9-2012

Strategies to Improve the Performance of Antioxidants in Oil-in-Water Emulsions

Atikorn Panya
University of Massachusetts Amherst, apanya@foodsci.umass.edu

STRATEGIES TO IMPROVE THE PERFORMANCE OF ANTIOXIDANTS IN OIL-IN-WATER EMULSIONS

A Dissertation Presented

by

ATIKORN PANYA

Submitted to the Graduate School of the

University of Massachusetts Amherst in partial fulfillment

of the requirements for the degree of

DOCTOR OF PHILOSOPHY

September 2012

The Department of Food Science

STRATEGIES TO IMPROVE THE PERFORMANCE OF ANTIOXIDANTS IN OIL-IN-WATER EMULSIONS

A Dissertation Presented

by

ATIKORN PANYA

Approved as to style and content by:

_____

Eric A. Decker, Chair

_____

D. Julian McClements, Member

_____

Paul M. Lahti, Member

_____

Eric A. Decker, Department Head

Department of Food Science

DEDICATIONS

To my mom and dad, and all my friends, and my destiny in science

ACKNOWLEDGMENTS

I would like to express my thanks to my advisor, Dr. Eric A. Decker, for accepting me as his Ph.D. student, offering me the great opportunity to conduct this remarkable research topic, and supporting me with his kind guidance, intelligence and strong experiences. I would like to thank Dr. Julian McClements for his kind suggestion on this research and his enthusiasm to come up with new research ideas. I would like to thank Dr. Micha Peleg and Dr. Mark Normand for their suggestion and hard effort to help me developing a simulation mathematical model that I can use in my future research.

Also, I would like to extend special thanks to Dr. Paul M. Lahti in the chemistry department, Umass Amherst for being a member of my committee. I appreciated for his donating valuable times to teach and suggest me on developing EPR techniques used in this research.

I would like to express my appreciation to Jean Alamed for her continuous assistance in my research as well as her friendship. Many thanks to Bingcan, Get, Dao and Mickaël for their continuing and unconditional friendship and support over the years; I could not have done this without their helps. I would also like to thank all faculty, staff and lab members with special thanks to Dr. Xiao, Fran and Dan. In addition, I would like to thank all my Thai friends in Umass Amherst. I've never been missing my hometown.

I would like to thank all staffs in the Royal Thai government and office of educational affairs in Thailand and USA for the scholarship and support during my Ph.D. study.

ABSTRACT

STRATEGIES TO IMPROVE THE PERFORMANCE OF ANTIOXIDANTS IN OIL-IN-WATER EMULSIONS

SEPTEMBER 2012

ATIKORN PANYA, B.S., KHONKAEN UNIVERSITY, KHONKAEN, THAILAND
M.S., MAHIDOL UNIVERSTY, BANGKOK, THAILAND

Ph.D., UNIVERSITY OF MASSACHUSETTS AMHERST MA

Directed by: Professor Eric A. Decker

Due to the limited number of approved antioxidants for food applications, several alternative strategies to improve antioxidant performance have been developed by focusing on synergistic antioxidant interactions. Susceptibility to lipid oxidation in food systems is the result of the summation of antioxidative and prooxidative mechanisms. Understanding the sometimes paradoxical behavior of antioxidants and prooxidants is a vital key to design synergistic antioxidant systems suitable for particular foods. This research focused on 3 main strategies to improve the performance of antioxidant activity in oil-in-water emulsions.

The first part of this research has been focused on inhibition of lipid oxidation by a combination of the modification of liposomal surfaces by chitosan-coating techniques along with addition of rosmarinic acid esters of varying polarity. Repelling metal ions away from the interface of positively charged liposomes can inhibit lipid oxidation

(induced by Fe^{2+}), and also reduce antioxidant loss by Fe^{3+} reduction. As a result, lipid oxidation can be inhibited synergistically because of a reduction in the prooxidant activity of iron.

Second, understanding non-linear antioxidant behavior (the cut-off effect) of antioxidant esters in oil-in-water (O/W) emulsions was also studied to determine how the distributions and locations of antioxidants impacted their antioxidant activity. Antioxidant activity of rosmarinic acid was improved by esterification with alkyl chain lengths between 4 to 12 carbons due to increased ability to partitioning at the interface in oil-in-water emulsions. Surfactant micelles which could increase or decreased the concentration of the antioxidants at the emulsion droplet interface altered antioxidant activity.

In the last part of this research, rosmarinic acid and its esters were found to be an excellent tool for studying how antioxidant location could impact its ability to interact with α-tocopherol in O/W emulsions. Synergistic, additive, and antagonistic effects were observed in the combinations between the rosmarinate esters with α-tocopherol. Increases in alkyl chain lengths of rosmarinic acid have influenced both the partitioning of the rosmarinate esters as well as their ability to they interact with α-tocopherol at the interface of oil-in-water emulsions. Fluorescence quenching and EPR studies showed that water soluble rosmarinic acid (R0) exhibited more interactions with α-tocopherol than any of the esters (R4-R20). Synergistic antioxidant interactions between rosmarinic acid and α-tocopherol could not be explained by electron transfer mechanisms, but formation of caffeic acid from rosmarinic acid. Due to the thermodynamic infeasibility and the fact

that increases in α-tocopherol degradation rates, α-tocopherol could not be regenerated efficiently by rosmarinic acid. This formation of caffeic acid was proposed to be responsible of the synergistic activity of R0 and α-tocopherol since the formation of an additional antioxidant could further increase the oxidative stability of the emulsion.

TABLE OF CONTENTS

Page

ACKNOWLEDGMENTS v

ABSTRACT..... vi

LIST OF TABLES.....xv

LIST OF FIGURES xvi

CHAPTER

1. INTRODUCTION 1

2. LITERATURE REVIEW 4

2.1 Principles of lipid oxidation mechanisms 4

2.2 Beyond the polar paradox theory.....6

2.3 Principles of synergistic antioxidant interactions 10

2.4 Free radical exchanges between antioxidants resulting synergistic antioxidant activity....12

2.4.1 Prediction of antioxidant interactions....14

2.4.1.1 Reduction potentials and free radical interactions....14

2.4.1.2 Bond dissociation enthalpy....19

2.4.1.3 Impact of physical structures on antioxidant interaction....22

2.4.1.4 Effects of pH and microenvironment solvents on reduction potentials of antioxidants....29

2.4.1.5 Effects of pH on regenerations of antioxidants....31

2.4.2 Combinations of free radical scavenging antioxidants....38

2.4.2.1 Synergistic antioxidant interactions between tocopherol homologs....38

2.4.2.2 Antioxidant interactions of flavonoids....40

2.4.2.3 Antioxidant interactions of carotenoids....44

2.4.2.4 Antioxidant interactions of phospholipids....47

2.5 Mechanisms of antagonistic antioxidant interactions....49

2.5.1 Bipolar antioxidant behaviors: antioxidant vs. prooxidant activity....49

2.5.1.1 Reduction of metals by free radical scavengers....50

2.5.1.2 Reverse chain-braking reactions by free radical scavengers....50

2.5.1.3 Direct decomposition of hydroperoxides....52

2.5.1.4 Increase of metal solubility 53

2.5.2 Other prooxidant hypotheses 54

2.5.2.1 Complex formation between antioxidants 54

2.5.2.2 Prooxidant activity of carotenoids 55

3. EFFECTS OF CHITOSAN AND ROSMARINATE ESTERS ON THE PHYSICAL AND OXIDATIVE STABILITY OF LIPOSOMES 56

3.1 Introduction 56

3.2 Materials and Methods 58

3.2.1 Materials 58

3.2.2 Synthesis of rosmarinate esters 59

3.2.3 Preparation of liposomes 60

3.2.4 Liposomal charge and size measurements 61

3.2.5 Measurements of lipid hydroperoxides 62

3.2.6 Measurement of hexanal 62

3.2.7 Determination of antioxidant partitioning 63

3.2.8 Statistics 63

3.3 Results and discussion 64

3.3.1 Effect of chitosan and EDTA on liposomal stability 64

3.3.2 Effect of rosmarinic acid ester on the oxidative stability of liposomes 68

3.3.3 Effect of rosmarinic acid esters hydrocarbon length on the oxidative stability of coated liposomes....71

4. AN INVESTIGATION OF THE VERSATILE ANTIOXIDANT MECHANISMS OF ACTION OF ROSMARINATE ALKYL ESTERS IN OIL-IN-WATER EMULSIONS....75

4.1 Introduction....75

4.2 Materials and Methods....77

4.2.1 Synthesis of rosmarinate esters....78

4.2.2 DPPH scavenging activity....78

4.2.3 Emulsion preparation....79

4.2.4 Measurements of Lipid Hydroperoxides....80

4.2.5 Measurement of hexanal....81

4.2.6 Determination of antioxidant partitioning....81

4.2.7 Front-face fluorescence measurements....82

4.2.8 Determination of interfacial rosmarinate esters....83

4.2.9 Statistical Analysis....84

4.3 Results and discussion....84

4.3.1 Influence of esterification on the DPPH scavenging activity of rosmarinic acid....84

4.3.2 Effects of alkyl chain lengths of rosmarinates on oxidation stability of stripped soybean oil-in water (O/W) emulsions....85

