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Review

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Antioxidant Activity of Essential Oils

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ABSTRACT: Essential oils (EOs) are liquid mixtures of volatile compounds obtained from aromatic plants. Many EOs have antioxidant properties, and the use of EOs as natural antioxidants is a field of growing interest because some synthetic antioxidants such as BHA and BHT are now suspected to be potentially harmful to human health. Addition of EOs to edible products, either by direct mixing or in active packaging and edible coatings, may therefore represent a valid alternative to prevent autoxidation and prolong shelf life. The evaluation of the antioxidant performance of EOs is, however, a crucial issue, because many commonly used "tests" are inappropriate and give contradictory results that may mislead future research. The chemistry explaining EO antioxidant activity is discussed along with an analysis of the potential in food protection. Literature methods to assess EOs' antioxidant performance are critically reviewed.

KEYWORDS: *essential oils, antioxidant, oxidation, food, hydroperoxide, peroxyl radicals*

INTRODUCTION

Essential oils (EOs) are liquid mixtures of volatile compounds obtained from aromatic plants, most commonly by steam distillation. They constitute what is called the "essence" of a plant and usually have pleasantly scented fragrances. Aromatic plants and EOs have been used for millennia for their health benefits, well documented in ancient literature.[1] Some of the purported beneficial properties, for example, antiseptic, antioxidant, and anti-inflammatory, have been supported by recent scientific investigation.[2,3] Hundreds of compounds (secondary metabolites) with relatively low boiling points have been identified in EOs, and the large chemical diversity of their constituents influences the oxidative stability of EOs. On the other hand, several essential oils have been attributed good antioxidant properties, which can be exploited to protect other materials, such as food, from rancidity.[4]

Antioxidant properties play also a pivotal role in some of EOs' biological activities, which is justified by the involvement of oxidative stress in pathology.[5] These attributes are due to the inherent ability of some of their components, particularly phenols, to stop or delay the aerobic oxidation of organic matter, although the procedure by which the oil is obtained from the raw material (distillation) limits the content of phenolics in the final matrix because many such compounds are nonvolatile. However, there are phenol-free EOs that express antioxidant behavior. As we will discuss in the following sections, this is due to the radical chemistry of some terpenoids and other volatile constituents (e.g., sulfur-containing components of garlic).[1]

The search for natural antioxidants with the virtue of being nontoxic has given rise to a large number of studies on the antioxidant potential of EOs. This is particularly relevant because most common synthetic antioxidants (such as butylated hydroxyanisole (BHA) or butylhydroxytoluene (BHT)) are suspected to be potentially harmful to human health.[6,7] On the other hand, reports on EOs' antioxidant properties from different scientific fields or from different laboratories are sometimes contradictory, often because of diverse experimental settings, which make difficult any comparison among the results. Some of the methods used to assess EOs' antioxidant performance suffer from limitations that, if not adequately addressed, may compromise the significance of results.

The aim of this review is not to offer a comprehensive survey of the literature but to analyze the chemistry and mechanisms at the basis of EOs' antioxidant activity, highlighting their potential and usefulness, with particular focus on their application for the protection of food. Methods used to assess their antioxidant performance will be critically reviewed, and arguments for the selection of the most appropriate methods will be discussed, on the basis of the experience developed in our groups.

ANTIOXIDANTS: DEFINITION AND MECHANISMS

It is common practice in the study of natural compounds to identify antioxidants as "molecules able to react with radicals" or provided of reducing power so as to counteract the oxidative stress caused by radicals. This approach is witnessed by the chemistry of several tests developed to assay the antioxidant activity of natural extracts or isolated phytochemicals, which are based on the reaction of the potential antioxidant with some colored persistent radical (e.g., $DPPH^{\bullet}$ or $ABTS^{\bullet+}$) or with some oxidizing nonradical species such as Fe^{3+} ions (e.g., FRAP assay). This approach is not entirely correct.

By definition, antioxidants are compounds capable of slowing or retarding the oxidation of an oxidizable material, even when used in very modest amount (<1%, commonly 1−1000 mg/L) as compared to the amount of material they have to protect. Focusing on processes of relevance in biological systems or in food science, the materials to protect are most commonly

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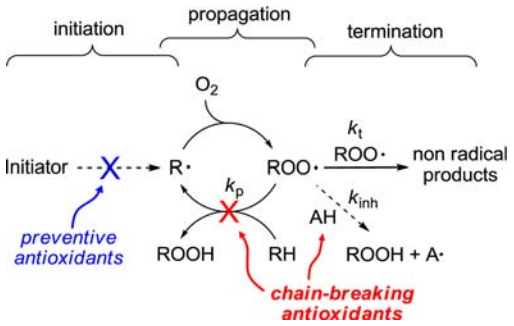
lipids, proteins, carbohydrates, and, to a minor extent, other organic molecules that compose animal or vegetal tissues. Their oxidation occurs by a radical chain reaction mediated by peroxyl radicals (ROO$^{\bullet}$) that parallels the autoxidation of hydrocarbons.[8]

The process, generalized in Scheme 1, is initiated by some radical species that, regardless of its origin or structure, is able

Scheme 1. Simplified Mechanism of Hydrocarbon Autoxidation and Antioxidant Protection



to react with the (lipid) substrate RH (most commonly by H-atom abstraction) to yield an alkyl radical R$^{\bullet}$, which will react at diffusion-controlled rate with oxygen to form a peroxyl radical (ROO$^{\bullet}$). Cyclically, ROO$^{\bullet}$ attacks another molecule of the substrate to yield a hydroperoxide ROOH (the oxidized substrate) and another radical. The chain reaction proceeds for many cycles before two radical species incidentally quench each other in a, so-called, termination step. The number of cycles occurring between initiation and termination is named "chain length".

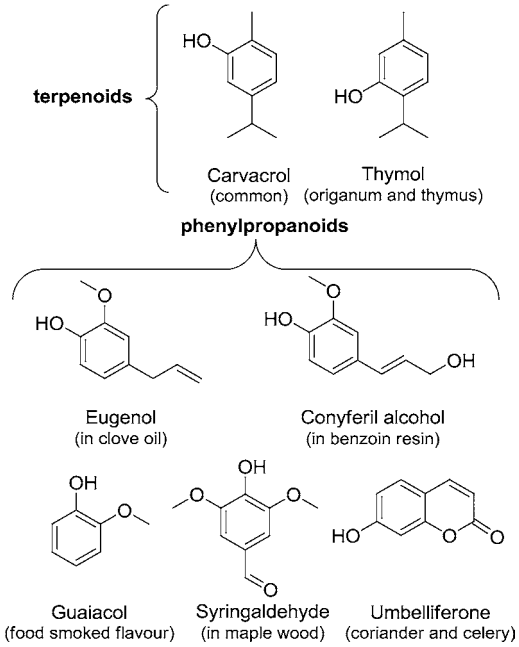
Compounds capable of impairing this radical chain reaction are called *direct antioxidants* and are divided into two main groups depending on their mechanism of interference.[8] *Preventive antioxidants* interfere with the initiation process; that is, they retard the initial formation of radical species.[8] Examples of such are the enzyme catalase and metal chelators such as phytic acid. (By blocking redox active metal ions (e.g., Fe^{2+}) in an oxidized form (e.g., Fe^{3+}), metal chelators may prevent the occurrence of Fenton-type chemistry, which is one of the most important radical initiation processes.) *Chain-breaking antioxidants* slow (or block) autoxidation by competing with the propagation reactions; that is, they react with peroxyl radicals more rapidly than the oxidizable substrate to form species that *do not propagate the oxidation chain*.[8] Because preventive antioxidants are completely ineffective after the process has started, chain-breaking antioxidants are by far the most important direct antioxidants: their efficacy primarily relates to the *kinetics of* reaction *with peroxyl radicals* (the actual chain-carrying species), which has to be compared to the rate of propagation, that is, the rate of RH + ROO$^{\bullet}$. The fact that a compound can react with "some radical species" does not mean it is an antioxidant, unless (i) the radical species is actually involved in oxidative chain carrying, that is, it is a peroxyl radical; (ii) the reaction is much faster than the reaction of the radical with the material to protect, for example, unsaturated lipids; and (iii) the reaction products are species unable to propagate the chain- reaction.[8] Phenols are the prototypical chain-breaking antioxidants.[9]

