Chemical Methods To Evaluate Antioxidant Ability

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1. Introduction

Antioxidants are able to hinder oxidation when only a small amount of them is used. Antioxidants, also called bioantioxidants, attract much attention since aging and degenerative diseases are related to the oxidation of biological components induced by reactive oxygen species (ROS).[1] Recently, aging, cancer, atherosclerosis, and some other serious diseases have been confirmed to correlate with low-density lipoprotein (LDL), cell membranes, and DNA exposed to oxidative stress.[2,3] Supplementations of antioxidants to maintain health and to cure diseases are an important strategy in therapy, called antioxidant therapy.[4] Even dietary foods are able to prevent the occurrence of cancer owing to abundant antioxidants contained in a plant diet.[5] Antioxidants

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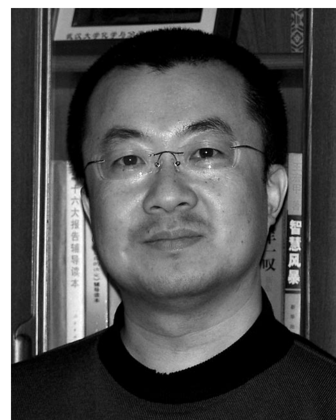

Zai-Qun Liu (born April 24, 1968, Changchun, China) studied at Jilin University (Changchun, China) from 1986 to 1996 and received his B.Sc. in 1990, M.Sc. in 1993 (major in organic synthesis under microwave irradiation), and Ph.D. in 1996 (major in the template effect of amine on the synthesis of aluminophosphate molecular sieves). He was a Postdoctorate Fellow at Lanzhou University (Lanzhou, China) from 1996 to 1998 and began with research work on antioxidants. He became Associate Professor in 1999 and Professor in 2001 in the Department of Organic Chemistry, College of Chemistry, Jilin University. He has published about 40 papers on antioxidants and 1 textbook on organic chemistry.

are composed of a large amount of enzymatic and nonenzymatic compounds. For example, superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione reductase, and glutathione transferase are enzymatic antioxidants in human plasma and erythrocytes.[6] Nowadays, peroxiredoxin (Prdx1) is found to play an antioxidative role in erythrocytes to defend against tumors.[7] Nonenzymatic antioxidants involve many small molecular organic compounds such as α-tocopherol (TOH),[8] ascorbic acid,[9] ubiquinol,[10] β-carotene,[11] etc. Organic chemists are mainly concerned with small molecular antioxidants because they can explore the mechanism of these antioxidants to protect biological systems,[12] extract natural compounds from plants to inspect the antioxidant activities,[13] and synthesize antioxidants with novel structures.[14]

ROS involve a series of oxidants such as hydrogen peroxide (H_2O_2), lipid peroxides (LOOH), singlet oxygen ($^1O_2$), hydroxyl radical ($^{\bullet}OH$), peroxyl radical ($ROO^{\bullet}$), peroxynitrite (^-OONO), etc.,[15] among which the radicals attract much attention because they can drive carcinogenesis by damaging DNA and proteins[16] and cause cardiovascular diseases by oxidizing LDL.[17] Thus, some works are devoted to investigating the radical-scavenging properties of the presented drugs to enlarge the application of these drugs as antioxidants.[18−20] Other works focus on the extraction of natural compounds from plants to identify the valid ingredients for scavenging radicals.[21−26] These works provide a

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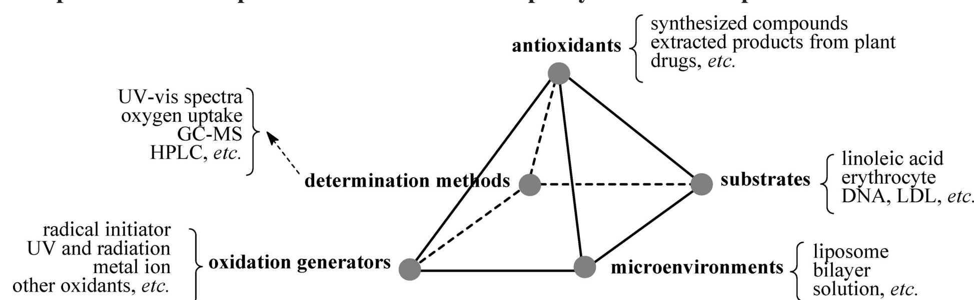
Scheme 1. Relationship between the Exploration of Antioxidant Capacity and Other Aspects



large amount of antioxidants for further pharmacological research and clarify the antioxidant mechanism for designing novel drugs. In particular, it is necessary for an organic laboratory to set up various convenient methods to test the antioxidant effectiveness of the prepared and extracted compounds since the quantitative structure−activity relationship (QSAR) of the antioxidant gives abundant potential for the synthesized or extracted compounds to be antioxidants. The antioxidant activity expressed by chemical indexes leads to a deep understanding of the nutritive effect of diets.[27] In addition, it is of importance for pharmacologists to inspect the antioxidant activities of the known drugs to enlarge the applications. For example, diclofenac, a nonsteroidal anti-inflammatory drug,[28] is able to inhibit oxidation induced by hydroxyl radical.[29]

Many methods have been set up to evaluate the antioxidant effectiveness based on biological experimental materials. For example, the phagocytosis of erythrocytes is increased by diamide-induced oxidation, leading to cross-linking band 3.[30] This experimental system can be used to test whether an antioxidant can hinder this cross-linkage. Lipid peroxidation in LDL induced by 15-lipoxygenase plays an important role in atherogenesis. Thus, 15-lipoxygenase-mediated peroxidation of LDL is an in vitro experimental system to test antioxidant activity.[31] In addition, metal ion-mediated oxidation of lipids[32] and utilization of biological samples as experimental materials[33] are appropriate for mimicking in vivo oxidation. However, it is not very easy for a chemical laboratory to set up these experimental systems with biological characteristics.

A large body of experimental data have been accumulated to describe the kinetic process of radical-induced oxidation, to calculate the bond dissociation energy (BDE) of O−H and N−H in antioxidants, and to measure the redox potential of radicals.[34] These results help us to understand the mechanism for an antioxidant to scavenge radicals from a chemical kinetic point of view. Since there is no uniform way to give a definite expression of antioxidant activity, many methods are exploited to test the activity of an antioxidant to avoid shortcomings resulting from a one-dimensional evaluation method.[35] The chemical ways to evaluate antioxidant activity are of importance for chemists to get detailed information on the synthesized or extracted antioxidants. Hence, some convenient experimental systems to evaluate antioxidant capacity should be introduced to organic laboratories. Recently, a review has summarized the multifaceted aspects of antioxidants, the kinetic models for autoxidation inhibited by antioxidants, and the chemical principles for determining antioxidant capacities.[36] The treatment of the results from biological samples leads to insights into the antioxidative process within biological samples.[37]

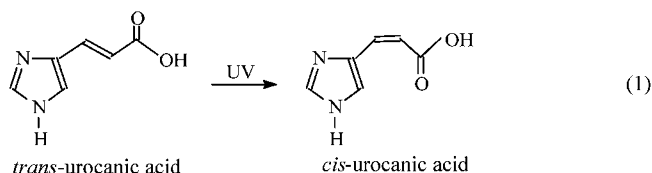
The aim of this review is not only to sum up some chemical methods for testing antioxidant capacity, but also to introduce some biological materials as experimental materials in evaluating antioxidant capacity chemically. Scheme 1 illustrates the mode for exploring antioxidant capacity. The basis of this pyramid contains four factors. Substrates are compounds that are susceptible to oxidation, and the microenvironment imitates the biological surroundings. For example, linoleic acid (LH) as the substrate is dissolved in the micelles of cetyltrimethylammonium bromide (CTAB), sodium dodecyl sulfate (SDS), or Triton X-100 to mimic polyunsaturated fatty acid (PUFA) located in the cationic, anionic, and neutral microenvironment.[38] In addition to LH as the substrate, some biological materials are also applied as substrates. The cellular membrane and LDL contain many PUFAs and proteins that are subject to oxidative stress.[39,40] DNA is also sensitive to attack from oxidants, leading to the formation of carbonyl species eventually.[41] The applications of erythrocytes, DNA, and LDL combine the substrate with the microenvironment. Meanwhile, treatment of the results from these biological samples with chemical kinetics reveals molecular information on the biological samples. To imitate oxidative stress, radical initiators, UV radiation, metal ions, and other oxidants are applied to initiate the oxidation of chemical agents or biological samples. The oxidative process is followed by various valid methods. As a result, an in vitro experimental system is set up to evaluate the ability of an antioxidant to inhibit oxidation.

2. Resources of Oxidants and Radicals

The simplest way to test the ability of an antioxidant is to directly expose the antioxidant to ROS resources such as UV light, metal ions, $^1O_2$, ^-OONO, etc. The ability of an antioxidant to scavenge radicals is another topic in the characterization of the antioxidant capacity. The interactions between antioxidants and radicals give direct evidence of the ability of antioxidants to trap radicals. Thus, if some stable radicals or some methods to generate radicals readily are available in an organic laboratory, the radical-scavenging property of the synthesized or extracted compounds can be explored promptly. $^•OH$ can be generated in physical and chemical ways. Azide radical ($N_3^•$) and trichloromethyl peroxyl radical ($Cl_3COO^•$) can be regarded as derivatives of $^•OH$. 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonate) cationic radical ($ABTS^{•+}$), 2,2′-diphenyl-1-picrylhydrazyl (DPPH), and galvinoxyl radical are stable radicals at room temperature.

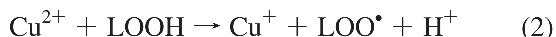
2.1. UV Light

trans-Urocanic acid in the human epidermis generates *cis*-urocanic acid under UV light at 200−290 nm as shown in eq 1, which is a model reaction for immunosuppression.[42] The isomers of urocanic acid as well as the byproducts are detected readily by high-performance liquid chromatography (HPLC). Thus, the conversion from *trans*- to *cis*-urocanic acid is an experimental system to test the ability of an antioxidant to hinder UV-induced isomerization. Visible light from a high-pressure mercury lamp (250 W) is able to oxidize human LDL in the presence of diphenyl ketone or disodium 3,3′-disulfodiphenyl ketone as a photosensitizer.[43] Thus, this experimental system connects LDL with photooxidation.