4.3.3 Effect of Tween 20 micelles on the physical location and antioxidant activity of the rosmarinic acid esters in stripped soybean O/W emulsions 89

4.3.4 Measuring the chemical microenvironments of the rosmarinate esters in O/W emulsions by Front-Face Fluorescence.....93

4.3.5 Evidence of rosmarinate esters at oil-water interfaces using a surface active probe. 97

5. COMPLEX MECHANISMS OF ANTIOXIDANT INTERACTIONS BETWEEN ROSMARINATE ESTERS AND α-TOCOPHEROL IN STRIPPED SOYBEAN OIL-IN-WATER EMULSIONS 104

5.1 Introduction.....104

5.2 Materials and methods 106

5.2.1 Chemicals and materials 106

5.2.2 Synthesis of rosmarinate esters.....107

5.2.3 Emulsions preparation 108

5.2.4 Measurements of particle size of emulsions 109

5.2.5 Measurements of lipid hydroperoxides.....110

5.2.6 Measurements of hexanal 111

5.2.7 Calculation of antioxidant interaction indexes of rosmarinate esters and α-tocopherol combinations.....111

5.2.8 Determination of antioxidant partitioning 112

5.2.9 Determination of antioxidant decomposition during oxdation studies 113

5.2.10 Front-face fluorescence quenching measurements 115

5.2.11 Cyclic voltammetry measurements.....116

5.2.12 Measuring the efficiency of the regeneration of α-tocopherol by rosmarinic acid and its esters by electron paramagnetic resonance (EPR) spectroscopy 116

5.2.13 Statistical analysis 118

5.3 Results and discussion 119

5.3.1 Antioxidant activities of rosmarinic acid esters and α-tocopherol combinations in stripped soybean O/W emulsions 119

5.3.2 Partitioning of the rosmarinic acid esrers and α-tocopherol in O/W emulsions 122

5.3.3 Interactions between antioxidants in O/W emulsions as determined by front-face quenching measurements 124

5.3.4 Electrochemical properties of the rosmarinic acid esters and α-tocopherol 126

5.3.5 Regeneration efficiency of the rosmarinic acid esters to reduce α-tocopheroxyl radical in homogeneous and heterogeneous systems 129

5.3.6 Effects of various concentrations of the rosmarinic acid esters on antioxidant interactions with α-tocopherol 131

6. CONCLUSION 141

BIBLIOGRAPHY 145

LIST OF TABLES

Table Page

2.1 The secondary rate constants of tocopherols regenerated from ascorbic acid and ascorbate monoanions in EtOH/H_2O at 25^oC 14

2.2 The one electron reduction potential (E°') of the radical form of reactive oxygen species, phenolic antioxidants, carotenoids, and flavonoids. 16

2.3 Bond dissociation enthalpies (BDEs) of antioxidants and related compounds 21

2.4 Efficiency of natural phenolic compounds regenerating α-tocopherol in homogenous and heterogeneous systems 28

2.5 The secondary rate constants of the regeneration of α-tocopherol by green tea flavonoids and other phenolics. 42

2.6 The rate constants of the reverse reactions of α-tocopheroxyl radicals and various fatty acids at 25^oC 51

2.7 Rate constants of decomposition reaction of cumyl hydroperoxide by phenols in benzene at 413^oK 53

4.1 Initial secondary rate constants of 16-ArN_2+ (170 μM) consumption in the presence of rosmarinate esters (30 μM) in washed oil-in-water emulsions with and without 2.5% Tween 20. 99

5.1 Electrochemical parameters of tested antioxidants in acetonitrile, 50 mM phosphate buffer (pH 7), and 1% Tween 20 in 50 mM phosphate buffer (pH 7) solutions at 25^oC obtained from cyclic voltammetry (CV). 128

LIST OF FIGURES

Figure Page

2.1 The polar paradox theory in food emulsions 7

2.2 Schematic demonstration of the cut of effect of antioxidants. Relative antioxidant activity changes (%) were obtained from conjugated autoxidizable triene (CAT) method 9

2.3 Redox recycling of α-tocopherol by water soluble antioxidants 13

2.4 Schematic demonstrations of antioxidant interactions in homogeneous and heterogeneous systems impacted by physical locations 24

2.5 Dynamic localization of α-tocopherol and peroxyl radicals which allows for regeneration of α-tocopherol by ascorbic acid in liposomal membranes 26

2.6 The regeneration of α-tocopherol (R^1) from α-tocopheroxyl radical by phenolic compounds (R^2).....29

2.7 The pH-dependent (pH 2-7) differential pulse volammograms of gallic acid and butyl gallate. 31

2.8 Effect of pH on second-order rate constants (Ks) of some green tea flavonoids and vitamin C with the aroxyl radical in 5.0% Triton X-100 micelles at 25.0^{o}C 34

2.9 Different molecular forms of EC (EC-H_2, EC-H-, and EC^{2-}) and ascorbic acid ((VO-H_2, VO-H-, and VO^{2-}) as the effect of pH in aqueous solution.....34

2.10 Examples of relationships between second-order rate constants (Ks) and mole fraction influenced by pH for EC and Vitamin C 37

2.11 Free radical exchanges among the mixture of tocopherols and lipid peroxyl radicals in homogeneous systems 39

2.12 Antioxidant interaction mechanisms of ascorbic acid, caffeic acid and α-tocopherol 43

2.13 Probable antioxidant mechanisms of carotenoids with tocopherol and ascorbic acid based on the protection of β-carotene by α-tocopherol.....46

2.14 Probable antioxidant mechanisms of carotenoids with tocopherol and ascorbic acid based on the protection of α-tocopherol by β-carotene.....47

2.15 Possible interactions occurring in quercetin/caffeic acid complex and quercetin/rosmarinic acid complex 54

3.1 Physical stability of liposomes (pH 3.0 and 55^{o}C) during storage as determined by particle diameter (a) and droplet charge (b).....66

3.2 Oxidative stability of uncoated (lecithin with and without EDTA) and chitosan coated (lec-chi) liposomes during storage (pH 3.0 and 55^{o}C) as determined by formation of hydroperoxides (a) and hexanal (b).....67

3.3 Oxidative stability of uncoated and chitosan coated liposomes in the presence and absence of 40 μM octadecyl rosmarinic acid ester (RA) during storage at 55^{o}C at pH 3.0. Lipid oxidation was monitored by measuring hydroperoxide (a) and hexanal (b). 69

3.4 Influence of various concentrations of octadecyl rosmarinic acid ester on oxidative stability of chitosan coated liposomes during storage at 55oC at pH 3.0. Lipid oxidation was monitored by measuring hydroperoxide (a) and hexanal (b). 70

3.5 Influence of different aliphatic side chains on rosmarinic acid esters on oxidative stability of chitosan coated liposomes during storage (pH 3.0 and 55^{o}C). Lipid oxidation was monitored by measuring hydroperoxide (a) and hexanal (b). 73

3.6 Concentration of rosmarinic acid and its esters in the aqueous phase of chitosan-coated liposomes. 74

4.1 DPPH scavenging activity of the rosmarinic acid and its alkyl esters in methanol. Data represents means (n=3) ± standard deviations.....85

4.2 Hydroperoxide (A) and hexanal (B) formation in 1% stripped soybean oil-Tween 20 emulsions at 25^{o}C in the presence of rosmarinic acid and its alkyl esters at equivalent molarities (30µM). Data represents means (n=3) ± standard deviations. 87

4.3 Hydroperoxide (A) and hexanal (B) formation in 1% stripped soybean oil-Tween 20 emulsions at 25^{o}C in the presence of rosmarinic acid and its alkyl esters at equivalent DPPH radical scavenging activity. Data represents means (n=3) ± standard deviations. 88

4.4 Hydroperoxide (A) and hexanal (B) formation in 1% stripped soybean oil-Tween 20 emulsions at 25^{o}C as a function of increasing Tween 20 concentrations (0.1, 0.5, 1.0, and 2.5% w/w). Data represents means (n=3) ± standard deviations. 91

4.5 Hydroperoxide (A) and hexanal (B) formation in 1% stripped soybean oil-Tween 20 emulsions at 25^{o}C as a function of increasing Tween 20 concentrations (0.1, 0.5, 1.0, and 2.5% w/w) in the presence of butyl rosmarinate ester (R4). Data represents means (n=3) ± standard deviations. 92

4.6 Hydroperoxide (A) and hexanal (B) formation in 1% stripped soybean oil-Tween 20 emulsions at 25^{o}C as a function of increasing Tween 20 concentrations (0.1, 0.5, 1.0, and 2.5% w/w) in the presence of dodecyl rosmarinate ester (R12). Data represents means (n=3) ± standard deviations. 94