Several compounds not provided of relevant antioxidant behavior, for example, in the protection of lipids in model systems or food products, do nonetheless increase the antioxidant defenses in living systems, for example, by inducing the expression or enhancing the activity of antioxidant enzymes. These compounds are called *indirect antioxidants* with relevant examples among natural products.[8]

DIRECT ANTIOXIDANT ACTIVITY OF ESSENTIAL OILS

To rationalize the mechanism of antioxidant activity expressed by essential oils, it is necessary to briefly address their composition. Despite the observed large chemical diversity, the main components of common essential oils can be classified in two structural families with respect to hydrocarbon skeleton: terpenoids, formed by the combination of two (monoterpene), three (sesquiterpene), or four (diterpene) isoprene units, and phenylpropanoids. Both terpenoid and phenylpropanoid families comprise phenolic compounds, sometimes accounted among principal components of several EOs. Some common structures are illustrated in Scheme 2.

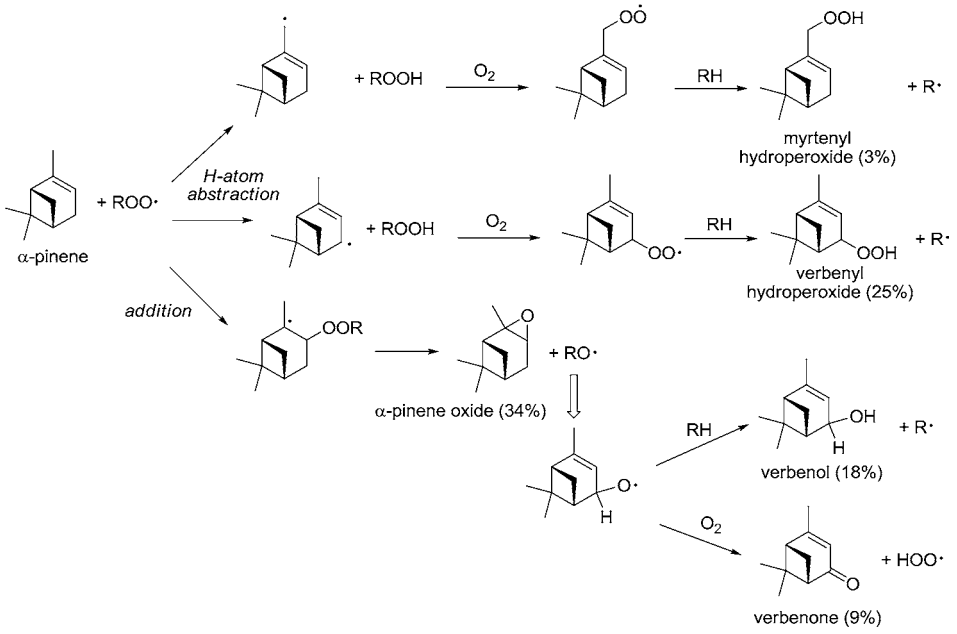
Scheme 2. Some Common Phenolic EO Components



In general, phenolic compounds, both natural (e.g., α-tocopherol) or synthetic (e.g., BHA), act as antioxidants due to their high reactivity with peroxyl radicals, which are disposed of by formal hydrogen atom transfer (eq 1).[10] The actual rate of reaction is not known for most EO components; however, it has been measured as $k_{inh} = 5 \times 10^3$ M^{-1} s^{-1} at 303 K for guaiacol[11], and it can be estimated as $k_{inh} > 10^4$ M^{-1} s^{-1} for most other components, such as those collected in Scheme 2, by comparison with structurally related phenols.[8] Due to its stability, the product phenoxyl radical will not propagate the radical chain, but rather "wait" for a second peroxyl radical and quench it in a very fast radical−radical reaction (eq 2). The number n of peroxyl radicals (or oxidative chains) quenched by one molecule of antioxidant is called the "stoichiometric factor" (n = 2 for phenols such as guaiacol or α-tocopherol).

$$\mathrm{PhOH} + \mathrm{ROO}^{\bullet} \xrightarrow{k_{inh}} \mathrm{PhO}^{\bullet} + \mathrm{ROOH} \quad (1)$$

Scheme 3. Autoxidation of α-Pinene at 90 °C under Pure O_2 (Reprinted with Permission from Reference 12. Copyright 2010 Wiley)[a]



[a]The pathways leading to the formation of the most abundant oxidation products are indicated. RH = α-pinene, R$^\bullet$ = α-pinene alkyl radical, RO$^\bullet$ = α-pinene alkoxyl radical, HOO$^\bullet$ = hydroperoxyl radical (superoxide radical neutral form).

$$PhO^\bullet + ROO^\bullet \xrightarrow{fast} \text{nonradical products} \quad (2)$$

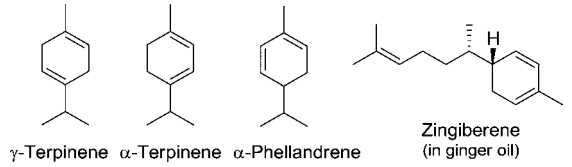
Other terpenoid components of essential oils can react rapidly with peroxyl radicals; however, the reaction yields a reactive alkyl radical (from terpene hydrocarbon skeleton), which, in the presence of oxygen, forms a peroxyl radical that propagates the oxidative chain. In other words, nonphenolic terpenoids, particularly unsaturated ones, would autoxidize similarly to unsaturated lipids. The process can be illustrated in the case of ubiquitous α-pinene, which has been described in detail (Scheme 3). [12]

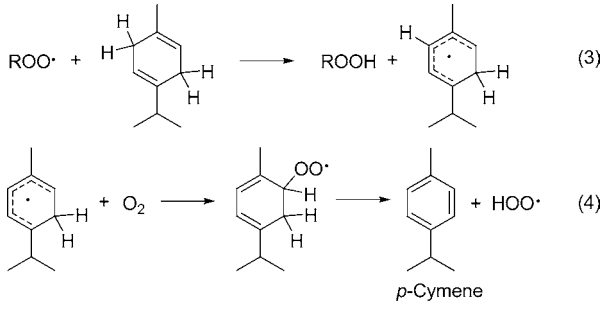
When α-pinene or similar EO components are mixed with an oxidizable material such as unsaturated lipids, both the lipids and the EO components will undergo autoxidation, and will be subjected to similar degradation. In other words, the substrate to be protected (the lipid) and the potential antioxidant (the EO componets) will co-oxidize. No protection can be expected from the essential oil because products arising from reactions with chain-carrying peroxyl radicals are reactive species able to propagate the oxidative chain. There are, however, relevant exceptions to the above general statement, as will be shown in the following.

