all affect the handling of lutein samples. It is therefore highly recommended that all samples be kept with minimal exposure to light, air and high temperature.

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The high lipophilicity of lutein and related esters determines their solubility in the extracting solvent, thus the efficiency of extraction of lutein from various sample matrices may vary. Hydrolysis or saponification is often performed during or after the extraction in order to simplify the chromatographic profile. This can be done chemically using alkali or enzymatically using esterases. We examined several commercially available hydrolases and esterases, and found that bovine cholesterol esterase (E.C. 3.1.1.13) was the only one effective in hydrolyzing lutein diesters into free lutein (Fig. 1).

Although lipophilic solvents such as hexane are usually used in carotenoid extraction (*33,34*), xanthophylls including lutein, have been extracted with relatively polar solvents such as polyethylene glycol (*35*) and diethyl ether (*36*), to increase the recovery. Tetrahydrofuran (THF) and methanol may also be added to the food sample for better extraction efficiency (*37*).

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In recent years, alternative methods such as supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE) have increasingly become popular in extracting phytochemicals, including lutein and carotenoids. In SFE, supercritical CO_2 is perhaps the most widely used solvent for lutein and carotenoids in general. Supercritical CO_2 is apolar, therefore an ideal solvent for lipophilic carotenoids like lutein (*38-41*). Depending on the sample, solvent modifies such as ethanol may be essential to the high recovery of lutein in SFE (*34*). The profiles and yields from marigold flower were similar between the SFE and Soxhlet extracts (Fig. 2). PLE or ASE (accelerated solvent extraction) is another technique in the area of bioactive phytochemicals. Organic solvents are still used in PLE extraction, however, conditions such as high pressure and elevated temperature significantly reduce the use of solvent and enhance extraction efficiency. Breithaupt (*4*) compared the extraction efficacy of ASE with that of a solvent extraction, and found that the recovery of lutein was 97.6% by ASE, only slightly lower than that by manual solvent extraction (99.8%).

Currently, separation and detection of lutein is carried out mainly by chromatography, particularly high performance liquid chromatography (HPLC). Other chromatographic techniques are relatively disadvantaged for lutein analysis. Both normal phase (NP) and reversed-phase (RP) HPLC have been used in the separation of lutein. The lipophilic characteristics of free lutein, its stereo-isomers, mono- and diesters, and the regioisomers of esters have made the NP-HPLC a favorable choice of separation by some researchers (*2,27,42*), however, perhaps a greater number of excellent RP-HPLC methods have been developed for lutein and carotenoids separation. C18 columns have proven to be well suited for separating lutein from other carotenoids (*43-45*), and free lutein from its mono- and diesters and isomer (*3,31,46*). Higher sensitivity was achieved using 2 mm column as compared to the regular 4.6 mm column (*47*). Further, compared with the C18 columns, C30 column is particularly a good choice for separation of geometric isomers of carotenoids including lutein,

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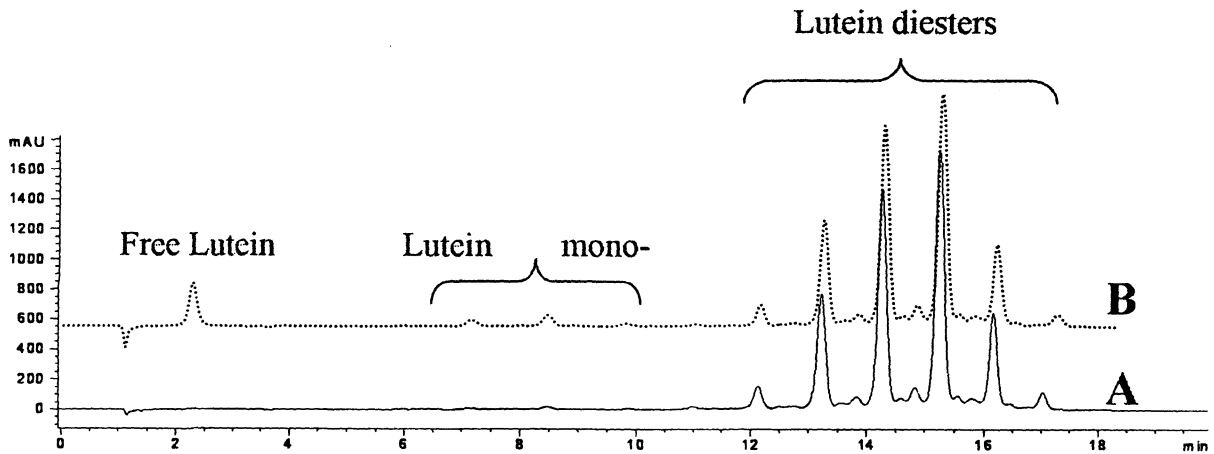

Figure 1. Enzymatic hydrolysis of lutein diesters in marigold flower extract. A: Crude extract of marigold flower before hydrolysis; B: Hydrolysis by a commercially available enzyme (Sigma-Aldrich Company), bovine Cholesterol esterase (E.C. 3.1.1.13) at pH 7.0 and 37°C for 48 h. Procedures followed manufacturer's suggestion. HPLC conditions were the same as in (3).

In Antioxidant Measurement and Applications; Shahidi, F., el al.;
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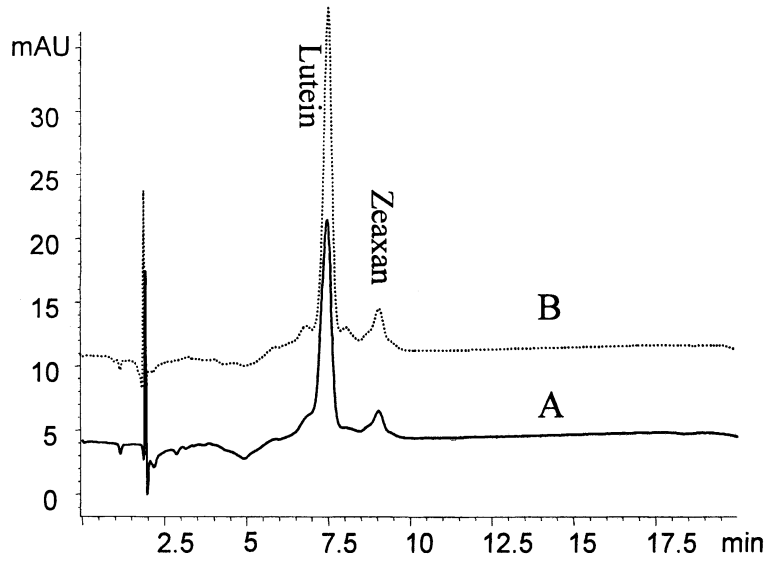

Figure 2. High performance liquid chromatographic profile of marigold flower extracts by solvent and supercritical fluid extraction (SFE). A: SFE using supercritical carbon dioxide. A Hewlett-Packard Model 7680T supercritical fluid extractor was used. Pump rate: 2 mL/min, Marigold flower powder (Tagetes erecta) (0.5 g, particle size < 90 μm) was treated for 5 min of static extraction followed by 50 min of dynamic extraction at a constant pressure and temperature of (350 bar and 40 °C). Yield of oleoresin, 5.5% by SFE. B: Soxhlet extraction using hexane (12 h). Yield of oleoresin, 5.7 by Soxhlet extraction. Data was reported at the Third International Conference on Nutraceuticals and Functional Foods, November 16-20, 2002, San Diego, CA.

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especially for more complex samples such as those high in esters (*2,48-50*). An excellent separation of lutein and zeaxanthin stereoisomers was achieved using C30 RP-HPLC (*51*). Packed capillary HPLC is particularly useful for samples with low lutein concentrations (*52*). Detection and quantification of lutein have largely been relying on online ultraviolet/visible (UV/Vis) detection using photodiode array detector (DAD) and mass spectrometry (MS) (*3*). Use of LC-DAD-MS has become increasingly used in the separation and detection of lutein, although other detection techniques have been reported (*53-55*)

One of the biggest obstacles in research on nutraceuticals and natural health products is the need for large quantities of highly pure bioactive ingredients for animal and human clinical trials. Preparative chromatographic techniques have been used, however, it is costly and labour-intensive. High-speed counter-current chromatography (HSCCC) is a relatively new separation technique that is based on the partition coefficient (*K*) of a compound in two different solvents. It is generally recognized that the *K* value of the target analyte be in the range of 0.2-5 in a given two-phase system in order to obtain good separation, preferably between 0.5-1 (*56,57*). Only a few HSCCC methods have been developed for lutein. Wei *et al.* (*58*) developed a method for purifying lutein from the crude extracts of fresh marigold flower. A method using hexane-ethanol-water (6:4.5:1.5, v/v/v) was also developed in our laboratory and found to be a simple and rapid approach to gram-level pure lutein production (*28*) (Fig. 3).

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Human health benefits

Antioxidant activities of lutein

Lutein and zeaxanthin are known to effectively scavenge reactive oxygen species (ROS), especially singlet oxygen and peroxyl radicals. In quenching singlet oxygen, the ground state lutein molecule receives the energy from the singlet oxygen, leading to the formation of ground state oxygen and a triplet excited lutein. The energy of triplet state lutein is then readily dissipated to the surrounding environment, resulting in intact lutein for re-use (*59*). Such transfer of energy from singlet oxygen to lutein is highly efficient because the energy levels of the triplet oxygen and lutein and other structurally related carotenoids are close (*60*). Similarly, lutein and carotenoids are also the most efficient scavengers of peroxyl radicals, products of lipid peroxidation in cellular membranes. The activity of lutein against peroxidation caused by peroxyl radicals is attributed to its highly conjugated double bond system which stabilizes the radical adducts (*60*). The *in vitro* antioxidant activity is considered to be strongly dependent on the nature of the oxidizing radical species and less dependent on the carotenoid structure (*59,60*).

In the biological system, while vitamin A precursors such as β-carotene are strong antioxidants and essential to human health, many non-provitamin A

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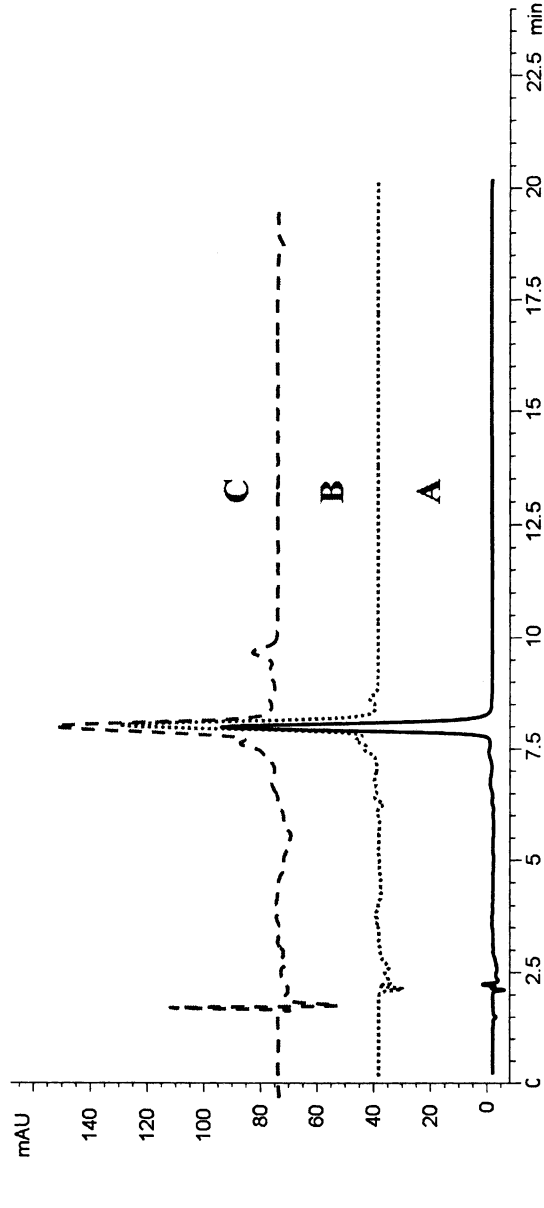

Figure 3. HPLC chromatograms of lutein: A, purified using HSCCC; B, standard; C, saponification mixture of marigold flower extract. See Experimental section for HPLC conditions. Reproduced from Reference (28).

carotenoids, particularly xanthophylls such as lutein and zeaxanthin, have been found to have even higher antioxidant activities (*12,13*). Miki (*12*) found that lutein, although less strong than astaxanthin, had a strong quenching effect against singlet oxygen, and a stronger scavenging effect against free radicals than β-carotene. We also found that lutein was a more powerful antioxidant against superoxide radical in the photochemiluminescence assay (Fig. 4). The extensive conjugated double bonds system in all carotenoids again is considered to play an important role in the antioxidant activity, however, structurally different individual carotenoids differed in their antioxidant potential in humans (*61*). Sundelin and Nilsson (*62*) studied the antioxidative role of lutein, zeaxanthin and other carotenoids using retinal cell culture and an oxidative marker lipofuscin. They reported significantly reduced formation of lipofuscin in cells treated with antioxidant substances including lutein and zeaxanthin. They concluded that since the result is not dependent on their protective effect against photo-oxidative reactions, the chain breaking abilities of these antioxidants in peroxidative reactions of lipid membranes and quenching of free radicals were of importance for inhibition of lipofuscin formation. Lutein and zeaxanthin have also been found to protect lipid membranes against free radical attack in a study by Seddon *et al.* (*63*). They found that UV-induced lipid oxidation was slowed down by lutein and zeaxanthin, and the orientation how the molecule is aligned with the membrane affected the protective efficacy of lutein and zeaxanthin (*63*). The antioxidative protection of lutein and zeaxanthin was also confirmed by Khachik *et al.* (*14*) who identified oxidation products of lutein and zeaxanthin in the macular region of the human and monkey retinas. Lutein and β-carotene showed strong antioxidant activity *in vitro* by quenching peroxyl radicals, and the presence of lutein or lycopene produced the strongest synergistic effect (*64*). The antioxidant activity of lutein is a strong evidence for its physiological effects, particularly chronic diseases that are known to be caused by free radicals.

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Effect of lutein on vision

It is well known that intense light can produce damage in the retina. The action spectrum for light-induced damage shows a distinct maximum at wavelengths between 400 and 450 nm, consistent with the absorption spectrum of the macular pigment, lutein and zeaxanthin. Several studies have shown clear evidence that macular pigment attenuates photic damage in the human retina (*4*). The protection of the retina from photic damage has been postulated to occur through two different functional roles. The first of these is through absorption of blue light as it enters the inner retinal layers thereby attenuating the intensity and potential for photo-oxidation of reactive unsaturated lipid components of photoreceptor disk membrane. However, lutein and zeaxanthin are considered

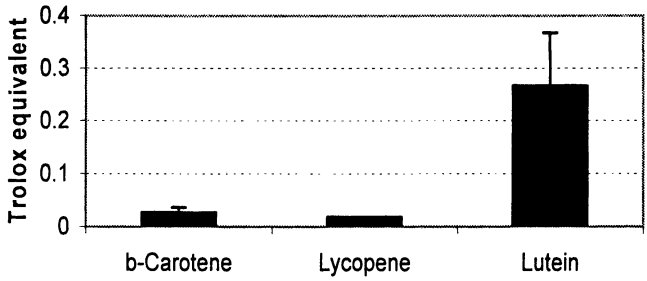

Figure 4. Antioxidant activity measured by the PCL method. Compounds were tested at 0.1 mM levels and expressed as trolox equivalent.

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to be photosensitizers and antioxidants in the inner segment where they play a more important role in neutralizing singlet oxygen (*4*).

Lutein and zeaxanthin have been identified as the major constituents of the macular pigment of the human retina (*19*), and have been considered to play most critical roles in protecting human macula (*17,18,20*), particularly from risk of AMD (*4,5*) and cataract (*65*).

An experiment using monkey's eye showed that diet free of xanthophylls resulted in lutein and zeaxanthin in serum and no detectable macular pigment deposition in monkeys, however, repletion with lutein and zeaxanthin in the diet was found to quickly restore to the normal level of macular pigment in those monkeys, even after life-long xanthophyll deficiency (*66*). Deposition of lutein and zeaxanthin seemed to be through binding to a specific protein termed XBP (xanthophylls-binding protein) from the retina of human eyes (*67*). Lower lutein and zeaxanthin concentrations were found in retinas from AMD donors than from normal donors in the controls. An inverse association between the incidence of AMD and the macular pigment density was confirmed (*20*). The levels of lutein in the macular region of people with AMD were also significantly lower than those without (*47*). Lutein and zeaxanthin are perhaps the most important xanthophylls for human, as they have been detected in human plasma and breast milk (*14,15*). Bone *et al.* (*68*) compared the effects of a range of lutein doses (2.4- 30 mg/d), as well as a high zeaxanthin dose (30 mg/d), on the serum and macular pigment in human subjects, and found serum lutein concentrations in each subject reached a plateau that was correlated with the dose, and the rate of increase in macular pigment optical density was correlated with the plateau concentration of carotenoids in the serum. A review by Beatty *et al.* (*4*) revealed vast evidence that supports the hypothesis that macular pigment (lutein and zeaxanthin) may protect against AMD and age related maculopathy (ARM) and AMD, but also acknowledged that further investigation was necessary. Khachik *et al.* (*69*) found that lutein acts as an

antioxidant in the macular region of the retina by identifying the presence of an oxidation product of lutein in the macula.

Olmedilla *et al.* (*65*) supplemented cataract patients with 15 mg of lutein three times a week for up to two years, and found that the lutein-supplemented patients' visual acuity improved nearly one line on the Snellen visual acuity chart compared with the placebo controls. Similarly, Richer *et al.* (*70*) found that after 12 months of 10 mg of lutein or 10 mg of lutein plus antioxidant supplementation, visual acuity in AMD patients improved by 5.4 and 3.5 letter, respectively, on Snellen chart. Those receiving a placebo showed no improvement in acuity.

Effects of lutein on cancer and heart diseases

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Although lutein is best known for its protective role against AMD and other vision related deteriorations, it has also been found to contribute to the lower risks of several other chronic diseases such as atherosclerosis and colon cancer (*10,11*). Lutein and other non-provitamin carotenoids were found to be more active than β-carotene in enhancing cell-mediated and humoral immune response in animals and humans (*71*), Alzheimer disease and vascular dementia (*72*).

Sumantran *et al.* (*73*) examined the effects of lutein and all-*trans* retinoic acid (ATRA), on apoptosis and chemosensitivity in primary normal human mammary epithelial cells, SV40 transformed mammary cells, and MCF-7 human mammary carcinoma cells. Lutein and ATRA selectively induced apoptosis in transformed but not normal human mammary cells. In addition, both compounds protected normal cells, but not transformed cells, from apoptosis induced by the chemotherapy agents etoposide and cisplatin. Furthermore, lutein and ATRA selectively increased the ratio of Bcl-xL:Bax protein expression in normal cells but not transformed mammary cells, equal suggesting a possible mechanism for selective modulation of apoptosis. The differential effects of lutein and ATRA on apoptotic pathways in normal *versus* transformed mammary epithelial cells may have important implications for chemoprevention and therapy. In a study with mice, Park *et al.* (*74*) showed that dietary lutein as low as 0.002% inhibited mammary tumor incidence, growth and latency, and their more recent study found that inhibitory effect on mammary cancer growth by dietary lutein was by selectively increasing tumor cell apoptosis and decreasing blood lymphocyte apoptosis, and by inhibiting angiogenesis (*71*). Mice fed with lutein had tumors that were 30 to 40% smaller on day 50 post-inoculation compared to unsupplemented mice, and the lowest tumor volume was found in mice fed 0.002% lutein (*71*). These studies have led to the examination of the relations between dietary intake of α-carotene, β-carotene, β-cryptoxanthin, lycopene, and lutein + zeaxanthin and breast cancer risk in a large cohort study of Canadian women (*75*), however, the study found

no clear association between the intake of any of the studied carotenoids and breast cancer risk in the study population as a whole or in subgroups defined by smoking status, relative body weight (assessed by body mass index), intakes of total fat, energy, alcohol, or folic acid, family history of breast cancer, or menopausal status. They concluded that prospective cohort studies of carotenoids in relation to breast cancer are scarce and further studies are warranted.