4.7 Hydroperoxide (A) and hexanal (B) formation in 1% stripped soybean oil-Tween 20 emulsions at 25^{o}C as a function of increasing Tween 20 concentrations (0.1, 0.5, 1.0, and 2.5% w/w) in the presence of eicosyl rosmarinate ester (R20). Data represents means (n=3) ± standard deviations. 95

4.8 The effect of surfactant concentrations on the antioxidant partitioning of butyl (R4), dodecyl (R12) and eicosyl (R20) rosmarinate esters (100 µM) into aqueous phase of in oil-in-water emulsions. Data represents means (n=3) ± standard deviations. Error bars lie within data points. 96

4.9 Fluorescence emission scans of butyl rosmarinate ester (30 µM) in various solvents with different polarities.....96

4.10 Front-face fluorescence measurement (λ_{ex} 323, λ_{em} 420) of rosmarinic acid and its butyl (R4), dodecyl (R12) and eicosyl (R20) esters in washed O/W emulsions with varying Brij concentrations. Data represents means (n=3) ± standard deviations. 98

4.11 Schematic demonstration of the distribution of the rosmarinate esters in the interface region of O/W emulsions and in non-ionized surfactant micelles. In the O/W emulsions the rosmarinate esters can exist as monomers, micelles (or reverse micelles) or co-micelles. A and B show medium chain rosmarinate esters (4-12 carbons) in low and high surfactant concentration. In this case, the rosmarinate esters are mainly at the oil-water interface of the emulsion droplet. C and D show long chain rosmarinate ester (20 carbons) in low and high surfactant concentration. In this case, a higher proportion of the rosmarinate ester is in the interior and/or aggregated outside of the emulsion droplet in the absence of excess surfactant micelles.....101

5.1 Hydroperoxide (A) and hexanal (B) formation in 1% stripped soybean oil-Tween 20 emulsions at 25^{o}C in the presence of individual of rosmarinic acid and its alkyl esters and α-tocopherol (30μM). Data points and error bars represent means (n=3) ± standard deviations.....120

5.2 Hydroperoxide (A) and hexanal (B) formation in 1% stripped soybean oil-Tween 20 emulsions at 25^{o}C in the presence of combinations of rosmarinic acid and its alkyl esters with α-tocopherol (30μM+30 μM). Data points and error bars represent means (n=3) ± standard deviations.. 121

5.3 Interaction indexes of the combinations of rosmarinic acid and its esters with α-tocopherol in 1% stripped soybean oil-Tween 20 emulsions at 25^{o}C. Data were calculated from oxidation lag time obtained from hydroperoxide and hexanal formation. 123

5.4 The antioxidant partitioning of rosmarinic acid (R0), butyl- (R4), dodecyl- (R12) and eicosyl- (R20) rosmarinate esters and α-tocopherol (100 μM) into aqueous phase of in oil-in-water emulsions. Data points and error bars represent means (n=3) ± standard deviations. 124

5.5 Fluorescence quenching of α-tocopherol (λ_{em}= 325 nm) by the rosmarinic acid esters in O/W emulsions with/without influences of surfactant micelles 125

5.6 Regeneration efficiencies of the rosmarinic acid esters to reduced α-tocopheroxyl radicals in homogenous (ethanol) and heterogeneous (Tween 20) micelle solutions. Dash line indicates ratios of regeneration efficiency of Tween 20 and ethanol. Data points and error bars represent means (n=5) ± standard deviations. 130

5.7 Effects of different concentrations of antioxidants (15, 30 and 60 μM) on decompositions of α-tocopherol (A), rosmarinic acid (B) and dodecyl rosmarinate ester (C), and hexanal formation during the oxidation of O/W emulsions at 25^{o}C.....133

5.8 Effects of various concentrations (15, 30 and 60 μM) of rosmarinic acid (R0) on decompositions of rosmarinic acid and hexanal formation (A), and decompositions of α-tocopherol and tocopheroxyl quinone (TQ) formation (B) in 1% stripped soybean oil-Tween 20 emulsions at 25^{o}C in the combination with α-tocopherol (30μM). Data points and error bars represent means (n = 3) ± standard deviations.. 135

5.9 Effects of various concentrations (15, 30 and 60 μM) of dodecyl rosmarinate ester (R12) on decompositions of dodecyl rosmarinate ester and hexanal formation (A), and decompositions of α-tocopherol and tocopheroxyl quinone (TQ) formation (B) in 1% stripped soybean oil-Tween 20 emulsions at 25^{o}C in the combination with α-tocopherol (30μM). Data points and error bars represent means (n = 3) ± standard deviations.. 136

5.10 Structures of main antioxidant products observed during oxidation. A) represents reduced form of rosmarinic acid and dodecyl rosmarinate. B) represents the quinone formed on the 2-oxyphenylpropanonyl moiety of dodecyl rosmarinate (the quinone of rosmarinic acid was not detected). C) represents caffeic acid observed in the oxidation od rosmarinic acid and dodecyl rosmarinate.. 137

5.11 Accumulation (A330) of caffeic acid in R0 (A) and R12 (B), and the quinone in R12 (C) observed in the individual and the combinations with α-tocopherol at different concentrations during the oxidation in O/W emulsions.....139

CHAPTER 1

INTRODUCTION

Lipid oxidation in food and biological systems has been a concern in various fields of science because it is related to both food quality deterioration and health complications such as cardiovascular diseases and cancers. In the food industry, the use of free radical scavenging antioxidants is one of the main strategies to delay the occurrence of rancidity by inhibiting the initiation and propagation steps of lipid oxidation. Over the past decades, there have not been any major new antioxidants available to the food industry besides natural rosemary extracts. It seems likely that future synthetic antioxidants will be even less acceptable for food applications since food related organizations and consumers are concerned of possible health risks and the regulatory process for approval of synthetic antioxidants is more expensive than the antioxidant market can bear. In fact some of the already approved synthetic antioxidants might be lost from the market since butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) may pose a potential risk as carcinogens (*1*). Therefore, it has been challenging for food scientists to improve the oxidative stability of products under the limitation of existing approved antioxidants.

Due to the complexity of the lipid oxidation process, the selection of antioxidants for various applications based on their intrinsic physical (e.g. polarity) and chemical properties (e.g. free radical scavenging rate and stoichiometry of electron transfer) has proven to be inefficient for predicting antioxidant activity in real food systems (*2*). To

improve the antioxidative performance of existing antioxidants, other strategies could be applied if new knowledge was obtained on the behavior of antioxidants in different types of food matrices. In addition, antioxidants could be used more efficiently if their interactions with other antioxidants to produce synergistic, additive, and antagonistic antioxidative effects were better understood.

Ideally, free radical scavengers should be localized in the microenvironments where lipid radicals are generated for maximum effectiveness. With regard to this matter, the polar paradox hypothesis was developed in an attempt to predict the antioxidant activity of compounds based on their polarity in different lipid media (*3*). Accordingly, nonpolar antioxidants are more effective than their polar homologues in oil-in-water emulsion. This hypothesis was later utilized by Frankel et al. (1994) to explain how the physical location of free radical scavengers impacts their antioxidant activity in heterogeneous systems. Even though a number of studies seemingly confirmed the antioxidant polar paradox theory (*3*, *4*), several recent publications have shown cases where the polar paradox theory does not accurately predict antioxidant behavior. This loophole in the polar paradox theory has been commonly observed when one manipulates the polarity of antioxidants by esterification with various alkyl chain lengths. This means that the polar paradox theory does not uniformly predict antioxidant activity in foods by simply matching the solubility of an antioxidant in different mediums such as oil and water.

Without understanding interactions between antioxidants and other food components, it is difficult to accurately predict the performance of an antioxidant in a

food system. Interactions between combinations of antioxidants might result in superior antioxidant activity compared to individual antioxidants, known as synergistic effects. This beneficial phenomenon can be obtained from chemical and/or physical interactions, or even indirect interactions among multi-component antioxidants. Among various antioxidant interactions, one of the most effective mechanisms is radical transfer, or so called redox recycling that explains the regeneration of one antioxidant by another. For instance, radical transfer between α-tocopherol and ascorbic acid, which is one of the most effective synergistic interactions, has been widely reported *in vivo* and *in vitro* in both biological and food systems (*5-8*). Generally, it has been accepted that α-tocopherol is regenerated from α-tocopheroxyl radical by ascorbic acid. Moreover, the same is true with other combinations of antioxidants, for instance, carotenoids with flavonoids (*9*) and tocopherol with green tea polyphenolic antioxidants (*10-12*). These radical transfer reactions are influenced by different factors including thermodynamic properties, their inherent antioxidant activity, prooxidant properties, polarity, localization in microenvironments and partitioning and distribution in particular food systems like homogenous solutions, micelles, emulsions, and liposomal membranes. However, in certain conditions, combinations of antioxidants can result in antagonistic effects. Thus, in addition to the need to comprehend an individual antioxidant's mechanisms in a particular food system, understanding the possible mechanisms by which antioxidants cooperate together in particular systems is essential to being able to systematically design a more powerful antioxidant system, and obtain the full benefits of antioxidants in food systems.