Terpenoids having a cyclohexadiene structure, such as γ-terpinene typical of tea-tree oil but found in a variety of conifers, α-phellandrene typical of dill and eucalyptus, and others (Scheme 4) undergo autoxidation characterized by a very fast termination process, with bimolecular rate constant for the decay of peroxyl radicals 1 or 2 orders of magnitude larger than recorded, for instance, with PUFA and as much as 1000-fold that of saturated or monounsaturated lipids.[8,13]

The mechanism of chain termination during the oxidation of γ-terpinene has been investigated in detail by one of us, and can be summarized by eqs 3–6.[13,14] The driving force for the overall process is the formation of aromatic *p*-cymene and the very fast decay of neutral superoxide radical (eqs 5 and 6).

Scheme 4. Some Common Mono- and Sesquiterpenes Bearing the Cyclohexadiene Core





When molecules such as γ-terpinene are mixed with an unsaturated lipid in sufficient amount, they will cause an overall increase in the rate of oxidative chain termination, thereby shortening the chain length and reducing the overall rate of oxidation, as assessed from the rate of oxygen consumption or the rate of formation of oxidized products. These compounds therefore behave as antioxidants, although they do not fall into the categories outlined in the previous section. They cannot be defined as chain-breaking antioxidants because the products of their reaction with peroxyl radicals do propagate the oxidative chain, albeit with a reduced overall efficiency. For simplicity we

will call them *termination-enhancing* antioxidants to distinguish them from *chain-breaking* antioxidants.

Inhibition by one or the other mechanism can have a significantly different outcome in the protection of oxidizable material such as lipids, as illustrated in Figure 1. Chain-breaking

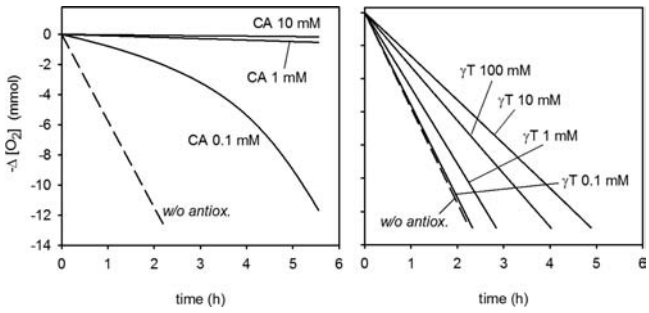


Figure 1. Simulated traces of oxygen consumption during the thermally initiated (50 mM AIBN, 30 °C) autoxidation of a sample of linoleic acid (1 M) in an apolar solvent without inhibitor (dotted line) or in the presence of growing concentrations of carvacrol (CA, left panel) or γ-terpinene (γT, right panel), being respectively examples of chain-breaking and termination-enhancing antioxidants found in essential oils. Simulations were made with Gepasi 3.0 software using known rate constants from the literature.[13]

antioxidants dramatically inhibit autoxidation already at low concentration ($<10^{-3}$ molar ratio with respect of the oxidizable material), even in the presence of a radical initiator, until they are consumed; when used in sufficient concentration, they normally give a neat inhibition period, the extension of which is proportional to their concentration. On the other hand, as much as 1 mM γ-terpinene was found necessary to reduce by about 50% the rate of oxidation of 30 mM linoleic acid (in cyclohexane containing ∼1 mM 2,2′-azobis(isobutyronitrile) (AIBN) as radical initiator) at 50 °C.[13] When autoxidation is forcibly initiated by some controlled radical source (e.g., AIBN), no real inhibition period is observed for termination-enhancing antioxidants even at high concentrations, and there is no linear (or even no monotonic) dependence between the antioxidant performance and the concentration of the antioxidant.

RAW ESSENTIAL OILS VERSUS ISOLATED COMPONENTS

Because natural essential oils are mixtures of several components, the different types of antioxidants or oxidizable terpenoid components previously described often coexist. When a natural EO is used to protect some material, one could expect that the most effective antioxidant components dominate, and the overall oxidative protection offered by the oil is mostly that due to such components. This is true in some cases, but many exceptions have been observed.[4] The overall performance as antioxidant is, in fact, the result of the complex interplay among components and the oxidizable material to be protected. In general, depending on the exact EO composition and experimental conditions, synergistic or antagonistic behavior is to be expected. An interesting overview of the different possibilities is offered in a study by Kulisic et al.[15] The investigators analyzed the spontaneous oxidation of lard, in the presence/absence of some EOs or their components and fractions using the Rancimat test. Whereas unprotected lard heated at 100 °C showed measurable oxidation starting after 5.2 h, the induction time was increased upon the addition of 0.16% (w/w) standard antioxidants (α-tocopherol, BHA, BHT), phenolic EOs' components thymol or carvacrol, or several EOs containing such components. However, comparison of the performance of each raw oil with its isolated hydrocarbon fraction (lacking phenolic components) or with its oxygenated fraction (comprising mainly phenolic components) allows the most interesting discussion, as summarized in Figure 2.

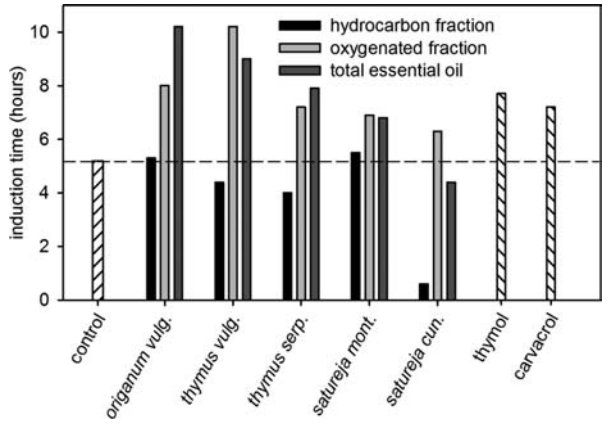
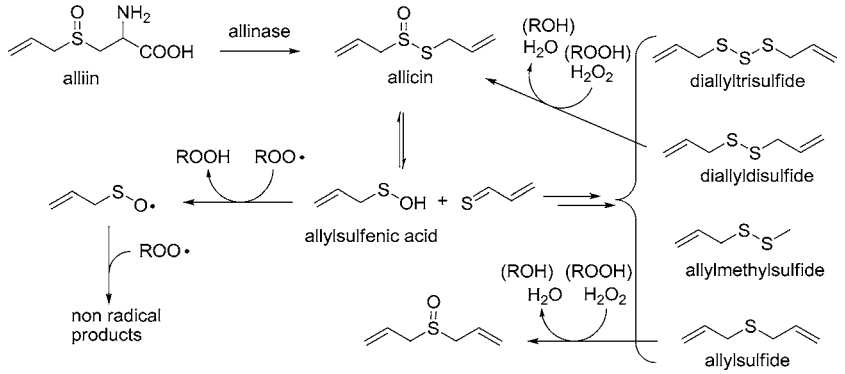


Figure 2. Induction time for the peroxidation of lard at 100 °C in the absence (control) and in the presence of 0.16% (w/w) of the essential oils of *Origanum vulgare* L. spp. *hirtum*, *Thymus vulgaris* L., *Thymus serpyllum* L., *Satureja montana* L., and *Satureja cuneifolia* Ten. or of their hydrocarbon and phenolic fractions. Data were taken from ref 15.

