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ANALYSIS OF ANTIOXIDANT SYNERGISM AND ITS MECHANISMS IN DIFFERENT FOOD SYSTEMS

A Dissertation Presented

by

IPEK BAYRAM

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Food Science

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DEDICATION

Dedicated to my family and friends, who supported me during this amazing journey.

ACKNOWLEDGMENTS

First and foremost, I would like to thank my academic advisor, Dr. Eric A. Decker, for his invaluable guidance, support, and patience throughout my degree, as well as for being very encouraging and considerate at all times. I am extremely fortunate to have such a wonderful mentor! I would also like to thank my committee members, Dr. David Julian McClements, Dr. Jiakai Lu, and Dr. Pierre Villeneuve, for their helpful suggestions and advice. I would like to express my gratitude to Jean Alamed, who has always been extremely helpful, kind, and friendly. A gigantic thank you to the University of Massachusetts Amherst and the Department of Food Science for providing an atmosphere for numerous career advancement and collaboration opportunities. In addition, many thanks to my coworkers, lab mates, all past & present professors and teachers for their support, guidance, and friendship.

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ABSTRACT

ANALYSIS OF ANTIOXIDANT SYNERGISM AND ITS MECHANISMS IN DIFFERENT FOOD SYSTEMS

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Lipid oxidation results in off-flavors, toxic aldehydes, and co-oxidation of proteins and color compounds. Combining antioxidants to achieve synergistic interactions has been practiced for decades to improve oxidative stability. Nevertheless, synergism mechanisms have been poorly understood and rarely studied. This dissertation examines the mechanisms of antioxidant synergism in a model system with α-tocopherol (α-TOC) and myricetin (MYR). The interactions between α-tocopherol and taxifolin (TAX) were also tested because it has structural similarities to myricetin but has a higher redox potential.

The first part of this research focused on the antioxidant interactions between α-tocopherol and myricetin in stripped soybean oil-in-water emulsions at pH 4.0 and pH 7.0. At pH 7.0, α-tocopherol: myricetin ratios of 2:1 and 1:1 yielded interaction indices of 3.00 and 3.63 for lipid hydroperoxides, and 2.44 and 3.00 for hexanal formation, indicating synergism. Myricetin's ability to regenerate oxidized α-tocopherol and slow its degradation was identified as the synergism mechanism. At pH 4.0, an antagonistic interaction was observed, which was associated with high ferric-reducing activity of myricetin in acidic environment. α-Tocopherol and taxifolin exhibited non-synergistic effects both in acidic

and neutral emulsions. This was due to taxifolin's inability to recycle α-tocopherol at both pHs, unlike myricetin, and taxifolin's high ferric-reducing activity at pH 4.0, similar to myricetin.

The second part of this research focused on antioxidant interactions between α-tocopherol and myricetin in stripped soybean oil. α-Tocopherol and myricetin ratios of 5:1, 2:1, 1:1, 1:2, and 1:5 resulted in interaction indexes of 1.14, 1.50, 1.55, 1.30, and 1.16, showing synergistic activity for both lipid hydroperoxide and hexanal formation. Synergism was also observed in phospholipid-containing bulk oils, both in the absence and presence of reverse micelles. α-Tocopherol and taxifolin, however, had additive effect at all antioxidant ratios. Antioxidant degradation results showed that myricetin delayed α-tocopherol oxidation, whereas taxifolin did not. These results revealed that myricetin's lower redox potential allowed it to cause synergism through regenerating oxidized α-tocopherol rather than by decreasing oxidation by metal chelation since both myricetin and taxifolin chelated iron. Thus, this study advises combining α-tocopherol with myricetin in bulk oils and neutral oil-in-water emulsions to improve oxidative stability and reduce food waste.

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LIST OF ABBREVIATIONS

MYR (myricetin)

TAX (taxifolin)

α-TOC (alpha-tocopherol)

TQ (α-tocopherylquinone)

SSO (stripped soybean oil)

MCT (medium chain triacylglycerols)

HPLC (high performance liquid chromatography)

GC (gas chromatography)

o/w emulsion (oil-in-water emulsion)

PTFE (polytetrafluoroethylene)

DI water (deionized water)

Na-EDTA (ethylenediaminetetraacetic acid (disodium salt))

E° (one-electron reduction potential)

BDE (bond dissociation enthalpy)

BHT (butylated hydroxytoluene)

BHA (butylated hydroxyanisole)

TBHQ (tert-butylhydroquinone)

ECG (epicatechin gallate)

EGC (epigallocatechin)

EGCG (epigallocatechin gallate)

DPPH (2,2-diphenylpicrylhydrazyl)

LDL (low density lipoprotein)

BSA (bovine serum albumin)

DC_4PC (1,2-dibutyryl-sn-glycero-3-phosphocholine)

DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine)

CHAPTER 1

INTRODUCTION

Despite more than 150 years of research, lipid oxidation remains a major challenge for the food industry due to the complexity of food products and the multiple elements that influence oxidation. Controlling lipid oxidation is crucial since it has a number of detrimental effects on food quality. The lipid oxidation reactions produce volatile chemicals such as aldehydes, ketones, and alcohols, which cause rancidity. The free radicals and aldehydes produced by lipid oxidation also promote co-oxidation of other molecules, particularly proteins, vitamins, and pigments, influencing the nutritional quality, texture and appearance of the product. Aside from food quality concerns, current research has also suggested that lipid oxidation products can be toxic to biological tissues and can have a negative impact on gut microbiota (Lei et al., 2021).

Antioxidants are any molecules that can inhibit the deterioration of food products by minimizing lipid oxidation. Antioxidants can serve different actions in food, including scavenging free radicals, chelating metal ions and quenching singlet oxygen. Biological tissues have highly efficient endogenous antioxidant systems emerging from the need to protect the aerobic organisms against oxygen and its reactive species. However, many food processing operations can remove or degrade endogenous antioxidants in foods. For example, heating destroys antioxidant enzymes, steam strips out phenolic antioxidants and refining removes beneficial antioxidants from oil. Therefore, the incorporation of exogenous antioxidants into the food systems is often necessary, especially as food manufacturers try to make healthier products that are high in unstable unsaturated fatty

acids. Consumer desires for clean labels and increase in health concerns related to synthetic antioxidants have prompted the food industry to find alternative ways to minimize lipid oxidation to improve food quality and safety.

One of the most effective strategies used by the food industry is combining antioxidants. A combination of antioxidants results in three different types of interactions: synergism, additive effect, and antagonism. Synergism refers to the concept where combination of antioxidants enhances oxidative stability more efficiently than the sum of the individual antioxidant effects. Antagonism, on the other hand, refers to the concept that combining antioxidants makes them less effective than the sum of the individual compounds. Additive effect refers to the scenario in which combining antioxidants has no additional harm or benefit on the oxidative stability compared to the sum of the individual antioxidants. It should be noted that synergistic combinations are those in which one compound has little antioxidant activity but can increase the activity of the second antioxidant (e.g., phospholipid-tocopherol combinations). Combinations where both compounds have antioxidant activity can also be synergistic (e.g., myricetin-tocopherol combinations). While the former may show stronger synergism, the latter can produce better overall antioxidant activity because both antioxidants provide protection. Therefore, calculation of synergism by formulas such as interaction index (Panya et al., 2012; Culler et al., 2022), does not always indicate which antioxidant combinations are the most effective. Obtaining antioxidant synergism is beneficial for the food industry as effective combinations could: (1) prolong shelf-life; (2) protect foods with lower antioxidant concentrations which could lower costs, especially where one of the antioxidants may already be present in the food (e.g., tocopherols) and (3) improve nutritional quality and

food safety. Therefore, the food industry is testing a variety of antioxidant mixtures to obtain synergistic interactions.

Synergism is influenced by multiple factors, including type of food matrix, pH, antioxidant type/concentration/ratio, interactions with other food components, and processing conditions. Therefore, it is very difficult to determine which antioxidant combinations will exhibit synergistic effects in a given food system. Hundreds of papers on antioxidant synergism have been published so far, but the majority of them either fail to explain the reason for the synergistic activity or only suggest underlying mechanisms without providing experimental evidence. A few examples from research articles in which authors identified synergistic interactions but failed to provide an explanation for their occurrence include catechin/resveratrol (Skroza et al., 2015), kaempferol/myricetin (Hidalgo et al., 2010), and lycopene/vitamin E (Shi et al., 2007). It is essential to understand the mechanisms of synergism and the factors that can produce this synergism as this will facilitate the identification of which antioxidant combinations will work in different foods versus conducting numerous studies to screen antioxidant mixtures.

This dissertation research focuses on the mechanisms underlying synergistic antioxidant interactions in both heterogeneous (oil-in-water emulsions) and homogeneous (bulk oil) systems, employing model systems such as α-tocopherol and myricetin and α-tocopherol and taxifolin. To better understand the interactions between these antioxidant couples in various food systems, this study focused on (1) determining antioxidant synergism by analyzing the oxidative stability of foods by measuring lipid hydroperoxide and headspace hexanal formation and, calculating interaction indices based on lag phases, (2) determining whether myricetin (redox potential=360 mV) and taxifolin (redox

potential=500 mV) can recycle oxidized α-tocopherol (redox potential=500 mV) back to its original form, and understanding the effect of redox potentials on synergism, (3) determining the antioxidant degradation behavior of myricetin, taxifolin, and α-tocopherol in relation to fatty acid oxidation rates in order to determine potential antioxidant interactions, and (4) determining whether myricetin and taxifolin could chelate iron in both food systems, and whether this could help the synergism due to metal chelation, or whether it could cause antagonism if myricetin and taxifolin had ferric reducing activities to produce more pro-oxidant ferrous iron.

In complex food systems, it is often difficult to predict which antioxidant combinations will work synergistically. Multiple factors, including the chemical structure, concentration and ratio of antioxidants, type of food system, interaction of antioxidants with other food components, storage conditions, and external environmental factors may influence the type of antioxidant interaction. Understanding the mechanisms of synergism will aid the food industry in the production of effective antioxidant mixtures to improve oxidative stability and shelf-life, as well as in the development of simple, rapid, and reliable methods for determining and evaluating synergism. Being better able to predict synergisms will aid in the development and production of foods with unstable lipids (e.g., omega-3 fatty acids) and thus could improve the health and wellness attributes of the food supply and decrease food waste.

CHAPTER 2

LITERATURE REVIEW: LIPID OXIDATION

2.1 Importance of lipid oxidation and its consequences

Lipid oxidation is a series of reactions that affect food quality and consumer acceptance. The oxidation of lipids poses a significant challenge in the food industry during food production, handling, and storage because the reactions are difficult to control once they begin. Lipid oxidation reactions produce hundreds of molecules with varying physical and chemical properties. Each molecule has its own chemical reactivity, polarity, partitioning behavior, surface/interfacial activity, or thermal stability (McClements & Decker, 2000). These unique changes in the food matrix cause lipid oxidation to influence food products' overall quality by changing the flavor, texture, color, and nutrition. As a result, the food industry is looking for natural and cost-effective ways to reduce lipid oxidation reactions to improve product quality and safety while also extending shelf-life.

2.1.1 Impact on flavor

Lipid oxidation produces hundreds of small, volatile compounds that cause rancidity-related off-flavors. For example, linoleic acid oxidation produces hexanal, which imparts a fresh-cut grass flavor to the oil (Van Aardt et al., 2005). Similarly, the oxidation of omega-3 fatty acids produces *cis*-4-heptanal and *trans, cis*-2,4-heptadienal, which are responsible for the undesirable fishy aroma (Hartvigsen et al., 2000). Lipid oxidation may result in the formation of desired flavor compounds in some cases. Some oxidation is desired during frying, for example, because linoleic acid oxidation produces *trans, trans*-2,4-decadienal, which gives the food a deep-fried aroma (Mottram, 1998). Similarly, some

lipid oxidation is desired in cheese products to provide a distinct flavor and aroma profile (Nawar, 1996). However, most lipid oxidation reactions diminish the overall quality of the product; therefore, reactions should be minimized. Many of the end products of lipid oxidation have low threshold values in the range of parts per million and parts per billion, indicating that the undesirable flavor is easily detectable by humans. For instance, fish oil oxidation generates *trans, trans*-2,6-nonadienal and *trans, cis*-2,6-nonadienal, which have extremely low threshold values (0.001-0.002 ppm); thus, even slight oxidation will result in off-flavor and be detectable by the consumer (Frankel, 1984). Therefore, it is essential to minimize lipid oxidation reactions in order to preserve the food's original flavor.

Table 2.1. Flavor description of some lipid oxidation end-products. Adapted from Belitz and Grosch (1999).

End-products	Flavor Description
Hexanal	Tallowy, green, leafy
Nonanal	Tallowy, Soapy-fruity
2-*trans*-hexanal	Apple
2-*trans*-nonenal	Tallowy, cucumber
2-*trans*-decanal	Tallowy, orange
2,4-*trans, trans*-heptadienal	Fatty, oily
2,4-*trans, trans*-decadienal	Frying odor
4,5,2-*trans*-epoxy-*trans*-decenal	Metallic

2.1.2 Impact on texture

The oxidation of lipids results in the formation of highly reactive free radicals. This high energy may cause co-oxidation of other molecules by abstracting electrons from other compounds. Secondary lipid oxidation products, mainly unsaturated aldehydes, can react with primary amino groups on proteins (Burcham & Kuhan, 1996). These interactions

influence the hydrophobicity, solubility, water-holding capacity, and aggregation properties of proteins, thereby influencing the texture and functionality of the product (Hematyar et al., 2018). Toyosaki (2007) discovered that linoleic acid hydroperoxides interact with gluten proteins in dough, thereby accelerating gluten denaturation and tightening the network. In contrast, Patrignani et al. (2014) demonstrated that the formation of even minute quantities of hydroperoxides due to lipid oxidation increases the hardness of biscuits. Therefore, lipid oxidation must be regulated to ensure and preserve the food product's desired texture.

2.1.3 Impact on color

Pigments are the chemical compounds responsible for imparting color. Most pigments in food systems are susceptible to oxidation because they contain multiple double bonds (Rodriguez-Amaya, 1999). Co-oxidation of color pigments may result from lipid oxidation, leading to either color loss or the formation of new color compounds. This will negatively affect food quality and consumer acceptance. For instance, phenolics in food can donate hydrogen to lipid radicals generated by lipid oxidation, resulting in the formation of quinones (Parveen et al., 2010). These quinones are capable of polymerizing into melanin compounds, which are responsible for the brown color. Unsaturated aldehydes in meat can react with myoglobin to form metmyoglobin, which gives the product an unattractive dark color (Faustman et al., 2010). Similarly, the conjugated double bond structure in carotenoids is susceptible to destruction by free radicals, resulting in color loss (Vieira et al., 2017). Antioxidants have been found to preserve the color of meat products during storage by preventing lipid and protein oxidation. Jia et al. (2012) demonstrated that

the use of blackcurrant extracts inhibited oxidation in pork burgers, resulting in enhanced preservation of the meat's natural red color.

2.1.4 Impact on nutrition

Oxidation of lipids diminishes the nutritional value of food. Especially, vitamins are very susceptible to degradation during oxidation reactions because of their antioxidant effects, meaning that they sacrifice themselves to be oxidized before fatty acids. Tocopherol (Vitamin E) is a potent antioxidant in fats and oils because it can readily donate its phenolic hydrogen to lipid radicals to prevent their oxidation (Seppanen et al., 2010). This results in the rapid consumption of the original form of vitamin E, leaving the food deficient in tocopherols. Likewise, depending on the properties of the food system, ascorbic acid (Vitamin C) can function as an antioxidant. Ascorbic acid is rapidly consumed during lipid oxidation reactions in the presence of α-tocopherol because ascorbic acid regenerates α-tocopheroxyl radicals (Niki et al., 1984), which reduces the vitamin C content of the food matrix. Lipid oxidation reactions also impact the nutritional value of the food due to the loss of essential fatty acids such as α-linolenic acid and linoleic acid (Domínguez et al., 2019; Shahidi & Zhong, 2010). Secondary oxidation products, primarily aldehydes, can react with amino acids to form other complexes, resulting in a significant loss of essential amino acids (Guyon et al., 2016).

2.1.5 Impact on toxicity

Lipid oxidation reactions in food have created a significant concern due to the possibility of toxic effects in biological tissues, observed in various cell studies and animal

models. Chang et al. (1978) demonstrated that deep-frying produces hundreds of different lipid oxidation products, many of which have toxic effects. Some lipid oxidation products, such as malondialdehydes, have been shown to interact with DNA and amino acids, causing tissue damage by forming fluorescent brown pigments (Horton et al., 1987).

The oxidation of linoleic acid at high temperatures can form acrolein, a high-priority toxic chemical, and has been shown to cause blood vessel dysfunction and enhanced myocardial oxidative stress in mice models (Ismahil et al., 2011). Similarly, cholesterol oxidation produces oxycholesterols, which are associated with cytotoxicity, mutagenicity, and atherogenicity (Smith & Johnson, 1989). For example, an increase in plasma levels of oxidized LDL is linked to coronary heart disease due to the modification of proteins caused by lipid oxidation (Holvoet, 2004). Although significant results are showing the toxic effects of lipid oxidation products, the influence on human health is still inconclusive, possibly due to the natural defense mechanism of the human body and the presence of antioxidants in food and biological tissues to reduce the oxidative damage (Vieira et al., 2017).

2.2 Mechanisms of lipid oxidation

Lipid oxidation is a complex series of reactions. The lipid oxidation mechanisms and the factors triggering/minimizing the oxidation reactions depend on whether the system is bulk oil or emulsion. In general, lipid oxidation can be categorized into three main steps: initiation, propagation, and termination (Nawar, 1996).

During the initiation stage, the first step is the formation of free radicals from the fatty acid molecules (L-H) due to hydrogen abstraction. This hydrogen abstraction

(formation of radicals) can be due to external factors like high temperature, UV radiation, interactions with hydroxyl groups, or metal catalysts. The hydrogen abstraction on fatty acid molecules generally occurs from the methylene interrupted carbon between the double bonds because the energy of the carbon-hydrogen bond at this particular location is lower than the bond energy of a regular carbon-hydrogen bond, making hydrogen abstraction thermodynamically more favorable. When hydrogen is removed from fatty acids, free radicals, molecules with unpaired electrons, are produced. The free radicals formed from fatty acids are known as alkyl radicals (L•), and those are the lowest energy radicals among other radicals formed during the lipid oxidation process. The energy of alkyl radicals is 600 mV, which is expressed in terms of reduction potential. However, alkyl radicals are still high in energy and thermodynamically unstable, making them chemically reactive. This fastens lipid oxidation reactions and leads to the propagation step.

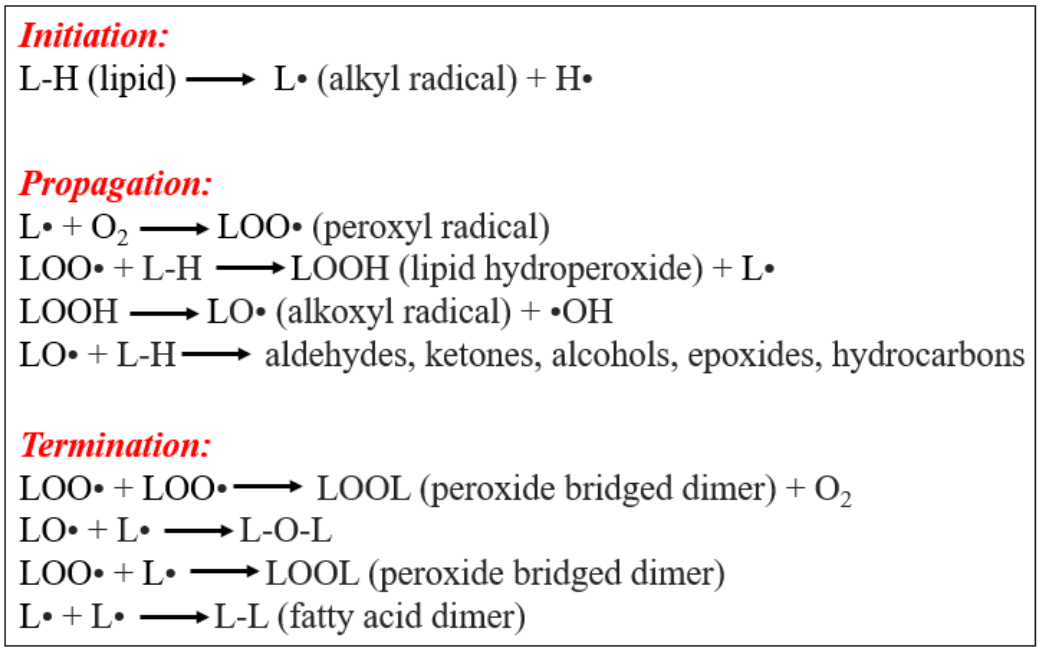


Figure 2.1. Mechanisms of lipid auto-oxidation.

During the propagation stage, the oxygen reacts with alkyl radicals that are formed during the initiation stage. Oxygen is a bi-radical compound with two unpaired electrons. The reactions between radicals are diffusion-limited, meaning that almost no activation energy is needed for the reaction between alkyl and oxygen radicals. This means that the reaction speed is dictated by how fast oxygen and alkyl radicals diffuse into close proximity (McClements & Decker, 2017). After the reaction, there is still one remaining unpaired electron on the oxygen atom. This resultant structure is known as peroxyl radical (LOO•). The peroxyl radicals are higher in energy (1000 mV) compared to alkyl radicals (600 mV); therefore, peroxyl radicals can abstract hydrogen from another fatty acid molecule to form lipid hydroperoxides (LOOH), whereas alkyl radicals are not able to do it directly due to their lower energy. This reaction is a very crucial part of the lipid oxidation process because it results in the formation of additional alkyl radicals and lipid hydroperoxides, which later breakdown into small molecules to create rancidity. In general, lipid hydroperoxides are compounds with a large molecular weight and a high boiling point, so they do not cause off-flavors in food systems. Nevertheless, the detection of primary oxidation products, lipid hydroperoxides, is essential for determining the extent of lipid oxidation. After peroxyl radical (LOO•) abstracts hydrogen from another fatty acid molecule to form lipid hydroperoxides (LOOH), new alkyl radicals (L•) also form as in the initiation stage. These new alkyl radicals react with oxygen once more, causing peroxyl radicals to cleave another hydrogen from another fatty acid to form hydroperoxides, thereby generating new alkyl radicals. This recurring process is called auto-oxidation. Once oxidation begins, it continues without need of any external factors, posing a significant problem for the food industry.

Following the formation of hydroperoxides during the propagation phase, the hydroxyl group is removed due to external factors, resulting in a structure known as an alkoxyl radical (LO•). Alkoxyl radicals are high-energy (1600 mV); therefore, they do not need to find another fatty acid molecule to abstract hydrogen, as peroxyl radicals do. Alkoxyl radicals can directly abstract the electron from the covalent bond next to it. The destruction of the covalent bond leads to the fatty acid breakdown. These β-scission reactions are very significant for food quality because they cause the decomposition of hydroperoxides into small, volatile molecules responsible for rancidity. The resulting low molecular weight molecules are known as secondary lipid oxidation products and can be hydrocarbons, aldehydes, ketones, alcohols, or epoxides. Usually, one alkyl radical forms one alkoxyl radical during the propagation step; thus, the auto-oxidation rate is linear. However, β-scission reactions generate two radicals from a single alkoxyl radical; this explains why the lipid oxidation rate is exponential and occurs so rapidly once oxidation has begun. The secondary lipid oxidation products formed at the end of β-scission reactions are highly dependent on the type of fatty acids that have been oxidized. The oxidation of omega-3 fatty acids produces propanal and ethane, whereas omega-6 fatty acids produce hexanal and pentane. As a result, specific methods should be developed depending on the nature of the lipid in the product to understand the extent of volatile compound formation.

Two lipid radicals react to form non-radical species during the termination stage of lipid oxidation, which ends the lipid oxidation reactions. This reaction will produce dimers, which will increase the viscosity of the oil. The termination stage is insignificant in food systems because the product is already rancid during the propagation stage, implying that lipid oxidation should be prevented before reaching the termination stage. Because of this,

the food industry employs free radical scavenging antioxidants to reduce oxidation reactions during the initiation and propagation stages.

2.3 Factors affecting lipid oxidation

2.3.1 Lipid content, type and position of fatty acids on triacylglycerols

Lipids contain triacylglycerols (TAGs), phospholipids, and cholesterols, all prone to lipid oxidation. Contrary to popular belief, the amount of lipids in the system has little effect on lipid oxidation rates. The presence of even a small amount of oil in emulsions, for example, produces significant off-flavors. Ang and Young (1992) demonstrated that increasing the fat content of cooked chicken thigh patties from 5% to 20% did not increase lipid oxidation as measured by malondialdehyde formation.

TAGs are composed of three fatty acids attached to a glycerol backbone. Fatty acids can be either saturated or unsaturated, depending on the absence or presence of double bonds in their structures. Double bonds are the primary reaction sites for lipid oxidation reactions, so the degree of unsaturation is a crucial factor influencing lipid oxidation. Due to the lower bond strength of the carbon-hydrogen bond, it is thermodynamically easier to abstract hydrogen from a neighboring carbon of a double bond than from a single bond, meaning that unsaturated fatty acids are required for oxidation reactions (Decker et al., 2012). Similarly, the oxidation rate increases as the number of double bonds in the TAG structure increases. Gunstone (1984) has shown that relative oxidation rates of oleic acid (18:1), linoleic acid (18:2), and linolenic acid (18:3) were 1:27:77 due to the presence of more reaction sites when the number of double bonds increases.

One of the first approaches that the food industry adopted to increase oxidative stability was to substitute unsaturated fatty acids with saturated fatty acids. However, this approach is not feasible in many cases because it is healthier to have less saturated fatty acids and more polyunsaturated fatty acids (PUFAs) in the human diet (Watkins & German, 1998). Similarly, saturated fats have a crystalline structure that affects physical and sensory properties; thus, in most cases, PUFAs cannot be substituted for saturated fats (McClements & Decker, 2000). That is why, instead of using saturated fats, the food industry replaces PUFAs with mono-unsaturated fatty acids (MUFAs), which have higher oxidative stability. This is typically accomplished by using high oleic seed oils, olive oil, or canola oil, all of which are high in oleic acid (18:1) (Decker & Bayram, 2021). However, MUFAs are still susceptible to lipid oxidation, and some PUFAs, such as omega-3 fatty acids, are more beneficial to human health; thus, substituting is not always the preferred option. Given these facts, food scientists are constantly looking for new ways to protect PUFA-containing food systems by incorporating effective antioxidants, chelators, or oxygen scavengers.

Aside from unsaturation, a few studies have found that the location of fatty acids on the TAG structure is also important for oxidative stability. Endo et al. (1997) investigated the effect of regio-isomers on oxidative stability by changing the location of eicosapentaenoic acid (EPA) on glycerol backbone. The EPA was found to be more oxidatively stable when it was in the sn-2 position. Similarly, Wijesundera et al. (2018) discovered that TAGs became more resistant to oxidation when docosahexaenoic acid (DHA) was located at the sn-2 position as opposed to the sn-3 position. The food industry can alter the location of fatty acids through specific chemical reactions. Interesterification

reactions are generally used to modify the physical characteristics of fats by rearranging fatty acids on the TAG structure. This strategy can be utilized to position oxidation-vulnerable lipids at the sn-2 position to produce oxidatively stable food products.

2.3.2 Oxygen

Oxygen is one of the primary components of lipid oxidation reactions; therefore, one of the most effective ways to minimize oxidation reactions is to remove as much oxygen as possible from food (Chen, McClements et al., 2011). There is no kinetic or thermodynamic barrier between oxygen bi-radical and alkyl radicals at the initiation stage of lipid oxidation, making the unpaired electrons react very quickly (Dunford, 1987). Andersson and Lingnert (1999) discovered that lowering headspace oxygen concentrations below 0.5% significantly reduced peroxide formation in rapeseed oil. Similarly, Fujisaki et al. (2000) discovered that decreasing headspace oxygen from 20% to 2% during frying of high oleic safflower oil significantly inhibited carbonyl formation and protected endogenous tocopherols over 30 hours. However, determining only headspace oxygen is insufficient for drawing practical conclusions. The amount of dissolved oxygen is more important in food systems than headspace oxygen because the proximity of oxygen to the lipid phase promotes the lipid oxidation reactions. Masella et al. (2010) demonstrated that high dissolved oxygen concentrations were a catalyst for the auto-oxidation reactions in extra virgin olive oil.

In general, oxygen concentration does not play a significant role in the rate of lipid oxidation, as even a small amount of oxygen is sufficient for lipid oxidation reactions to proceed. Johnson et al. (2017) discovered that the only way to retard lipid oxidation by

altering the oxygen concentration is to remove nearly all oxygen from the environment. They demonstrated that more than 93% of oxygen had to be removed from fish-oil-in-water emulsions to achieve meaningful oxidative protection. It is easy to remove oxygen from low moisture foods by simple nitrogen flushing because the most reactive oxygen is in the headspace. However, commercial strategies such as nitrogen flushing or purging are insufficient to remove large quantities of oxygen from emulsions or bulk oils because unit operations dissolve oxygen within the food, not the headspace (Johnson et al., 2017). Only long-term nitrogen flushing can effectively eliminate oxygen; however, this is impractical in food processing operations. To significantly increase the shelf-life of lipid-containing foods without the use of food additives, the food industry must develop methods for efficiently removing large quantities of oxygen.

2.3.3 Singlet Oxygen

Triplet oxygen, or atmospheric oxygen, is a biradical containing two unpaired electrons in two different orbitals with identical spin directions. Singlet oxygen, on the other hand, has two unpaired electrons either in a single orbital or two orbitals with opposite spin directions. Singlet oxygen is highly electrophilic due to its empty orbital, and it can directly react with electron-rich double bonds to form lipid hydroperoxides. Therefore, singlet oxygen-induced lipid oxidation does follow a radical-initiated process, which is the primary distinction between singlet oxygen and triplet oxygen in lipid oxidation in foods. Temperature and degree of unsaturation play a lesser role in singlet oxygen-induced oxidation compared to triplet oxygen (Van Dyck, 2010).

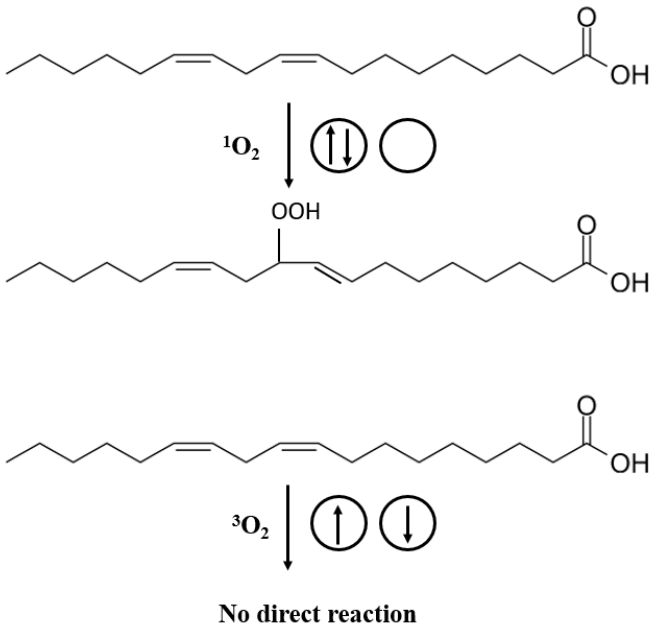


Figure 2.2. The differences between formation of lipid hydroperoxides from singlet and triplet oxygen.

Photosensitizers are required for singlet oxygen production. In the presence of light, photosensitizers transfer energy from light to triplet oxygen. The high energy causes the triplet oxygen's spin direction to reverse, resulting in the formation of highly reactive singlet oxygen (Korycka-Dahl et al., 1978). Chlorophyll and riboflavin are two common photosensitizers found in food systems. One of the primary purposes of the bleaching step in oil refining is to eliminate chlorophyll from the environment to reduce light-induced lipid oxidation. Non-refined oils, such as extra virgin olive oil, are stored in dark green bottles to minimize the formation of singlet oxygen. Riboflavin, also known as vitamin B2, is another photosensitizer that is abundant in dairy products. For instance, even though ice-cream is high in saturated fats and stored at freezing temperatures, exposure to light can still cause rancidity due to the presence of riboflavin. Similarly, oxidized α-tocopherol

(Kim et al., 2007), and certain food colorants (Pan et al., 2005) also act as photosensitizers and therefore accelerate the light-induced lipid oxidation.

The most efficient ways to decrease light-induced lipid oxidation are using light-blocking bottles, removing photosensitizers, using metal chelating agents, eliminating oxygen, and incorporating natural singlet oxygen quenchers. β-carotene is one of the most effective singlet oxygen quenchers employed in food products. According to Foote and Denny (1968), one molecule of β-carotene can quench up to 250-1000 molecules of singlet oxygen. Carotenoids with less than seven double bonds cannot function as singlet oxygen quenchers, but their effectiveness increases as the number of double bonds increases (Lee & Min, 1990). Polyphenols can also be used to quench singlet oxygen. For example, tea catechins in ethanol (Mukai et al., 2005) or the plant extract of Piper Betel (Bhattacharya et al., 2007) found to have singlet oxygen quenching properties. Superoxide anion is a reducing agent formed during the synthesis of singlet oxygen from photosensitizers. Superoxide anions also speed up lipid oxidation by converting ferric iron to more reactive ferrous iron, which causes lipid hydroperoxides to degrade into small, volatile compounds. By binding superoxide anions, superoxide dismutase (SOD) could also effectively reduce light-induced oxidation.

There are different methods for the identification of singlet oxygen and singlet oxygen-induced lipid oxidation. The lipid hydroperoxide formation occurs at different carbon numbers in singlet oxygen and atmospheric oxygen-induced reactions. For instance, lipid hydroperoxide formation occurs at carbon numbers 9 and 13 during the auto-oxidation of linoleic acid. However, singlet oxygen can also form additional lipid hydroperoxides at carbon numbers 10 and 12 (Frankel et al., 1979). Therefore, singlet oxygen could produce

seven carbon aldehydes (e.g., *trans*-2-heptanal) after β-scission reactions, which cannot be produced during auto-oxidation. Thus, the final secondary lipid oxidation products can be analyzed using GC-MS to understand if lipid oxidation is due to singlet oxygen or triplet oxygen. It is hard to detect singlet oxygen directly because the excited state has a very short life. The indirect measurement of singlet oxygen can be performed using spectrophotometric methods by reacting singlet oxygen with 1,3-diphenylisobenzofuran (Kochevar, & Redmond, 2000). Similarly, electron spin resonance spectroscopy (ESR) can be used to detect free radicals, i.e., singlet oxygen, using spin trapping agents (Whang & Peng, 1988).

2.3.4 Lipoxygenase

Lipoxygenase is an enzyme found in plant tissues that contains iron in its structure. In the presence of hydroperoxides, inactive ferrous ions are oxidized into active ferric ions (Wang & Hammond, 2010). For this reason, lipoxygenase activity requires the presence of hydroperoxides in the food matrix. Since most food systems already contain metal ions and hydroperoxides as a result of food processing, lipoxygenase-induced lipid oxidation is common. Active ferric ions abstract hydrogen from the pentadiene system of unsaturated lipids. The resulting alkyl radicals combine with oxygen to produce additional hydroperoxides.

Soybeans, rice bran, meat, fruits, vegetables, and dairy products are the foods most susceptible to lipoxygenase-induced lipid oxidation. Blanching is one of the most common methods for the inactivation of lipoxygenase enzyme due to its heat sensitivity. The presence of peroxidase in the food matrix is used to determine the efficacy of blanching,

as peroxidase is the most stable enzyme and its inactivation results in the inactivation of lipoxygenase as well. Lipoxygenase can cause rancidity even in low-fat foods at freezing temperatures; therefore, lipoxygenase inactivation is essential in food systems. Breeding is another method for minimizing the lipoxygenase activity. For instance, even though soy protein isolates contain very little fat (approximately 1%), lipid oxidation is a significant issue since high temperature alone is insufficient to deactivate lipoxygenase. Therefore, breeding techniques with low lipoxygenase-soybeans were implemented to reduce hexanal formation (Narvel et al., 1998). Changes in pressure (Mukhopadhyay & Chakraborty, 2007), gamma irradiation (Henderson & Eskin, 1990), and antioxidants that inhibit lipoxygenase activity, such as rosemary extract (Chen et al., 1992), can also be utilized to reduce lipoxygenase-induced lipid oxidation. In certain circumstances, however, lipoxygenase activity is desired because it can generate desirable flavors and aromas. For instance, the combination of lipoxygenase and hydroperoxide lyase produces volatiles with six to nine carbons, which give olive oil its distinctive, desired flavor (Salas et al., 1999).

2.3.5 Free fatty acids

Triacylglycerols (TAG) are the main component of oils and are composed of three fatty acids bound to a glycerol backbone. The hydrolysis reactions result in the formation of free fatty acids from TAG structure. For instance, the lipase enzyme becomes active when cell organelles decompartmentalize, leading to enzymatic hydrolysis of the triacylglycerols. In general, lipase originates from the digestive tissues; thus, it is highly available in rendered food products. Once fatty acids are isolated from the glycerol backbone, they oxidize significantly faster than triacylglycerols (Kinsella et al., 1978).

Additionally, free fatty acids impart a soapy and bitter flavor to oils and lower their smoke and flash points. Refsgaard et al. (2000) discovered that the addition of free fatty acids to fresh salmon mince altered sensory properties and imparted a metallic flavor to the product.

Although the primary reason why free fatty acids accelerate oxidation reactions is unknown, three hypotheses exist to explain this phenomenon. First, acid groups can initiate the decomposition of hydroperoxides, resulting in the formation of more secondary lipid oxidation products. Second, because free fatty acids increase the acidity of the matrix, metal ions become more soluble and speed up the oxidation of lipids. Finally, free fatty acids can bind metal ions and transport them close to oil, resulting in metal-induced lipid oxidation (Waraho et al., 2011). The neutralization step is carried out during the oil refining process to reduce excess free fatty acids from the oil. However, it is impossible to remove free fatty acids completely from food because high-temperature and high-pH food processes, such as frying and extrusion, cause triacylglycerol hydrolysis. In general, refined oils of high quality should contain no more than 0.05% free fatty acids, whereas frying oils may contain up to 2% free fatty acids (Chaiyasit et al., 2007) because the formation of free fatty acids at high temperatures is harder to control.

2.3.6. Transition metals

Iron is the second most abundant metal ion on the earth (Pantopoulos, 2012). It is highly present in biological tissues because it plays an essential role in the biosynthesis of DNA and is required as a cofactor for many proteins (Yiannikourides & Latunde-Dada, 2019). Because of its vital functions in the human body, iron is considered an essential micronutrient in the human diet. The intake of 10-20 mg iron per day is recommended

depending on different age groups to meet the daily iron requirement (Institute of Medicine, 2001) because iron has low bioavailability in the body, ranging from 5% to 18% depending on the source of iron (Aggett 2012). Heme iron (mostly in meat products) is more bioavailable than non-heme iron (mostly in vegetables and cereals). The presence of compounds such as polyphenols, tannins, and phytates in vegetables and grains reduce iron bioavailability by binding non-heme iron (Singh et al., 2016). The bioavailability issues and low consumption of iron through diet affect 30% of the population worldwide, making iron deficiency the most prevalent nutrient deficiency (DeMaeyer & Adiels-Tegman, 1985). More recently, the food industry started to fortify foods with iron in the form of ferrous sulfate or ferrous gluconate to overcome deficiency problems (Lysionek et al., 2003); however, the incorporation of iron in food significantly increases the lipid oxidation reactions, especially in dispersed lipids and bulk oils.