CHAPTER 2

LITERRATURE REVIEW

2.1 Principles of lipid oxidation mechanisms

Lipid oxidaiton in biological and food systems is driven by high-energy free radical species. These unstable radicals steal electrons and abstract hydrogen from lipids causing lipid oxidation. In general, lipid oxidation mechanisms is catagorized into three main pathways: initiation, propagation, and termination.

In the initiation step, the first free radicals are formed. Potential pathways for free radicals formation are the reaction of Fe^{2+} with H_2O_2, the so called as Fenton raction, which is mainly considered in biological systems (Reaction 1), and also the Haber-Weiss reaction (Reaction 2-3) (*13*) where reducing agents convert iron to its more reactive ferrous state. However, in food systems, the reaction of Fe^{2+} and Fe^{3+} with existing lipid hydroperoxides (LOOH), a Fenton-like reaction, has been considered most important (Reaction 4-5). Several high-energy radical species such as hydroxyl radicals (OH•), lipid alkoxyl radicals (LO•), and lipid peroxyl radicals (LOO•) are generated from hydroperoxide decomposition. The fatty acid radical (L•) is formed by the abstraction of its hydrogen by these radicals (X•) (Reaction 6).

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH\bullet \quad (1)$$

$$Fe^{3+} + O_2\bullet^- \rightleftharpoons Fe^{2+} + O_2 \quad (2)$$

$2O_2\bullet^- + 2H^+ \rightleftharpoons H_2O_2 + O_2$ (3)

$Fe^{2+} + LOOH \longrightarrow Fe^{3+} + LO\bullet + OH^-$ (4)

$Fe^{3+} + LOOH \longrightarrow Fe^{2+} + LOO\bullet + H^+$ (5)

$LH + X\bullet \longrightarrow L\bullet + X\text{-}H$ (6)

Where X• representsLO•, LOO•, OH• and etc.

In the propagation step, lipid peroxyl radaicals (LOO•) and lipid hydroperoxides (LOOH) are accumulated. These chain reaction proceses originate from L•, which quickly reacts with O_2 to form LOO• (Reaction 7) and then slowly turns into lipid hydroperoxides (LOOH) by hydrogen abstration from another unsaturated fatty acid (Reaction 8). Most chain breacking antioxidants (AH) are able to inhibit the propagation process by inactivation of the longer lived lipid radicals such as LOO• and LO• (Reaction 9).

$L\bullet + O_2 \longrightarrow LOO\bullet$ (7)

$k_5 = 3 \times 10^8\ M^{-1}\ s^{-1}$

$LOO\bullet + LH \longrightarrow LOOH + L\bullet$ (8)

$k_6 = 10\ M^{-1}\ s^{-1}$

$LOO\bullet + AH \longrightarrow LOOH + A\bullet$ (9)

In the termination step, the accumulated radicals from the propagation step can be terminated by self-interactions to form non-radical species such as oxidized polar/ non polar dimers or trimers of lipids (Reaction 10-12).

$$L\bullet + L\bullet \longrightarrow L\text{-}L \quad (10)$$

$$LO\bullet + L\bullet \longrightarrow LO\text{-}L \quad (11)$$

$$LOO\bullet + L\bullet \longrightarrow LOO\text{-}L \quad (12)$$

2.2 Beyond the polar paradox theory

Dating back to the 1980's, the first insight of paradoxical behaviors of antioxidants were observed by Porter (*3*). For the first time, the word "polar paradox" was introduced into the world of lipid oxidation from the observations that polar antioxidants tend to be more active than nonpolar antioxidants in nonpolar environments like bulk oil and visa versa. The hypothesis successfully explained and predicted the activity of antioxidants in bulk oil and oil-in-water emulsions containing oils stripped of their minor components. This version of polar paradox explained this phenomenon through the different locations of polar and nonpolar antioxidants in bulk oil and water. Later in 1994, Frankel and coworkers (*4*) took the polar paradox to the next step by taking surface activity of antioxidants into consideration as illustrated in Figure 2.1. This idea has been generally accepted since suitable antioxidants should be localized into interfacial regions where many lipid oxidation reactions occur. For example, in oil-in-water emulsions, antioxidants should be concentrated at the oil/water interfacial region

instead of diluted into the aqueous phase. Originally, the oxidation site in bulk oils was assumed to be the air/oil interfacial region. However, recent research has shown that polar antioxidants do not concentrate at the air/oil interfacial region and that bulk oils contain physical structures composed of naturally occurring water and surface active compounds. Therefore, in bulk oils it is also possible that the activity of antioxidants is strongly influence by water-oil interfaces (*14*, *15*).

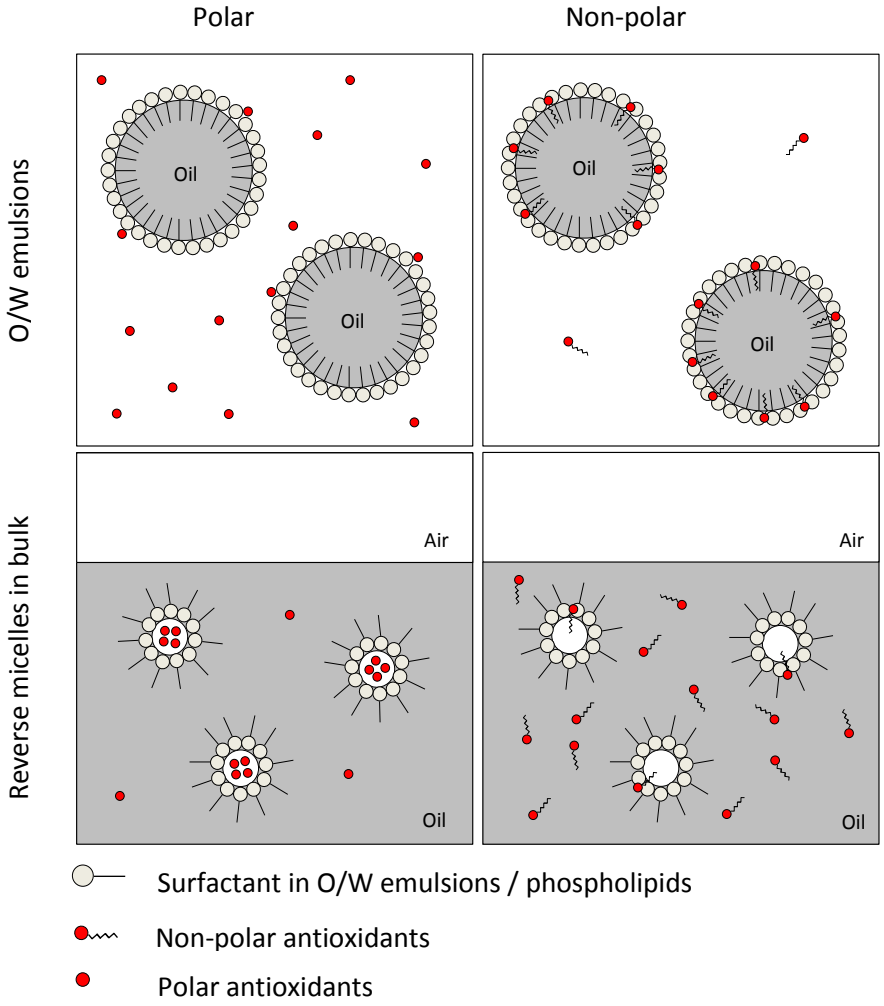


Figure 2.1 The polar paradox theory in food emulsions (Adapted from Laguerre et al., 2011 and Chen et al., 2010)

In general, the polar paradox theory has been considered as a main pillar of the principle of lipid oxidation in O/W emulsions and bulk oils. The theory could be used to explain the activity of many antioxidants in simple model systems and in some food applications. However, a loophole of the polar paradox has been observed when antioxidants of varying polarity were synthesized in order to more closely examine the polar paradox theory. These studies are unique since antioxidant polarity could be altered without interfering with the hydroxyl groups of the phenol structures. These esterified phenolic antioxidants with various alkyl chain lengths, the so called "Phenolipids" have provided new insights that challenge the polar paradox hypothesis.

A series of recent papers examined the activity of antioxidants (chlorogenic acid, rosmarinic acid, hydroxytyrosol, dehydrocaffeic acid and rutin) whose polarity was modified by esterification to alkyl chains of varying length (1-20 carbons) (*16-19*). In these studies, a nonlinear relationship of antioxidant effectiveness in oil-in-water emulsions as a function of polarity was observed presenting a challenge to the antioxidant polar paradox hypothesis. From a general perspective, as the alkyl chain length of the alkyl group was increased up to a medium chain length (typically 8-12 carbons), antioxidant activity increased in oil-in-water emulsions as would have been predicted by the polar paradox hypothesis. However, antioxidant activity then sharply declined with a further increase in the size of the alkyl chain even though this would have made the antioxidant even more nonpolar (Figure 2.2). This unexpected "cut-off" effect suggests us that the polar paradox theory should be reexamined utilizing the new knowledge gained from the studies utilizing these surface active antioxidants.