Oregano (*Origanum vulgare* L.) EO, containing 67% thymol + carvacrol and ∼14% terpinene (sum of α- and γ-), offers an example of synergism among EO components. Whereas its isolated hydrocarbon fraction offered no protection to lard and the oxygenated fraction (containing ∼94% thymol + carvacrol) performed not differently from isolated carvacrol or thymol, the whole oil protected lard from oxidation more efficiently than any fraction or component used alone at the same total concentration. On the other hand, savory (*Santureja montana*) EO, containing ∼50% thymol + carvacrol and only 6% γ-terpinene, afforded the same protection as its oxygenated fraction (∼ 70% carvacrol + thymol), which was slightly less effective than pure carvacrol or thymol, whereas the hydrocarbon fraction afforded negligible protection. In other words, savory's antioxidant behavior was simply that expected from the content of most effective components. EOs from two thymus species, *Thymus vulgaris* and *Thymus serpillum*, containing ca. 80% thymol + carvacrol (in different ratio) and ∼5.5% γ-terpinene each, had somewhat intermediate behavior (see Figure 2). At the opposite end was another savory species, *Satureja cuneifolia*, containing only 13% of phenols, no γ-terpinene, and a wealth of unsaturated terpenoids such as linalool. Its hydrocarbon fraction was largely pro-oxidant, counteracting the antioxidant behavior of the oxygenated fraction, so that the whole EO resulted in clear pro-oxidant behavior.

In general, care should be taken before assuming that the antioxidant property of EOs is simply that of one characteristic component. However consideration of its composition can allow roughly predicting the antioxidant potential: good antioxidant behavior can be expected from EOs having a large content in phenolics and modest content in unsaturated terpenes; even higher protection could come when the oil

Scheme 5. Formation of the Sulfides and Polysulfides Contained in the Garlic EO[a]



[a]The sulfenic acid formed during the transformation of allicin into the various allyl sulfides has a strong chain-breaking antioxidant activity, whereas sulfides and disulfides have preventive antioxidant activity due to their ability to reduce peroxides and hydroperoxides.

contains both large amounts of phenolics and good amounts of cyclohexadiene-like components (e.g., γ-terpinene). Oils having no or modest content in phenolic and cyclohexadiene-like components are likely to offer modest or no protection when mixed with edible fats.

One additional point should be considered concerning the antioxidant activity of EOs. Besides the botanical source, environmental (e.g., climatic) factors may affect the actual composition, reflecting different antioxidant activity with respect to literature data obtained with different specimens. This also applies to oils obtained with different extraction techniques. For instance, the content in eugenol in the oil from the leaves of *Pimenta dioica* was 77.4% when the EO was obtained by supercritical fluid extraction (SFE), whereas it was only 45.4% by hydrodistillation of the same specimen.[16] Although systematic investigations under comparable settings are lacking in this regard, a few studies have shown that the antioxidant activity of oils obtained from the same plant (e.g., savory) by different SFE methods might outperform that of EOs obtained by conventional steam distillation.[17] This can be explained by the modest volatility and partial water-solubility of phenolic components that are partly lost during hydrodistillation.

GARLIC AND SULFUR-CONTAINING VOLATILES

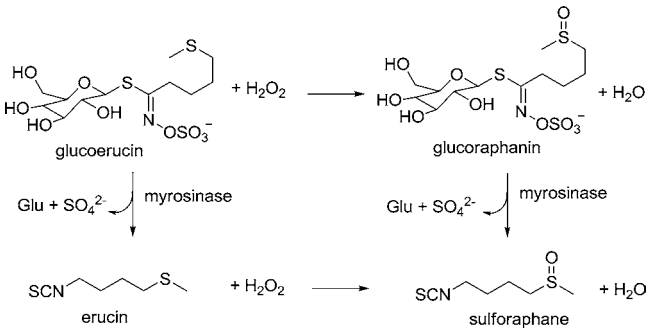
Allium species such as garlic (*Allium sativum*), onions (*Allium cepa*), shallots (*A. cepa* var. *aggregatum*), leeks (*Allium ampeloprasum*), scallions (*Allium fistulosum*), and others yield (better by extraction) oils having chemical composition largely different from common EOs, which are often not listed among essential oils due to the unpleasant flavor and lack of interest in the preparation of fragrances. They are mainly composed of sulfur-containing volatiles that have been reported to possess a wealth of biological properties attributed to their antioxidant activity,[18,19] which, in turn, has been confirmed in different model systems (although the actual performance seems to largely depend on the chosen system and the method of EO preparation).[20,21] Many such volatile constituents are not originally present in the plant, for example, in garlic, but they are formed during chopping of garlic cloves by the action of the enzyme allinase, which transforms the amino acid alliin in the thiosulfinate allicin (Scheme 5). Allicin is rather unstable and decomposes to a variety of sulfur-containing compounds through the formation of allylsulfenic acid as transient intermediate.[22] The composition of garlic extracts depends strictly on the preparation procedures.[23] Garlic EO obtained by water and steam distillation contains principally diallyl trisulfide (~50%), diallyl disulfide (~25%), methylallyl di- and trisulfides, and diallyl sulfide in smaller amounts.[20] When used as inhibitors of the controlled autoxidation of isopropylbenzene or styrene, diallyl disulfide and allylmethyl sulfide did not show any relevant antioxidant activity, suggesting that they are oxidized together with the oxidizable substrate.[24] On the other hand, allicin, contained in fresh garlic homogenates, has been demonstrated to have very strong antioxidant activity, due to the formation of its unstable degradation product allylsulfenic acid, which is an excellent radical-trapping agent.[25,26] Similar antioxidant chemistry has been described for other thiosulfinates, such as (*S*)-benzyl phenylmethanethiosulfinate found in *Petiveria alliaceae*, that serve as a dynamic reservoir of unstable sulfenic acids, the "true" antioxidants able to quench peroxyl radicals with $k_{inh} > 10^7$ M^{-1} s^{-1}.[25,27] Therefore, as summarized in Scheme 5 the antioxidant chemistry of sulfur-containing EOs from *Allium* and related genera is due to a direct chain-breaking activity that is expressed only upon conversion of the inactive components into thiosulfinates that ultimately yields the "active" sulfenic acid. The antioxidant activity is also supported by a preventive mechanism, the reduction of hydroperoxides and hydrogen peroxide, that could otherwise initiate peroxidation.