Iron and copper are not freely available in biological tissues and are bound to the proteins in the form of ferritin, lactoferrin, or transferrin; therefore, their reactivity is controlled. However, the metal ions are being released from the tissues during food processes such as harvesting or slaughtering, resulting in highly reactive free metal ions. The presence of metal ions in the food matrix causes the breakdown of lipid hydroperoxides (LOOH) into peroxyl (LOO•) or alkoxyl (LO•) radicals and the production of reactive oxygen species. The resulting radicals promote a chain reaction causing further oxidation. The effect of metal ions on lipid oxidation is very prominent, especially in food emulsions and bulk oils. Hydroperoxides are surface-active molecules; therefore, they locate themselves at the oil-water interface in these systems. Thus, the metal ions

associated with the aqueous or lipid phase can easily interact with hydroperoxides to decompose them into free radicals, resulting in enhanced lipid oxidation.

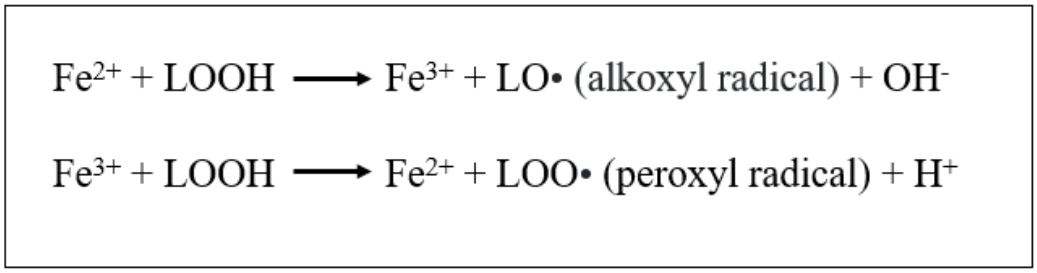


Figure 2.3. Reaction scheme of lipid hydroperoxide decomposition in the presence of ferrous and ferric ions.

Even meager amounts of metal ions (around ten ppb) can promote lipid oxidation in food systems. Metal ions are present naturally in most foods such as muscle foods, powdered foods, cereals, eggs, nuts, fruits, vegetables, and beverages; therefore, metal ion-promoted lipid oxidation can be a challenge to ensure food quality. In addition, water and processing equipment can result in metal ion migration to the product; therefore, it is tough to remove metal ions entirely from food. Although copper is more reactive than iron for hydroperoxides decomposition, copper is still not as big a problem as iron in food systems since copper ion concentrations tend to be much lower in biological tissues than iron (Rowley and Halliwell, 1983).

Iron is present in two different forms in food systems: ferrous (Fe^{2+}) and ferric iron (Fe^{3+}). Ferric ions can directly attack fatty acids to initiate lipid oxidation by producing alkyl radicals. On the other hand, the ferrous ion can indirectly form alkyl radicals by producing hydroxyl radicals through the Fenton reaction. Although ferric iron is more common in foods, ferrous iron possesses a more significant challenge for the food industry

because its 10^{13} to 10^{17}-fold more solubility makes it more reactive than ferric ion (Zumdahl, 1989). This accelerates the breakdown of lipid hydroperoxides by more than 10^5 fold, thus causing lipid oxidation reactions to happen much faster in the presence of ferrous iron (Dunford, 1987). The higher reactivity of ferrous iron has been shown by Mozuraityte et al. (2008) in a liposome system where the addition of ferrous ions accelerated the lipid oxidation by decreasing the dissolved oxygen amount in the environment significantly compared to the ferric ion. In general, the ferrous form of iron is not very typical in food systems; however, reducing agents can donate an electron to ferric ion to reduce it to ferrous iron, resulting in increased lipid oxidation. For example, ascorbic acid itself is an antioxidant; however, it may behave as a prooxidant in emulsions because ascorbic acid can cause redox cycling of iron. Similar phenomena were observed with gallic acid by Mei et al. (1999). Although low concentrations of gallic acid behave as an antioxidant at neutral pH, it showed reducing activity at acidic pH because of the prooxidant effect caused by reducing the oxidation state of iron in oil-in-water emulsions.

2.3.7 Heme proteins

Heme proteins, which contain iron in their structure, are highly available in muscle tissues. The primary function of iron-containing proteins is to determine the color of muscle foods. Myoglobin, hemoglobin, cytochrome C and catalase are examples of heme-proteins available in foods (Richards, 2010). Iron-containing proteins can promote lipid oxidation by themselves, but they can also serve as a source of free metal ions once proteins get denatured during cooking, slaughtering, and processing. Similarly, oxidation of oxymyoglobin to metmyoglobin results in formation of superoxide anions, which reduce

ferric ions into more reactive ferrous ions and promote lipid oxidation. Heme proteins trigger lipid oxidation by causing hydroperoxide decomposition or alkyl radical formation due to the ferryl species.

Dark muscle tissues have more hemeproteins than light muscle tissues. For example, chicken thighs contain more heme protein than chicken breast, so the oxidation reactions are much faster. Beef oxidizes more rapidly than pork and chicken for the same reason (Rhee & Ziprin, 1987). Lipid oxidation in heme proteins causes color change, off-flavor, and vitamin loss. Furthermore, consuming oxidized muscle tissues has negative health consequences, primarily leading to vascular diseases (Spiteller, 2005). Consumption of grape seed extract (Kuffa et al., 2009) and red wine (Gorelik et al., 2008) might prevent further oxidation in the stomach and suppress harmful malonaldehyde formation in human plasma. In addition, oxidation of heme proteins has a negative effect on meat texture (Kanner, 1994), because free radicals oxidize sulfhydryl groups on proteins, forming crosslinks via disulfide bonding and decreasing solubility. To minimize lipid oxidation of heme proteins, it is recommended to use free radical scavengers, incorporate metal chelators, and remove oxygen from meat packaging (Richards, 2010). Similarly, optimizing acidity reduces lipid oxidation, and curing meat with sodium nitrite stabilizes hemoglobin and myoglobin activity by binding nitric oxide (Ohshima et al., 1988).

2.3.8 Environmental factors

2.3.8.1 Temperature

Storage and processing temperatures have a massive impact on the rate of lipid oxidation. In general, temperatures higher than 60°C causes rapid hydroperoxide

decomposition. Similarly, high temperatures cause prooxidant metal ions to be released from proteins and accelerate the consumption of endogenous antioxidants, hastening lipid oxidation. During the frying process, the bubbling of oil creates foams which increases the contact between oxygen and oil, resulting in a significant rise in lipid oxidation. As a result, defoaming agents are used as a processing aid in the food industry to increase oil quality by reducing lipid oxidation reactions and improve safety because foams are very flammable. In general, it is highly recommended to store oils at refrigeration or freezing temperatures to prevent rancidity. Fish oil, for instance, is abundant in omega-3 fatty acids, which are highly susceptible to oxidation; therefore, it is always advised to store fish oil supplements at freezing temperature.

2.3.8.2. Water activity

Many foods exhibit minimal oxidation at water activities around 0.3-0.4. In general, as water activity decreases, so does the rate of lipid oxidation. As the amount of free water in a system decreases, the mobility of catalysts decreases, making it more difficult for molecules to react. However, if the water level is reduced to an insufficient level, lipid oxidation is likely to increase. Typically, lipid hydroperoxides are surrounded by a layer of water that stabilizes the hydroperoxides in the system. Once hydroperoxides are devoid of water, they become unstable and rapidly decompose into secondary lipid oxidation products. For instance, breakfast cereals have a low water content to achieve a crunchy texture. Therefore, rancidity is a significant problem in breakfast cereals, reducing their shelf life.

Lipid oxidation is a complex process influenced by numerous variables. Other factors that influence lipid oxidation include the physical properties of fats, irradiation, oil refining, quality of the raw materials, oxidation history, etc. Due to the impossibility of preventing lipid oxidation due to the aforementioned factors, the food industry uses free radical scavengers, metal chelators, and singlet oxygen quenchers to reduce rancidity in food formulations. The following chapter (Chapter 3) discusses antioxidants, their synergistic/antagonistic interactions, and the underlying mechanisms of these interactions.

CHAPTER 3

LITERATURE REVIEW: UNDERLYING MECHANISMS OF SYNERGISTIC ANTIOXIDANT INTERACTIONS DURING LIPID OXIDATION

The part of this review paper "Underlying mechanisms of synergistic antioxidant interactions during lipid oxidation" was published in Trends in Food Science & Technology.

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3.1 Abstract

Unsaturated lipids undergo radical-initiated oxidative deterioration during lipid oxidation, which is one of the most significant food quality and waste issues. Lipid oxidation results in off-flavors, toxic aldehydes, and the co-oxidation of proteins and color compounds. Various antioxidant strategies are utilized by the food industry to reduce lipid oxidation and increase shelf-life. Combining antioxidants to achieve synergistic interactions has been practiced for decades to improve oxidative stability. Nevertheless, underlying mechanisms of synergistic interactions between antioxidants are poorly understood and rarely studied. This review examines the main hypothesized mechanisms of antioxidant synergism, which include: 1) Regeneration of an oxidized antioxidant by another compound, 2) Differences in antioxidant partitioning in homogeneous and heterogeneous systems, 3) Combination of free radical scavenging and metal chelating activities to provide two distinct protection pathways, and 4) Formation of additional antioxidant compounds, such as phenolics, dimers, or adducts, upon oxidation which can further inhibit lipid oxidation. In complex food systems, it is often difficult to predict which

antioxidant combinations will work synergistically. Understanding the mechanisms of synergism will aid the food industry in the production of effective antioxidant mixtures to improve oxidative stability and shelf-life, as well as in the development of simple, rapid, and reliable methods for determining and evaluating synergism.

3.2 Antioxidant regeneration due to redox cycling

Free radical scavenging antioxidants are widely used in the food industry because of their ability to slow down the initiation and propagation stages of lipid oxidation. Their ability to inhibit lipid oxidation is determined by their reduction potential and structure, which influence their ability to reduce lipid radicals as well as the energy of the antioxidant radical, which is typically reduced in phenolic antioxidants due to resonance delocalization. Antioxidants with low redox potentials (e.g., <600 mV) (Decker et al., 2010) are thermodynamically capable of donating hydrogens to convert alkyl, alkoxyl and peroxyl free radicals to nonradical species such that the free radicals are not able to further oxidize unsaturated lipids. Thus, free radical scavengers, such as tocopherols, are depleted during storage as they scavenge free radicals by hydrogen donation, and hence, lose their ability to protect unsaturated fatty acids. If a secondary free radical scavenger with a substantially lower redox potential than that of the primary antioxidant (e.g., tocopherols) exists in the food matrix, it may be able to thermodynamically reduce the oxidized primary free radical scavenger back to its original form, a phenomenon called antioxidant regeneration. However, just regenerating one antioxidant with another might not result in synergistic activity. This is because when antioxidants are combined, the total antioxidant activity is related to the total number of antioxidant molecules in the system. Thus, if both antioxidants are equally effective at scavenging free radicals, their combination should

produce an additive effect. One way to achieve synergistic regeneration reactions is for one antioxidant to partition into the site of lipid oxidation (e.g., cell membrane) and the second antioxidant to regenerate the antioxidant that is preferentially oxidized in the lipid. This is the case for ascorbic acid and tocopherols in phospholipid bilayers, including cell membranes. The reaction scheme of α-tocopherol regeneration by ascorbic acid is shown in Figure 3.1. This reaction involves the formation of the α-tocopheroxyl radical due to lipid radical scavenging (Step 1), followed by the hydrogen transfer from the most acidic hydroxyl group of ascorbic acid (carbon 4) to α-tocopheroxyl radical, resulting in the formation of α-tocopherol and semihydroascorbate radical (Fujisawa et al., 2006) (Step 2).

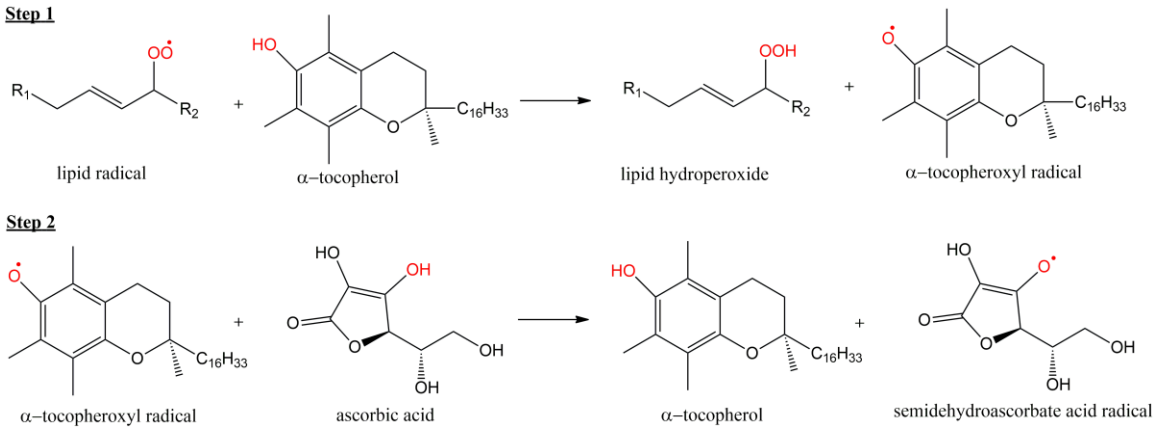


Figure 3.1. Reaction scheme of α-tocopherol regeneration by ascorbic acid. Step 1: Formation of α-tocopheroxyl radical, Step 2: Reduction of α-tocopheroxyl radical by ascorbic acid.

Ascorbic acid's ability to reduce and regenerate α-tocopheroxyl radical has been demonstrated using pulse radiolysis (Packer et al., 1979) and electron spin resonance spectroscopy (Niki et al., 1982). In heterogeneous systems, such as soybean phosphatidylcholine liposomes, Niki et al. (1985) and Thomas et al. (1992) demonstrated the synergistic activity of ascorbic acid and α-tocopherol, emerging from the different partitioning of the antioxidants within the liposome structure. α-Tocopherol is lipid-

soluble, but its hydroxyl group and phytol chain give it amphiphilic properties. Therefore, α-tocopherol partitions at the interface of biological tissue cell membranes to provide protection against oxidation and membrane dysfunction. When free radicals are present within the membrane, α-tocopherol is oxidized to form more polar α-tocopheroxyl radical, which can interact with water-soluble ascorbic acid and initiate the electron transfer required to regenerate α-tocopherol back to its original form (Fukuzawa et al., 1993) (Figure 3.2). Water soluble ascorbic acid may not be an effective antioxidant in biological tissues in this case because water soluble free radicals, such as hydroxyl radicals, are difficult to scavenge due to their high energy, which allows them to oxidize the first molecule they encounter. Ascorbic acid is synergistic although it is not very effective on its own due to its inability to penetrate through the membrane to inhibit lipid oxidation but can maintain the antioxidant activity of α-tocopherol in the phospholipid bilayer. In addition, oxidized ascorbic acid is regenerated enzymatically by glutathione and NAD(P)H in biological tissues, which maintain ascorbic acid concentrations to further regenerate more α-tocopherol (Pullar et al., 2017).

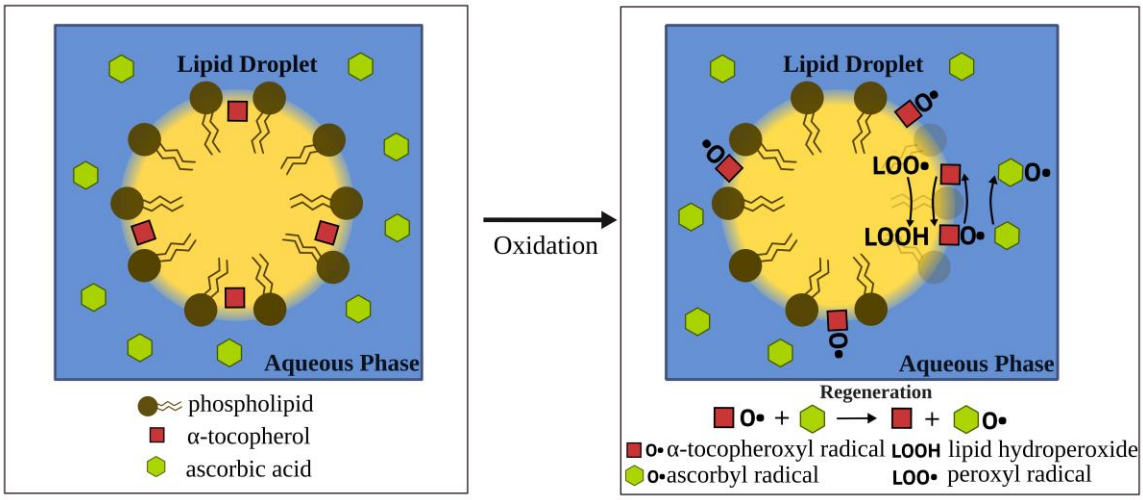


Figure 3.2. Schematic representation of α-tocopherol regeneration by ascorbic acid in heterogeneous systems due to differences in antioxidant partitioning.

Niki et al. (1984) also reported a synergistic interaction between α-tocopherol and ascorbic acid during the oxidation of methyl linoleate in *tert*-butyl alcohol/methanol solution. This discovery is intriguing because if both antioxidants were equally effective in a homogeneous system, the number of electrons to interact with the fatty acid radicals would be expected to produce an additive effect. It is possible that the methyl linoleate system is not homogeneous and instead has physical structures like association colloids that partition the α-tocopherol and ascorbic acid into different phases, allowing them to behave as they do in phospholipid bilayers. It is also possible that antioxidants regenerate each other in stoichiometries that are not 1:1, resulting in synergistic antioxidant interactions in homogeneous systems. An antioxidant with multiple redox active groups, for instance, may regenerate more than one primary antioxidant, or the oxidation product of an antioxidant may still possess reduction potential, allowing it to reduce another primary antioxidant in the system.

Ascorbic acid-tocopherol synergism may be applicable to foods where oxidation occurs in the cell membrane (e.g., muscle foods). However, in muscle foods, ascorbic acid levels are typically low and decrease rapidly during post-mortem (Decker & Hultin, 1990). Supplementing livestock with vitamin E (Delosière et al., 2019) and adding exogenous ascorbic acid to muscle foods (Ham et al., 2019) have been found to effectively inhibit oxidation; but it is unknown what role ascorbic acid/tocopherol synergism plays in these antioxidant strategies. Ascorbic acid/tocopherol synergism might not be effective in other food systems due to the insolubility of ascorbic acid in bulk oils, and the ability of ascorbic acid to increase the prooxidant activity of transition metals by redox cycling in oil-in-water emulsions (Jacobsen et al., 1999), thus making ascorbic acid-tocopherol combinations less

effective than in biological tissues where iron reactivity is tightly controlled by chelation to proteins (Decker & Bayram, 2021).

It is possible for combinations of antioxidants other than ascorbic acid-tocopherols to be synergistic through redox cycling. The ability of antioxidants to regenerate each other can be estimated by comparing their one electron reduction potential (E°), which explains the potential of substances to transfer electrons. E° of antioxidants have been widely documented in the literature (Decker et al., 2010) utilizing various techniques such as flash photolysis, pulse radiolysis, or cyclic voltammetry. It is critical to note that E° of an antioxidant derived by different approaches should be compared carefully due to differences in notating the results (e.g., potential versus half-potential). Similarly, E° is strongly influenced by system characteristics such as pH, ionic strength, and solvent type; thus, comparing E° values of antioxidants requires identical experimental conditions. The ability of ascorbic acid to regenerate α-tocopherol is due to its lower redox potential (ascorbate monoanion, AscH- / semidehydroascorbate radical, Asc•-, 282 mV) than α-tocopherol (α-tocopherol, TOH / α-tocopheroxyl radical, TO•, 500 mV) (Buettner, 1993). Therefore, it is possible to estimate the likelihood of antioxidant regeneration by comparing E° of different antioxidants. Quercetin-quercetagetin-fisetin (330 mV), myricetin (360 mV), and epigallocatechin (EGC)-epigallocatechin gallate (EGCG) (430 mV), for example, have lower redox potentials than α-tocopherol (500 mV) (Decker et al., 2010), which could produce synergistic interactions due to α-tocopherol regeneration. This logic is consistent with previous studies that found synergistic interactions between α-tocopherol/quercetin in methyl linoleate *tert*-butyl alcohol solution (Pedrielli & Skibsted, 2002), α-tocopherol/myricetin in stripped sunflower oil (Marinova et al., 2008), and α-

tocopherol/EGCG in linoleic acid *tert*-butyl alcohol/water solution (Jia et al., 1998). The synergistic interaction between α-tocopherol and green tea extract was also observed during oxidation of linoleic acid in sodium dodecyl sulfate (SDS) micelles (Dai et al., 2008), where the synergism was attributed to EGCG-mediated α-tocopherol regeneration. This system resembles the ascorbic acid and α-tocopherol synergism in that EGCG is highly water-soluble and, like ascorbic acid, is a potentially weak antioxidant on its own due to its inability to interact with lipid radicals formed in the lipid phase. The analysis of α-tocopherol degradation rates revealed that EGCG, like ascorbic acid, can reduce α-tocopheroxyl radical, resulting in synergism. On the other hand, the regeneration of α-tocopherol is thermodynamically infeasible by antioxidants with E° greater than 500 mV, such as kaempferol (750 mV), rutin (600 mV), catechin-epicatechin (570 mV), chlorogenic acid (550 mV), and caffeic acid (534 mV) (Decker et al., 2010). This logic is consistent with previous studies that reported antagonistic/additive interactions between α-tocopherol and caffeic acid in aqueous dispersions of linoleic acid (Peyrat-Maillard et al., 2003), α-tocopherol and catechin/epicatechin in stripped sunflower oil (Yin et al., 2012), and α-tocopherol and chlorogenic acid in phosphatidylcholine liposomes (Neunert et al., 2015), where additive effect results due to the absence of any interaction between antioxidants, and antagonism may result from the regeneration of a less potent antioxidant by a more potent antioxidant.

Although E° provides a decent approximation, it does not always guarantee that antioxidant combination will result in regeneration reactions. Some reactions that are thermodynamically favorable may not be kinetically feasible. Combinations of water- and lipid-soluble antioxidants, for instance, may not always be synergistic if emulsifiers at the

lipid-water interface reduce the ability of antioxidants to undergo electron transfer due to their charge, which could repel oppositely charged antioxidants, or the thickness of the interface, which could sterically hinder antioxidant interactions (Barouh et al., 2022). Also, the structure of an antioxidant may be such that the antioxidant radical is sterically protected, making it difficult to interact with a regenerating antioxidant. These could explain why contradictory results were reported for the same antioxidant pairs in similar food systems. For example, while Dai et al. (2008) discovered synergistic antioxidant interactions between α-tocopherol and EGCG during the oxidation of linoleic acid in sodium dodecyl sulfate (SDS) micelles, Durand et al. (2015) discovered non-synergistic interactions between α-tocopherol and EGCG during the oxidation of methyl eleostearate in sodium caseinate-stabilized emulsions. This may be due to differences in interfacial properties as well as variations in tocopherol-EGCG proximity, as tocopherol can interact with EGCG at the micelle interface much more readily than in emulsions due to the micelle's smaller interfacial thickness compared to an emulsion stabilized by a large protein, such as casein.

Another approach to estimate the antioxidant regeneration would be to compare bond dissociation enthalpy (BDE), which is a thermodynamic property showing the energy required for bond cleavage. Numerous experimental measurements and theoretical calculations have been performed to determine the BDE of a wide range of phenolic antioxidants (Decker et al., 2010). The comparison of these values may suggest the probability of hydrogen transfer from one antioxidant to another, analogous to E°; thus, it may provide insight into the likelihood of antioxidant regeneration. However, the results of E° and BDE may differ depending on the system conditions. For example, quercetin and

α-tocopherol have E° values of 330 and 500 mV, respectively (Decker et al., 2010), implying that quercetin could thermodynamically donate an electron to reduce oxidized α-tocopherol. However, quercetin and α-tocopherol have BDE values of 81.98 and 78.87 kcal/mol, respectively (Decker et al., 2010), implying that it is more difficult for quercetin to donate hydrogen because more energy is required to break the bond. Similarly, ascorbic acid has a lower E° (282 mV), but a higher BDE value (83.00 kcal/mol) than α-tocopherol (Decker et al., 2010). The observed synergistic interactions between α-tocopherol and quercetin in methyl linoleate *tert*-butyl alcohol solution (Pedrielli & Skibsted, 2002) and α-tocopherol and ascorbic acid in methyl linoleate *tert*-butyl alcohol/methanol solution (Niki et al., 1984) were attributed to α-tocopherol regeneration, indicating that E° might provide a more accurate estimation than BDE. In addition, one-electron reduction potential (E°) is reversible whereas bond dissociation enthalpy (BDE) is not, so it is thought that reduction potentials more accurately describe antioxidant regeneration (Warren et al., 2010). These contradictory values may also result from different measurement techniques, type of solvent, pH of the system, or the complexity of the calculation methods if the estimation is theoretical.

Besides thermodynamic estimations, the regeneration of antioxidants can be directly measured using experimental techniques such as cryogenic electron paramagnetic resonance or high performance liquid chromatography. The former can be employed by monitoring the loss of the primary antioxidant radical signal in the presence of a secondary antioxidant (Panya et al., 2012). The latter can be used if an oxidation product of the primary antioxidant can be measured (e.g., quinone) and then determining if a secondary antioxidant can convert the oxidation product back to the original primary antioxidant

structure (Cui et al., 2015). There are many factors influencing the radical transfer between antioxidants, especially in complex food systems, such as antioxidant/prooxidant activity, thermodynamic properties, solubility and polarity of the antioxidants, and antioxidant distribution throughout the food matrix owing to the presence of micelles, membranes, and so on. As a result, it is critical to look for different antioxidant regenerating pairs that may be suited for a variety of different food applications including bulk oils, emulsions, and low moisture food products.

3.3 Antioxidant regeneration through other mechanisms

Phospholipids are amphiphilic lipids composed of two fatty acids and a phosphate group attached to a glycerol backbone. Phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylethanolinositol (PI), and phosphatidic acid (PA) are major phospholipid types found in food systems. During the degumming process, the majority of phospholipids are removed from bulk oil to prevent cloudy appearance, darkening at high temperatures, and eliminate contamination with excess metal ions carried by phospholipids (Weng & Gordon, 1993). However, due to their amphiphilic properties, phospholipids are added to food systems for their emulsifying and crystal modification properties. In recent years, the synergistic interactions between phospholipids and tocopherols have been a promising research area for the enhancement of oxidative stability, but the mechanisms underlying these interactions are unclear. Some researchers claimed simple electron transfer between antioxidants (Hudson & Lewis, 1983a) similar to the ascorbic acid/α-tocopherol system, while others claimed due to the phospholipids having metal chelating activity (Dacaranhe & Terao, 2001), or regenerating lipid radicals with

their acidic protons. Weng and Gordon (1993) demonstrated that PE was capable of converting α-tocopherylquinone to α-tocopherol, resulting in synergistic interactions in lard. Prior to this discovery, only the regeneration of α-tocopherol by ascorbic acid, which occurred through the reduction of α-tocopheroxyl radicals, was known (Packer et al., 1979). The process begins with the two-electron oxidation of α-tocopherol in food systems due to free radical scavenging activity, which results in the formation of α-tocopherylquinone (Figure 3.3). Doert et al. (2012) demonstrated that the reduction of α-tocopherylquinone by phospholipids is not caused by simple electron transfer due to differences in redox potentials, but rather by rearrangement reactions. The authors showed that PE and PS form complexes with α-tocopherylquinone, which later generate PE-PS-α-tocopherones through amino-carbonyl reactions. PE-PS-α-tocopherones then undergo a series of rearrangements, including decarboxylation (for PS) and acid heterolysis (for PE), resulting in regeneration of α-tocopherol (Figure 3.3). On the other hand, authors did not find any α-tocopherol regeneration with PC, which was associated with the absence of amine group. Studies analyzing the synergistic interactions between α-tocopherol and phospholipids in bulk oils support this conclusion. For instance, Takenaka et al. (2007) demonstrated synergistic antioxidant activity between α-tocopherol and PE in stripped bonito oil, whereas PC had no synergistic effect. Similarly, Cui et al. (2015) found that the addition of PE to store-bought soybean oil containing endogenous tocopherols increased shelf-life, whereas the addition of PC had the opposite effect. Xu et al. (2019) concluded the same synergistic activity of tocopherols with PE in stripped soybean oil, but they discovered an additive effect with PS. On the other hand, Samdani et al. (2018) discovered that while PS acts synergistically with α-tocopherol in stripped soybean oil-in-water

emulsions, PE produced an additive effect, which is the exact opposite effect compared to bulk oils. This might be due to differences in partitioning at the emulsion droplet interface because of varying phospholipid and surfactant interactions. Synergisms in these systems occur because the phospholipids themselves have little to no antioxidant activity; therefore, any increase in tocopherol activity by phosphatidylethanolamine (PE) or phosphatidylserine (PS) results in synergism.

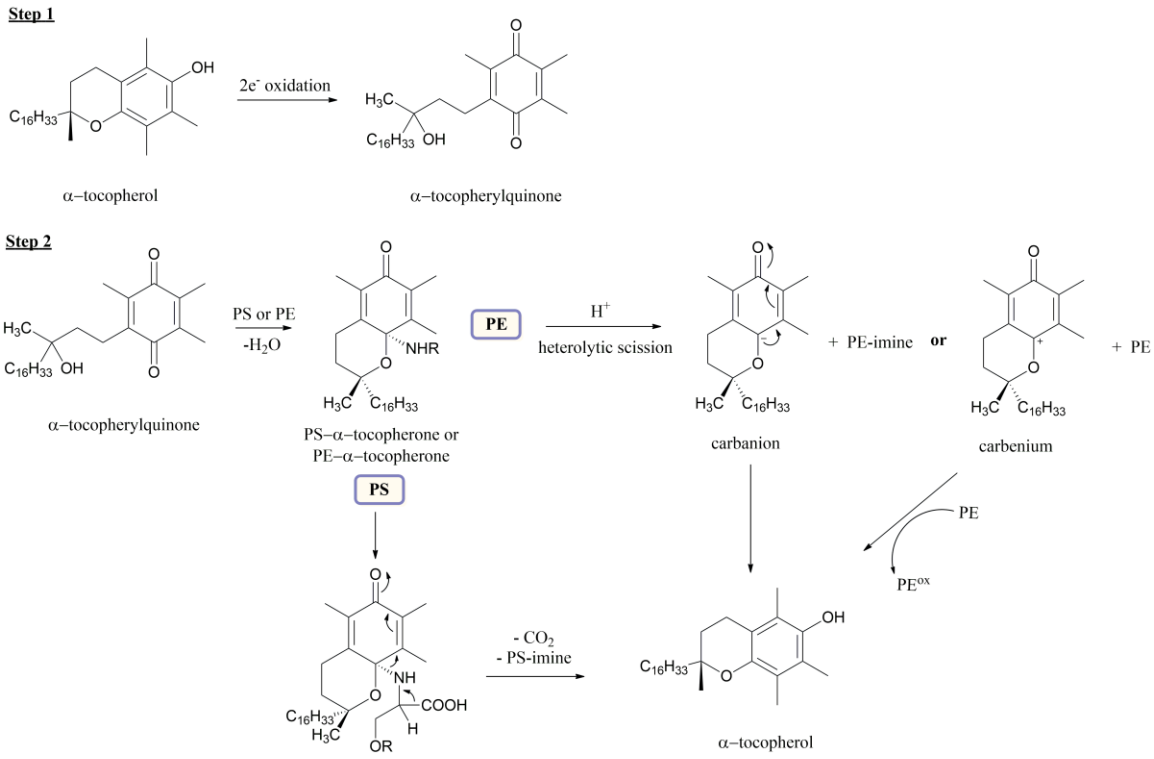


Figure 3.3. Reaction scheme of α-tocopherol regeneration by phospholipids. Step 1: Formation of α-tocopherylquinone as a result of antioxidant activity, Step 2: Reduction of α-tocopherylquinone by phosphatidylethanolamine (PE) and phosphatidylserine (PS) through series of rearrangement reactions. Adapted by Doert et al. (2012).

The vast majority of studies demonstrating PE and PS synergism with tocopherols employ research-grade phospholipids. The commercially available lecithins are a mixture

of various phospholipid types, including prooxidant PC. However, PC in lecithins can be transformed to PE or PS by the enzyme phospholipase D through hydrolysis and transphosphatidylation reactions. The resulting high-PE and high-PS lecithins could be combined with tocopherols to obtain synergism. For instance, Arora et al. (2022) converted high-PC (94%) soybean lecithin to high-PS (92%) soybean lecithin, which showed synergistic interactions with α-tocopherol in stripped soybean oil-in-water emulsions. Similarly, Culler et al. (2022) obtained synergistic activity both in oil-in-water emulsions and stripped soybean oil when researchers converted high-PC (96%) soybean lecithin to high-PE (72%) soybean lecithin. Compared to the direct use of purified phospholipids, this conversion permits the food industry to produce more cost-effective methods for enhancing the oxidative stability of bulk oils and emulsions.

Phenolic antioxidants may also regenerate each other by forming stable complexes as a result of π-π stacking rather than simple redox cycling, potentially resulting in synergism. This was observed between rosmarinic acid and quercetin, where the formation of a stable complex between cinnamic acid and flavonol due to the π-π stacking of the aromatic ring of the phenolic and the B ring of the flavonol resulted in synergistic activity (Peyrat-Maillard et al., 2003). This alters the structure and bonding between molecules, making the complex more stable than the parental compounds in terms of hydrogen-donating capacity for regeneration reactions.

3.4 Differences in antioxidant partitioning within food

The ability of antioxidants to interact synergistically may also occur as a result of their different partitioning within the food matrix. The different localization of antioxidants

in emulsions, micelles, liposomes, or association colloids could promote antioxidant regeneration, hence synergism, as observed in α-tocopherol/ascorbic acid combinations in heterogeneous systems (Niki et al., 1985; Thomas et al., 1992). Therefore, it is important to know the location and efficacy of antioxidants in order to design synergistic antioxidant interactions.

It was first proposed in the 1980s that nonpolar antioxidants are more active in polar settings, such as oil-in-water emulsions, while polar antioxidants are more active in nonpolar environments, such as bulk oils due to their distinct partitioning in the area where lipid oxidation is prevalent (Porter et al., 1989). Trolox, for example, was shown to be a stronger antioxidant in corn oil triacylglycerols than α-tocopherol due to its more polar nature, as suggested by the polar paradox (Huang et al., 1996). This theory has been one of the most influential guidelines for estimating the antioxidant activity in simple model systems. However, multiple studies have demonstrated that the polar paradox theory does not always accurately predict antioxidant activity, particularly in systems in which antioxidants are modified with different alkyl chain lengths to alter their polarities and, consequently, their partitioning and antioxidant activity. This has been observed in oil-in-water emulsions containing rosmarinic acid (Laguerre et al., 2010) and hydroxytyrosol (Medina et al., 2009). In general, while antioxidant activity increased in emulsions with increasing hydrophobicity (C2 to C8 or C12 alkyl chain length) as predicted by the polar paradox hypothesis, there was a "cut-off" effect for longer alkyl chains (> C8-C12), where antioxidant activity decreased as molecules became more nonpolar. This cut-off effect was caused by multiple factors, including 1) molecules becoming extremely nonpolar after a certain alkyl chain length, resulting in partitioning in the oil phase as opposed to the

interface, and (2) more nonpolar antioxidants forming micelles with emulsifiers, thereby migrating away from the emulsion droplets. From a synergism standpoint, Panya et al. (2012) demonstrated that only water soluble rosmarinic acid devoid of a linked alkyl chain exhibited synergism with α-tocopherol due to their maximum interaction, as observed by fluorescence quenching. This suggests that rosmarinic acid was only synergistic when it was in the water phase versus coexisiting with tocopherol in the emulsion droplet. This demonstrates the importance of using antioxidants to achieve synergistic antioxidant interactions based not only on their polarities and efficacies, but also on their interaction/physical contact with each other.

Until now, several water- and lipid-soluble antioxidants have been studied in heterogeneous systems without taking the antioxidant partitioning into consideration; consequently, contradictory results regarding antagonistic and synergistic interactions have been published. The interactions between antioxidants should be investigated individually in homogeneous (e.g., solvents) and heterogeneous (e.g., liposomes, micelles) systems, as the same antioxidants may function synergistically in one system but antagonistically in another. For instance, it was found that while quercetin and α-tocopherol had antagonistic interactions in stripped sunflower oil, they acted synergistically in methyl linoleate emulsions (Becker et al., 2007). This may be due to the fact that the presence of a lipid-water interface may have increased regeneration-induced synergism in comparison to a homogeneous system. However, this could also be due to the fact that quercetin can chelate metals. This was also observed with other phenolic compounds, where heterogeneous systems had higher α-tocopherol regeneration rates than in pure hexane due to their distinct partitioning (Iglesias et al., 2009). Therefore, it is essential to have knowledge of the

physical locations of antioxidants in heterogeneous systems to determine whether antioxidants could operate synergistically. This could be accomplished by measuring the partition coefficient of antioxidants (log P), which reflects a molecule's tendency to partition in water or lipid phases, giving a notion of their hydrophilicity versus lipophilicity. This is often performed using immiscible octanol-water systems. The partition coefficients of some antioxidants are summarized in Table 3.1.

Table 3.1. The partition coefficients (log P) of some antioxidants

Compound	Log P	References	Compounds	Log P	References
Tocopherol	12.10*	Jamshidian et al. (2012)	Sesamol	1.34*	Geetha et al. (2015)
	589	Liao & Yin (2000)		1.29	Geetha et al. (2015)
Carnosol	4.58*	Logan et al. (2015)	Taxifolin	1.22*	Dičkancaitė et al. (1998)
	15.90**	Huang et al. (1997)		1.12	Shatalin & Shubina (2014)
Carnosic Acid	5.13*	Logan et al. (2015)	EGCG	0.39	Shibusawa et al. (2005)
	10.20**	Huang et al. (1997)		1.67*	Shibusawa et al. (2005)
				1.41**	Kajiya et al. (2001)
BHT	6.60*	Jamshidian et al. (2012)	Hydroxytyrosol	1.07	Peng et al. (2015)
Myricetin	5.27	Liao & Yin (2000)	Caffeic Acid	0.56	Liao & Yin (2000)
	3.18**	Chuang et al. (2017)		1.30*	Son & Lewis (2002)
				1.29**	Noubigh et al. (2009)
Trolox	3.83**	Huang et al. (1997)	Ferulic Acid	1.58*	Son & Lewis (2002)
	3.20*	Son & Lewis (2002)		0.49	Sohn & Oh (2003)
Resveratrol	3.09*	Yang et al. (2017)	Propyl Gallate	1.20*	Jamshidian et al. (2012)
				0.85**	Huang et al. (1997)

Quercetin	3.84 3.32** 2.74*	Liao & Yin (2000) Chuang et al. (2017) Dičkancaitė et al. (1998)	Rutin	0.76** 0.85	Chuang et al. (2017) Pedriali et al. (2008)
Thymol	3.04	Schwarz et al. (1996)	Catechin	0.32 0.86* 0.38**	Shibusawa et al. (2005) Shibusawa et al. (2005) Kajiya et al. (2001)
BHA	3.40* 2.95	Jamshidian et al. (2012) Schwarz et al. (1996)	Epicatechin	0.13 0.86* 0.30**	Shibusawa et al. (2005) Shibusawa et al. (2005) Kajiya et al. (2001)
TBHQ	3.00*	Jamshidian et al. (2012)	EGC	-0.50 0.43* 0.40**	Shibusawa et al. (2005) Shibusawa et al. (2005) Kajiya et al. (2001)
Rosmarinate	2.07* 0.48** 2.50	Logan et al. (2015) Huang et al. (1997) Huang et al. (2019)	Gallic Acid	0.02** -0.89	Huang et al. (1997) Schwarz et al. (1996)
ECG	1.06 2.10* 1.79**	Shibusawa et al. (2005) Shibusawa et al. (2005) Kajiya et al. (2001)	Chlorogenate	-0.77* -0.90	Maisuthisakul et al. (2006) Bonarska-Kujawa et al. (2015)
Vanillic Acid	1.43* 1.42**	Kamaya et al. (2005) Noubigh et al. (2010)			

* indicated the estimated log P obtained through computation, ** indicated the experimental log P obtained through chromatographic methods and, no asterisk indicated the experimental log P obtained through spectrophotometric analysis. Aside from the techniques, the log P values of the same chemicals vary depending on the type of solvent (octanol-water versus oil-water), pH and the presence of other components.