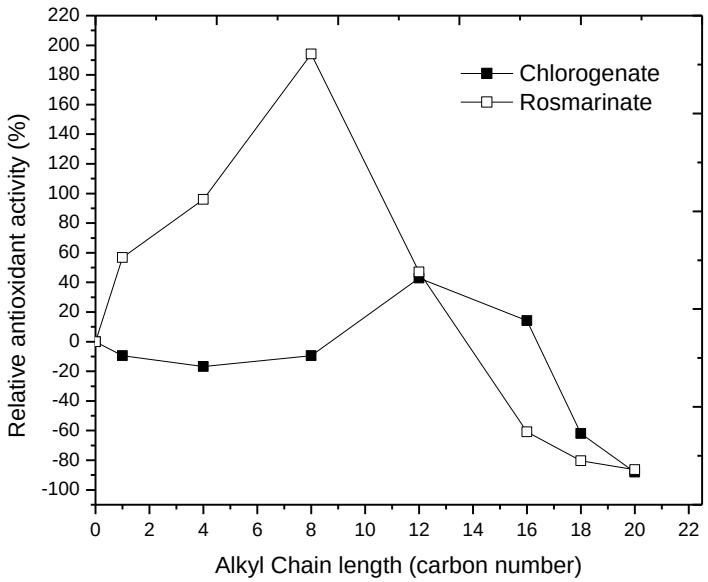


Figure 2.2 Schematic demonstration of the cut of effect of antioxidants. Relative antioxidant activity changes (%) were obtained from conjugated autoxidizable triene (CAT) method (Adapted from Laguerre et al., 2011)

One possible explanation of this cut-off effect is that the sudden decreases in antioxidant activity with longer alkyl chains was due to their increased hydrophobicity which led to their partitioning into the oil phase rather than at the emulsion droplet interface. In addition, it has been suggested that the more nonpolar antioxidants might form mixed micelles with emulsifiers used to prepare the emulsions resulting in their migration away from the emulsion droplet (*16, 17, 20*). Unfortunately, there is limited information on how the length of the alkyl chain of esterified phenolics impacts their partitioning inside emulsion droplets or in mixed micelles, and how dynamic exchange and distribution of antioxidants in each phase would result in the overall ability of antioxidants to inhibit lipid oxidation in food emulsions.

The failure of the polar paradox to universally predict antioxidant behavior suggests that other factors impact the ability of antioxidants to inhibit lipid oxidation in food systems. These other factors could include the ability of combinations of antioxidants to synergistically or antagonistically inhibit oxidation. Synergistic or antagonistic relationships can occur by both chemical and physical pathways.

2.3 Principle of synergistic antioxidant interactions

A factor that is not taken into consideration for the net activity of an antioxidant in the polar paradox hypothesis is the ability of antioxidants to synergistically interact to inhibit lipid oxidation. Lipid oxidation in foods is governed by various physical, chemical and enzymatic prooxidants (e.g. oxygen, light, radiation, metal ions, lipid hydroperoxides, lipoxygenases and other minor components) (*21-24*). Synergistic antioxidant interaction can occur via antioxidant regeneration and/or combinations of multiple antioxidants with different mechanisms (e.g. metal chelation, singlet oxygen quenching, and hydroperoxide decomposition) or by physical effects (*21*, *25*, *26*).

The first synergistic mechanism is antioxidant regeneration via the recycling of a stronger (primary) antioxidant with a weaker (secondary) antioxidant. By this principle, the primary antioxidant is spared by the secondary antioxidant Synergistic activity occurs because the primary antioxidant partition near the site where lipid oxidation is occurs thus increasing its effectiveness. For example, α-tocopherol is the primary antioxidant in synergistic interactions with ascorbic acid because α-tocopherol is in the lipid phase. Not only does the regeneration of α-tocopherol occur with ascorbic acid, but it also interacts

with phenolics, β-carotene, amino acids, peptides, and phospholipids (*27-33*). The electron transfer mechanisms among antioxidants may be influenced by many physical and chemical parameters (e.g. physical location of antioxidants, pH, and presence of prooxidants).

The second mechanism that can produce synergistic antioxidant interactions is due to control of prooxidants such as metal ions. Phenolic antioxidants can exhibit synergistic effects with metal chelating agents such as EDTA (*34, 35*), citric acid (*13*), flavonoids (*36-38*), amino acids and peptides, (*39-41*), Maillard reaction products (*42*), and phospholipids (*43-50*). The inactivation of metal ions is perhaps one of the most effective strategies to synergistically increase the activity of free radical scavenging antioxidants because metal ions can directly inactivate antioxidants and reduction of metals by antioxidants can increase the reactivity of the metals as described above in reactions.

The activity of antioxidants can also be enhanced by physical aspects. It has been postulated that phospholipids enhance the antioxidant activity of α-tocopherol in bulk oil by aiding the ability of tocopherol to partition into water-lipid interfaces where lipid oxidation is prevalent (*14, 15, 51, 52*) Therefore, providing suitable microenvironments for the partitioning of antioxidants at the site of lipid oxidation reactions can enhance its ability to scavenge free radicals.

2.4 Free radical exchange reactions between antioxidants resulting in synergistic antioxidant activity

Initial studies of synergistic antioxidant interactions were observed in biological membrane systems when ascorbic acid (vitamin C) as found to maintain the concentration of α-tocopherol (vitamin E) and synergistically increase the oxidation stability of tissue membranes. In natural tissue membranes, the amount of α-tocopherol is significantly higher than ascorbic acid. However, only α-tocopherol can act as a potent antioxidant in tissue membranes against lipid radicals because of its lipophilic properties allow it to concentrate in the membrane (*53*). Since α-tocopherol concentrates in the membrane, it has been suggested that the synergistic interaction resultsfrom α-tocopherol acting as the primary antioxidant to scavenge lipid radicals and the resulting α-tocopheroxyl radicals then reacting with ascorbic acid as the secondary antioxidants to regenerate α-tocopherol (*54*).

Packer and his collages (1979) reported the first direct observation of free radical transfers between α-tocopherol and ascorbic acid by a pulse radiolysis technique (*55*). Niki and coworkers had also confirmed the regeneration of α-tocopherol by ascorbic acid and glutathione by electron spin resonance spectroscopy (ESR) (*56*). In this study, the amount of α-tocopheroxyl radicals was determined to be 10^{-8} M in liposomes after induction of oxidation by an Fe-ADP complex. These tocopheroxyl radicals were not detected when ascorbic acid was added to the system. The rate of regeneration of tocopherols by ascorbic acid was reported to be $2x10^{5}$ M^{-1} s^{-1} at pH 7 (*57*).

The simple schematic of free radical transfer reactions among lipid peroxyl radicals and antioxidants (ascorbic acid and glutathione) in biological systems had been proposed as shown in Figure 2.3.

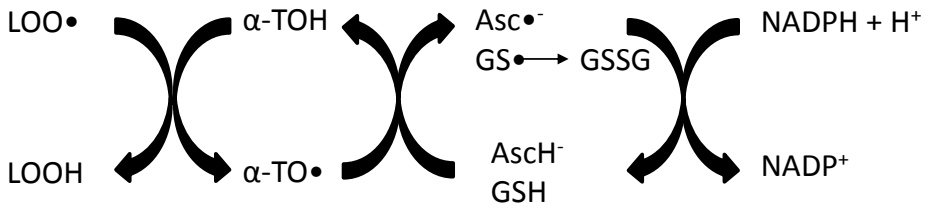


Figure 2.3 Redox recycling of α-tocopherol by water soluble antioxidants (After Packer et al. (1979) and Niki et al. (1982) (*55, 56*))

Synergistic antioxidant interactions have also been reported with ascorbic acid and other forms of tocopherols as showed in Table 2.1. The secondary rate constants of the regeneration of tocopherols by ascorbic acid showed that γ-tocopherol has the fastest rate compared to β-, and α-tocopherol respectively (*58*). Results implied the potential stronger synergistic interaction between γ-tocopherol and ascorbic acid. Unfortunately, there are no other reports on the interactions in *in vivo* and *in vitro* systems especially in food emulsions systems.

Table 2.1 The secondary rate constants of tocopherols regenerated from ascorbic acid and ascorbate monoanions in $EtOH/H_2O$ at 25°C (*58*, *59*)

Reactions	*k* at 25°C ($M^{-1}s^{-1}$)
α-Toc• + Na^+ AsH^-	2.73×10^6 (4.97×10^4)[a]
β-Toc• + Na^+ AsH^-	3.65×10^6
γ-Toc• + Na^+ AsH^-	3.81×10^6

[a] with ascorbic acid in SDS micelles

A better understanding of antioxidant interactions could be obtained by examining factors such as the direction of electron transfer of two or more antioxidants, the rate constant of the regeneration, the thermodynamics of radical reactions and structure-related bond energies by determining the one-electron reduction potential ($\Delta E^{ó}$) and OH-bond dissociation enthalpies (BDEs).