Other natural substances containing divalent sulfur atoms may similarly act as preventive antioxidants by decomposing hydroperoxides by a nonradical pathway. Glucosinolates from daikon (*Raphanus sativus* L.)[28,29] and rocket (*Eruca sativa* Mill.)[30] are able to reduce H_2O_2 and organic hydroperoxides to water and alcohols, respectively, at the expense of a S(II) atom, which is oxidized to sulfoxide S═O (see Scheme 6). The same chemistry is responsible for the direct (preventive) antioxidant activity of volatile isothiocyanates (ITCs) released from Brassicaceae upon chopping or grinding the vegetables or seeds, by enzymatically (myrosinase) induced decomposition of glucosinolates.[31]

INDIRECT ANTIOXIDANTS IN ESSENTIAL OILS

Volatile ITCs characteristic of brassica seeds, sprouts, and mature vegetables[32] have been shown to possess a wealth of biological properties, including anti-inflammatory, detoxifying, and cancer-protecting, mainly attributed to their antioxidant activity.[33] Their antioxidant activity is, however, only minimally explained by their preventive decomposition of hydroperoxides

Scheme 6. Decomposition of Hydrogen Peroxide by Glucoerucin, a Glucosinolate Found in Rocket (*Eruca sativa* Mill.), and by the Derived Volatile Isothiocyanate Erucin



(vide supra), which is only possible for those ITCs such as erucin having a divalent sulfur in the hydrocarbon chain; hence, it cannot be expressed by some of the most active components such as sulforaphane (from broccoli).[30] It was instead clarified that this arises in living systems from transcriptional induction of antioxidant enzymes such as the NAD(P)H:quinone oxidoreductase (NQO1), glutathione peroxidase (GPx), glutathione reductase (GR), thioredoxine reductase (THR), and others, through the Keap1-Nrf2-antioxidant responsive element (ARE) signaling pathway, with modulation of cellular glutathione levels.[31] Similar indirect antioxidant activity, based on the same signaling pathway, has been shown for garlic volatiles.[34] Nuclear factor erythroid-2-related factor 2 (Nrf2), a transcription factor, is normally inactivated by binding with Kelch-like ECH-associated protein-1 (Keap1) in the cytoplasm. ITCs and garlic volatiles such as ajoene cause the release of Nrf2 that translocates in the nucleus and binds to ARE elements of target genes, activating the transcription, hence increasing the level of antioxidant enzymes. Several other essential oils, with prevailing terpeneoid/phenylpropanoid composition, have been found to modulate enzyme activity (including phase I and phase II systems) involved in cellular redox homeostasis. For instance, caraway EO was found to modulate cytochrome P4501A1 and glutathione *S*-transferase (GST) in rats,[35] *Wedelia chinensis* (Osbeck) EO was shown to enhance the activity of catalase (CAT), superoxide dismutase (SOD), and GPx, as well as glutathione levels in mice,[36] whereas the same activity was recently shown for lavender EO in rats[37] and for the EO of *Alpiniae zerumbet* (ginger family) in human endothelial cells.[38] GPx activity is also enhanced in vivo by fennel.[39] Although these activities are very relevant in determining EOs' biological properties and medicinal potential, they cannot be exploited in the antioxidant protection of food or oxidizable materials; therefore, they will not be further discussed.

METHODS USED TO MEASURE ANTIOXIDANT ACTIVITY

The capability of a compound to inhibit spontaneous oxidative degradation of a substrate is described by two *distinct* parameters: the stoichiometric factor (also termed by some as "antioxidant capacity"), which is the number of radicals trapped by one antioxidant molecule, and the reactivity, the most important in determining the antioxidant activity, which depends on the rate constant of the reaction between antioxidants and the chain-carrying radicals.[8,40] In principle, the best antioxidant test consists of evaluating the effects that a compound can have on the oxidation of a substrate which is subjected *to the same conditions found in real systems*. However, spontaneous oxidations are usually slow at room temperature, and thus it may take weeks or even months to see any appreciable effects on the oxidation kinetics. The study of the inhibited process would likely be unpractical in many cases. Therefore, several methods have been put forward to have an estimate of the antioxidant activity, and their differences often consist of the degree of simplifications made with respect to real oxidative processes.

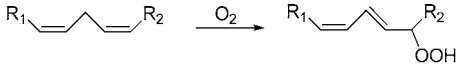
Methods Based on Inhibited Autoxidation. These methods, which resemble more closely real autoxidation processes, are based on the measurement of the rate of oxidation of a lipid/substrate in the presence and absence of antioxidants. The rates of oxidation provide the reactivity of antioxidants toward the peroxyl radicals, whereas the stoichiometry of the antioxidant can be derived from the length of the inhibition period.[9,26] The oxidizable substrate may be represented by pure compounds (e.g., linoleic acid, styrene, isopropylbenzene) or by natural lipid mixtures, such as those present in egg yolk and lard. In the latter case, a certain degree of unpredictability could arise from the variability of the composition of the oxidizing mixture and from the presence of endogenous antioxidants (unless they are removed in advance).[40] Spontaneous initiation is an uncontrolled process that depends on the presence of traces of hydroperoxides and transition metals and on the exposure to light and/or heat. To reduce the reaction time and improve the reproducibility, initiation can be accelerated by increasing the temperature and/or by adding Fe^{2+} or Cu^{2+} ions, H_2O_2 or azoinitiators, such as lipid soluble AIBN, or water-soluble AAPH ((2,2′-azobis(2-amidinopropane) dihydrochloride).[40] The latter are particularly useful because their homolytic decomposition proceeds at a constant rate, which depends on the temperature only, and therefore, azoinitiators provide a constant initiation rate during the entire course of autoxidation.[8,9]

Autoxidations can be followed by measuring either the disappearance of one of the reagents (usually O_2) or the formation of the products (primarily hydroperoxides and other molecules formed by decomposition of hydroperoxides). The main methods based on inhibited autoxidation are summarized below.

Oximetry methods rely on the determination of O_2 uptake in a closed system by using either a pressure gauge[26,41,42] or a polarographic probe[8,43] and are particularly convenient because they allow one to measure exactly the rate of oxidation. Most of the kinetic data relative to the reactivity of antioxidants with peroxyl radicals have been obtained in this way.[8,10,14]

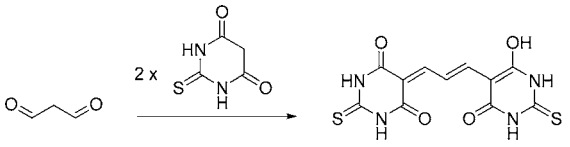
Hydroperoxides, early end-products of the lipid oxidation, can be quantified by iodometric titration (eq 7)[44] or by reaction with triphenylphosphine. Recently, a triphenylphosphine–coumarin conjugate has been used as a fluorescent probe to measure hydroperoxide formation.[25,45] In the case of the autoxidation of natural polyunsaturated lipids, the formation of the typical absorption band of conjugated dienes (eq 8) can be monitored by spectrophotometry.[46] HPLC-UV analysis of the mixture under oxidation may represent a more powerful alternative to simple spectrophotometry.[47,48]

$$2\,I^- + H_2O + ROOH \longrightarrow ROH + 2\,OH^- + I_2 \quad (7)$$



(8)

The TBARS (thiobarbituric acid reactive species) method is based on the spectrophotometric measurement of the pink-colored adduct of 2-thiobarbituric acid ($\lambda_{max} \approx 532$ nm) with malondialdehyde, which is one of the several end-products formed by the further oxidation and decomposition of polyunsaturated lipid hydroperoxides (eq 9).[49,50] However, this method suffers from limitations due to reactions of TBA with other compounds not related to lipid peroxidation, so that overestimation of malondialdehyde may occur.[51]



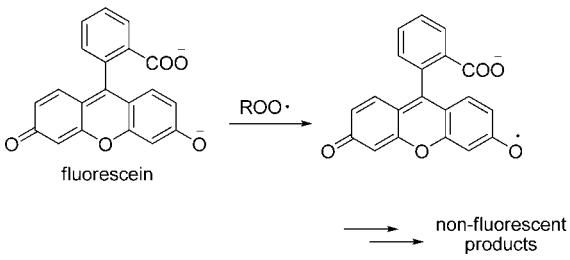
(9)

Volatile oxidation products such as hexanal, which are formed after the decomposition of unsaturated lipid hydroperoxides, can be analyzed by headspace gas chromatography monitoring the progress of autoxidation.[52,53] Similarly, 4-hydroxynonenal (4-HNE) can be analyzed by GC-MS or HPLC after appropriate derivatization.[54,55]

The Rancimat apparatus measures the release of volatile acids, formed upon advanced oxidation of fats (usually lard) under an air stream at 90−120 °C, by a conductometric method.[15,50] The antioxidant activity is normally described as a function of the induction time observed in the oxidation profiles. Thus, this method provides only an estimate of the antioxidant stoichiometry combined with threshold reactivity. The high temperature might cause the loss of low-boiling antioxidants, resulting in underestimation of their activity.