However, measuring hydrophilicity/lipophilicity alone is insufficient to estimate the partitioning site because some antioxidants have amphiphilic properties and preferentially locate at the oil-water interface, where most lipid oxidation reactions occur. Therefore, the “polar paradox” theory was expanded to take into account the impact of antioxidants' surface activity (Frankel et al. 1994). Since free radicals are mainly generated at oil-water interfaces due to surface active lipid hydroperoxide decomposition, interfacial antioxidants may be better able to suppress lipid oxidation. This is especially true in emulsion systems, where interfacial antioxidants are far more powerful than water-soluble antioxidants partitioning directly into the aqueous phase. In oil-in-water emulsions, for example, amphiphilic ascorbyl palmitate and propyl gallate partitioned at higher concentrations at the interface than tocopherol, resulting in enhanced oxidative stability (López-Martínez & Rocha-Uribe, 2017). Similarly, even though bulk oil appears to be a homogeneous system, the same hypothesis applies. Bulk oils contain physical structures known as association colloids, which are formed when amphiphilic molecules, such as phospholipids, enclose water molecules to create micelle-like structures. Consequently, it is anticipated that the oil-water interfaces are also the principal lipid oxidation sites for bulk oils, thus, surface active antioxidants are good choices to inhibit oxidation. α-Tocopherol, for example, is an effective antioxidant in bulk oils because 73% of α-tocopherol partitions at the interface and 26% in the hydrophobic phase (Gunaseelan et al., 2006). From a synergism standpoint, surface-active antioxidants may have more synergistic interactions compared to their non-surface active counterparts due to their ease of interacting with other antioxidants located in aqueous and lipid phases, which may lead to antioxidant regeneration. On the other hand, if regenerating

antioxidants could not interact at all due to the partitioning at different phases (aqueous and lipid) rather than at the interface, an additive or antagonistic effect may be observed. Therefore, knowing which antioxidants locate at the interface can help predict whether an antioxidant combination will act synergistically. The antioxidants' interfacial activity can be measured using fluorescence with the interfacial 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE), where the change in NBD-PE intensity in the presence of antioxidants demonstrates the ability of antioxidants to locate at the interface (Cui et al., 2015). Similarly, a 4-hexadecylarenediazonium ion probe, which partitions only at the interfacial layer of the emulsions and reacts with the antioxidants located at the interface, can be used to determine the distributions of antioxidants (Gunaseelan et al., 2006). Interfacial activity of antioxidants can also be estimated by measuring the interfacial tension using equipments such as the drop shape analyzer, which records the image of the drop in the absence and presence of an antioxidant to calculate the interfacial tension (Sasaki et al., 2010). The interfacial activity of some antioxidants is summarized in Table 3.2. The interfacial activity was determined by comparing the interfacial tensions of the control group without antioxidants to the samples after antioxidant addition. The different antioxidant concentrations and their resulting interfacial tensions were summarized.

Table 3.2. The interfacial activity of some antioxidants

Interface-Active Compounds	System	Concentration (mmol/kg)	Interfacial Tension of Control (mN/m)	Interfacial Tension of Antioxidant (mN/m)	References
Tocopherol	Hexadecane:water	1.0-5.0	43.3	41.5-35.0	Chaiyasit et al. (2007)

TBHQ	Hexadecane:water	0.25-1.0	43.3	43.0-41.6	Chaiyasit et al. (2007)
Trolox	Hexadecane:water	0.2-1.0	43.3	34.8-28.0	Chaiyasit et al. (2007)
Propyl Gallate	Hexadecane:water	1.0	43.3	34.6	Chaiyasit et al. (2007)
Octyl Chlorogenic Acid	Hexadecane:water	0.13-0.65	44.4	42.2-34.9	Sasaki et al. (2010)
Dodecyl Chlorogenic Acid	Hexadecane:water	0.13-0.65	44.4	39.1-17.8	Sasaki et al. (2010)
Catechin	Refined olive oil:buffer	0.01-10.0	72.8	57.3-53.5	Di Mattia et al. (2010)
Quercetin	Refined olive oil:buffer	0.01-10.0	72.8	59.1-56.1	Di Mattia et al. (2010)
Syringic Acid	Extra virgin olive oil:water:Tween 20	5.05	41.0	32.5	Katsouli et al. (2017)
Caffeic Acid	Extra virgin olive oil:water:Tween 20	5.55	41.0	36.4	Katsouli et al. (2017)
Vanillic Acid	Extra virgin olive oil:water:Tween 20	5.95	41.0	36.2	Katsouli et al. (2017)
Rutin	Soybean oil:buffer:soy protein isolate	0.08-0.32	7.4	6.5-5.1	Cui et al. (2014)
Non Interface-Active Compounds	**System**	**Concentration (mmol/kg)**	**Interfacial Tension of Control (mN/m)**	**Interfacial Tension of Antioxidant (mN/m)**	**References**
EGCG	Corn oil:water	0.04-0.62	26.6	25.5-25.3	Mura et al. (2017)
BHT	Hexadecane:water	1.0-5.0	43.3	43.7-43.7	Chaiyasit et al. (2007)
Chlorogenic Acid	Hexadecane:water	0-0.65	44.4	44.4-44.3	Sasaki et al. (2010)
Gallic Acid	Soybean oil:water	5.9-205.7	14.0	14.0-13.0	Gomes et al. (2016)

The interfacial tension of control groups varies based on the measurement technique and the presence of other compounds in the testing phase, such as emulsifiers (e.g. Tween 20), proteins (e.g. soy protein isolate), or other antioxidant compounds (e.g. phenolics in extra virgin olive oil). The mmol/kg unit represents the mmol of added antioxidants per kilogram of phase containing the solubilized antioxidants.

3.5 Combination of free radical scavenging and metal chelating activities

The food industry employs antioxidant combinations with different mechanisms of action, such as free radical scavenging activity, metal chelating ability and singlet oxygen quenching activity. Combining two antioxidants with distinct molecular mechanisms can increase oxidative stability via synergistic effects. Two commonly used antioxidants in food systems are free radical scavengers and metal chelators; therefore, it is critical to understand their synergistic/antagonistic interactions in order to develop systems with improved oxidative stability.

In vivo and *in vitro* lipid oxidation occurs via free radical chain mechanism in which external factors produce free radicals that trigger the chain reactions. The presence of metal ions in food systems, particularly iron and copper, is one of the primary external factors forming the free radicals. Iron and copper are normally bound to proteins in biological tissues; hence, their reactivity is typically under control. However, these bound metal ions can be released during harvesting, slaughtering, salting, cooking and pH changes during food processing operations, resulting in more reactive free metal ions. Released metal ions can partition into aqueous phase and promote lipid hydroperoxide decomposition into peroxyl or alkoxyl radicals at the interface, which promote further oxidation by reacting with more unsaturated fatty acids. Although free radical scavengers are added to deactivate these free radicals in food systems, they eventually become depleted and antioxidant activity is lost. Decreasing radical generation can be achieved by inhibiting metal activity with chelators. The inhibition of prooxidant metal activity can slow down the depletion rate of free radical scavengers, resulting in a longer shelf-life.

The food industry uses metal chelators as food additives to reduce the metal ion-mediated

lipid oxidation reactions. Metal chelators can slow lipid oxidation by varying the reduction potential of the transition metals (Timoshnikov et al. 2022), supplying a steric effect to avoid direct contact between transition metals and hydroperoxides (Gulcin & Alwasel, 2022), modifying the partitioning of the metal ions within food matrix (Yi et al., 2014), and reducing the metal ion solubility (Sun et al., 2021). Organic acids (e.g., citric acid), ethylenediaminetetraacetic acid (EDTA), polyphosphates, and proteins are common food chelators used in the food industry, and their efficiency is determined by the chelator: metal ion ratio and food characteristics such as pH. EDTA is one of the most effective chelators due to its high affinity for the majority of metal ions, potent activity at low concentrations, and superior chelating ability under acidic conditions compared to most organic acids. Concerns exist regarding clean labeling when EDTA is used, and other natural chelators (e.g., organic acids, proteins, peptides) are not as effective due to their low solubility in certain food systems and decreased activity at low pH as a result of protonation (Díaz et al., 2003). Therefore, it is crucial to examine the possibility of using other natural compounds, such as flavonoids, as food chelators.

The rate of metal-induced lipid oxidation reactions varies depending on the nature of the food product; therefore, it is critical to determine whether a metal chelator will be effective in a specific food system and, if so, which concentrations or types of chelators will provide the best protection. For example, the rate of metal-promoted lipid oxidation is significantly higher in emulsions than low moisture foods as the presence of water-oil interface increases the contact between lipid hydroperoxides and prooxidative metals. Some food systems, such as low moisture crackers do not require the use of metal chelating agents. Barden et al. (2015) showed that iron addition into crackers had no effect on the

lag phase of hydroperoxide and hexanal formation, nor did the addition of EDTA extend shelf-life. This is likely due to the inability of metals to diffuse into the lipids in low moisture conditions.

Besides chelators, metal reactivity can be influenced by factors that impact the ability of metals to interact with water-lipid interfaces. For example, using positively charged molecules at the interface such as cationic surfactants, carbohydrates, and proteins can repel positively charged prooxidant metal ions and prevent contact with the oil phase, thereby reducing oxidation. On the other hand, negatively charged interfaces attract positively charged metal ions and can increase oxidation. Since the majority of food emulsion droplets are negatively charged, metal chelating agents such as negatively charged proteins and peptides can bind and remove metal ions away from the emulsion droplet surface thereby improving oxidative stability.

If the food industry intends to combine free radical scavengers and natural metal chelators to achieve synergistic interactions, it is crucial to evaluate the ability of metal-chelator complexes to inhibit lipid oxidation, as metal chelating ability does not always guarantee the antioxidant effect. Natural metal chelators, such as phenolics and organic acids, may exhibit prooxidant effects following chelation if the chelated metal ion is more soluble and reactive than the unchelated metal. Many phenolic compounds reduce ferric iron into more soluble ferrous iron to be able to bind the metal ions (Mira et al., 2002). This could cause lipid oxidation reactions to proceed much faster as reduced metals are more prooxidative. For instance, caffeic acid addition led to prooxidant activity in fish oil-in-water emulsions as a result of the conversion of endogenous ferric ions to more reactive ferrous ions irrespective of pH and type of the emulsifier (Sørensen et al., 2008). Given

this prooxidant activity, synergism with free radical scavengers is unlikely in these systems unless other mechanisms are involved. This is due to the fact that faster lipid oxidation results in faster consumption of the free radical scavenger, resulting in decreased protection.

Several researchers, on the other hand, believe that even if a metal ion is transformed to a more active state, the complex can retain antioxidant activity as a result of chelation. For example, although Mira et al. (2002) showed iron reducing activity of quercetin and taxifolin, Hudson and Lewis (1983b) have reported antioxidant activity in lard, where authors explained the reason to be the copper chelating ability. It is important to note, however, that the antioxidant/prooxidant activity of metal chelating phenolics is highly dependent on the system conditions. Most phenolics, for example, have significantly higher iron reducing activity in acidic pH than in neutral pH; thus, antioxidants that act synergistically in neutral pH due to metal chelating ability may act antagonistically in acidic food formulations due to metal reduction. Therefore, it may be possible to achieve synergistic interactions through the combination of free radical scavenging and metal chelating activities only when the metal chelator can inhibit the prooxidant activity of metal ions upon chelation. Yao et al. (2012) discovered synergistic antioxidant interactions between quercetin and resveratrol, hypothesizing that metal chelating activity could be one of the mechanisms involved. However, it is difficult to determine whether synergism is caused by the chelating ability of phenolics, as these molecules can also scavenge free radicals. Metal chelators with no free radical scavenging activity, such as EDTA, citric acid, or polyphosphates, could thus be combined with free radical scavengers to better understand if this combination can result in synergistic interactions. For example, Frankel

et al. (1959) identified antioxidant synergism between tocopherols and citric acid in soybean oil. Similarly, combining tocopherols and sodium polyphosphate resulted in increased antioxidant activity in heated palm oil when compared to individual compounds (Chang, 1989). However, a similar synergism was not observed when EDTA and tocopherols were combined. This combination is difficult to assess because EDTA is highly effective at delaying lipid oxidation on its own, resulting in additive but not synergistic interactions (Let et al., 2007). Figure 3.4 gives a schematic representation of combined actions of free radical scavenging and metal chelating activities during inhibition of lipid oxidation.

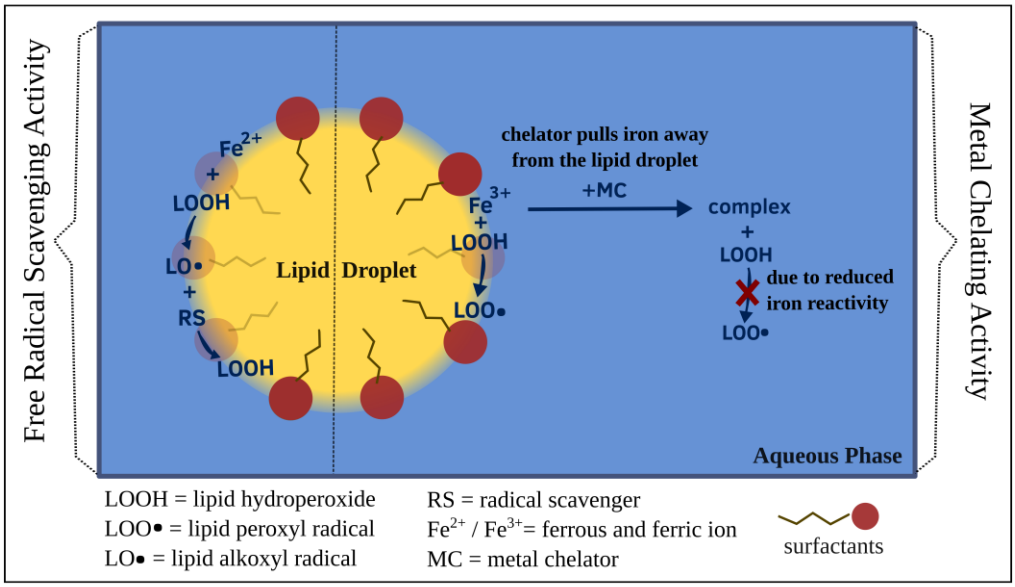


Figure 3.4. Synergistic antioxidant combinations of free radical scavenging and metal chelating activities during lipid oxidation in oil-in-water emulsions.

Both synergism or antagonism are possible depending on the type of metal chelator, type and oxidation state of the metal ion, and the matrix used to perform the experiments. As a result, selecting the metal chelator based on the features of the food system is critical for achieving enhanced oxidative stability as well as synergistic interactions with the free

radical scavenger. This can be accomplished by determining the metal chelating activity in certain food systems using techniques such as UV-VIS spectroscopy, electrospray mass spectrometry, Raman spectroscopy, and nuclear magnetic resonance. After assuring the metal chelating capacity of the compound, the oxidative stability can be analyzed by lipid hydroperoxide and volatile analysis in the absence and presence of the chelator. High performance liquid chromatography might also be used to test the degradation rate of the free radical scavenger in the absence and presence of the metal chelator, which is a good indicator of how well the metal chelator spares the free radical scavenger. Given all of these considerations, developing an effective free radical scavenger and natural metal chelator mixture could be a promising method for extending the shelf-life of lipid-based food products through synergistic interactions.

3.6 Formation of additional antioxidant compounds

Aside from the mechanisms described above, the interactions between antioxidants may cause them to react with one another to form complexes/adducts or to degrade into smaller compounds, resulting in the formation of new antioxidant molecules. If the formed compounds such as intermolecular complexes, adducts, dimers, and/or phenolics have a higher antioxidant activity in the food than the parental antioxidants, it could retard the formation of rancidity and result in synergistic interactions (Olszowy-Tomczyk, 2020). There has not been much research done to determine whether synergism or antagonism in binary systems is caused by the formation of a tertiary compound. However, this mechanism appears to be very likely because antioxidants go through multiple chemical

changes during lipid oxidation and can induce each other's degradation and complexation processes especially in complex food systems due to the presence of other compounds.

Antioxidants or their quinone forms may undergo breakdown processes and form new antioxidant molecules. This was previously observed in oil-in-water emulsions containing α-tocopherol and rosmarinic acid, where rosmarinic quinone fragmented into caffeic acid, and the presence of α-tocopherol increased the caffeic acid formation (Panya et al., 2012). The generation of caffeic acid resulted in synergism due to the presence of a third phenolic compound that could scavenge additional free radicals. Similarly, the synergism between butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) has been attributed to the formation of adducts between compounds and the subsequent formation of new phenolic compounds as a result of their interaction (Omura, 1995).

In addition to the formation of an entirely new antioxidant compound, synergism may result from the formation of intermolecular complexes, adducts, or dimers between antioxidant molecules. 2,2-diphenylpicrylhydrazyl (DPPH) radical scavenging activity was used to determine synergistic antioxidant interactions between glutathione and taxifolin /lutein /quercetin /catechin (Pereira et al., 2013). The synergism was found to be due to the formation of antioxidant monoglutathionyl and diglutathionyl adducts from glutathione and the quinone methide on B ring of the phenolics. In these cases, if the resulting complex has diminished antioxidant properties, the formation of an adduct or additional bonding between antioxidants may result in antagonistic activity. For example, the same authors discovered that the complex formed between glutathione and galangin on ring A resulted in antagonistic activity because the complex formation accelerated glutathione degradation (Pereira et al., 2013). Similarly, catechin and ellagic acid had

antagonistic interactions during low density lipoprotein (LDL) oxidation because hydrogen bonding between the carbonyl group of ellagic acid and the ortho-dihydroxy group of catechin blocked catechin's hydrogen donating capacity (Meyer et al., 1998). Pinelo et al. (2004) also observed antagonistic interactions between resveratrol, catechin, and quercetin, as the polymerization reactions between antioxidants decreased the availability of hydroxyl groups for hydrogen transfer. Therefore, as a rule of thumb, it is possible to expect synergistic interaction if the resulting complex or adduct has more antioxidant activity than the parental compounds, possibly due to its higher stability. On the other hand, if complexation occurs through the hydroxyl groups on molecules, we would expect antagonism because they are the main groups of hydrogen transfer during antioxidant protection.

In addition to the formation of adducts between antioxidant molecules, numerous studies have also examined the effect of antioxidant-protein adducts on synergism. For instance, bovine serum albumin (BSA) demonstrated synergistic activity with green tea catechins in stripped sunflower oil-in-water emulsions (Almajano et al., 2007). The researchers discovered that catechin can bind to BSA irreversibly, and that the resulting protein-catechin adducts have higher antioxidant activity than the parental compounds. The adduct, in this case, retained its antioxidant activity due to the presence of free hydroxyl groups even after complexation. However, depending on the system conditions, such as the type of antioxidant, protein, and food matrix, the formation of antioxidant-protein adducts may have antagonistic or additive effects as well. For example, researchers discovered that complexes formed between casein and tea catechin masked the antioxidant activity of the

tea catechins due to the reduction of optimum free radical scavenging activity (Arts et al., 2002).

In light of these interactions between antioxidant molecules, it is essential to investigate the formation of additional compounds using experimental methods such as liquid chromatography coupled with mass spectrometry. This will aid in the analysis of any unknown molecules in the matrix and possibly determine if they are the cause of the synergistic interaction. Of course, it is impossible to detect and analyze every single component within the matrix for antioxidant activity; thus, it is a smart approach to search for the chemistry and thermodynamics of the parental antioxidants to determine what the possible degradation products or complexes/adducts are. In general, the most oxidizable carbon on the molecule would be the most susceptible to breakdown as a result of the electron-withdrawing effects exerted by neighboring atoms (Panya et al., 2012). This causes the closest bond to undergo scission reactions, leading to the formation of new compounds. Therefore, it could be possible to estimate the newly formed compound by looking at the molecular structures of the parental compounds.

3.7 Conclusion and future perspectives

Antioxidant interactions may have a positive or negative impact on the oxidative stability of food systems, resulting in synergism or antagonism. The most likely synergistic free radical scavenging antioxidant combinations would be an antioxidant physically located at the site of oxidation and a second antioxidant with a lower reduction potential capable of accessing and regenerating the antioxidant at the site of oxidation. In addition, free radical scavenger and metal chelator combination can be effective if the chelator

decreases free radical generation and thus antioxidant degradation. Multiple factors, including the chemical structure, concentration and ratio of antioxidants, type of food system, interaction of antioxidants with other food components, storage conditions, and external environmental factors may influence the type of antioxidant interaction. As a result, understanding the mechanisms underlying synergistic antioxidant interactions is critical for designing food systems with increased antioxidant activity. Understanding the mechanisms will assist the food industry in more rapidly developing effective antioxidant mixtures to extend shelf-life. Being better able to predict synergisms will aid in the development and production of foods with unstable lipids (e.g., omega-3 fatty acids) and thus could improve the health and wellness attributes of the food supply and decrease food waste.

Although synergistic antioxidants are a promising approach for reducing lipid oxidation in foods, some challenges must be addressed. Because antioxidant interactions are extremely complex due to the complexity of food, more extensive research in various food matrices is required to develop synergistic combinations suitable for a specific product. Despite considerable number of research and advances in antioxidant synergism, more detailed research in the following areas is required to gain a better understanding of synergism mechanisms: (1) Development of new techniques for analyzing the antioxidant regeneration ability of a wide range of antioxidants in different systems, such as bulk oils, emulsions, and low-moisture foods; (2) Screening and testing the metal chelating and reducing activities of a wide range of natural plant phenolics in different food systems under varying system conditions (such as pH); (3) Creating fast and reliable techniques for assessing antioxidant interactions and the formation of additional antioxidant compounds.

CHAPTER 4

SYNERGISTIC MECHANISMS OF INTERACTIONS BETWEEN MYRICETIN OR TAXIFOLIN WITH α-TOCOPHEROL IN OIL-IN-WATER EMULSIONS

The part of this research article "Synergistic mechanisms of interactions between myricetin or taxifolin with α-tocopherol in oil-in-water emulsions" was published in Journal of Agricultural and Food Chemistry.

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4.1 Abstract

The antioxidant interactions between α-tocopherol and myricetin in stripped soybean oil-in-water emulsions at pH 4.0 and pH 7.0 were analyzed. At pH 7.0, α-tocopherol (α-TOC): myricetin (MYR) ratios of 2:1 and 1:1 yielded interaction indices of 3.00 and 3.63 for lipid hydroperoxides, and 2.44 and 3.00 for hexanal formation, indicating synergism. Myricetin's ability to regenerate oxidized α-tocopherol and slow its degradation was identified as the synergism mechanism. Antagonism was observed at pH 4.0 due to high ferric-reducing activity of myricetin in acidic environment. The interaction between α-tocopherol and taxifolin (TAX) was also investigated due to structural similarities of myricetin and taxifolin. α-Tocopherol and taxifolin combinations exhibited antagonism at both pH 4.0 and pH 7.0. This was associated with taxifolin's inability to recycle α-tocopherol while still increasing the prooxidant activity of iron. The combination of α-tocopherol and myricetin was found to be an excellent antioxidant strategy for oil-in-water emulsions at pH values near neutrality.

4.2 Introduction

Lipid oxidation is one of the most important chemical reactions affecting food quality, and it can have negative effects on human health through the production of toxic byproducts and the destruction of essential nutrients. Researchers have focused their efforts on the inhibition of lipid oxidation to prolong shelf-life and reduce food loss. The food industry has used synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) for decades to solve this problem; however, a perception that these additives pose a health risk has led researchers to seek natural alternatives (Samdani et al., 2018; Xu et al., 2019). Natural antioxidants such as vitamin E (tocopherols), vitamin C (ascorbic acid), carotenoids, and phenolic compounds are now frequently added to food products to protect the sensory and nutritional quality. Single antioxidants are sometimes not effective at all stages of food preservation because of the complexities of lipid oxidation reactions in heterogeneous foods, such as oil-in-water emulsions. Therefore, it is a good strategy to use antioxidant combinations which produce a synergistic effect to improve antioxidant performance (Judde et al., 2003; Jaswir et al., 2004). Combining different antioxidants not only increases the effectiveness of food preservation but can also reduce the amount of food additive required, resulting in reduced production costs and improved health and sustainability benefits.

Tocopherols are widely distributed antioxidants in food products as they are naturally present in seed oils and foods with cell membrane lipids. The synergistic interaction of tocopherols with other compounds has been the subject of numerous studies to improve antioxidant activity, in which synergism occurred through various mechanisms. For instance, several studies have revealed that tocopherols and ascorbic acid have one of

the most powerful synergistic interactions in biological tissues due to the ability of ascorbic acid to reduce tocopheroxyl radicals in cell membranes to regenerate oxidized α-tocopherol back to its active state (Niki et al., 1985; Thomas et al., 1992; Jorgensen et al., 1999). Furthermore, Panya et al. (2012) found that α-tocopherol has synergistic effects with rosmarinic acid in oil-in-water emulsions due to the formation of an additional antioxidant, caffeic acid, from the decomposition of rosmarinic acid. In addition, strong synergistic interactions of tocopherols with phosphatidylserine in oil-in-water emulsions (Samdani et al., 2018) and with phosphatidylethanolamine in bulk oils (Xu et al., 2019) have been reported through the formation of tocopherones and regeneration via a series of rearrangement reactions (Doert et al., 2012).

Myricetin (3, 3′, 4′, 5, 5′, 7 hexahydroxyflavone) is a naturally occurring antioxidant abundant in fruits, vegetables, tea, berries, and red wine. Myricetin has demonstrated anticancer (Holder et al., 2007), anti-inflammatory (Wang et al., 2010), antibacterial (Xu & Lee, 2001), and antidiabetic (Li & Ding, 2012) activities due to its unique chemical structure. Chobot and Hadacek (2011) have discovered that the mechanism of myricetin's antioxidant effect involves the inactivation of reactive oxygen species and chelation of iron ions, making it a candidate for synergistic interactions with tocopherols. Previous studies have shown the antioxidant activity of myricetin in bulk oil and oil-in-water emulsions (Chen et al., 1996; Wanasundara & Shadidi, 1998; Roedig-Penman & Gordon, 1998) as well as in liposomes (Gordon & Roedig-Penman, 1998). The synergistic antioxidant activity of α-tocopherol and myricetin in stripped sunflower oil has also previously been reported (Marinova et al., 2008); however, to our knowledge, no study has investigated the synergistic activity in oil-in-water emulsions.

Direct evidence of the mechanisms responsible for synergistic antioxidant activity with different antioxidant combinations remains lacking. Different authors have suggested that antioxidant synergism can be explained by: (i) regeneration of an antioxidant that partitions at the site of oxidation (e.g. tocopherols in cell membranes) by a lower reduction potential antioxidant (e.g. ascorbic acid); (ii) formation of complexes between antioxidants which exhibit higher antioxidant activity than that of parent compounds; (iii) differences in the phase distributions of different antioxidants (e.g. lipid and water soluble antioxidants in biphasic systems) to allow for inactivation of different radical types; and (iv) combination of different antioxidants with different mechanisms such as combination of the free radical scavenging and metal chelating activities (Bayram & Decker, 2023).

This study first investigated whether myricetin and α-tocopherol were synergistic in oil-in-water emulsions as they have been reported to be in bulk oils (Marinova et al., 2008). This was performed at pH 4.0 and 7.0 which are common pH values for food emulsions. Soybean oil was chosen specifically because it is highly unsaturated, with a fatty acid composition of 55% linoleic acid, 22% oleic acid, and 6% linolenic acid (Cherif & Slama, 2022). Second, mechanisms producing synergism were evaluated by analyzing myricetin's metal chelating activity and tocopherol regeneration. The results were compared to taxifolin (3, 3′, 4′ 5, 7 pentahydroxyflavanone or dihydroquercetin), a flavonoid structurally similar to myricetin (Figure 4.1). Myricetin has a gallol group on its B ring (3′, 4′, 5′-OH), whereas taxifolin has a catechol group (3′, 4′-OH) and thus, taxifolin is missing one hydroxyl group. In addition, while myricetin's C ring contains a C2-C3 double bond, taxifolin has a saturated bond. Previous research has demonstrated that these differences in structure have a significant effect on metal chelating (Mladěnka et al., 2011),

metal reducing (Mira et al., 2002) and free radical scavenging activities (Gendaram et al., 2011) and therefore, may also have varying impacts on antioxidant synergism. We specifically chose taxifolin as a comparison with myricetin because according to earlier studies, it chelates metal ions (Mira et al., 2002; Topral et al., 2015) but has a higher one-electron reduction potential (500 mV) than myricetin (360 mV) suggesting that it might not be able to regenerate α-tocopherol (500 mV) (Decker et al., 2010). Therefore, the aim of this study was to determine the synergistic antioxidant activity of myricetin and α-tocopherol in oil-in-water emulsions to develop an alternative antioxidant preservation strategy for the food industry. In addition, mechanisms underlying synergistic interactions were evaluated to better predict the properties of antioxidants that might produce synergism.

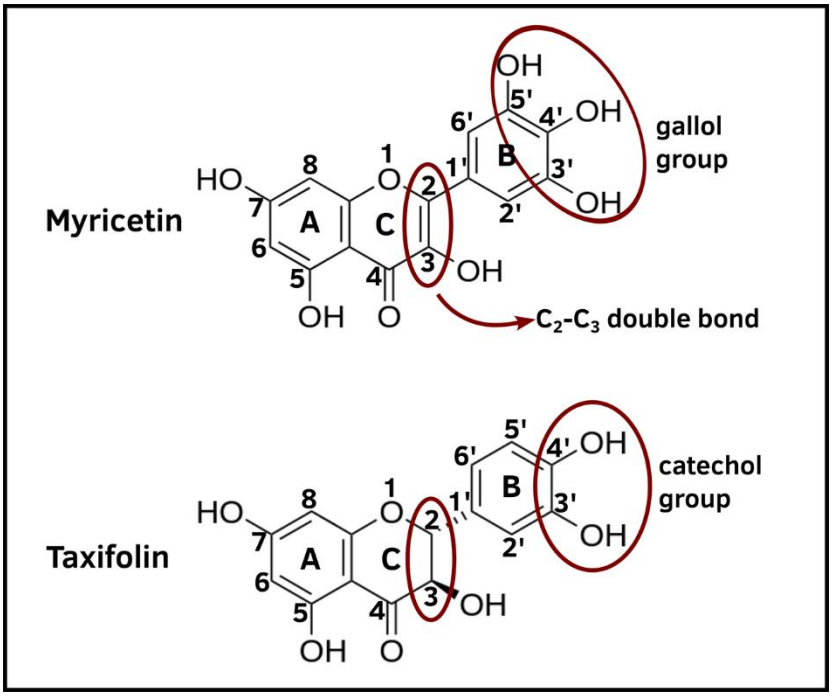


Figure 4.1. Structural differences between myricetin and taxifolin (dihydroquercetin).

4.3 Materials and methods

Soybean oil from a retail store in Hadley, MA was kept at -80 °C until use. Activated charcoal, silicic acid, imidazole, sodium acetate anhydrous, Tween 20, iron (II) sulfate heptahydrate, barium chloride dihydrate, 1,4-dioxane, ethanol, iron (II) chloride, iron (III) chloride, hexanal, cumene hydroperoxide, (±)-α-tocopherol, D-α-tocopherylquinone and 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid sodium salt (ferrozine) were obtained from Sigma-Aldrich (St. Louis, MO). Ascorbic acid, taxifolin, and myricetin were purchased from Combi-Blocks Inc. (San Diego, CA). Ammonium thiocyanate, hydrochloric acid, methanol, 1-butanol, isopropanol, isooctane, hexane, and ethylenediaminetetraacetic acid (disodium salt) were bought from Fisher Scientific (Fair Lawn, NJ).

4.3.1 Oil stripping

Oil stripping was performed to remove minor compounds from soybean oil that can impact oxidation kinetics, such as tocopherols, phospholipids, and transition metals, using the technique described by Boon et al. (2008) Briefly, silicic acid was washed 7-8 times with deionized water to flush out impurities before being dried for 24 hours at 125 °C. The three layers of a chromatographic column (3.0 cm ID x 35 cm L) were created using 22.5 g of silicic acid, 5.25 g of activated charcoal, and another 22.5 g of silicic acid by dissolving each layer in ~40 mL of hexane, preceded by elution of the hexane from the column. The column was loaded with a mixture of 30 g of soybean oil and 30 mL of hexane, and then 270 mL of hexane was passed through the column to retrieve the stripped soybean oil. Stripped soybean oil (SSO) was captured in a round-bottom flask placed in an ice bath and

coated with aluminum foil to avoid light-induced oxidation. Hexane was removed by a vacuum rotary evaporator (Model RE 111, Buchi, Flawil, Switzerland) at a temperature of 27 °C, followed by 10 minutes of nitrogen flushing to eliminate any residual solvent. SSO was kept in an amber bottle at -80 °C until use. High performance liquid chromatography (HPLC) was performed to confirm tocopherol removal after stripping, as described in section 4.3.6, by dissolving 125 μL of SSO in 1 mL of hexane.

4.3.2 Emulsion preparation and storage conditions

Stripped soybean oil-in-water emulsions were prepared using 1% (wt) SSO and 0.1% (wt) Tween 20 as a surfactant. The pH of a 10 mM sodium acetate-imidazole buffer was adjusted to 4.0 and 7.0 with 6N hydrochloric acid. A handheld homogenizer (Model M133/1281-0, BioSpec Products Inc., Bartlesville, OK) was used for 2 minutes to blend the oil, surfactant, and buffer to form a coarse emulsion. The coarse emulsion was then passed through a microfluidizer three times at 39 psig (Model M-110L Microfluidics, Newton, MA) to further reduce the particle size. The microfluidizer chamber and coil were kept cold with an ice-water mixture to minimize lipid oxidation. The final emulsion samples with no antioxidant addition were used as a control group. Ethanolic stock solutions of α-tocopherol, myricetin, and taxifolin were added to the emulsions to give a final α-tocopherol concentration of 30 μM and final myricetin or taxifolin concentrations of 15 or 30 μM. The samples were mixed with a magnetic stirrer at 300 rpm for 4 hours at 4 ºC to ensure homogeneous distribution. Emulsion samples (1 mL) were pipetted into 10 mL GC headspace vials (Supelco), closed with aluminum caps with PTFE/silicone septa, and kept at 37 °C in light-blocking containers throughout the oxidation study. Three vials

from each treatment were taken on a regular basis to assess the formation of primary and secondary lipid oxidation products as well as α-tocopherol concentrations.

4.3.3 Measurement of particle size distribution and zeta potential

Emulsions were characterized by measuring the droplet size distribution via light scattering and zeta potential via micro-electrophoresis with a Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, United Kingdom). Each emulsion was diluted 100 times with continuous phase buffers (pH 4.0 and 7.0) to obtain the best attenuation. The measurements were performed at 25 ºC with 60 seconds of equilibration time. Particle size distribution and zeta potential were determined immediately after emulsion preparation and during the lag phase of oxidation of samples to ensure stability during the storage experiments. The emulsion particle size ranged between 180.9 ± 1.0 nm and 192.8 ± 0.8 nm depending on the sample treatment, and there was no increase in particle size during 35-days of storage, as well as no visible phase separation or creaming. Similarly, the zeta potential ranged between -12.3 ± 0.2 mV and -7.5 ± 0.6 mV for pH 7.0 emulsions, and between 3.9 ± 0.8 mV and 5.4 ± 1.4 mV for pH 4.0 emulsions, with no significant change during 35-days of storage.

4.3.4 Measurement of lipid hydroperoxides

The formation of lipid hydroperoxides were monitored using a modified version of the method described by Shantha and Decker (1994). Shortly, 0.3 mL of emulsion samples were dissolved in 1.5 mL of isooctane: isopropanol (3:1 v/v) solution and centrifuged at 1785*g* for 3 minutes (CL10 centrifuge, Thermo Fisher Scientific, Inc., Waltham, MA). The

upper clear layer (0.2 mL) was pipetted into 2.8 mL of methanol: 1-butanol (2:1 v/v) solution. The samples were vortexed for 30 seconds after addition of 15 μL of a 3.94 M aqueous solution of ammonium thiocyanate and 15 μL of a 0.072 M ferrous solution. To prepare the ferrous solution, 0.144 M iron (II) sulfate heptahydrate ($FeSO_4.7H_2O$) in water and 0.132 M barium chloride dihydrate ($BaCl_2.2H_2O$) in 0.4 M hydrochloric acid (HCl) were mixed at 1:1 ratio and then centrifuged at 1785*g* for 5 minutes to collect the supernatant. A methanol: 1-butanol (2:1 v/v) solution containing no emulsion samples was used as blank. After 20 minutes of reaction time in a light-blocking container at room temperature, the absorbance was recorded at 510 nm using a UV-VIS spectrophotometer (Genesys 20, Thermospectronic, Waltham, MA). Samples having absorbance readings greater than 1.2 were diluted 10 times with methanol: 1-butanol (2:1 v/v) solution. Lipid hydroperoxide concentration was calculated by preparing a calibration curve of cumene hydroperoxide in methanol: 1-butanol (2:1 v/v) solution with concentrations ranging from 0.1 to 0.5 mM. The results of lipid hydroperoxides were expressed as "mmol/kg oil."

4.3.5 Measurement of headspace hexanal

The formation of headspace hexanal was monitored on a regular basis using a Shimadzu Gas Chromatograph (GC)-2010 with an AOC-6000 autosampler, and a flame-ionization detector (FID) (Shimadzu, Kyoto, Japan). The GC vials containing emulsion samples were initially preheated in the autosampler heating block at 55 °C for 10 minutes before injection. A stable flex solid-phase microextraction (SPME) fiber needle (50/30 μm divinylbenzene/ carboxen/ polydimethylsiloxane) (Supelco, Bellefonte, PA) was introduced into the headspace of GC vials for 2 minutes to absorb volatile compounds at

the end of the preheating process. The fiber needle was then placed into the GC injector port at 250 ℃ to desorb the volatile compounds for 3 minutes at a split ratio of 1:7. Helium (carrier gas), hydrogen and air (detector) were used at a flow rate of 30 mL/min, 40 mL/min, and 400 mL/min, respectively. The chromatographic separation of compounds was performed at 65 °C during 10 minutes analysis with a fused-silica capillary column (30 m × 0.32 mm ID × 1 μm) coated with 100% poly (dimethylsiloxane) (Equity DB-1, Supelco). The peak for headspace hexanal was detected by a flame ionization detector (FID) at 250 ℃ with a retention time of ~4.8 min. The headspace hexanal concentration was calculated by preparing a calibration curve of emulsions containing different concentrations of standard hexanal (2-800 μM). The results of headspace hexanal were expressed as "μmol/kg oil."