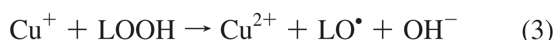


2.2. Metal Ions

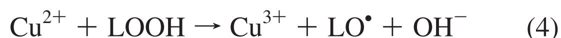
Copper ion (Cu^{2+}) is often used to initiate the oxidation of LDL because, as shown in the following equations, Cu^{2+} and Cu^{+} are able to react with the residue hydroperoxide of LH (LOOH) in LDL, resulting in further oxidation of LH to form oxidized LDL containing the peroxyl radical (LOO•) or alkoxyl radical (LO•) of LH:



$$Cu^{2+} + LOOH \rightarrow Cu^{+} + LOO^{\bullet} + H^{+} \quad (2)$$

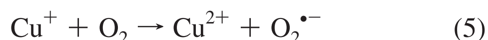


$$Cu^{+} + LOOH \rightarrow Cu^{2+} + LO^{\bullet} + OH^{-} \quad (3)$$

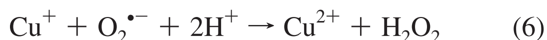


$$Cu^{2+} + LOOH \rightarrow Cu^{3+} + LO^{\bullet} + OH^{-} \quad (4)$$

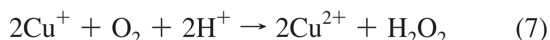
Copper species can also react with oxygen species to form hydroperoxide and hydroxyl radical (•OH) as shown in the following equations:



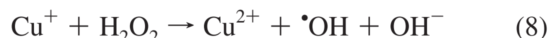
$$Cu^{+} + O_2 \rightarrow Cu^{2+} + O_2^{\bullet -} \quad (5)$$



$$Cu^{+} + O_2^{\bullet -} + 2H^{+} \rightarrow Cu^{2+} + H_2O_2 \quad (6)$$



$$2Cu^{+} + O_2 + 2H^{+} \rightarrow 2Cu^{2+} + H_2O_2 \quad (7)$$



$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + {}^{\bullet}OH + OH^{-} \quad (8)$$

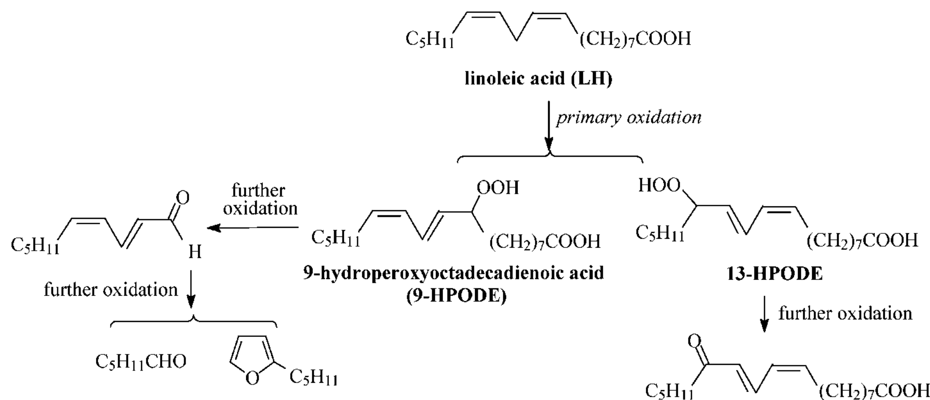
The physiological meaning of the aforementioned reactions on the oxidation of LDL has been reviewed.[44] After methyl esters of PUFAs are prepared in the presence of diazomethane at pH 2, 54 products from Cu^{2+}-mediated oxidation of human LDL are identified by gas chromatography combined with electron impact mass spectrometry (GC/MS).[45] Solid-phase microextraction (SPME) is used to extract oxidative products prior to the determination by GC/MS. Scheme 2 outlines the possible process of the oxidation of LH in LDL.[46] Cu^{2+} is able to break DNA strand scission in the presence of bleomycin[47] or resveratrol and its analogues.[48] When Cu^{2+} is reduced to form Cu^{+}, N−H in bleomycin and O−H in resveratrol convert to N- and O-centered radicals, respectively, that are able to damage the DNA strand. The aforementioned methods of metal ion-induced oxidation of biological samples can be used to inspect antioxidant activities since the addition of antioxidants lags the formation of oxidative products from LDL and DNA.

The most convenient method to test the antioxidant activity is to mix the antioxidant with complexes of copper or iron.[49,50] Both Cu(I) and Fe(II) can form a complex with the same ligand, but the λ_{max} of the complex formed by Cu(I) and Fe(II) differs from that with Cu(II) and Fe(III) as shown in Scheme 3. The absorbance of the complexes changes because the antioxidant has the ability to decrease the amount of Cu(II) or Fe(III).

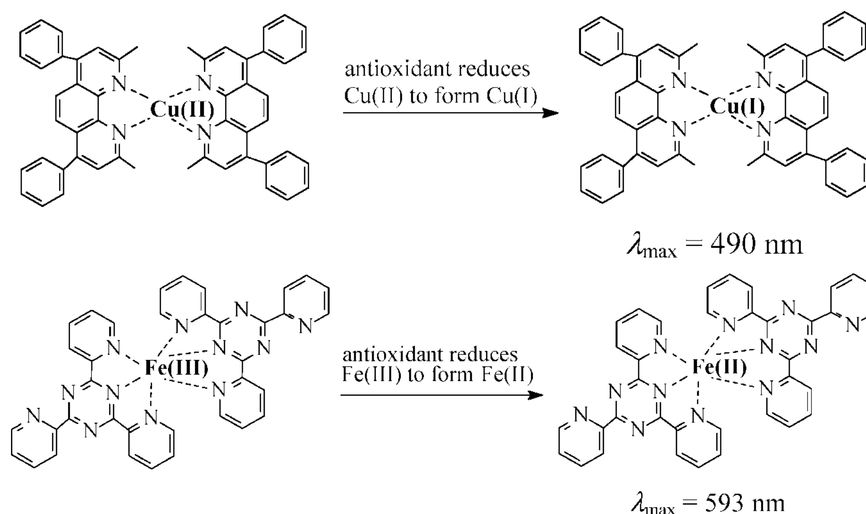
2.3. $^1O_2$

The spontaneous dismutation of superoxide anion forms singlet oxygen ($^1O_2$), a major oxidant of biological samples.[51] The guanine in DNA is the preferable site for $^1O_2$-induced oxidation, whereas oxidation cannot occur at the deoxyribose moiety.[52] $^1O_2$ induces lysis of erythrocytes, monocytes (THP1), and macrophages (J774) when it is derived from the decomposition of H_2O_2 in the presence of hypochlorite and hypobromite.[53] In the process of lysis of the above cells, N-centered radicals are observed by electron paramagnetic resonance (EPR) in the presence of 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) as a spin labeler to trap radicals. The formation of N-centered radicals may be attributed to the decomposition of chloramines or bromamines within the cell or in the cell membrane. $^1O_2$ can be conveniently obtained by light radiation when methylene blue acts as a photosensitizer.[54] Chemical methods to generate $^1O_2$ are mainly composed of decomposing H_2O_2 catalyzed by metal ions.[55] In addition to mineral compounds employed to generate $^1O_2$, the same concentrations of NaOCl and histidine stimulate the decomposition of H_2O_2 to form $^1O_2$ in sodium phosphate buffer (pH 7.1) at 30 °C.[23] The yield of $^1O_2$ generated from the above mixture is detected by the decrease of the

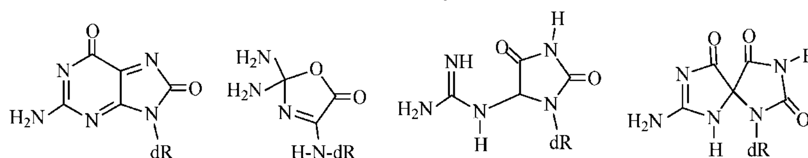
Scheme 2. Oxidative Products in Cu^{2+}-Mediated Oxidation of LH in LDL



Scheme 3. Antioxidants Reduce Cu(II) and Fe(III) in the Complexes



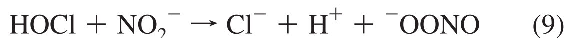
Scheme 4. Products of Guanine in DNA Derived from Oxidation by $^{-}$OONO



absorbance for *N*,*N*-dimethyl-*p*-nitrosoaniline at 440 nm because *N*,*N*-dimethyl-*p*-nitrosoaniline can be bleached in the oxidation of $^1O_2$. Some commonly used antioxidants such as Trolox (6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid), ascorbate, and amino acid are used to quench $^1O_2$.[56] Tetra-*tert*-butylphthalocyanine is employed as a chemiluminescent probe (λ_{max} = 703 nm as the emission wavelength) in the interaction between antioxidants and $^1O_2$.[57]

2.4. $^{-}$OONO

Hypochlorous acid (HOCl) reacts with nitrite to form peroxynitrite ($^{-}$OONO; λ_{max} = 302 nm, ε = 1670 M^{-1} cm^{-1}) as shown in the following equation:

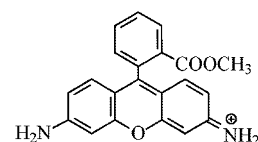


$$HOCl + NO_2^- \rightarrow Cl^- + H^+ + {}^-OONO \quad (9)$$

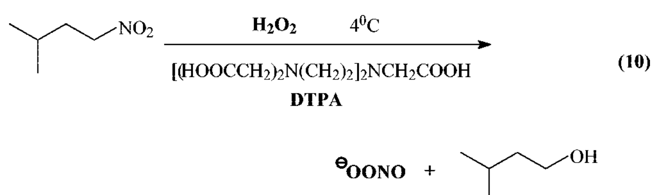
HOCl can be generated in neutrophils, implicating that the above reaction may take place in vivo. Human LDL is oxidized by a mixture of HOCl and NO_2^- (molar ratio 1:1) at a rate of (7.4 ± 1.3) × 10^3 M^{-1} s^{-1} at 25 °C.[58] It has been demonstrated that adenosine and guanosine in DNA are susceptible to $^{-}$OONO.[59] Especially, as shown in Scheme 4, four oxidation products including 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), 2,2-diamino-4-[(2-deoxy-*β*-D-*erythro*-pentafuranosyl)amino]-5(2*H*)-oxazolone (oxazolone), spiroiminodihydantoin, and N^1-(*β*-D-*erythro*-pentofuranosyl)-5-guanidinohydantoin (guanidinohydantoin) are isolated from the oxidation of guanine in DNA with different dosages of $^{-}$OONO employed.[60]

As shown in eq 10, $^{-}$OONO is readily obtained by oxidizing isoamyl nitrite with H_2O_2 under basic conditions with diethylenetriaminepentaacetic acid (DTPA) as the surfactant.[61] In general, NaOH is applied in the preparation of $^{-}$OONO, so Na^+ is the positive ion in the solution of $^{-}$OONO. After the organic byproduct isoamyl alcohol is extracted by CH_2Cl_2 or $CHCl_3$, and the residue H_2O_2 is decomposed by flowing through a column filled with MnO_2,

Scheme 5. Structure of Rhodamine 123



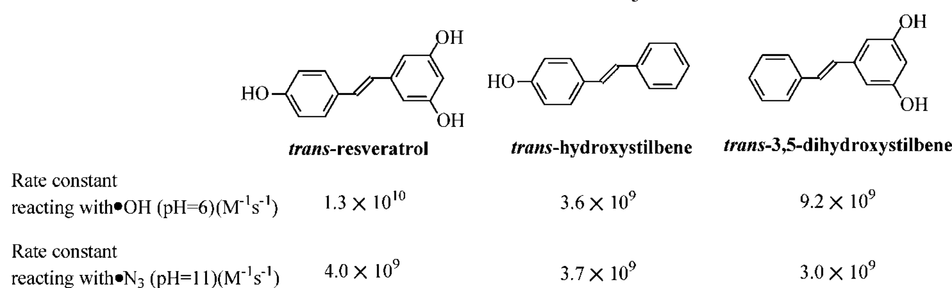
the $^{-}$OONO aqueous solution can be preserved for 2−4 weeks at −20 °C. Thus, $^{-}$OONO can be used as an oxidant to determine the antioxidant capacity. For example, $^{-}$OONO breaks the DNA strand observed by electrophoresis and oxidizes DNA to form 8-hydroxy-2′-deoxyguanosine (8-OHdG) and 2′-deoxyguanosine (2′dG) detected by HPLC.[62] However, the concentration of $^{-}$OONO formed in cells is too low to be detected directly. Dihydrorhodamine 123, a fluorescent probe, is oxidized by $^{-}$OONO to form rhodamine 123 (structure in Scheme 5) with the excitation wavelength at 500 nm and the emission wavelength at 536 nm. Rhodamine 123 generated from the oxidation of dihydrorhodamine 123 by $^{-}$OONO is linearly related to the concentration of $^{-}$OONO ranging from 0 to 1000 nM.[63]



2.5. *β*-Carotene-Bleaching Test in Linoleic Acid Emulsion

The oxidation of LH can be inhibited by *β*-carotene. An emulsion containing LH and *β*-carotene is prepared by dissolving *β*-carotene, LH, and Tween in $CHCl_3$ first. Then $CHCl_3$ is evaporated under a stream of N_2, water is added, and the mixture is shaken vigorously to form an emulsion (λ_{max} = 470 nm). The oxygen dissolved in water oxidizes