Methods Based on Competitive Probe Bleaching. In the quest for more practical ways to study antioxidant activity, several methods based on the study of the kinetics of nonchain radical processes have also been suggested. In these systems, antioxidants compete for the peroxy radical with a reference free radical scavenger, which can be easily detected by UV−vis or fluorescence spectroscopies. A common limitation of these methods derives from the practice to calculate the antioxidant activity from the area below the curve of fluorescence or absorbance versus time or from a single measurement after a fixed time lapse. Some authors have emphasized that these indices have no clear physical meaning as they depend both on the reactivity and on the stoichiometry of the antioxidant.[40]

ORAC (oxygen-radical antioxidant capacity) is a popular method used to estimate the content of antioxidants in food. The antioxidant competes with a fluorescent probe (e.g., phycoerythrin or fluorescein) for quenching peroxyl radicals generated from AAPH, a water-soluble thermal azoinitiator (eq 10).[40]

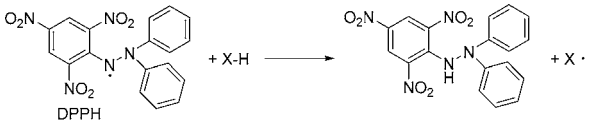


(10)

The β-carotene bleaching test consists in measuring the decay (after a fixed time) of the absorption at 470 nm due to β-carotene under a flux of free radicals and in the presence or absence of antioxidants.[14,40,56]

Indirect Methods. In these methods, colored persistent radicals or metal cations are used as probes. They are reduced by the antioxidant, and the color change in the solution is measured by UV−vis spectrophotometry. The main limitations of these tests are that the probes are chemically very different from the radicals responsible for the autoxidation of real systems and because of the absence of any oxidizable substrate (i.e., the substrate that should be protected by the antioxidant). The result indicates a "radical trapping power" rather than true antioxidant activity. This means that molecules able to reduce these probes are not necessarily able to stop the oxidative chain. Single-point measurements of absorbance decay after a fixed time (which usually arbitrarily varies from laboratory to laboratory) further reduce the meaning of these methods.[40]

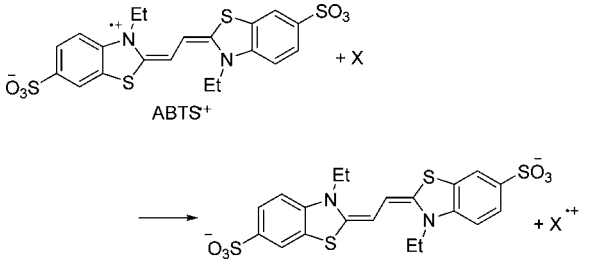
DPPH Test. An antioxidant, or any molecule with a weak X−H bond, reacts with the colored and persistent radical DPPH (2,2-diphenyl-1-picrylhydrazyl, $\lambda_{max} \sim 520$ nm) causing discoloration of the solution (eq 11).[49,50] Results are commonly expressed as IC_{50}, defined as the concentration of the potential antioxidant needed to decrease by 50% the initial absorbance of the colored radical. Because it depends on the reaction time, taken alone this parameter does not provide meaningful information of the actual reactivity of the antioxidant; furthermore, data can only be compared when obtained under identical settings.



(11)

Due to the similar electronic configuration between DPPH and peroxyl radicals, the significance of this method could be greatly improved under appropriate settings, particularly by monitoring the entire time evolution of the reaction[8] instead of performing single-point measurements (see next section).

TEAC Test (Trolox-Equivalent Antioxidant Capacity). The antioxidant, or any reducing agent X, reacts with the colored and persistent radical $ABTS^{\bullet+}$ (2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)), which has a strong absorption band in the range 600−750 nm (eq 12). Discoloration is compared with that produced by Trolox.[40] It suffers from limitations similar to those of the DPPH test.



(12)

FRAP Test (Ferric Reducing Antioxidant Power). The antioxidant reduces Fe^{3+} to Fe^{2+}, which forms a colored complex (590 nm) with 2,4,6-tripyridyl-*s*-triazine.[50]

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Folin−Ciocalteu Test. The antioxidant is oxidized in a basic medium formed by a mixture of tungstate and molybdate (Folin−Ciocalteu reagent) with the consequent formation of colored (750 nm) molybdenium ions, MoO^{4+}. Gallic acid is used as reference.[50] In this, like in the previous (FRAP) test, no radical reaction occurs; hence, these tests simply indicate some reducing ability of the potential antioxidant.

SELECTION OF THE TESTING METHOD: THE CASE STUDY OF ROSEMARY ESSENTIAL OIL

Because not all of the methods summarized above have the same chemical soundness, knowledge of their limitations is required to have meaningful results. To illustrate the problem, we collected from the literature the results of several studies on the antioxidant performance of *Rosmarinus officinalis* EO, determined by different methods (Table 1).[49,50,56−58]

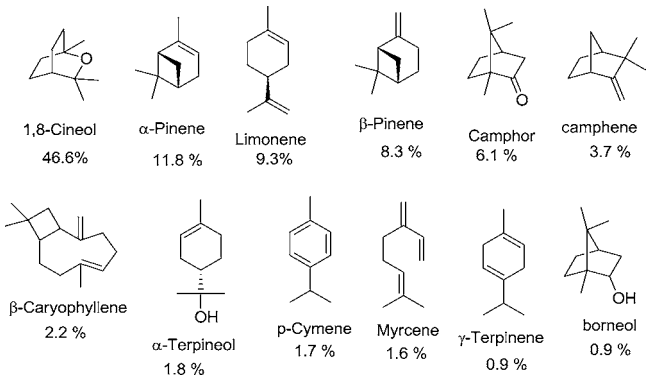
Table 1. Results from Different Tests To Measure the Antioxidant Activity of *R. officinalis* Essential Oil

assay	antioxidant effect	ref
DPPH bleaching	yes, stronger than BHT	49
	yes, but weaker than BHT	50
	yes, slightly weaker than thymus EO	56
deoxyribose oxidation by Fe^{2+}/H_2O; TBARS[a]	yes, stronger than BHT	49
oxidation of membrane lipids initiated by Fe^{2+}/H_2O, TBARS[a]	yes, but weaker than BHT	49
oxidation of membrane lipids initiated by Fe^{2+}/ascorbate; TBARS[a]	yes, stronger than BHT	49
β-carotene bleaching	yes, slightly weaker than thymus EO	56
FRAP (ferric reducing antioxidant power)	yes, but weaker than BHT	50
oxidation of egg yolk phospholipids initiated by ABAP,[b] TBARS[a]	yes, but lower than BHT	57
spontaneous oxidation of hexanal	no, absent or modest inhibition compared to BHT	58
oxidation of methyl linoleate, conjugated dienes detection	no, absent or modest inhibition compared to BHT	58
oxidation of phospholipids initiated by Fe^{2+}, TBARS[a]	no, much lower than BHT	50
Rancimat	no, very low or prooxidant	50
spontaneous oxidation of egg yolk phospholipids, TBARS[a]	no, much lower than BHT	57
spontaneous oxidation of rat liver homogenate, TBARS[a]	no, much lower than BHT	57
oxidation of rat liver homogenate initiated by ABAP,[b] TBARS[a]	no, absent or modest inhibition compared to BHT	57

[a]Detection of the thiobarbituric reactive species. [b]2,2′-Azobis(2-amidinopropane) dihydrochloride, a water-soluble azoinitiator.

