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PERSPECTIVES

How To Standardize the Multiplicity of Methods To Evaluate Natural Antioxidants

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A great multiplicity of methods has been used to evaluate the activity of natural antioxidants by using different techniques of inducing and catalyzing oxidation and measuring the end point of oxidation for foods and biological systems. Antioxidant in vitro protocols for foods should be based on analyses at relatively low levels of oxidation under mild conditions and on the formation and decomposition of hydroperoxides. For antioxidant in vivo protocols, widely different methods have been used to test the biological protective activity of phenolic compounds. Unfortunately, many of these protocols have been based on questionable methodology to accurately measure oxidative damage and to assess relevant changes in biological targets. Many studies testing the ex vivo activity of phenolic compounds to inhibit human low-density lilpoprotein (LDL) oxidation have been difficult to evaluate because of the structural complexity of LDL particles and because a multitude of markers of oxidative damage have been used. Although studies with animal models of atherosclerosis have demonstrated the antioxidant effect of phenolic compounds in delaying the progress of this disease, human clinical trials of antioxidants have reported inconsistent and mixed results. Complex mixtures of plant polyphenols have been shown to be absorbed to varying degrees as metabolites in the intestine, but little is known about their interactions, bioavailability, and their in vivo antioxidant activity. Several metabolites identified in human plasma after consuming flavonoids need to be tested for possible nonantioxidant activities. More research and better-designed human studies are required to clarify the complex questions of bioavailability of polyphenols and the factors affecting their in vivo activities. Until we know what relevant in vivo activities to measure, any claims on the biological and health protective effects of natural polyphenolic compounds in our diet are premature.

KEYWORDS: Antioxidants; methods; protocols; activity; foods; biological systems; metabolites; low-density lipoproteins

INTRODUCTION

The literature on natural antioxidants has exploded in the past decade because they may account for the recognized and potential heath benefits of fruits and vegetables in the diet. Natural antioxidants from plants are generally multifunctional in foods and biological systems. In addition to scavenging free radicals, the multiple activities of antioxidants include inactivating metal catalysts by chelation, reducing hydroperoxides into stable hydroxyl derivatives, and interacting synergistically with other reducing compounds (*1, 2*). A great multiplicity of protocols has been used to evaluate the activity of natural antioxidants by using a wide variety of free radical generating systems, different methods of inducing oxidation, and measuring end points of oxidation (*3–14*). Unfortunately, variable and confusing results have been reported depending on the protocols, methods, and conditions used to test the antioxidant activity.

In a general overview of phenolic compounds in fruits, Robards et al. (*3*) tabulated a list of 38 methods published between 1983 and 1998 to assess the antioxidant activity for both food and biologically relevant substrates, using a wide variety of "initiators", substrates, and end point measurements. They distinguished between four inhibition assays, measuring inhibition at a fixed time point, reaction rate, or lag phase (and discoloration with no data in the table), but they did not discuss the relative merits of the methods or approaches. The same group published another review (*6*) tabulating common tests

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for antioxidant activity measurements, grouping together methods to measure oxidation and antioxidant activity that require a substrate target.

A later review by Laguerre et al. (*12*) discussed the advantages and drawbacks of existing rapid in vitro tests for lipid oxidation and direct methods using "lipid-like substrates" and some of the problems created by the different oxidation conditions used. More emphasis was placed on the first two nonspecific measurement strategies dealing with oxygen depletion and substrate loss than the more relevant and specific methods dealing with primary and secondary lipid oxidation products. The loss of lipid substrate is an insensitive method because it measures a small change (less than 1%) as compared to a very large amount of stating material. Oxidative damage of lipids can be observed before significant changes in lipid can be measured (above 5%). Volatile decomposition products formed from oxidized linoleate- and linolenate-containing vegetable oils can be detected by sensory panels in the range of parts per million, and those from long-chain *n*-3 polyunsaturated fatty acid (PUFA) from fish oils and algae can be detected in the range of parts per billion. Obviously, much more useful and sensitive methods are needed than those based on oxygen depletion and substrate loss. For example, analyses of hexanal in infant milk powder by solid-phase microextraction−mass spectrometry can be achieved in the range of parts per billion [(*1*), Chapter 6, Table 6.7, p 152]. Advanced instrumentation techniques to evaluate lipid oxidation in foods or oil-based products include a wide range of gas chromatography (GC) with capillary columns and high-performance liquid chromatography linked with different forms of mass spectrometry (HPLC-MS) (electron impact, chemical ionization, atmospheric pressure chemical ionization, and coordination ion spray) and tandem mass spectrometry (MS/MS) [(*1*), Chapter 6].