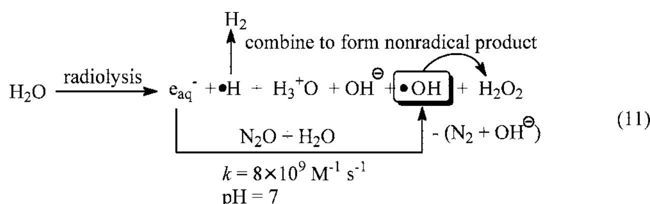
Scheme 6. Rate Constants of Stilbene Derivatives in Reaction with $^{\bullet}OH$ and $N_3^{\bullet}$



LH, and β-carotene prohibits the oxidation of LH. As a result, the absorbance at 470 nm decreases with increasing time. If the decrease of the absorbance is inhibited by an antioxidant, the antioxidant is regarded to protect LH against autoxidation.[64] This method can even be simplified to only methyl linoleate being employed, in which the conjugated diene formed from the autoxidation of methyl linoleate is followed by measuring the increase of the absorbance at 234 nm.[65] Moreover, the Folin−Ciocalteu method is utilized to evaluate the antioxidant effectiveness of total flavonoids colored by 5% $NaNO_2$ and 10% $AlCl_3$ in 1 M NaOH aqueous solution, whose maximum wavelength locates at 415 nm.[66] These methods are usually used to test the antioxidant capacity of a mixture extracted from plants. However, these methods just give a relative antioxidant capacity because 2,6-di-*tert*-butyl-4-methoxyphenol (BHT) is employed as a standard antioxidant capacity in this case.[67]

2.6. $^{\bullet}OH$

$^{\bullet}OH$ is derived from the radiolysis of water with a hydrated electron (e_{aq}^-), a H atom, and some ions generated as byproducts. e_{aq}^- converts into $^{\bullet}OH$ rapidly by reacting with N_2O. Thus, eq 11 indicates a way to generate $^{\bullet}OH$. The antioxidant actions of *trans*-stilbene derivatives are explored by interacting with $^{\bullet}OH$ from the radiolysis of water.[68] By means of a chemical experiment, $^{\bullet}OH$ is generated from the decomposition of H_2O_2 catalyzed by Fe^{2+}.[69] An antioxidant may chelate Fe^{2+} before Fe^{2+} catalyzes H_2O_2 to form $^{\bullet}OH$. Thus, it is difficult to identify whether an antioxidant really scavenges $^{\bullet}OH$ or chelates Fe^{2+} in the mixture of the Fenton reaction. At present, $^{\bullet}OH$ is generated in the mixture of Fe^{3+}−EDTA−H_2O_2−deoxyribose in the aqueous phase or tetrachlorohydroquinone−H_2O_2 in the organic phase.[70] The applications of these methods to generate $^{\bullet}OH$ avoid metal ions being chelated by antioxidants.[71]

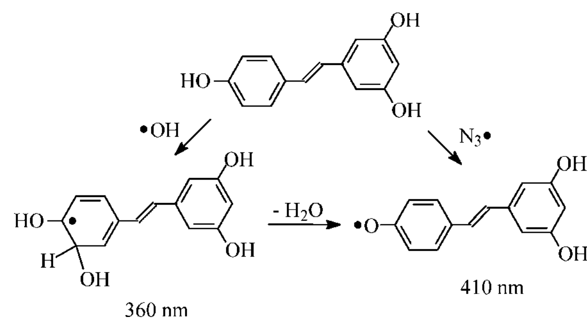


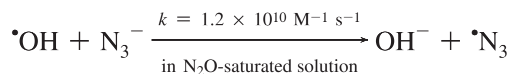
(11)

2.7. $N_3^{\bullet}$

As shown as eq 12, $N_3^{\bullet}$ is derived from the oxidation of N_3^- by $^{\bullet}OH$.[72] Both $^{\bullet}OH$ and $N_3^{\bullet}$ are powerful oxidants with similar rate constants in oxidizing stilbene derivatives as shown in Scheme 6.[68] As shown in Scheme 7, it is possible for $^{\bullet}OH$ to add to the benzene ring in resveratrol and to abstract the H atom from O−H, but $N_3^{\bullet}$ can only abstract the H atom from O−H.[68]

Scheme 7. Difference of $^{\bullet}OH$ and $N_3^{\bullet}$ in Interaction with Resveratrol



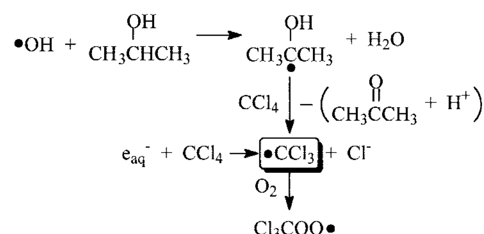


$$^{\bullet}OH + N_3^- \xrightarrow[\text{in } N_2O\text{-saturated solution}]{k\,=\,1.2\,\times\,10^{10}\ M^{-1}\ s^{-1}} OH^- + {}^{\bullet}N_3 \quad (12)$$

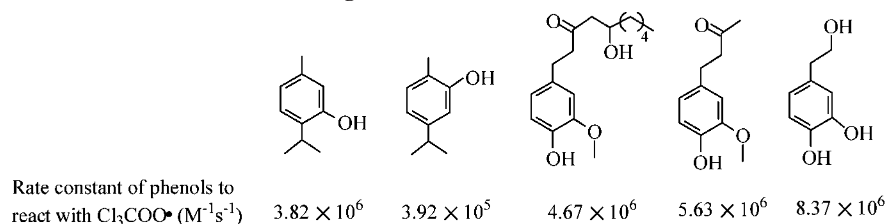
2.8. $Cl_3COO^{\bullet}$

Like $^{\bullet}OH$ and $N_3^{\bullet}$, trichloromethyl peroxyl radical ($Cl_3COO^{\bullet}$) is also employed to test the radical-scavenging property of antioxidants. $Cl_3COO^{\bullet}$ is generated in a mixture containing 1% CCl_4 (v/v) and 50% $(CH_3)_2CHOH$ (v/v) in 10 mM KH_2PO_4−KOH buffer at pH 7.4. It is also regarded as the derivative from $^{\bullet}OH$ as shown in Scheme 8.[73] When $Cl_3COO^{\bullet}$ is applied to react with some natural phenols, the rate constants are measured and are illustrated in Scheme 9.[73] $N_3^{\bullet}$ and $Cl_3COO^{\bullet}$ are formed by interacting corresponding agents with $^{\bullet}OH$ generated by radiolysis. These radicals are not stable enough to be preserved at room temperature. Recently, $Cl_3COO^{\bullet}$ was employed to interact with antioxidants such as retinoids[74] and lycopene[75] extracted from food. The operations for measuring the rate constants of antioxidants to trap $^{\bullet}OH$, $N_3^{\bullet}$, and $Cl_3COO^{\bullet}$ are not very convenient, so the three radicals discussed in the three following sections are often utilized to test the antioxidant capacity since they are stable radicals at room temperature.

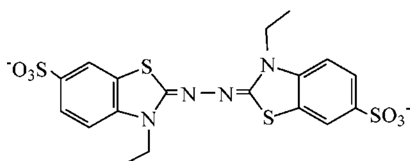
Scheme 8. Formation of $Cl_3COO^{\bullet}$



Scheme 9. Rate Constants of Natural Phenols Reacting with $Cl_3COO^•$



Scheme 10. Structure of $ABTS^{2-}$



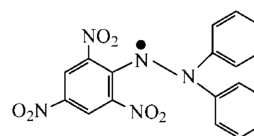
2.9. $ABTS^{•+}$

2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonate) ($ABTS^{2-}$; structure in Scheme 10) is a colorless dianion salt of sodium or ammonium and can form a colorful cationic radical ($ABTS^{•+}$, $\lambda_{max} = 734$ nm) under oxidation by $K_2S_2O_8$ at room temperature for 16 h. The mixture is then diluted by ethanol to give an absorbance at 0.70 ± 0.02, defined as the reference absorbance (A_{ref}).[76,77] A_{ref} decreases to a stable value (A_{detect}) when $ABTS^{•+}$ is mixed with an antioxidant. The percentage of an antioxidant needed to trap $ABTS^{•+}$ is calculated by $(1 - A_{detect}/A_{ref}) \times 100$. This is the simplest application of $ABTS^{•+}$ to inspect the ability of the total antioxidants in food[78] and in blood samples.[79] The interaction between an antioxidant and $ABTS^{•+}$ exhibits the overall reductive ability of the whole molecule of the antioxidant; therefore, it cannot give a quantitative result to express the ability of a single hydroxyl group in the antioxidant to reduce $ABTS^{•+}$. Trolox is always assigned to be a reference antioxidant to interact with $ABTS^{•+}$, and then other antioxidants react with $ABTS^{•+}$ under the same experimental conditions. The results from other antioxidants in reducing $ABTS^{•+}$ are compared with those of Trolox, expressed as the Trolox equivalent antioxidant capacity (TEAC).[80] In addition to the simplest usage of $ABTS^{•+}$, flow injection analysis (FIA) (see Scheme 11) is applied to identify the TEAC of individual or mixed samples. The *clean* $ABTS^{•+}$ is formed online by electrochemical oxidation of $ABTS^{2-}$ in the FIA apparatus. The flow rate, injection volume, and ratio between $ABTS^{•+}$ and the carrier solution need to be optimized in determining the abilities of antioxidants to reduce $ABTS^{•+}$.[81]

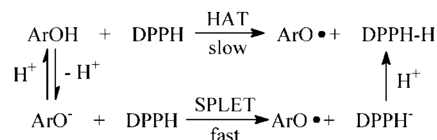
2.10. DPPH

DPPH ($\lambda_{max} = 517$ nm, structure in Scheme 12) is a stable N-centered radical at room temperature and is always employed to inspect the radical-scavenging properties of antioxidants.[82] DPPH is dissolved in ethanol to give an absorbance around 1.0 (A_{ref}) at 517 nm, and the addition of antioxidants decreases the absorbance of the DPPH solution to a stable value (A_{detect}). The ability of the antioxidant to trap DPPH (%) is also calculated by $(1 - A_{detect}/A_{ref}) \times 100$. The abilities of natural compounds such as derivatives of caffeic acid,[83] (−)-matairesinol,[84] anthocyanins,[85] etc. to scavenge radicals are identified by interacting with DPPH. The H atom in C−H in β-carotene derivatives can even be abstracted by DPPH.[86] The concentration of an antioxidant needed to trap 50% DPPH or $ABTS^{•+}$ is designated as IC_{50} to express the antioxidant capacity. A low value of IC_{50} for an antioxidant indicates that the antioxidant behaves as a strong radical scavenger. Therefore, DPPH is often adopted to inspect the radical-scavenging properties of extractions from plants and medicinal herbs. The synthesized antioxidants are also applied to react with DPPH to elucidate their radical-scavenging properties.[87]

Scheme 12. Structure of DPPH

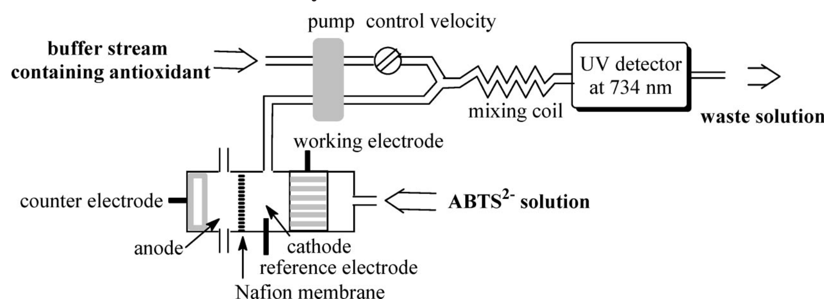


Scheme 13. Mechanisms of DPPH To Interact with Phenolic Compounds (ArOH): HAT and SPLET