Rosemary EO has been the subject of intense study, because, thanks to its characteristic scent, it may be used to enrich the flavor while prolonging the shelf life of meats. Consideration of the composition of the oil, as reported by Ruberto et al. (Scheme 7),[57] reveals no component with predictable high antioxidant activity (e.g., phenols). The absence of phenolic components (such as thymol, carvacrol, or eugenol) and the very limited content of cyclohexadiene derivatives (α- and γ-terpinene) are constant features of rosemary EO specimens employed in all of the studies collected in Table 1. Actually, such a composition fully justifies the results of the work by Lee and co-workers,[58] who conclude that rosemary EO is not an antioxidant, at variance with rosemary hydroalcoholic extract that has a powerful antioxidant effect, due to the nonvolatile phenols carnosol, carnosic acids, and rosmarinic acid, which are completely removed during EO preparation. Surprisingly, it can be seen in Table 1 that only 7 of 14 tests give the correct answer. Because it is common practice in the scientific literature to classify an essential oil as an antioxidant if the majority among three to four tests gives a positive result, rosemary EO may be judged as antioxidant in some papers whereas the opposite conclusion is reached in others, depending on the chosen tests.

Scheme 7. Main Components of Rosemary EO Accounting for 91.6% of EO's Ccomposition (Data Taken from Reference 57)



From a closer look at Table 1, tentative explanations for such confusing outcomes can be put forward. Rosemary EO seems to have some reactivity toward the colored DPPH radical, although smaller than that of thyme EO, which contains phenols with proven antioxidant power. The reactivity of rosemary EO with DPPH is not surprising, considering that DPPH is able to react also with hydrocarbons, by abstracting an H atom from C−H bonds with a sufficiently low bond dissociation enthalpy (BDE). This, however, does not indicate antioxidant activity, as carbon-centered radicals, formed after H atom abstraction by DPPH (or by $ROO^{\bullet}$ in real settings), react with O_2 to form peroxyl radicals that propagate the oxidative chain. Therefore, the discoloration of DPPH in the presence of rosemary EO does not indicate antioxidant activity, but rather the presence of highly oxidizable compounds in this EO, such as α- and β-pinene or limonene. For instance, the BDE of the weakest C−H bond of α-pinene has been estimated to be very similar to that of the N−H bond of DPPH-H (80.7 and 78.9 kcal/mol, respectively),[4] indicating that the H atom transfer is thermodynamically feasible. The interference given by hydrocarbon oxidation is amplified by the common practice to follow DPPH bleaching for long periods (usually for about 30 min) as the reaction of DPPH with C−H groups is intrinsically much slower than that with "true" antioxidants. For instance, the second-order rate constant at 298 K in CCl_4 for reaction of DPPH with α-tocopherol, having and O−H BDE of 77.2 kcal/mol,[8] was measured as $k = 3.6 \times 10^3$ M^{-1} s^{-1},[59] whereas the corresponding reaction with 1,4-cyclohexadiene, having almost identical enthalpy change (BDE_{C-H} = 326.3 kJ/mol corresponding to 77.9 kcal/mol),[60] proceeded with $k = 1.3 \times 10^{-3}$ M^{-1} s^{-1} under identical settings,[61] being over 1,000,000-fold slower. We suggest that this problem may be circumvented, or at least minimized, by following DPPH discoloration for a short

Table 2. Antioxidant Activity of Selected Essential Oils

essential oil	main components[a]	assay[b]	activity	ref
oregano (*Origanum vulgare* L.)	thymol, carvacrol, γ-terpinene	Rancimat	good, but lower than BHT	15, 50
		TBARS	good, comparable to BHT	50, 64
thyme (*Thymus vulgaris* L.)	thymol	Rancimat	good, but lower than BHT	15, 50
		TBARS	good, comparable to BHT	50
		volatile aldehydes	medium	53
		TBARS, hydroperoxides	good, but lower than BHT	81
wild thyme (*Thymus serpyllum* L.)	carvacrol, thymol, γ-terpinene, *p*-cymene	Rancimat	medium	15
winter savory (*Santureja Montana* L.)	thymol, *p*-cymene, γ-terpinene, carvacrol	Rancimat	medium	15
cuneate Turkish savory (*Santureja cuneifolia* Ten.)	linalool, germacrene D, α-pinene, thymol	Rancimat	low	15
clove (*Syzygium aromaticum* L.)	eugenol	Rancimat	good	50, 65
		TBARS	good, comparable to BHT	50
		volatile aldehydes	good	53
		TBARS, hydroperoxides	good, but lower than BHT	81
sage (*Salvia officinalis* L.)	α-thujone, camphor, viridiflorol	Rancimat	very low or pro-oxidant	50,65
		TBARS	low	50
		TBARS, hydroperoxides	low, much lower than BHT	81
rosemary (*Rosmarinus officinalis* L.)	α-pinene, limonene, camphor	Rancimat	very low or prooxidant	50
		TBARS	very low	50
		TBARS, hydroperoxides	very low, lower than sage	81
sweet basil (*Ocimum basilicum* L.)	linalool, estragole, methyl cynnamate, eugenol	Rancimat	very low	65
		volatile aldehydes	very low	53
bush-basil (*Ocimum minimum* L.)	eugenol, α-terpinolene, 1,8-cineole	TBARS	good	82
coriander (*Coriandrum sativum* L.)	linalool, α-pinene, camphor, *p*-cymene	TBARS	low (lower than bush-basil)	81
celery (*Apium graveolens* L.)	β-selinene, phellandral, limonene	TBARS	very low (lower than coriander)	81
fennel (*Foeniculum vulgare* Mill.)	estragole, α-pinene, α-phellandrene	Rancimat	no (prooxidant)	65
		volatile aldehydes	very low	53
green anise (*Pimpinella anisum* L.)	*trans*-anethol, estragole	volatile aldehydes	very low	53
tarragon (*Artemisia dracunculus* L.)	estragole, *p*-cymene	volatile aldehydes	very low	53
parsley (*Petroselinum sativum* Hoffm.)	apiol, myristicine, α-pinene	volatile aldehydes	very low	53
marjoram (*Marjorana hortensis* Moench.)	terpinen-4-ol, γ-terpinene, α-terpinene, α-terpineol	Rancimat	no	65
mint (*Mentha piperita* L.)	isomenthone, neomenthol, 1,8-cineole	Rancimat	no (prooxidant)	65
caraway (*Carum carvi* L.)	carvone	TBARS, hydroperoxides	low, much lower than BHT	81
black cumin (*Nigella sativa* L.)	thymoquinone,[c] carvacrol	TBARS	very good (better than quercetin)	83
cumin (*Cumin cyminum* L.)	cuminaldehyde	TBARS, hydroperoxides	very low, lower than sage	81

[a]Compositions of essential oils are from refs 15, 49, 53, 57, 65, 81, and 82. [b]See text for a description. [c]Results may have been due to reduction of thymoquinone to the corresponding hydroquinone by ascorbate during the assay.

time period (2−5 min) and by using relatively small quantities of EO. In this way, only fast reactions occurring with "true" antioxidants are detected. Even better, the actual kinetics of reaction should be investigated by monitoring the full time evolution of DPPH absorbance,[62] in place or reporting the percent discoloration after a fixed time or IC_{50} values. Bleaching of the $ABTS^{\bullet+}$ radical and reporting data as percent bleaching compared, for instance, to Trolox (e.g., TEAC test) suffer from similarly large limitations and should be discouraged.