An epidemiological study in the Pacific Islands indicated that people with a high intake of all three compounds (β-carotene, α-carotene and lutein) had the lowest risk of lung cancer (*76*). Lutein showed antitumor activity in a two-stage carcinogenesis experiment in the lung of ddY mice, initiated with 4-nitroquinoline-1-oxide (4NQO) and promoted with glycerol. Treatment with lutein showed a decrease tendency for lung tumor formation (*76*).

Lutein was found to inhibit the development of aberrant crypt foci in Sprague-Dawley (SD) rat colon induced by N-methyl-nitrosourea (MNU) (*77*). Slattery *et al.* (*10*) also found that among the younger colon cancer patients, there was an inverse association between lutein intake and the cancer risk. Lutein was also found to protect HepG2 human liver cells against free radical-induced damage in lipid peroxidation, and such effect was independent of provitamin A activity (*78*).

Recent data provide support for a protective role of lutein in the development or progression of atherosclerosis. Dwyer *et al.* (*11*) found the formation of atherosclerotic lesions in the aortic arch to be significantly lower in lutein-supplemented apolipoprotein E null mice than in controls. Additionally, in a cohort of men and women, plasma lutein concentrations at baseline were inversely associated with carotid intima-media thickness progression over 18 months. However, the results in relation to the association between lutein intake and cardiovascular diseases have been inconsistent. In a recent study, Sesso *et al.* (*79*) identified 499 cases of patients with cardiovascular diseases and followed up the plasma concentrations of several carotenoids for 2.1 y, and found that higher concentrations of plasma lutein/zeaxanthin and retinol might moderately increase the CVD risk. Lutein has protective effects against lipid peroxidation and damages of singlet oxygen (*80,81*). In a review by Ribaya-Mercado and Blumberg (*82*), they discussed about the potential contribution of lutein and zeaxanthin to the prevention of heart disease and stroke. Even as the evidence for the role of lutein and zeaxanthin in disease prevention continues to evolve, particularly from human studies directed to their bioavailability, metabolism, and dose-response relationships with intermediary biomarkers and clinical outcomes, they concluded that recommendations to consume foods rich in xanthophylls are consistent with current dietary guidelines. Despite these recent discoveries, direct evidences for the effects for lutein on cancer and heart diseases are yet to be found.

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Bioavailability of lutein

For lutein to be active *in vivo*, it has to be successfully absorbed and delivered to the target organs. However, many factors affect how lutein is absorbed in biological systems, including humans (*1*). It was found that the bioavailability of lutein from spinach was higher than that of β-carotene, however, food matrix of spinach (cell wall structure) is only a limiting factor in determining the bioavailability of β-carotene and to a lesser extent of lutein in humans (*83*). Lutein can exist in the free form or esterified to fatty acids depending on different plants, therefore the bioavailability of lutein may also vary. Currently, lutein supplementation is through preparations made from marigold flowers, some of which are high in lutein diesters. Bowen *et al.* (*84*) found that lutein diester formulation was more bioavailable than the free lutein formulation, and dissolution appeared to be more important to bioavailability than the presence or absence of esterified fatty acids, as they found a substantial amount of free lutein in the circulation when diesters were consumed. Sufficient fat in the meal to supplement consumption may be important for maximizing bioavailability (*84,85*). On the other hand, different results were found by other researchers. The bioavailability of lutein from egg was found to be higher than that from other sources such as lutein, lutein ester supplements, and spinach, and there were no significant differences in lutein bioavailability among lutein, lutein ester supplements, and spinach (*86*).

The complexity of the bioavailability of lutein and related compounds is far from fully understood. Environmental factors, food processing, food matrix, structural differences and the interaction among other food components all have an effect on their efficiency of uptake and absorption (*1*).

Kostic *et al.* (*87*) found that when human subjects were given mixtures of lutein and β-carotene at equimole doses, the absorption of lutein was significantly reduced to 54-61% of the control by β-carotene. They concluded that carotenoids interact with each other during intestinal absorption, metabolism, and serum clearance, although individual responses can differ markedly.

Other biological effects of lutein

Lutein has also been studied for other physiological functions. Schünemann *et al.* (*88*) observed that serum levels of carotenoids were positively associated with forced expiratory volume in 1 second (FEV1) and forced vital capacity (FVC) as indicators of lung function in a general population sample. The strongest association was found for the serum carotenoids β-cryptoxanthin and lutein/zeaxanthin (*89*). More recently, the association of lung function (FEV1 and FVC) with these carotenoids in the diet of the general population was investigated, and positive association of vitamin C, vitamin E, and lutein/zeaxanthin intake with pulmonary function was found. Lutein/zeaxanthin

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had the strongest association using FVC. They also found further evidence that smokers may show stronger associations between dietary antioxidants and lung function (*88*).

High dose of carotenoids may cause adverse effects. Certain carotenoids such as β-carotene have been found to cause a negative effect at high dosages, especially among smokers (*21,22*). However, the effects of pure lutein at high dosage is not clear (*6*). Our recent *in vitro* study showed no evidence that lutein causes any mutagenic effect at all doses (lutein at 334, 668 and 1335 μg/plate) in standard Ames test in the presence and absence of S9 mix; on the contrary, an anti-mutagenic effect in a dose-dependent manner was found (Fig. 5). These results provide important information towards the use of a high dose of pure lutein for human disease prevention.

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Conclusions

Lutein is a powerful antioxidant with significant biological importance to human health. Currently accumulated knowledge on lutein requires further studies on several aspects including the mechanisms of its various bioactivities in the biological system, and how it is absorbed and transported to the site of action. All these require good separation, quantification and detection methods for low concentrations of lutein. Samples containing trace amounts of lutein in biological tissues resulting from animal and human clinical trials are particularly challenging. There are various analytical methods available, however, HPLC, particularly when it is coupled with MS can be a powerful tool in quantitative and qualitative analysis of lutein. On the other hand, pure lutein is currently very costly, and it is difficult for scientists to obtain highly purified lutein at gram levels. HSCCC offers a good semi-preparative method for purification of high purity lutein. Lutein has been associated with several human chronic diseases, particularly AMD, many of which are considered to be caused by excess oxidative stress from free radicals. Its activity against cancer has not been widely studied, however, its protective role against mutation was evidenced in our recent study and suggests that even at relatively high doses, at least *in vitro*, lutein does not cause mutation, not only that, it actually inhibited mutation caused by known mutagens in a dose-dependent manner. Such data points toward a need for further investigations.

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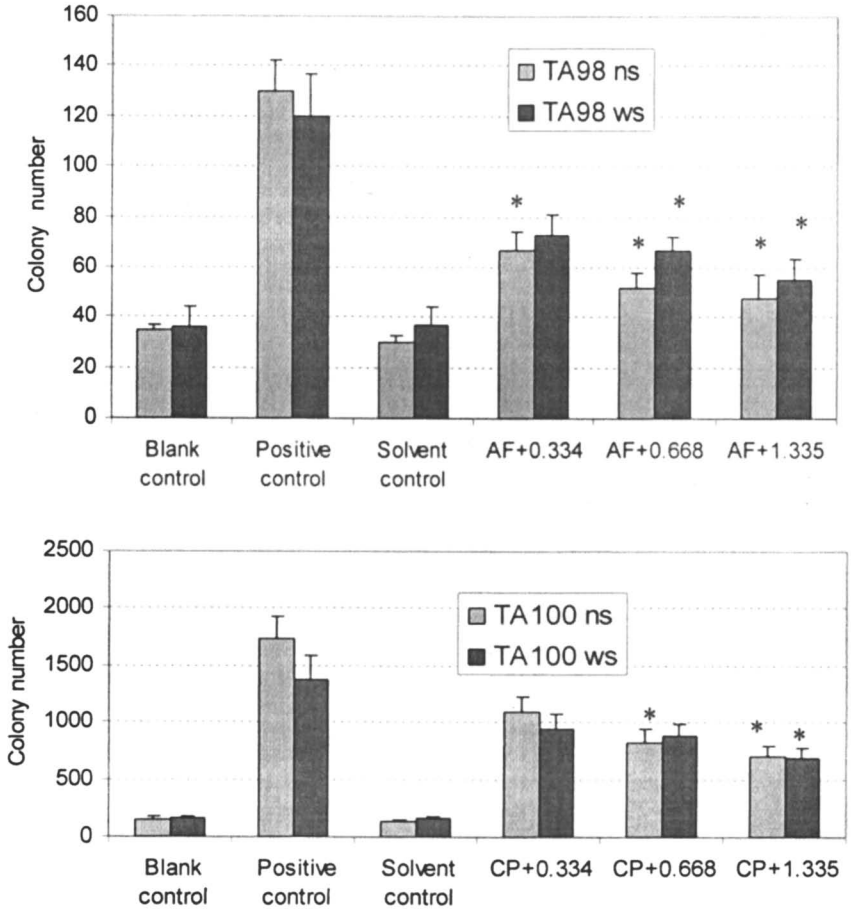

*Figure 5. Anti-mutagenic activity of lutein on mutagenicity induced by 2-aminofluorene (2-AF) and cyclophosphamide (CP) in S. typhimurium TA_{98} and TA_{100} in the absence and the presence of S9 mix. For TA_{98}, 2-aminofluorene and dexon were used as positive controls in the absence or the presence of S9; for TA_{100}, cyclophosphamide and sodium azide were used in the absence or the presence of S9. *significant reduction (two times or more reduction) when compared to positive controls.*

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Chapter 24

Vitamin C Protects Against Hydrogen Peroxide-Induced Inhibition of Gap-Junction Intercellular Communication through the Blocking Phosphorylation of Connexin-43 and ERK1/2 in Rat Liver Epithelial Cells

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Antioxidants are known to protect cells from oxidative DNA damage, but the effect of antioxidants on the tumor-promotion process has not yet been clarified. The present study found that vitamin C exhibited antioxidant effects similar to those of butylated hydroxyanisole (BHA) using 2,2-diphenyl-1-picryl-hydrazyl, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), and superoxide radical-scavenging assays. Vitamin C protected against the hydrogen peroxide (H_2O_2)-induced inhibition of GJIC, which is a tumor-promotion process, while BHA had no recovery effect, instead inhibiting synergistically. Vitamin C blocked H_2O_2-induced phosphorylation of connexin-43 and extracellular-signal-regulated protein kinase 1/2, which are critical regulators of GJIC. These findings suggest that antioxidants exert different effects on reactive oxygen species-mediated tumor promotion.

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Numerous antioxidative substances have been found to exhibit potential cancer chemopreventive activities, but the definition of the appropriate biomarkers to quantify their chemopreventive effects remains subjective (*1*). A precise understanding of the underlying biochemical and molecular mechanisms is the first step to identifying such biomarkers, and is essential for the successful implementation of chemopreventive strategies. Cellular enzymes and structural proteins, membranes, simple and complex sugars, and DNA and RNA are all susceptible to oxidative damage that leads to tumor initiation. Minimizing the exposure to diverse environmental carcinogens is one strategy for preventing the majority of human cancers, but the complete avoidance of exposure to etiologic factors that can initiate cancer may be unrealistic (*2*). Therefore, recent chemopreventive strategies have focused more on identifying substances possessing antipromoting or antiprogressive activities that can suppress the transformation of initiated or precancerous cells into malignant ones, rather than on searching for anti-initiators (*2*).

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Oxidation involves the withdrawal of energy by oxygen from reduced carbon-based molecules. The paradox is that this process of free-radical oxidation is both deleterious and life sustaining because it is coupled to electron transport in the mitochondria of living cells. The oxygen used by cells to generate energy represents a source of oxygen radicals and reactive oxygen systems. The action of carcinogens is often accompanied by oxidation reactions acting on DNA. Therefore, investigations of the carcinogenic effects of oxidative stress have primarily focused on genotoxicity, but reactive oxygen species (ROS) are also known to play a significant role in the promotional stage of carcinogenesis. In particular, several oxidants and free-radical generators are tumor promoters, and ROS – including the superoxide anion and hydrogen peroxide (H_2O_2) – are strongly associated with carcinogenic processes.

Cell-to-cell communication through gap-junction channels (GJIC) is essential for maintaining homeostasis via the modulation of cell proliferation and differentiation in multicellular organisms (*3*). Inhibition of GJIC is strongly related to carcinogenicit and particularly to tumor promotion. Normal, contact-inhibited fibroblasts and epithelial cells have functional GJIC, while most – if not all – tumor cells have dysfunctional homologous or heterologous GJIC. Cancer cells are characterized by a lack of growth control, inability to terminally differentiate or perform apoptosis under normal conditions, and an extended or immortalized life span. Chemical tumor promoters, growth factors, and hormones have been shown to inhibit GJIC. Several oncogenes have been shown to downregulate GJIC function, while antioncogene drugs and antitumor-promoting natural and synthetic chemicals have been shown to reinstate GJIC

and growth control with loss of tumorigenicity. Therefore, one hypothetically rational approach to identify antitumor-promoting chemopreventive drugs and anticarcinogenic treatments is to prevent the downregulation of GJIC by the tumor promoters and thereby restore GJIC in neoplastic cells (*4,5*). The tumor-promoting effects of H_2O_2 are supported by the formation of colonies in soft agar, appearance of foci in monolayer cultures, and disruption of GJIC (*6*). Since the inhibition of GJIC is strongly related to carcinogenic processes, particularly the tumor-promotion process, enhancers of GJIC are also anticipated to prevent cancer.

Dietary or pharmaceutical augmentation of the endogenous antioxidant defense capacity has been considered a plausible way to prevent ROS-mediated carcinogenicity. Although antioxidants are known to protect cells from oxidative stress by scavenging free radicals and quenching lipid-peroxidation chain reactions (which may cause DNA damage) and thereby block the initiation of carcinogenesis, the effect of antioxidants on the tumor-promotion process remains to be clarified. Furthermore, most free-radical scavengers act in reversible oxidation–reduction reactions, and some antioxidants can act both as antioxidants and prooxidants depending on their structure and the reaction conditions. Although antioxidant capacity can be evaluated using chemical methods that are easy to execute and exhibit high reproducibility, such methods do not represent what happens in living cells. Therefore, assays using *in vitro* living cells may be useful for assessing the antioxidant activity of compounds. The present study investigated the effects of vitamin C and butylated hydroxyanisole (BHA) (Figure 1) on ROS-mediated carcinogeneis *in vitro*.

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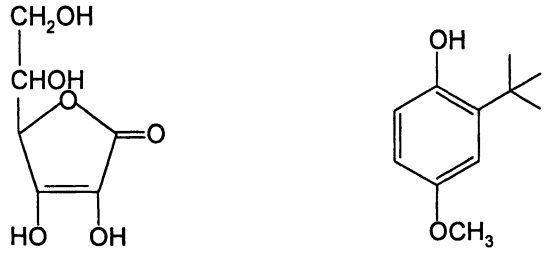

Figure 1. Chemical structures of vitamin C (ascorbic acid) and BHA (butylated hydroxyanisole).

Materials and Methods

Chemicals

Vitamin C, BHA, ammonium phosphate monobasic ($NH_4H_2PO_4$), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), dimethyl sulfoxide (DMSO), H_2O_2, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), lucifer yellow, xylenol orange, ammonium ferrous sulfate, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), sodium dodecyl sulfate (SDS), acrylamide, and Tris-HCl were obtained from Sigma Chemical (St. Louis, MO). Triton X-100 was obtained from AMRESCO (Solon, OH). Antibodies of ERK and p-ERK were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-connexin-43 (anti-Cx43) antibody was obtained from Zymed Laboratories (San Francisco, CA). All other chemicals used were of analytical or HPLC grade (Fisher, Springfield, NJ).

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DPPH Radical-Scavenging Activity Assay

The DPPH radical-scavenging activities of vitamin C and BHA were measured using the method described by Brand-Williams *et al.* with minor modifications (*7,8*). DPPH radical was dissolved in 80% aqueous methanol. Varying concentrations of vitamin C and BHA (0.1 mL) were added, individually, to 2.9 mL of the DPPH radical solution. The mixture was then shaken vigorously and allowed to stand at 23°C in the dark for 30 min, at which time the decrease in absorbance at 517 nm was measured using a spectrophotometer (Hitachi, Japan).

ABTS Radical-Scavenging Activity Assay

A method developed by van den Berg *et al.* was used, with slight modification, to assess the ABTS radical-scavenging activities of vitamin C and BHA (*8,9*). AAPH (1.0 mM) was mixed with 2.5 mM ABTS as diammonium salt in a phosphate-buffered saline (PBS) solution (100 mM potassium phosphate buffer, pH 7.4, containing 150 mM NaCl). The mixture was heated in a 68 °C water bath for 13 min, and the concentration of the resulting blue-green ABTS radical solution was adjusted to an absorbance of 0.650 ± 0.020 at 734 nm. The solution with varying concentrations of vitamin C and BHA (20 μL) was added, individually to 980 μL of the resulting blue-green ABTS radical solution. The mixture was incubated in the dark in a 37 °C water bath for 10 min, at which time the decrease in absorbance at 734 nm was measured.

Superoxide-Anion Radical-Scavenging Activity Assay

HL-60 human promyelocytic leukemia cells (HL-60 cells) were suspended at a density of 1×10^6 cells/ml in RPMI 1640 medium containing 10% fetal bovine serum and 1.3% DMSO, and incubated for 7 days at 37 °C in an incubator containing 5% CO_2. Cells were harvested by centrifugation, washed with PBS, and then resuspended in PBS at a density of 1×10^6 cells/mL. After preincubation with varying concentrations of vitamin C and BHA, respectively, for 15 min, TPA (8 μM) and cytochrome *c* (60 μM) were added to the reaction mixture. After an additional incubation at 37 °C for 30 min, superoxide-anion generation was determined by measuring the absorbance of reduced cytochrome *c* at 550 nm.

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GJIC Bioassay

WB-F344 rat liver epithelial cells (WB-F344 cells) were kindly provided by Dr. J. E. Trosko at Michigan State University (USA). WB-F344 cells were cultured in Dulbecco's modified Eagle's medium (GIBCO BRL), supplemented with 10% fetal bovine serum (GIBCO BRL) and penicillin/streptomycin (GIBCO BRL), in a 37 °C humidified incubator (Forma Scientific, Mariotta, OH) containing 5% CO_2 and 95% air. The GJIC was measured using the scrape-loading/dye-transfer technique (*10*). Briefly, WB-F344 cells were preincubated with noncytotoxic doses of the test compounds and 500 μM H_2O_2. After incubation, the cells were washed twice with 2 ml of PBS. Lucifer yellow was added to the washed cells, and three scrapes were made with a surgical steel-bladed scalpel at low light intensities. Each scrape traversed a large group of confluent cells. After 3 min of incubation, the cells were washed four times with 2 ml of PBS and then fixed with 2 ml of a 4% formalin solution. The number of communicating cells indicated by the dye was counted under an inverted fluorescence microscope (Olympus Ix70, Okaya, Japan).

Cell Viability

Cell viability was measured using the MTT assay as described previously (*11*). Briefly, cells were cultured in 96-well plates at 10,000 cells/well in media for 24 h. Each well was filled with fresh media containing varying amounts of each compound, and the cells were incubated for an additional 24 h at 37 °C, followed by treatment with MTT for 4 h. The medium was removed and DMSO was added to dissolve the blue formazan residue. The optical density at 570 nm of each well was then measured using a microplate reader (Molecular Devices,

Sunnyvale, CA). All data are from at least three replications for each prepared sample.

Western Blot Analysis

Western blot analysis was performed for Cx43, ERK1/2, and p-ERK1/2. Briefly, sample cells were resuspended into 4× sample buffer (8% SDS, 20% glycerin, 250 mM Tris-HCl, pH 7.5, 0.2% bromophenol blue, 40 mM DTT), heated for 5 min, and separated by 12.5% SDS-polyacrylamide gel electrophoresis. The proteins were then transferred to a 0.45-μm polyvinylidene fluoride transfer membrane (Gelman Laboratory, Ann Arbor, MI). Blots were blocked with Tween-20 Tris-buffered saline (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20) containing 5% skim milk, and then probed with antibodies according to the manufacturer's instructions and with HRP-goat antimouse IgG (Zymed Laboratories, San Francisco, CA) coupled to peroxidase. Blots were developed using an enhanced chemiluminescence system (Amersham Biosciences, UK).

Statistical Analysis

Data in the figures are presented as mean ± SD values. Statistical significance was assessed using one-way analysis of variance followed by two-tailed Dunnett's *t*-test, with the level of significance set at $p<0.01$.