IN VITRO ANTIOXIDANT PROTOCOLS FOR FOODS

The large amount of effort expended in testing new and natural antioxidants emphasizes the need for improved and more consistent methods and their standardization. Because of a wide divergence of results of natural antioxidants in foods systems, more valid and rigorous guidelines and assay protocols are required to bring some order and agreement to this important field. Our understanding of the effects of antioxidant compounds can only be improved if more specific methodology is used and is capable of defining what products are formed and inhibited by antioxidants depending on conditions, systems, and targets of protection.

The activity and mechanism of complex natural antioxidants are affected by many factors including the particular system used, the conditions of oxidation, the kinetics and the composition of the system, the partitioning properties of the antioxidants between different phases and their interface, and the physical state of the substrate (*4, 15*). Of particular importance are the conditions used to accelerate oxidation by raising the temperature, by using metal catalysts or other types of initiators, by increasing the surface, and by exposing them to light. Therefore, antioxidant protocols must be carefully designed on the basis of the specificity of the analytical methods used to follow the progress of oxidation and choosing a proper end point relevant to the protection of foods against oxidation.

Because many natural antioxidants such as tocopherols and flavonoids inhibit both the formation and the decomposition of hydroperoxides [(*1*), Chapters 2 and 4; (*4, 15*)], it is essential to measure several parameters of oxidation. Yet many recent antioxidant studies use only one method to measure primary oxidation products such as peroxide value or conjugated dienes or reactivities toward artificial radical initiators [(*2*), Chapter 4; (*4*)]. The selection of valid reference compounds for relative in vitro activity of natural antioxidants is also a difficult and controversial problem (*16*).

The common use of Trolox as a reference, a synthetic hydrophilic carboxylic acid analogue of α-tocopherol, is questionable to evaluate the relative activity of flavonoids and other natural antioxidants. In many studies, the results are generally expressed as "Trolox equivalents", which cannot be interpreted reliably because the mechanisms of natural antioxidants vary widely according to their hydrophilic and lipophilic properties in different multiphase substrates [(*1*), Chapter 5; (*2*), Chapter 4; (*15*)]. It is therefore difficult to develop a single measure that will account for the multiple mechanisms of natural antioxidants. Several antioxidant protocols used in foods consist of oxidizing different lipid substrates under standardized conditions and assessing activity by various methods to determine how much oxidation is inhibited. Many of these test systems overlook the effects of composition and interfacial phenomena that affect antioxidant performance from multiple components in real food systems. Valid protocols to test the activity of natural antioxidants require careful design to clarify the mechanisms of antioxidants by employing relevant substrates and relatively mild oxidation conditions.

Choice of in Vitro Protocols. Significant variations in test results obtained by questionable methods have created much confusion. The judicious choice of in vitro protocols should be designed to obtain meaningful interpretation of antioxidant action and to answer the following questions:

(a) What are the protective properties of antioxidants?
(b) What substrates are protected from oxidation and what products of oxidation are inhibited? Measure the correct extent of oxidation and inhibition and choose an appropriate end point of oxidation.
(c) In a multiphase food system, is the antioxidant located where oxidation takes place?
(d) What are other interacting components that may affect the results in real-life applications? Determine any possible adverse prooxidant effects by using a range of concentrations.

Each antioxidant evaluation should thus be carried out under various conditions of oxidation, using several methods to measure different products of oxidation related to real food quality.

Required Paramaters and Analyses. Well-designed testing protocols for antioxidants (*4*) should include the following:

(a) A suitable food substrate (triacylglycerols or phospholipids) in more than one system including bulk, emulsion, or liposome systems. Free fatty acids should be avoided because they form micelles in which antioxidants behave differently than triacylglycerols.
(b) Relatively mild conditions of oxidation (below 60 °C) to minimize changes in mechanism due to oxygen solubility and avoidance of artificial diazo initiators that are not relevant to either food or biological oxidation. Select conditions that simulate the storage of real foods as closely as possible.
(c) Analyses at relatively low levels of oxidation (below 1%) of both initial products (hydroperoxides, peroxide value, and conjugated dienes) and secondary decomposition products (aldehydes and volatile carbonyls compounds), at several time periods to include the initiation and early stages of propagation phases of oxidation.