4.3.6 Measurement of α-tocopherol degradation

α-Tocopherol concentrations in emulsions were measured periodically to determine the antioxidant degradation kinetics using a Shimadzu Prominence-i LC-2030C HPLC (Shimadzu, Kyoto, Japan) coupled with a Supelcosil LC-Diol analytical column (25 cm x 4.0 mm x 5μm, Bellefonte, PA), a Supelguard LC-Diol guard column (2 cm x 4.0 mm x 5μm) and a fluorescence detector (RF-20 Axs, Shimadzu, Kyoto, Japan). Emulsions (0.5 mL) were added to 1.3 mL of isooctane: isopropanol (3:1 v/v) solution to break the emulsions and solubilize α-tocopherol. The samples were centrifuged at 2080*g* for 5 minutes (CL10 centrifuge, Thermo Fisher Scientific, Inc., Waltham, MA) to separate the organic phase from emulsion buffer. The upper layer organic phase containing α-tocopherol (0.5 mL) was evaporated under nitrogen and, hexane (0.5 mL) was added to

solubilize α-tocopherol in a solvent that was compatible with the HPLC mobile phase. After adding the hexane, the samples were passed through 0.22 μm PTFE syringe filters (Fisher Scientific, Pittsburgh, PA) to remove particulates, and then placed into HPLC vials for analysis. The detection of α-tocopherol was performed using normal phase separation technique described by Xu et al. (2015). The mobile phases for the analysis were 95% hexane and 5% 1,4-dioxane, with an isocratic solvent flow rate of 1 mL/min. Twenty μL of each sample was injected into the column, and the analysis was carried out at 37 °C. α-Tocopherol was detected by a fluorescence detector at excitation and emission wavelengths of 290 and 330 nm, respectively, with a retention time of ~4.3 min. The following equation was used to calculate the percentage of remaining α-tocopherol on a specific day.

$$\text{α-Tocopherol \% on day 'X'} = \frac{\text{Peak area on day 'X'}}{\text{Peak area on day 0}} \times 100$$

4.3.7 α-Tocopherol regeneration by myricetin and taxifolin

Myricetin and taxifolin were tested for their ability to reduce α-tocopherylquinone (TQ) to α-tocopherol. One percent (wt) stripped soybean oil-in-water emulsions (at pH 4.0 and pH 7.0) were made according to the emulsion preparation method described in section 2.2. α-Tocopherylquinone (at 1000 μM final concentration) was combined with myricetin or taxifolin (at 1000 μM final concentration) in oil-in-water emulsions and mixed on a magnetic stirrer at 300 rpm for 4 hours at 4 °C to ensure uniform distribution. The samples (1 mL) were pipetted into 10 mL GC headspace vials (Supelco), sealed with aluminum caps with PTFE/silicone septa, and stored at 37 °C in light-blocking containers to repeat the same conditions as the oxidation studies. Three vials were taken from each treatment at regular intervals to determine α-tocopherol concentrations (if present) using the HPLC

method described in section 4.3.6. An emulsion calibration curve was prepared with different concentrations of standard α-tocopherol (2-100 μM) to calculate the concentration of regenerated α-tocopherol.

4.3.8 Determination of ferrous ion chelating activity of flavonoids

The combination of ferrozine and ferrous ions forms a magenta-colored complex. The addition of antioxidants with metal chelating activity causes the antioxidants and ferrozine to compete for binding the ferrous ion, resulting in a loss of color of the ferrozine-ferrous complex. The metal chelating activity of myricetin and taxifolin was determined in 10 mM sodium acetate-imidazole buffer (pH 4.0 and 7.0) using the ferrozine method described by Mladěnka et al. (2011). Briefly, myricetin and taxifolin stock solutions were prepared in methanol due to their limited solubility in water, and then diluted into the final concentrations of 5 to 80 μM using pH 4.0 and pH 7.0 buffers. Twenty μL of 10 mM ferrous chloride solution (in DI water) and 100 μL of 5 mM ferrozine solution (in DI water) were added separately into 5 mL of each antioxidant solution. After vortexing, the samples were incubated at room temperature in the dark for 10 minutes, and the absorbance was recorded at 562 nm with a UV-VIS spectrophotometer (Genesys 20, Thermospectronic, Waltham, MA). Ethylenediaminetetraacetic acid (disodium salt) (Na-EDTA) was used as a standard metal chelator to compare the chelating activity of myricetin and taxifolin. The following equation was used to calculate the percent ferrous ion chelating activity of the antioxidants at given concentrations.

$$\text{Ferrous ion chelating activity \%} = \frac{Abs_{control} - (Abs_{Wsample} - Abs_{Osample})}{Abs_{control}} \times 100$$

where $Abs_{control}$ denotes absorbance without antioxidants but with ferrozine and iron,

$Abs_{Wsample}$ denotes sample absorbance with antioxidant, ferrozine and iron, and $Abs_{Osample}$ denotes sample absorbance with antioxidants but without ferrozine and iron. The results were presented as the half maximal effective concentration value (EC_{50}), which is the concentration of antioxidants needed to change the absorbance of the ferrozine-iron control by 50%. The EC_{50} value was found by plotting the linear regression curve of antioxidant concentration against the percent ferrous ion chelating activity.

4.3.9 Ferric ion reducing activity of flavonoids by modified ferrozine assay

The iron reducing activity of myricetin and taxifolin was measured in 10 mM sodium acetate-imidazole buffer at pH 4.0 and pH 7.0 using a modified ferrozine assay (Shubina & Shatalin, 2017). Briefly, myricetin and taxifolin stock solutions were prepared in methanol due to the limited solubility in water, and then diluted into final concentrations of 15 and 30 μM using pH 4.0 and pH 7.0 buffers. Fifty μL of 10 mM ferric chloride solution (in DI water) was added into the 5 mL of each antioxidant solution to give final ferric ion concentration of 100 μM. Then, 130 μL of 40 mM ferrozine stock solution (in DI water) was added. After vortexing, the samples were incubated at room temperature in the dark for 10 minutes, and the absorbance was recorded at 562 nm with a UV-VIS spectrophotometer (Genesys 20, Thermospectronic, Waltham, MA). Briefly, since ferrozine only binds ferrous ions, color formation would not be anticipated when only ferric ions are present. As a result, the formation of any color during incubation indicates the presence of ferrous ions and demonstrates the antioxidants' iron reducing activity. The concentration of final ferrous ions was calculated by a calibration curve prepared with different concentrations of ferrous chloride (10-100 μM) in pH 4.0 and pH 7.0 buffers.

Ascorbic acid was utilized as the standard due to its known ability to reduce ferric ions.

4.3.10 Statistical analyses

All measurements were taken in triplicate. Data were presented in the form of means ± standard deviations. Minitab 19 (State College, PA) was used to perform one-way analysis of variance (ANOVA), and Tukey's HSD test was employed to compare the differences between mean values ($p < 0.05$). Lipid hydroperoxide and headspace hexanal lag phases were determined based on one-tailed Dunnet's *post hoc* test. The lag phase was defined as the first data point that differed statistically from the day zero reading of the experiment ($p < 0.05$). The interaction indices were calculated using the lag phases to determine whether the interaction between antioxidants was synergistic, antagonistic, or additive. Interaction index > 1 indicates synergism, interaction index = 1 indicates additive effect, and interaction index < 1 indicates antagonism.

$$\text{Interaction index} = \frac{\text{Experimental lag phase}}{\text{Theoretical lag phase}} = \frac{\text{(Lag phase of antioxidant combination)}}{\text{(Lag phase of antioxidant}_1\text{) + (Lag phase of antioxidant}_2\text{)}}$$

4.4 Results and discussion

4.4.1 Antioxidant interactions of α-tocopherol and myricetin or taxifolin in pH 7.0 oil-in-water emulsions

The interaction between α-tocopherol and myricetin was investigated by evaluating lipid hydroperoxides and headspace hexanal formation during storage at 37 °C in 1% stripped soybean oil-in-water emulsions stabilized by Tween 20 at pH 7.0. All antioxidant treatments increased the lag phase of lipid hydroperoxides and headspace hexanal generation compared to the control group that received no antioxidant treatment (Figure

4.2). α-Tocopherol treated emulsion samples had a lag phase of 6 days for lipid hydroperoxides and 7 days for headspace hexanal production, compared to the control group's lag phases of 0 and 1 day, respectively. Emulsions treated with 15 and 30 μM myricetin alone exhibited lag phases of 1 and 2 days for lipid hydroperoxides and 2 and 3 days for headspace hexanal formation, respectively, indicating that myricetin is a weaker antioxidant than α-tocopherol in stripped oil-in-water emulsions. However, combining 15 and 30 μM myricetin with 30 μM α-tocopherol enhanced lag phase of the emulsions considerably, with lipid hydroperoxide lag phases of 21 and 29 days, and hexanal lag phases of 22 and 30 days, respectively. α-Tocopherol: myricetin ratios of 2:1 and 1:1 resulted in interaction indices of 3.00 and 3.63 for lipid hydroperoxides and, 2.44 and 3.00 for headspace hexanal formation. For instance, while 30 μM α-tocopherol alone exhibited 7 days of lag phase and 30 μM myricetin alone exhibited 3 days of lag phase for hexanal formation, the combination resulted in 30 days of lag phase, indicating a very prominent increase in shelf-life. The synergism was also assessed by comparing the lag phase of combination samples (30 μM α-tocopherol + 30 μM myricetin) with 60 μM α-tocopherol and 60 μM myricetin separately to evaluate synergism at same total antioxidant concentration. The results showed that emulsions treated with 60 μM myricetin only and 60 μM α-tocopherol only had lag phases of 2 and 3 days for lipid hydroperoxide formation, and 3 and 4 days for headspace hexanal formation (data not shown), compared to the 30-days of hexanal lag phase of the combination of 30 μM α-tocopherol + 30 μM myricetin. These findings suggested that combining α-tocopherol and myricetin in neutral pH emulsions could be a powerful strategy to reduce the formation of oxidative rancidity.

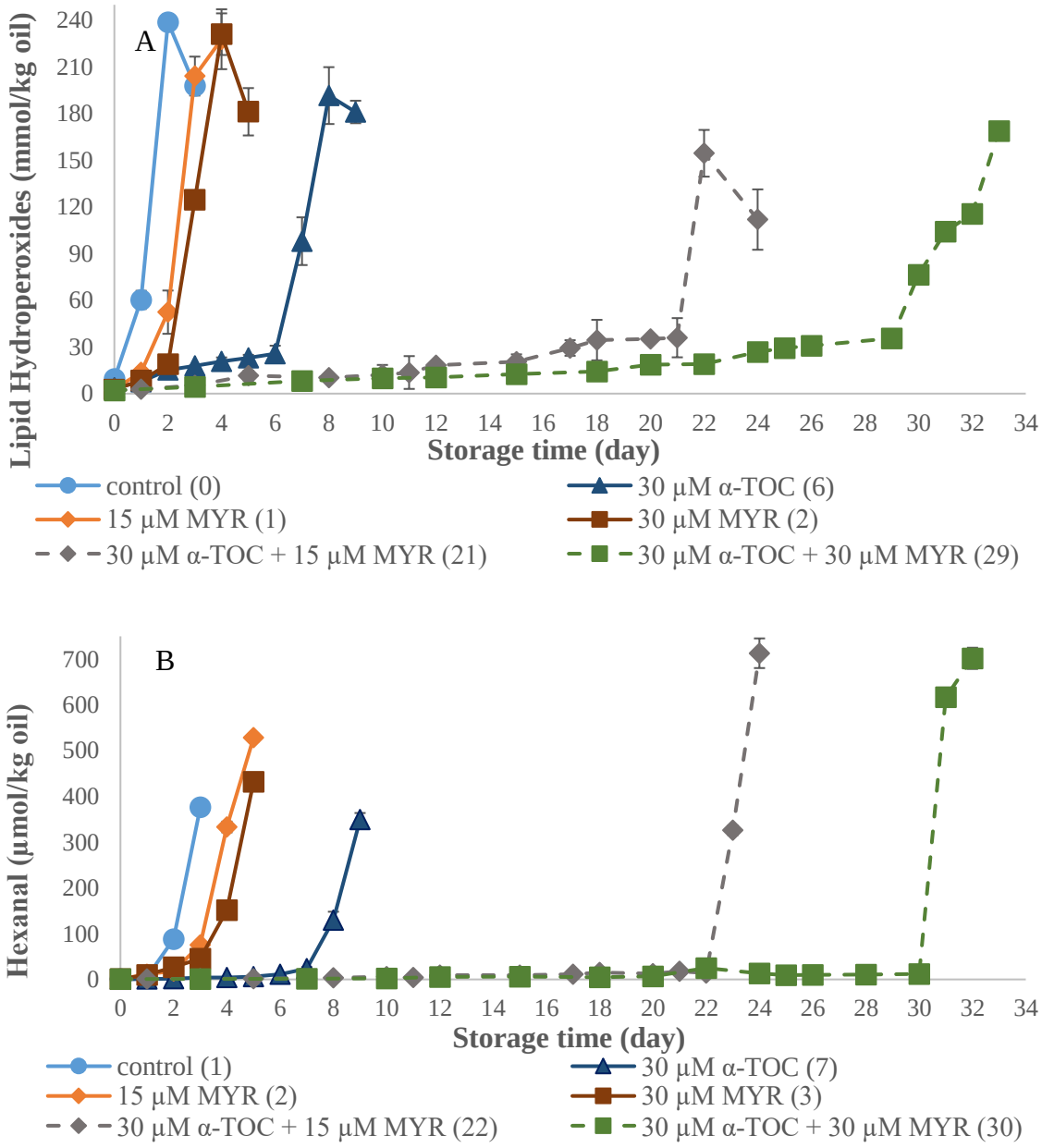
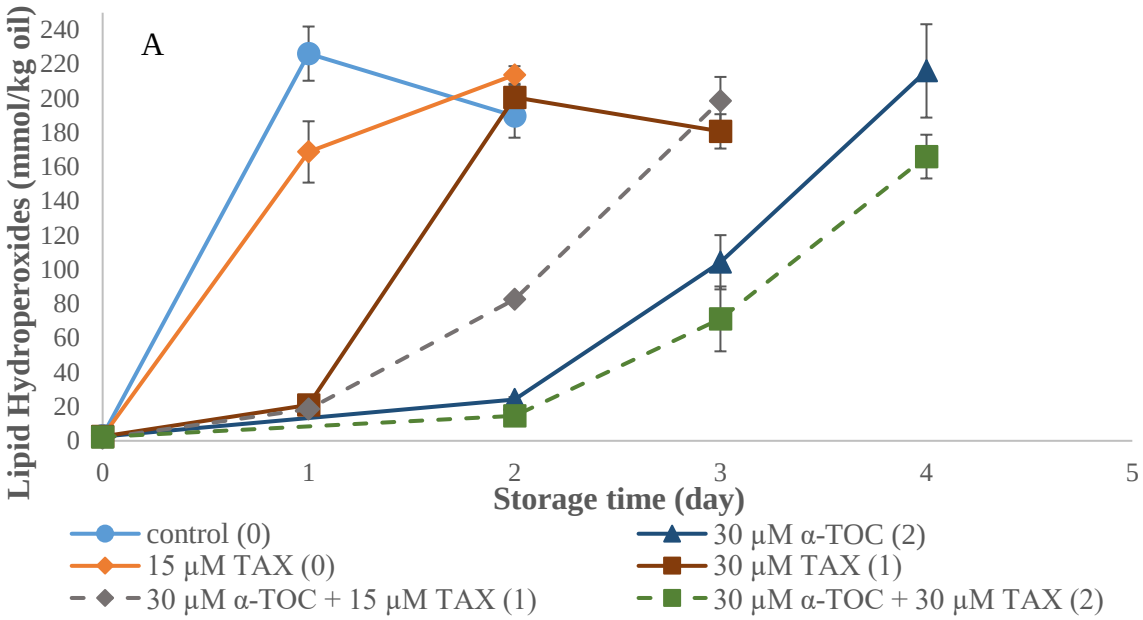


Figure 4.2. Formation of lipid hydroperoxides (A) and headspace hexanal (B) in 1% stripped soybean oil-in-water emulsions (at pH 7.0) stabilized by Tween 20 containing 30 µM α-tocopherol (α-TOC) and 15 or 30 µM myricetin (MYR), and their respective combinations during storage at 37 °C. Data points show means (n=3) ± standard deviation. Some error bars lie within the data points. Lag phases for sample treatments are shown in parentheses in the figure legend.

The synergistic activity of myricetin and α-tocopherol was compared to the activity of taxifolin and α-tocopherol at pH 7.0 since both myricetin and taxifolin can chelate metals but they have very different redox potentials. Identical antioxidant concentrations for α-tocopherol (30 μM) and taxifolin (15 or 30 μM) were tested. Taxifolin treatments failed to inhibit lipid oxidation as the hydroperoxide and hexanal lag phases were the same as the control group (Figure 4.3), indicating that taxifolin was not an effective antioxidant in stripped soybean oil-in-water emulsions at pH 7.0. α-Tocopherol: taxifolin ratios of 2:1 and 1:1 exhibited antagonistic activity with interaction indices of 0.50 and 0.67 for lipid hydroperoxides and 0.50 and 1.00 for headspace hexanal formation, respectively. These results indicated that the combination of α-tocopherol and taxifolin in pH 7.0 emulsions did not further improve the oxidative stability as was seen for α-tocopherol and myricetin combinations.



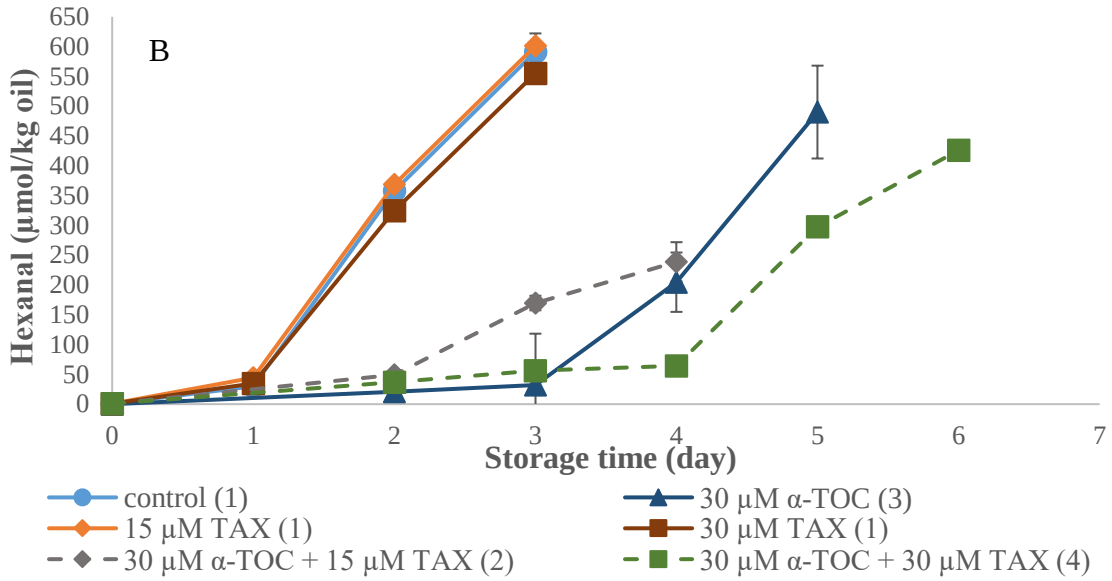
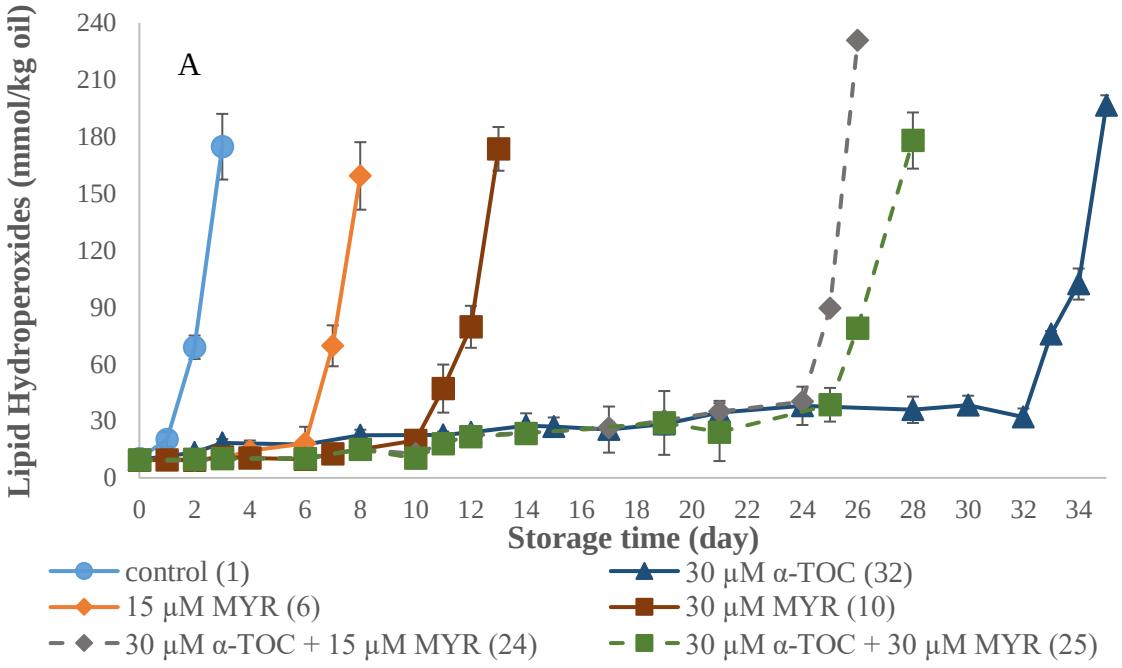


Figure 4.3. Formation of lipid hydroperoxides (A) and headspace hexanal (B) in 1% stripped soybean oil-in-water emulsions (at pH 7.0) stabilized by Tween 20 containing 30 µM α-tocopherol (α-TOC) and 15, 30 µM taxifolin (TAX), and their respective combinations during storage at 37 °C. Data points show means (n=3) ± standard deviation. Some error bars lie within the data points. Lag phases for sample treatments are shown in parentheses in the figure legend.

4.4.2 Antioxidant interactions of α-tocopherol and myricetin or taxifolin in pH 4.0 oil-in-water emulsions

The investigation of antioxidant activity at pH 4.0 is critical because many food emulsions, including salad dressings and condiments have a pH range of 2.9-4.4 (Culler et al., 2022). Figure 4.4 showed that α-tocopherol (30 µM) treated emulsion samples had lag phases of 32 days for lipid hydroperoxides and 33 days for headspace hexanal production, compared to lag phases of 1 and 2 days for the control group. Emulsions containing 15 and 30 µM myricetin had lag phases of 6 and 10 days for lipid hydroperoxides, and 6 and 11 days for headspace hexanal formation, respectively. Combining 15 and 30 µM myricetin with 30 µM α-tocopherol at pH 4.0 exhibited 24 and 25 days of lag phase for lipid

hydroperoxides and 25 and 26 days of lag phase for headspace hexanal. The lag phases for the combinations were lower than those of emulsions containing α-tocopherol alone. This resulted in interaction indices of 0.63, and 0.60 for lipid hydroperoxides and 0.64 and 0.59 for headspace hexanal formation for α-tocopherol: myricetin ratios of 2:1 and 1:1 respectively, showing antagonistic interactions at pH 4.0.



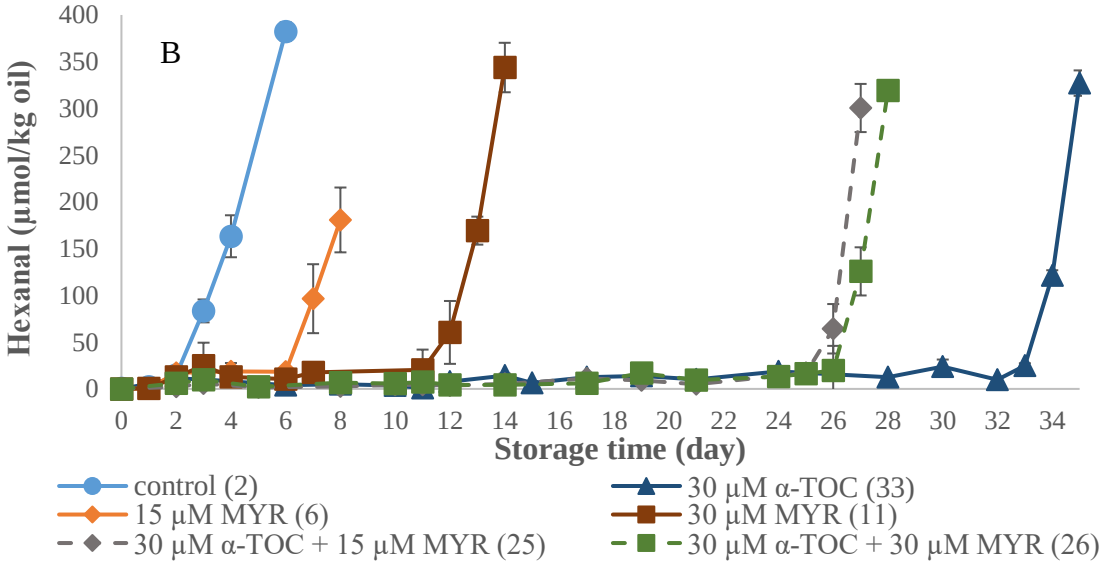
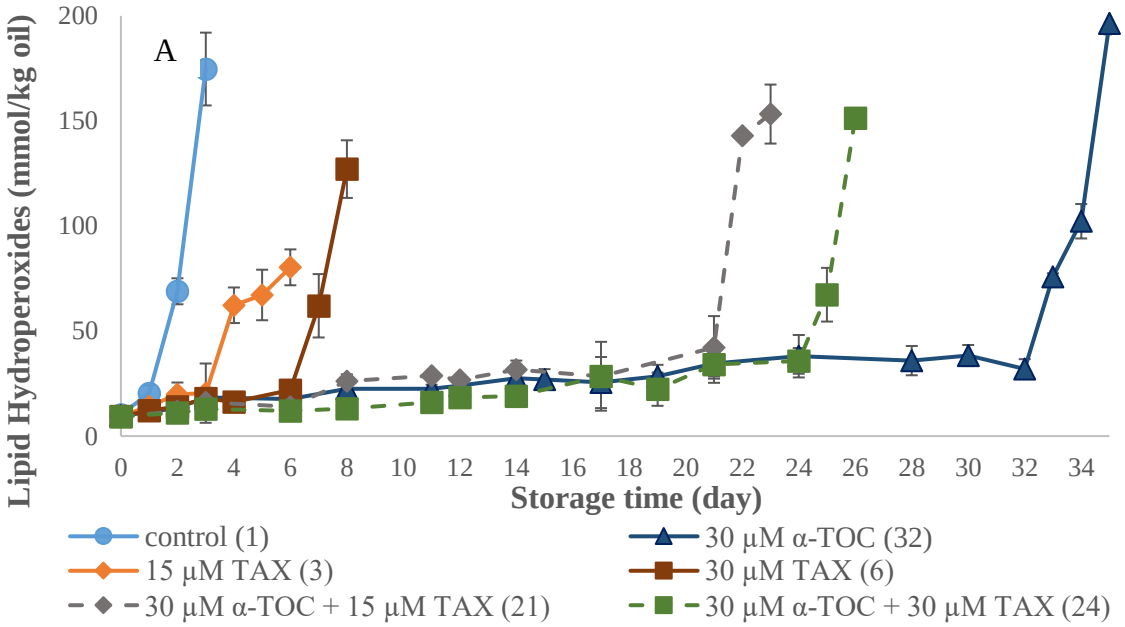


Figure 4.4. Formation of lipid hydroperoxides (A) and headspace hexanal (B) in 1% stripped soybean oil-in-water emulsions (at pH 4.0) stabilized by Tween 20 containing 30 μM α-tocopherol (α-TOC) and 15, 30 μM myricetin (MYR), and their respective combinations during storage at 37 °C. Data points show means (n=3) ± standard deviation. Some error bars lie within the data points. Lag phases for sample treatments are shown in parentheses in the figure legend.

Emulsions containing 15 and 30 μM taxifolin had lag phases of 3 and 6 days for lipid hydroperoxides and 4 and 6 days for hexanal formation, respectively compared to lipid hydroperoxide and hexanal lag phases of 1 and 2 days for the control group at pH 4.0 (Figure 4.5). Combining 15 and 30 μM taxifolin with 30 μM α-tocopherol resulted in lag phases of 21 and 24 days for lipid hydroperoxides and 23 and 25 days for headspace hexanal formation, respectively, which were shorter than those of emulsions containing 30 μM α-tocopherol alone. This resulted in interaction indices of 0.60 and 0.63 for lipid hydroperoxides and 0.62 and 0.64 for headspace hexanal for α-tocopherol: taxifolin ratios of 2:1 and 1:1 respectively, concluding that the combination of taxifolin and α-tocopherol did not act synergistically neither at pH 7.0 (Figure 4.3) nor at pH 4.0 (Figure 4.5).



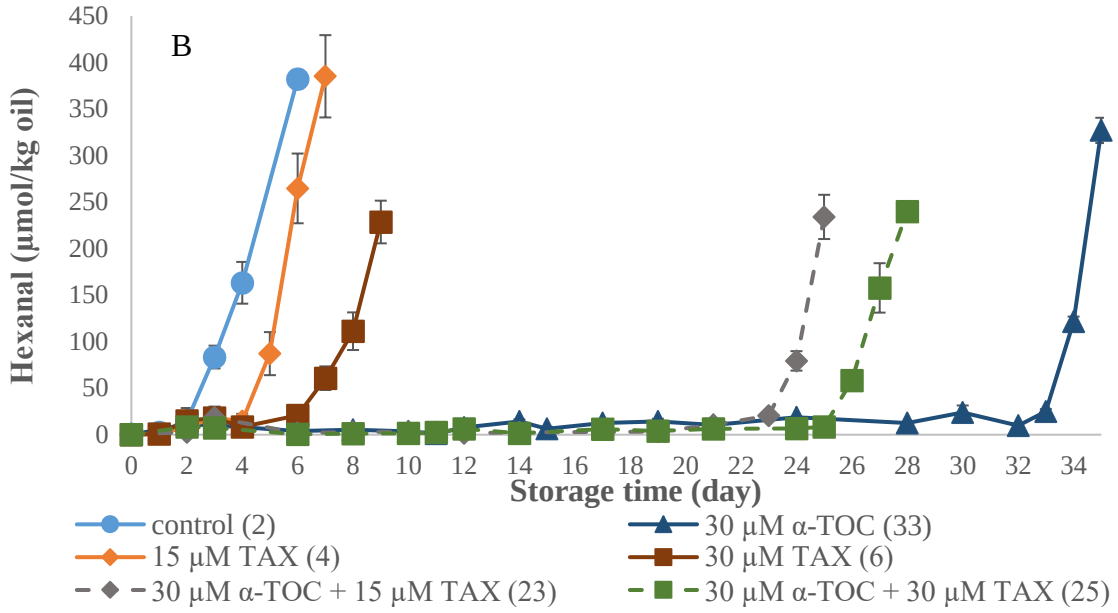


Figure 4.5. Formation of lipid hydroperoxides (A) and headspace hexanal (B) in 1% stripped soybean oil-in-water emulsions (at pH 4.0) stabilized by Tween 20 containing 30 µM α-tocopherol (α-TOC) and 15, 30 µM taxifolin (TAX), and their respective combinations during storage at 37 °C. Data points show means (n=3) ± standard deviation. Some error bars lie within the data points. Lag phases for sample treatments are shown in parentheses in the figure legend.

4.4.3 α-Tocopherol consumption during the storage of pH 4.0 or pH 7.0 oil-in-water emulsions

Analyses of α-tocopherol consumption rates were conducted to identify potential interactions between α-tocopherol and myricetin or taxifolin. At pH 7.0, the combination of α-tocopherol and myricetin slowed the degradation rate of α-tocopherol relative to α-tocopherol-only emulsions (Figure 4.6A). For instance, α-tocopherol alone was depleted after 8 days whereas α-tocopherol combined with 15 and 30 µM myricetin was depleted after 22 and 30 days of storage. On the other hand, α-tocopherol degradation rate in α-tocopherol and myricetin combined samples at pH 4.0 was faster than the α-tocopherol degradation rate alone (Figure 4.6B). For example, α-tocopherol was completely degraded

after 34 days of storage, whereas it was completely degraded after 24 and 25 days in the presence of 15 and 30 μM myricetin, respectively. The rate of α-tocopherol degradation in α-tocopherol and taxifolin combined samples was faster than to the rate of α-tocopherol degradation alone in emulsions at either pH 4.0 or 7.0 (Figure 4.7), suggesting that taxifolin could not interact with α-tocopherol and decrease its rate of degradation.

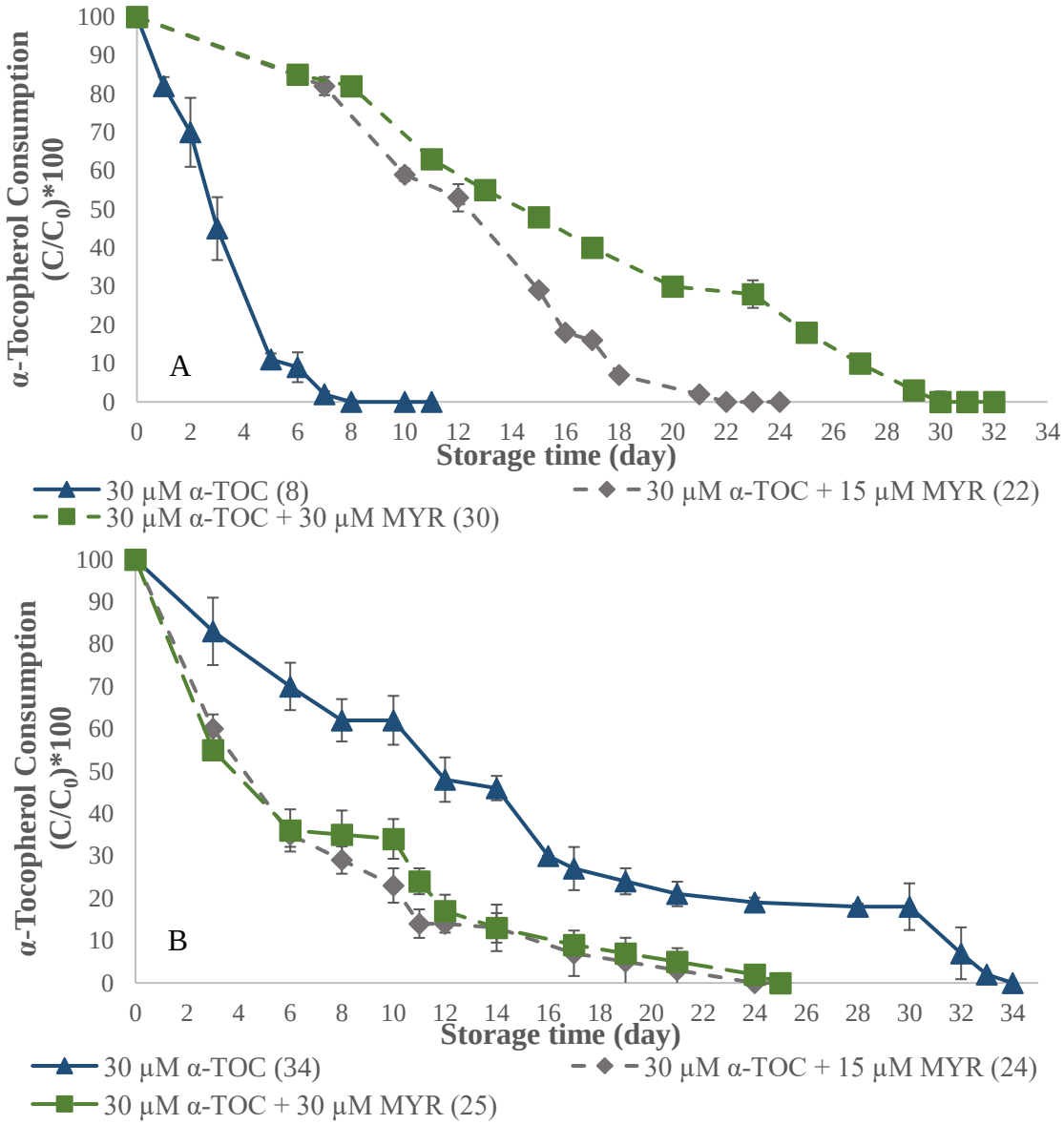


Figure 4.6. Consumption of α-tocopherol in 1% stripped soybean oil-in-water emulsions at pH 7.0 (A) and pH 4.0 (B) stabilized by Tween 20 containing 30 μM α-tocopherol (α-TOC) and its combinations with 15 and 30 μM myricetin (MYR) during storage at 37 °C.

Data points show means (n=3) ± standard deviation. Some error bars lie within the data points. Days for α-tocopherol concentration to fall below 95% of day zero are indicated in parentheses in the figure legend.

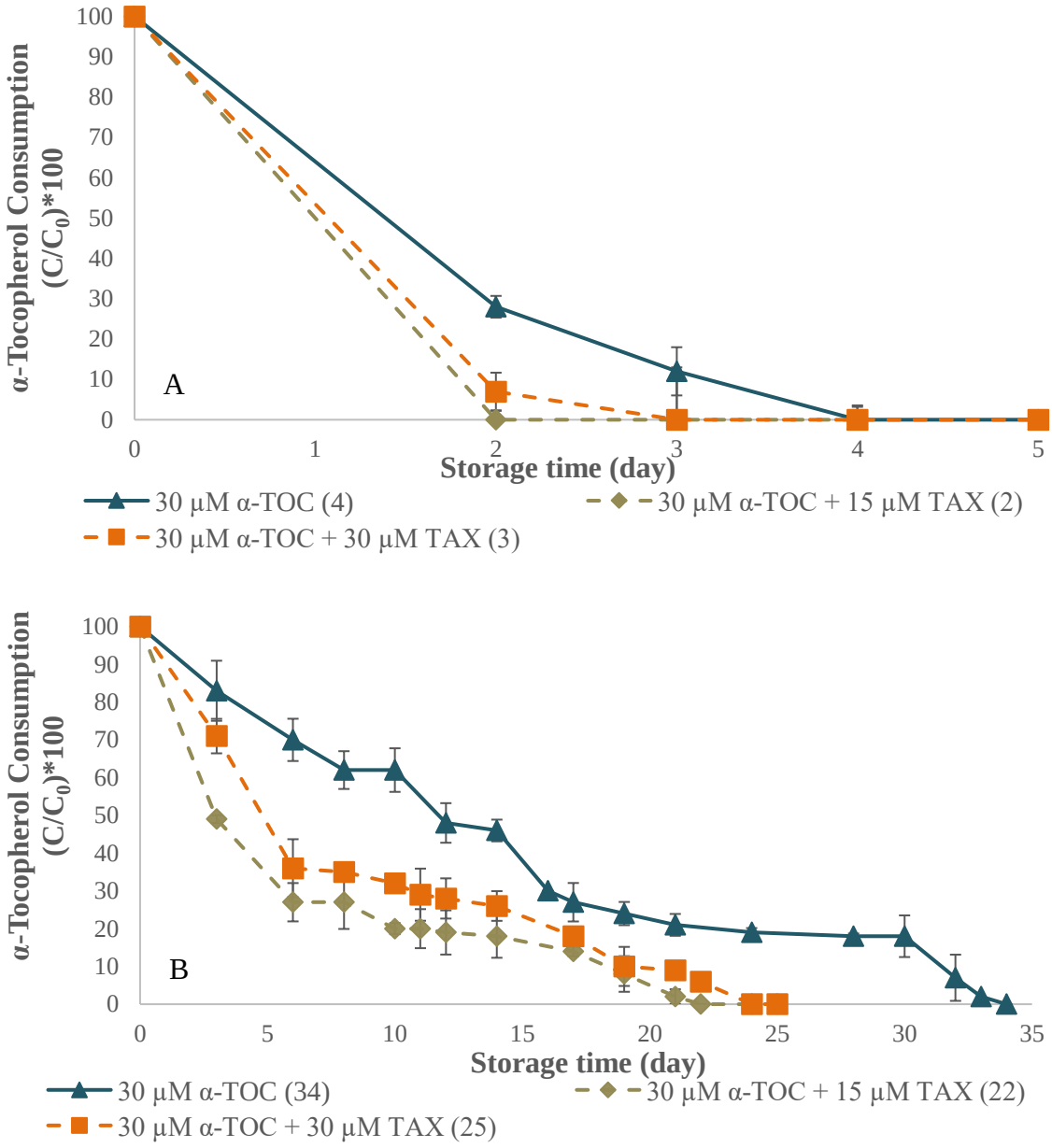


Figure 4.7. Consumption of α-tocopherol in 1% stripped soybean oil-in-water emulsions at pH 7.0 (A) and pH 4.0 (B) stabilized by Tween 20 containing 30 μM α-tocopherol (α-TOC) and its combinations with 15 and 30 μM taxifolin (TAX) during storage at 37 °C. Data points show means (n=3) ± standard deviation. Some error bars lie within the data points. Days for α-tocopherol concentration to fall below 95% of day zero are indicated in parentheses in the figure legend.