Results

Free-Radical-Scavenging Activities of Vitamin C and BHT

DPPH and ABTS can generate stable free radicals in aqueous buffer and ethanol solution, respectively. Preliminary studies were performed to determine the optimal concentration of the radicals and the reaction times (*8,12*). Similar to our previous observations with dietary antioxidants and food (*8,12*), vitamin C and BHA exhibited dose-dependent free-radical-scavenging activities (Figure 2). The free-radical-scavenging activity of BHA was found to be similar to that of vitamin C, as determined by both the DPPH (Figure 2A) and ABTS (Figure 2B) radical-scavenging assays.

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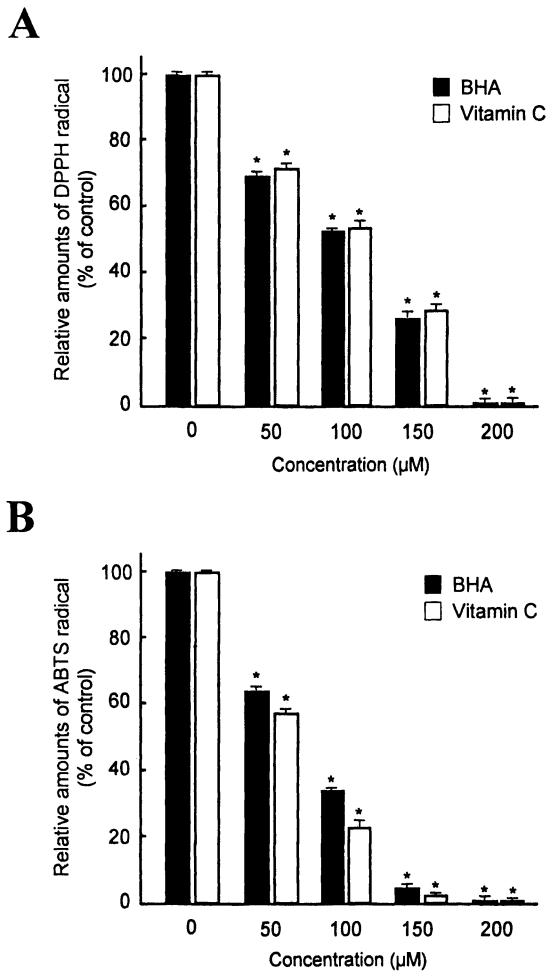

*Figure 2. Free-radical-scavenging activities of vitamin C and BHA. The radical-scavenging activities of vitamin C and BHA were evaluated by both DPPH (A) and ABTS (B) radical-scavenging assays as described in Materials and Methods. Data are mean ± SD values for six independent experiments. *, Significantly different from untreated control group ($p < 0.01$).*

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Inhibitory Effects of Vitamin C and BHA on Superoxide-Anion-Radical Generation

Tumor promoters, in particular TPA, have been reported to trigger the generation of the superoxide-anion radical in epithelial cells and leukocytes via the xanthine and NADPH oxidase systems, respectively. We investigated the inhibitory effects of vitamin C and BHA on the generation of the superoxide anion by measuring its ability to reduce cytochrome *c* in a TPA-treated HL-60 cell culture system. Treatment with vitamin C and BHA suppressed TPA-induced generation of the superoxide anion in cultured HL-60 cells in a dose-dependent manner without affecting cell viability (Figure 3).

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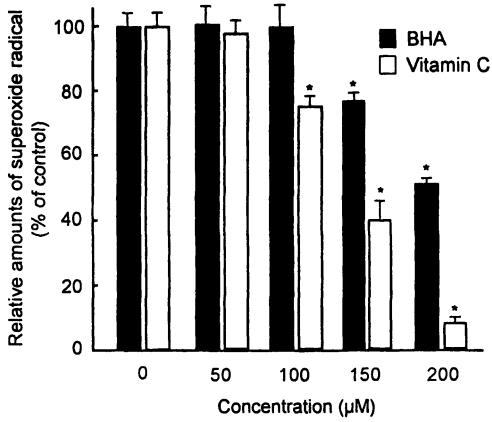

*Figure 3. Superoxide-anion-radical-scavenging activity of vitamin C and BHA. The inhibitory effects of vitamin C and BHA on the generation of the superoxide anion were evaluated by measuring its ability to reduce cytochrome c in a TPA-treated HL-60 cell culture system as described in Materials and Methods. Data are mean ± SD values for six independent experiments. *, Significantly different from the control group ($p < 0.01$).*

Vitamin C, but not BHA, Protected Against H_2O_2-induced Inhibition of GJIC in WB-F344 Cells

To screen and verify the antitumor-promoting activity of antioxidants *in vitro*, nontumorigenic WB-F344 rat liver epithelial cells (WB-F344 cells) were used as a model system. The effects of vitamin C and BHA on GJIC in WB-

F344 cells were investigated using the scrape-loading/dye-transfer technique. H_2O_2 dose-dependently inhibited GJIC (Figure 4A), and the inhibition of GJIC recovered almost completely after 2 h (Figure 4B). Vitamin C significantly recovered this inhibition of GJIC, as expected, while BHA had no recovery effect, instead inhibiting synergistically (Figure 5). The protective activities of vitamin C against the inhibition of GJIC by H_2O_2 were dose-dependent at the concentrations used in this study (Figure 5). Therefore, subsequent experiments were performed with vitamin C.

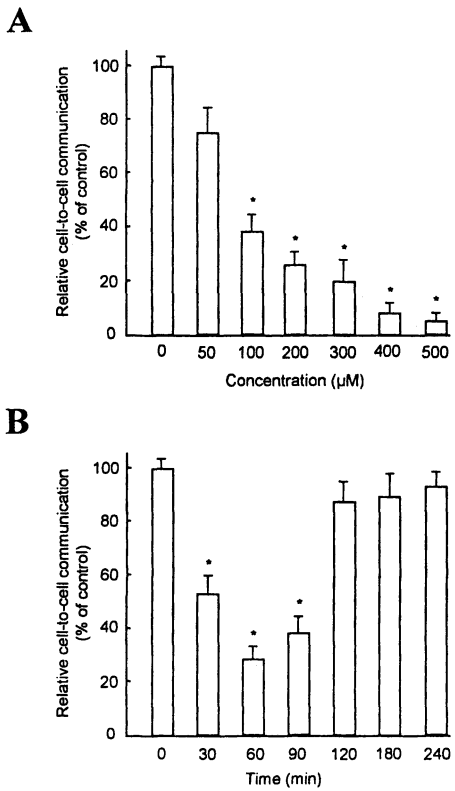

*Figure 4. H_2O_2 inhibited dose-dependently and reversibly GJIC in WB-F344 rat liver epithelial cells (WB-F344 cells). (A) A dose-dependant inhibition of GJIC by H_2O_2. The cells were exposed to the indicated concentration of H_2O_2. (B) A reversible inhibition of GJIC by H_2O_2. The cells were exposed to the 300 μM H_2O_2 for the indicated time. GJIC was assessed using the scrape-loading/dye-transfer method as described in Materials and Methods. Relative recovery rates were determined by measuring the numbers of communicating cells. Data are mean ± SD values for three independent experiments. *, Significantly different from the control group ($p < 0.01$).*

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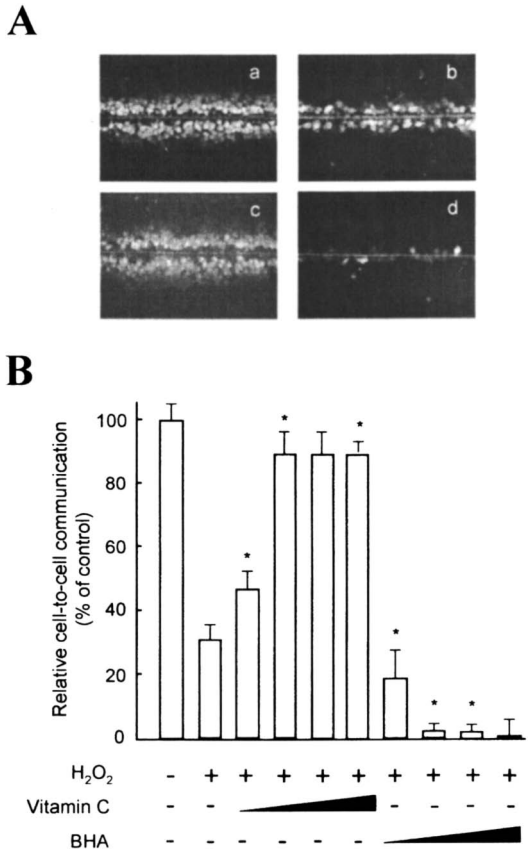

*Figure 5. Vitamin C, but not BHA, protected against the inhibition of GJIC induced by H_2O_2. (A) The cells were exposed to the indicated concentration of vitamin C and BHA, respectively, in the presence or not of 300 μM H_2O_2 for 1 h, and GJIC was assessed using the scrape-loading/dye-transfer method. a, control (ddH_2O as vehicle); b, H_2O_2; c, H_2O_2 plus 100 μM vitamin C; d, H_2O_2 plus 100 μM BHA. The data shown are representative of at least three independent experiments. (B) The cells were exposed to the indicated concentration (50, 100, 150, and 200 μM) of vitamin C and BHA, respectively, in the presence or not of 300 μM H_2O_2 for 1 h. Relative recovery rates were determined by measuring the numbers of communicating cells. Data are mean ± SD values for six independent experiments. *, Significantly different from the group treated with H_2O_2 alone ($p < 0.01$).*

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Vitamin C Blocked H_2O_2-induced Phosphorylation of Cx43 and ERK1/2 in WB-F344 Cells

Cx43 plays a key role in the modulation of GJIC. Several previous studies have shown that H_2O_2 induces the hyperphosphorylation of Cx43 in WB-F344 cells that is the mechanism responsible for the inhibition of GJIC. We performed Western blotting with antibodies specific to Cx43 to examine the changes in phosphorylation resulting from treating the cells with vitamin C (Figure 6). Three major bands (P0, P1, and P2) were detected in untreated WB-F344 cells, and mobility shifts from band P0 or P1 to higher-molecular-weight band P2 or P3 indicated the phosphorylation of Cx43. Cells treated with H_2O_2 exhibited band shifts to band P2. The treatment of vitamin C with H_2O_2 decreased the phosphorylation ratio (P2/P0) of Cx43 by H_2O_2. The results from a previous study suggest that the H_2O_2-induced inhibition of GJIC is mediated by phosphorylation of Cx43 via activation of mitogen-activated protein kinase, particularly extracellular signal-regulated protein kinases (ERK)1/2 (*13*). Treating the cells with vitamin C blocked the phosphorylation of ERK1/2 by H_2O_2 (Figure 7).

Discussion

The carcinogenicity of oxidative stress is primarily attributable to the genotoxicity of ROS, but ROS are also known to play a significant role in the promotional stage of carcinogenesis. In particular, several oxidants and free-

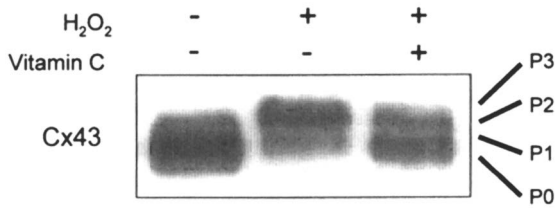

Figure 6. Vitamin C blocked the phosphorylation of Cx43 by H_2O_2 in WB-F344 cells. The cells were exposed to 300 μM H_2O_2 for 1 h in the absence or presence of vitamin C. The phosphorylation status of Cx43 was analyzed by Western blot analysis as described in the Materials and Methods. The data shown are representative of three independent experiments. Lane 1, control (ddH_2O as vehicle); lane 2, H_2O_2; lane 3, H_2O_2 plus 100 μM vitamin C. P0 and P1 are the phosphorylation patterns of Cx43 in untreated cells; P2 and P3 are hyperphosphorylation patterns of Cx43 in cells treated with H_2O_2.

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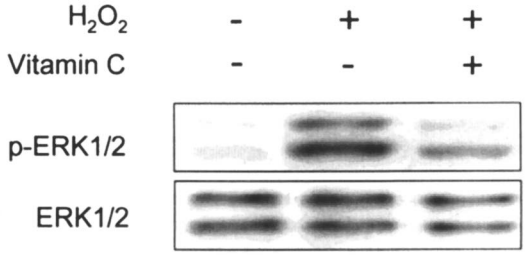

Figure 7. Vitamin C inhibited the phosphorylation of ERK1/2 by H_2O_2 in WB-F344 cells treated with H_2O_2. The cells were exposed to 300 μM H_2O_2 for 30 min in the absence or presence of vitamin C. The levels of phosphorylated and total ERK1/2 were determined by Western blot analysis as described in the Materials and Methods. The data shown are representative of three independent experiments. Lane 1, control (ddH_2O as vehicle); lane 2, H_2O_2; lane 3, H_2O_2 plus 100 μM vitamin C.

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radical generators are tumor promoters. Some studies link oxidative stress induced by ROS to tumor promotion in mouse skin and other tissues (*14,15*). Many tumor promoters generate ROS, and the involvement of ROS – particularly H_2O_2 – in tumor promotion is supported by both *in vivo* and *in vitro* studies (*14,16*). The topical application of tumor promoters to mouse skin decreases the production of H_2O_2 in the epidermis, which correlates with their tumor-promoting potential (*17*). The involvement of H_2O_2 in carcinogenesis has been shown in both *in vivo* and *in vitro* studies. A recent study suggests that the carcinogenicity of H_2O_2 is attributable to the inhibition of GJIC (*6*).

Many studies focused on the antimutagenic or anticarcinogenic activities of antioxidative substances. Antioxidants have generally been considered to have chemopreventive effects, but it is possible that each antioxidant can have a different effect depending on its structure and dosage. In the present study, we found that vitamin C prevented the inhibition of GJIC by H_2O_2 (*Figure 5*). Mitogen-activated proteins kinases (MAPKs), which include the ERK1/2, the c-Jun N-terminal kinase (JNK) and the p38 subfamilies, are important regulatory proteins that regulate signal transduction pathways that control many aspects of mammalian cellular physiology including cell growth, differentiation and cell death. In particular, it has been documented that the phosphorylation of ERK1/2 plays an important role in the inhibition of GJIC through phosphorylation of Cx43 in several cell lines. We found that treating the cells with vitamin C blocked the phosphorylation of Cx43 (Figure 6) and ERK1/2 (Figure 7) by H_2O_2. However, BHA had no effect on the H_2O_2-induced inhibition of GJIC, instead inhibiting synergistically (Figure 5). A previous study also showed that two other antioxidants, propylgallate and Trolox, did not prevent the H_2O_2-mediated inhibition of GJIC (*18*). Furthermore, green tea polyphenols, both

epigallocatechin gallate (*19*) and gallic acid (*10*) at a concentration of 20 μg/mL or more inhibited GJIC in the absence of H_2O_2. Several reports also suggest that some antioxidative phenolic substances, such as gallic acid and EGCG, induce DNA damage (*20,22*), and that their damaging effects are probably due to the generation of ROS. Several studies have shown that metal-mediated autooxidation of some antioxidants, including phenolics, generates semiquinone radicals, resulting in the enhancement of redox activity to produce ROS, including H_2O_2 (*23,24*). Thus, the effect of dietary phenolic phytochemicals on GJIC is considered to vary with the structure of the individual compounds and their dosage. Some antioxidants may also exert antimutagenic and antitumor-promoting activities at relatively low dosages, whereas some excess antioxidant remaining in a free form may instead exhibit toxicity or carcinogenicity.

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Vitamin C reportedly exerts substantial cancer chemopreventive effects mainly due to its strong antioxidant activities against oxidative DNA damage. This antioxidant has also been used as a dietary supplement to prevent oxidative-stress-mediated chronic diseases such as cancer, cardiovascular disease, hypertension, stroke, neurodegenerative disorders, and aging. In 1997, expert panels at the World Cancer Research Fund and the American Institute for Cancer Research asserted that vitamin C can reduce the risk of stomach, mouth, pharynx, esophagus, lung, pancreas, and cervical cancers. However, several studies have shown that vitamin C exhibits prooxidant activity under certain conditions such as in the presence of transition-metal ions or alkalis (*25*). A recent study showed that even a moderate daily dose of supplementary vitamin C (200 mg) induces the formation of genotoxins from lipid hydroperoxides, which results in DNA damage and the initiation of carcinogenesis (*25*). Despite questionable experimental designs and the numerous contradicting reports, the current consensus from epidemiological and human studies is that a low risk of cancer is more strongly related to diets rich in multiple antioxidants than to dietary supplementation with individual antioxidants (*26*).

A recent theory (*2,4,27*) on epigenetics also suggests that greater attention must be paid to those multistage carcinogenesis processes that do not involve DNA damage. Cancer prevention strategies that involve intervention at the tumor-promotion stage (a reversible and long-term process) are more practical than those intervening at the tumor-initiation stage (an irreversible and short-term process). Since tumor promotion is closely linked to oxidative and inflammatory processes, it can be efficiently reversed and suppressed by vitamin C (*26*). Rosenkranz *et al.* (*28*) indicates that inhibition of GJIC in rodents is strongly linked to carcinogenicity, possibly by influencing inflammatory and developmental processes. The inhibition of GJIC is involved in the nongenotoxic induction of cancer and in tumor promotion (*28*). Our results indicate that the cancer chemopreventive effects of vitamin C are linked to its protective effects against epigenetic mechanisms, such as the inhibition of GJIC, as well as its antioxidant activities.

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Chapter 25

Formation of Off-Odorants during Light Exposure of Milk and Its Inhibition by Antioxidants

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Potent off-odorants formed during light exposure of milk were identified and the effects of added antioxidants on their formation investigated. The ultra-high temperature (UHT) milk purchased from a local market was stored under fluorescent light (15,000 lux) or in the dark for 16 h at 10 °C. The flavor differences between the light-exposed and non-exposed milk were monitored by aroma extract dilution analysis using the aroma extracts prepared from the milk. Off-flavor formation in the milk upon light exposure can be attributed to the formation of nine potent odorants, namely hexanal, (*Z*)-4-heptenal, 1-octen-3-one, methional, (*E*)- and (*Z*)-2-nonenal, (*E,Z*)-2,6-nonadienal, (*E,E*)-2,4-nonadienal, and (*E,E*)-2,4-decadienal. The contribution of these odorants to the light-induced off-flavor was further confirmed by flavor reconstitution experiments using the reference substances. Among the tested antioxidants, (-)-epicatechin and chlorogenic acid were the most effective inhibitors of the formation of the identified off-odorants during light exposure of milk and also most effective for reducing off-flavor intensities of light-exposed milk.

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Off-flavor formation in milk due to light exposure has already been extensively studied. Sulfur-containing volatiles such as methional, methanethiol, dimethyl sulfide, and dimethyl disulfide have been identified in milk exposed to light (*1,2*). These compounds probably result from the degradation of the sulfur-containing amino acids of the serum (whey) proteins (*3*), and are considered to be responsible for the off-flavor components described as cabbage, burnt protein, or "activated" (*4*). On the other hand, carbonyl compounds such as 2-alkenals, 2-alkanones, acetaldehyde, and alkanals are formed by the light-induced lipid oxidation in milk (*5,6*), and are considered to be responsible for the off-flavor components described as metallic and tallowy (*4*). Recently, Cadwallader and Howard employed GC-sniffing techniques to detect potent odorants formed in milk exposed to light (*7*). They revealed that methional, 2-acetyl-1-pyrroline, pentanal, hexanal, 1-hexen-3-one, 1-octen-3-one, and (*Z*)-1,5-octadien-3-one are the major contributors of light-induced off-flavor. Thus, many odorants are suggested to be responsible for the off-flavor formation in milk due to light exposure. However, it has not been clarified whether the mixture of these odorants actually represents the flavor character of the light-exposed milk.

Only a few studies have reported the effects of added antioxidants on the formation of off-flavor in milk during light exposure. Jung et al. reported that the addition of L-ascorbic acid reduced the formation of dimethyl disulfide and improved the sensory quality of light-exposed skim milk (*8*). Muranishi et al. reported that phenolic antioxidants, such as catechins and rosmarinic acid inhibited the formation of methional, dimethyl disulfide, and dimethyl trisulfide in an aqueous solution of methionine under riboflavin photo-sensitized conditions (*9*).

The aims of the present study were (1) to identify the potent off-odorants formed during light exposure of milk, (2) to verify the analytical results by flavor reconstitution using reference substances, and (3) to clarify the effect of added antioxidants on the formation of off-odorants during light exposure of milk.