(d) Different levels of antioxidants as compared to the same molar concentrations of active components. With crude plant extracts, the total phenolic contents and compositional data are necessary to compare samples.
(e) Calculations based on induction period, percent inhibition or rates of hydroperoxide formation or decomposition, or antioxidant concentration required to obtain an appropriate level of inhibition.
(f) Sensory evaluations for foods, vegetable, and fish oils containing linolenic acid and long-chain PUFAs (EPA, eicosapentaenoic acid; and DHA, docosahexaenoic acid) that produce fishy responses at very low levels of oxidation.

A technical committee of the American Oil Chemists' Society (AOCS) developed procedures to assess the effects of antioxidants in oils and fats published in its "Official Methods and Recommended Practice" (*17*), including the preparation of purified triacylglycerols to use as substrates by stripping vegetable oils of their natural tocopherols and other minor constituents, oxidation temperatures between 25 and 60 °C, analyses of primary oxidation products (hydroperoxides, peroxides, and conjugated dienes) and secondary decomposition products (carbonyl and volatile compounds), and sensory evaluation of odor and flavor to confirm results from instrumental and chemical analyses.

See also the following examples of good practice in ref *1* [Figures 9.7 and 9.8, p 232; Figures 9.10 and 9.11, p 236; Table 9.11, p 238; Table 9.20, p 251, corn oil (stripped of tocopherols)], the corresponding references, and in ref *2* (Table 5.7, p 116, refs 2a,b and 7).

The thiobarbituric acid (TBA) test has been commonly used to measure lipid oxidation in some foods and biological systems. Because the colored complex due to TBA-reactive substances (TBARS) was originally characterized as the condensation adduct between TBA and malonaldehyde (MDA), which was used as a standard, the results are expressed as mg MDA per kg of sample. However, MDA is very unstable and often either is not detected in many oxidized lipids or is a minor secondary oxidation product. When the formation of pure MDA from different lipid oxidation precursors was tested by GC analyses of the stable tetramethylacetal derivatives, the results were significantly different than those from the TBA test (*18*). The TBA test is also notoriously unspecific and is influenced by a multitude of factors, including heating conditions, pH, metal ions, and antioxidants. This test is therefore unsuitable to study the effects of antioxidants and not acceptable for complex food materials (e.g., meat and fish) and biological systems containing nonlipid constituents contributing to the color reaction.

Model vs Real Food Systems. In simplified model systems, interfacial phenomena may be overridden when interpreting antioxidant mechanisms and activity that appear strongly influenced by complex interfacial and phase distribution properties (*4*). In complex real food systems, accelerated oxidation tests are difficult to standardize. Each antioxidant test should be calibrated for each type of lipid or food, and oxidation conditions should be close to the storage conditions under which the food is to be protected. Ultimately, antioxidants should be evaluated on the food itself.

When testing the activity of potential food antioxidants, the first aim may be to develop a model system, where basic chemical principles can be deduced. On the other hand, the true effectiveness of antioxidants cannot be properly assessed unless the conditions, that is, the complexity of the system, are as close as practically possible to the conditions under which protection against oxidation is required. Targeting of antioxidants to prevent particular free radical formation steps and oxidative deterioration processes requires detailed understanding of the mechanisms of hydroperoxide formation and decomposition. Specific lipid model systems should mimic the food or physiological target systems to be protected as close as practically possible.

IN VIVO ANTIOXIDANT PROTOCOLS

What Is a Biological Antioxidant? Extensive studies of oxidation and antioxidants in biological systems have added a new dimension of complexity. The use of the term "antioxidant" has been abused. This term has assumed a broad meaning in biology, nutrition, and health sciences to include repair systems (iron transport proteins and antioxidant enzymes) and vascular factors affecting homeostasis (physiological chemical functions), signal transduction (transfer of genetic material by redox signaling), and regulation of gene expression of detoxifying enzymes (*11*).

The evaluation of "total antioxidant capacity" (TAC) (*5, 13, 14, 19*) of foods has become a popular and common index, which is purported to be a measure of the nutritional and health effects of fruits and vegetables. In a solvent extraction study of fruits and vegetables, phenolic compounds in the water and acetone extracts contributed the most to TAC, while the chloroform extract did not contribute to TAC (*13*). On this basis, the authors concluded that the extraction procedure was more effective for foods rich in water-soluble than those rich in lipid-soluble phenolic compounds. Regarding the TAC concept, Sies (*14*) commented that "Neither the term *total* nor the term *capacity* are applicable to the *in vitro* assays using an arbitrarily selected oxidant generator and assaying a sample removed from its biological context." He concluded that there is little evidence in support of the association between the TAC and the health effects of fruits and vegetables.