Overall, myricetin was able to synergistically interact with α-tocopherol at pH 7.0 (Figure 4.2) but not at pH 4.0 (Figure 4.4) while taxifolin was not synergistic with α-tocopherol at either pH (Figure 4.3 and Figure 4.5). The ability of myricetin and α-tocopherol to interact synergistically could be due to several different mechanisms. First, the metal chelating activity of myricetin could decrease metal-promoted free radical generation which would decrease the consumption of α-tocopherol. If this was the case, then we would have expected taxifolin to also be synergistic since it also chelates metals (Topal et al., 2015), but it was not. In addition, myricetin and taxifolin at pH 4.0 were antagonistic in the presence of α-tocopherol (Figure 4.4 and Figure 4.5), suggesting that other factors such as their ability to increase the prooxidant activity of metals could also be impacting their effectiveness. Second, myricetin could be more effective than taxifolin due to its ability to convert α-tocopheroxyl radical back to its original α-tocopherol form due to its lower reduction potential (360 mV for myricetin vs. 500 mV for taxifolin). This is supported by the ability of myricetin to decrease the rate of α-tocopherol degradation at pH 7.0 (Figure 4.6A) where there was synergistic activity (Figure 4.2). However, it is unclear why myricetin was antagonistic and was not able to decrease α-tocopherol degradation rates at pH 4.0 if α-tocopherol regeneration was the main reason for synergism. Therefore, it seems like the ability of myricetin and taxifolin to interact with both metal ions and α-tocopherol were impacting their ability to inhibit lipid oxidation. In the subsequent sections of this article, we conducted additional evaluations of the ability of myricetin and taxifolin to interact with α-tocopherol and metals in an attempt to gain a better understanding of the factors that can impact synergistic interactions with α-tocopherol.

4.4.4 Regeneration of α-tocopherol by myricetin and taxifolin

One of the mechanisms that could be used to obtain synergistic antioxidant interactions to improve shelf-life is the ability of one antioxidant to regenerate another. This can happen when one of the antioxidants has a lower one-electron reduction potential (E°) than the other and is thus able to regenerate the oxidized antioxidant. The one-electron reduction potentials of α-tocopherol, myricetin, and taxifolin at pH 7.0 are reported to be 500 mV, 360 mV, and 500 mV, respectively (Buettner, 1993; Jovanovic et al., 2000). Therefore, thermodynamics suggests that α-tocopherol could be regenerated by myricetin, but not by taxifolin. To directly observe this phenomenon, equal concentrations of α-tocopherylquinone (oxidized α-tocopherol) and myricetin or taxifolin were combined in the emulsions, and the reduction of the α-tocopherylquinone to regenerate α-tocopherol was determined using HPLC. The results showed that at both pH 4.0 and 7.0, myricetin was able to reduce α-tocopherylquinone back to α-tocopherol structure via two-electron reduction; however, taxifolin was unable to produce any α-tocopherol from α-tocopherylquinone at either pH (Figure 4.8). This was attributed to structural differences between myricetin and taxifolin, with myricetin containing a C2-C3 double bond and a gallol group that decreases its one electron reduction potential and increases its ability to donate hydrogen to α-tocopherylquinone. The fact that myricetin had higher α-tocopherylquinone regeneration rates at pH 4.0 than 7.0 could be due to the greater reducing power of myricetin at low pH. However, it is unclear why myricetin was synergistic at pH 7.0 but not at 4.0 where it more rapidly regenerated α-tocopherol (Figure 4.8).

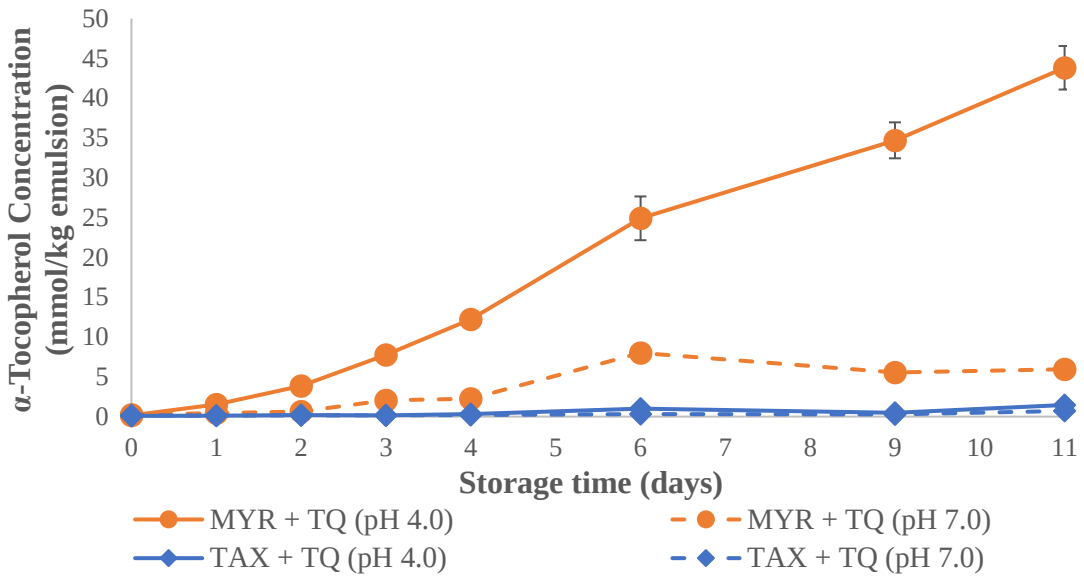


Figure 4.8. Regeneration of α-tocopherol in 1% stripped soybean oil-in-water emulsions at pH 4.0 and pH 7.0 stabilized by Tween 20 containing "1000 μM myricetin (MYR)+1000 μM α-tocopherylquinone (α-TQ)" and "1000 μM taxifolin (TAX)+1000 μM α-tocopherylquinone (α-TQ)". Data points show means (n=3) ± standard deviation. Some error bars lie within the data points.

4.4.5. Interactions between iron and myricetin or taxifolin

Transition metals are among the most significant prooxidants in food systems due to their ubiquitous presence in biological tissues and foods and due to their ability to form alkyl radicals and decompose lipid hydroperoxides to peroxyl and alkoxyl radicals (Schaich, 1992). Metal chelators are commonly employed in food systems to reduce iron-induced lipid oxidation. The metal chelators inhibit lipid oxidation by preventing the redox cycling of metal ions, sterically impeding the contact between metal ions and hydroperoxides, decreasing the solubility of iron, and blocking all metal-binding sites (Burkitt & Gilbert, 1990; Graf, 1990). However, most metal chelators used in the food industry are either synthetic (such as EDTA) or not very effective in oil-in-water emulsions (such as citric acid and phosphates) (Hu et al., 2004). Consequently, the food industry seeks natural alternatives to minimize metal-induced oxidation, especially in emulsion systems

where iron activity is highly prominent. It has been demonstrated that many flavonoids chelate metal ions, making them potent options; however, the effect of their chelating ability on oxidation reactions in emulsions is not always clear. This is because, depending on the type and concentration of flavonoids used, flavonoid-iron complexes may make iron more soluble, allowing greater access of iron to the emulsion droplets. In addition, flavonoids with low redox potential can reduce the oxidation state of iron, making it a stronger prooxidant.

The ferrozine assay was utilized to examine the ferrous ion chelating activity of myricetin and taxifolin at both pH 4.0 and pH 7.0. The ferrous ion was chosen because of its strong prooxidant activity due to its high solubility and reactivity (Dunford, 1987). The ferrous ion chelating activity of myricetin and taxifolin were compared to Na-EDTA because it is one of the most frequently used metal chelators in food formulations. Table 4.1 demonstrated that Na-EDTA had comparable iron chelating activity both at pH 4.0 and pH 7.0, with EC_{50} values of 25.16 ± 1.26 μM and 27.53 ± 1.32 μM, respectively. Na-EDTA's ability to chelate both at pH 4.0 and pH 7.0 was because of its wide range of pKa values of 1.7, 2.6, 6.3, and 10.6 (Decker et al., 2010), that allows it to have deprotonated groups that can bind metal ions even at pH 4.0.

Table 4.1. Ferrous ion chelating activity (EC_{50} = half maximal effective concentration) and ferric ion reducing capacity of myricetin (MYR) and taxifolin (TAX) in 10 mM sodium acetate-imidazole buffer at pH 7.0 and pH 4.0. The efficiency of ferrous ion chelating activity and ferric ion reducing capacity was compared with Na-EDTA and ascorbic acid standards, respectively. The initial concentration of ferric chloride was 100 μM for testing the ferric ion reducing activity. Data points show means (n=3) ± standard deviation.

Ferrous ion chelating activity (EC_{50} value)		
	pH 7.0	**pH 4.0**
Myricetin	10.41 ± 0.49 μM	-
Taxifolin	42.63 ± 2.45 μM	-
Na-EDTA	27.53 ± 1.32 μM	25.16 ± 1.26 μM

Ferric ion reducing activity (Concentration of ferrous ions formed)		
	pH 7.0	**pH 4.0**
Myricetin (15 µM)	1.72 ± 0.19 µM	57.27 ± 0.40 µM
Myricetin (30 µM)	1.27 ± 0.09 µM	83.93 ± 0.30 µM
Taxifolin (15 µM)	0.83 ± 0.12 µM	20.44 ± 0.11 µM
Taxifolin (30 µM)	0.44 ± 0.08 µM	43.43 ± 0.28 µM
Ascorbic Acid (15 µM)	4.17 ± 0.20 µM	30.67 ± 0.11 µM
Ascorbic Acid (30 µM)	5.66 ± 0.16 µM	56.35 ± 4.38 µM

Both myricetin and taxifolin were able to chelate ferrous ions at pH 7.0 (Table 4.1). According to our findings, the ferrous ion chelating activity of myricetin (EC_{50}=10.41 ± 0.49 µM) was greater than that of Na-EDTA (EC_{50}=27.53 ± 1.32 µM) while taxifolin (EC_{50}=42.63 ± 2.45 µM) was less than both myricetin and EDTA. It is likely that the reason why myricetin exhibited greater iron chelating activity than taxifolin at pH 7.0 was due to its lower pKa value and the presence of a C2-C3 double bond, which facilitates chelation by increasing the planarity of the molecules. For instance, Mladěnka et al. (2011) compared the iron chelating activity of flavonoids and concluded that the presence of the C2-C3 double bond improved the metal chelating activity of quercetin at neutral pH compared to taxifolin since double bond in quercetin keeps the A and C rings coplanar, allowing the 3-OH/4-oxo and 5-OH/4-oxo groups to be closer for chelation. Similar results were discovered by Mira et al. (2002) who found that apigenin was highly effective at chelating copper ions, whereas naringenin with the same structure but lacking a C2-C3 double bond was not as effective.

Neither myricetin nor taxifolin were able to chelate ferrous ions at pH 4.0 (Table 4.1). Álvarez-Diduk et al. (2013) determined the experimental pKa values for the six hydroxyl groups of myricetin to be 6.33, 7.57, and 9.65 for its 4′, 7, 3-OH groups and

theoretical values of 11.84, 12.77, and 14.04 for its 5′, 5, 3′-OH groups, respectively. In the case of taxifolin, no analysis of its five different hydroxyl groups has been reported. However, SciFinder estimated the most acidic pKa values of myricetin and taxifolin to be 6.30 ± 0.40 and 7.39 ± 0.60, respectively. This suggests that neither myricetin nor taxifolin have deprotonated groups and thus lack metal chelation sites at pH 4.0.

The antioxidant activity of flavonoids is also influenced by their metal-reducing capacity (Moran et al., 1997). This reduction causes the formation of a more soluble and reactive state of the metal ions, which more rapidly promotes the formation of hydroxyl radicals from hydrogen peroxide (Koppenol, 1993) and peroxyl and alkoxyl radicals from fatty acid hydroperoxides (Tadolini et al., 1997). Therefore, the potential prooxidant activity of myricetin and taxifolin via reduction of ferric ions at pH 4.0 and pH 7.0 was determined by exposing different concentrations of myricetin and taxifolin (15 and 30 μM) to 100 μM ferric chloride and measuring the binding of the resulting ferrous ions to ferrozine.

According to the results, both myricetin and taxifolin had iron-reducing activity at pH 4.0 (Table 4.1), and myricetin had a higher iron-reducing capacity than taxifolin. For example, 30 μM myricetin and 30 μM taxifolin produced 83.93 ± 0.30 μM and 56.35 ± 0.38 μM of ferrous ions, respectively. At pH 7.0, myricetin and taxifolin had almost no iron reducing activity, with less than 2 μM ferrous ions formed from 100 μM ferric ions (Table 4.1). It is important to note that not all the reduced iron contributes to color formation at neutral pH because myricetin and taxifolin also chelate the ferrous ions (Table 4.1). As a result, the concentrations of reduced ferrous ions at pH 7.0 may be slightly higher than observed. Mei et al. (1999) made a similar observation on pH effect, where gallic acid

and methyl gallate had 44-55 times more iron reducing activity at pH 3.0 than at pH 7.0.

According to previous research, ascorbic acid can reduce two equivalents of transition metal ions (Shubina & Shatalin, 2017). Our findings supported this data, as the addition of 15 µM and 30 µM ascorbic acid resulted in the formation of 30.67 ± 0.11 µM and 56.35 ± 4.38 µM ferrous ions, respectively, indicating an ascorbic acid: reduced iron ratio of 1:2. A similar conclusion was reached for myricetin and taxifolin which were able to produce more ferrous ions on a molar basis than added antioxidants, implying that they would be reducing more than one equivalent of iron. For example, 15 and 30 µM myricetin produced ferrous ions concentrations of 57.27 ± 0.40 and 83.93 ± 0.30 µM, respectively, at pH 4.0 (Table 4.1), indicating that more than three equivalents of ferric ions were reduced. This could be due to the presence of six different hydroxyl groups that could potentially reduce iron. Similarly, 15 and 30 µM taxifolin led to the formation of 20.44 ± 0.11 and 43.43 ± 0.28 µM ferrous ions, respectively, at pH 4.0 (Table 4.1), which is slightly less than the reducing power of ascorbic acid. The significance of the C2-C3 double bond on iron-reducing activity has been previously reported (Mira et al., 2002; Shubina & Shatalin, 2017) and was postulated to be due to the delocalization of electron density between A, B and C rings.

When all the mechanistic studies are correlated with the lipid oxidation findings, the following conclusions can be drawn about the study. Synergistic activity was observed between α-tocopherol and myricetin at pH 7.0 (Figure 4.2). This seemed to be due to the ability of myricetin to regenerate α-tocopherol as shown by its ability to regenerate α-tocopherylquinone (Figure 4.8) and chelate metals (Table 4.1), which in turn could have decreased the degradation rate of α-tocopherol (Figure 4.6A). Even though myricetin was

able to reduce large amounts of α-tocopheroxyl radical at pH 4.0 compared to pH 7.0 (Figure 4.8), it showed antagonistic interactions with α-tocopherol (Figure 4.4) and also did not decrease α-tocopherol consumption rates (Figure 4.6B). This was potentially due to myricetin's increased iron reducing activity, as well as its diminished ability to chelate iron (Table 4.1) due to its protonated hydroxyl groups at pH 4.0 both of which could increase metal promoted free radical generation and increase the rate of tocopherol degradation. These results suggest that the ability of myricetin to reduce the oxidation state of iron and increase the activity of prooxidant metals was probably more important than its α-tocopherol regenerating activity in its overall antioxidant activity at pH 4.0.

Taxifolin did not synergistically interact with α-tocopherol either at pH 7.0 (Figure 4.3) or pH 4.0 (Figure 4.5). This was attributed to the higher redox potential of taxifolin, which prevented it from reducing α-tocopherylquinone at either pH (Figure 4.8), thereby preventing it from decreasing α-tocopherol degradation rates (Figure 4.7). In addition, taxifolin had weaker iron chelating activity at pH 7.0 than myricetin (Table 4.1) which also could impact its ability to interact synergistically with α-tocopherol. At pH 4.0, taxifolin did not chelate iron but was able to promote iron reduction (Table 4.1), both of which may have contributed to its inability to enhance the activity of α-tocopherol.

4.5 Conclusion

Overall, this study demonstrated that the combination of myricetin and α-tocopherol in oil-in-water emulsion at pH 7.0 produced synergistic antioxidant activity and thus could be a novel method for enhancing the oxidative stability of food. It also suggests that combining α-tocopherol with other flavonoids with reducing potentials that would

allow them to regenerate oxidized tocopherols could also produce synergistic antioxidant activity. If another antioxidant that could regenerate oxidized tocopherols but did not reduce iron as strongly as myricetin, it is possible that synergistic antioxidant activity could be produced at lower pH values.

CHAPTER 5

ANALYSIS OF THE MECHANISM OF ANTIOXIDANT SYNERGISM BETWEEN α-TOCOPHEROL AND MYRICETIN IN BULK OIL

5.1 Abstract

α-Tocopherol (α-TOC) and myricetin (MYR) synergistically inhibit lipid oxidation in bulk oil but the mechanism underlying this effect is unknown. In this research, stripped soybean oil (SSO) was treated with α-tocopherol (50 μM), myricetin (10-250 μM), and their combinations. Taxifolin (TAX) was also tested because it has structural similarities to myricetin but with a higher redox potential. Tocopherol:myricetin ratios of 5:1, 2:1, 1:1, 1:2, and 1:5 resulted in extended lag phases ranging from 16 to 99 days, with lag phase increasing with increasing myricetin concentrations. Synergism between myricetin and tocopherol was also seen in phospholipid-containing bulk oils, both in the absence and presence of reverse micelles. Myricetin (redox potential=360 mV) delayed the oxidation of α-tocopherol (redox potential=500 mV) whereas taxifolin (redox potential=500 mV) did not. Both myricetin and taxifolin were able to chelate iron as determined by UV-VIS spectroscopy. These results suggest that the lower redox potential of myricetin allowed it to produce synergistic antioxidant activity by regenerating oxidized α-tocopherol and through its ability to decrease oxidation by metal chelation.

5.2 Introduction

Lipid oxidation is a series of chemical reactions where unsaturated fatty acids react with oxygen to form lipid hydroperoxides. These lipid hydroperoxides further break down into small, volatile molecules such as aldehydes and ketones. This leads to the formation

of undesired aroma and flavor in food, the production of toxic compounds, and the co-oxidation of other food molecules such as proteins and pigments, impacting the texture, functionality, and appearance (Vieira et al., 2017). Therefore, the food industry adapts different strategies in food to overcome the negative consequences of lipid oxidation. One of the very promising approaches to retard oxidative rancidity is combining natural antioxidants to produce synergistic antioxidant interactions.

The interaction between antioxidants exhibits synergistic, additive, or antagonistic effects depending on the type of food system, nature of antioxidants, and their respective ratios. Synergism is a concept where the combined antioxidant action is greater than the sum of each antioxidant action separately (Olszowy-Tomezyk, 2020). Synergistic antioxidant interactions have multiple benefits, including prolonged shelf-life, improved food quality, reduced production costs, and decreased food waste (Tsao, 2015). Since synergistic antioxidant combinations could give the same or better antioxidant stability as commercially used synthetic antioxidants such as butylated hydroxytoluene (BHT) or tert-butylhydroquinone (TBHQ), they could be incorporated into food systems to obtain clean-labeled products (Culler et al., 2021).

Synergistic antioxidant interactions could be more widely used strategies to improve oxidative stability in foods if how and where they work can be better understood and predicted. Understanding the mechanism behind the synergistic activity is crucial to establishing protocols to screen multiple antioxidants and testing for potential synergistic interactions. The synergistic antioxidant mechanisms are not often well understood but have been postulated to be due to the regeneration of one antioxidant by another, the

combination of free radical scavenging and metal chelating activities, and the different partitioning of antioxidants within the food matrix (Bayram & Decker, 2023).

The food industry utilizes free radical scavenging antioxidants such as tocopherols because they can interact with fatty acid radicals and decrease their ability to promote further oxidation. However, free radical scavengers such as tocopherols are eventually depleted as they scavenge free radicals and thus, their ability to protect unsaturated fatty acids is lost (Arora et al., 2022). Antioxidant regeneration is a phenomenon where a compound donates an electron to an oxidized free radical scavenger to return the antioxidant to its original state so that it can scavenge additional free radicals (Decker & Bayram, 2021). The common example of this phenomenon has been observed between α-tocopherol (α-TOC) and ascorbic acid, where ascorbic acid reduces α-tocopheroxyl radical back to α-tocopherol to prolong its scavenging activity (Niki et al., 1984), leading to synergistic actions. This electron transfer between α-tocopherol and ascorbic acid is thermodynamically favored due to the differences in redox potentials (500 mV for α-tocopherol, 282 mV for ascorbic acid) (Decker et al., 2010).

Combining free radical scavenging and metal chelating antioxidants is another strategy to improve oxidative stability due to their different inhibition pathways. Metal chelators inhibit lipid oxidation by interacting with transition metals such as iron and copper to decrease their ability to decompose lipid hydroperoxides into chain reaction-promoting free radicals. By decreasing free radical generation rates, free radical scavenging antioxidants are consumed slower and can inhibit lipid oxidation for longer periods of time. Ethylenediaminetetraacetic acid (EDTA) is one of the most effective metal chelators, but it is not a preferred antioxidant in food systems since it is not natural and is not considered

a “clean-label” ingredient (Decker & Bayram, 2021). Therefore, substituting EDTA with natural metal chelators and combining them with free radical scavenging antioxidants could be a promising way to improve shelf-life due to the different inhibition mechanisms.

Myricetin (MYR) is an excellent antioxidant to analyze the synergistic activity with tocopherols since it has lower redox potential that might lead to tocopherol regeneration (Decker et al., 2010), and has multiple reactive hydroxyl groups that might lead to metal chelation (Cherrak et al., 2016). It is also naturally present in fruits, vegetables, and nuts (Ross & Kasum, 2002), and suitable to incorporate in food systems due to its anti-cancer, anti-diabetic, and anti-infectious activities (Park et al., 2016). The previous studies have shown that myricetin can be used to extend the shelf-life of stripped sunflower oil (Roedig-Penman & Gordon, 1998), canola oil (Chen et al., 1996), and marine oil (Wanasundara & Shadidi, 1998).

Much of the literature focuses primarily on combining antioxidants and determining whether they act synergistically or antagonistically, with very little research focusing on the mechanisms by which antioxidants interact. Therefore, there are currently few systematic approaches for predicting if antioxidant combinations will result in synergistic, additive, or antagonistic activity. Therefore, this research focuses on determining if synergistic antioxidant activity between α-tocopherol and myricetin is due to antioxidant regeneration, metal chelating activity or both. The activity of myricetin was compared to taxifolin (TAX) which is structurally similar to myricetin. Both molecules have 3-OH/4-CO, 4-CO/5-OH, and catechol groups, suggesting they can chelate metals, but taxifolin does not have C2-C3 double bond and pyrogallol group, which increases its redox potential to 500 mV compared to 360 mV for myricetin (Decker et al., 2010).

Previous research has demonstrated that myricetin and α-tocopherol exhibit synergistic activity during the autooxidation of stripped sunflower oil (Marinova et al., 2008). The article analyzed the oxidative stability of oil at 100 °C by measuring the formation of the primary oxidation products, peroxides and conjugated dienes. Since aldehydes are the primary causes of food spoilage and rancidity, our research focuses on the measurement of both primary (lipid hydroperoxides) and secondary (headspace hexanal) oxidation products. We also used 60 °C temperature to more accurately simulate the oxidation of oil during storage, as lipid oxidation results are misleading at temperatures above 60 °C for vegetable oils, which could lead to inaccurate interpretation as expressed by Frankel (2005). Marinova et al. (2008) stated that myricetin was regenerated by α-tocopherol by comparing the consumption rates of the antioxidants; however, this comparison may be misleading because thermodynamically, α-tocopherol should not be able to regenerate myricetin because of its higher redox potential. Oppositely, our previous research has shown the ability of myricetin to reduce α-tocopherylquinone back to α-tocopherol both in acidic and neutral oil-in-water emulsions (Bayram et al., 2023).

In the current study, different methods were also utilized to determine the mechanisms behind the synergistic activity, including high-performance liquid chromatography (HPLC) to measure myricetin/α-tocopherol/taxifolin degradation kinetics and UV-VIS spectroscopic analysis to detect the presence of iron-myricetin complex. Mechanistic knowledge gained from these investigations may provide a more systematic approach to determine which antioxidant combinations in bulk oils are synergistic, additive, or antagonistic.

5.3 Materials and methods

Soybean oil and sunflower oil were purchased from a retail store in Hadley, MA, and medium-chain triacylglycerol (MCT) was obtained from Warner Graham Company (Baltimore, MD). Myricetin and taxifolin were purchased from Combi-Blocks Inc. (San Diego, CA). Silicic acid, activated charcoal, barium chloride dihydrate, iron (II) sulfate heptahydrate, iron (II) chloride, cumene hydroperoxide, hexanal, (±)-α-tocopherol, 1,4-dioxane, and ethanol were purchased from Sigma-Aldrich (St. Louis, MO). Hexanes, 1-butanol, methanol, hydrochloric acid and ammonium thiocyanate were purchased from Fisher Scientific (Fair Lawn, NJ). 1,2-dibutyryl-sn-glycero-3-phosphocholine (DC_4PC) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were obtained from Avanti Polar Lipids (Birmingham, AL).

5.3.1 Flavonoid-metal complexation by UV-VIS spectroscopy

The spectra of free and iron-complexed myricetin and taxifolin in non-oxidizable MCT oil were recorded from 230 nm to 800 nm at room temperature on a UV-VIS spectrophotometer (Genesys 20, Thermospectronic, Waltham, MA) using the method of Cornard et al. (2001) with minor modifications. MCT oil with constant concentrations of myricetin and taxifolin (40 μM) and varying concentrations of ferrous chloride (0-600 μM) were prepared to yield iron: flavonoid ratios of 0, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 1, 1.5, 2, 2.5, 5, 10, and 15. The shift in maximum absorbance wavelength of the flavonoid upon addition of ferrous chloride was recorded to determine the presence of iron: flavonoid chelates. The molar ratio method (Yoe & Jones, 1944) was used to calculate the iron chelation stoichiometry of flavonoids by plotting iron: flavonoid molar ratio vs. absorbance at

maximum absorbance wavelength after complexation, yielding a curve with two straight lines. The intersection of two asymptotic lines indicated the iron chelation stoichiometry after interpolating to the molar ratio x-axis.

5.3.2 Isolation of soybean oil triacylglycerols

Oil stripping was performed to remove naturally occurring minor compounds from the oil, including tocopherols, phospholipids, mono and diacylglycerols, free fatty acids, and metal ions, according to the method of Boon et al. (2008) with minor modifications. In short, silicic acid was washed seven to eight times with deionized water to remove impurities, and then dried at 125 °C for 24 hours for activation. The layers of a chromatographic column (3.0 cm in diameter and 35 cm in height) were created by adding 22.5 g of silicic acid, 5.25 g of activated charcoal, and another 22.5 g of silicic acid by dissolving each layer in 40 mL of *n*-hexane, followed by elution of excess *n*-hexane from the column. Soybean oil (30 g) mixed with 30 mL of *n*-hexane was loaded onto the column and then 270 mL of *n*-hexane was passed through the column to recover stripped soybean oil (SSO). The stripped oil was collected in a round-bottom flask placed in an ice bath and covered with aluminum foil to prevent light-induced oxidation. *n*-Hexane was evaporated using a vacuum rotary evaporator (Model RE 111, Buchi, Flawil, Switzerland) at 27 °C, followed by 10 min of nitrogen flushing to remove any remaining trace solvent. The final stripped soybean oil was stored in amber glass at -80 °C until use. Verification of tocopherol removal after stripping was determined using HPLC by the method (Section 5.3.7)

5.3.3 Sample preparation for oxidation study

A 50:50 mixture of stripped soybean oil (SSO) and non-oxidizable medium-chain triacylglycerol (MCT) were blended to decrease excessive oil stripping to reduce the amount of waste solvents. In the first part of the experiments, ethanol stock solutions of α-tocopherol, myricetin, and/or taxifolin were added into 1:1 SSO:MCT oil blend at a final α-tocopherol concentration of 50 μM and final myricetin and taxifolin concentrations of 10, 25, 50, 100, 250 μM. In the second part of the experiments, final α-tocopherol concentration of 25 μM and final myricetin concentration of 25 μM were combined in the presence of 1000 μM 1,2-dibutyryl-sn-glycero-3-phosphocholine (DC_4PC) or 1000 μM 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 200 ppm of water to produce oil systems with reverse micelles. In the final part of the experiments, different concentrations of myricetin (50, 100, and 250 μM) were added into store-bought soybean oil and sunflower oil to analyze the improvement in oxidative stability.

After addition of antioxidants, the samples were magnetically stirred overnight at 4 °C to obtain a homogeneous distribution of the antioxidants. Samples (0.5 mL) were aliquoted into 10 mL GC headspace vials (Supelco), sealed with aluminum caps with PTFE/silicone septa, and stored at 60 °C in the dark throughout the oxidation study. Periodically, three vials were taken from each group to examine the formation of lipid hydroperoxides and hexanal, as well as degradation of α-tocopherol and flavonoids.

5.3.4 Measurement of lipid hydroperoxides

Lipid hydroperoxides were assessed periodically according to the method of Shantha and Decker (1994) with slight modifications. Briefly, 20 μL of the oil sample was

dissolved in 2.8 mL of methanol: 1-butanol (2:1 v/v) solution. Fifteen μL of 3.94 M ammonium thiocyanate solution in deionized water and fifteen μL of ferrous solution were added and samples were vortexed for 30 seconds. The ferrous solution was prepared by mixing equal volumes of 0.144 M ferrous sulfate ($FeSO_4$) in deionized water and 0.132 M barium chloride ($BaCl_2$) in 0.4 M hydrochloric acid, and collecting the supernatant after centrifuging at 1785*g* for 5 min (CL10 centrifuge, Thermo Fisher Scientific Inc., Waltham, MA). A mixture containing no oil sample was prepared as blank. After 20 min of incubation at room temperature in the dark, the absorbance was measured by a UV-VIS spectrophotometer (Genesys 20, Thermospectronic, Waltham, MA) at 510 nm. Samples with a high absorbance (> 1.2) were diluted with methanol: 1-butanol (2:1 v/v) solution. The concentration of lipid hydroperoxides was calculated using a standard curve of cumene hydroperoxide ranging from 0.2 to 5 mM, and the results were expressed as "mmol/kg oil."

5.3.5 Measurement of headspace hexanal

Headspace hexanal was assessed periodically using a Shimadzu GC-2010 coupled with an AOC-6000 autosampler and flame-ionization detector (FID) (Shimadzu, Kyoto, Japan). Volatile compounds were separated with a fused-silica capillary column (30 m × 0.32 mm ID × 1 μm) coated with 100% poly (dimethylsiloxane) (Equity- 1, Supelco). Headspace vials were initially preheated at 55 ℃ for 10 min in the autosampler heating block. A stable flex solid-phase microextraction (SPME) fiber needle (50/30 μm divinylbenzene/ carboxen/ polydimethylsiloxane) (Supelco, Bellefonte, PA) was injected into the vial headspace for 2 min to absorb volatiles. The fiber needle carrying the volatiles was then injected into the 250 ℃ injector port for 3 min with a 1:7 split ratio to desorb the

compounds. Volatiles were then separated in the 65 °C column during a 10 min run for each sample treatment. Helium was used as a carrier gas at a flow rate of 30 mL/min, and hydrogen and air were used for the ionization flame. A flame ionization detector (FID) at 250 ℃ was used to detect hexanal with a retention time of ~4.8 min. The hexanal concentration was determined using a calibration curve of MCT oil spiked with different concentrations of authentic hexanal ranging from 2 to 800 μM, and the results were expressed as "μmol/kg oil."

5.3.6 Determination of lipid hydroperoxide and headspace hexanal lag phases and calculation of interaction indexes for antioxidant combinations

Lag phase (shelf-life) was calculated for both lipid hydroperoxide and headspace hexanal formation for each sample treatment. The lag phase was determined as the first data point that is statistically distinct from day zero readings ($p < 0.05$). The interaction index was calculated to determine the type of antioxidant interaction, which is defined as the ratio of the observed lag phase (experimental) to the expected lag phase (theoretical) of the antioxidant combination with the following formula: Interaction index = (Lag phase of antioxidant combination)/ [(Lag phase of antioxidant$_1$) + (Lag phase of antioxidant$_2$)], where interaction index > 1 is synergistic, interaction index = 1 is additive, and interaction index < 1 is antagonistic.

5.3.7 Measurement of α-tocopherol, myricetin, and taxifolin loss

The concentrations of α-tocopherol, myricetin, and taxifolin were periodically measured using a Shimadzu Prominence-i LC-2030C HPLC system (Shimadzu, Kyoto,

Japan) equipped with a Supelcosil LC-Diol column (25 cm x 4.0 mm, particle size 5µm, Bellefonte, PA), a Supelguard LC-Diol security guard column (2 cm x 4.0 mm, particle size 5µm) and a fluorescence detector (RF-20 Axs, Shimadzu). Samples (125 µL) used for α-tocopherol analysis were dissolved in 1 mL of hexane, and samples (125 µL) used for myricetin and taxifolin analysis were dissolved in 1 mL of 1,4-dioxane. The samples were filtered with 0.22 µm PTFE syringe filters (Fisher Scientific, Pittsburgh, PA) to minimize the passage of any particulates into the analytical column.

A normal phase separation method with minor modifications was used to detect α-tocopherol (Xu et al., 2015). Hexane and 1,4-dioxane (95:5 v/v) were used as isocratic mobile phase at a flow rate of 1 mL/min. Each sample (20 µL) was injected with an autosampler into the analytical column at 37 °C. α-Tocopherol was detected using a fluorescence detector with an excitation wavelength of 290 nm and an emission wavelength of 330 nm, and its retention time was determined to be ~4.3 min.

Hexane and 1,4-dioxane (40:60 v/v) were used as isocratic mobile phase at a flow rate of 1 mL/min to detect myricetin and taxifolin. Each sample (40 µL) was injected with an autosampler into the analytical column at 37 °C. A photodiode array (PDA) detector was used to detect taxifolin at 290 nm and myricetin at 373 nm based on their maximum absorbance wavelengths. Taxifolin and myricetin retention times were determined to be ~3.7 and ~3.9 min, respectively.

The percentage of antioxidants on a specific day (X) was calculated using the following formula based on the readings from day zero: Antioxidant % = (Peak area at day X/ Peak area at day 0) x 100.

5.3.8 Statistical analysis of data

All analyses were conducted in triplicate measurements. Data were presented as means ± standard deviations. Results were analyzed using a one-way analysis of variance (ANOVA) in Minitab 19 (State College, PA). Oxidation lag phases were calculated using a one-tailed Dunnet's *post hoc* test to determine when lipid hydroperoxide and headspace hexanal concentrations were significantly higher than day zero concentrations.

5.4 Results and discussion

5.4.1 Determination of flavonoid-metal complexation by UV-VIS spectroscopy

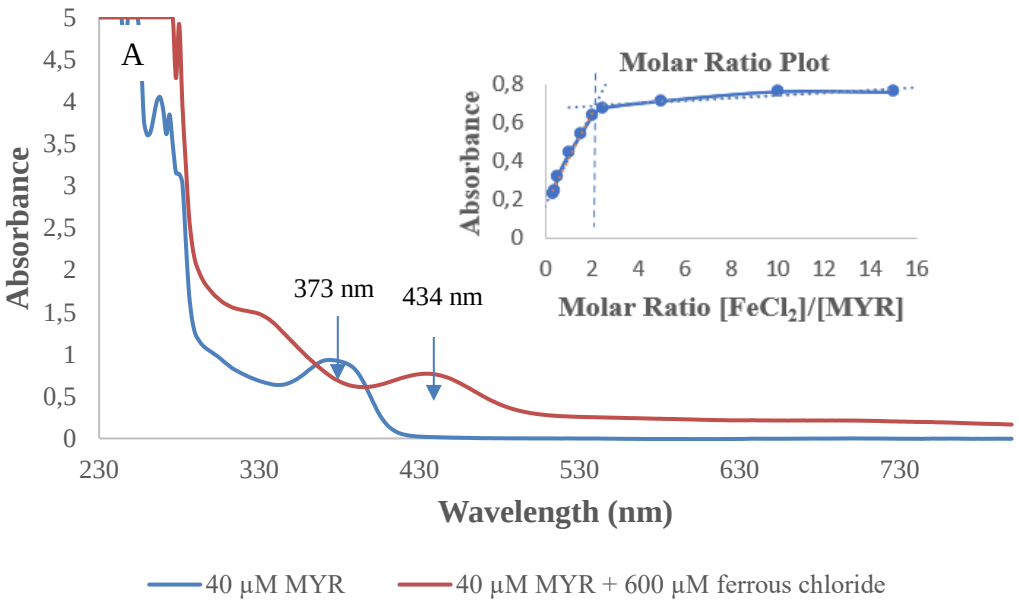
The majority of flavonoids have metal chelating activity due to the presence of numerous hydroxyl groups in their structures. This metal chelating activity has been suggested to be involved in the inhibition of lipid oxidation (Kejík et al., 2021), as free metal ions are responsible for the decomposition of lipid hydroperoxides into fatty acid decomposition products that cause rancidity. Our previous research has shown that both myricetin and taxifolin are potent ferrous ion chelators in emulsions as detected by ferrozine assay, with myricetin being a stronger chelator (Bayram et al., 2023). However, Dimitrić Marković et al. (2011) found that the ability of flavonoids to form metal chelates are highly dependent on the media, including the type of solvent, pH, and oxidizing agents. As there is no information on the ability of myricetin and taxifolin to chelate metals in bulk oils, we used a UV-VIS spectroscopic approach to determine metal interactions. The iron was selected as a metal ion source for this experiment since iron is generally at higher concentrations in food systems than other prooxidant metals such as copper, and thus can

be a more important prooxidant. Medium chain triglycerols (MCT) were used as the bulk oil to minimize complications that could arise from metal promoted lipid oxidation.

Figure 5.1 depicted the absorption spectra of myricetin and taxifolin in MCT oil after adding various concentrations of ferrous chloride to determine the ability of the flavonoids to chelate metal ions. Myricetin is a flavonoid consisting of six hydroxyl groups and one carbonyl group. Four possible chelation sites exist on myricetin molecules, including two catechol moieties on ring B and two hydroxyl groups (3-OH and 5-OH) next to the carbonyl group. UV-VIS spectrum of myricetin alone in MCT oil had a prominent absorption peak at 373 nm (Figure 5.1A). The addition of ferrous chloride resulted in a new maximum absorbance peak at 434 nm due to the myricetin-ferrous ion complexation (Figure 5.1A). The maximum peak absorbance represented a red shift from the original peak of ~61 nm, indicating that the cinnamoyl ring system was involved in myricetin-metal complexation via its 3-OH and 4-CO groups (Jomová et al., 2019). The molar ratio plot for $[FeCl_2]$/[MYR] versus absorbance at complex formation wavelength (434 nm) indicated the iron:myricetin complexation stoichiometry of 2:1. Since there would be a strong steric hindrance upon complexation of ferrous ions via the 3-OH and 4-CO site, it is unlikely for the 4-CO and 5-OH group to participate as a second complexation site (Cornard & Merlin, 2002). Therefore, catechol groups in the B-ring of the myricetin molecule might be responsible for the binding of the second iron molecule.