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Materials and Methods

Materials

Ultra-high-temperature (UHT) treated whole milk was purchased from a local market. All chemicals were of the highest grade commercially available and were used without any further purification.

Light Exposure of Milk

One hundred grams of milk were sealed in a 180-mL glass bottle with a plastic-lined aluminum cap. The samples were stored under fluorescent light

(15,000 lux) at 10 °C for 16 h in an Eyela LST-300 light box (Tokyo Rikakikai Co. Ltd., Tokyo, Japan). When using an antioxidant, 1.0 g of a solution containing 10 mg of the antioxidant and 100 mg of Tween 20 in water were added to 99 g of the milk. The milk containing an antioxidant was exposed to light under the same conditions as described earlier.

Preparation of Aroma Extracts

The volatile fraction was isolated from 100 g of the milk by high-vacuum distillation using a solvent-assisted flavor evaporation (SAFE) apparatus as previously described by Engel et al. (*10*). The obtained distillate was spiked with 100 μL of an internal standard solution containing 2-octanol in dichloromethane (50 mg/kg), and then extracted with dichloromethane (50 mL × 2). The extract was dried over sodium sulfate, concentrated *in vacuo* to ~5 mL, and further concentrated under a stream of nitrogen to ~50 μL.

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Gas Chromatography-Olfactometry (GC-O)

An Agilent 6850 series gas chromatograph equipped with a thermal conductivity detector (TCD) and a DB-WAX fused silica capillary column (30 m × 0.25 mm i.d.; film thickness of 0.25 μm; J&W Scientific was used. The outlet from the detector was connected to a sniffing port flushed with humidified air at 100 mL/min. The operating conditions were as follows: detector temperature, 250 °C; nitrogen carrier gas flow rate, 1 mL/min; oven temperature program, 40 °C, raised at 5 °C/min to 210 °C (60 min); 1 μL of sample was injected in the splitless mode. An aroma extract dilution analysis (*11*) was conducted using serial 1:3 dilutions of the original aroma extract with dichloromethane.

Gas Chromatography-Mass Spectrometry (GC-MS)

An Agilent 6890 N gas chromatograph equipped with an Agilent Model 5973 N series mass spectrometer and a DB-WAX fused silica capillary column (60 m × 0.25 mm i.d.; film thickness of 0.25 μm; J&W Scientific was used. The operating conditions were as follows: injector temperature, 250 °C; helium carrier gas flow rate, 1 mL/min; oven temperature program, 40 °C, raised at 3 °C/min to 210 °C (60 min); 1 μL of sample was injected in the splitless mode; ionization voltage, 70 eV; ion source temperature, 140 °C.

Sensory Evaluation

The flavor profiles of the milk were evaluated by fourteen trained assessors consisting of three females and eleven males. They were asked to rate the

intensities of the five odor attributes ("freshness," "fatty," "metallic," "dusty," and overall off-odor) using a linear scale ranging from 1 (= absent) to 7 (= very strong). Ranking tests were conducted according to a method described by Meilgaard et al. (*12*). Twenty untrained panelists consisting of eight females and twelve males were asked to rank a series of six samples from first to sixth in the increasing order of off-flavor intensities. The statistical significance was evaluated by Friedman's test.

Results and Discussion

Identification and Quantification of Off-Odorants

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Flavor changes in milk due to light exposure were monitored by a comparative aroma extract dilution analysis using the aroma extracts prepared from the milk. Figure 1 shows the flavor dilution (FD) chromatograms obtained from the milk stored under florescent light (Figure 1a) and stored in the dark (Figure 1b). At an FD factor of sixteen and above, a total of fifteen odorants were detected. Among them, the FD factors of nine odorants, i.e., peak numbers 2, 3, 4, 7, 8, 9, 10, 12, and 14 increased with light exposure, whereas those of the other odorants did not change or decreased with light exposure. These results indicate that the off-flavor formation in the light-exposed milk can be attributed to the increase in these nine odorants.

Based on GC-MS and GC-Olfactometry analyses using reference substances, the nine odorants whose FD factors increased with light exposure were identified as listed in Table I. With the exception of (*Z*)-2-nonenal, whose MS spectrum was too small to be quantified, the amounts of the identified odorants in the milk were determined using calibration curves obtained by the addition of the reference compounds to the milk. In agreement with the values of the FD-factor, the concentration of the nine odorants significantly increased with light exposure (Table I).

Reconstitution of the Light-Induced Off-Flavor

In order to verify the analytical results, flavor reconstitution experiments using the reference substances were attempted. Differences in the quantified amounts of off-odorants between the light-exposed and non-exposed milk (Table I) were added to the non-exposed milk. Sensory evaluation was then performed to compare the flavor profiles. Figure 2 shows the results of the flavor profiling analysis among the light-exposed milk, non-exposed milk, and non-exposed milk with the added off-odorants. The difference between the light-exposed milk and non-exposed milk (Figure 2a, b) indicates that the "freshness" of the milk almost totally deteriorated and "fatty," "metallic," and "dusty" off-odors developed with light exposure. With the addition of the identified odorants

Table I. Concentrations and Flavor Dilution Factors of Potent Off-Odorants in Light-Exposed and Non-Exposed Milk

peak no.[a]	*RI*[b]	*compound*	*odor description*	*FD-factor*		*concn in milk*[c] *(μg/kg)*	
				LP[d]	*NP*[e]	*LP*[d]	*NP*[e]
2	1089	hexanal	green	16	< 16	37.0	5.7
3	1237	(*Z*)-4-heptenal	tomato-like	16	< 16	0.3	0.0
4	1296	1-octen-3-one	metallic	256	16	0.9	0.0
7	1447	methional	potato-like	64	< 16	7.6	3.6
8	1516	(*Z*)-2-nonenal	earthy	16	< 16	nd[f]	nd[f]
9	1525	(*E*)-2-nonenal	fatty	256	< 16	8.3	0.4
10	1575	(*E,Z*)-2,6-nonadienal	green	16	< 16	1.0	0.2
12	1689	(*E,E*)-2,4-nonadienal	fatty	64	< 16	1.6	0.1
14	1805	(*E,E*)-2,4-decadienal	fatty	64	16	2.4	0.2

[a] Numbers correspond to those in Figure 1. [b] Retention index on DB-WAX (60 m). [c] Each value is the mean of three experiments. [d] Light exposed milk: milk was stored under fluorescent light (15,000 lux) at 10 °C for 16 hr. [e] Non-exposed milk: milk was stored in the dark at 10 °C for 16 hr. [f] Not determined.

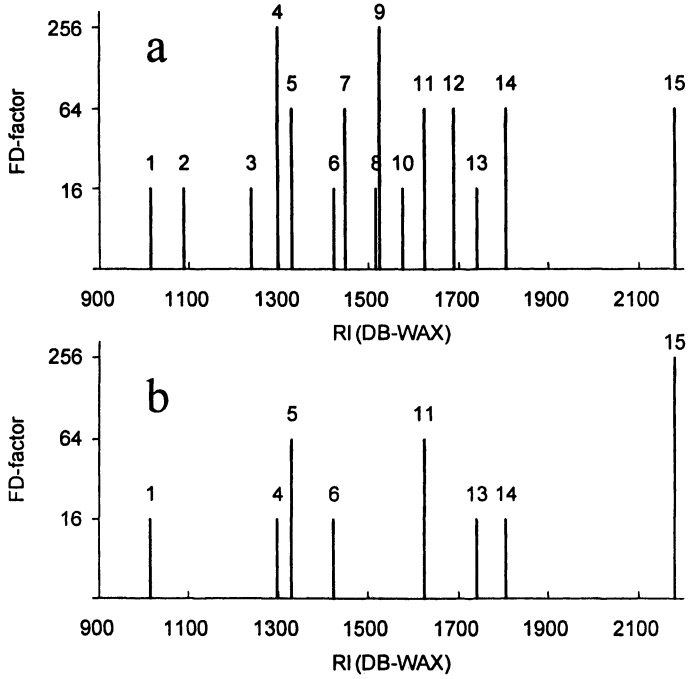

Figure 1. Flavor dilution chromatograms obtained by an aroma extract dilution analysis of milk (a) stored under florescent light and (b) stored in the dark.

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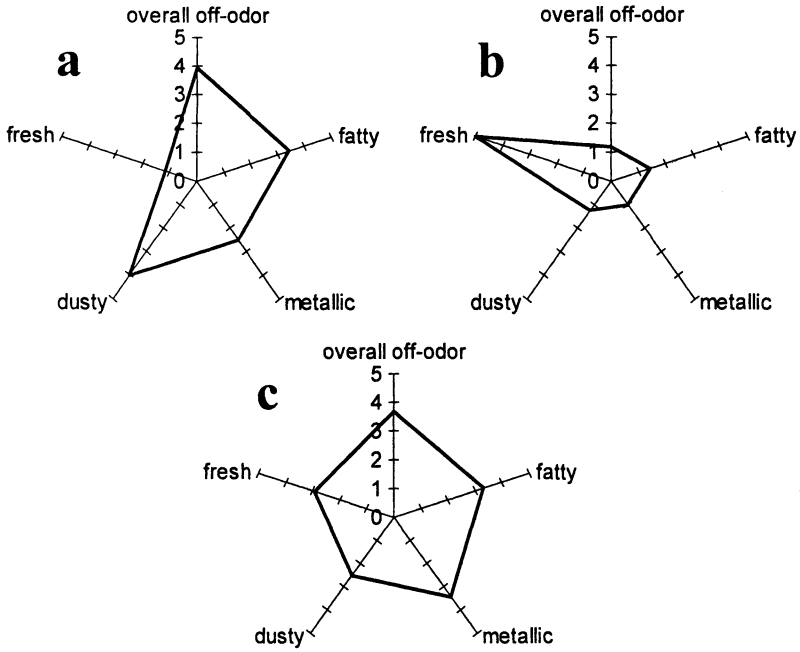

Figure 2. Comparative flavor profiling analysis among (a) light-exposed milk, (b) non-exposed milk, and (c) non-exposed milk with added off-odorants.

to the non-exposed milk, the light-induced off-odors were well reproduced except for the "dusty" off-odor (Figure 2c). No significant difference was observed in the intensities of the "fatty," "metallic," and "overall off-odor" between the light-exposed milk and non-exposed milk with added off-odorants, whereas a significant difference was observed in the intensity of the "dusty" off-odor between them (Figure 2a, c). These results indicate that unknown compounds can contribute to the "dusty" off-odor of the light-exposed milk.

Effects of Antioxidants

In order to inhibit light-induced off-flavor formation in milk, we tested the addition of five antioxidants, (-)-epicatechin, chlorogenic acid, α-tocopherol, L-ascorbic acid, and Trolox C. Figure 3 shows the effects of these antioxidants on the formation of the off-odorants whose FD-factors were greater than 64 in the light-exposed milk (Table I). Among the tested antioxidants, (-)-epicatechin and chlorogenic acid showed the strongest inhibitory effects against most of the off-odorants. These two antioxidants almost completely inhibited the formation of (*E,E*)-2,4-nonadienal, (*E*)-2-nonenal, and (*E,E*)-2,4-decadienal (Figure 3a-c). On the other hand, the inhibitory effects of (-)-epicatechin and chlorogenic acid on

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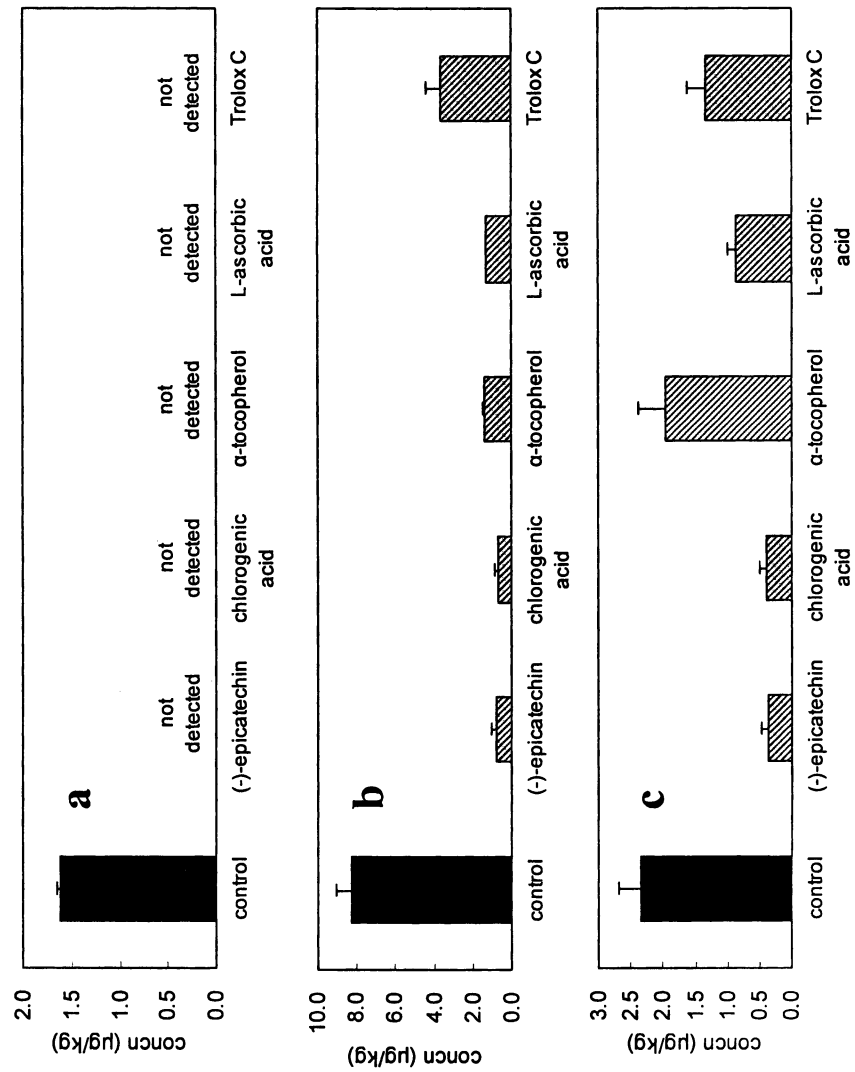

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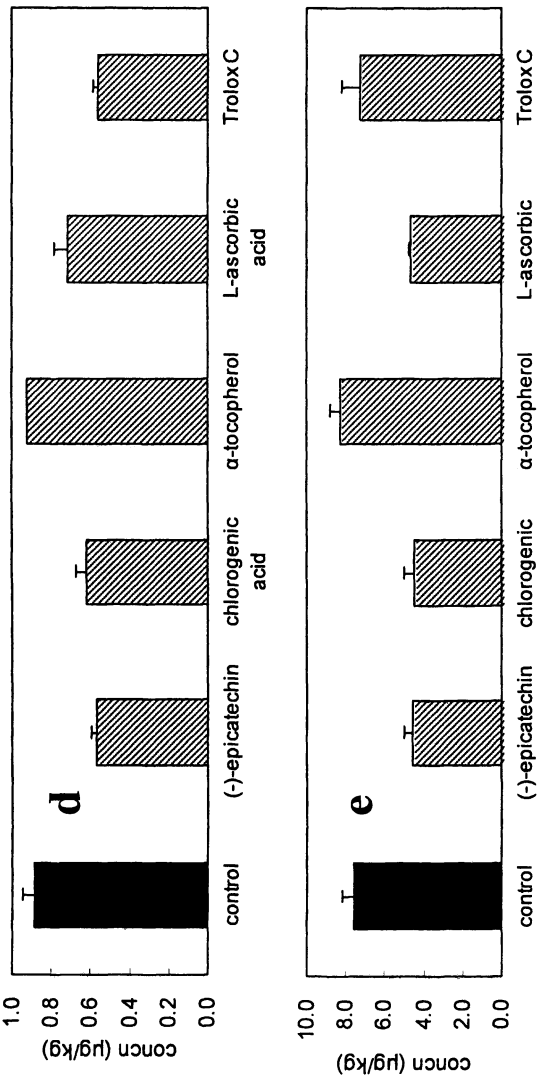

Figure 3. Effects of added antioxidants (100 mg/Kg) on the formation of (a) (E,E)-2,4-nonadienal, (b) (E)-2-nonenal, (c) (E,E)-2,4-decadienal, (d) 1-octen-3-one, (e) methional during light exposure of milk. The error bar represents standard deviation (n = 3)

the formation of 1-octen-3-one and methional were moderate, but were still equal to or stronger than the effects of the other antioxidants (Figure 3d, e). Both (-)-epicatechin and chlorogenic acid were reported to inhibit the formation of methional from methionine in an aqueous solution under riboflavin photosensitized conditions (9). The results in this study demonstrate that (-)-epicatechin and chlorogenic acid are also effective for inhibiting the formation of lipid-derived carbonyl compounds during the light-exposure of milk.

The results of the sensory ranking tests according to the off-flavor intensities of the milk are shown in Figure 4, in which the higher ranking sums indicate the stronger off-flavor intensities. The addition of (-)-epicatechin followed by chlorogenic acid show the lowest ranking sum, indicating the weakest off-flavor intensity. Thus, the effectiveness of (-)-epicatechin and chlorogenic acid on the inhibition of the light-induced off-flavor was demonstrated in both the instrumental and sensory analyses.

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Mechanisms of Antioxidant Actions

All the tested antioxidants are well-known radical scavengers, which can inhibit lipid oxidation proceeding via a free-radical chain mechanism. This might explain the inhibitory effects of the tested antioxidants on the formation of the lipid-derived odorants such as (*E,E*)-2,4-nonadienal, (*E*)-2-nonenal, and (*E,E*)-2,4-decadienal (Figure 3a-c). However, all the tested antioxidants including (-)-epicatechin and chlorogenic acid could not fully inhibit the formation of 1-octen-3-one and methional (Figure 3 d, e). It has been proposed that 1-octen-3-one in oxidized lipids comes from 1-octen-3-ol (*13*), which is a unique product whose formation is clearly from the oxidation of linoleic acid with singlet oxygen (*14*). Regarding the formation of methional, a mechanism like Strecker degradation in which methionine as a free amino acid reacts with the excited triplet riboflavin has been proposed (*15*). Therefore, the moderate inhibitory effects of (-)-epicatechin and chlorogenic acid on the formation of 1-octen-3-one and methional might be due to their lack of ability to quench both singlet oxygen and the excited triplet riboflavin.

Conclusions

This study provides new information regarding light-induced off-flavor formation in milk and its prevention using antioxidants. The results from this study indicated that nine off-odorants, hexanal, (*Z*)-4-heptenal, 1-octen-3-one, methional, (*E*)- and (*Z*)-2-nonenals, (*E,Z*)-2,6-nonadienal, (*E,E*)-2,4-nonadienal, and (*E,E*)-2,4-decadienal are the major contributors of light-induced off-flavor in milk. In addition, the use of antioxidants, especially (-)-epicatechin and chlorogenic acid, were found to effectively inhibit the formation of these off-odorants. The added antioxidants might inhibit the riboflavin-sensitized photo-

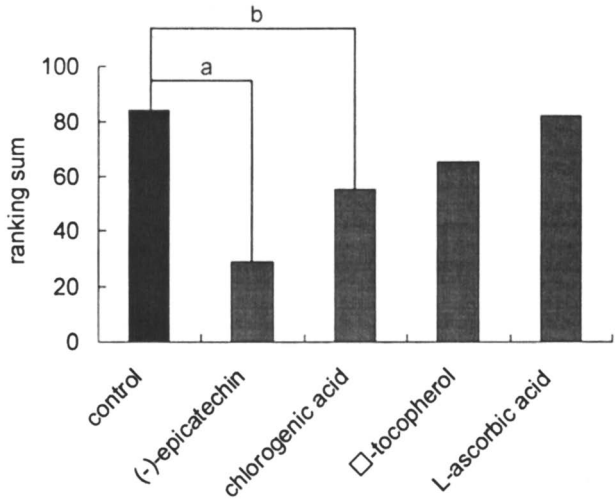

Figure 4. Sum of sensory ranking among milk exposed to light with and without added antioxidants. Samples were ranked in the increasing order of off-flavor intensities.

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oxidation of lipids and proteins in three possible ways: (1) by scavenging free radicals, (2) by quenching singlet oxygen, and (3) by quenching the excited triplet riboflavin. Further studies are needed to clarify the antioxidant actions of the tested compounds.