Many health benefits of tocopherols and flavonoids are now being explained by a multitude of "nonantioxidant" complex activities and specific effects that do not involve free radical inhibition. α-Tocopherol can inhibit cell, platelet adhesion and aggregation, specific scavenger receptors formation of inflammatory mediators of cyclooxygenase-2 and 5-lipoxygenase, and inhibition of specific scavenger receptors [(*2*), Chapter 6, H]. Flavonoids have anti-inflammatory properties related to their inhibition of enzymes that produce eicosanoids by a complex cascade of reactions catalyzed by cycooxygenase and leukotrienes catalyzed by lipoxygenses [(*1*), Figures 13.11 and 13.12, pp 425−426]. Flavonoids are also involved in regulating a complex network of of inflammatory cytokines (tumor growth factors). Other nonantioxidant activities of flavonoids include regulating signaling pathways for their cellular neuroprotective, cardioprotective, and chemopreventive properties, transferring genetic information, and controlling the gene survival or death [(*2*), Chapter 6, H, p 182]. These and other complex biological activities cannot be evaluated by the oversimplified antiradical methods such as ORAC (oxygen radical absorbance capacity) and TEAC (Trolox equivalent antioxidant capacity) that are claimed to be in vivo (*19*), because they are only based on relative inhibition toward artificial diazo free radical initiators, AAPH [2,2′-azobis-(2-amidino-propane) dihydrochloride] and $ABTS^{\bullet}$ [2,2′-azinobis-(3-ethylbenzothiazoline 6-sulfonate)].

In Vitro vs ex Vivo and in Vivo Antioxidant Activities. Studies comparing in vitro vs ex vivo and in vivo testing of antioxidants generally have shown significantly divergent results [(2), Chapter 6, D, pp 150−155]. Antioxidants have been

commonly evaluated by determining their relative concentrations in blood after supplementation. However, such analyses can be confounded by oxidative stress that causes an up-regulation of antioxidant enzymes. Widely different methods have been used to test the relative antioxidant activity of phenolic antioxidants to scavenge peroxyl, hydroxyl, or superoxide radicals.

The results of in vitro and in vivo studies to assess the effects of phenolic antioxidants are difficult to interpret because questionable methodology has been used to accurately measure oxidative damage and to assess significant and relevant changes in biological targets. Protocols for biological antioxidant using in vivo systems have been especially controversial because the complex mechanisms of their protection are not well-understood. The antioxidant potency of polyphenols evaluated by a number of in vitro and some in vivo tests is commonly based on their activity using artificial azo initiators of free radical oxidation, ABTS and AAPH; radicals produced by oxidation of linoleic acid; or reducing ferric ions in plasma (*14*). Antioxidant methods that commonly use these artificial radical model systems provide no information on what biological targets are protected. These initiating processes are also not relevant to either food or biological systems, and the damage caused cannot be used to estimate the in vitro and in vivo activity of natural antioxidants. The general assumption that these processes represent accurate models is completely fallacious and misleading. To determine the real effects of polyphenolic antioxidants, it is important to obtain specific information on the type of oxidation products that are inhibited and their biological source(s). Several specific assays are needed to elucidate products causing oxidative damage in biological tissues. Until comparative results of the various methods can be related to significant influence in biological systems, the results must be questioned. Several specific assays are therefore needed to elucidate products causing oxidative damage in biological tissues. The several metabolites of phenolic compounds (see next section) identified in plasma from human studies of flavonoids should also be tested for possible nonantioxidant properties including anti-inflammatory and other activities discussed above.

Considerable research has shown that the development of atherosclerosis proceeds by the accumulation of low-density lipoprotein (LDL) particles in macrophages, after oxidation of PUFA lipids within these particles [*(1)*, Chapter 13, E]. On the basis of this lipid oxidation mechanism, the prevention of LDL oxidation by antioxidants has been considered as an important means of decreasing the risk of heart disease. However, atherosclerotic lesions develop slowly by a complex series of cellular reactions involving lipid oxidation resulting from oxidative stress at several stages of lesion formation. Dietary antioxidants may not be effective in the presence of high levels of oxidation products at the more advanced stages of this disease.

Many studies have tested phenolic compounds for their ex vivo activity to inhibit human LDL oxidation catalyzed with Cu because the same properties were exhibited by the oxidative modification of LDL in vivo induced with endothelial cells. The potent antioxidant activity of phenolic compounds found in red wine toward oxidation of human LDL was previously advanced to explain the "French Paradox", the apparent relationship of a high-fat diet with a low incidence of coronary atherosclerosis (*20*). This research on wine antioxidants supporting the hypothesis that human health is influenced by the efficiency of many protection systems against oxidative damage led to a worldwide increase in the literature on biological antioxidants.