A second problem arises with the use of Fe^{2+} (or other transition metal) salts as radical sources, usually in combination with peroxides or reducing agents, to initiate the oxidation of unsaturated lipids. Although these tests are formally based on lipid peroxidation, and are therefore expected to afford more meaningful results than indirect methods (see previous section), the harsh process used to initiate the reaction reduces their meaningfulness. Via the Fenton reaction, they produce $HO^{\bullet}$ radicals, which are known for being extremely reactive

toward almost any organic substrate. Rosemary EO can therefore be co-oxidized with unsaturated lipids and may show an apparent antioxidant activity, especially when used in large concentrations.

Indeed, the third major warning is about the concentration of EOs used in these tests. By definition, an antioxidant or the EO under study should be used at concentrations much lower than that of the unsaturated lipids it is supposed to protect, normally at micromolar to millimolar levels, however, normally not larger than 1%. (On the basis of our own experience, this is also the largest concentration used to stabilize lipids in foods that would maintain acceptable organoleptic properties.) When tests are performed using unrealistic larger amounts, EOs having highly oxidizable components (such as rosemary) can be co-oxidized with unsaturated lipids, and this would result in an apparent inhibition. One example of such a kinetic mislead has recently been discussed by one of us.[24] Besides these major points, the outcome of antioxidant activity tests can be influenced also by the solvent[63] and by partition of the antioxidant in the reaction mixture, which often consists of biphasic or emulsified systems.

On the basis of previous discussion, the best way to estimate the antioxidant activity is to measure the rate of oxidation of polyunsaturated lipids under controlled conditions both in the absence and in the presence of *small* amounts of the investigated essential oils. The oxidation should start spontaneously or be initiated by azo compounds under controlled conditions and may be followed by measuring the consumption of O_2[41] or the formation of oxidized products (see previous section). In case the temperature has to be raised (e.g., in the Rancimat test), care should be taken to minimize the evaporation of EOs' components.

Methods that should be used with caution, only as a preliminary screening, are those based on indirect methods and competitive probe bleaching. Tests based on redox reactions occurring in the absence of both radical species and oxidizable lipids (e.g., FRAP or Folin−Ciocalteu) should be avoided to assess antioxidant activity.

ANTIOXIDANT ACTIVITY OF SELECTED ESSENTIAL OILS

In line with the considerations expressed in the previous section, we have summarized in Table 2 selected recent studies on the antioxidant activity of essential oils performed with meaningful techniques based on the inhibited oxidation of lipids.[24,50,64−66] Results are expressed qualitatively using BHT as common reference, because, being obtained with different methods, numerical comparison is not possible.

PRACTICAL APPLICATIONS AND TESTS IN REAL SYSTEMS

Essential oils are promising food stabilizers in those cases when their aroma is not in contrast with the organoleptic characteristics of food.

Goulas et al. showed that oregano EO, in addition to modified atmosphere and salting, was able to extend the shelf life of sea bream and to reduce the formation of volatile amines and of TBAR compounds.[67] A 1:1 mixture of thymol and carvacol and pretreatment with electrolyzed NaCl solutions extended the shelf life of carp fillets from 4 to 16 days at 5 °C.[68] Oregano EO was also shown to protect extra virgin olive oil from oxidation during storage.[69]

In the previous section we have discussed predictive tests of antioxidant activity based on (simplified) model systems, such as homogeneous lipid solutions. Although those tests allow the most accurate characterization of antioxidant behavior, they might be quite far from real systems, such as food. Hence, direct testing on food products could bring additional information. We suggest, however, to be careful in drawing conclusions from tests on the protection of complex food systems, especially if they have not been preceded by meaningful studies in model systems, as illustrated in the following examples.

In the case of porcine and bovine meat, essential oils seem to protect food from oxidation almost irrespectively of their direct (chain-breaking) antioxidant activity. For example, Fasseas and co-workers found that minced meat samples were protected from autoxidation, assessed by measuring TBARS formation, by both oregano and sage EOs.[70] This observation is intriguing considering that whereas oregano is rich in phenolic antioxidants, sage EO has no chain-breaking antioxidant activity. From a mechanistic perspective, we suggest that the antioxidant protection shown by sage EO is indirect, being actually a consequence of its well-known bacteriostatic and fungistatic activities,[71] thereby preventing food spoilage (including oxidation) caused by microorganisms. Indeed, Estevez and co-workers investigated the effect of essential oils on spoilage of porcine liver pate and frankfurters, finding that sage and rosemary EOs (both without direct antioxidant activity) were able to reduce the formation of TBARS and volatile compounds during storage of the meat samples.[72] Interestingly, on the other hand, one different study revealed a pro-oxidant effect in consequence of the addition of high levels of rosemary EO to meat.[73] In discussing food preservation (as opposed to antioxidant activity in simpler systems), it should not be overlooked that there is a deep connection between oxidative spoilage and bacterial/fungal metabolism. Consequently, it is often difficult to distinguish between different preserving mechanisms, antimicrobial versus antioxidant, and observed oxidative protection often arises from the interplay between the two activities.

Recent and promising applications of EOs are in active packaging and edible coatings. Active packaging refers to systems having active functions beyond the containment and protection of the product. Essential oils rich in eugenol, thymol, and carvacrol (as well as the isolated terpenes) were proposed as components of active packaging to reduce the microbial decay and to preserve the antioxidant characteristics of table grapes,[74] strawberries,[75] and bayberries.[76] In these experiments, a small amount of the investigated essential oils was introduced inside the packaging, so that only vapors of EOs were in contact with food. Another possibility is to introduce antioxidant EOs directly in the polymer films, so that it can diffuse in the inner atmosphere, as recently reported, for instance, by Park et al.[77] Controlled transfer of some EO components into the food is yet another more sophisticated approach in active packaging, which could be used to induce antimicrobial as well as antioxidant protection together with amelioration of the aroma.[78]

Edible coatings are constituted by polysaccharides, proteins, and lipids that are used to preserve the freshness of the product, and the incorporation of active compounds in these matrices allows bioactive coatings to be obtained.[79] Edible films of chitosan incorporated with the EO of thymus showed good antioxidant as well as antibacterial effect.[80]

In conclusion, from the above examples it is clear that the antioxidant activity of some EOs, particularly oregano and thymus (together with the phenolic components thymol, carvacrol, and eugenol), does have the potential for actual application in real food systems. The use of essential oils as natural antioxidants is a field of growing interest, especially in food science and in complementary medicine. On the other hand, there is a need for a more rational and standardized approach in experimental design so as to generate meaningful data that can be compared among different research groups and easily transferred to the actual application of EOs for the preservation of food as well as the manufacture of health-oriented products.

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Notes

The authors declare no competing financial interest.

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