2.4.1 Prediction of antioxidant interactions

2.4.1.1 Reduction potentials and free radical interactions

To predict the hierachy of free radical electron transfer reactions, it is necessory to understand the thermodynamic properties of the reactions (*60*). Buettner (1993) had proposed the prediction of a pecking order or hierachy by using one eletron reduction potentials. Reduction potential is a valuable thermodynamic property, giving straightforward information about the oxidation/reduction ability of a compound. In general, the standard one-electron reduction potential (E°) of compounds has been measured by using flash photolysis and pulse radiolysis methods, however, reduction

potentails can also be obtained from other electrochemical methods such as cyclic voltammetry (CV) . The cyclic voltammetric study of compounds have been reported as half-peak potential ($E_{P/2}$), half wave potential ($E_{1/2}$), and peak potential (E_{Pc} or E_{Pa}). Thus, the data from the cyclic voltammetric method should not be compared directly to the one-electron reduction potential data from various literatures without knowing the actual experimental conditions for further calculations.

Besides the standard electron reduction potential (E°), one eletron reduction potential applied at pH 7.0, so called $E^{ó}$ or E_7 are commonly measured. The $E^{ó}$ value is considered as more relevent and useful for biological and biochemical approaches. Buettner (1993) pulished a valuable set of the $E^{ó}$ value of free radicals and antioxidants related to lipid oxidation. In this chapter, we have further abulated the $E^{ó}$ values of other intersting antioxidants including carotenoids and flavonoids in order to provide the useful data evalutating the direction of the reactions based on the thermodynamic property of these anitoxidants. As illustrated in Table 2.2, the $E^{ó}$ values are listed in order from high to low. The higher the reduction potential, the higher the ability to steal an electron (or hydrogen atom) from those with a lower reduction potential (*60*). For example, α-tocopherol (480 mV) and ascorbic acid (280 mV) have lower the reduction potential than polyunsaturated fatty acid (600 mV) and alkylperoxide (~1000 mV) radicals, so it is thermodynamically feasible for these antioxidants to donate an electron to fatty acid radicals.

Table 2.2 The one electron reduction potential ($E^{\acute{o}}$) of the radical form of reactive oxygen speices, phenolic antioxidants, carotenoids, and flavonoids.

Compounds	$E^{\acute{o}}$ (mV)	Compounds	$E^{\acute{o}}$ (mV)
H_2O	2310[a]	pyrogallol	575[j]
CH_3CH_3	1900[a]	Catechin	570[g]
ROH (aliphatic alkoxyl radical)	1800[a]	3,4-dihydroxyphenylalanine (DOPA)	570[g]
H_2O_2 ($HOO^{\bullet}$, H^+)	1060[a]	EC	570[i]
β-carotene in TX-100	1060[b]	Gallic acid	560[k]
Canthaxanthin in TX-100	1041[b]	Methyl gallate	560[k]
Zeaxanthin in TX-100	1031[b]	Sinapic acid	556[h]
Astaxanthin in TX-100	1030[b]	Chlorogenic acid	550[h]
β-carotene in TX-405/TX-100	1028[b]	ECG	550[i]
ROOH (alkyl peroxyl radical)	1000[a]	3,4-dihydroxylcinnamic acid	540[j]
Lycopene in TX-405/TX-100	980[a]	Theaflavin digallate	540[l]
Astaxanthin	970[c]	Caffeic acid	534[h]
Canthaxanthin	950[c]	Catechol	530[a]
H_2O_2 ($O_2^{\bullet-}$, $2H^+$)	940[a]	4-Methylcatechin	520[f]
Glutathione	920[d]	4-methylcatehol	520[k]
Zeaxanthin	850[c]	Theaflavin	510[l]
β-carotene	840[c]	Taxifolin	500[e]
Resorcinol	810[j]	α-tocopherol	500[a]
Kaempferol	750[e]	Trolox	480[a]
Hesperidin	720[f]	EGC	430[f]
Galangin	620[e]	EGCG	430[j]
3,4-dihydroxylbenzoic acid	600[g]	Myricetin	360[e]
Luteolin	600[e]	Fisetin	330[j]
Morin	600[e]	Quercetagetin	330[e]
PUFA (bis-allylic-H)	600[a]	Quercetin	330[f]
Rutin	600[g]	Ascorbic acid	282[a]
o-Coumaric acid	596[h]	Fe(II) EDTA	120[a]
Ferulic acid	595[h]	Fe(II) (aqueous)	110[a]
b-coumaric acid	590[h]	Fe(II) citrate	100[a]
p-Coumaric acid	590[h]	Ubiquinone	-36[a]
Uric acid	590[i]	Dehydroascorbic acid	-174[a]

[a] Buettner, 1993 (*60*); [b] Burke, *et al*., 2001 ; [c] Han, *et al*., 2010 (*9*); [d] Sharma and Buettner, 1993 (*61*); [e] Jovanovic *et al*., 2000 (*62*); [f] Jovanovic, *et al*., 1996 (*63*); [g] Jovanovic, *et al*. 1994 (*64*), [h] Foley, *et al*., 1999 (*65*); [i] Luczaj et al., 2005 (*66*); [j] Rice-Evans *et al*., 2003 (*67*); [k] Jovanovic, *et al*., 1995 (*68*); [l] Jovanovic, *et al*., 1997 (*69*)

Thermodynamics of lipid oxidation mechanisms could be explained by using one electron reduction potentials (*60*). Possitive values ($\Delta E^{\acute{o}} > 0$) indicate thermodynamically favorable reactions. Based on this principle, ascorbic acid and some antioxidants such as epigallocatechin gallate (EGCG from green tea) and quercetin can thermodynamically regenerated α-tocopherol (Reaction 13-15). Synergistic interaction of these antioxidants by redox recycling have been reported in *in vivo* and *in vitro* model systems (*7, 10, 33, 66, 70-73*)

$$TO\bullet + AscH^{-} \longrightarrow TOH + Asc^{-}\bullet \quad (13)$$

$\Delta E^{\acute{o}} \approx + 200$ mV, $k_{10} = 2 \times 10^{5}\ M^{-1}\ s^{-1}$

$$TO\bullet + EGCG\text{-}H \longrightarrow TOH + EGCG\bullet \quad (14)$$

$\Delta E^{\acute{o}} \approx + 70$ mV

$$TO\bullet + Quercetin\text{-}H \longrightarrow TOH + Quercetin\bullet \quad (15)$$

$\Delta E^{\acute{o}} \approx + 170$ mV

However, the use of the reduciton potential to predict antioxidant interactions does have limitation. Reduction potential relies on electron transfer mechanism, however, other mechanims may be prefered, for example an addition reaction of $O_2\cdot^{-}$ to the double bonds of polyunsaturated fatty acid (*60*). In addition, the thermodynamically prefered reaction might not be kinetically feasible due to the influenced of physical barreirs of heterogeneous systems such as liposomes, micelles and emulsions on reaction rates. For example. it might be difficult for a hydrophobic antioxidant to interact with a hydrophilic

antioxidant due to their partitioning into different solvents. Some thermodynamically unfeasible reactions can also occur if the conditions allow for the disappearance of the reaction product (*60, 70, 73*). For instance, the thermodynamically unfeasible reaction of α-tocopherol (500 mV) regenerated by caffeic acid (CA); (534 mV), gallic aicd (GA); (560 mV), and epicatechin (EC); (570 mV) (reaction 13) have been reported indicating that some radical exchange mechanisms are a reversible process. The the efficiency of the regeneration of α-tocopherol from these antioxidants (mole of α-tocoperoxyl radical reduced per mole of antioxidant) are 1.4×10^{-4}, 4.3×10^{-4}, and 4.5×10^{-4}, respectively (Pazos, 2007). These values are relatively low compared to the regenerating efficiency by ascorbyl palmitate of 0.93 which is a thermodynamically favorable reactions (*73*).

In addition, it is important to keep in mind that we compare the $E^{\circ\prime}$ value at the same pH condition. In fact, reduction potentials are highly dependent on pH, solvent type and ionic strength. This means that the location of antioxidants in foods might impact their reduction potentail depnding on if they are located in the aqueous phase or in hydrophobic media such as emulsions, micelles and liposomes. For instance, α-tocopherol known as a lipid soluble antioxidant generally locate itself below the interface of liposomal membranes. In that particular location, it has been suggested that the reduction potential of α-tocopherol under the apolar environment of the membrane might be higher, which is speculated to be the driving force for the regenerating of α-tocopherol by by antioxiadnts that have thermodynamic unfeasible reactions (*74*). The influence of microenvironments on the reduction potential of antioxidant should be further investigated.