Studies of LDL oxidation have been extremely difficult to evaluate because of the great structural complexity of the LDL particles varying greatly in heterogeneity. A wide variety of methods have been used to prepare human LDL particles and conditions to induce LDL oxidation that greatly influenced the degree of oxidation. A multitude of markers for oxidative damage produced by LDL oxidation include linoleate and cholesteryl linoleate hydroperoxides, aldehydes, core aldehydes (also referred to as aldehydo-glycerides), aldehyde−protein adducts, reactive carbonyls, oxysterols, hydroxyl-enals, isoprostanes (cyclic peroxides derived from arachidonic acid), fluorescence, and oxidized proteins or protein carbonyls. More advanced methods have been used recently to elucidate the mechanism of LDL oxidation. Isoprostanes used as an index of oxidative status in biological samples required GC-MS or LC-MS analyses after purification to remove impurities or more selective MS/MS analyses.

The hydroperoxides of linoleate and arachidonate are the principal precursors of aldehydes causing damage to LDL by formation of Schiff bases with the lysine amino group of apoprotein B moiety of LDL [(*1*), Chapter 13, E, 2b, pp 410−414]. Static headspace GC proved to be useful to determine specific volatile oxidation products from *n*-6 PUFAs (pentane and hexanal) and *n*-3 PUFAs (propanal) in LDL samples from hypertriglyceridemic human subjects fed fish oil supplements [(*1*), Chapter 13, F, 2b, pp 428−433 and Figure 13.15, p 431].

Comparative measurements of isoprostanes and oxidized LDL in human plasma showed no change whether the subjects were on a flavonoid-rich diet or flavonoid-poor diet (*21–23*). Similarly, supplementation with rutin (quercetin-3-rutinoside) had no effect on the urinary concentration of 8-hydroxy-2′-deoxyyguanosine, a biomarker of oxidative DNA damage (*24*). Inconsistent results were also reported on the effects of fruit juice consumption on plasma isoprostane and oxidative DNA damage markers (*25–28*), while diets rich in soy were shown to decrease isoprostane concentrations (*29*). In a review of methods to evaluate health promotion by flavonoids, tocopherols, and other phenols, Halliwell et al. (*30*) concluded that isoprostanes and 8-hydroxy-2′-deoxyy-guanosine are not ideal biomarkers and that "the data are confusing and self-contradictory."

In contrast to a large number of studies showing that natural phenolic antioxidants can reduce the oxidative modification of LDL in vitro or ex vivo, only a few studies with animal models have shown that they could inhibit atherosclerosis because they were induced under oxidative stress. Sies (*14*) concluded that in vitro data do not apply to a compound or extract if it is not taken up in the gastrointestinal tract or if it is metabolized to products that are not redox-active. Although studies with animal models of atherosclerosis have demonstrated the antioxidant effect of vitamin E and phenolic compounds in delaying the progress of this disease, human clinical trials of antioxidants have given inconsistent and mixed results. The accumulation of circulating oxidized LDL in the artery and the development of lesions within a few months have been demonstrated in various animal models on special diets. However, in humans, lesions develop much more slowly over periods of decades. For example, a study using as models hamsters fed for 16 weeks a special hypercholesterolemic diet showed that catechin and vitamin E significantly lowered the accumulation of aortic fatty streaks (*31*). Of course, such a study indicating that catechin and vitamin E could prevent fatty streak in aorta could not be performed with humans. Therefore, the results of animal testing of antioxidants may not agree with those of human studies because they are based on significantly different end points.

Relatively little is known about the in vivo antioxidant activity of flavonoids in humans because of the great difficulty of demonstrating this activity with phenolic compounds from plants, which contain other potentially beneficial constituents. Plant foods contribute a multitude of other compounds that may impart beneficial health effects, including fibers, B vitamins, folic acid, and modulating agents for nitric acid production. In vitro studies generally employed concentrations of phenolic compounds that far exceed the in vivo concentrations that might be reached in the body. For example, dietary quercetin is not expected to affect platelet aggregation in vivo because effective concentrations would never be reached in plasma at the concentration of quercetin-containing foods, which seldom exceed 1 μM and are much lower than those required in in vitro studies: 2−20 times to inhibit LDL oxidation and 250−2500 times to inhibit platelet aggregation [*(2)*, Table 6.1, p 151]. Furthermore, quercetin is metabolized in plasma as methoxy, glucuronides, and sulfate derivatives that have significantly lower antioxidant activity than the parent quercetin [*(2)*, Figure 6.3, p 152, and Table 6.2, p 153; (*31*)]. At the same concentration of 0.5 μM, the antioxidant activity in inhibiting LDL oxidation catalyzed by Cu was significantly diminished by the methyl, sulfate, and glucuronide derivatives of quercetin as compared to the quercetin aglycone (*31*). After 1 h of consumption of 120 mL of red wine containing 35 mg of catechin, this major flavonoid was converted in human blood plasma almost entirely into its metabolite 3′-*O*-methyl catechin, in levels of only 81−91 nmol/L, present as both sulfate conjugate and a conjugate containing both glucuronide and sulfate residues (*32*). The levels of metabolites were independent of ethanol. The authors concluded that "if flavonoids are protective nutrients, the active forms are likely to be metabolites, which are far more abundant in plasma than the forms that exist in foods." More human studies are needed to better evaluate the risks resulting from too high a polyphenol consumption.