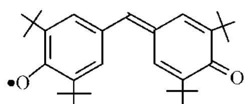


The reaction of a phenolic compound (ArOH) with DPPH is expressed by the mechanisms of hydrogen atom transfer (HAT) and sequential proton loss electron transfer (SPLET). The H atom in O−H may transfer from ArOH to DPPH directly in the HAT mechanism. Alternatively, the SPLET mechanism means that ArOH first deprotonates to form phenolic anion (ArO^-), and then DPPH accepts an electron from ArO^-. Scheme 13 illustrates the comparison between HAT and SPLET.[88] Curcumin is prone to form an anion by losing a H atom from phenolic OH, and then the curcumin

Scheme 11. Apparatus of FIA To Generate $ABTS^{•+}$ by Electrochemical Oxidation Online



Scheme 14. Structure of Galvinoxyl



anion transfers an electron to DPPH, following the SPLET mechanism. In addition, the solvent applied for ArOH to scavenge DPPH may act as a hydrogen bond donator or hydrogen bond acceptor, which affects the reaction between an antioxidant and DPPH during the HAT mechanism. The abilities of the solvent to donate or accept a hydrogen bond are quantitated by Abraham's parameters α_2^H and β_2^H, respectively. As shown in the following equation, the rate constant of the reaction between an antioxidant and DPPH (k^{HAT}) is equal to the rate constant for the reaction occurring in a neutral solvent (k^0), eliminating the influence from the ability of the solvent to donate or accept a hydrogen bond:[89,90]

$$\log k^{HAT} = \log k^0 - 8.3\alpha_2^H\beta_2^H \quad (13)$$

2.11. Galvinoxyl Radical

Galvinoxyl radical (structure in Scheme 14, $\lambda_{max} = 428$ nm) is a stable O-centered radical at room temperature. The application of galvinoxyl radical is the same as that of DPPH to screen the radical-scavenging abilities of antioxidants. One molecule of TOH can trap one molecule of galvinoxyl radical at a rate constant of 2.4×10^3 M^{-1} s^{-1} detected by the stopped-flow technique on a visible spectrometer.[91] Antioxidants attenuate the electron spin resonance (ESR) signal of galvinoxyl radical or DPPH; thus, the rate constants of the reaction between galvinoxyl radical or DPPH and the antioxidant can be measured accurately. For example, the rate constants of α-tocopheryl hydroquinone and ubiquinol-10 in trapping galvinoxyl radical are 1.0×10^4 and 6.0×10^3 M^{-1} s^{-1}, respectively.[92] Quantum calculation on galvinoxyl radical by density function theory (DFT) reveals that galvinxoyl radical forms pairs, leading to the anomalous magnetic property at low temperature.[93]

3. Characterization of the Antioxidant Capacity Based on Chemical Kinetics

3.1. Oxidation of Linoleic Acid Based on Oxygen Exhaustion

LH (structure in Scheme 15) is the main ingredient of PUFAs in LDL and membranes that is susceptible to oxidation by radicals deriving from the decomposition of 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH, R−N=N−R; structure in Scheme 15). Hence, AAPH-induced oxidation of LH usually acts as a chemical model to investigate radical-induced oxidation of PUFAs. The initiation of AAPH-induced oxidation of LH is outlined in the following equations:

$$\text{R−N=N−R} \xrightarrow{k_d} 2\text{R}^\bullet + \text{N}_2 \quad (14)$$

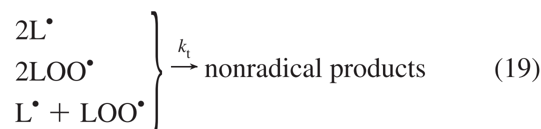
$$\text{R}^\bullet + \text{O}_2 \rightarrow \text{ROO}^\bullet \quad (15)$$

$$\text{ROO}^\bullet + \text{LH} \rightarrow \text{ROOH} + \text{L}^\bullet \quad (16)$$

The radical of LH (L$^\bullet$) combines with oxygen to form a peroxyl radical (LOO$^\bullet$), which abstracts the H atom from another LH. As shown in eqs 17 and 18, the process of radical propagation causes the oxidation of LH to form peroxide (LOOH) completely. Finally, the residues L$^\bullet$ and LOO$^\bullet$ combine with each other to terminate the radical reaction as shown in eq 19.

$$\text{L}^\bullet + \text{O}_2 \xrightarrow{k_O} \text{LOO}^\bullet \quad (17)$$

$$\text{LOO}^\bullet + \text{LH} \xrightarrow{k_p} \text{LOOH} + \text{L}^\bullet \quad (18)$$



$$\left.\begin{array}{l} 2\text{L}^\bullet \\ 2\text{LOO}^\bullet \\ \text{L}^\bullet + \text{LOO}^\bullet \end{array}\right\} \xrightarrow{k_t} \text{nonradical products} \quad (19)$$

Equations 14−19 are treated on the basis of the steady-state hypothesis, in which the concentrations of the radicals remain constant, namely, d[LOO$^\bullet$]/dt = d[L$^\bullet$]/dt = 0.[94] Consequently, as shown in the following equation, the rate of oxygen exhaustion during the oxidation of LH (R_p) is found to correlate with the rate of radical initiation (R_i) and the concentration of LH:[95]

$$-\text{d}[\text{O}_2]/\text{d}t = R_p = [k_p/(2k_t)^{0.5}]R_i^{0.5}[\text{LH}] \quad (20)$$

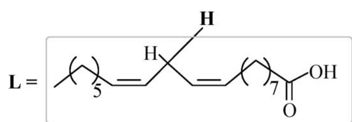
The coefficient in eq 20, $k_p/(2k_t)^{0.5}$, called the *oxidizability*, represents the susceptibility of LH to be oxidized. With an antioxidant (AH) added to the above radical propagation, AH may replace LH to be oxidized by LOO$^\bullet$ as shown in eq 21. If the antioxidant radical (A$^\bullet$) combines with LOO$^\bullet$ to form a nonradical product (LOOA) as shown in eq 22, the radical-induced oxidation of LH is inhibited until all the antioxidant is depleted. Therefore, the addition of AH generates an *inhibition period* (t_{inh}).

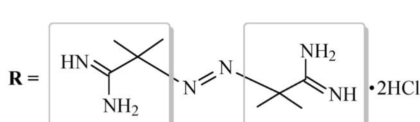
$$\text{LOO}^\bullet + \text{AH} \xrightarrow{k_{inh}} \text{LOOH} + \text{A}^\bullet \quad (21)$$

$$\text{LOO}^\bullet + \text{A}^\bullet \xrightarrow{k_{combine}} \text{LOOA} \quad (22)$$

The exhaustion rate of oxygen in the presence of AH is described as R_{inh} relating to the concentrations of AH and LOO$^\bullet$ as shown in the following equation:

Scheme 15. Structures of LH and AAPH





$$R_{inh} = k_{inh}n[AH][LOO^{\bullet}] \qquad (23)$$

The *stoichiometric factor*, n, can be regarded as the number of $LOO^{\bullet}$ molecules trapped by one molecule of AH as expressed in the following equation:

$$n = R_i t_{inh}/[AH] \qquad (24)$$

To treat eqs 21 and 22 on the basis of the steady-state hypothesis, R_{inh} is expressed by

$$-d[O_2]/dt = R_{inh} = k_p R_i[LH]/(nk_{inh}[AH]) \qquad (25)$$

After R_{inh}, R_p, and t_{inh} are determined by using the equipment as shown in Scheme 16,[96] the kinetic parameters including k_{inh}, k_p, and n are calculated by eqs 20, 24, and 25. Therefore, the ability of an antioxidant to protect LH against radical-induced oxidation is described by these chemical kinetic parameters.

The key factor for calculating k_{inh}, k_p, and n of an antioxidant is to obtain the rate of radicals to initiate the oxidation of LH (R_i). Since the absolute value of R_i is difficult to measure directly, a reference antioxidant is used to scale R_i when n for the reference antioxidant is known. As shown in the following equation, the decay rate of the reference antioxidant in protecting LH against radical-induced oxidation is designated as R_i divided by n of the reference antioxidant:

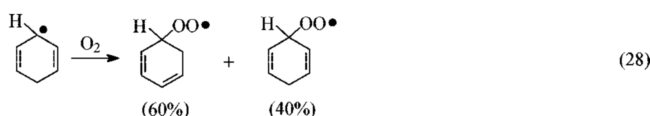
$$-d[AH]/dt = R_i/n \qquad (26)$$

For estimating R_i, TOH or Trolox is assigned to be the reference antioxidant whose n is taken as 2.0. R_i can be calculated after the decay rate of TOH or Trolox is measured.[97] Equation 26 is integrated to obtain eq 27; thus, the estimation of R_i is simplified just to measure t_{inh} generated from a certain concentration of TOH or Trolox. Then R_i is estimated by eq 27, equivalent in style to eq 24, when the n of TOH or Trolox is taken as 2.0.[98]

$$R_i = n[AH]_0/t_{inh} \qquad (27)$$

3.2. Analysis of Oxidative Products

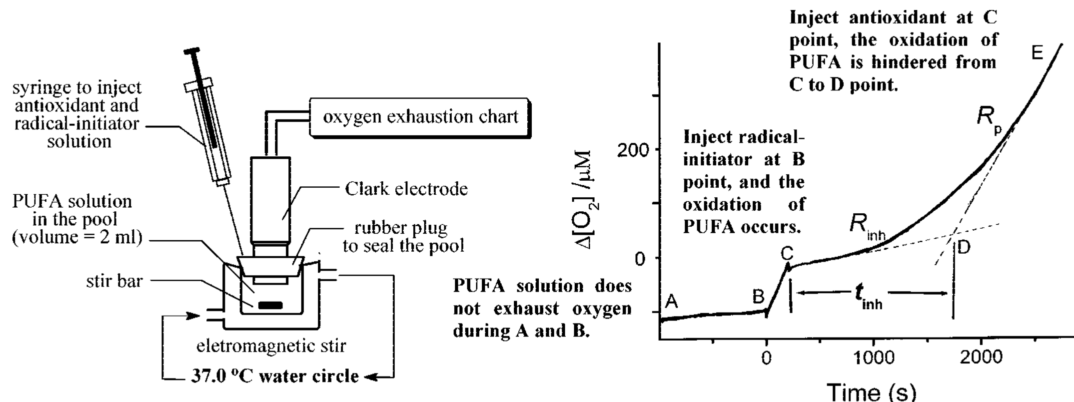
After the H atom at the bisallyl in LH is abstracted by radicals to form $LOO^{\bullet}$ in the presence of oxygen, the structure of $LOO^{\bullet}$ is identified by analyzing the oxidation products from the autoxidation of LH or its methyl ester. As shown in Scheme 17, four main products including *trans*,*cis*- and *trans*,*trans*-conjugated dienes are confirmed when the methyl ester of LH is oxidized in benzene/1,4-cyclohexadiene.[99] A calculation of the BDE of C−O in peroxyl radicals of 1,4-cyclohexadiene also suggests that, as shown in the following equation, oxygen is added preferentially to the allyl position at the end of the conjugated diene radical in cyclohexadiene:[100]



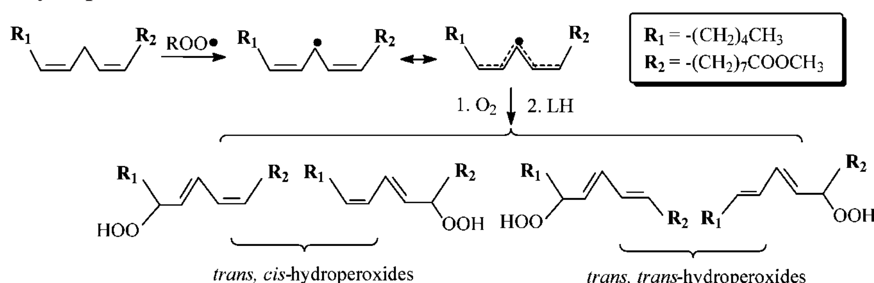
(28)