2.4.1.2 Bond dissociation enthalpy (BDEs)

Bond dissociation enthalpy, also called bond dissociation energy (BDE), is the enthalpy indicating the amount of energy required to cause hemolytic bond cleavage (*75*). The BDE is considered as an important thermodynamic quantity that determines the stability of free radicals (*76, 77*). Not only have the BDEs of antioxidants been used for the estimation of the antioxidant activity in order to measure the ability of a compound to donate a hydrogen atom to various lipid radical species thus inhibiting lipid oxidation, but it has been also used for determination of the potential of hydrogen transfer from one antioxidant to another antioxidant. As the driving force for hydrogen transfer, the minimum O-H bond dissociation enthalpy (OH-BDE_{min}) of the phenolic antioxidant is usually correlated to the rate constant of the ability of an antioxidant to inhibit chain propagation (*78-82*), as well as the efficiency and the rate of regeneration of antioxidants (*83, 84*).

In terms of regeneration or interaction among antioxidants, the BDE values are also provide useful thermodynamic information for predicting the hierarchy of the reactions, the rate constant and the efficiency of regeneration. Numerous experimental (*75, 85*) and theoretical studies (*80, 84*) have provided information on the BDEs of antioxidants. A number of theoretical BDE values of antioxidant have been reported, however, the experimental BDE values are considered as more reliable information. Theoretical BDE values have suffered from variations of calculating methods with different levels of complexity. For instance, AM1 (Austin model 1) and PM3 (parametric method number 3) methods are semi-empirical methods; while DFT (density functional

theory) methods are based on quantum mechanical theories for calculation. In general, DFT methods are popular, inexpensive and accurate, but time-consuming. In addition, the obtained BDEs for most DFT methods are usually underestimated and vary from system to system (*77*).

Due to the problems associated with theoretical BDE values, this section focuses on the experimental O-H BDE values (Table 2.2). A relationship between chemical structure, BDEs and the ability of a compound to inhibit lipid oxidation and regenerate antioxidant radicals has been proposed (*81*, *83*). For instance, a strong relationship exists between the antioxidant activity of tocopherols, BDEs and the number of methyl group substituted on the chromanol ring has been observed. α-Tocopherol is full methylated with 3 methyl groups, whereas β-, γ-, and δ-tocopherols contains 2, 2 and 1 methyl groups, respectively. Generally, α-tocopherol is well recognized as the most reactive form among tocopherols followed by β-, γ-, and δ-tocopherols with their estimated BDEs in $PhCH{=}CH_2$ solvent of 79.1, 80.2, 80.1, and 82.2 kcal/mol, respectively(*85*).

Table 2.3 Bond dissociation enthalpies (BDEs) of antioxidants and related compounds

Compounds	BDE (kcal/mol)	Compounds	BDE (kcal/mol)
Ascorbic acid[a,b]	69.00***, 83.00***	Luteolin[g]	81.91
Dehydroascorbic acid[b]	71.00***	Quercetin[g]	81.98
β-carotene[c]	73.85**	Gallic acid[g, i]	83.03*, 82.00
α-tocopherol[d, h, e]	77.10, 78.87, 79.30	Mesityl alcohol[e]	82.10
BHA[d]	77.20	Dihydroquercetin[g]	82.12
3,5-di-tert-butylcatehol[f]	79.30	Rutin[g]	82.15*
6,7-Dihydroxyflavone[g]	79.42*	Octyl gallic acid[f]	82.50
7,8-Dihydroxylflavone[g]	79.59*	Propyl gallic acid[f]	82.60
Propyl gallic acid[h]	79.97*	Hesperidin[g]	82.65
BHT[d]	80.00	Fisetin[g]	82.77
β-Glugalline[g]	80.07	Epicatechol[g]	82.84*, 83.13
γ-tocopherol[g,e]	80.04, 80.10	ρ-hydroquinone[h]	84.13
β-tocopherol[e]	80.20	tert-Butyl hydroperoxide[e]	84.20
Ubiquinol-10[e,g]	80.50, 82.53	Hesperidin[g]	84.56
3,5-di-tert-butylcatehol[f]	80.70	Chrysin[g]	85.35
Epigallocatechol[g]	80.71	o-Polyphenol (1,1-biphenyl-2-ol)[h]	86.50
2,5-di-tert-pentylhydroquinone[f]	80.80	Galangin[g]	86.78
Tannic acid[g]	80.81	Morin[g]	86.90
Epicatecholgallate (pH7, 9)[g]	81.64, 80.88	Hydroperoxides[e]	87.49
Pyrocatechol[h]	81.24	ρ-Polyphenol (1,1-biphenyl-4-ol)[h]	88.12
EC[i,g]	81.20, 82.03	Resorcinol[h]	88.19
Caffeic acid[g]	81.21*	Phenol[e]	90.40
Tocol[g]	81.29	Methanol[e]	105.20
Propyl gallate[g]	81.45	Ethanol[e]	105.40
Myricetin[g]	81.48*	H_2O[e]	118.00
δ-tocopherol[g, i]	81.62, 82.20		
Catechol[i,g]	81.80, 82.82*		

* indicated the BDEs observed in micelles, ** indicated the estimated C-H BDE, and *** indicated the BDEs from the DFT methods. Besides the techniques, the different BDEs of the same compounds are depended on the solvent used and pH of the systems.

[a] Wright, 2002(*86*); [b] Wang *et al*., 2009(*87*); [c] Mortensen *et al*., 1998(*88*); [d] Foti, 2007(*89*); [e] Luo, 2007(*90*); [f] Lucarini, *et al*., 2002(*91*); [g] Denisova *et at*., 2008(*85*); [h] Denisov *et al*., 2000(*92*); [i] Pazos, *et al*., 2007(*83*)

2.4.1.3 Impact of physical structures on antioxidant interaction

In the past decades, studies on antioxidant interactions in heterogeneous systems were randomly tested by various water and lipid soluble antioxidants without evaluating how physical location would impact antioxidant interactions. This could be why the results of antioxidant combination studies are inconsistent. In micelles, synergistic effects were observed in the mixtures of rosmarinic acid, quercetin and caffeic acid, however, antagonistic effects were observed in mixtures of α-tocopherol and caffeic acid, α-tocopherol and rosmarinic acid, catechin and caffeic acid, and caffeic acid and quercetin (*93*). Water soluble antioxidants such as chlorogenic acid and quercetin exhibited synergistic effects with α-tocopherol at 44.8% and 12% synergy in microemulsions containing methyl linoleate/styrene, Tween 20, n-butanol.(*94*). Mixtures of α-tocopherol and ascorbic acid were more active in bulk oils, while mixtures of α-tocopherol and ascorbyl palmitate were more active in O/W emulsions (*4*). The combination of δ-tocopherol, ascorbyl palmitate, and lecithin exhibited the best synergistic effect in oxidative stability of bulk oils (*95*).

These inconsistencies could be due to the fact that besides the role of thermodynamic properties of antioxidants influencing the oxidation hierarchy and possibility of the electron transfer reaction in homogeneous systems, different physical locations of individual antioxidant may play an important role in chemical kinetics resulting in synergistic, additive and antagonistic interactions. Several models have been developed in order to study antioxidant interactions. They can be classified into two main systems including homogenous (solvents) and heterogeneous (micelles, emulsions and

liposomes) systems. Interestingly, even though the same antioxidants were used, the results are often inconsistent between homogenous and heterogeneous systems. Thermodynamic feasible reactions of antioxidant regenerations in homogeneous solutions maybe not kinetically feasible in heterogeneous systems if antioxidants cannot pass through physical barriers. Thus, in heterogeneous systems, a non-surface active antioxidant could become a weaker antioxidant while a surface active antioxidant becomes stronger antioxidant. From this principle, it can be speculated that the degree of synergistic antioxidant interactions observed in heterogeneous systems seem to be higher compared to in homogeneous systems. As illustrated in Figure 2.4, a thermodynamic feasible reaction of antioxidant interactions may be impacted by physical locations of antioxidants and influence of surfactant types which result a variety of antioxidant interactions. Future research on antioxidant interactions should consider these standard models of antioxidant interactions along with electrochemical properties of antioxidants to better understand mechanisms of synergistic, additive, and antagonistic antioxidant interactions.