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Chapter 26

Control of Irradiation-Induced Lipid Oxidation and Volatile Sulfur Compounds Using Antioxidants in Raw Meat and Ready-to-Eat Meat Products

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Ionizing radiation is a non-thermal processing technology used for extending shelf-life and disinfestation of fruits and vegetables, and for inactivating foodborne pathogens and spoilage microorganisms of various foods. However, ionizing radiation can promote lipid oxidation, particularly during post-irradiation storage when exposed to oxygen, and induce development of an off-odor in meats. Free radicals, such as hydroxyl radicals and hydrated electrons, generated from radiolysis of water, attack food components (proteins, amino acids, lipids etc.), leading to an increased rate of lipid oxidation and production of volatile sulfur compounds. Most of the volatile sulfur compounds, such as hydrogen sulfide, methanethiol, methyl sulfide, dimethyl disulfide and dimethyl trisulfide have very low odor thresholds. Antioxidants applied either as additives, ingredients, or dietary supplementation inhibited lipid oxidation, but had a limited effect on production of volatile sulfur compounds, suggesting the mechanisms for irradiation-induced lipid oxidation and production of volatile sulfur compounds are different. Combination of antioxidants with packaging systems may be used to reduce both lipid oxidation and production of off-odor compounds.

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ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

Ionizing radiation is a non-thermal processing technology used for retarding fruit ripening, disinfecting fruits and vegetables, and for inactivating foodborne pathogens and spoilage microorganisms in many foods. Irradiation can kill, injure and inactivate foodborne pathogens and enhance food safety in meat and meat products. However, commercial use of the technology is limited partially due to concerns on adverse effects of irradiation on product quality. When foods are irradiated, particularly at high doses, an off-flavor can develop. Volatile sulfur compounds (VSCs) derived from proteins and sulfur-containing amino acids and compounds that are derived from lipid oxidation may contribute to the off-odor due to irradiation (*1*, *2*).

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Lipid Oxidation and Radiolysis of Lipids

Lipid oxidation is a process involving oxidation of unsaturated fatty acids in the presence of oxygen. Lipid oxidation is important for raw and cooked meats because it causes quality deterioration such as change in color, loss of nutritional values, and production of off-odors/off-flavors (rancid, warmed-over flavor, etc.) due to production of aldehydes, ketones and many other compounds (*3*, *4*). It is generally believed that three phases are involved in lipid oxidation: (1) initiation, the formation of free radicals; (2) propagation, the free-radical chain reactions; and (3) termination, the formation of stable products. The oxidation of fatty acids occurs via a free radical chain mechanism involving the abstraction of hydrogen atoms with subsequent attack by molecular oxygen, leading to formation of hydroperoxides. The hydroperoxides decompose to give up a variety of breakdown products including aldehydes, alcohols, ketones, hydrocarbons, etc. (*3*,*4*).

In foods such as meats that contain mostly water, irradiation exerts its effects mainly via free radicals generated from radiolysis of water. The primary free radicals generated from radiolysis of water are hydrated electrons (e_{aq}^-), hydroxyl radicals (OH) and hydrogen atoms (H), which in turn attack food components, such as proteins, lipids, amino acids, and induce chemical changes in raw and cooked meats (*5*). Exposure of fatty acids and lipids to irradiation in the presence of oxygen accelerates the lipid oxidation process. This is probably because irradiation enhances the following three reactions: formation of free radicals which can combine with oxygen, breakdown of hydroperoxides and destruction of antioxidants (*6*). For example, it has been shown that irradiation (3 kGy) caused a 15% reduction of free β-tocopherol and a 30% reduction of free α–tocopherol in chicken breast muscle (*7*). Obviously, reduction in the concentrations of antioxidants may increase the rate of lipid oxidation of meats during storage. In the absence of oxygen, irradiation directly causes cleavages at certain locations in the lipid molecules, leading to the formation of radiolytic compounds which are mainly dependent on the fatty acid composition of the fat

(*6*). The radiolytic compounds in the absence of oxygen are largely hydrocarbons.

Because lipid oxidation is a significant quality deterioration problem in meats, measurements of lipid oxidation have been used to indicate the stability and potential shelf-life of meats. Thiobarbituric acid reactive substances (TBARS) assay is a widely used method for the determination of lipid oxidation in meats and meat products (*8*). However, the TBARS measurement is not specific and does not directly measure volatile compounds that contribute to the off-odors. Aldehydes are the major lipid oxidation products contributing to oxidation flavor and rancidity in meats. Hexanal is the predominant oxidative volatile adehyde found in many meats and meat products (*3*). A linear relationship between hexanal content and sensory scores in cooked ground pork (*9*), and a correlation between hexanal and TBARS (*10*) have been found.

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There is an inconsistency in terms of whether irradiation induces lipid oxidation of raw meats measured immediately after irradiation. Many studies demonstrated that irradiation accelerated TBARS values (*11-13*) while in some studies irradiation had no effect on TBARS values (*14,15*). Generally speaking, the rate of irradiation-induced lipid oxidation was higher in aerobically packaged meats than in vacuum packaged samples. Most studies on raw meats have shown that irradiated meats had accelerated lipid oxidation during storage, particularly when stored in aerobic packages (*12,16*). When irradiated meats were cooked and stored, TBARS values in the cooked meats increased more rapidly than the raw meats.

In cooked ready-to-eat (RTE) meat products, TBARS can be either increased or decreased by irradiation. Many studies showed that irradiation increased TBARS values in RTE meats (*17-19*). Jo and others (*20*) found irradiation (4.5 kGy) increased TBARS values of cooked pork sausages, but the difference disappeared during 7 days of storage at 4°C as TBARS increased in all samples. On the other hand, TBARS values can be reduced by irradiation in RTE meats and the reduction increased with higher irradiation doses (*21,22*). Furthermore, TBARS values in some irradiated RTE meat products decreased during storage. For example, lipid oxidation in cooked beef exposed to air was inhibited by high dose (5-48 kGy) of irradiation and the TBARS decreased during storage (*23*). As a result, the reduced TBARS values by irradiation enhanced oxidative stability during storage (*24*).

Lipid oxidation can result in formation of many aldehydes. It has been shown that irradiation increased production of hexanal and pentanal in a number of meat products even though the increases may not always be significant (*11,25,26*). Hexanal was found in emulsions prepared from arachidonic acid, not in those from linolenic acid during a post-irradiation storage period (*27*), suggesting that hexanal can be produced from arachidonic acid (*27*) and/or linoleic acid (*28*) in meats in the presence of oxygen. Without oxygen, only n-1 alkanes and n-2 alkenes were produced. C_{15} hydroperoxide is first produced by the reaction of arachidonic acid with hydroxyl radicals and oxygen (Figure 1).

Hexanal is then synthesized from the cleavage of C_{14}-C_{15} bond of the C_{15} hydroperoxide radical, similar to the proposed formation of hexanal from linoleic acid (*28*). Other mechanisms of hexanal production may exisit, such as rearrangement of conjugated double bonds after abstraction of H followed by reaction with oxygen to form hydroperoxide radical. Pentanal and hexanal were highly correlated with TBARS values in irradiated meats (*29,30*). More often higher levels of aldehydes were produced in aerobically packaged meats than vacuum packaged samples. In addition, the production of aldehydes generally increased in aerobically packaged meats during storage, and the increase in irradiated meats was faster than non-irradiated ones (*31-33*). The levels of aldehydes in vacuum packaging had little change or decreased during storage (*20,29,33*).

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Contribution of Volatile Sulfur Compounds to the Off-Odor

Earlier studies using sterilization dose (20-60 kGy) described the irradiation-induced off-odor as 'metallic', 'sulfide', 'wet dog', 'wet grain', 'goaty' or 'burnt' (*34*). The odor has been called 'irradiation odor'. More recent studies using low doses (<10 kGy) described the off-odor as 'barbecued corn–like', 'steamed or rotten vegetables', and 'bloody'(*1, 2*), odors that are subtle and often not noticeable (*35-37*). Montgomery et al. (*38*) observed that irradiation (2 kGy) induced an increase in the scores of the off-odor in both aerobically and vacuum packaged beef patties. Lefebvre et al. (*39*) found that irradiation (2.5 and 5 kGy) induced an off odor and increased peroxide levels in raw ground beef, and the odor of irradiated cooked ground beef was slightly disliked by a nontrained panel.

Reducing or eliminating lipid oxidation had little effect on the development of the off-odor. For example, even though vacuum packaging reduced or eliminated lipid oxidation compared to aerobic packaging, irradiation induced the off-odor of ground pork in both packages (*32,38,40*). The intensity of irradiation odor of turkey patties was higher in the vacuum packaged than the aerobic packaged ones (*25*). The results suggested that volatiles other than those from lipid oxidation contributed mostly to the off-odor. Many studies have demonstrated that irradiation increased production of VSCs which are correlated to the development of the off-odor (*1,2,40*). Several earlier researchers suggested that H_2S and methanethiol were important for the development of the off-odor (*41-43*). Patterson and Stevenson (*44*), using GC-olfactory analysis, showed that dimethyl trisulfide was the most potent off-odor compound in irradiated raw chicken meats. Ahn and his colleagues (*1*) have identified carbon disulfide, methanethiol, dimethyl sulfide, dimethyl disulfide and dimethyl trisulfide in several types of irradiated raw meats using GC-FID and GC-MS. Using a more sensitive detector, a pulsed flame photometric detector, H_2S, carbon disulfide,

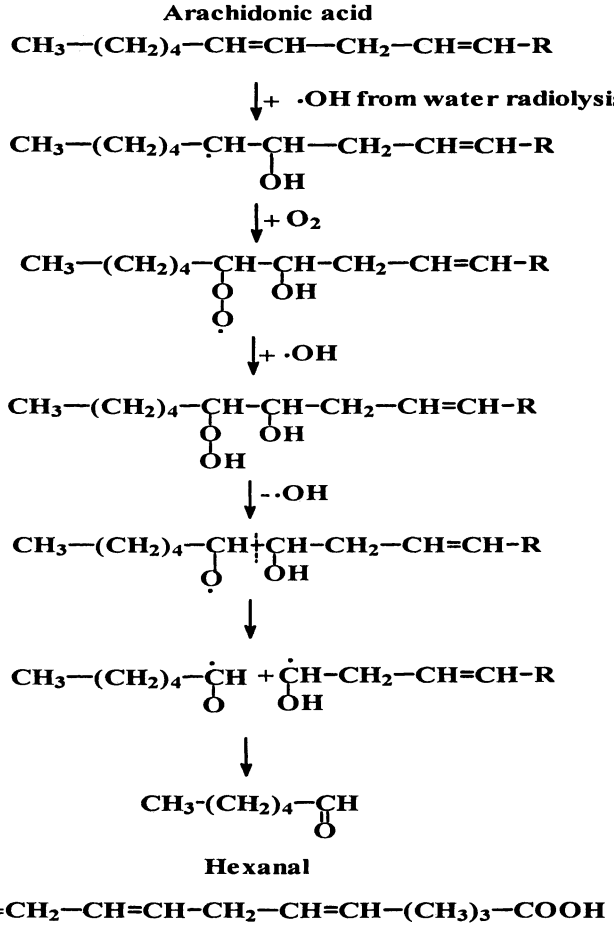

Figure 1. Proposed formation of hexanal from arachidonic acid in the presence of oxygen.

methanethiol, dimethyl sulfide, dimethyl disulfide and dimethyl trisulfide have been found in irradiated cooked turkey breasts (*45*). Most of the compounds increased with irradiation doses (Figure 2). However, CS_2 and dimethyl sulfide can decrease or increase with increasing doses depending on initial levels of the compounds. If the initial (non-irradiated) levels of these two compounds are high, irradiation decreases the levels of the compounds; when the levels of these two compounds are very low or below detectable limit, irradiation reduces the levels of the two compounds. Many commercially produced RTE meats have high levels of CS_2 which could be produced as a result of contamination during

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processing. A high level of CS_2 has been found in deionized water in laboratories, probably due to the contamination from the water lines (X. Fan, unpublished data).

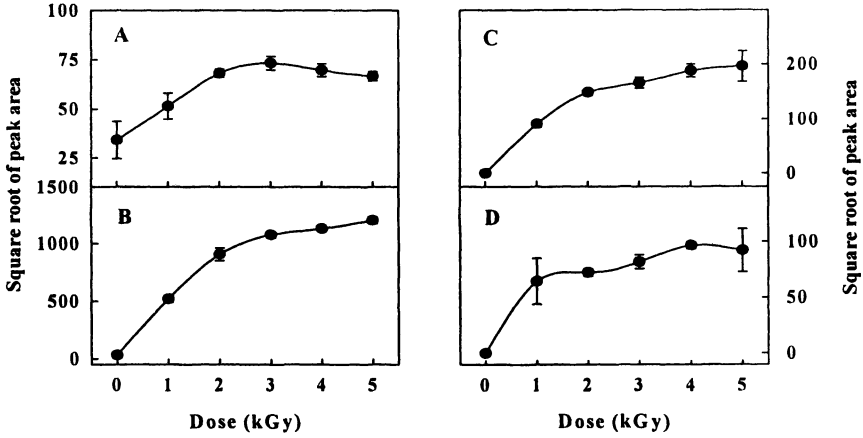

Figure 2. Effect of irradiation dose on the concentration of hydrogen sulfide (A), methanethiol (B), dimethyl disulfide (C), and dimethyl trisulfide (D) of cooked turkey breast. Concentrations of sulfur compounds were expressed as square root of peak area. Vertical bars represents standard deviation of means. Adopted from ref 45.

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Many of these compounds have very low odor thresholds (*2*), and the amounts of many VSCs (such as methanethiol and dimethyl sulfide) induced by irradiation exceeded the odor threshold by several hundredfold (*46*). Addition of VSCs at the levels similar to those induced by irradiation into food induced an off-odor (*46*). VSCs found in irradiated meat products are likely formed from sulfur containing compounds reacting with the free radicals generated from the radiolysis of water (*1*). These sulfur containing compounds may include amino acids in the form of either free amino acids (methionine and cysteine), peptides (glutathione and cystine) or proteins, and others (thiamine and coenzyme A). Irradiation of sulfur-containing amino acids produced similar off-odor as those produced in irradiated meats (1). Figure 3 shows a proposed scheme for the formation of VSCs from methionine. Attack of methionine by hydrated electrons from radiolysis of water results in formation of methanethiol radicals (*28*). Methanethiol radicals can produce dimethyl disulfide or methanethiol. Disproportionation of dimethyl disulfide generates dimethyl sulfide and dimethyl trisulfide.

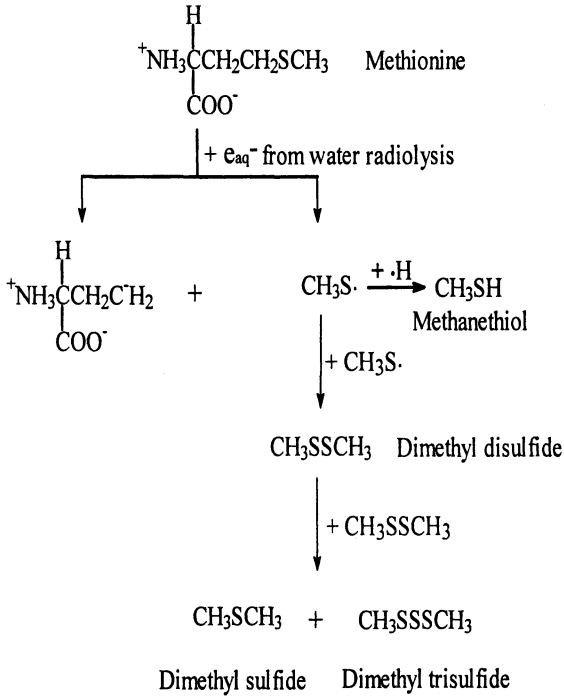

Figure 3. Proposed formation of methanethiol, methyl sulfide, dimethyl sulfide and dimethyl disulfide from methionine (Modified with permission from reference 28. Copyright 2003 Institute of Food Technologists.)

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There are many factors affecting lipid oxidation and development of the off-odor, such as irradiation dose, antioxidant levels, storage times, temperature at which irradiation is conducted, type of antioxidant (water soluble or oil soluble), packaging type, type of meat, and fat content. Some meats may be more sensitive to irradiation than others. Meats with a high proportion of unsaturated fats such as pork and poultry are particularly susceptible to oxidative rancidity (*47,48*). These types of meats are also more-susceptible to irradiation-induced off-odor (48).

VSCs are most likely responsible for the irradiation odor in many irradiated meats. However, lipid oxidation may also play an important role in the development of the off-odor. For example, an earlier study demonstrated that H_2S and methanethiol were partially responsible for the poor acceptability of irradiated cooked meats. But carbonyls, compounds including aldehydes from lipid oxidation, either enhanced the undesirable flavor effect of these VSCs or in combinations produced the off-odor (*49*). Not only could the aldehydes from lipid oxidation contribute to the off-odor, but also the irradiation-accelerated lipid oxidation during storage could increase oxidative changes, leading to the

typical development of rancid flavors. Therefore, techniques that can reduce both the off-odor and lipid oxidation are needed.

Use of Antioxidants to Control Lipid Oxidation and the Off-Odor

Antioxidants have been used for inhibiting lipid oxidation and off-odors in many types of foods (*50*). For inhibition of lipid oxidation and off-odor due to high dose irradiation, irradiation in the frozen state, exclusion of oxygen, and use of antioxidants have been recommended (*41*). Irradiation and storage in the frozen state (–40 °C) reduced but did not eliminate the irradiation-induced off-odor (*26*). For some products, being frozen can change their characteristics (texture, appearance, etc.), and freezing and thawing also increase cost. Therefore irradiated foods in the frozen state may not be feasible for all foods. Even though irradiation can be conducted in the absence of oxygen (vacuum-packaging) and volatiles from lipid oxidation are mostly prevented, the off-odor can not completely be inhibited due to production of volatile sulfur compounds whose production does not necessarily depend on the presence of oxygen (*1*). Therefore the use of antioxidants has been widely explored.

Antioxidants can be introduced into meats using different methods. Antioxidants can be added to animal feeds as a dietary supplement applied weeks or months before slaughter to boost the endogenous levels of antioxidants in meats. After slaughter, antioxidants can be mixed into ground meats as additives. For whole muscle meats or chucks, antioxidants may be applied using infusion. For RTE meat products such as bologna and frankfurter, antioxidants can be applied as ingredients in the meat emulsion mixture before cooking and irradiation, or as a post-processing dip for sliced RTE meat products.

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Application of Antioxidants in Raw Meats

Dietary Supplementation

Patterson and Stevenson (*44*) were the first to attempt to control irradiation-induced off-odors in chicken meat by dietary supplementation of antioxidants in chickens. They raised chickens on feed containing additional α-tocopheryl acetate and/or ascorbic acid (800 mg/kg each) for 7 weeks before slaughter, and found that dietary supplementation of α-tocopheryl acetate in combination with ascorbic acid reduced the yield of total volatiles while either vitamin alone had little effect. However, no result on individual volatiles and sensory evaluation of the off-odor was presented. Ahn *et al.* (*29*) raised turkeys with diets containing up to 100 IU (1 IU = 2/3 mg vitamin E) of α-tocopheryl acetate/kg diet from 1-

105 days followed with diet with higher tocopheryl acetate (up to 600 IU/kg) from 105 to 122 days of age. The authors found that dietary tocopherol of >200 IU/g reduced TBARS of the meat due to irradiation during storage at 4 °C. The dietary supplementation did not control lipid oxidation, production of aldehydes or off-odor generation in cooked meats stored under aerobic condition. In addition, dietary tocopherol supplements to turkey diets had little effect on irradiation-induced methanethiol, dimethyl sulfide or dimethyl disulfide of raw turkey meat (*51*). Inhibitory effect on dimethyl disulfide was only observed in previously frozen-stored (3 months) meats.

Formanek et al. (*16*) fed cattles with diets containing 20 and 2000 mg α–tocopheryl acetate/kg for approximately 50 days prior to slaughter. Ground beef prepared from the supplemented cattle were mixed with rosemary extract or other seasonings. The antioxidant supplementation of diet alone or in combination with rosemary extract as an additive resulted in reduced levels of lipid oxidation.