Furthermore, the BDE of $C-OO^{\bullet}$ and the rate of $LOO^{\bullet}$ to eliminate two oxygen atoms from $LOO^{\bullet}$ are measured as shown in Scheme 18.[101] The rate constant to eliminate oxygen from nonconjugated diene-type $LOO^{\bullet}$ (1.9×10^6 s^{-1}) approaches the diffusion-controlled rate because of a relatively low BDE of $C-OO^{\bullet}$ in $LOO^{\bullet}$ (3.8 kcal/mol). Contrarily, the rate constants to eliminate oxygen from conjugated diene-type $LOO^{\bullet}$ to form *cis*,*cis*- and *trans*,*cis*-radicals are much lower than that of the aforementioned reaction owing to a high BDE of the corresponding $C-OO^{\bullet}$ in $LOO^{\bullet}$ (9.9 kcal/mol). The addition of TOH to the oxidation of LH governs the amount and kind of products from the oxidation of LH because the rate constant of TOH to react with

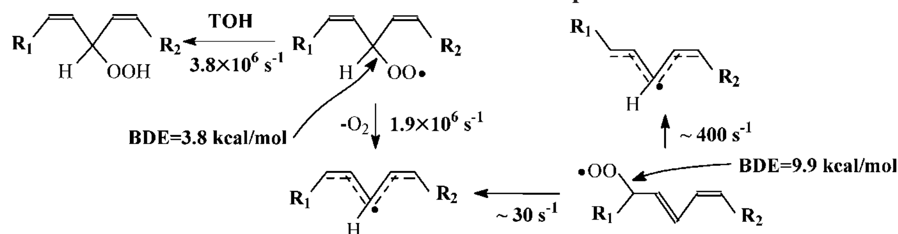
Scheme 16. Equipment To Measure the Oxygen Exhaustion and the Corresponding Chart



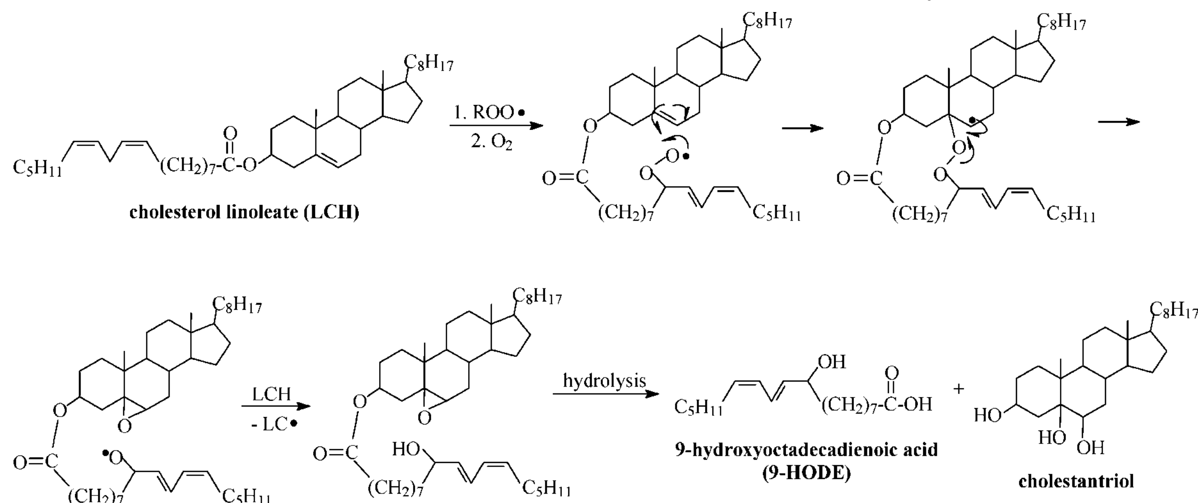
Scheme 17. Isomers of Hydroperoxides Derived from the Oxidation of LH



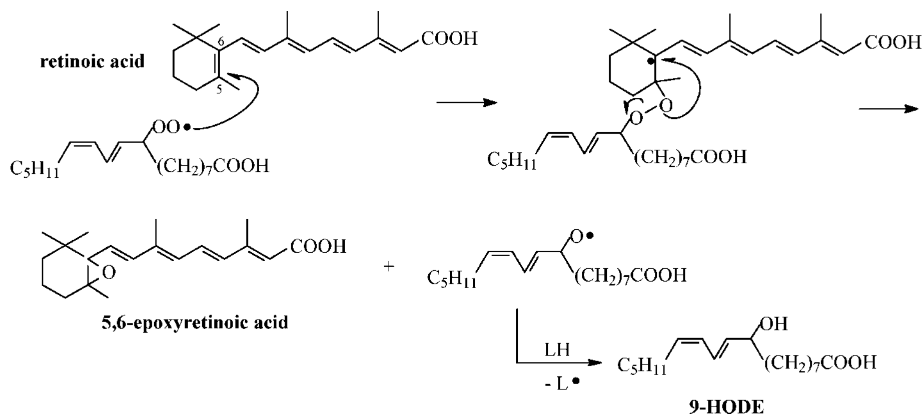
Scheme 18. BDE of C−OO• in LOO• and the Rate Constants of the Decomposition of LOO•



Scheme 19. Formation of 9-HODE and Cholestantriols from Cholesterol Linoleate via a Peroxyl Addition Reaction



Scheme 20. AAPH-Induced Oxidation of LH Stimulated by Retinoic Acid



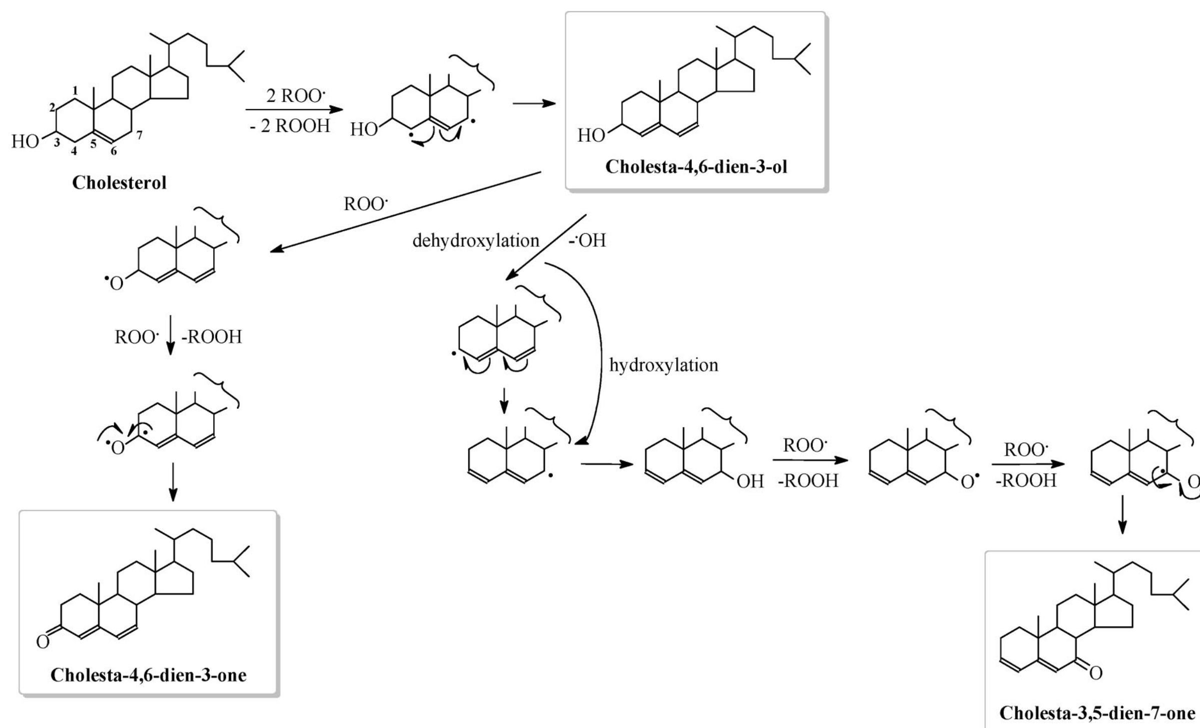
nonconjugated diene-type LOO• is as high as 3.8×10^6 M^{-1} s^{-1}.[101] The fast reaction between LOO• and TOH hinders the isomerization of C=C in LOO•.

LH forms cholesterol linoleate (LCH) in LDL. If LH in LCH is oxidized to form LOO•, C=C in cholesterol can be added by intramolecular LOO• to generate an epoxide of cholesterol. Eventually, cholestantriols and 9-hydroxyoctadecadienoic acid (9-HODE) are generated as shown in Scheme 19.[102]

As shown in Scheme 20, AAPH-induced oxidation of LH is accelerated by retinoic acid.[103] After LOO• adds to the C^5=C^6 bond in retinoic acid, the cleavage of the O−O bond forms 5,6-epoxyretinoic acid and the alkoxyl radical of LH (LO•), which converts into 9-HODE (or 13-HODE) eventually. Moreover, *all-trans*-retinol is an antioxidant to protect unilamellar soybean phosphatidylcholine against AAPH-induced oxidation. β-Carotene functions as an in vitro antioxidant only at a low partial pressure of oxygen (<150 Torr), while it behaves as autocatalytic and has a prooxidative effect at a high partial pressure of oxygen, particularly at the relatively high concentration used.[11] It was also found that a low partial pressure of oxygen and low concentration of retinol are beneficial for retinol to protect phosphatidylcholine by comparing t_{inh} and R_{inh}/R_p with the different pressures of oxygen applied. The generation of 5,6-retinol epoxide confirms that alkyl peroxyl radical generated from phosphatidylcholine adds to C^5=C^6 in retinol. Thus, the antioxidant ability of retinol to protect phosphatidylcholine depends on the concentration of retinol and the partial pressure of oxygen.[104]

Erythrocytes are susceptible to oxidation induced by ROS including AAPH, Cu^{2+}, and Fe^{2+}/H_2O_2, resulting in hemolysis. After erythrocytes are hemolyzed by ROS, and the proteins in the membranes are precipitated by $CHCl_3$/CH_3OH, it is found that cholesterol in the membrane of the erythrocytes is oxidized to form cholesta-4,6-dien-3-ol, cholesta-4,6-dien-3-one, and cholesta-3,5-dien-7-one. The oxidation process is shown in Scheme 21.[105]

Scheme 21. Formation of Cholesta-4,6-dien-3-ol, Cholesta-4,6-dien-3-one, and Cholesta-3,5-dien-7-one from the Oxidation of Cholesterol in the Membrane of Erythrocytes

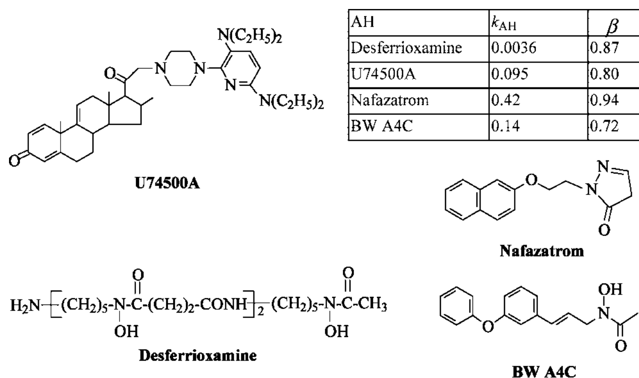


3.3. Improvement in Chemical Kinetic Deduction

In the oxidation of egg lecithin phosphatidylcholine (ELP) induced by *tert*-butyl hyponitrite (DBHN) in the water phase, the rate of oxygen exhaustion (R_p) is the same as that of oxidation occurring in chlorobenzene. ELP dissolved in water forms a liposome with a bilayer, but it forms a homogeneous solution in chlorobenzene. The rate of the oxidation of ELP in chlorobenzene is the same as that in water, demonstrating that the chemical kinetic expression for the radical-induced oxidation of PUFA in homogeneous solution is suitable for that in liposomes or micelles.[106] This provides a theoretical basis for introducing LH into CTAB, SDS, and Triton X-100 to mimic PUFA in the cationic, anionic, and neutral microenvironments and to obtain the oxidizability, k_{inh}, and n of antioxidants in micelles.[38] Moreover, R_p and R_{inh} can also be measured by the formation of LOOH detected by HPLC.[107] When γ-terpinene is oxidized by 2,2′-azobis(isobutyronitrile) (AIBN), the formation rate of *p*-cymene, the only oxidative product, is utilized to express the oxidation rate of γ-terpinene.[108,109] Furthermore, the experimental systems for oxidations of PUFAs are also composed of AIBN-induced oxidation of tetralin,[110] Cu^{2+}-induced oxidation of yolk lipoprotein,[111] AAPH-induced oxidation of glycerol trioleate,[112] etc.