Taxifolin is a flavonoid that contains five hydroxyl groups and one carbonyl group. There are three potential chelation sites on taxifolin molecules, including a catechol moiety on ring B and two hydroxyl groups adjacent to the carbonyl group (3-OH and 5-OH). The UV-VIS spectra of taxifolin in MCT oil revealed a prominent absorption peak at 290 nm

(Figure 5.1B). The addition of ferrous chloride created a second absorption peak at 336 nm due to the complexation of taxifolin with ferrous ions (Figure 5.1B). The C2-C3 saturated bond in taxifolin structure impacts the delocalization of the π-system between the A-C rings and the B-ring (unlike myricetin) and deconjugates the rings (Jomová, et al., 2019). The interaction between ferrous ions and taxifolin via 3-OH and 4-CO groups is, therefore, less favorable. As a result, the formation of a new peak corresponding to a red shift of ~46 nm from the original peak suggested the complexation of taxifolin with iron via its 4-CO and 5-OH groups (Jomová, et al., 2019). The molar ratio plot for [$FeCl_2$]/[MYR] versus absorbance at the wavelength of complex formation (336 nm) suggested a complexation stoichiometry of 2:1, similar to that of myricetin. As a result, the catechol group in the B-ring of the taxifolin molecule might be responsible for the binding of the second iron molecule, like in myricetin. Overall, these findings demonstrated that both myricetin and taxifolin chelate ferrous ions in a lipid matrix.



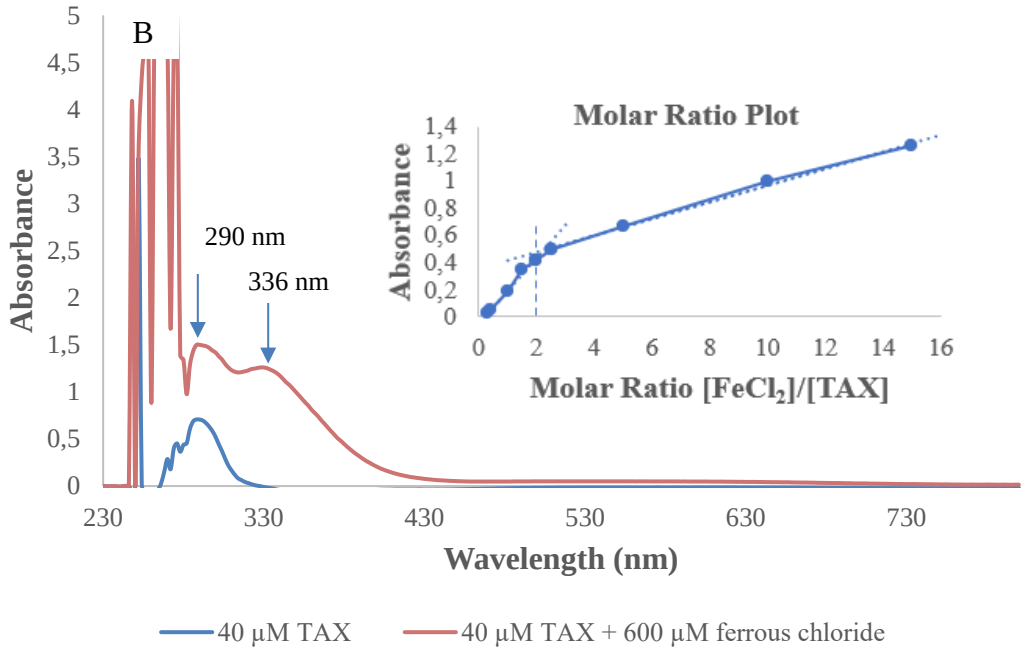
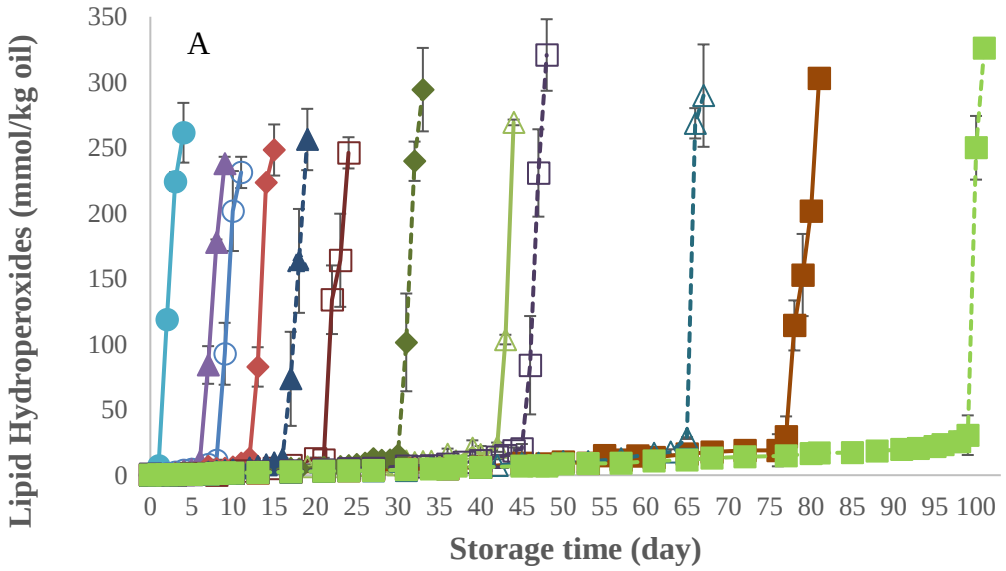


Figure 5.1 UV-VIS spectra of myricetin (MYR) (A) and taxifolin (TAX) (B) in MCT oil in the absence and presence of FeCl2 and, the molar ratio plots at λmax (myricetin complex) = 434 nm, and λmax (taxifolin complex) = 336 nm for chelation stoichiometry.

5.4.2 Antioxidant activity of α-tocopherol-myricetin and α-tocopherol-taxifolin combinations in stripped soybean oil

The ability of myricetin and α-tocopherol to inhibit lipid oxidation in 1:1 SSO:MCT oil blend was tested by measuring lipid hydroperoxides and headspace hexanal formation during storage at 60 °C. The α-tocopherol concentration (50 µM) was kept constant to observe the impact of antioxidant ratios on activity by varying the myricetin concentrations (10, 25, 50, 100, 250 µM). All samples with antioxidant treatments were able to retard the lipid hydroperoxide and headspace hexanal formation compared to the control group (Figure 5.2). α-Tocopherol alone increased the hydroperoxide and hexanal lag phases to 8 days compared to 1 day for the control. Myricetin alone produced hydroperoxide and hexanal lag phases ranging from 6 to 76 days for concentrations from 10 µM to 250 µM (Figure 5.2). Combining α-tocopherol and myricetin produced hydroperoxide and hexanal

lag phases ranging from 16 to 99 days (Figure 5.2). For example, 50 µM α-tocopherol had an 8-day hexanal lag phase, and 50 µM myricetin had a 21-day lag phase; however, combining the antioxidants at these concentrations extended the lag phase to 45 days (Figure 5.2). α-Tocopherol and myricetin ratios of 5:1, 2:1, 1:1, 1:2, and 1:5 resulted in interaction indexes of 1.14, 1.50, 1.55, 1.30, and 1.16, showing synergistic activity (interaction index >1) for both lipid hydroperoxide and headspace hexanal formation. Even though the interaction index decreased with increasing myricetin concentrations above the 1:1 ratio, increasing myricetin in the presence of α-tocopherol continued to increase the lag phase of both hydroperoxides and headspace hexanal. This shows that while the efficiency of synergism as measured by the interaction index should not be confused with synergistic antioxidant combinations that can maximize the shelf-life of foods. A previous study investigated the synergism of α-tocopherol and myricetin in stripped sunflower oil at 100 °C by measuring peroxides and conjugated dienes, finding that equal ratios of antioxidants resulted in maximum synergism and increasing myricetin concentration improved induction period, similar to our conclusions (Marinova et al., 2008).



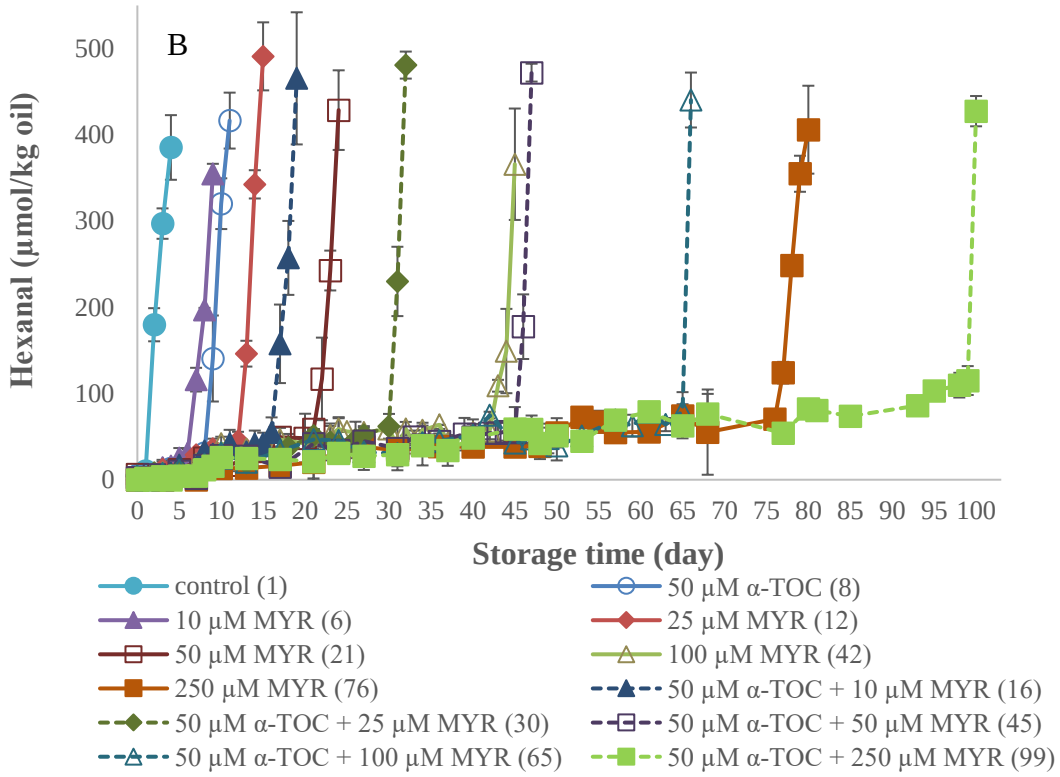


Figure 5.2 Formation of lipid hydroperoxides (A) and headspace hexanal (B) in 1:1 stripped soybean oil: MCT oil blend containing 50 µM α-tocopherol (α-TOC) and 10, 25, 50, 100 or 250 µM myricetin (MYR), and their respective combinations during storage at 60 °C. Data points represent means (n=3) ± standard deviation. Some error bars lie within the data points. Lag phases for each sample treatment are shown in parentheses.

Taxifolin, also known as dihydroquercetin, is a flavonoid antioxidant found in various foods, including onions, tomatoes, grapes, strawberries, citrus fruits, peanuts, and pine seeds (Turck et al., 2017). There has not been comprehensive research on the antioxidative ability of taxifolin in food systems, but previous studies have shown that taxifolin could be used to extend the shelf-life of lard and cottonseed oil (Kurth, 1951) and linoleic acid emulsions (Topal et al., 2015). Taxifolin differs from myricetin in that it lacks the 5-OH group and C2-C3 double bond, resulting in an increase in redox potential from 360 mV to 500 mV, which is the similar to the redox potential of α-tocopherol (Decker et

al., 2010). Therefore, we compared taxifolin's antioxidant activity to myricetin in the presence and absence of α-tocopherol.

As in the myricetin study, α-tocopherol concentration (50 μM) was kept constant and taxifolin concentrations were varied from 10 to 250 μM. All samples with antioxidant treatments (except 10 μM taxifolin) were able to retard the lipid hydroperoxide and headspace hexanal formation compared to the control group (Figure 5.3). α-Tocopherol alone increased the lipid hydroperoxide and hexanal lag phases to 6 days compared to 1 day for the control (Figure 5.3). Taxifolin alone produced lipid hydroperoxide and hexanal lag phases ranging from 1 to 10 days for concentrations from 10 μM to 250 μM (Figure 5.3). Combining α-tocopherol and taxifolin produced hydroperoxide and hexanal lag phases ranging from 7 to 16 days (Figure 5.3). For example, 50 μM α-tocopherol had an 6-day hexanal lag phase, and 50 μM taxifolin had a 3-day lag phase; however, combining the antioxidants at these concentrations resulted in 9-day of lag phase (Figure 5.3). α-Tocopherol and taxifolin ratios of 5:1, 2:1, 1:1, 1:2, and 1:5 all resulted in interaction indexes of 1.00, indicating an additive effect for both lipid hydroperoxide and headspace hexanal formation. This indicated that although α-tocopherol and taxifolin individually protected the oil from oxidation, their combination did not further improve the shelf-life. This suggests that antioxidant regeneration may be the cause of synergistic activity between α-tocopherol and myricetin due to their different redox potentials; and additive effect between α-tocopherol and taxifolin may be due to their identical redox potentials, resulting in thermodynamic unfavorability for radical transfer from one antioxidant to another.

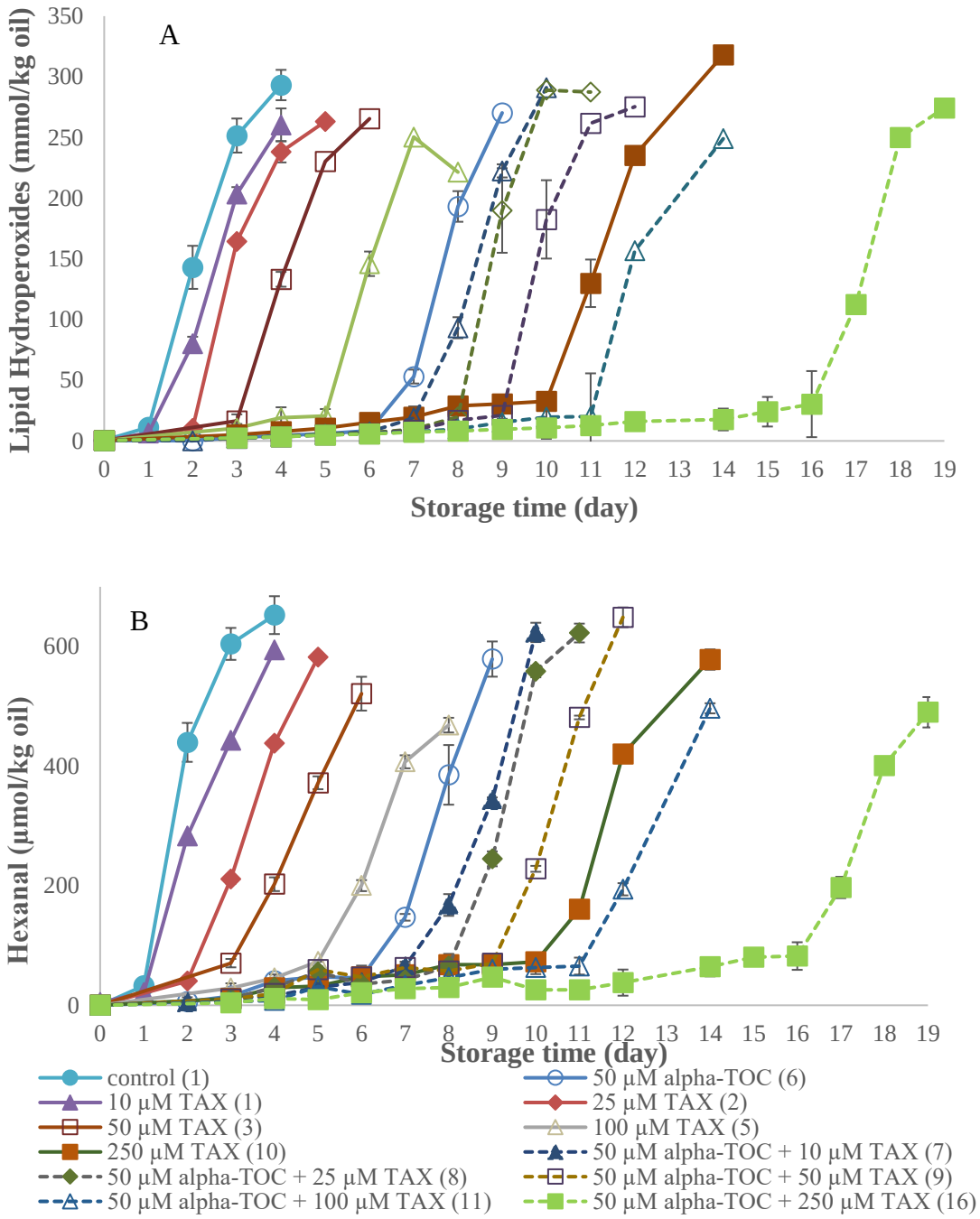


Figure 5.3 Formation of lipid hydroperoxides (A) and headspace hexanal (B) in 1:1 stripped soybean oil: MCT oil blend containing 50 µM α-tocopherol (α-TOC) and 10, 25, 50, 100 or 250 µM taxifolin (TAX), and their respective combinations during storage at 60 °C. Data points represent means (n=3) ± standard deviation. Some error bars lie within the data points. Lag phases for each sample treatment are shown in parentheses.

5.4.3 Depletion of α-tocopherol-myricetin and α-tocopherol-taxifolin during oxidation of stripped soybean oil

Decomposition of α-tocopherol and myricetin was measured to assess antioxidant degradation kinetics in relation to fatty acid oxidation rates and determine the potential interactions between α-tocopherol and myricetin. Hexanal lag phase for each treatment, α-tocopherol depletion, and myricetin depletion were summarized in Table 5.1.

Table 5.1 Summary of hexanal lag phase and antioxidant depletion (>95%) in 1:1 stripped soybean oil: MCT oil blend containing 50 μM α-tocopherol (α-TOC) and 10, 25, 50, 100, 250 μM myricetin (MYR), and their respective combinations during storage at 60 °C.

Sample	Hexanal lag phase (days)	α-Tocopherol depletion (days)	Myricetin depletion (days)
Control	1	-	-
50 μM α-TOC	8	9	-
10 μM MYR	6	-	7
25 μM MYR	12	-	13
50 μM MYR	21	-	22
100 μM MYR	42	-	43
250 μM MYR	76	-	77
50 μM α-TOC + 10 μM MYR	16	17	13
50 μM α-TOC + 25 μM MYR	30	31	26
50 μM α-TOC + 50 μM MYR	45	46	41
50 μM α-TOC + 100 μM MYR	65	66	61
50 μM α-TOC + 250 μM MYR	99	100	95

When α-tocopherol and myricetin were evaluated individually, oxidation of the fatty acids to form hexanal began at a similar time to when either α-tocopherol or myricetin were mostly degraded (Table 5.1). For instance, 50 μM α-tocopherol was mostly degraded after 9 days with an 8-day hexanal lag phase, and 50 μM myricetin was mostly depleted after 22 days with a 21-day hexanal lag phase (Table 5.1). The hexanal lag phase was 45 days when 50 μM α-tocopherol and 50 μM myricetin were combined, with myricetin and α-tocopherol being depleted after 41 and 46 days, respectively (Table 5.1). These results

were not unexpected since free radical scavengers donate electrons to fatty acid radicals to prevent further oxidation; therefore, fatty acids did not oxidize until the majority of antioxidants were depleted. However, it did provide some insights to the kinetics of α-tocopherol and myricetin oxidation as myricetin degraded faster than α-tocopherol and hexanal formation did not start until the α-tocopherol was mostly degraded (see Figure 5.4 for an example of α-tocopherol and myricetin depletion and hexanal formation).

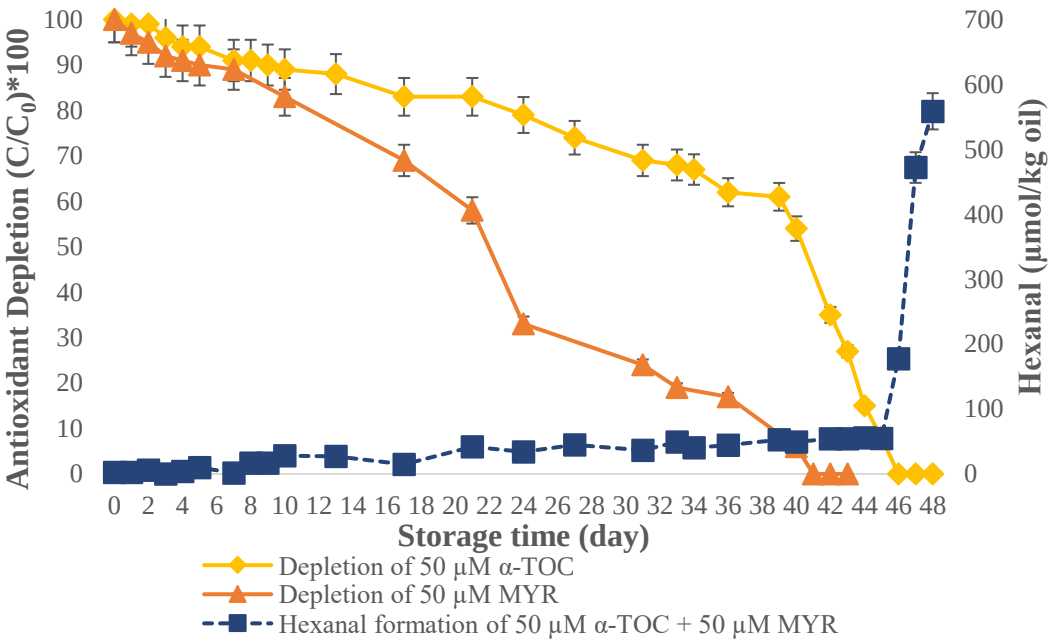


Figure 5.4 α-Tocopherol and myricetin depletion and hexanal formation in 1:1 stripped soybean oil: MCT oil blend containing the combination of 50 µM α-tocopherol (α-TOC) and 50 µM myricetin (MYR) during storage at 60 °C. Data points represent means (n=3) ± standard deviation. Some error bars lie within the data points.

The reasons why myricetin degraded faster than α-tocopherol in combination samples (Table 5.1) could be due to several phenomena. For example, myricetin's lower one-electron reduction potential (360 mV) compared to α-tocopherol (500 mV) (Decker et al., 2010) could allow it to be oxidized by free radicals faster than α-tocopherol. In addition,

myricetin's lower redox potential would also allow it to donate an electron to oxidized α-tocopherol resulting in α-tocopherol regeneration. Thus, myricetin could degrade faster because it is being consumed by both interactions with fatty acid radicals and oxidized tocopherol. In addition, despite the fact that both myricetin and taxifolin are metal chelators in lipids as shown in Figure 5.1, myricetin is a more potent metal chelator than taxifolin as demonstrated in emulsion systems (Bayram et al., 2023). Therefore, it is possible that myricetin inhibits the ability of metals to degrade lipid hydroperoxides and prevents the formation of free radicals that degrade tocopherol further (Figure 5.4) by chelating more metal ions than taxifolin, resulting in synergistic activity (Figure 5.2).

The decomposition kinetics of α-tocopherol and taxifolin was also evaluated to assess their relationship to fatty acid oxidation rates and determine the potential interactions between α-tocopherol and taxifolin. Hexanal lag phase for each treatment, α-tocopherol depletion, and taxifolin depletion were summarized in Table 5.2.

Table 5.2 Summary of hexanal lag phase and antioxidant depletion (>95%) in 1:1 stripped soybean oil: MCT oil blend containing 50 µM α-tocopherol (α-TOC) and 10, 25, 50, 100, 250 µM taxifolin (TAX), and their respective combinations during storage at 60 °C.

Sample	Hexanal lag phase (days)	α-Tocopherol depletion (days)	Taxifolin depletion (days)
Control	1	-	-
50 µM α-TOC	6	7	-
10 µM TAX	1	-	2
25 µM TAX	2	-	3
50 µM TAX	3	-	4
100 µM TAX	5	-	6
250 µM TAX	10	-	11
50 µM α-TOC + 10 µM TAX	7	7	8
50 µM α-TOC + 25 µM TAX	8	8	9
50 µM α-TOC + 50 µM TAX	9	9	10
50 µM α-TOC + 100 µM TAX	11	10	11
50 µM α-TOC + 250 µM TAX	16	16	17

As observed with myricetin and α-tocopherol, fatty acid oxidation began once the majority of antioxidants were degraded (Table 5.2). For example, 50 µM taxifolin was depleted after 4 days with a 3-day hexanal lag phase (Table 5.2). However, unlike myricetin, when α-tocopherol and taxifolin were combined, both antioxidants were depleted at similar rates (Table 5.2). For example, the hexanal lag phase was 9 days when 50 µM α-tocopherol and 50 µM taxifolin were combined, with taxifolin and α-tocopherol being depleted after 10 and 9 days, respectively (see Figure 5.5 for an example of α-tocopherol and taxifolin depletion and hexanal formation).

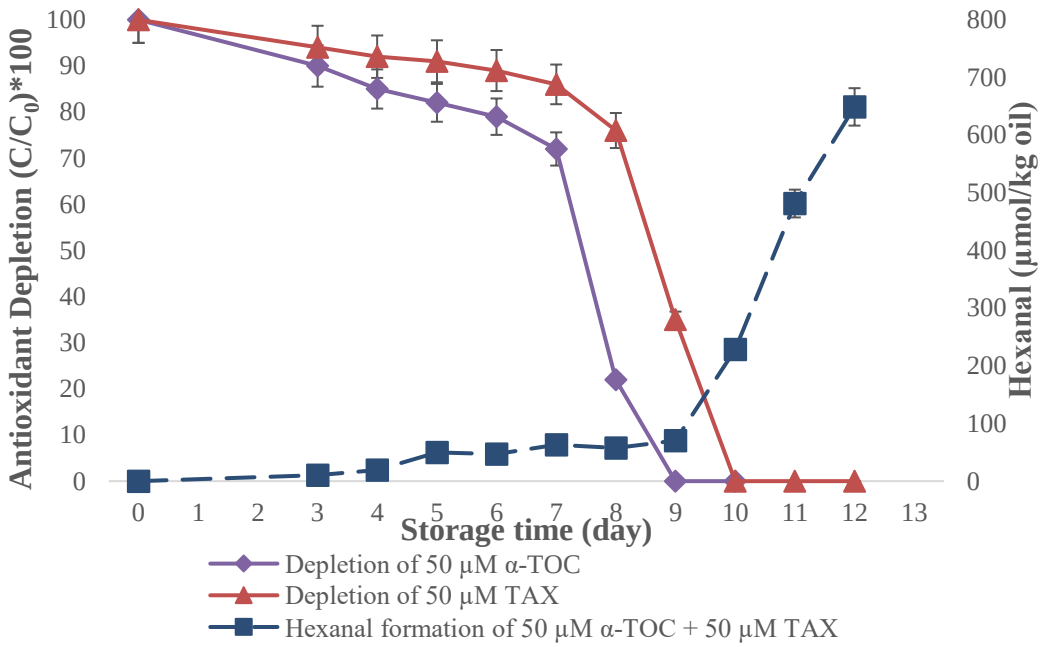


Figure 5.5 α-Tocopherol and taxifolin depletion and hexanal formation in 1:1 stripped soybean oil: MCT oil blend containing the combination of 50 µM α-tocopherol (α-TOC) and 50 µM taxifolin (TAX) during storage at 60 °C. Data points represent means (n=3) ± standard deviation. Some error bars lie within the data points.

The reasons why α-tocopherol and taxifolin showed similar degradation behavior when added together could be due to several phenomena. For example, the equal redox

potential of α-tocopherol and taxifolin (500 mV) (Decker et al., 2010) could allow both antioxidants to be oxidized by free radicals at a similar rate. In addition, unlike myricetin, taxifolin's equal redox potential as α-tocopherol might prevent electron donation between taxifolin and α-tocopherol as previously shown in emulsions (Bayram et al., 2023), minimizing the possibility of antioxidant regeneration. Moreover, the lower metal chelating efficiency of taxifolin compared to myricetin (Bayram et al., 2023) may be another reason why taxifolin was unable to reduce the rate of α-tocopherol depletion (Figure 5.5), resulting in an additive effect (Figure 5.3).

5.4.4 Antioxidant activity of α-tocopherol-myricetin in the presence of phospholipids in stripped soybean oil

After demonstrating the synergistic activity of α-tocopherol and myricetin in stripped soybean oil, additional research was conducted to examine the effect of phospholipid reverse micelles on α-tocopherol and myricetin interactions. In a previous study, Chen et al. (2010) found that 1000 μM 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) forms reverse micelles in stripped soybean oil, while 1,2-dibutyryl-sn-glycero-3-phosphocholine (DC_4PC), which contains short chain fatty acid, do not form reverse micelles. Therefore, these two phospholipids were used in this study so that the oils would have equal concentrations of phospholipids with one having reverse micelles (DOPC) and the other not having reverse micelles (DC_4PC). We did not perform additional research for α-tocopherol and taxifolin combinations in this section of the experiment because synergism was not observed in stripped soybean oils, as shown in Figure 5.3. We used phosphatidylcholine (PC) instead of phosphatidylserine (PS) or phosphatidylethanolamine

(PE) because PE and PS contribute to the synergistic activity by regenerating tocopherols in bulk oil and emulsion systems, whereas PC shows no effect in oxidative stability in the presence of tocopherols (Takenaka et al., 2007; Doert et al., 2012; Cui et al., 2015; Culler et al., 2022).

The formation of lipid hydroperoxides and headspace hexanal was analyzed in the presence of 1000 μM DC_4PC with α-tocopherol and myricetin (Figure 5.6). The results showed that the lag phases were remarkably comparable to those observed in stripped soybean oil, as illustrated in Figure 5.2. For example, 50 μM α-tocopherol had an 8-day lag phase for both lipid hydroperoxide and hexanal formation in stripped soybean oil, and a 7-day lag phase for lipid hydroperoxides and an 8-day lag phase for hexanal formation in stripped soybean oil with DC_4PC. The results were quite similar for myricetin as well, where 50 μM myricetin had a lag phase of 21 days for both lipid hydroperoxide and hexanal formation in stripped soybean oil (Figure 5.2), and 20 days for both lipid hydroperoxide and hexanal formation in stripped soybean oil with DC_4PC (Figure 5.6). The system with DC_4PC was quite equivalent to stripped soybean oil because DC_4PC did not form any reverse micelles, and there were no physical structures to lead to antioxidant partitioning; therefore, the antioxidant activity was unaffected. Similarly, the interaction index for the combination of 25 μM α-tocopherol and 25 μM myricetin was calculated as 1.43, suggesting that the presence of phospholipids (without micelle formation) still led to synergism.

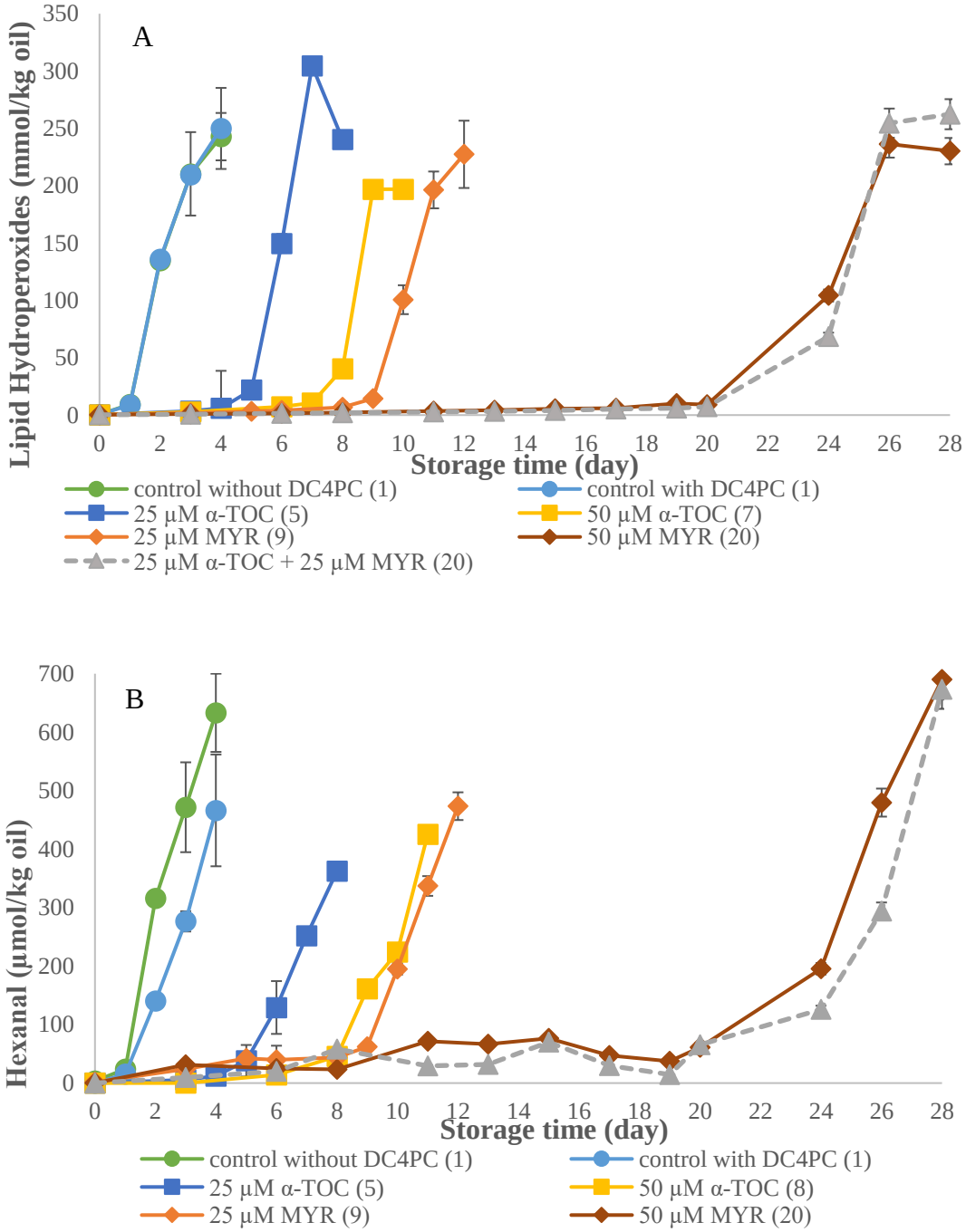


Figure 5.6 Formation of lipid hydroperoxides (A) and headspace hexanal (B) in 1:1 stripped soybean oil: MCT oil blend containing 1000 µM 1,2-dibutyryl-sn-glycero-3-phosphocholine (DC_4PC) with 25, 50 µM α-tocopherol (α-TOC) and 25, 50 µM myricetin (MYR), and their combinations during storage at 60 °C. Data points represent means (n=3) ± standard deviation. Some error bars lie within the data points. Lag phases for each sample treatment are shown in parentheses.

The formation of lipid hydroperoxides and headspace hexanal was analyzed in the presence of 1000 μM DOPC with α-tocopherol and myricetin (Figure 5.7). The results showed that the lag phases of α-tocopherol containing samples were slightly different when compared to system without any phospholipids or with DC_4PC. For example, 50 μM α-tocopherol had an 8-day lag phase for both lipid hydroperoxide and hexanal formation in stripped soybean oil without reverse micelles (Figure 5.2), and a 6-day lag phase for lipid hydroperoxides and an 7-day lag phase for hexanal formation in stripped soybean oil with reverse micelles (Figure 5.7). This slight prooxidative activity could be attributed to the formation of a negatively charged interface in the presence of DOPC, which draws positively charged metal ions to the same location where lipid hydroperoxides are present, resulting in the faster breakdown of lipid hydroperoxides and increase lipid oxidation rates (Chen, Han, et al., 2011). However, the activity of myricetin changed in the presence of the DOPC micelles. For example, 50 μM myricetin had a 20-day lag phase for both lipid hydroperoxide and hexanal formation in stripped soybean oil with DC_4PC (no micelles) (Figure 5.6), whereas a 4-day lag phase in stripped soybean oil with DOPC (with micelles) (Figure 5.7). According to our previous research, myricetin has ferric ion reducing activity that can promote lipid oxidation in oil-in-water emulsions (Bayram et al., 2023). Therefore, positively charged endogenous ferric ions may be converted to more prooxidant ferrous ions by myricetin. If this happens at the negatively charged reverse micelle interface where lipid hydroperoxides also exist, this could help explain the decreased antioxidant activity of myricetin in the presence of DOPC micelles. However, the interaction between α-tocopherol (25 μM) and myricetin (25 μM) remained synergistic with an interaction index of 1.50 for lipid hydroperoxides and 1.40 for headspace hexanal. This indicates that the

presence of phospholipids (with micelle formation) still led to synergism but had a substantial effect on the total antioxidant activity by reducing the lag phase.

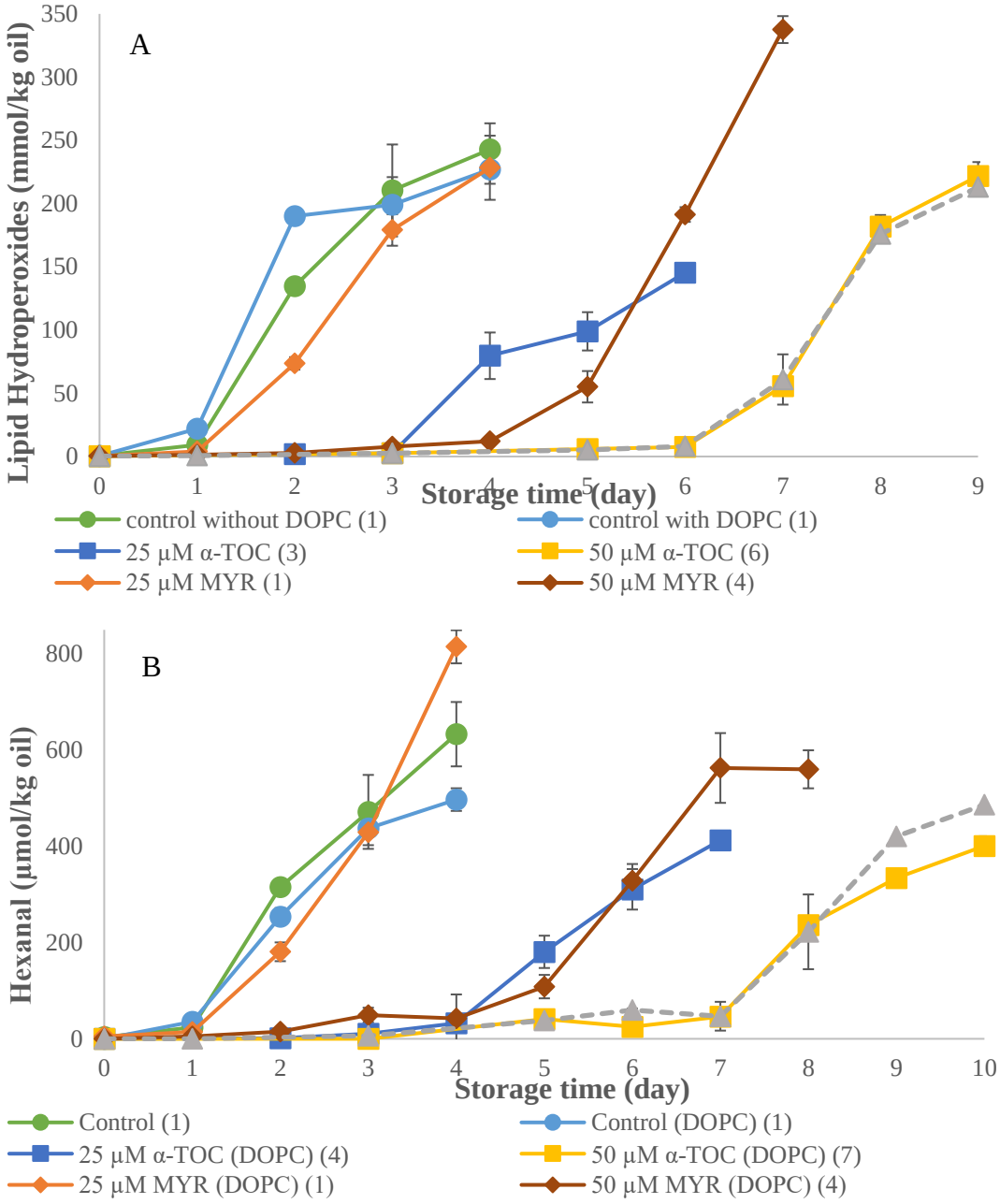


Figure 5.7 Formation of lipid hydroperoxides (A) and headspace hexanal (B) in 1:1 stripped soybean oil: MCT oil blend containing 1000 µM 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) with 25, 50 µM α-tocopherol (α-TOC) and 25, 50 µM myricetin (MYR), and their combinations during storage at 60 °C. Data points represent means (n=3) ± standard deviation. Some error bars lie within the data points. Lag phases for each sample treatment are shown in parentheses.