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Direct Addition in Ground Meats

Chen *et al.* (*30*) added sesamol, quercetin and BHT to ground pork, and demonstrated that rosemary oleoresisn and rutin were effective in the reduction of lipid oxidation for the first 3 days of storage at 4°C. Hexanal and other aldehydes were reduced by sesamol and quercetin. Nam and Anh (*11*) found that the combination of sesamol with α-tocopherol or with Trolox (final concentration 200 μM each) in ground pork only slightly reduced TBARS or production of VSCs such as dimethyl disulfide or dimethyl trisulfide, but dramatically reduced production of pentanal and hexanal (Table I). In another study, Lee and Ahn (*25*) tried the compounds in higher concentrations (1 mM) in ground turkey breast and found that sesamol, tocopherol or Trolox alone or in combination reduced TBARS, volatile production and off-odor intensity in turkey breast homogenates or patties. However, these antioxidants were much more effective in the inhibition of hexanal production than production of dimethyl disulfide. For example, a combination of sesamol and Trolox reduced hexanal production by 90%, while reduced only about 50% for dimethyl disulfide in the raw meat homogenates. Lee *et al.* (*52*) added several combinations of antioxidants to ground turkey breast, and found antioxidants had no effect on the production of VSCs, color change or off-odor intensity of irradiated meats even though sesamol+α-tocopherol or gallate+α-tocopherol reduced TBARS and production of aldehydes. Lee *et al.* (*53*) found that far-infrared-treated rice hull extract significantly reduced the amount of dimethyl disulfide, aldehydes, and lipid oxidation in irradiated raw turkey meat during storage. However, addition of rice hull changed the characteristic color and odor of turkey meat.

Table I. Effect of Antioxidant Combination on Irradiation (4.5 kGy)-Induced Volatile Compounds in Pork Homogenates

Volatiles	*Control*	*S+G*	*S+T*	*S+E*	*G+T*	*T+E*
DMS	599 a	596 a	605 a	390 b	511 a	576 b
DMDS	3774 a	2168 b	1862 b	2124 b	2238 b	2394 b
Pentanal	184 a	0 b	17 b	0 b	45 b	0 b
Hexanal	650 a	29 b	147 b	0 b	131 b	95 b
TBARS	0.79 a	0.64 b	0.65 b	0.65 b	0.70 b	0.63 b

Means with same letters within a row are not significant difference (P>0.05). S: sesamol; G: Gallic acid; T: Trolox; E: Tocopherol. (Modified with permission from reference 11. Copyright 2003 Elsevier.)

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Marinate and Infusion

Marinating chicken legs with a mixture containing ground rosemary and thyme reduced irradiation–induced oxidation of unsaturated fatty acid in the meat (*54*). Although marinating is a common practice used by the meat industry to add flavoring and tenderizing compounds in meats, it takes a long time for antioxidants to diffuse into meats. Therefore, infusion either using pressure/vacuum or direct injection have been applied. Green tea extract, grape seed extract or both (3000 and 6000 ppm based on fat content) were infused into chicken breasts before irradiation (*13*). It is found that addition of green tea extract, grape seed extract or both inhibited lipid oxidation measured by the TBA methods, and non-volatile carbonyl content (*13*). However, TBHQ was generally more effective than the plant extract. Wong *et al.* (55) infused cow carcasses with sodium ascorbate (17.5 L of 500 mM per half carcass) or saline (as a control), ground beef, prepared from these animals, was frozen at –30 °C and then irradiated (5 and 10 kGy). The authors found sodium ascorbate, served as prooxidant, increased lipid oxidation during storage at 4°C.

Application of Antioxidants in RTE Meat Products

The effects of antioxidants added in raw meats prior to irradiation, on lipid oxidation and the off-odor of cooked meats have been investigated. Chen *et al.* (*30*) found sesemol, quercetin and BHT, added in raw ground pork, were effective in inhibiting lipid oxidation of cooked pork patties. Lee *et al.* (*56*) added 200 ppm BHA, ascorbyl palmitate, α-tocopherol and β-carotene to formulation of ground beef patties before irradiation and cooking, and found all antioxidants were effective in inhabiting TBARS values. Ascorbyl palmitate had the strongest antioxidant effect. In another study, Lee *et al.* (*53*) applied far-infrared treated rice hull extract, sesamol and rosemary oleoresin in ground

turkey meat before cooking. They found all treatments reduced production of volatile aldehydes (hexanal, pentenal), and TBARS values of cooked meats, but did not significantly affect dimethyl disulfide or other VSCs. Rababah *et al.* (*13*) infused green tea extract and grape seed extract (3000 and 6000 ppm) and combination of the two into chicken breasts. The samples were then irradiated and cooked. All treatments inhibited irradiation–induced TBARS numbers and non-volatile carbonyl compounds in the cooked samples. Some studies have shown that antioxidants applied in raw meats were not effective in inhibiting lipid oxidation of cooked meats even though they were effective for raw meats (*30*), probably due to the rapid rate of lipid oxidation in cooked meats under aerobic conditions (*29, 30*).

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As Ingredients in Formulation

Many RTE meats are manufactured from ground meats with other ingredients. Antioxidants are added to control the oxidative stability of the products. Cured meats contain an effective antioxidant, sodium nitrite; Uncured meats typically depend on synthetic compounds such as BHA and BHT. Many RTE meats also contain seasonings, some of which have antioxidant capacity.

Du and Ahn (*21*) applied several antioxidants in turkey sausage formulations, and found sesamol had the highest antioxidant effect in terms of TBARS reduction; Rosemary extract had the weakest antioxidant effect. The amount of total volatiles was decreased significantly by the antioxidants, but the antioxidants had minimal effects on the off-flavor induced by irradiation. Addition of 3.5% soy protein concentrate into formulation, increased antioxidant activity by almost threefold, but did not reduce the increase in TBARS in beef bologna (*17*). Fan *et al.* (22) added sodium nitrite, sodium erythorbate, and rosemary extract in meat emulsion of turkey bologna before cooking. The cooked bologna was then irradiated at 3 kGy. Rosemary extract and sodium nitrite were very effective in inhibiting TBARS values while erythorbate slightly increased lipid oxidation (Figure 4). None of the antioxidants was able to reduce levels of VSCs from irradiated RTE products (Figure 5). H_2S and methanethiol were slightly promoted by the addition of the antioxidants (*22*).

Applied as Immersion

To eliminate post-processing contamination of food-borne pathogens present on the surface of RTE meat products, RTE meat products were immersed in a solution that inhibits the growth of pathogens during storage. To study whether VSCs can be reduced by antioxidant treatments, similar to the antimicrobial dipping, sliced turkey bologna was dipped into water, 20 mM of ascorbic acid, vitamin E, and sesamol, and 0.75% of rosemary extract for 2 min.

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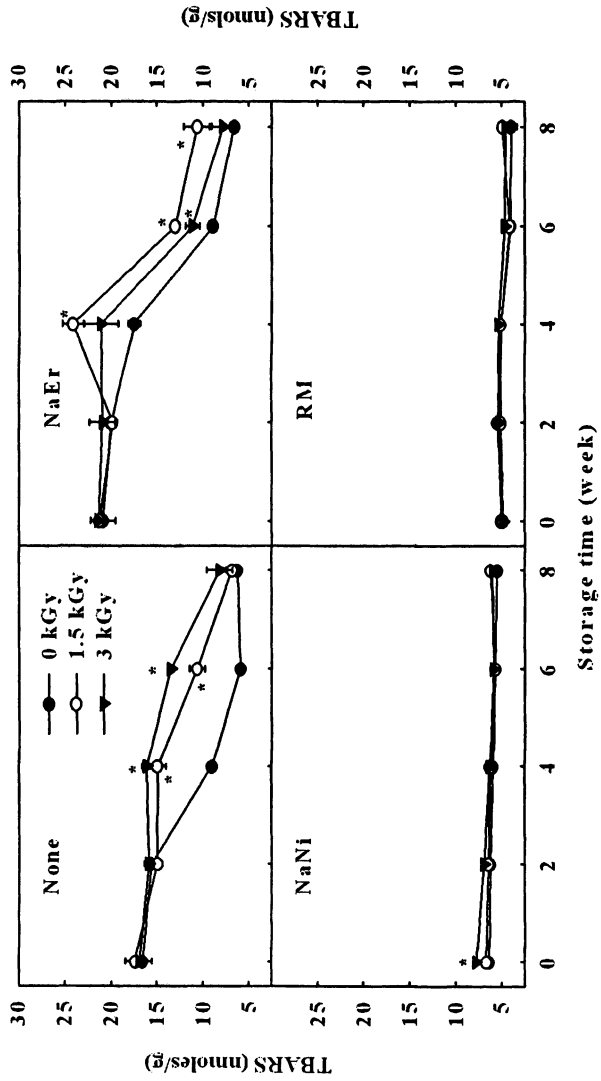

*Figure 4. Effect of antioxidants, radiation dose and storage on lipid oxidation of ready-to-eat turkey bologna. Sliced bologna made from ground turkey breast containing no antioxidant (None), sodium erythorbate (NaEr), sodium nitrite (NaNi) and rosemary extract (RM) were gamma irradiated at 0, 1.5 and 3.0 kGy, and then stored at 5 °C for 8 weeks. Vertical bars represent standard errors (n=4). * indicates significant (P<0.05) difference from the non-irradiated samples. Adapted from reference 22.*

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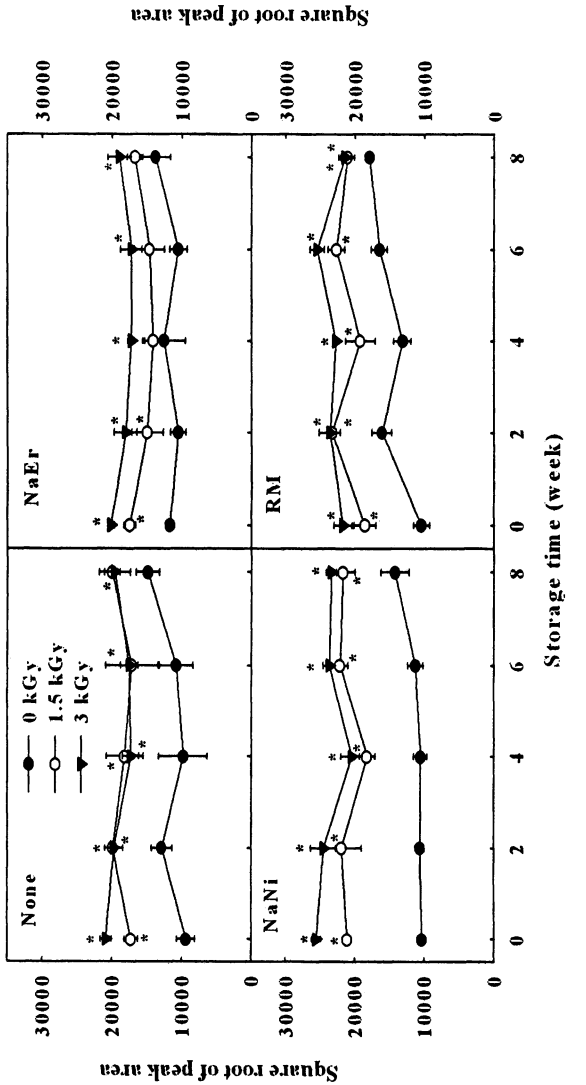

*Figure 5. Effect of antioxidants and radiation dose on total amount of volatile sulfur compounds of ready-to-eat turkey bologna. Sliced bologna made from ground turkey breast containing no antioxidant (None), 500 ppm sodium erythorbate (NaEr), 200 ppm sodium nitrite (NaNi) and 0.075% rosemary extract (RM) were gamma irradiated at 0, 1.5 and 3.0 kGy, and then stored at 5 °C for 8 weeks. Vertical bars represent standard errors (n=4). * indicates significant ($P<0.05$) difference from the non-irradiated samples. Total volatile sulfur compounds are the sum of H_2S, methanethiol, CS_2, dimethyl sulfide, dimethyl disulfide, and dimethyl trisulfide.*

The samples were then irradiated to 3 kGy at 4°C. Irradiation induced formation of methanethiol, dimethyl disulfide, dimethyl trisulfide and reduced production of dimethyl sulfide and CS_2 (Table II). The antioxidants had no effect on irradiation-induced VSCs.

Overall, antioxidants are relatively effective in inhibiting irradiation-induced lipid oxidation, but are ineffective or inconsistent in the reduction of the off-odor and VSCs. Other means to control the off-odor and production of VSCs are needed. The levels of many VSCs decreased during storage (*22*), and irradiated meats packaged in air permeable bags had much lower VSCs levels compared to those in oxygen-barrier bags after storage, suggesting that the VSCs are either oxidized or volatilized in air permeable bags. Correspondingly, off-odor intensity also decreased more rapidly in aerobic packages than in vacuum packages (*32, 33*). A double packaging technique has been developed by Ahn and his colleagues based on the above concept (*1, 57*). The double bagging consists of an outer air-impermeable bag and an inner air-permeable film bag (*57*). After irradiation, the outer bag can be torn to allow escape and oxidation of VSCs. Because antioxidants inhibit irradiation-induced lipid oxidation while irradiation-induced VSCs evaporate in air permeable packages, combination of antioxidants and aerobic packages should reduce the off-odor. Combination of antioxidants with the double packaging systems may be a better approach for eliminating the off-odor and lipid oxidation (*58*).

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Table II. Effect of Antioxidants on Irradiation-induced Volatile Sulfur Compound Production of Turkey Bologna

Dose (kGy)	*Antioxidant*	*H_2S*	*CS_2*	*MT*	*DMS*	*DMDS*	*DMTS*
0	None	483 b	4973 a	247 c	505 abc	318 d	107 d
	Ascorbic acid	419 b	4224 ab	205 c	553 ab	163 d	80 d
	Vitamin E	354 b	4273 ab	210 c	521 abc	175 d	76 d
	Sesamol	445 b	4322 ab	255 c	589 a	176 d	78 d
	Rosemary	370 b	4172 ab	232 c	522 abc	174 d	85 d
3	None	522 ab	3524 bc	2126 a	266 bc	4522 ab	418 bc
	Ascorbic acid	759 a	3145 c	1410 b	233 c	3521 c	331 c
	Vitamin E	396 b	2744 c	1776 ab	233 c	3958 bc	348 c
	Sesamol	268 b	3477 bc	1807 ab	366 bc	4972 a	562 a
	Rosemary extract	407 b	3202 c	2103 a	277 bc	4763 a	495 ab

Sliced turkey bologna was dipped in water (none) or different antioxidant solutions for 3 min before irradiated at 3 kGy. The antioxidants were 20 mM of ascorbic acid, vitamin E, and sesamol, and 0.75% of rosemary extract. Volatile compounds were measured the next day, and expressed as square root of peak area count (n=4). Means with same letters within a column are not significant difference (P>0.05). Modified from reference *2*.

Issues and Future Research

Antioxidants have limited effects on irradiation–induced VSCs, even though most antioxidants are able to inhibit lipid oxidation. The reason for the difference in response to antioxidants may be due to different mechanisms in the formation of VSCs and lipid oxidation. Although free radicals generated from radiolysis of water are involved in both lipid oxidation and VSC formation, hydroxyl radicals are implicated in the attack on lipids while hydrated electrons react with sulfur-containing amino acids (Figures 1, 3). For irradiation-induced lipid oxidation, hydroxyl radicals attack unsaturated fatty acids producing fatty acid radicals. The radicals then combine with oxygen and generate hydroperoxides, which undergo further decomposition reactions (Figure 1). VSCs are believed to be produced via hydrated electrons (Figure 3). The attack of hydrated electrons on sulfur-containing amino acids produces sulfur compound radicals (such as methanethiol radicals) which directly generate VSCs. To inhibit lipid oxidation, antioxidants can terminate the free-radical chain reactions by donating hydrogen or electrons to free radicals and convert them to more stable products (primary antioxidants), or serve as oxygen scavengers, chelating agents, etc. (secondary antioxidants). However, to inhibit production of VSCs, antioxidants have to react with hydrated electrons or with sulfur compound radicals, which are more difficult to control. Compared with VSC formation, there are more steps in lipid oxidation, which provides antioxidants more opportunities to interrupt the process. Furthermore, a much higher rate of the increase in VSC formation than lipid oxidation as a function of radiation also makes complete control of VSC formation difficult.

Approval of any antioxidant for food use by regulatory agencies requires extensive toxicological studies. As a result, only a few have been accepted as "generally recognized as safe" (GRAS) status for food use, even though a large number of compounds have been shown to possess antioxidant activity. For antioxidants added to meats prior to irradiation, it would be even more difficult to get approval because controversies exist regarding the technology. The fate of antioxidants after irradiation has to be studied before regulatory agencies can approve the use. To date, no antioxidant has been approved by U. S. federal regulatory agencies for use to control quality changes of meats prior to irradiation.

Due to changes in consumers' attitudes and concerns on toxicological aspects of synthetic antioxidants, there has been increasing interest in identification and use of natural antioxidants, or plant extracts to minimize lipid oxidation and off-odors. Many natural antioxidants have been studied in terms of inhibiting irradiation-induced off-odor. Most of the antioxidants demonstrated an inhibitive effect on lipid oxidation, and some could prevent irradiation-induced color change. These natural antioxidants include rosemary extract, thyme

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powder or extract, sesamol, rice hull extract, green tea, vitamin C and E. Crude plant extracts (such as dry herbs and spices) have been approved by regulatory agencies at doses up to 30 kGy for control decay and for disinfestations (*59*). Therefore, it may be easier to get approval for use of plant extracts as antioxidants in meats and meat products intended for irradiation.

Studies are needed to investigate the mechanisms of VSC formation. Although it has been proposed that hydrated electrons generated from the radiolysis of water are involved in generation of VSCs, the mechanism has not been confirmed. In some studies, methanethiol is produced while in other studies dimethyl disulfide and dimethyl trisulfide are mostly detected. The reasons for the variations is unknown. Furthermore, irradiation can degrade some VSCs (such as dimethyl sulfide and CS_2). The fate of these compounds is unclear. Understanding the mechanisms will help us to develop measures to reduce production of VSCs and the off-odor.

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Chapter 27

Role of Plant-Based Binders on Lipid Stability and Color of Stored Minced Beef

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The effect of non-meat ingredients (*i.e.*, plant-based binders) on the stability of hemoprotein pigments and unsaturated lipids was investigated using an *in vitro* liposome model and fresh-minced beef systems. The binders studied were of wheat, soy and mustard origin, as these are permitted in Canada for incorporation into fresh meat products. Incorporation of soy protein ingredients and deheated mustard flour, at levels typically employed by the meat industry, resulted in a reduction in the formation of secondary lipid oxidation products by 32-55% and 80-88%, respectively; however, autoxidation of oxymyoglobin was not retarded. Both the *in vitro* and minced beef studies revealed that most of the binders examined had a negative effect on the stability of oxymyoglobin, thereby resulting in deterioration of the fresh meat color. On the other hand, soy protein concentrate and whole wheat flour imparted protection against oxymyoglobin oxidation compared to that of the control by 10-34% and 10-12%, respectively. This study indicated that soy- and wheat-based binders do influence fresh color as well as the development of off-flavors in fresh-minced beef products; moreover, their effect is a function of the specific binder in question and its constituents.

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The minced form of bovine muscle tissue is the most popular fresh beef product in North America. According to the Beef Information Centre (BIC) in 2003, Canadian retail production of fresh minced beef accounted for 51% of the kilogram volume and 30% of the dollar volume for all beef products (*1*). Minced meat is converted to end products like burgers, patties and sausages after it has been mixed with functional non-meat ingredients; these products are available to the customer in both fresh and frozen forms, although fresh products are preferred (*2*). The non-meat ingredients are typically protein and/or carbohydrate-rich plant materials; they are often referred to as fillers or binders and are widely used in combination with spices and flavorants to enhance the sensory characteristics of the final product. In Canada, flours and protein products (*e.g.*, isolates) derived from plants may be incorporated into burger-type fresh minced meat products (*3*). These ingredients offer beneficial characters to the final processed product such as an enhanced taste, juiciness and a reduction in purge during thermal processing. They also provide an advantage to the manufacturer by reducing production costs. The choice of binder is primarily based on price, so in North America they tend to be mostly of wheat flour or soy protein origin. Although the addition of plant binders to comminuted meats has been practiced by the Canadian fresh meat product industry for many years, we are not aware of any scientific peer-reviewed study that has looked at the effect of binders on the retention of fresh meat color in the final processed product.