Some antioxidants (structure in Scheme 22) just decrease the exhaustion rate of oxygen rather than generate t_{inh} when they protect LH against radical-induced oxidation of PUFAs.[113] If t_{inh} cannot be obtained, it is impossible to calculate R_i by eq 27. Consequently, other kinetic parameters cannot be calculated. Therefore, it is necessary to improve the deduction for the kinetic process on the basis of all the radicals including R$^•$, L$^•$, A$^•$, and LOO$^•$ at the steady state. Their concentrations remain constant, namely, d[R$^•$]/dt = d[L$^•$]/dt = d[A$^•$]/dt = d[LOO$^•$]/dt = 0. Experimentally, the rates of oxygen exhaustion are measured in the presence of various concentrations of an antioxidant (R_{inh}) and compared with that in the absence (R_p) of the antioxidant. The ratio between R_{inh} and R_p is expressed by

Scheme 22. Values of k_{AH} of Some Antioxidants and β in Protecting LH against Oxidation



AH	k_{AH}	β
Desferrioxamine	0.0036	0.87
U74500A	0.095	0.80
Nafazatrom	0.42	0.94
BW A4C	0.14	0.72

$$R_{inh}/R_p = 1 - \beta\{(k_{AH}[AH] + 1) - (k_{AH}^2[AH]^2 + 1)^{0.5}\} \quad (29)$$

where

$$\beta = k_p[LH]/\{(4k_dk_t[R{-}N{=}N{-}R])^{0.5} + k_p[LH]\} \quad (30)$$

and

$$k_{AH} = k_{inh}/(4k_dk_t[R{-}N{=}N{-}R])^{0.5} \quad (31)$$

The constant k_{AH} is a measure of the radical-scavenging ability of the antioxidant, whereas β is a constant independent of the nature of the antioxidant. The constants are evaluated from eq 29 by an iterative fitting procedure in which the residual sum of squares is minimized.[113] Together with the chemical kinetic equations in section 3.1, the ability of the

antioxidant can be estimated kinetically no matter whether t_{inh} can be generated.

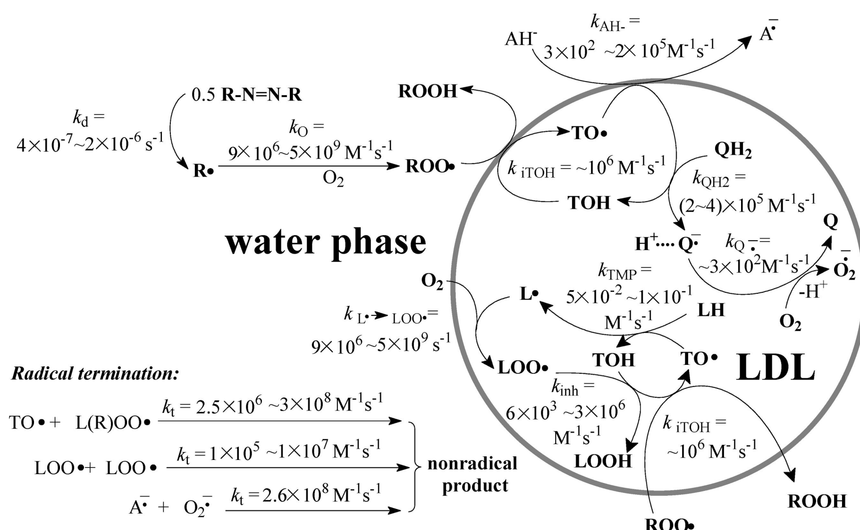
4. Applications of Biological Tissues To Inspect Antioxidant Activity

4.1. Low-Density Lipoprotein

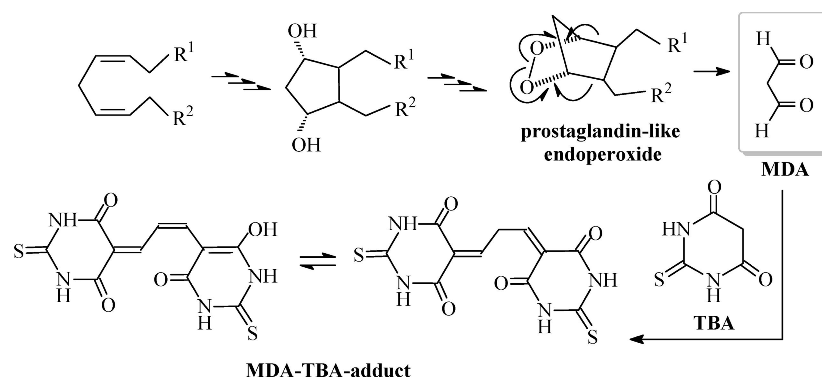
The oxidized LDL is a cytotoxin that causes atherosclerosis eventually.[114] The endogenous antioxidants in LDL are able to protect LDL against ROS-induced oxidation. Chemically, LDL is appropriate for the exploration of antioxidant capacity because it contains abundant PUFAs and various endogenous antioxidants simultaneously. For instance, the antioxidant capacities of polyphenols extracted from green tea[115] and synthetic hydroxyl-substituted coumarin[116] are investigated in AAPH-induced oxidation of LDL by measuring the variation of the concentration of oxygen. Therefore, k_{inh} and n of these antioxidants are calculated by eqs 20, 24, and 25. On the other hand, endogenous TOH is found to be a prooxidant[117] in the absence of ubiquinol-10 (QH_2).[118] The chemical kinetics reveals the relationships between the concentrations of LDL, AAPH, and endogenous TOH. It is found that a low concentration of AAPH, a high concentration of endogenous TOH, and the absence of other antioxidants stimulate TOH to form a radical ($TO^\bullet$), initiating the additional oxidative propagation within the particle of LDL, called *tocopherol-mediated peroxidation* (TMP).[119] TMP takes place intrinsically within LDL; it can be inhibited by endogenous QH_2 and exogenous ascorbate (AH^-). Scheme 23 collects the rate constants of the reactions among various antioxidants within and outside of LDL.[120]

Besides the measurement of the rate of oxygen exhaustion, the oxidation of LDL can be followed by detecting the formations of malondialdehyde (MDA),[121] diene, cholesteryl linoleate hydroxide (Ch18:2-OH), and cholesteryl linoleate hydroperoxide (Ch18:2-OOH).[122] Traditionally, MDA is measured to characterize the oxidative extent of LDL since the nonconjugated diene of PUFAs in LDL converts into prostaglandin-like endoperoxide and generates MDA eventually as shown in Scheme 24. The C=O groups in MDA can form a colorful adduct (λ_{max} = 535 nm) with thiobarbituric acid (TBA); thus, the oxidation of LDL can be followed by detecting TBA reactive substance (TBARS).[123] As shown in Scheme 17, the nonconjugated diene of LH in LDL first transforms to a conjugated diene (λ_{max} = 234 nm) when LDL is subjected to oxidation; therefore, the increase of the absorbance at 234 nm exhibits the oxidative extent of LDL induced by Cu^{2+}.[124] This method is not appropriate for AAPH-induced oxidation of LDL since AAPH has an absorbance at 234 nm. Detection of Ch18:2-OH or Ch18:2-OOH by HPLC gives the amount of oxidative products directly. LDL is isolated from plasma by ultracentrifugation at 140000g for 6 h when the density of plasma is adjusted to 1.4 g/mL by dissolving NaBr, and a 0.9% NaCl aqueous solution is added onto the layer of plasma as shown in Scheme 25.[125] Hence, LDL is a facile biological material employed to inspect antioxidant capacity.

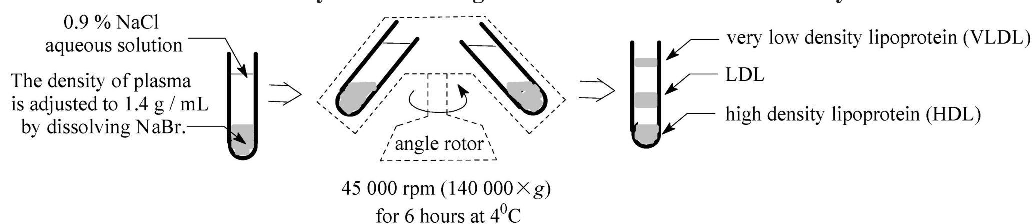
Scheme 23. Chemical Kinetic Model of Radical-Induced Oxidation of LDL and the Protective Effects of Endogenous and Exogenous Antioxidants on LDL



Scheme 24. MDA Generated from the Oxidation of LDL and Detected by Reacting with TBA To Form TBARS



Scheme 25. Isolation of LDL from Plasma by Ultracentrifugation with a Discontinuous Density Gradient



4.2. DNA

DNA is another biological material usually employed to inspect antioxidant activity when it is oxidized by metal ions, peroxyl radical, irradiation, etc. The interaction between DNA and ROS transforms the supercoiled DNA into a single-stranded and linear form. This process can be observed by electrophoresis.[126] For example, in the presence of (9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoic acid, the cleavage of pBR 322 plasmid DNA is observed by electrophoresis.[127] The usage of green tea phenols in combination with Trolox protects DNA against AAPH-induced oxidation efficiently because of no cleavage fragments of DNA observed by electrophoresis.[128] On the contrary, electrophoresis exhibits more fragments deriving from DNA in the presence of hydroxycinnamic acid and Cu^{2+}, revealing that hydroxycinnamic acid in combination with Cu^{2+} plays a prooxidative role to break DNA.[129] The technique of ^{32}P-labeled DNA allows the obvious observation of the fragments of DNA by electrophoresis. For example, after 3′-phosphoglycolate, the terminus of DNA, is labeled by ^{32}P, the breaks of the DNA strand can be observed by electrophoresis more manifestly when DNA is oxidized by $^{\bullet}$OH or calcium-activated nucleases.[130] Because DNA is a polymer, and the oxidative products are small organic molecules, their densities and viscosities are different. A bulk acoustic wave impedance sensor is applied to monitor the variations of the density and viscosity for the DNA solution during oxidation induced by vitamin C and Fe^{3+}, indicating that the oxidation of DNA follows the first-order kinetic law.[131] A small volume of mitochondrial DNA is labeled with an intercalating dye, YOYO-1, to improve the sensitivity for detecting the oxidative products of DNA by fluorescence correlation spectroscopy equipped with a dichroic mirror (>510 nm) and a bandpass filter (515−560 nm).[132] Moreover, the measurement of 8-hydroxy-2′-deoxyguanosine (8-OHdG) by HPLC gives a quantitative expression for the damage of DNA.[133]

After many methods have been set up to inspect the oxidation of DNA, the ways to induce the oxidation of DNA are taken into consideration. Some chemical agents are regarded as cytotoxic because the metabolites from them can initiate the oxidation of DNA. For instance, benzene is a strong carcinogen to animals and humans because the metabolites of benzene including benzoquinone, catechol, and 1,2,4-benzenetriol result in the formation of $O_2^{\bullet-}$ and other oxygen radicals to damage DNA in the presence of metal ions,[134] especially Cu^{2+}.[135] Moreover, when ^{32}P-labeled human DNA is exposed to *o*-aminophenol, a metabolite from *o*-anisidine, it is found that *o*-aminophenol causes an increase of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG). The addition of Cu^{2+} or CuZn-SOD enhances the damage of DNA and alters the sequence of DNA simultaneously, and a radical signal is detected in the mixture of *p*-aminophenol (5 mM) and Cu^{2+} (0.5 mM) in the ESR spectrum, which is in agreement with the calculated signal of *p*-aminophenoxyl radical.[136]