5.4.5 Oxidative stability of non-stripped soybean oil and sunflower oil in the presence of myricetin

After demonstrating the synergistic activity of myricetin and α-tocopherol in stripped soybean oil with and without phospholipids and its micelles, we decided to directly incorporate myricetin into tocopherol-containing store-bought oils. This is due to the fact that bulk oils are heterogeneous systems because they contain surface active compounds (e.g. phospholipids, free fatty acids, mono- and diacylglycerols and lipid hydroperoxides) and water, resulting in the formation of physical structures known as association colloids (Kittipongpittaya et al., 2014). Association colloids can result in differences in antioxidant partitioning and thus could impact antioxidant and lipid radical interactions (Chen, McClements, et al., 2011). On the other hand, stripped soybean oil lacks amphiphilic surface-active components and thus does not contain association colloids that could alter antioxidant location (Kittipongpittaya et al., 2016).

Sunflower oil was chosen in this experiment because it has high levels of α-tocopherol which was used in the previous experiments, while soybean oil was tested since it was used in the stripped oil studies. Soybean oil had a total tocopherol concentration of 1136 mg/kg oil (111 mg/kg alpha, 832 mg/kg gamma, and 193 mg/kg delta) while sunflower oil had a total tocopherol concentration of 324 mg/kg oil (248 mg/kg alpha, 17 mg/kg beta, 48 mg/kg gamma, and 11 mg/kg delta). The results showed that soybean oil had shorter lag phases for both lipid hydroperoxide and hexanal formation than sunflower oil, despite having higher tocopherol concentrations (Figure 5.8). This could be because soybean oil has a higher linolenic acid content (6.07%) than sunflower oil (1.90%), making it more susceptible to oxidation (Talpur et al., 2009). In the presence of myricetin, both

soybean oil and sunflower oil had improved lag phases, which increased with increasing myricetin concentrations. Incorporating 250 μM myricetin, for example, extended the hexanal lag phase of soybean oil from 13 days to 33 days and sunflower oil from 24 days to 47 days. It is unclear why myricetin was not more effective in soybean oil, which contained more total tocopherols than sunflower oil, but this may have been due to the presence of linolenic acid or differences in the tocopherol homolog composition, if myricetin regenerated one tocopherol homolog more effectively than others. The improvement in lag phases indicates that myricetin can be added to bulk oils as a secondary antioxidant to extend shelf-life due to its free-radical scavenging and tocopherol-regenerating properties.

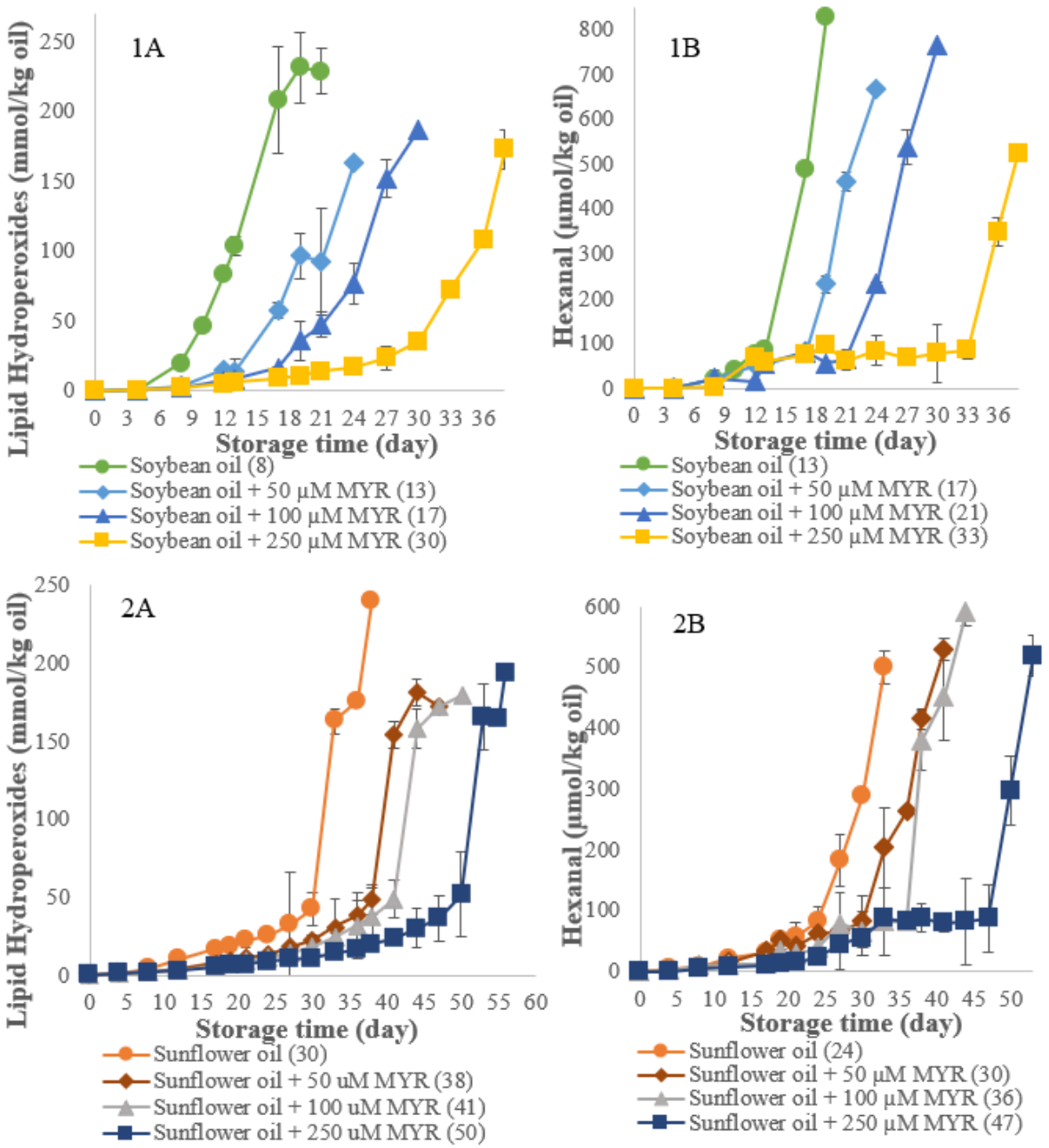


Figure 5.8 Formation of lipid hydroperoxides (A) and headspace hexanal (B) in non-stripped soybean oil (1) and non-stripped sunflower oil (2) containing 50, 100 and 250 µM myricetin (MYR) during storage at 60 °C. Data points represent means (n=3) ± standard deviation. Some error bars lie within the data points. Lag phases for each sample treatment are shown in parentheses.

5.5 Conclusion

The synergistic effect of α-tocopherol and myricetin suggests an alternative protection strategy for the food industry to prolong shelf-life and improve the quality of oil products. α-Tocopherol and myricetin synergism appears to be due to myricetin's ability to regenerate oxidized α-tocopherol due to its low redox potential. Taxifolin, on the other hand, was not able to decrease the rate of α-tocopherol degradation, which is likely due to its similar redox potential as α-tocopherol, which would thermodynamically prevent it from reducing oxidized α-tocopherol. Despite the fact that both taxifolin and myricetin bind metal ions, one had an additive effect with tocopherol and the other had a synergistic effect, suggesting that metal chelating activity may not be the primary mechanism for synergism.

CHAPTER 6

CONCLUSION

Unsaturated fats experience oxidative deterioration initiated by radicals during lipid oxidation, which poses a significant challenge to food quality and food waste. This process leads to unpleasant tastes, harmful aldehydes, and the co-oxidation of proteins and color compounds. The food industry employs various antioxidant strategies to combat lipid oxidation and extend the shelf-life of products. One long-standing approach is combining antioxidants to achieve synergistic effects and enhance oxidative stability. However, the underlying mechanisms behind antioxidant interactions are not well comprehended and have received limited research attention. The primary proposed mechanisms of antioxidant synergism are: 1) The regeneration of a depleted antioxidant by another compound, 2) Differences in antioxidant distribution in homogeneous and heterogeneous systems, 3) The combination of scavenging free radicals and chelating metal ions to provide two distinct protective pathways, and 4) The formation of additional antioxidant compounds, such as phenolics, dimers, or adducts, during oxidation, which further inhibit lipid oxidation. The purpose of this thesis was to examine how α-tocopherol (α-TOC) and myricetin (MYR) interact in distinct food systems (bulk oils and oil-in-water emulsions), as well as the mechanisms underlying synergistic and antagonistic interactions. The findings were compared to taxifolin, also known as dihydroquercetin, which is a flavonoid that shares a similar structure with myricetin. Myricetin possesses a gallol group on its B ring (3′, 4′, 5′-OH), whereas taxifolin has a catechol group (3′, 4′-OH), resulting in taxifolin lacking one hydroxyl group. Furthermore, myricetin's C ring contains a double bond between C2 and

C3, whereas taxifolin has a saturated bond in the same position. Because of these structural differences, myricetin has a redox potential of 360 mV, whereas taxifolin has a redox potential of 500 mV, which is equivalent to α-tocopherol and thus influences antioxidant interactions.

We first examined the way α-tocopherol and myricetin, two antioxidants, interacted in stripped soybean oil-in-water emulsions at pH 4.0 and pH 7.0. When the pH was 7.0, the ratios of α-tocopherol (α-TOC) to myricetin (MYR) of 2:1 and 1:1 resulted in interaction indices of 3.00 and 3.63 for lipid hydroperoxides, and 2.44 and 3.00 for hexanal formation, respectively. These findings indicated that there was a synergistic effect between the two antioxidants. We identified that the mechanism behind this synergism was myricetin's ability to reduce oxidized α-tocopherol (α-tocopherylquinone) back to original α-tocopherol structure and slow down its degradation as shown by HPLC. On the other hand, when the pH was 4.0, an antagonistic effect was observed. This was due to the acidic environment increasing the ferric-reducing activity of myricetin, which accelerated the depletion rate of α-tocopherol. The combination of α-tocopherol and taxifolin exhibited antagonism at both pH 4.0 and pH 7.0. This antagonism was attributed to taxifolin's inability to recycle α-tocopherol as shown by HPLC while simultaneously increasing the prooxidant activity of iron. Therefore, the combination of α-tocopherol and myricetin was identified as an excellent strategy for antioxidant protection in oil-in-water emulsions, particularly at near-neutral pH levels. This novel approach shows promise in enhancing the stability of food against oxidation. Additionally, our study suggested that combining α-tocopherol with other flavonoids that possess the ability to regenerate oxidized tocopherols could also lead to a synergistic antioxidant effect. If an alternative antioxidant capable of

regenerating oxidized tocopherols but with a weaker iron-reducing capacity than myricetin were employed, it might also be possible to achieve synergistic antioxidant activity at lower pH levels.

Second, our study examined the combined effects of α-tocopherol and myricetin on inhibiting lipid oxidation in bulk oils. To investigate this, stripped soybean oil was subjected to treatments with α-tocopherol (50 μM), myricetin (10-250 μM), and their combinations. Additionally, taxifolin (TAX), which shares structural similarities with myricetin but possesses a higher redox potential, was included in the tests. Various tocopherol: myricetin ratios, including 5:1, 2:1, 1:1, 1:2, and 1:5, were utilized, resulting in extended lag phases lasting from 16 to 99 days. The duration of the lag phase increased as the concentration of myricetin rose. The synergistic effects between myricetin and α-tocopherol were also observed in bulk oils containing phospholipids, both in the absence and presence of reverse micelles. While myricetin (redox potential=360 mV) delayed the oxidation of α-tocopherol (redox potential=500 mV), taxifolin (redox potential=500 mV) did not. Both myricetin and taxifolin exhibited the ability to chelate iron, as confirmed by UV-VIS spectroscopy. These findings indicate that the lower redox potential of myricetin allowed it to demonstrate synergistic antioxidant activity by regenerating oxidized α-tocopherol and reducing oxidation through metal chelation. Despite the metal ion binding properties of both taxifolin and myricetin, one exhibited an additive effect with α-tocopherol while the other displayed a synergistic effect. This indicates that the chelation of metal ions may not be the primary mechanism behind their synergistic action. The combined action of α-tocopherol and myricetin offers a potential alternative strategy for the food industry to enhance the shelf-life and quality of oil products.

Having a grasp of how synergism works will assist the food industry in creating antioxidant combinations that are more effective in enhancing oxidative stability and extending shelf-life. Additionally, knowing synergism mechanisms will contribute to the creation of easy, fast, and accurate techniques for identifying and assessing synergism. Improving our ability to forecast synergistic effects will be beneficial for developing and manufacturing food products containing unstable lipids, such as omega-3 fatty acids. This, in turn, has the potential to enhance the health and wellness benefits of the food supply while reducing food waste.

BIBLIOGRAPHY

Aggett, P. J. (2012). Iron. In J. W. Erdman, I. A. Macdonald, & S. H. Zeisel (Eds.), *Present knowledge in nutrition*. (pp. 506-20). Wiley-Blackwell, Washington, DC.

Almajano, M. P., Delgado, M. E., & Gordon, M. H. (2007). Albumin causes a synergistic increase in the antioxidant activity of green tea catechins in oil-in-water emulsions. *Food Chemistry, 102*(4), 1375-1382. https://doi.org/10.1016/j.foodchem.2006.06.06

Álvarez-Diduk, R., Ramírez-Silva, M. T., Galano, A., & Merkoçi, A. (2013). Deprotonation mechanism and acidity constants in aqueous solution of flavonols: A combined experimental and theoretical study. *The Journal of Physical Chemistry B, 117*(41), 12347-12359. https://doi.org/10.1021/jp4049617

Andersson, K., & Lingnert, H. (1999). Kinetic studies of oxygen dependence during initial lipid oxidation in rapeseed oil. *Journal of Food Science, 64*(2), 262-266. https://doi.org/10.1111/j.1365-2621.1999.tb15879.x

Ang, C. Y. W., & Young, L. L. (1992). Research note: Influence of fat content on oxidative stability of cooked chicken thigh patties during frozen storage. *Poultry Science, 71*(10), 1794-1796. https://doi.org/10.3382/ps.0711794

Arora, H., Culler, M. D., Decker, E. A. (2022). Production of a high-phosphatidylserine lecithin that synergistically inhibits lipid oxidation with α-tocopherol in oil-in-water emulsions. *Foods, 11*(7), 1014. https://doi.org/10.3390/foods11071014

Arts, M. J. T. J., Haenen, G. R. M. M., Wilms, L. C., Beetstra, S. A. J. N., Heijnen, C. G. M., Voss, H.-P., & Bast, A. (2002). Interactions between flavonoids and proteins: Effect on the total antioxidant capacity. *Journal of Agricultural and Food Chemistry, 50*(5), 1184-1187. https://doi.org/10.1021/jf010855a

Barden, L., Vollmer, D., Johnson, D., & Decker, E. (2015). Impact of iron, chelators, and free fatty acids on lipid oxidation in low-moisture crackers. *Journal of Agricultural and Food Chemistry, 63*(6), 1812-1818. https://doi.org/10.1021/jf5048018

Barouh, N., Bourlieu-Lacanal, C., Figueroa-Espinoza, M. C., Durand, E., & Villeneuve, P. (2022). Tocopherols as antioxidants in lipid-based systems: The combination of chemical and physicochemical interactions determines their efficiency. *Comprehensive Reviews in Food Science and Food Safety, 21*(1), 642-688. https://doi.org/10.1111/1541-4337.12867

Bayram, I., & Decker, E. A. (2023). Underlying mechanisms of synergistic antioxidant interactions during lipid oxidation. *Trends in Food Science and Technology, 133*, 219−230. https://doi.org/10.1016/j.tifs.2023.02.003

Bayram, I., Laze, A., & Decker, E. A. (2023). Synergistic mechanisms of interactions between myricetin or taxifolin with α-tocopherol in oil-in-water emulsions. *Journal of Agricultural and Food Chemistry, 71,* 9490-5000. https://doi.org/10.1021/acs.jafc.3c01226

Becker, E. M., Ntouma, G., & Skibsted, L. H. (2007). Synergism and antagonism between quercetin and other chain-breaking antioxidants in lipid systems of increasing structural organisation. *Food Chemistry, 103*(4), 1288-1296. https://doi.org/10.1016/j.foodchem.2006.10.03

Belitz, H. D., & Grosch, W. (1999). Food Chemistry, Springer-Verlag, Heidelberg, Berlin. http://dx.doi.org/10.1007/978-3-662-07281-3

Bhattacharya, S., Mula, S., Gamre, S., Kamat, J. P., Bandyopadhyay, S. K., & Chattopadhyay, S. (2007). Inhibitory property of Piper betel extract against photosensitization-induced damages to lipids and proteins. *Food Chemistry, 100*(4), 1474-1480. https://doi.org/10.1016/j.foodchem.2005.12.04

Bonarska-Kujawa, D., Cyboran-Mikołajczyk, S., & Kleszczyńska, H. (2015). Molecular mechanism of action of chlorogenic acid on erythrocyte and lipid membranes. *Molecular Membrane Biology, 32*(2), 46-54. https://doi.org/10.3109/09687688.2015.1031833

Boon, C. S., Xu, Z., Yue, X., McClements, D. J., Weiss, J., & Decker, E. A. (2008). Factors affecting lycopene oxidation in oil-in-water emulsions. *Journal of Agricultural and Food Chemistry, 56*, 1408-1414. https://doi.org/10.1021/jf072929+

Brittes, J., Lúcio, M., Nunes, C., Lima, J. L. F. C., & Reis, S. (2010). Effects of resveratrol on membrane biophysical properties: relevance for its pharmacological effects. *Chemistry and Physics of Lipids, 163*(8), 747-754. https://doi.org/10.1016/j.chemphyslip.2010.07

Buettner, G. R. (1993). The pecking order of free-radicals and antioxidants: Lipid peroxidation, alpha-tocopherol, and ascorbate. *Archives of Biochemistry and Biophysics, 300*(2), 535-543. https://doi.org/10.1006/abbi.1993.1074

Burcham, P. C., & Kuhan, Y. T. (1996). Introduction of carbonyl groups into proteins by the lipid peroxidation product, malondialdehyde. *Biochemical and Biophysical Research Communications, 220*(3), 996-1001. https://doi.org/10.1006/bbrc.1996.0521

Burkitt, M. J., & Gilbert, B. C. (1990). Model studies of the iron-catalyzed Haber-Weiss cycle and the ascorbate driven Fenton reaction. *Free Radical Research Communications, 10*(4-5), 265-280. https://doi.org/10.3109/10715769009149895

Chaiyasit, W., Elias, R. J., McClements, D. J., & Decker, E. A. (2007). Role of physical structures in bulk oils on lipid oxidation. *Critical Reviews in Food Science and Nutrition, 47*(3), 299-317. https://doi.org/10.1080/10408390600754248

Chang, H-K. (1989). Synergistic effect of tocopherol, citric acid and sodium polyphosphate on the oxidative stability of heated frying oil. *Journal of the Korean Applied Science and Technology, 6*(1), 15-19. https://doi.org/10.12925/jkocs.1989.6.1.3

Chang, S. S., Peterson, R. J., & Ho, C. T. (1978). Chemical reactions involved in the deep-frying of foods. *Journal of the American Oil Chemists' Society, 55*, 718-727. https://doi.org/10.1007/bf02665369

Chen, B., Han, A., Laguerre, M., McClements, D. J., & Decker, E. A. (2011). Role of reverse micelles on lipid oxidation in bulk oils: Impact of phospholipids on antioxidant activity of α-tocopherol and Trolox. Food and Function, 2, 302–309. https://doi.org/10.1039/c1fo10046g

Chen, B., Han, A., McClements, D. J., & Decker, E. A. (2010). Physical structures in soybean oil and their impact on lipid oxidation. *Journal of Agricultural and Food Chemistry, 58*, 11993–11999. https://doi.org/10.1021/jf102763p

Chen, B., McClements, D. J., & Decker, E. A. (2011). Minor components in food oils: a critical review of their roles on lipid oxidation chemistry in bulk oils and emulsions. *Critical Reviews in Food Science and Nutrition, 51*(10), 901-916. https://doi.org/10.1080/10408398.2011.606379.

Chen, Q., Shi, H., & Ho, C. T. (1992). Effects of rosemary extracts and major constituents on lipid oxidation and soybean lipoxygenase activity. *Journal of the American Oil Chemists' Society, 69*, 999-1002. https://doi.org/10.1007/BF02541065

Chen, Z. Y., Chan, P. T., Ho, K. Y., Fung, K. P., & Wang, J. (1996). Antioxidant activity of natural flavonoids is governed by number and location of their aromatic hydroxyl groups. *Chemistry and Physics of Lipids, 79*(2), 157-163. https://doi.org/10.1016/0009-3084(96)02523-6

Cherif, A., & Slama, A. (2022). Stability and change in fatty acids composition of soybean, corn, and sunflower oils during the heating process. Journal of Food Quality, 2022, 6761029. https://doi.org/10.1155/2022/6761029

Cherrak, S. A., Mokhtari-Soulimane, N., Berroukeche, F., Bensenane, B., Cherbonnel, A., Merzouk, H., Elhabiri, M. /2016). In vitro antioxidant versus metal ion chelating properties of flavonoids: A structure-activity investigation. *PLoS One, 11*, e0165575. https://doi.org/10.1371/journal.pone.0165575

Chobot, V., & Hadacek, F. (2011). Exploration of pro-oxidant and antioxidant activities of the flavonoid myricetin. *Redox Report, 16*(6), 242-247. https://doi.org/10.1179/1351000211Y.0000000015

Chuang, S.-Y., Lin, Y.-K., Lin, C.-F., Wang, P.-W., Chen, E.-L., & Fang, J.-Y. (2017). Elucidating the skin delivery of aglycone and glycoside flavonoids: How the structures affect cutaneous absorption. *Nutrients, 9*(12), 1304. https://doi.org/10.3390/nu9121304

Cornard, J. P., Boudet, A. C., & Merlin, J. C. (2001). Complexes of Al(III) with 3′4′-dihydroxy-flavone: Characterization, theoretical and spectroscopic study. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 57*, 591–602. https://doi.org/10.1016/s1386-1425(00)00412-1

Cornard, J. P., & Merlin, J. C. (2002). Spectroscopic and structural study of complexes of quercetin with Al(III). *Journal of Inorganic Biochemistry, 92*, 19–27. https://doi.org/10.1016/s0162-0134(02)00469-5

Cui, L., McClements, D. J., & Decker, E. A. (2015). Impact of phosphatidylethanolamine on the antioxidant activity of α-tocopherol and trolox in bulk oil. *Journal of Agricultural and Food Chemistry, 63*(12), 3288-3294. https://doi.org/10.1021/acs.jafc.5b00243

Cui, Z., Kong, X., Chen, Y., Zhang, C., & Hua, Y. (2014). Effects of rutin incorporation on the physical and oxidative stability of soy protein-stabilized emulsions. *Food Hydrocolloids, 41*, 1-9. https://doi.org/10.1016/j.foodhyd.2014.03.006

Culler, M. D., Bayram, I., Decker, E. A. (2022). Enzymatic modification of lecithin for improved antioxidant activity in combination with tocopherol in emulsions and bulk oil. *Journal of Agricultural and Food Chemistry, 70*, 13404-13412. https://doi.org/10.1021/acs.jafc.2c05182

Culler, M. D., Inchingolo, R., McClements, D. J., Decker, E. A. (2021). Impact of polyunsaturated fatty acid dilution and antioxidant addition on lipid oxidation kinetics in oil/water emulsions. *Journal of Agricultural and Food Chemistry, 69*, 750−755. https://doi.org/10.1021/acs.jafc.0c06209

Dacaranhe, C. D., & Terao, J. (2001). A unique antioxidant activity of phosphatidylserine on iron-induced lipid peroxidation of phospholipid bilayers. *Lipids, 36*(10), 1105-1110. https://doi.org/10.1007/s11745-001-0820-7

Dai, F., Chen, W.-F., & Zhou, B. (2008). Antioxidant synergism of green tea polyphenols with α-tocopherol and l-ascorbic acid in SDS micelles. *Biochimie, 90*(10), 1499-1505. https://doi.org/10.1016/j.biochi.2008.05.007

Decker, E. A. & Bayram, I. (2021). Why does lipid oxidation in foods continue to be such a challenge? *INFORM, 32*(5), 18-25. https://doi.org/10.21748/inform.05.2021.18

Decker, E. A., & Hultin, H. O. (1990). Nonenzymic catalysts of lipid oxidation in mackerel ordinary muscle. *Journal of Food Science, 55*(4), 951-953. https://doi.org/10.1111/j.1365-2621.1990.tb01572.x

Decker, E. A., Akoh, C. C., & Wilkes, R. S. (2012). Incorporation of (n-3) fatty acids in foods: Challenges and opportunities. *Journal of Nutrition, 142*(3), 5-8. https://doi.org/10.3945/jn.111.149328

Decker, E. A., Chen, B., Panya, A., & Elias, R. J. (2010). Understanding antioxidant mechanisms in preventing oxidation in foods. In E. A. Decker, R. J. Elias, & D. J. McClements (Eds.), *Oxidation in foods and beverages and antioxidant applications* (pp. 228). Woodhead Publishing, Cambridge, UK. https://doi.org/10.1533/9780857090447.2.225

DeMaeyer, E. M, & Adiels-Tegman, M. (1985). The prevalence of anemia in the world. *World Health Statistics Quarterly, 38*(3), 302-316.

Delosière, M., Durand, D., Bourguet, C., & Claudia Terlouw, E. M. (2019). Lipid oxidation, pre-slaughter animal stress and meat packaging: Can dietary supplementation of vitamin E and plant extracts come to the rescue? *Food Chemistry, 309*, 125668. https://doi.org/10.1016/j.foodchem.2019.125668

Di Mattia, C. D., Sacchetti, G., Mastrocola, D., Sarker, D. K., & Pittia, P. (2010). Surface properties of phenolic compounds and their influence on the dispersion degree and oxidative stability of olive oil O/W emulsions. *Food Hydrocolloids, 24*(6-7), 652-658. https://doi.org/10.1016/j.foodhyd.2010.03.007

Díaz, M., Dunn, C. M., McClements, D. J., & Decker, E. A. (2003). Use of caseinophosphopeptides as natural antioxidants in oil-in-water emulsions. *Journal of Agricultural and Food Chemistry, 51*(8), 2365-2370. https://doi.org/10.1021/jf025984l

Dičkancaitė, E., Nemeikaitė, A., Kalvelytė, A., & Čėnas, N. (1998). Prooxidant character of flavonoid cytotoxicity: Structure-activity relationships. *Biochemistry and Molecular Biology International, 45*(5), 923-930. https://doi.org/10.1002/iub.7510450510

Dimitrić Marković, J. M., Marković, Z. S., Brdarić, T. P., & Filipović, N. D. (2011). Comparative spectroscopic and mechanistic study of chelation properties of fisetin with iron in aqueous buffered solutions. Implications on in vitro antioxidant activity. *Dalton Transactions, 40*, 4560–4571. https://doi.org/10.1039/c0dt01834a

Doert, M., Jaworska, K., Moersel, J., & Kroh, L.W. (2012). Synergistic effect of lecithins for tocopherols: Lecithin based regeneration of alpha-tocopherol. *European Food Research and Technology, 235,* 915-928. https://doi.org/10.1007/s00217-012-1815-7

Domínguez, R., Pateiro, M., Gagaoua, M., Barba, F. J., Zhang, W., & Lorenzo, J. M. (2019). A comprehensive review on lipid oxidation in meat and meat products. *Antioxidants, 8*(10), 1-31. https://doi.org/10.3390/antiox8100429

Dunford, H. B. (1987). Free radicals in iron-containing systems. *Free Radical Biology and Medicine, 3*(6), 405-421. https://doi.org/10.1016/0891-5849(87)90019-0

Durand, E., Zhao, Y., Coupland, J. N., & Elias, R. J. (2015). Assessing interactions between lipophilic and hydrophilic antioxidants in food emulsions. *Journal of Agricultural and Food Chemistry, 63*(49), 10655-10661. https://doi.org/10.1021/acs.jafc.5b04152

Endo, Y., Hoshizaki, S., & Fujimoto, K. (1997) Autoxidation of synthetic isomers of triacylglycerol containing eicosapentaenoic acid. *Journal of the American Oil Chemists' Society, 74*, 543-548. https://doi.org/10.1007/s11746-997-0178-x

Faustman, C., Sun, Q., Mancini, R., & Suman, S. P. (2010). Myoglobin and lipid oxidation interactions: Mechanistic bases and control. *Meat Science, 86*(1), 86-94. https://doi.org/10.1016/j.meatsci.2010.04.025

Foote C. S., & Denny R. W. (1968). Chemistry of singlet oxygen. VII. Quenching by β-carotene. *Journal of the American Oil Chemists' Society, 90*, 6233-6235. https://doi.org/10.1021/ja01024a061

Frankel, E. N. (1984). Chemistry of free radical and singlet oxidation in lipids. *Progress in Lipid Research, 23*(4), 197-221. https://doi.org/10.1016/0163-7827(84)90011-0

Frankel, E. N. (2005). Stability methods. In E. N. Frankel (Ed.), *Lipid oxidation* (pp. 165–186). The Oily Press, Dundee, Scotland.

Frankel, E. N., Cooney, P. M., Moser, H. A., Cowan, J. C., & Evans, C. D. (1959). Effect of antioxidants and metal inactivators in tocopherol-free soybean oil. *Fette, Seifen, Anstrichmittel, 61*(10), 1036-1039. https://doi.org/10.1002/lipi.19590611046

Frankel, E. N., Huang, S.-W., Kanner, J., & German, J. B. (1994). Interfacial phenomena in the evaluation of antioxidants: Bulk oils vs emulsions. *Journal of Agricultural and Food Chemistry, 42*(5), 1054-1059. https://doi.org/10.1021/jf00041a001

Frankel E. N., Neff, W., & Bessler, T. (1979), Analysis of autoxidized fats by gas chromatography-mass spectrometry: V. Photosensitized oxidation. *Lipids, 14*, 961-967. https://doi.org/10.1007/BF02533431

Fujisaki, M., Mohri, S., Endo, Y., & Fujimoto, K. (2000). The effect of oxygen concentration on oxidative deterioration in heated high-oleic safflower oil. *Journal of the American Oil Chemists' Society, 77*(3), 231-234. https://doi.org/10.1007/s11746-000-0037-1

Fujisawa, S., Ishihara, M., Atsumi, T., & Kadoma, Y. (2006). A quantitative approach to the free radical interaction between alpha-tocopherol or ascorbate and flavonoids. *In Vivo, 20*(4), 445-452.

Fukuzawa, K., Ikebata, W., Sohmi, K. (1993). Location, antioxidant and recycling dynamics of alpha-tocopherol in liposome membranes. *Journal of Nutritional Science and Vitaminology, 39*, S9-S22. https://doi.org/10.3177/jnsv.39.supplement_s9

Geetha, T., Singh, N., Deol, P. K., & Kaur, I. P. (2015). Biopharmaceutical profiling of sesamol: physiochemical characterization, gastrointestinal permeability and pharmacokinetic evaluation. *RSC Advances, 5*(6), 4083-4091. https://doi.org/10.1039/c4ra10926k

Gendaram, O., Choi, Y. H., Kim, Y. S., & Ryu, S. Y. (2011). Anti-oxidative and antibacterial constituents from Sedum hybridum. *Natural Product Sciences, 17*(4), 279-284.

Gomes, A., Costa, A. L. R., de Assis Perrechil, F., & da Cunha, R. L. (2016). Role of the phases composition on the incorporation of gallic acid in O/W and W/O emulsions. *Journal of Food Engineering, 168*, 205-214. https://doi.org/10.1016/j.jfoodeng.2015.07.04

Gordon, M. H., & Roedig-Penman, A. (1998). Antioxidant activity of quercetin and myricetin in liposomes. *Chemistry and Physics of Lipids, 97*(1), 79-85. https://doi.org/10.1016/S0009-3084(98)00098-X

Gorelik, S., Ligumsky, M., Kohen, R. & Kanner, J. (2008). A novel function of red wine polyphenols in humans: Prevention of absorption of cytotoxic lipid peroxidation products. *Faseb Journal, 22*(1), 41-46. https://doi.org/10.1096/fj.07-9041com

Graf, E. (1990). Antioxidant functions of phytic acid. *Free Radical Biology and Medicine, 8*(1), 61-69. https://doi.org/10.1016/0891-5849(90)90146-a

Gulcin, I., & Alwasel, S. H. (2022). Metal ions, metal chelators and metal chelating assay as antioxidant method. *Processes, 10,* 132. https://doi.org/10.3390/pr10010132

Gunaseelan, K., Romsted, L. S., Gallego, M.-J. P., González-Romero, E., & Bravo-Díaz, C. (2006). Determining α-tocopherol distributions between the oil, water, and interfacial regions of macroemulsions: Novel applications of electroanalytical chemistry and the pseudophase kinetic model. *Advances in Colloid and Interface Science, 123-126*, 303-311. https://doi.org/10.1016/j.cis.2006.05.007

Gunstone, F. D. (1984). Reaction of oxygen and unsaturated fatty acids. *Journal of the American Oil Chemists' Society, 61*(2), 441-447. https://doi.org/10.1007/BF02678811

Guyon, C., Meynier, A., & de Lamballerie, M. (2016). Protein and lipid oxidation in meat: A review with emphasis on high-pressure treatments. *Trends in Food Science & Technology, 50,* 131-143. https://doi.org/10.1016/j.tifs.2016.01.026

Ham, Y.-K., Song, D.-H., Ha, J.-H., Park, S.-K., Kim, Y.-R., Chin, K. B., & Kim, H.-W. (2019). Efficacy of ascorbic acid on processing characteristics and lipid oxidation of pre-rigor salted chicken breasts during vacuum refrigerated storage. *LWT, 118,* 108691. https://doi.org/10.1016/j.lwt.2019.108691

Hartvigsen, K., Lund, P., Hansen, L. F., & Hølmer, G. (2000). Dynamic headspace gas chromatography/mass spectrometry characterization of volatiles produced in fish oil enriched mayonnaise during storage. *Journal of Agricultural and Food Chemistry, 48*(10), 4858-4867. https://doi.org/10.1021/jf991385b

Hematyar, N., Rustad, T., Sampels, S., & Kastrup Dalsgaard, T. (2019). Relationship between lipid and protein oxidation in fish. Aquaculture Research, 50(5), 1393-1403. https://doi.org/10.1111/are.14012

Henderson, H. M., Eskin, N. A. M. (1990). Buckwheat lipoxygenase: inactivation by gamma irradiation. *Food Chemistry, 38*(2), 97-103. https://doi.org/10.1016/0308-8146(90)90156-X

Hidalgo, M., Sánchez-Moreno, C., & de Pascual-Teresa, S. (2010). Flavonoid–flavonoid interaction and its effect on their antioxidant activity. *Food Chemistry, 121*(3), 691-696. https://doi.org/10.1016/j.foodchem.2009.12.09

Holder, S., Zemskova, M., Zhang, C., Tabrizizad, M., Bremer, R., Neidigh, J. W., & Lilly, M. B. (2007). Characterization of a potent and selective small-molecule inhibitor of the PIM1 kinase. *Molecular Cancer Therapeutics, 6*(1), 163-172. https.//doi.org/10.1158/1535-7163.MCT-06-0397

Holvoet P. (2004). Oxidized LDL and coronary heart disease. *Acta Cardiologica, 59*(5), 479-484. https://doi.org/10.2143/AC.59.5.2005219

Horton, A. A., Fairhurst, S., & Bus, J. S. (1987). Lipid peroxidation and mechanisms of toxicity. *CRC Critical Reviews in Toxicology, 18*(1), 27-79. https://doi.org/10.3109/10408448709089856

Hu, M., Julian McClements, D., & Decker, E. A. (2004). Impact of chelators on the oxidative stability of whey protein isolate-stabilized oil-in-water emulsions containing ω-3 fatty acids. *Food Chemistry, 88*(1), 57-62. https://doi.org/10.1016/j.foodchem.2004.01.02

Huang, J., Chen, P., Rogers, M., & Wettig, S. (2019). Investigating the phospholipid effect on the bioaccessibility of rosmarinic acid-phospholipid complex through a dynamic gastrointestinal in vitro model. *Pharmaceutics, 11*(4), 156. https://doi.org/10.3390/pharmaceutics11040156

Huang, S.-W., Frankel, E. N., Aeschbach, R., & German, J. B. (1997). Partition of selected antioxidants in corn oil-water model systems. *Journal of Agricultural and Food Chemistry, 45*(6), 1991-1994. https://doi.org/10.1021/jf9701695

Huang, S.-W., Frankel, E. N., Schwarz, K., & German, J. B. (1996). Effect of pH on antioxidant activity of α-tocopherol and trolox in oil-in-water emulsions. *Journal of Agricultural and Food Chemistry, 44*(9), 2496-2502. https://doi.org/10.1021/jf960262d

Huang, S.-W., Hopia, A., Schwarz, K., Frankel, E. N., & German, J. B. (1996). Antioxidant activity of α-tocopherol and trolox in different lipid substrates: Bulk oils vs oil-in-water emulsions. *Journal of Agricultural and Food Chemistry, 44*(2), 444-452. https://doi.org/10.1021/jf9505685

Hudson, B. J. F., & Lewis, J. I. (1983a). Polyhydroxy flavonoid antioxidants for edible oils. Phospholipids as synergists. *Food Chemistry, 10*(2), 111-20. https://doi.org/10.1016/0308-8146(83)90027-4

Hudson, B. J. F., & Lewis, J. I. (1983b). Polyhydroxy flavonoid antioxidants for edible oils. Structural criteria for activity. *Food Chemistry, 10*(1), 47-55. https://doi.org/10.1016/S0308-8146(83)80001-6

Iglesias, J., Pazos, M., Andersen, M. L., Skibsted, L. H., & Medina, I. (2009). Caffeic acid as antioxidant in fish muscle: mechanism of synergism with endogenous ascorbic acid and alpha-tocopherol. *Journal of Agricultural and Food Chemistry, 57*(2), 675-681. https://doi.org/10.1021/jf802888w

Institute of Medicine. (2001). *Dietary reference intakes for vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc.* National Academy Press. Washington, DC. https://doi.org/10.17226/10026

Ismahil, M.A., Hamid, T., Haberzettl, P., Gu, T., Chandrasekar, B., Srivastava, S., Bhatnagar, A., & Prabhu, S.D. (2011) Chronic oral exposure to the aldehyde pollutant acrolein induces dilated cardiomyopathy. *American Journal of Physiology-Heart and Circulatory Physiology, 301*(5), H2050-H2060. https://doi.org/10.1152/ajpheart.00120.2011

Jacobsen, C., Adler-Nissen, J., & Meyer, A. S. (1999). Effect of ascorbic acid on iron release from the emulsifier interface and on the oxidative flavor deterioration in fish oil enriched mayonnaise. *Journal of Agricultural and Food Chemistry, 47*(12), 4917-4926. https://doi.org/10.1021/jf990241u

Jamshidian, M., Tehrany, E. A., & Desobry, S. (2012). Antioxidants release from solvent-cast PLA film: Investigation of PLA antioxidant-active packaging. *Food and Bioprocess Technology, 6*(6), 1450-1463. https://doi.org/10.1007/s11947-012-0830-9

Jaswir, I., Kitts, D. D., Che Man, Y. B., & Hassan, T. H. (2004). Synergistic effect of rosemary, sage and citric acid on fatty acid retention of heated flaxseed oil. *Journal of Oleo Science, 53*(12), 581-591. https://doi.org/10.5650/jos.53.581

Jia, N., Kong, B., Liu, Q., Diao, X., & Xia, X. (2012). Antioxidant activity of black currant (ribes nigrum L.) extract and its inhibitory effect on lipid and protein oxidation of pork patties during chilled storage. Meat Science, 91(4), 533-539. https://doi.org/10.1016/j.meatsci.2012.03.010

Jia, Z.-S., Zhou, B., Yang, L., Wu, L.-M., & Liu, Z.-L. (1998). Antioxidant synergism of tea polyphenols and α-tocopherol against free radical induced peroxidation of linoleic acid in solution. *Journal of the Chemical Society, Perkin Transactions 2*, (4), 911–916. https://doi.org/10.1039/a706691k

Johnson, D. R., Gisder, J., Lew, L., Goddard, J. M., & Decker, E. A. (2017). Is oxygen reduction a viable antioxidant strategy for oil-in-water emulsions? *European Journal of Lipid Science and Technology, 119*, 1600285. https://doi.org/10.1002/ejlt.201600285

Jomová, K., Hudecova, L., Lauro, P., Simunkova, M., Alwasel, S. H., Alhazza, I. M., & Valko, M. (2019). A switch between antioxidant and prooxidant properties of the phenolic compounds myricetin, morin, 3',4'-dihydroxyflavone, taxifolin and 4-hydroxy-coumarin in the presence of copper (II) ions: A spectroscopic, absorption titration and DNA damage study. *Molecules, 24*, 4335. https://doi.org/10.3390/molecules24234335

Jorgensen, L. V., Madsen, H. L., Thomsen, M. K., Dragsted, L. O., & Skibsted, L. H. (1999). Regeneration of phenolic antioxidants from phenoxyl radicals: An ESR and electrochemical study of antioxidant hierarchy. *Free Radical Research, 30*(3), 207-220. https://doi.org/10.1080/10715769900300231

Jovanovic, S. V., & Simic, M. G. (2000). Antioxidants in nutrition. In C. C. Chiueh (Ed.), *Reactive oxygen species: From radiation to molecular biology* (pp. 326-334). New York Academy of Sciences. https://doi.org/10.1111/j.1749-6632.2000.tb06197.x

Judde, A., Villeneuve, P., Rossignol-Castera, A., & Le Guillou, A. (2003). Antioxidant effect of soy lecithins on vegetable oil stability and their synergism with tocopherols. *Journal of the American Oil Chemists' Society, 80*(12), 1209-1215. https://doi.org/10.1007/s11746-003-0844-4

Kajiya, K., Kumazawa, S., & Nakayama, T. (2001). Steric effects on interaction of tea catechins with lipid bilayers. *Bioscience, Biotechnology, and Biochemistry, 65*(12), 2638-2643. https://doi.org/10.1271/bbb.65.2638

Kamaya, Y., Fukaya, Y., & Suzuki, K. (2005). Acute toxicity of benzoic acids to the crustacean Daphnia magna. *Chemosphere, 59*(2), 255-261. https://doi.org/10.1016/j.chemosphere.2004.11

Kanner, J. (1994). Oxidative processes in meat and meat products: Quality implications. *Meat Science, 36*(1-2), 169-189. https://doi.org/10.1016/0309-1740(94)90040-X

Katsouli, M., Polychniatou, V., & Tzia, C. (2017). Influence of surface-active phenolic acids and aqueous phase ratio on w/o nano-emulsions properties; model fitting and prediction of nano-emulsions oxidation stability. *Journal of Food Engineering, 214*, 40-46. https://doi.org/10.1016/j.jfoodeng.2017.06.01

Kejík, Z., Kaplánek, R., Masařík, M., Babula, P., Matkowski, A., Filipenský, P., Veselá, K., Gburek, J., Sýkora, D., Martásek, P., & Jakubek, M. (2021). Iron complexes of flavonoids-antioxidant capacity and beyond. Int J Mol Sci. 2021;22:646. doi:10.3390/ijms22020646.