Studies on animals or cultured human cells indicate that polyphenols may be beneficial in the prevention of cardiovascular diseases, cancers, and degenerative diseases (*33*). However, these studies have often been conducted at concentrations far beyond those detected in humans. Inhibition of urokinase (a transphosphorylase enzyme shown to cause remission of cancer in mice) in vitro by epigallocatechin gallate (EGCG) has been attributed to the well-known anticancer activity of green tea (*34*). However, this inhibition required concentrations greater than 1 mmol/L, which is too high to be achieved in plasma because 9 cups of green tea produced only about 1 μmol/L of EGCG.

On one hand, in vitro studies showed that vitamin C and flavonoids were effective in inhibiting LDL oxidation induced by cells, macrophages, copper, and AAPH, a commonly used artificial azo free radical initiator (*35*). On the other hand, vitamin E was more effective with cells and macrophages than with copper, but it was a prooxidant with AAPH. Corresponding ex vivo studies showed that the hydrophilic vitamin C and flavonoids have no effect, because they were removed during preparation of LDL. In contrast, vitamin E supplementation improved LDL resistance to oxidation with cells and copper but was prooxidant with AAPH. These results emphasize the problems of using the questionable and misleading AAPH to induce LDL oxidation in vitro or ex vivo.

Although plant phenolic antioxidants in fruits and beverages may have a protective effect against coronary heart disease, the molecular mechanism of protection is not well-understood. The true impact of oxidation processes in biological systems is controversial due to a lack of reliable biomarkers of oxidative damage reflecting a biological end point and the complex properties of natural phenolic compounds in different food and biological systems.

Absorption, Metabolism, and Bioavailabilty of Antioxidants. Much research has been published on the absorption, metabolism, and bioavailability of flavonoids. In a review of flavonols, flavones, and flavanols, Hollman and Arts (*36*) reported that the flavonol levels found in vegetable and fruits are less than 10 mg/kg flavonol, including quercetin as the dominant component. According to these authors, the dietary intake of flavonols varies 10-fold between countries (6−60 mg/day), and the absorption of dietary quercetin glycosides in humans ranges between 20 and 50%.

In a later review, Hollman (*37*) reported that glucosides can only be absorbed from the small intestines, which is more efficient than from the colon and will lead to higher plasma concentrations. After absorption from the small intestine, flavonoids are efficiently conjugated with glucuronic acid or sulfate or *O*-methylated, and no free flavonoid aglycones can be found in plasma or urine, except for catechins. Janisch et al. (*38*) tested the most abundant plasma quercetin conjugates for their ability to inhibit Cu-induced oxidation of human LDL and to bind to human albumin, in comparison to free flavonoids and other quercetin conjugates. The lag time of LDL oxidation was increased by up to four times by low concentrations (<2 mM) of quercetin-3-glucuronide but was not affected by equivalent concentrations of quercetin-3′-sulfate.

Several metabolites of quercetin found in human plasma were chemically synthesized and tested for their ability to inhibit Cu-induced oxidation of human LDL (*38*). As compared to quercetin, its glucuronide conjugates prolonged the lag time of Cu-induced LDL oxidation in decreasing order: quercetin-7-glucuronide, quercetin, quercetin-3-glucuronide, and quercetin-4′-glucuronide. However, a previous report (*39*) showed that the lag phase of LDL oxidation in the presence of quercetin glucuronides was about half that of quercetin. Although the metabolites would be expected to have different biological properties than the corresponding aglycones from which they are derived, the biological activities of metabolite mixtures formed in different tissues are not well-established.