Now that Cu^{2+} is able to stimulate the generation of an O-centered radical from a phenolic compound, whether it can lead to a S-centered radical from thiols and the influence of the S-centered radical on DNA are the further concern with respect to the oxidation of DNA. As summarized in a previously published review,[52] it is complicated for thiols to inhibit or promote the damage of DNA. When DL-cysteine is added to the oxidative experimental system of DNA, the oxidant employed to oxidize DNA and the electron-transfer procedure govern DL-cysteine to be an antioxidant or a prooxidant.[137] A mixture of glutathione (GSH) and Cu^{2+} (both 0−5 mM) definitely causes the cleavage of plasmid DNA observed by electrophoresis, and the oxidation products are measured after reaction with TBA to form TBARS (ε_{532} = 158 000 M^{-1} cm^{-1}).[138] Scheme 26 illustrates the formation of MDA in $^{\bullet}$OH-induced oxidation of DNA.[139] The radical generated from the decomposition of AAPH can abstract the H atom from the C-4′ atom of DNA and cause strand breaks.[140] The further oxidative products can also be trapped by TBA to form TBARS. In the process of AAPH-induced oxidation of DNA, the addition of melatonin lags the formation of TBARS. The lag time (t_{inh}) is proportional to the concentration of melatonin applied.[141] The following equation, equivalent in style to eq 24, can be used to express the linear relationship between t_{inh} and the concentration of an antioxidant needed to protect DNA:

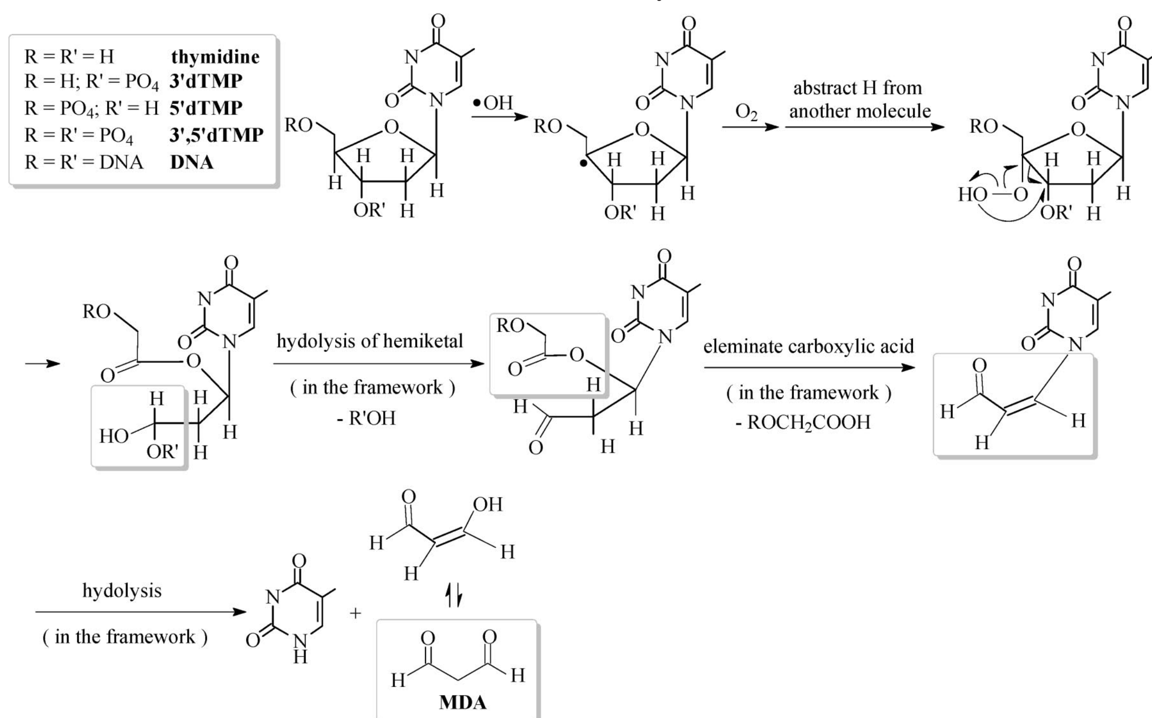
$$t_{inh} = (n/R_i)[AH] \quad (32)$$

R_i is a relative value by using Trolox or TOH as the reference antioxidant whose n is assigned as 2.0. However, both Trolox and TOH cannot generate t_{inh} in protecting DNA when TBARS is measured. Thus, R_i is assumed to be equal to the rate of radical generated from the decomposition of AAPH, $R_g = (1.4 \pm 0.2) \times 10^{-6}$ [AAPH] s^{-1}. On the basis of this assumption, it is found that one molecule of melatonin can trap almost two radicals in protecting DNA.[141] Although the physiological meaning of the n value of an antioxidant cannot be confirmed, n is still a quantitative index to compare the antioxidant ability in protecting DNA.

4.3. Erythrocytes

The abundant components of proteins and lipids in the membrane of erythrocytes make erythrocytes an in vitro substrate usually employed to screen antioxidant capacity. Oxidative stress on erythrocytes involves many aspects. For instance, the variation of pH affects the shape of the erythrocytes[142] and the rate of acid transfer into the cytosol.[143] Triton X-100 as a surfactant functions as an oxidative resource replacing phospholipids in the membrane.[144] Incubating erythrocytes with lecithin liposomes generates lipid peroxidation in the erythrocyte membrane, depletes TOH, and leads to hemolysis eventually.[145] The phospholipid domains, conformational order, and shape of the intact human erythrocytes have been investigated by

Scheme 26. Formation of MDA from the Oxidation of DNA Induced by •OH



analyzing the variations of peaks within 2050−2250 cm^{-1} in a second-derivative Fourier transform infrared (FT-IR) spectrum when 1,2-dilauroylphosphatidylethanolamine and 1,2-dipentadecanoylphosphatidylcholine with a perdeuterated acyl chain are incorporated preferentially into the inner or outer leaflets of the erythrocytes.[146] Nevertheless, AAPH-induced hemolysis of erythrocytes is the most suitable method to evaluate antioxidant activity.[147,148] Originally, the membrane protein is regarded as the critical target attacked by radicals in the erythrocyte membrane.[149] Hemolysis does not take place immediately when erythrocytes are incubated with AAPH in phosphate-buffered saline (PBS; 150 mM NaCl, 8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, pH 7.4) at 37 °C because of the protection from the endogenous antioxidants. After the endogenous antioxidants are depleted completely, hemolysis occurs with hemoglobin ($\lambda_{max} = 535$ nm) leaking out of the membrane and dissolving in PBS. Thus, after the residue erythrocytes are removed by centrifugation, the absorbance in the PBS phase (supernatant solution) reflects the hemolysis extent. The endogenous antioxidants lag the occurrence of hemolysis and generate an inhibition period (t_{inh}). Sometimes the absorbance in the supernatant solution from the erythrocyte suspension thoroughly hemolyzed by water is assigned as the reference value (Abs_{ref}); the absorbance of the supernatant solution at other time points (Abs_{detect}) is compared with Abs_{ref} to obtain the hemolysis percentage ($Abs_{detect}/Abs_{ref} \times 100$).[150] The addition of antioxidants may increase t_{inh}.[151] On the other hand, MDA can also be generated in the process of hemolysis.[152]

It is essential to obtain t_{inh} accurately. Some antioxidants affect the hemolysis rate other than prolong t_{inh}. Thus, the time at which 50% of the erythrocytes are hemolyzed is assigned as t_{inh}.[153] The variation of the absorbance during the whole process of hemolysis can be expressed by the Boltzmann equation:

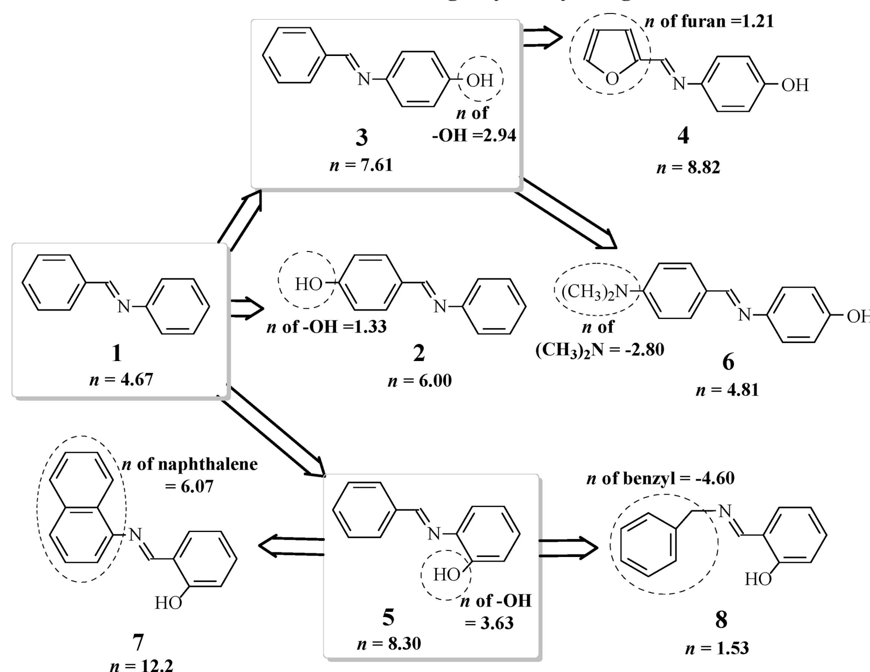
$$A = (A_{initial} - A_{final})/[1 + e^{(t-t_0)/dt}] + A_{final} \quad (33)$$

A in eq 33 indicates the absorbance at a certain time point t, $A_{initial}$ and A_{final} represent the absorbance at the beginning and end of hemolysis, and t_0 reveals the time at which half of the erythrocytes are hemolyzed.[154] When the values of A and the corresponding t are input into the software for data treatment, eq 33 is given automatically, and t_0 is obtained consequently. Since t_0 can be prolonged by additional antioxidants, the difference between the presence of the additional antioxidants and the control experiment is regarded as t_{inh}, which is proportional to the concentration of the exogenous antioxidants as expressed in eq 32. Trolox acts as a reference antioxidant in AAPH-induced hemolysis to measure R_i with n assigned as 2.0. It is found that R_i is equal to R_g, demonstrating that all the radicals generated from the decomposition of AAPH attack the erythrocytes directly.[155] This is due to the radical generated from AAPH locating at the same phase in which the erythrocytes are suspended. The relationship between t_{inh} and the concentration of other antioxidants is expressed by eq 32. As a result, the n value of other antioxidants is calculated with a known R_i.

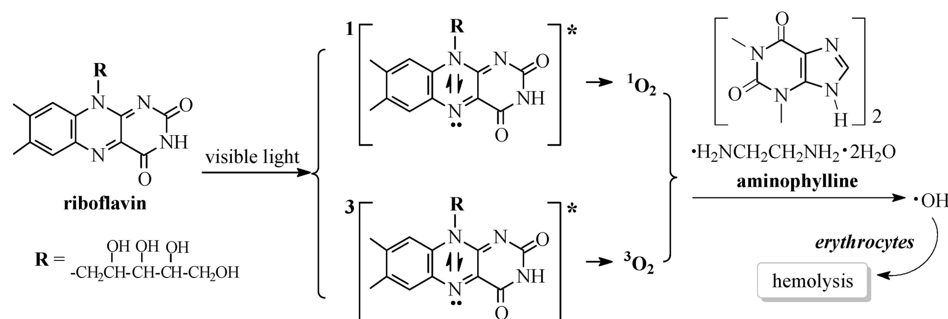
The n values of hydroxyl-substituted Schiff bases are measured and compared with each other as shown in Scheme 27.[156,157] When Schiff bases **1** and **2** protect erythrocytes against AAPH-induced hemolysis, the former one generates an n value of 4.67 and the latter one generates an n value of 6.00. The difference between the structures of Schiff bases **1** and **2** is a −OH attaching to the benzaldehyde in **2**. The difference in the n values between Schiff bases **1** and **2** (6.00 − 4.67 = 1.33) can be regarded as the contribution from the −OH in Schiff base **2**. Therefore, the contributions from substituents to the antioxidant abilities of Schiff bases are expressed by the difference value of n as shown in Scheme 27.