In meat, the post-slaughter changes occurring in muscle tissue initiated by oxidation reactions have a significant impact on the color of fresh meat. Oxidation of myoglobin and unsaturated lipid constituents are the prime contributory factors for reduced color and flavor acceptability of fresh, stored meat (*4,5*). Understanding how to limit oxidation (*i.e.*, of pigment and lipid) is important to the meat industry, because color is the main sensory attribute by which consumers evaluate the freshness of minced meat and retail cuts. Oxidation reactions of myoglobin and unsaturated lipids are accelerated after size reduction operations such as chopping and grinding/mincing, and these continue during refrigerated and frozen storage (*6,7*). Essentially, size reduction operations increase the surface area of meat thereby exposing more of it to air. Interaction of myoglobin/deoxymyoglobin, the chief pigment of muscle tissue, with atmospheric oxygen results in oxygenation of the ferrohemoprotein pigment and the formation of oxymyoglobin (oxyMb), the desired cherry-red colored pigment associated with fresh red meats. At the same time, interaction of meat constituents during size reduction operations with oxygen accelerates oxidation of unsaturated lipids, loss of intercellular endogenous reductants/antioxidant enzymes, and may result in an increased microbial burden/contamination. The end products prepared with fresh minced red meat have a short shelf-life during refrigerated retail display; this is chiefly due to the accelerated discoloration of oxyMb and partially due to off-flavor development (*7*).

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Introduction of non-meat ingredients to minced meat during product formulation and the subsequent mechanical operations to which it is subjected further alters the chemical and physical environment of the meat. Such environmental changes can affect the chemical reactions happening in muscle tissue that determines the primary sensory aspects of fresh meat, notably the red color. Although color is only one attribute contributing to the quality of the end product, it is this attribute by which the consumer judges fresh meat and ultimately dictates their purchasing decision (*8,* 9).

The capacity of antioxidants to delay oxidation of both lipids and myoglobin in meat has provided some insight into the relationship between these two major autoxidation reactions. Several research groups have reported that the addition of vitamin E, mainly α-tocopherol, exhibited an inhibitory effect towards both lipid and myoglobin oxidation (*10,11*). Though the action as to how lipid oxidation is inhibited by antioxidants has been established, the mechanism for inhibition of oxyMb oxidation remains under debate (*12-17*). Plant-based ingredients used as fillers/binders in meat formulations, such as soy protein concentrate and wheat flour, are known to possess antioxidant properties. There is a considerable amount of information available on the antioxidative activity of wheat- and soy-based products in the literature (*18,19*) and some pertaining to their effectiveness as suppressors of meat lipid oxidation (*20,21*). It is hypothesized, if oxidation of oxyMb and unsaturated lipids in fresh minced beef is "coupled," then retardation of lipid oxidation by the added binders should slow down the rate or extent of oxyMb oxidation. Binders like soy protein concentrate and mustard flour contain known antioxidative compounds that can retard lipid oxidation (*21*); consequently, they should be able to slow oxyMb oxidation and the discoloration of fresh minced beef.

The objective of the present study was to assess the effect of adding soy-, mustard- and wheat-based binders on the stability of color and unsaturated lipids of fresh minced beef during refrigerated retail storage. This study was based on the hypothesis that antioxidants and endogenous reductants present in plant binders aught to have a positive effect on slowing the discoloration of the fresh red color of minced beef muscle.

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Materials and Methods

Chemicals

All chemicals employed were of analytical grade or better. Ultra-high purity water was used in the preparation of all reagents. All phospholipids (1,2-dioleoyl-, 1,2-dilinoleoyl- and 1,2-dilinolenoyl-*sn*-glycero-3-phosphocholine) were purchased from Avanti Polar Lipids Inc. (Albaster, AL). Horse heart metmyoglobin, cholesterol, dicetylphosphate, ascorbic acid, 2-thiobarbituric acid, 1,1,3,3-tetramethoxypropane, methanol and chloroform were acquired from Sigma-Aldrich Canada Ltd. (Oakville, ON).

Binders

Commercial wheat flour (CWF), commercial wheat gluten (CWG), toasted wheat crumb (*i.e.*, leavened dough baked and ground to fine particles; TWC), soy protein isolate (SPI), soy protein concentrate (SPC) and deheated mustard flour (*i.e.*, flour that has been thermally treated to inactive the enzyme myrosinase and hence the pungency of the mustard; DMF) were obtained from Newly Weds Foods, Edmonton, AB. Laboratory-milled wheat flour (variety Elsa, harvested in 2000, 70% extraction; LWF) was obtained from the Crop Development Centre, University of Saskatchewan, Saskatoon, SK. Pre-cooked or micronized whole wheat flour (MWWF) is the flour from infrared (IR) light-treated wheat grain, and whole wheat flour (WWF) is the flour from the same lot of seeds without IR treatment; both were kindly provided by InfraReady Foods, Saskatoon, SK.

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Preparation of Liposomes

Oxymyoglobin (oxyMb) was prepared by reducing metmyoglobin (metMb) followed by an oxygenation step according to the procedures described by Brown and Mebine (*22*) and Yin and Faustman (*23*). Briefly, a solution of metMb (5 mg/mL), prepared in a citrate buffer (pH=6.4, 4 mM), was chemically reduced by adding sodium hydrosulfite (0.1 mg/mg of metMb) and then by bubbling laboratory-grade air (*i.e.*, for oxygenation) through it. Residual hydrosulfite was removed by passing the resultant solution through a Bio-Rad 1.5 × 12 cm polypropylene Econo-Pac® column containing an AG 501-X8 mixed-bed ion exchange resin (*i.e.*, consists of equivalent amounts of AG 1-X8 and AG 50W-X8; Bio-Rad Laboratories (Canada) Ltd., Mississauga, ON). The concentration of oxyMb and unreacted metMb was determined spectrophotometrically by measuring absorbance values at 525, 545, 565 and 572 nm. The absorbance observed at 730 nm was taken as zero for referencing. The concentration of oxyMb in the solution was then calculated from equations provided by Krzywicki (*24*). In the preparation of oxyMb liposomes, the concentration of oxyMb was adjusted to 2.5 mg/mL.

Liposomes of multi lamellar vesicles containing unsaturated phospholipids and oxyMb were prepared as described by Yin and Faustman (*23*). Phospholipids (30 mg, at a ratio of 140:10:1; dioleoyl:dilinoleoyl:dilinolenoyl), cholesterol (12 mg) and dicetylphosphate (3 mg) were dissolved in chloroform/methanol (15 mL, 2:1, v/v) in a 50-mL round bottom flask. The solvent was evaporated using a Büchi Rotavapor/water bath (Models EL 131 and 461, respectively, Brinkmann Instruments [Canada] Ltd., Toronto, ON) resulting in the formation of a thin lipid film. The oxyMb solution (10 mL, 2.5 mg/mL), a NaCl solution (1 mL, 0.1% w/v), three glass beads (3mm, diameter) and binder suspensions (1 mL. 4.5%, w/w) were added to the lipid film. The

oxyMb-liposomes were then formed by agitation using a wrist-action mechanical shaker for 30 min. Samples of liposomes were withdrawn at specified time intervals for analysis. For the control, the binder suspension was replaced by 1 mL of distilled water.

Preparation of Beef Burgers

Fresh beef inside rounds (aged for 21 d) from Canada Grade A carcasses were purchased from a beef packer (XL Meats, Calgary, AB) and frozen at –30°C until used. Meat was thawed at –1°C for 24 h and then transferred to a wet processing area maintained at ~4°C for product preparation. The *semimembranosus* muscles were removed, and then trimmed of all visible fat and connective tissue before comminution. Large pieces of sectioned muscle were first passed through a ½″ plate and later through a ⅜″ plate with a four-blade knife Hobart meat grinder (Hobart MFG Co., Troy, OH). The temperature of the meat was maintained at 0-2°C during grinding and further processing.

The fresh minced beef so obtained was portioned into equal quantities. In a Hobart food mixer (Hobart Manufacturing Co Ltd., Don Mills, ON) the prepared mince (85%, w/w) was combined with a binder (4.5%, w/w), NaCl (1.0%, w/w) and ice water (9.5%, w/w). During mixing, the temperature of the beef was maintained below 2°C. Burgers (120 g) were formed from each preparation (*i.e.*, those containing different binders) using a OMAS hamburger press (OMCAN Manufacturing and Distributing Inc., Oakville, ON). Patties were placed on individual Styrofoam® trays and wrapped in a clear, oxygen-permeable membrane (30,000 $cm^3/m^2/24$ h, Vitafilm, Huntsman Film Products of Canada, Toronto, ON) and then transferred to a retail display case. The display case was maintained at 4°C and an average illumination of 980 lux was applied. Burger samples held in the retail display case were withdrawn every 24 h over a 4-day period (*i.e.*, 96 h in total) for analyses.

Chemical Analyses

Proximate analysis of meat and binders

The following chemical constituents were determined for the starting beef and plant binders according to Official Methods of Analysis of AOAC International (*25*): moisture content by air drying *ca.* a 2-g test sample at 102°C to a constant weight (950.46 B, see p. 39.1.02); ash content by igniting *ca.* a 3 to 5-g test sample in a muffle furnace at 550°C until light grey ash results (920.153, see p. 39.1.09); crude protein content by the classical macro Kjeldahl method using cupric sulfate Kjeltabs instead of mercuric oxide ones (981.10, see p. 39.1.19); and fat (crude) content by petroleum ether extraction using a Goldfisch apparatus (960.39 (a), see p. 39.1.05).

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Oxymyoglobin content

Aliquots (0.25 mL) of liposomes for each treatment were collected, passed through an Acrodisc® 13-mm syringe filter with a 0.45-μm nylon membrane (Pall Corporation, Ann Arbor, MI) and then diluted with the citrate buffer (pH=6.4, 4 mM) to 1 mL. For burgers, the oxyMb content of meat was determined after its extraction from the muscle tissue. The outer surface of burger samples (*i.e.*, the top 3-mm thick layer only) was scraped off. Hemoprotein pigments were then extracted from a known quantity of the meat (5.0 g) with 0.04 M phosphate buffer (1:3, w/v; pH=6.8; 4°C) using a Stomacher Lab-Blender 400 (Model BA6021, A.J. Seward, Co., Bury St. Edmunds, UK). The tissue homogenate was centrifuged at 10,000 × *g* for 10 min at 2°C to remove insoluble particles. The supernatant containing extracted pigments was saved for analysis. Absorbances of the liposome and meat pigment preparations were scanned between 400 and 600 nm using a UV-VIS photodiode array spectrophotometer (HP 8453, Agilent Technologies Inc., Wilmington, DE). Based upon the absorbance readings and the equations described by Krzywicki (*24*), the forms of myoglobin were calculated as total, oxy- and metmyoglobin.

Lipid oxidation

2-Thiobarbituric acid reactive substances (TBARS) were determined at different periods of storage to assess the extent of lipid oxidation in the liposomes and meat tissue extracts. The following modifications were introduced to the protocol described by Schmedes and Hølmer (*26*): an aliquot of liposome or tissue extract was added to 20% (w/v) trichloroacetic acid at a ratio of 1:3 (v/v) to precipitate proteins, and then centrifuged (10,000 × *g* for 10 min at 4°C) to recover the supernatant-containing TBARS. An aliquot of the recovered supernatant was mixed with 0.2 M 2-thiobarbituric acid at a ratio of 1:1 (v/v) and then incubated for 30 min at 85°C. The extent of chromophore development was determined by measuring the absorbance at 532 nm and then quantifying it from a standard curve constructed with 1,1,3,3-tetramethoxypropane. Results were expressed as nmol malondialdehyde (MDA) equivalents per mL of liposome or g of meat.

Antioxidant potential of binders

Binder suspensions (2.5%, w/v) were prepared in distilled water and used in the following assays: hydroxyl radical ($HO^{\bullet}$) scavenging capacity of the binder suspensions was determined by the deoxyribose assay according to Halliwell *et al.* (*27*); the content of total phenolics in the suspensions was

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determined by the classical Folin Denis assay and expressed as ferulic acid equivalents (*28*); and reducing power of the suspensions was determined according to Oyaizu (*29*) with modifications described by Amarowicz *et al.* (*30*), and results expressed relative to ascorbic acid.

Surface color of the burgers

The surface color of prepared burgers were measured as CIE – L*, a*, b* values using a Hunter Lab colorimeter (Miniscan XE, Hunter Associates Laboratory Inc., Reston, VA). The instrument was initially calibrated with black and white tiles and then with a secondary one: a pink tile covered with a piece of the Vitafilm overwrap. The Hunter Lab values (*i.e.*, L*, a* and b*) of burger samples were obtained at two randomly selected spots and were monitored throughout the display storage period.

Experimental design and statistical analysis

Three separate trials were conducted for burgers and liposomes, and for each trial all types of binders were investigated. Data analysis was carried out with SAS (version 9.0) software.

Results and Discussion

Composition of Meat and Binders

The beef used for this study was extra lean with only a 2% lipid content. Compositional analysis indicated that the binders consisted of varying amounts of protein and lipid. All binders, except for DMF, contained very low quantities of lipid with a contribution of 0.02-0.07 g of lipids per 100 g burger at the 4.5% (w/w) addition level. On the other hand, DMF contributed ~1.4 g of lipid to the burger formulation. Soy protein-based binders contain a considerable amount of protein and these markedly increased the total protein level in the burgers. This was not the case for the 3 wheat flour ingredients and TWC, whose protein contents did not differ significantly from that of WWF. At the 4.5% (w/w) addition level, the contribution of protein from SPC, SPI and CWG was 1.2, 3.6 and 3.2 g while from the wheat flours it varied only from 0.53 to 0.65 g per 100 g product, respectively. Additionally, DMF did not contribute greatly to the protein level in the burger products derived therefrom. The amount of beef in all burgers was held constant so as to maintain very similar levels of hemoprotein pigments in each formulation.

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Oxymyoglobin Oxidation

Liposomes

The liposome model system study revealed that binders do have a marked effect on the autoxidation of both unsaturated polar lipids and oxyMb when each of these oxidizing substrates is examined separately with the non-meat ingredients. Table I reports the depletion in the oxyMb content for liposomes comprised of different binders over time. The data collected indicate that all of the binders exerted an "accelerated" effect on oxyMb oxidation in the presence of unsaturated polar lipids. Most interesting were those liposome systems containing no binders and lipids, as they exhibited the least extent of oxyMb oxidation (*NB*, the only exception being the system containing TWC and no added unsaturated lipids). Presence of unsaturated polar lipids in the model system resulted in a considerable increase in the degree of oxyMb oxidation, thereby suggesting that lipid oxidation products can enhance pigment oxidation. When comparing all of the wheat-based ingredients, WWF- and its micronized (MWWF) product-containing liposomes exhibited a lesser degree of oxyMb oxidation relative to the CWF or CWG treatment. The TWC-containing system, however, was most surprising as it performed best in its retardation of oxyMb oxidation when unsaturated polar lipids were absent from the system. Soy protein ingredient (*i.e.*, SPC and SPI) and DMF-containing liposomes demonstrated a similar extent of "accelerated" oxyMb oxidation compared to those of the controls; this occurred even for the systems devoid of unsaturated polar lipids.

Burgers

The oxymyoglobin content on the bloomed surface of all burgers before they were placed in the retail display case (*i.e.*, time = 0 h) ranged from 60 to 83% of the total heme pigments. The only exception, however, was that of DMF-treated burgers, which had an oxyMb content of ~35% at 0 h (Figure 1). The color of fresh meat in contact with atmospheric oxygen is predominantly dictated by the relative amounts of deoxymyoglobin, oxyMb and metMb. It is well documented that the conversion of deoxymyoglobin to the oxy- and met-forms is affected by several intrinsic (*e.g.*, pH, muscle type, animal age, breed gender, diet) and extrinsic (*e.g.*, temperature, oxygen availability, type of lighting, surface microbial growth and storage conditions such as air, modified atmosphere and vacuum) factors (*8,31*). In this study, the burgers containing DMF exhibited a much lower content of oxyMb compared to other treatments and that of the control at all sampling periods. Clearly, incorporation of DMF into the beef pattie had a detrimental effect on the stability of oxyMb. The repercussions due to premature discoloration of fresh meat during storage and refrigerated retail display translate in to a significant economic loss for the meat

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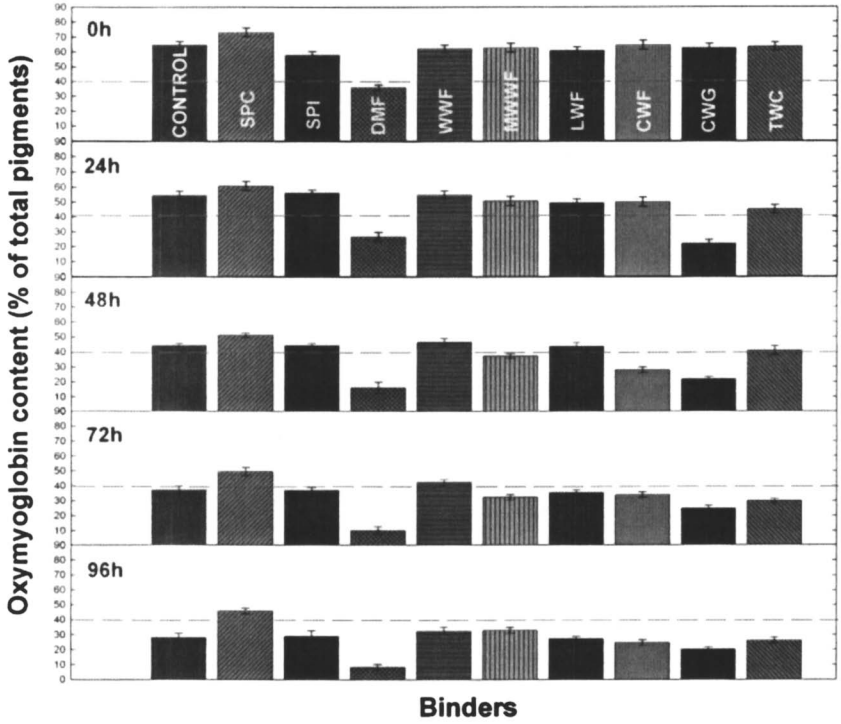

Figure 1. Change in oxymyoglobin content of the surface meat of burgers containing different binders during storage in a 4°C retail display case.

industry. It has been estimated that in the US ~15% of beef sold in supermarkets is discounted by up to 20% because of premature discoloration even though the meat is quite suitable for consumption (*9*). The concentration of oxyMb on the surface of burgers did not change appreciably due to addition of any wheat-based binders during processing or when the individual samples on Styrofoam® trays were first placed into the retail display case (*i.e.*, 0 h samples). In fact, the oxyMb content remained close to the level observed for that of the control. Quite interesting was that SPC addition to the formulation exhibited a higher percentage of oxyMb relative to the control at 0 h. The average pigment level for SPI-treated samples, on the other hand, was slightly less that that of the control. During the first 24 h of display storage, all treatments except for SPC resulted in lower oxyMb contents compared to the control. This pattern was again observed at the 48- and 72-h sampling times. Among the wheat-based binders, WWF and MWWF retained the highest oxyMb levels throughout the display storage, and by the end of the 96-h storage period they, in fact, recorded higher percentages of oxyMb than the control.

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According to Renerre (*31*), after 8 days of refrigerated storage the percentage of metMb at the surface of beef can vary from 25 to 50% of the total heme pigment content. Our study was quite different in that the impact of soy- and wheat-based ingredients, which are routinely added to comminuted meat products, on fresh meat color was examined as an "external" factor, because these non-meat ingredients can influence the consumer-desired color and hence salability of fresh minced beef products.

Lipid Oxidation

Liposomes

Oxidation of unsaturated lipids in the liposomes containing different binders is presented in Table II as TBARS values (nmol MDA eq./mL liposome) at the 28-h point. Presence of oxyMb in the control system significantly enhanced oxidation of unsaturated polar lipids even though no binders were present. This indicates that oxidation products of hemoprotein pigments have an influence on lipid oxidation, as described by Kanner *et al.* (*6*) and Decker and Xu (*32*). Addition of CWF, CWG and LWF to the liposome model devoid of oxyMb markedly enhanced lipid oxidation resulting in high TBARS values. In the presence of oxyMb, TBARS values for systems containing these binders were even greater. Surprisingly, the 2 other wheat additives; that is, WWF and MWWF, did not promote lipid oxidation to the same degree as that of CWF, CWG and TWC. In fact, TBARS values for WWF- and MWWF-treated systems, both in the presence and absence of oxyMb, were less than that of the control. For the lipoxomes containing both oxyMb and a binder, the lowest TBARS values were observed for preparations containing the soy additives (*i.e.*, SPC and SPI); SPI performed slightly better than SPC at curbing lipid oxidation in the liposome system; however, the reverse was noted for counterpart systems absent of oxyMb.