Many studies are currently aimed at clarifying the bioavailability of plant flavonoids from different foods [(*2*), Chapter 4]. Manach et al. (*40*) reviewed the data from 97 bioavailability studies and concluded that the most well-absorbed polyphenols are gallic acid and isoflavones, followed by catechins, flavanones, and quercetin glucosides, but with different kinetics. The least well-absorbed are the proanthocyanins, the galloylated tea catechin, and the anthocyanins. Williamson and Manach (*41*) reviewed the data from 98 intervention studies on polyphenols in humans in vivo based on short-term changes in biomarkers. Quercetin affected some cancer markers and antioxidant biomarkers claimed to be in vivo. Among 14 intervention studies on flavonols or flavonol-containing foods reported, only three measured antioxidant activities; among 20 intervention studies on catechin-containing foods, studies reported only six measured antioxidant activities; and among 41 intervention studies of procyanidins or procyanidin-containing foods, 20 measured antioxidant activities. However, many of the markers for in vivo activity have not been validated for cardiovascular disease and carcinogenesis. The authors concluded that as compared with the effects of polyphenols in vitro, any significant effects in vivo are more limited due to lack of validatation and understanding of biomarkers and lack of long-term studies.

Although human consumption of flavanol-rich cocoa products inhibited LDL oxidation [(*2*), Table 6.1, p 151], dimers of proanthocyanidins in chocolate are slowly absorbed and poorly bioavailable. The geometric configuration of catechin also influences its absorption in rats (*42*). In contrast to fruits and wine containing the (+)-enantiomer of catechin, chocolate mainly contains (−)-catechin and (−)-epicatechin, which are poorly bioavailable when consumed in chocolate or other cocoa-containing products.

The most important factors determining the absorption of polyphenols and its metabolites in the gut are defined as "the amount of compound reaching the enterocyte (intestinal cells) in a form suitable for absorption" (*43*). These factors include the nature of the sugar moiety and solubility as modified by ethanol, fat, and emulsifiers. The absorption of green tea catechins and other flavanols is affected by epimerization reactions that occur during food processing and the presence of lipid and carbohydrate. The absorption of polyphenols also depends on their release from the food matrix and increases with dose either linearly or by reaching saturation. For example, vitamin E from milk is more efficiently absorbed into human plasma than vitamin E from other foods or from vitamin E capsules dispersed in regular milk (*44*). There is therefore a lack of systematic information on the effects of other food components on the bioavailability of polyphenols and natural antioxidants.

The bioavailability of polyphenols is highly controlled by the capacity of the intestine and the liver to secrete conjugated metabolites. For example, major circulating quercetin metabolites in plasma have opposing effects on angiogenesis (physiological growth of new blood vessels from pre-existing vessels) (*45*). While quercetin and quercetin-3-glucuronide inhibited the vascular endothelial growth factor-induced endothelial cell functions and angiogenesis, quercetin-3′-sulfate promoted these endothelial functions. The ratio of these metabolites could be shifted by their metabolism in vivo. Rat intestinal perfusion model studies (*46*) showed that genistein and hesperetin were less available than ferulic acid for peripheral tissues because of a high intestinal and biliary secretion of their conjugates. Genistein and hesperetin appeared less available than ferulic acid for peripheral tissues because of a high intestinal and biliary secretion of their conjugates. Therefore, a high polyphenol intake appeared to improve their bioavailability by the saturation of the intestinal secretion of conjugates.

Most human intervention studies have used cultured cells to test plant polyphenols as aglycones and at relatively high micromolar concentrations rather than their in vivo complex metabolic conjugates that have different biological properties found in plasma at much lower nanomolar concentrations (*32, 42, 47*). Many intervention studies were based on questionable measures of lipid oxidation, including the notoriously unspecific TBA test for urinary MDA (*48*) and most recently to test the effect of red wine polyphenols in human plasma (*49*). The literature in this field continues to be controversial and confusing on the nature and properties of the multitude of flavonoid metabolite mixtures because of their complexity found in vivo and their different distribution patterns in human tissues and cells.

Health Benefits of Natural Antioxidants. Although the well-recognized health benefits of fruits and vegetables were attributed to their flavonoid content (*36–41*), Manach et al. (*40*) reported total metabolites ranging from 0 to 4 mol/L with an intake of 50 mg aglycone equivalents and relative urinary excretion ranging from 0.3 to 43% of the ingested dose. Many claims are confusing and unjustified when a multitude of health benefits of plant flavonoids are based on in vitro testing of aglycones rather than their corresponding metabolites and at significantly higher concentrations than found in human plasma. The increase in plasma antioxidant capacity, based on ferric reducing antioxidant potential (FRAP), in humans after apple consumption was shown to be due mainly to urate, a well-known metabolic effect of fructose, and not due to apple-derived flavonoids (*50, 51*). Although uric acid is derived from a complex degradation pathway of purine nucleotide, it cannot be referred to as a "physiological antioxidant", (*50*) on the basis of unspecific measurements of FRAP. This ferric reducing potential method reflects only the total phenolic concentration and not the protective properties of the antioxidant, in the absence of an oxidizable substrate.