Other methods are also applied to initiate hemolysis. Visible light causes hemolysis of 0.5% erythrocytes suspended in 10 mM Tris−HCl (pH 7.4) with aminophylline (80 μg/mL) and riboflavin (50 μM) as photosensitizers. As

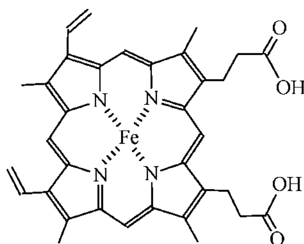
Scheme 27. *n* Values of Substituents of Schiff Bases in Protecting Erythrocytes against AAPH-Induced Hemolysis



Scheme 28. Photoinduced Hemolysis of Erythrocytes in the Presence of Riboflavin



Scheme 29. Structure of Hemin



shown in Scheme 28, $^1O_2$ and triplet oxygen ($^3O_2$) are formed in the case of irradiation of riboflavin by visible light and then oxidize aminophylline. As a result, $^{\bullet}OH$ is formed in the oxidation of aminophylline and leads to hemolysis eventually.[158] Experimental evidence reveals the loss of K^+ in the membrane prior to hemolysis; thus, the chelation of K^+ by these photosensitizers may be another reason for hemolysis. Hemin (structure in Scheme 29) initiates hemolysis by accelerating the loss of K^+ in the erythrocyte membrane. Therefore, if an antioxidant can protect erythrocytes against hemin-induced hemolysis, it can be regarded as a protector to stabilize the erythrocyte membrane. TOH[159] and ginsenosides[160] are efficient membrane stabilizers to inhibit hemin-induced hemolysis. Moreover, xanthine oxidase (XO) is used to initiate the oxidation of LDL and the hemolysis of erythrocytes, and the antioxidant capacities are inspected in these experimental systems as well.[161]

5. Other methods

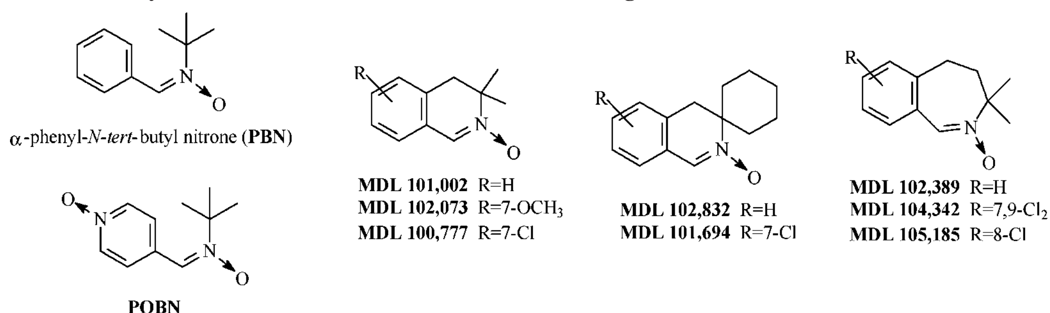
5.1. ESR

The single electron in the radical can be detected by ESR. Thus, ESR is the most important experimental technique to *observe* radicals directly and to inspect the antioxidants to trap radicals. The antioxidant abilities of cyclic nitrone spin traps (structures in Scheme 30) are characterized by ESR spectroscopy.[162] The decrease of the ESR signals indicates that the inhibition of Cu^{2+}-induced oxidation of LDL is dependent upon the ability of the N→O bond to chelate Cu^{2+} and to trap radicals in LDL. Moreover, the N→O compounds are usually applied to test the rate constant of antioxidants to trap radicals chemically. As shown in Scheme 31, the radical generated from TOH can be repaired by ebselen[122] and polyphenols extracted from green tea.[163] The addition of 5,5-dimethylpyrroline (DMPO) traps radicals in AAPH-induced hemolysis.[148] *p*-Dinitrobenzenide is reduced by potassium to form an ion pair with an ESR signal, validating that the ion pair is a radical.[164]

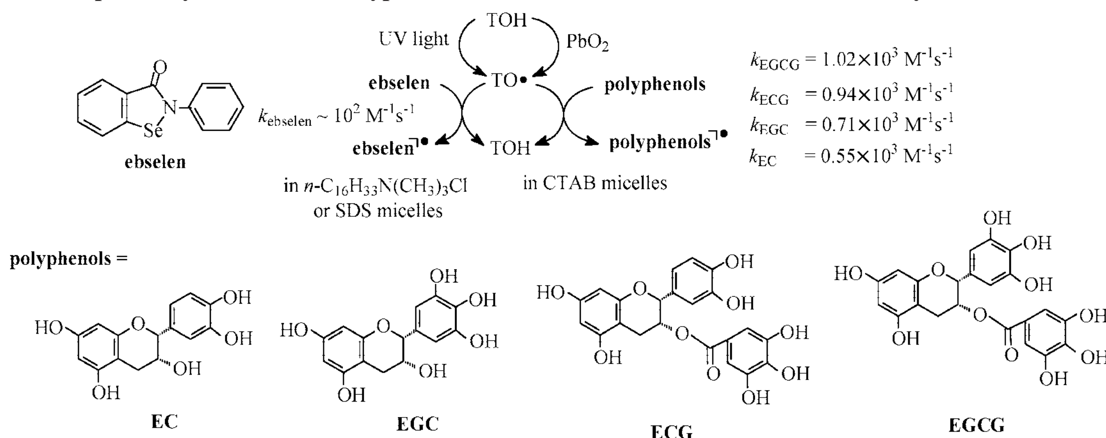
5.2. Quantum Calculation

Since some radicals are very difficult to detect experimentally, quantum calculation plays an important role in expressing the properties of these radicals, in which enthalpy,

Scheme 30. Structures of Cyclic Nitrone with Abilities To Protect LDL against Cu^{2+}- or AAPH-Induced Peroxidation

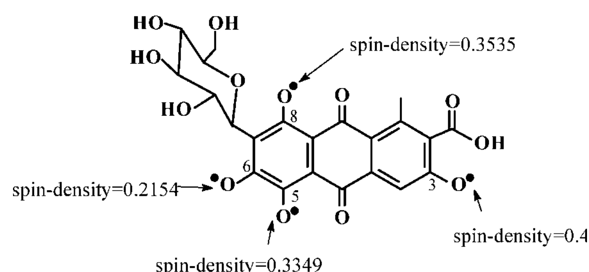


Scheme 31. TO• Repaired by Ebselen and Polyphenols with Different Rate Constants Measured by ESR



entropy, bond energy, and heat capacity are the thermodynamic parameters employed usually. The radicals derived from vinyl alcohols (enols) are regarded as the intermediates in the oxidation of hydrocarbons and are not readily trapped. Alternatively, the structures of all the possible radicals are optimized at the B3LYP/6-31G(d,p) level and at the same harmonic vibrational frequencies and zero-point vibrational energies. Theoretical calculation provides the possibility for comparing the enthalpy ($\Delta_f H°_{298}$) and entropy ($S°_{298}$) during the formation of these radicals. As a result, the values of $\Delta_f H°_{298}$ for $^•CH{=}CHOH$, $^•CH{=}CHOCH_3$, and $CH_2{=}C^•OCH_3$ formed in the gas phase are around 30−40 kcal/mol, whereas the values of $\Delta_f H°_{298}$ for *anti*-$CH_2{=}C^•OH$ and $CH_2{=}CHOCH_2^•$ range from 20 to 30 kcal/mol, demonstrating that the latter radicals are more stable than the former ones.[165] The influences of the *ortho*-substituted groups on the radical formed at the phenolic hydroxyl group are evaluated by calculating the BDE of O−H.[166] Furthermore, the BDE values of the N−H bond in phenothiazine derivatives are similar to that of O−H in TOH, revealing the reason why phenothiazine derivatives have antioxidant capacities similar to that of TOH.[167] The spin density on the atom with the single electron should be taken into consideration in evaluating the stabilization of radicals. Four hydroxyl groups in carminic acid have similar BDEs of O−H; however, as shown in Scheme 32, the spin densities on the O atoms are quite different when carminic acid forms radicals. A low spin density implies that the single electron can be dispensed perfectly to the benzene ring, resulting in a relatively stable radical.[168] Some radicals have comparable stability, but the corresponding compounds are not good antioxidants. For example, the bond strengths of phenol and toluene are quite similar, as are the spin densities in the resulting radicals. Phenol may play an antioxidant role, whereas toluene cannot. There are a couple of theories to explain the low barrier for H atom transfer between phenols and oxygen-centered radicals such as triplet repulsion in the transitions state[169] and proton-coupled electron transfer (PCET).[170]

Scheme 32. Spin Densities on O Atoms When O−H in Carminic Acid Forms Radicals



6. Conclusion

Some convenient methods employed to explore antioxidant capacities are introduced to organic laboratories majoring in the synthesis or extraction of antioxidants. These methods are composed of inspecting antioxidant capacities by interacting with oxidants, evaluating antioxidant behaviors by using biological experimental materials, and calculating the stabilization of radicals derived from antioxidants by quantum chemical methods. These methods are beneficial for researchers to investigate the antioxidant capacities of the extraction from natural resources rapidly and to explore the structure−activity relationships of the synthetic antioxidants conveniently.

7. Abbreviations

AAPH	2,2′-azobis(2-amidinopropane hydrochloride)
$ABTS^{•+}$	2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) cationic radical
AH^-	ascorbate
AIBN	2,2′-azobis(isobutyronitrile)

ArOH	phenolic compound
ArO^-	phenolic anion
BDE	bond dissociation energy
Ch18:2-OH	cholesteryl linoleate hydroxide
Ch18:2-OOH	cholesteryl linoleate hydroperoxide
CTAB	cetyltrimethylammonium bromide
DBHN	*tert*-butyl hyponitrite
DFT	density functional theory
2′dG	2′-deoxyguanosine
DMPO	5,5-dimethylpyrroline
DPPH	2,2′-diphenyl-1-picrylhydrazyl
DTPA	diethylenetriaminepentaacetic acid
ELP	egg lecithin phosphatidylcholine
ESR	electron spin resonance
FIA	flow injection analysis
GSH	glutathione
HAT	hydrogen atom transfer
HODE	hydroxyoctadecadienoic acid
HPODE	hydroxyperoxyoctadecadienoic acid
LCH	cholesterol linoleate
LDL	low-density lipoprotein
LH	linoleic acid
LOOH	peroxide of linoleic acid
$LOO^\bullet$	peroxyl radical of linoleic acid
$LO^\bullet$	alkoxyl radical of linoleic acid
MDA	malondialdehyde
8-OHdG	8-hydroxy-2′-deoxyguanosine
8-oxodG	8-oxo-7,8-dihydro-2′-deoxyguanosine
PCET	proton-coupled electron transfer
PUFA	polyunsaturated fatty acid
QH_2	ubiquinol-10
QSAR	quantitative structure−activity relationship
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
SOD	superoxide dismutase
SPLET	sequential proton loss electron transfer
t_{inh}	inhibition period
TEAC	Trolox equivalent antioxidant capacity
TMP	tocopherol-mediated peroxidation
TOH	α-tocopherol
$TO^\bullet$	TOH radical
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substance
XO	xanthine oxidase

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