Kim, H. J., Lee, H. O., & Min, D. B. (2007). Effects and prooxidant öechanisms of oxidized α-tocopherol on the oxidative stability of soybean oil. *Journal of Food Science, 72*(4), C223-C230. https://doi.org/10.1111/j.1750-3841.2007.00339.x

Kinsella, J. E., Shimp, J. L., & Mai, J. (1978). The proximate composition of several species of fresh water fishes. *Food and Life Sciences Bulletin, 69*, 1-20.

Kittipongpittaya, K., Panya, A., Cui, L., McClements, D. J., & Decker, E. A. (2014). Association colloids formed by multiple surface active minor components and their effect on lipid oxidation in bulk oil. *Journal of the American Oil Chemists' Society, 91,* 1955–1965. https://doi.org/10.1007/s11746-014-2541-z

Kittipongpittaya, K., Panya, A., & Decker, E. A. (2016). Role of water and selected minor components on association colloid formation and lipid oxidation in bulk oil. *Journal of the American Oil Chemists' Society, 93*, 83–91. https://doi.org/10.1007/S11746-015-2752-Y

Kochevar, I., & Redmond, R. (2000). Photosensitized production of singlet oxygen. In L., Packer, H., Sies (Eds.). *Methods in enzymology*. (pp. 20-28). Academic Press. https://doi.org/10.1016/s0076-6879(00)19004-4

Koppenol, W.H. (1993). The centennial of the Fenton reaction. *Free Radicad Research, 15*, 645-651. https://doi.org/10.1016/0891-5849(93)90168-t

Korycka-Dahl, M. B., Richardson, T., & Foote, C. S. (1978). Activated oxygen species and oxidation of food constituents. *Critical Reviews in Food Science and Nutrition, 10*(3), 209-241. https://doi.org/10.1080/10408397809527250

Kuffa, M., Priesbe, T. J., Krueger, C. G., Reed, J. D. & Richards, M. P. (2009) Ability of dietary antioxidants to affect lipid oxidation of cooked turkey meat in a simulated stomach and blood lipids after a meal. *Journal of Functional Foods, 1*(2), 208-216. https://doi.org/10.1016/j.jff.2009.01.010

Kurth, E. F., & Chan, F. L. (1951). Dihydroquercetin as an antioxidant. *Journal of the American Oil Chemists' Society, 28*, 433–436. https://doi.org/10.1007/BF02589681

Laguerre, M., Giraldo, L. J. L., Lecomte, J., Figueroa-Espinoza, M. C., Baréa, B., Weiss, J., Decker, E. A., & Villeneuve, P. (2010). Relationship between hydrophobicity and antioxidant ability of "phenolipids" in emulsion: a parabolic effect of the chain length of rosmarinate esters. *Journal of Agricultural and Food Chemistry, 58*(5), 2869-2876. https://doi.org/10.1021/jf904119v

Lee, S. H., & Min, D. B. (1990). Effects, quenching mechanisms, and kinetics of carotenoids in chlorophyll-sensitized photooxidation of soybean oil. *Journal of Agricultural and Food Chemistry, 38*(8), 1630-1634. https://doi.org/10.1021/jf00098a002

Lei, L., Yang, J., Zhang, J., & Zhang, G. (2021). The lipid peroxidation product EKODE exacerbates colonic inflammation and colon tumorigenesis. *Redox Biology, 42*, 101880. https://doi.org/10.1016/j.redox.2021.101880

Let, M. B., Jacobsen, C., & Meyer, A. S. (2007). Ascorbyl palmitate, γ-tocopherol, and EDTA affect lipid oxidation in fish oil enriched salad dressing differently. *Journal of Agricultural and Food Chemistry, 55*(6), 2369-2375. https://doi.org/10.1021/jf062675c

Li, Y., & Ding, Y. (2012). Minireview: Therapeutic potential of myricetin in diabetes mellitus. *Food Science and Human Wellness, 1*(1), 19-25. https://doi.org/10.1016/j.fshw.2012.08.002

Liao, K., & Yin, M. (2000). Individual and combined antioxidant effects of seven phenolic agents in human erythrocyte membrane ghosts and phosphatidylcholine liposome systems: Importance of the partition coefficient. *Journal of Agricultural and Food Chemistry, 48*(6), 2266-2270. https://doi.org/10.1021/jf990946w

Logan, A., Nienaber, U., & Pan, X.S. (Eds.). (2015). *Lipid oxidation: Challenges in food systems*. Academic Press and AOCS Press.

López-Martínez, A., & Rocha-Uribe, A. (2017). Antioxidant hydrophobicity and emulsifier type influences the partitioning of antioxidants in the interface improving oxidative stability in o/w emulsions rich in n-3 fatty acids. *European Journal of Lipid Science and Technology, 120*(1), 1700277. https://doi.org/10.1002/ejlt.201700277

Lu, Z., Nie, G., Belton, P. S., Tang, H., & Zhao, B. (2006). Structure–activity relationship analysis of antioxidant ability and neuroprotective effect of gallic acid derivatives. *Neurochemistry International, 48*(4), 263-274. https://doi.org/10.1016/j.neuint.2005.10.010

Lysionek, A. E., Zubillaga, M. B., Salgueiro, M. J. Caro, R. A., Leonardi, N. M., Ettlin, E., & Boccio, J. R. (2003). Stabilized ferrous gluconate as iron source for food fortification. *Biological Trace Element Research, 94*(1), 73-77. https://doi.org/10.1385/BTER:94:1:73

Maisuthisakul, P., Pongsawatmanit, R., & Gordon, M. H. (2006). Antioxidant properties of teaw (cratoxylum formosum dyer) extract in soybean oil and emulsions. *Journal of Agricultural and Food Chemistry, 54*, 2719-2725. https://doi.org/10.1021/jf052396+

Marinova, E., Toneva, A., & Yanishlieva, N. (2008). Synergistic antioxidant effect of α-tocopherol and myricetin on the autoxidation of triacylglycerols of sunflower oil. *Food Chemistry, 106*(2), 628-633. https://doi.org/10.1016/j.foodchem.2007.06.02

Masella, P., Parenti, A., Spugnoli, P., & Calamai, L. (2010). Nitrogen stripping to remove dissolved oxygen from extra virgin olive oil. *European Journal of Lipid Science and Technology, 112*(12), 1389-1392. https://doi.org/10.1002/ejlt.200900277

McClements, D .J., & Decker, E. A. (2017). Lipids. In S., Damodarin, K., Parkin, O. R., Fennema (Eds.), *Fennema's Food chemistry*. CRC Press, Boca Raton, FL. https://doi.org/10.1201/9781315372914

McClements, D. J., & Decker, E. A. (2000). Lipid oxidation in oil-in-water emulsions: Impact of molecular environment on chemical reactions in heterogeneous food systems. *Journal of Food Science, 65*(8), 1270-1282. https://doi.org/10.1111/j.1365-2621.2000.tb10596.x

Medina, I., Lois, S., Alcantara, D., Lucas, R., & Morales, J. C. (2009). Effect of lipophilization of hydroxytyrosol on its antioxidant activity in fish oils and fish oil-inwater emulsions. *Journal of Agricultural and Food Chemistry, 57*(20), 9773-9779. https://doi.org/10.1021/jf9023867

Mei, L., McClements, D. J., & Decker, E. A. (1999). Lipid oxidation in emulsions as affected by charge status of antioxidants and emulsion droplets. *Journal of Agricultural and Food Chemistry, 47*(6), 2267-2273. https://doi.org/10.1021/jf980955p

Meyer, A. S., Heinonen, M., & Frankel, E. N. (1998). Antioxidant interactions of catechin, cyanidin, caffeic acid, quercetin, and ellagic acid on human LDL oxidation. *Food Chemistry, 61*(1-2), 71-75. https://doi.org/10.1016/s0308-8146(97)00100-3

Mira, L., Tereza Fernandez, M., Santos, M., Rocha, R., Helena Florêncio, M., & Jennings, K. R. (2002). Interactions of flavonoids with iron and copper ions: A mechanism for their antioxidant activity. *Free Radical Research, 36*(11), 1199-1208. https://doi.org/10.1080/1071576021000016463

Mladěnka, P., Macáková, K., Filipský, T., Zatloukalová, L., Jahodář, L., Bovicelli, P., Silvestri, I. P., Hrdina, R., & Saso, L. (2011). In vitro analysis of iron chelating activity of flavonoids. *Journal of Inorganic Biochemistry, 105*(5), 693-701. https://doi.org/10.1016/j.jinorgbio.2011.02.003

Moran, J. F., Klucas, R. V., Grayer, R. J., Abian, J., & Becana, M. (1997). Complexes of iron with phenolic compounds from soybean nodules and other legume tissues: Prooxidant and antioxidant properties. *Free Radical Biology and Medicine, 22*, 861-870. https://doi.org/10.1016/s0891-5849(96)00426-1

Mottram, D. S. (1998). Flavour formation in meat and meat products: A review. *Food Chemistry, 62*(4), 415-424. https://doi.org/10.1016/S0308-8146(98)00076-4

Mozuraityte, R., Rustad, T., & Storrø, I. (2008). The role of iron in peroxidation of polyunsaturated fatty acids in liposomes. *Journal of Agricultural and Food Chemistry, 56*(2), 537-543. https://doi.org/10.1021/jf0716073

Mukai, K., Nagai, S., & Ohara, K. (2005). Kinetic study of the quenching reaction of singlet oxygen by tea catechins in ethanol solution. *Free Radical Biology and Medicine, 39*(6), 752-761. https://doi.org/10.1016/j.freeradbiomed.2005

Mukhopadhyay, M., & Chakraborty, A. (2007). Lipoxygenase, lipase and trypsin inhibitor inactivated and sterilized legumes and cereal products without loss of nutrients enhanced shelf life. Indian Patent Applications, IN 2005MU00540 A 20070608.

Mura, E., Yagi, M., Kizaki, Y., Matsumiya, K., Matsumura, Y., & Hayashi, Y. (2017). Analysis of active components on oral fat sensations in oolong tea. *Food Science and Technology Research, 23*(1), 71-78. https://doi.org/10.3136/fstr.23.71

Narvel, J. M., Fehr, W. R., & Welke, G. A. (1998). Agronomic and seed traits of soybean lines lacking seed lipoxygenases. *Crop Science, 38*, 926-928. https://doi.org/10.2135/cropsci1998.0011183x0

Nawar, W. W. (1996). Lipids. In: O,R., Fennema (Eds.), *Food chemistry* (pp. 225-319). New York:Marcel Dekker.

Neunert, G., Górnaś, P., Dwiecki, K., Siger, A., & Polewski, K. (2015). Synergistic and antagonistic effects between alpha-tocopherol and phenolic acids in liposome system: spectroscopic study. *European Food Research and Technology, 241*(6), 749-757. https://doi.org/10.1007/s00217-015-2500-4

Niki, E., Kawakami, A., Yamamoto, Y., & Kamiya, Y. (1985). Oxidation of lipids. VIII. Synergistic inhibition of oxidation of phosphatidylcholine liposome in aqueous dispersion by vitamin E and vitamin C. *Bulletin of the Chemical Society of Japan, 58*(7), 1971-1975. https://doi.org/10.1246/bcsj.58.1971

Niki, E., Noguchi, N., Tsuchihashi, H., & Gotoh, N. (1995). Interaction among vitamin C, vitamin E and beta-carotene. *The American Journal of Clinical Nutrition, 62*(6) 1322-1326. https://doi.org/10.1093/ajcn/62.6.1322S

Niki, E., Saito, T., Kawakami, A., & Kamiya, Y. (1984). Inhibition of oxidation of methyl linoleate in solution by vitamin E and vitamin C. Journal of Biological Chemistry, 259(7), 4177-4182. https://doi.org/10.1016/s0021-9258(17)43026-2

Niki, E., Tsuchiya, J., Tanimura, R., & Kamiya, Y. (1982). Regeneration of vitamin E from α-Chromanoxyl radical by glutathione and vitamin C. *Chemistry Letters, 11*(6), 789-792. https://doi.org/10.1246/cl.1982.789

Noubigh, A., Abderrabba, M., & Provost, E. (2009). Salt addition effect on partition coefficient of some phenolic compounds constituents of olive mill wastewater in 1-octanol-water system at 298.15 K. *Journal of the Iranian Chemical Society, 6*(1), 168-176. https://doi.org/10.1007/bf03246517

Noubigh, A., Mgaidi, A., & Abderrabba, M. (2010). Temperature effect on the distribution of some phenolic compounds: An experimental measurement of 1-octanol/water partition coefficients. *Journal of Chemical & Engineering Data, 55*(1), 488-491. https://doi.org/10.1021/je900271h

Ohshima, T., Wada, S. & Koizumi, C. (1988). Influences of heme pigment, non-heme iron, and nitrite on lipid oxidation in cooked mackerel meat. *Nippon Suisan Gakkaishi, 54*(12), 2165-2171. https://doi.org/10.2331/suisan.54.2165

Olszowy-Tomczyk, M. (2020). Synergistic, antagonistic and additive antioxidant effects in the binary mixtures. *Phytochemistry Reviews*, 19(5), 63-103. https://doi.org/10.1007/s11101-019-09658-4

Omura, K. (1995). Antioxidant synergism between butylated hydroxyanisole and butylated hydroxytoluene. *Journal of the American Oil Chemists' Society, 72*(12), 1565-1570. https://doi.org/10.1007/bf02577855

Packer, J. E., Slater, T. F., & Willson, R. L. (1979). Direct observation of a free radical interaction between vitamin E and vitamin C. *Nature*, *278*(5706), 737-738. https://doi.org/10.1038/278737a0

Pan, X., Ushio, H., & Ohshima, T. (2005). Effects of molecular configurations of food colorants on their efficacies as photosensitizers in lipid oxidation. *Food Chemistry*, *92*(1), 37-44. https://doi.org/10.1016/j.foodchem.2004.07.01

Pantopoulos, K., Porwal, S. K., Tartakoe, A., & Devireddy, L. (2012). Mechanisms of mammalian iron homeostasis. *Biochemistry, 51*(29), 705-5724. https://doi.org/ 10.1021/bi300752r

Panya, A., Kittipongpittaya, K., Laguerre, M., Bayrasy, C., Lecomte, J., Villeneuve, P., McClements, D. J., & Decker, E. A. (2012). Interactions between α-tocopherol and rosmarinic acid and its alkyl esters in emulsions: Synergistic, additive, or antagonistic effect? *Journal of Agricultural and Food Chemistry, 60*(41), 10320-10330. https://doi.org/10.1021/jf302673j

Park, K.-S., Chong, Y., & Kim, M. K. (2016). Myricetin: biological activity related to human health. *Applied Biological Chemistry, 59*, 259–269. https://doi.org/10.1007/s13765-016-0150-2

Parveen, I., Threadgill, M. D., Moorby, J. M., & Winters, A. (2010). Oxidative phenols in forage crops containing polyphenol oxidase enzymes. *Journal of Agricultural and Food Chemistry, 58*(3), 1371-1382. https://doi.org/10.1021/jf9024294

Patrignani, M., Conforti, P. A., & Lupano, C. E. (2014). The role of lipid oxidation on biscuit texture during storage. International *Journal of Food Science & Technology, 49*(8), 1925-1931. https://doi.org/10.1111/ijfs.12550

Pedriali, C. A., Fernandes, A. U., Bernusso, L. de C., & Polakiewicz, B. (2008). The synthesis of a water-soluble derivative of rutin as an antiradical agent. *Química Nova, 31*(8), 2147-2151. https//doi.org/10.1590/s0100-40422008000800039

Pedrielli, P., & Skibsted, L. H. (2002). Antioxidant synergy and regeneration effect of quercetin, (-)-epicatechin, and (+)-catechin on alpha-tocopherol in homogeneous solutions of peroxidating methyl linoleate. *Journal of Agricultural and Food Chem*istry, *50*(24). 7138-7144. https://doi.org/10.1021/jf020437l

Peng, S., Zhang, B., Yao, J., Duan, D., & Fang, J. (2015). Dual protection of hydroxytyrosol, an olive oil polyphenol, against oxidative damage in PC12 cells. *Food & Function, 6*(6), 2091-2100. https://doi.org/10.1039/c5fo00097a

Pereira, R., Sousa, C., Costa, A., Andrade, P., & Valentão, P. (2013). Glutathione and the antioxidant potential of binary mixtures with flavonoids: Synergisms and antagonisms. *Molecules, 18*(8), 8858-8872. https://doi.org/10.3390/molecules18088858

Peyrat-Maillard, M. N., Cuvelier, M. E., & Berset, C. (2003). Antioxidant activity of phenolic compounds in 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH)-induced oxidation: Synergistic and antagonistic effects. *Journal of the American Oil Chemists' Society, 80*(10), 1007. https://doi.org/10.1007/s11746-003-0812-z

Pinelo, M., Manzocco, L., Nuñez, M. J., & Nicoli, M. C. (2004). Interaction among phenols in food fortification: Negative synergism on antioxidant capacity. *Journal of Agricultural and Food Chemistry, 52*(5), 1177-1180. https://doi.org/10.1021/jf0350515

Porter, W. L., Black, E. D., & Drolet, A. M. (1989). Use of polyamide oxidative fluorescence test on lipid emulsions: contrast in relative effectiveness of antioxidants in bulk versus dispersed systems. *Journal of Agricultural and Food Chemistry, 37*(3), 615-624. https://doi.org/10.1021/jf00087a009

Pullar, J., Carr, A., & Vissers, M. (2017). The roles of vitamin C in skin health. *Nutrients, 9*(8), 866. https://doi.org/10.3390/nu9080866

Refsgaard, H. H. F., Brockhoff, P., & Jensen, B. (1998). Sensory and chemical changes in farmed atlantic salmon (*Salmo salar*) during frozen storage. *Journal of Agricultural and Food Chemistry, 46*, 3473-3479. https://doi.org/10.1021/jf980309q

Rhee, K. S. & Ziprin, Y. A. (1987). Lipid oxidation in retail beef, pork and chicken muscles as affected by concentrations of heme pigments and nonheme iron and microsomal enzymic lipid peroxidation activity. *Journal of Food Biochemistry, 11*(1), 1-15. https://doi.org/10.1111/j.1745-4514.1987.tb00109.x

Richards, M. P. (2010). Heme proteins and oxidation in fresh and processed meats. In E. A. Decker, R. J. Elias & D. J. McClements (Eds.), *Oxidation in foods and beverages and antioxidant applications* (pp.105-121). Woodhead Publishing, Cambridge, UK. https://doi.org/10.1533/9780857090447.1.77

Rodriguez-Amaya, D.B. (1999). Changes in carotenoids during processing and storage of foods. *Archivos Latinoamericanos de Nutricion, 49*, 38S-47S

Roedig-Penman, A., & Gordon, M. H. (1998). Antioxidant properties of myricetin and quercetin in oil and emulsions. *Journal of the American Oil Chemists' Society, 75*(2), 169-180. https://doi.org/10.1007/s11746-998-0029-4

Ross, J. A., & Kasum, C. M. (2002). Dietary flavonoids: bioavailability, metabolic effects, and safety. Annual Review of Nutrition, 22, 19–34. https://doi.org/10.1146/annurev.nutr.22.111401.144957

Rowley and Halliwell (1983). Superoxide-dependent and ascorbate-dependent formation of hydroxyl radicals in the presence of copper salts: A physiologically significant reaction? *Archives of Biochemistry and Biophysics, 225*(1), 279-284. https://doi.org/10.1016/0003-9861(83)90031-0

Salas, J. J., Williams, M., Harwood, J. L., & Sanchez, J. (1999). Lipoxygenase activity in olive (*Olea europaea*) fruit. *Journal of the American Oil Chemists' Society, 76*(10), 1163-1168. https://doi.org/10.1007/s11746-999-0090-7

Samdani, G. K., McClements, D. J., & Decker, E. A. (2018). Impact of phospholipids and tocopherols on the oxidative stability of soybean oil-in-water emulsions. *Journal of Agricultural and Food Chemistry, 66*(15), 3939-3948. https://doi.org/10.1021/acs.jafc.8b00677

Sasaki, K., Alamed, J., Weiss, J., Villeneuve, P., López Giraldo, L. J., Lecomte, J., Figueroa-Espinoza, M.-C., & Decker, E. A. (2010). Relationship between the physical properties of chlorogenic acid esters and their ability to inhibit lipid oxidation in oil-in-water emulsions. *Food Chemistry, 118*(3), 830-835. https://doi.org/10.1016/j.foodchem.2009.05.07

Sato, K., Niki, E., & Shimasaki, H. (1990). Free radical-mediated chain oxidation of low density lipoprotein and its synergistic inhibition by vitamin E and vitamin C. *Archives of Biochemistry and Biophysics, 279*(2), 402-405. https://doi.org/10.1016/0003-9861(90)90508-v

Schaich, K.M. (1992). Metals and lipid oxidation. Contemporary issues. *Lipids 27*(3), 209-218. https://doi.org/10.1007/BF02536181

Schwarz, K., Frankel, E. N., & German, J. B. (1996). Partition behaviour of antioxidative phenolic compounds in heterophasic systems. *Lipid/Fett, 98*(3), 115-121. https://doi.org/10.1002/lipi.19960980306

Seppanen, C. M., Song, Q., & Saari Csallany, A. (2010). The antioxidant functions of tocopherol and tocotrienol homologues in oils, fats, and food systems. *Journal of the American Oil Chemists' Society, 87*(5), 469-481. https://doi.org/10.1007/s11746-009-1526-9

Shahidi, F., & Zhong, Y. (2010). Lipid oxidation and improving the oxidative stability. *Chemical Society Reviews, 39*(11), 4067-4079. https://doi.org/10.1039/b922183m

Shantha, N. C., & Decker, E. A. (1994). Rapid, sensitive, iron-based spectrophotometric methods for determination of peroxide values of food lipids. *Journal of AOAC International, 77*, 421-424. https://doi.org/10.1093/jaoac/77.2.421

Shatalin, Y. V., & Shubina, V. S. (2014). Partitioning of taxifolin-iron ions complexes in octanol-water system. *Biophysics, 59*(3), 351-356. https://doi.org/10.1134/s000635091403021x

Shi, J., Qu, Q., Kakuda, Y., Xue, S. J., Jiang, Y., Koide, S., & Shim, Y.-Y. (2007). Investigation of the antioxidant and synergistic activity of lycopene and other natural antioxidants using LAME and AMVN model systems. *Journal of Food Composition and Analysis, 20*(7), 603-608. https://doi.org/10.1016/j.jfca.2007.03.004

Shibusawa, Y., Shoji, A., Yanagida, A., Shindo, H., Tagashira, M., Ikeda, M., & Ito, Y. (2005). Determination of log po/w for catechins and their isomers, oligomers, and other organic compounds by stationary phase controlled high-speed countercurrent chromatography. *Journal of Liquid Chromatography & Related Technologies, 28*(17), 2819-2837. https://doi.org/10.1080/10826070500225317

Shubina, V. S., & Shatalin, Y. V. (2017). Antioxidant and iron-chelating properties of taxifolin and its condensation product with glyoxylic acid. *Journal of Food Science and Technology, 54*, 1467-1475. https://doi.org/10.1007/s13197-017-2573-0

Singh, A., Bains, K., & Kaur, H. (2016). Relationship of dietary factors with dialyzable iron and in vitro iron bioavailability in the meals of farm women. *Journal of Food Science and Technology, 53*(4), 2001-2008. https://doi.org/10.1007/s13197-015-2153-0

Skroza, D., Generalić Mekinić, I., Svilović, S., Šimat, V., & Katalinić, V. (2015). Investigation of the potential synergistic effect of resveratrol with other phenolic compounds: A case of binary phenolic mixtures. *Journal of Food Composition and Analysis, 38*, 13-18. https://doi.org/10.1016/j.jfca.2014.06.013

Smith, L.L., & Johnson, B.H. (1989) Biological activities of oxysterols. *Free Radical Biology and Medicine, 7*, 285-332. https://doi.org/10.1016/0891-5849(89)90136-6

Sohn, Y. T., & Oh, J. H. (2003). Characterization of physicochemical properties of ferulic acid. *Archives of Pharmacal Research, 26*(12), 1002-1008. https://doi.org/10.1007/bf02994749

Son, S., & Lewis, B. A. (2002). Free radical scavenging and antioxidative activity of caffeic acid amide and ester analogues: Structure−activity relationship. *Journal of Agricultural and Food Chemistry, 50*(3), 468-472. https://doi.org/10.1021/jf010830b

Sørensen, A.-D. M., Haahr, A.-M., Becker, E. M., Skibsted, L. H., Bergenståhl, B., Nilsson, L., & Jacobsen, C. (2008). Interactions between iron, phenolic compounds, emulsifiers, and pH in omega-3-enriched oil-in-water emulsions. *Journal of Agricultural and Food Chemistry, 56*(5), 1740-1750. https://doi.org/10.1021/jf072946z

Spiteller, G. (2005). The relation of lipid peroxidation processes with atherogenesis: A new theory on atherogenesis. *Molecular Nutrition & Food Research, 49*(11), 999-1013. https://doi.org/10.1002/mnfr.200500055

Sun, M., He, Z., Jaisi, D. P. (2021) Role of metal complexation on the solubility and enzymatic hydrolysis of phytate. *PLoS ONE 16*(8), e0255787. https://doi.org/10.1371/journal.pone.0255787

Tadolini, B., Cabrini, L., Menna, C., Pinna, G. G., & Hakim, G. (1997). Iron (III) stimulation of lipid hydroperoxide dependent lipid peroxidation. *Free Radical Research, 27*, 563-576. https://doi.org/10.3109/10715769709097860

Takenaka, A., Hosokawa, M., & Miyashita, K. (2007). Unsaturated phosphatidylethanolamine as effective synergist in combination with α-tocopherol. *Journal of Oleo Science*, *56*(10), 511-516. https://doi.org/10.5650/jos.56.511

Talpur, M. Y., Sherazi, S. T. H., Mahesar, S. A., & Kandhro, A. A. (2009). Effects of chicken frying on soybean, sunflower and canola oils. *Pakistan Journal of Analytical & Environmental Chemistry*, 10, 59–66.

Thomas, C. E., McLean, L. R., Parker, R. A., & Ohlweiler, D. F. (1992). Ascorbate and phenolic antioxidant interactions in prevention of liposomal oxidation. *Lipids, 27*(7), 543-550. https://doi.org/10.1007/bf02536138

Timoshnikov, V. A., Selyutina, O. Y., Polyakov, N. E., Didichenko, V., Kontoghiorghes, G. J. (2022). Mechanistic insights of chelator complexes with essential transition metals: Antioxidant/pro-oxidant activity and applications in medicine. *International Journal of Molecular Sciences, 23*(3), 1247. https://doi.org/10.3390/ijms23031247

Topal, F., Nar, M., Gocer, H., Kalin, P., Kocyigit, U. M., Gulcin I., & Alwasel, S. H. (2016). Antioxidant activity of taxifolin: An activity-structure relationship. *Journal of Enzyme Inhibition and Medicinal Chemistry, 31*(4), 674-683. https://doi.org/10.3109/14756366.2015.1057723

Toyosaki, T. (2007). Effects of hydroperoxide in lipid peroxidation on dough fermentation. *Food Chemistry, 104*(2), 680-685. https://doi.org/10.1016/j.foodchem.2006.12.031

Tsao, R. (2015). Synergistic interactions between antioxidants used in food preservation. In F. Shahidi (Ed.), *Handbook of antioxidants for food preservation*. (p. 339). Woodhead Publishing.

Turck, D., Bresson, J.-L., Burlingame, B., Dean, T., Fairweather-Tait, S., Heinonen, M., Hirsch-Erns, K. I., Mangelsdorf, I., McArdle, H. J., Naska, A., Neuhauser-Berthold, M., Nowicka, G., Pentieva, K., Sanz, Y., Siani, A., Sjödin, A., Stern, M., Tome, D., Vinceti, M., . . .Loveren, H. V. (2017). Scientific opinion on taxifolin-rich extract from Dahurian Larch (*Larix gmelinii*). *EFSA Journal, 15*, e04682. https://doi.org/10.2903/j.efsa.2017.4682

Van Aardt, M., Duncan, S. E., Marcy, J. E., Long, T. E., O'Keefe, S. F., & Nielsen-Sims, S. R. (2005). Aroma analysis of light-exposed milk stored with and without natural and synthetic antioxidants. *Journal of Dairy Science, 88*(3), 881-890. https://doi.org/10.3168/jds.S0022-0302(05)72754-5

Van Dyck, S. (2010). The impact of singlet oxygen on lipid oxidation in foods. In E. A. Decker, R. J. Elias, & D. J. McClements (Eds.), *Oxidation in foods and beverages and antioxidant applications* (pp. 57-75). Woodhead Publishing. Woodhead Publishing, Cambridge, UK. https://doi.org/10.1533/9780857090447.1.57

Vieira, S., Zhang, G., & Decker, E. (2017). Biological implications of lipid oxidation products. *Journal of the American Oil Chemists' Society, 94*(3), 339-351. https://doi.org/10.1007/s11746-017-2958-2

Wanasundara, U. N., & Shahidi, F. (1998). Stabilization of marine oils with flavonoids. *Journal of Food Lipids, 5*(3), 183-196. https://doi.org/10.1111/j.1745-4522.1998.tb00119.x

Wang, S. J., Tong, Y., Lu, S., Yang, R., Liao, X., Xu, Y. F., & Li X. (2010). Anti-inflammatory activity of myricetin isolated from Myrica rubra Sieb. et Zucc. leaves. *Planta Medica, 76*, 1492-1496. https://doi.org/10.1055/s-0030-1249780

Wang, T., & Hammond, E.G. (2010). Lipoxygenase and lipid oxidation in foods. In E. A. Decker, R. J. Elias & D. J. McClements (Eds.), *Oxidation in foods and beverages and antioxidant applications* (pp.105-121). Woodhead Publishing, Cambridge, UK. https://doi.org/10.1533/9780857090447.1.105

Waraho, T., Cardenia, V., Rodriguez-Estrada, M. T., McClements, D. J., & Decker, E. A. (2009). Prooxidant mechanisms of free fatty acids in stripped soybean oil-in-water emulsions. *Journal of Agricultural and Food Chemistry, 57*(15), 7112-7117. https://doi.org/10.1021/jf901270m

Waraho, T., McClements, D. J., & Decker, E. A. (2011). Impact of free fatty acid concentration and structure on lipid oxidation in oil-in-water emulsions. *Food Chemistry, 129*(3), 854-859. https://doi.org/10.1016/j.foodchem.2011.05.03

Warren, J. J., Tronic, T. A., & Mayer, J. M. (2010). Thermochemistry of proton-coupled electron transfer reagents and its implications. *Chemical Reviews, 110*(12), 6961-7001. https://doi.org/10.1021/cr100085k

Watkins, S. M., & German, J. B. (1998). Omega fatty acids. In: C. C, Akoh & D. B., Min (Eds.), *Food lipids: chemistry, nutrition and biotechnology* (pp. 463-494). New York: Marcel Dekker.

Weng, X. C., & Gordon, M. H. (1993). Antioxidant synergy between phosphatidyl ethanolamine and α-tocopherylquinone. *Food Chemistry, 48*(2), 165-168. https://doi.org/10.1016/0308-8146(93)90051-g

Whang, K., & Peng, I. (1988). Electron paramagnetic resonance studies of the effectiveness of myoglobin and its derivatives as photosensitizers in singlet oxygen generation. *Journal of Food Science, 53*(6), 1863-1865. https://doi.org/10.1111/j.1365-2621.1988.tb07862.x

Wijesundera, C., Ceccato, C., Watkins, P., Fagan, P., Fraser, B., Thienthong, N., & Perlmutter, P. (2008). Docosahexaenoic acid is more stable to oxidation when located at the sn-2 position of triacylglycerol compared to sn-1(3). *Journal of the American Oil Chemists' Society, 85*(6), 543-548. https://doi.org/10.1007/s11746-008-1224-z

Xu, H. X., & Lee, S. F. (2001). Activity of plant flavonoids against antibiotic-resistant bacteria. *Phytotherapy Research, 15*, 39-43. https://doi.org/10.1002/1099-1573(200102)15:1<39::aid-ptr684>3.0.co;2-r

Xu, N., Shanbhag, A., Li, B., Angkuratipakorn, T., & Decker, E. (2019). Impact of phospholipid-tocopherol combinations and enzyme modified lecithin on the oxidative stability of bulk oil. *Journal of Agricultural and Food Chemistry, 67*, 7954-7960. https://doi.org/10.1021/acs.jafc.9b02520

Xu, Z., Harvey, K. A., Pavlina, T. M., Zaloga, G. P., & Siddiqui, R. A. (2015). Tocopherol and tocotrienol homologs in parenteral lipid emulsions. *European Journal of Lipid Science and Technology, 117*, 15-22. https://doi.org/10.1002/ejlt.201400182

Yang, S.-C., Tseng, C.-H., Wang, P.-W., Lu, P.-L., Weng, Y.-H., Yen, F.-L., & Fang, J.-Y. (2017). Pterostilbene, a methoxylated resveratrol derivative, efficiently eradicates planktonic, biofilm, and intracellular MRSA by topical application. *Frontiers in Microbiology, 8,* 1103. https://doi.org/10.3389/fmicb.2017.01103

Yao, Y., Luong, T. N., Lepik, M., Aftab, N., Fong, V. H., & Vieira, A. (2012). Synergism of antioxidant phytochemicals: Comparisons among purified polyphenols and dietary plant extracts. *Acta Horticulturae*, (939), 121-127. https://doi.org/10.17660/actahortic.2012.939

Yi, J., Zhu, Z., McClements, D. J., & Decker, E. A. (2014). Influence of aqueous phase emulsifiers on lipid oxidation in water-in-walnut oil emulsions. *Journal of Agricultural and Food Chemistry, 62*(9), 2104-2111. https://doi.org/10.1021/jf404593f

Yiannikourides, A., & Latunde-Dada, G. O. (2019). A short review of iron metabolism and pathophysiology of iron disorders. *Medicines, 6*(3), 85. https://doi.org/10.3390/medicines6030085

Yin, J., Becker, E. M., Andersen, M. L., & Skibsted, L. H. (2012). Green tea extract as food antioxidant. Synergism and antagonism with α-tocopherol in vegetable oils and their colloidal systems. *Food Chem*istry, *135*(4), 2195-2202. https://doi.org/10.1016/j.foodchem.2012.07.025

Yoe, J. H., & Jones, A. L. (1944). Colorimetric determination of iron with disodium-1,2-dihydroxybenzene-3,5-disulfonate. *Industrial and Engineering Chemistry Analytical Edition, 16*, 111–115. https://doi.org/10.1021/i560126a015

Zumdahl, S. S. (1989). *Chemistry*. Cengage Learning.