Many studies have reported the neuroprotective, cardioprotective, and chemopreventive actions of dietary flavonoids (*52*). It is now becoming apparent that flavonoids, and their in vivo metabolites, do not only act as conventional hydrogen-donating antioxidants but may also exert "nonantioxidant" activities in cells through actions at protein kinases and lipid signaling pathways. Inhibitory or stimulatory actions at these pathways may affect cellular function by altering the phosphorylation of proteins and modulating gene expression. The influence of flavonoid metabolites on these properties may be key to their activities as anticancer agents, cardioprotectants, and inhibitors of neurodegeneration.

For a long time, vitamin E has been recognized to have an important physiological protective role as a lipid-soluble antioxidant in human nutrition. More recently, the biological properties of vitamin E (*53*) have assumed a wider context including possible pro-oxidant activities and nonantioxidant functions such as signaling molecule, regulating gene expression, and preventing cancer and atheriosclerosis. There is however a scarcity of evidence on the potential health benefits of different tocopherol homologues (γ and δ) and of vitamin E supplementation.

FUTURE RESEARCH

Complex mixtures of different polyphenols from plant foods and beverages are absorbed in the intestine, but very little is known about their interactions and possible consequences on their bioavailability. To better clarify the complex interactions between dietary polyphenols and health effects, future research is needed to address the following questions in food nutrition and biology:

(a) How do we develop and improve protocol methods to measure health benefits with better and noninvasive biomarkers of cardiovascular and degenerative disease?
(b) How do we determine and evaluate mixtures of bioactive phenolics to improve our understanding of their nutritional effects in complex foods?
(c) How does the intake of polyphenols influence their bioavailability and their saturation of the intestinal secretion of corresponding conjugated metabolites?
(d) How do we better define specific health effects of foods, fruits and vegetables, and supplements of concentrated extracts of polyphenol mixtures?
(e) What are the interactions and synergies between polyphenolic antioxidants and other nutrients in food plants?
(f) Can food plant processing be improved to increase the bioavailability of dietary components from plant matrix? The relevance and utility of in vitro antioxidant assays could be improved by providing useful health-related information by screening and testing the effects of food processing. However, these assays would be misleading

and have little utility to compare antioxidant results of food sources for health benefits.

Future research will need to give more emphasis on the many known metabolites of flavonoids that will require sensitive GC/MS analyses at the same ranges of concentrations found in blood. Clinical studies will also require better biomarkers used under experimental conditions relevant to human nutrition and health. Nutritional claims regarding antioxidant supplements cannot be justified until their benefits to humans are better understood. The biological effects of the flavonoid metabolites produced after absorption and their distribution in tissues in the body are not well understood, mainly because of lack of reliable in vivo testing protocols. If these metabolites have diminished antioxidant activity, appropriate tests are needed to determine how their nonantioxidant in vivo activities can be beneficial to health. The interaction of many components of the diet and phenolic antioxidants may attenuate or modify their activity. Therefore, nutrient interactions with phenolic antioxidants need to be explored to more fully understand their mechanism in nutrition.

It is premature to propose and recommend any methodology to assess the in vivo activity of natural polyphenolic compounds in our diet until more research is carried out to obtain a better understanding of (a) the complex questions of the bioavailability of dietary polyphenols; (b) the effects and interactions of other food components on tissue distribution, cellular uptake, and metabolism; and (c) until more and better designed human studies are carried out to clarify the factors affecting their absorption in vivo.

In view of the wide divergence of test results on natural antioxidants in foods and biological systems, more valid guidelines and protocols are urgently needed to bring some order in this important field of health and nutrition. With our limited state of knowledge of in vivo antioxidant activities in humans, any present claims made in the literature are premature and unjustified that we could assess nutritional and possible health benefits from complex mixtures of dietary flavonoids in plant foods and beverages. Until more well-designed human studies can provide clear evidence of health protective effects, future papers in the fields of food antioxidants and nutrition will have to be carefully screened to avoid premature claims of in vivo benefits of polyphenolic compounds found in plants and beverages.

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