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Burgers

As depicted in Figure 2, increasing TBARS values during retail storage denote a progression of lipid oxidation for the various burger preparations. At the onset of the experiment, the TBARS values for burgers ranged from 2.3 to 5.9 with the control at 3.4 nmol MDA eq./g meat. Burgers devoid of binder generally exhibited higher TBARS values over the course of the study than those containing binders; this point is clearly illustrated in Table III, especially at the 48-, 72- and 96-h sampling periods.

It was expected, lipid oxidation of the beef patties would follow a similar pattern to that of oxyMb oxidation, but this was not the case. The extent or degree of oxidation was varied and depended on the type of binder added to the burger formulation. For example, addition of DMF had a clear and definite inhibitory effect toward lipid oxidation; TBARS values of 2.3 and 5.3 nmol

Table I. Extent of oxymyoglobin oxidation in the liposome model system

Binder	*Time (h) required to see a 50% reduction in the initial oxymyoglobin concentration*	
	Without lipids[a]	*With lipids*[b]
Control – No binder	24	8
Whole wheat flour (WWF)	20	6
Micronized whole wheat flour (MWWF)	20	6
Laboratory-prepared wheat flour (LWF)	10	6
Commercial wheat flour (CWF)	6	2
Commercial wheat gluten (CWG)	10	5
Toasted wheat crumbs (TWC)	28	6
Commercial soy protein concentrate	6	3
(SPC)	8	3
Commercial soy protein isolate (SPI)	8	3
Deheated mustard flour (DMF)		

[a] 0.70 mg/mL for the initial oxymyoglobin content.

[b] 0.71 mg/mL for the initial oxymyoglobin content.

Table II. Extent of lipid oxidation in the liposome model system

Binder	*TBARS formation nmol MDA eq./mL (after 28 h)*	
	Without oxymyoglobin	*With oxymyoglobin*[a]
Control – No binder	3.06	7.90
Whole wheat flour (WWF)	2.56	5.51
Micronized whole wheat flour (MWWF)	2.83	5.95
Laboratory-prepared wheat flour (LWF)	7.06	16.0
Commercial wheat flour (CWF)	16.0	20.0
Commercial wheat gluten (CWG)	15.0	16.5
Toasted wheat crumbs (TWC)	6.00	8.12
Commercial soy protein concentrate (SPC)	2.09	4.72
Commercial soy protein isolate (SPI)	2.43	3.80
Deheated mustard flour (DMF)	2.50	5.08

[a] 0.71 mg/mL for the initial oxymyoglobin content.

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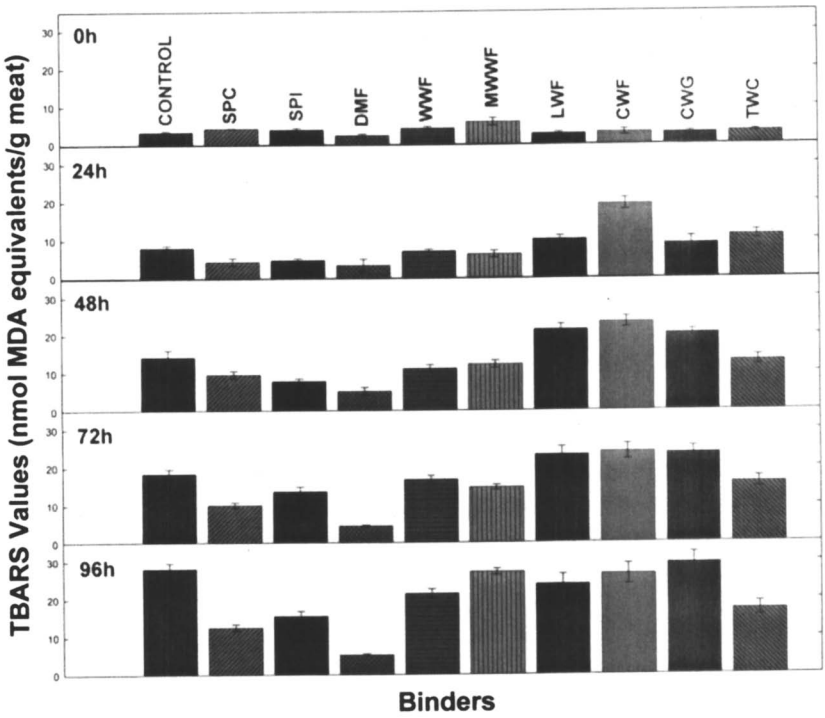

Figure 2. Progression of lipid oxidation on the surface meat of burgers containing different binders during storage in a 4°C retail display case.

MDA eq./g meat were recorded at the beginning (t = 0 h) and end (t = 96 h) of the study, respectively. Although not as effective as DMF at suppressing lipid oxidation, both soy protein-based binders afforded a beneficial effect to lipid stability; this is evident in Figure 2. It is important to recall, however, that the DMF and soy protein-based binders did not delay oxyMb oxidation like some of the other binders, which actually retarded brown color formation (*i.e.*, met Mb) on the surface of the burgers.

Among the wheat-based binders, the story is quite different: WWF, which is the milled whole grain of wheat, imparted a mild inhibitory effect toward the progression of lipid oxidation; in most cases the TBARS values were slightly better than those for WWF's micronized counterpart (*i.e.*, MWWF). The addition of LWF, CWF and CWG to burgers afforded higher TBARS values relative to the control at the 24-h point onward, thereby indicating a pro-oxidant effect from constituents within the binders. Burgers containing TWC showed varied results: TWC seemed to have a slight pro-oxidant effect during the first 2 d, but thereafter was slightly antioxidative compared to the control. Relative to

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DMF and the soy protein-based additives, the TWC treatment was not effective at retarding lipid oxidation in this study. It is likely that antioxidative constituents present in the DMF, SPI and SPC were able to suppress autoxidation of unsaturated meat lipids that had been compromised during the size reduction operations and subsequent aerobic handling processes.

Although the details are not described in this contribution, evaluations by a consumer-type sensory panel indicated that TBARS values below 15 nmol MDA eq./g meat correlated to surface color and hence the purchasing decision of the burger product. This correlation is applicable only for the soy protein- and wheat-based binders: DMF-treated samples were excluded. Table III nicely highlights the performance of the binders over time as being acceptable (*i.e.*, as denoted by shading) and those deemed as unacceptable. Furthermore, the range of data collected for all burger preparations at the various sampling periods is provided.

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Changes in CIE – L*, a*, b* values

Although the CIE – L*, a* and b* values were monitored and recorded for all burgers, only the mean a* values are reported in this work (Figure 3), as they indicate redness of the product. The binder-treated burgers had a* values similar to that of the control at the onset of the experiment; the surface of all burgers exhibited a bright red color thereby denoting a sufficient amount of oxyMb. As storage continued, progressively declining a* values were noted for all burgers. This was particularly evident for DMF and to a lesser extent for SPI-treated meat. Addition of DMF had a detrimental effect on the surface color of burgers: a* values of 33.1 and 8.9 were determined at the beginning (t = 0 h) and end (t = 96 h) of the study, respectively. This is quite interesting considering that the DMF and SPI were most effective at curbing lipid oxidation in burgers.

After 24 h of storage all samples showed lower a* values than their fresh analogues, and the values ranged from 24.9 to 30.9. All wheat-based binders afforded burgers with fairly similar a* values throughout the course of the study. On average, the LWF treatment was slightly superior at preserving the redness of the surface meat. Table IV reports the performance of the binders relative to one another and that of the control over time. The consumer-type sensory panel indicated that burger CIE – a* values equal to or greater than 19 units for all binder treatments except that of DMF were acceptable for purchase. The burgers containing soy protein- and wheat-based binders of an acceptable color are highlighted in Table IV. It seems that the results pertaining to the change in oxyMb content in the control did not parallel the change in CIE – a* value, which is an objective measurement of the redness perceived by human eye. According to the data, only SPC, WWF and MWWF were able to provide an acceptable color throughout the storage period tested. Furthermore, the range of data collected for all burger preparations at the various sampling periods is indicated.

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Table III. Extent of lipid oxidation based on addition of plant binders to fresh minced beef

Storage time, h	*Performance according to TBARS values (nmol MDA eq./g meat)* [a] ← *lowest* … *highest* →	*Range of data*
0	DMF>LWF>CWG>CWF>TWC≈**CONTROL**>SPI>WWF≈SPC>>MWWF	2.3-5.9
24	DMF>SPC>SPI>MWWF>WWF>**CONTROL**>CWG>LWF>TWC>>CWF	3.4-19.3
48	DMF>SPI>SPC>WWF>MWWF>TWC>**CONTROL**>>CWG>LWF>CWF	5.0-24.5
72	DMF>>SPC>SPI>MWWF>TWC>WWF>**CONTROL**>>LWF≈CWG≈CWF	4.5-26.6
96	DMF>SPC>SPI>TWC>WWF>LWF>CWF>MWWF>**CONTROL**≈CWG	5.2-30.8

[a] Mean TBARS values within 0.2 nmol MDA eq./g meat are indicated by ≈. Values less than or equal to 15 nmol MDA eq./g meat for all binder treatments except that of DMF are highlighted. This upper limit was derived from a correlation between consumer-type sensory evaluations for an acceptable red surface color and purchasing decisions based on that color.

Table IV. Colorimetric data for the performance of binders in burgers at retaining fresh meat color

Storage time, h	*Performance based on observed CIE – a* values* [a] ← *highest* … *lowest* →	*Range of data*
0	CWF>**CONTROL**≈TWC>LWF>CWG>MWWF≈WWF>SPI>SPC>DMF	38.0-33.1
24	LWF≈**CONTROL**>WWF>SPC>MWWF≈CWG≈TWC≈CWF>SPI>>DMF	30.9-24.9
48	LWF≈SPC>WWF>**CONTROL**>CWF>CWG>TWC≈MWWF≈SPI>>DMF	26.1-17.5
72	WWF>SPC>LWF>MWWF>**CONTROL**>CWF>CWG>TWC>SPI>>DMF	23.4-11.9
96	SPC>WWF≈MWWF>SPI>LWF≈CWF≈TWC>**CONTROL**>CWG>>DMF	21.6-8.9

[a] Mean CIE – a* values within 0.2 units of each other are indicated by ≈. CIE – a* values above 19 units are highlighted and these burgers are considered as purchasable based upon a consumer-type sensory panel. *NB*, the DMF treatment is excluded.

In Antioxidant Measurement and Applications; Shahidi, F., el al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

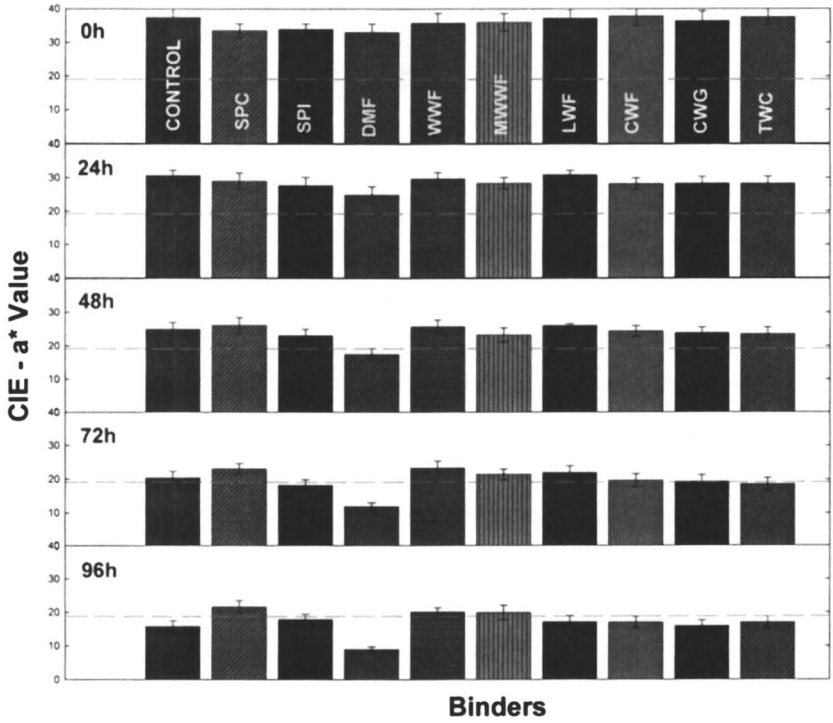

Figure 3. CIE – a values of burgers containing different binders during storage in a 4°C retail display case.*

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Antioxidative properties of the binders

Endogenous compounds, such as carotenoids and tocopherols present in the muscle, are recognized as having antioxidative activity and the potential to interfere with the oxidation of unsaturated lipids and hemoprotein pigments (*e.g.*, oxyMb). Other constituents of muscle tissue, namely carnosine/aniserine, ceruloplasmin, metallothionein and uric acid, may also function as antioxidants by a metal chelation mechanism (*i.e.*, they serve as secondary antioxidants). Antioxidant enzymes, which include superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase, help to breakdown/inhibit reactive oxygen species (ROS). Thus, these endogenous enzymes of muscle tissue assist in preventing ROS from inducing and accelerating myoglobin oxidation. Copper and iron (*i.e.*, in the free form) are present in trace amounts and can act as pro-oxidants. The successful employment of vitamin E (or α-tocopherol) supplementation in feed lot animals to enrich tissue antioxidant pools has

assisted in reducing the discoloration of post-mortem muscle (*9,11*) and is an extension of the rationale described above.

The plant binders used in the present investigation are known to contain constituents with proven antioxidant activity based on *in vitro* and *in vivo* studies. The components include phenolic acids, phenolic acid esters, tocopherols, tocotrienols, carotenoids, phytic acid and flavonoids, to name a few. These endogenous antioxidative compounds can react with free radicals (*i.e.*, lipid or other) by donating hydrogen atoms or electrons, by participating in redox reactions (*e.g.*, electron transfer) or by eliminating radicals. In wheat, phenolic acids are concentrated in the bran and aleurone fractions (*33*). Though ferulic, vanillic and *p*-coumaric acids were major phenolics identified in wheat bran extracts, along with other free phenolics including caffeic, chlorogenic, gentisic, syringic and *p*-hydroxybenzoic acids (*34*), they are also present in the flours but at reduced levels. Baublis *et al.* (*35*) reported that free ferulic acid and its esterified derivatives were the most antioxidative compounds in aqueous extracts of whole wheat products. In the present study, it is evident that whole wheat flour possesses a higher antioxidative capacity than the other wheat-based products such as flour or proteins when the bran fraction has been reduced. In their study, Zhou *et al.* (*36*) found that micronization increased the antioxidant activity of the aleurone fraction of wheat relative to its untreated counterpart. These authors attributed this finding to an increased surface area *via* the processing, thereby improving the availability of wheat antioxidants. Micronization of WWF (*i.e.*, MWWF) did not seem to offer any improvement in antioxidant efficacy in this work, based on the model system studies employed. Fibers of cereal flours including wheat have been found to inhibit lipoxygenase-catalyzed oxidation of linoleic acid emulsions (*37*).

Soy protein isolate contains a mixture of antioxidants including phenolics, saponins, and copper, a component of a number of antioxidant enzymes (*38*). In soy protein products, the phenolic compounds which provide antioxidant activity are mainly in the form of isoflavones (*i.e.*, daidzein and genistein) and phytic acid (*39*). Antioxidant activity of mustard flour is mostly attributed to its phenolic constituents, namely *p*-hydroxybenzoic and sinapic acids, their esterified derivatives, flavones/flavanols and condensed tannins (*20,40*). In DMF, free phenolic acids and their simple esters may be the antioxidative compounds which offer protection against lipid oxidation in products such as the minced beef burgers.

The data obtained from the liposome model and beef burgers indicated that each binder exhibits a different anti- or pro-oxidative potential against the oxidation of lipids and oxyMb. An enhanced degree of oxyMb oxidation when unsaturated lipids were present (*i.e.*, without binders) indicated that products of lipid oxidation have a profound pro-oxidative effect on oxyMb oxidation. It has been reported that oxyMb in the presence of oxidizing lipids of liposomes (*10,23*), muscle (*41-43*) and microsomes (*44*) can oxidize rapidly to metMb. Monahan *et al.* (*45*) reported that lipid and oxyMb oxidation is not always

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positively correlated: the level of dissolved oxygen in the oxidizing system is a critical factor at determining whether or not oxyMb oxidizes.

The antioxidant potential of binders as determined by the $HO^{\bullet}$ scavenging capacity, reducing power and total phenolic content are presented in Table V. DMF with the highest content of total phenolics showed the strongest $HO^{\bullet}$ scavenging capacity and reducing power of all binders examined. This capability was best exhibited by suppressing oxidation of unsaturated lipids in the burger model; the lowest TBARS values for the surface meat were observed throughout the storage period. Nevertheless, these antioxidative components may not be appropriate when protection against oxidation is required for oxyMb: DMF enhanced the oxidation of oxyMb! Unfortunately, the employment of DMF is not suitable in fresh meat products if the fresh red color is an important quality attribute to be preserved. DMF and SPC were very effective in suppressing lipid oxidation in fresh meat at the level employed in this study, and this was further exhibited in their thermally-processed counterparts (*20,21*).

Among the wheat-based ingredients, the whole wheat grain flours gave a moderate antioxidant potential compared to the other ingredients. When considering the overall results, the binders exhibiting the weakest effect on accelerating oxyMb oxidation had a moderate antioxidative potential. This suggests antioxidants that are strong inhibitors toward the oxidation of unsaturated lipids may be promoters/enhancers of oxyMb oxidation. On the other hand, a moderate antioxidatve activity may be able to balance both the integrity and stability of lipids and oxyMb.

Conclusions

When reviewing the indicators of lipid and pigment (*i.e.*, oxymyoglobin) oxidation, soy-, mustard- and wheat-based binders impacted these parameters to varying degrees. The results demonstrated that plant binders comprise a mixture of constituents, which influence lipid and hemoprotein pigment oxidation in some cases quite differently. This nullified the hypothesis put forward, if oxidation of oxyMb and unsaturated lipids in fresh minced beef is "coupled," then retardation of lipid oxidation by the added binders should slow down the rate or extent of oxyMb oxidation. In burgers, the profile of constituents in the binders may have had some impact on the efficacy of endogenous antioxidant enzymes in the muscle tissue. In other words, the mechanism(s) of lipid and oxyMb oxidation is further complicated when plant binders are present in the systems. In this work, SPC and WWF were best at retaining the desired red color in fresh minced beef while suppressing the formation of lipid oxidation products. DMF was detrimental to the color stability of fresh minced beef, but was an extremely good inhibitor of lipid oxidation.

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Table V. Antioxidative potential of binders based on three assays

Binders[a]	*HO˙ scavenging capacity, %*[b]	*Total phenolic content*[c]	*Reducing power*[d]
Whole wheat flour (WWF)	62.0±1.2b[e]	34.2±1.5e	20.2±1.2d
Micronized whole wheat flour (MWWF)	36.6±2.0d	36.8±0.9d,e	20.8±2.0d
Laboratory-prepared wheat flour (LWF)	28.8±2.1e	30.0±1.4f	11.7±2.1e
Commercial wheat flour (CWF)	63.1±1.8b	31.5±1.7e,f	13.3±1.8e
Commercial wheat gluten (CWG)	44.2±2.2c,d	30.5±2.0f	12.8±2.2e
Toasted wheat crumbs (TWC)	26.7±1.0e	26.7±1.2f	13.3±1.0e
Commercial soy protein concentrate (SPC)	73.2±1.3a	119.5±2.9c	41.3±1.3c
Commercial soy protein isolate (SPI)	66.2±1.5b	137.6±1.6b	56.8±1.5b
Deheated mustard flour (DMF)	71.2±1.4a	348.4±2.0a	163±1.4a

[a] Binders employed for the assay were as 2.5% (w/v) suspensions in distilled water.
[b] Deoxyribose assay (*27*).
[c] Data are expressed as μg ferulic acid equivalents using the classical Folin Denis assay.
[d] Data are relative to ascorbic acid which has a 100% reducing power.
[d] Means in a column having the same letter are not significantly ($p > 0.05$).

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