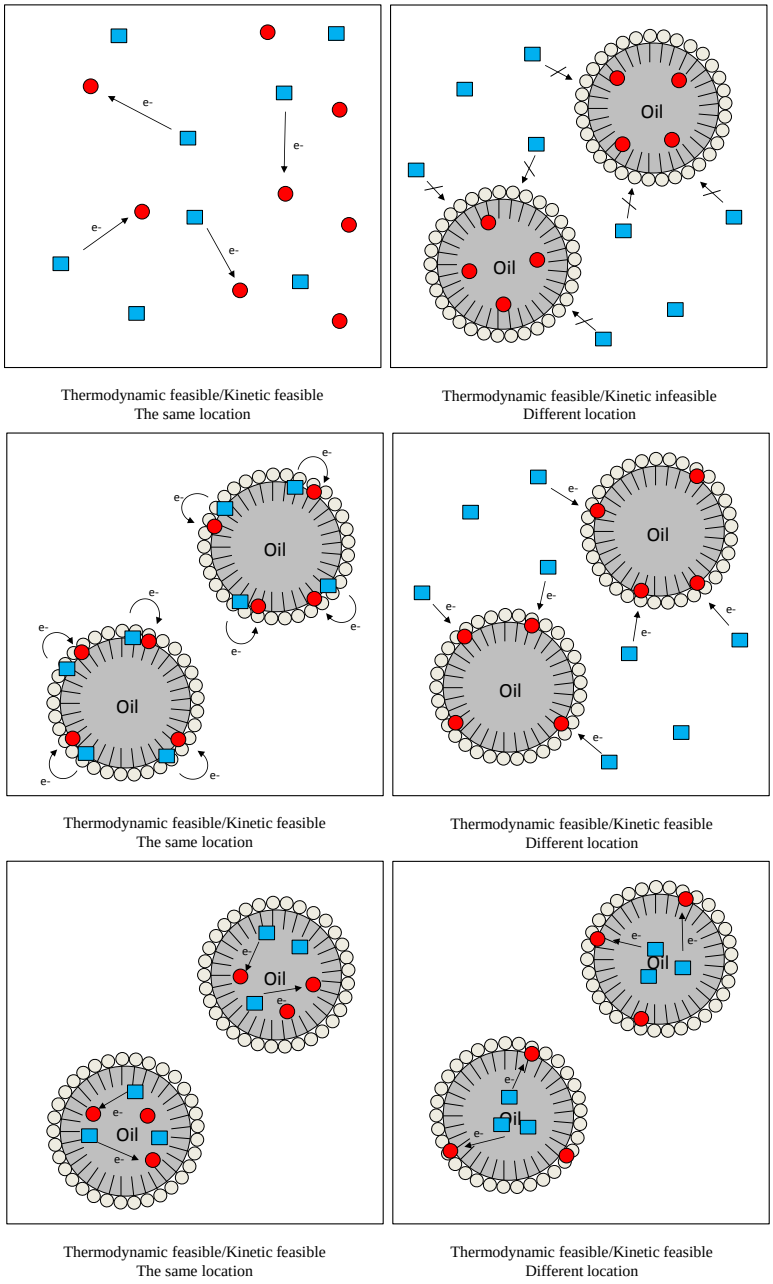


Figure 2.4 Schematic demonstrations of antioxidant interactions in homogeneous and heterogeneous systems impacted by physical locations

A good example of antioxidant interactions in heterogeneous systems is the regeneration of α-tocopherol by ascorbic acid in liposomes where ascorbic acid (water soluble) is located in aqueous phase, and α-tocopherol (lipid soluble) is incorporated into liposomal membranes. Since lipid radicals are being generated in the liposomal membrane, only α-tocopherol most effectively reacts with radicals formed inside the membranes. Since, ascorbic acid is unable to penetrate thought the membrane to inhibit the lipid oxidation. As illustrated in Figure 2.5, dynamic antioxidant actions of α-tocopherol and its regeneration by ascorbic acid in liposomal membranes were described by Fukazawa et al (*96, 97*). Dynamics locations of α-tocopherol in liposomes are near the surface of the membranes. Only small amounts of its OH-groups are actually exposed to the surface according to the study with nitroxide spin probe at 5, 7, 12, and 16 NS positions (*97*). It was suggested that interactions between OH-group of α-tocopherol and the ester carbonyl group of phosphatidylcholine inhibit the migration of α-tocopherol to the surface of the membrane (*98, 99*). Oxidation intermediates of tocopherols (e.g. tocopheroxyl radical and tocopheron cation) are more hydrophilic and interact less with the carbonyl group of phosphatidylcholine allowing tocopherols move further out into aqueous phase of the membrane-water interface (*99*). Since ascorbic acid cannot penetrate through neutral and negatively charged liposomal membranes (*96*), this migration of α-tocopherol to the interface allows ascorbic acid to regenerate α-tocopherol.

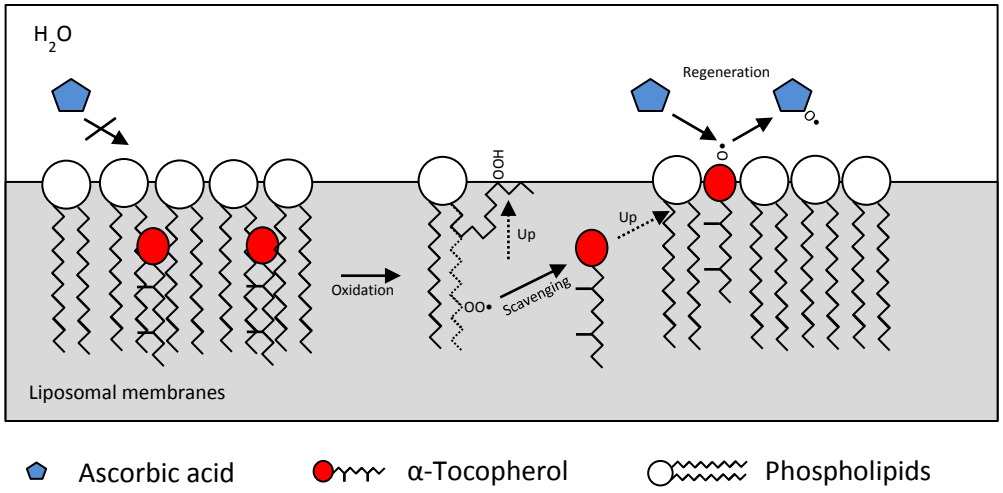


Figure 2.5 Dynamic localization of α-tocopherol and peroxyl radicals which allows for regeneration of α-tocopherol by ascorbic acid in liposomal membranes (Adapted from Fukuzawa et al. (1993) (*96*).

With the exception of ascorbate and α-tocopherol, there is very limited information on interactions between antioxidants at water-lipid interfaces of micelles, oil-in-water, and water-in-oil emulsions. This is partially because antioxidant partitioning food emulsions are far more complex than α-tocopherol and ascorbate in liposomes because antioxidants often partition in three different phases (e.g. lipid, oil-water interface, and aqueous phases). For example, a kinetic model was developed to determine that in both tributyrin- and octane-in-water emulsions, α-tocopherol partitioned in the interfacial, (70-90%), oil (7-17%) and aqueous (1-4%) regions (*100-102*).. Partitioning of Trolox (a water soluble derivative of α-tocopherol) in water phase of corn oil emulsified with Tween 20 at pH 6-7 is around 93-98% (*103*). An addition challenge in studying

interactions between antioxidants in food systems is that the antioxidants can interact with other components. For example, antioxidants can self-interact to form clusters (*25*), interact another antioxidant (free radical transfer), form hydrogen bonding with polar head group of surfactants (*104, 105*), and reduce metal ions exhibiting prooxidant effect (*23, 106, 107*). These complex interactions may play vital roles on synergistic, additive, and antagonistic interactions in food emulsions.

The potential for the partitioning of flavonoids into and near the membranes and micelles have also been suggested to increase their ability to interact with α-tocopherol in micelles compared to the more polar ascorbic acid (*108, 109*) In addition, the presence of a water-lipid interface seems to increase the efficiencies of α-tocopherol regeneration by flavonoids since higher regenerations rates are seen in protic solutions (SDS micelles in phosphate buffer solutions at pH 6.8) than in aprotic non polar solution (Hexane) (Pazos and coworkers as showed in Table 2.4).

Table 2.4 Efficiency of natural phenolic compounds regenerating α-tocopherol in homogenous and heterogeneous systems (*73, 83, 110*)

Phenolic compounds	Efficiencies (mol of tocopheroxyl radical reduced/mol of compounds)	Systems
Caffeic acid	1.4×10^{-4}	Hexane
	1.83×10^{-3}	SDS micelles (pH 6.8)
Propyl gallate	3.7×10^{-4}	Hexane
	2.17×10^{-2}	SDS micelles (pH 6.8)
EGCG	0.066	Hexane
	0.73	SDS micelles (pH 6.8)
Gallic acid	4.3×10^{-4}	Hexane
EC	4.5×10^{-4}	Hexane
Ascorbyl palmitate	0.93	Hexane
Ascorbic acid	1.22	SDS micelles (pH 6.8)

Interestingly, although some flavonoids exhibit lower efficiency of regeneration because their reduction potentials are higher than α-tocopherol, a potentially thermodynamically unfavorable process, regeneration can still be observed under certain conditions. Direct observations of α-tocopherol regeneration by relatively higher reduction potential antioxidants have been determined in hexane and SDS micelles by electron spin resonance spectroscopy (ESR). The synergistic effect of the combinations of caffeic acid and endogenous α-tocopherol in fish muscle model was reported (*73*). Amorati et al. and Pazos et al. (*70, 83*) suggested a process where a oxidation intermediate (semiquinone radical) of flavonoids was reduced by α-tocopheroxyl radical thus pulling forward the overall regeneration of α-tocopherol and give ortho-quinone phenolic derivative as shown in Figure 2.6.

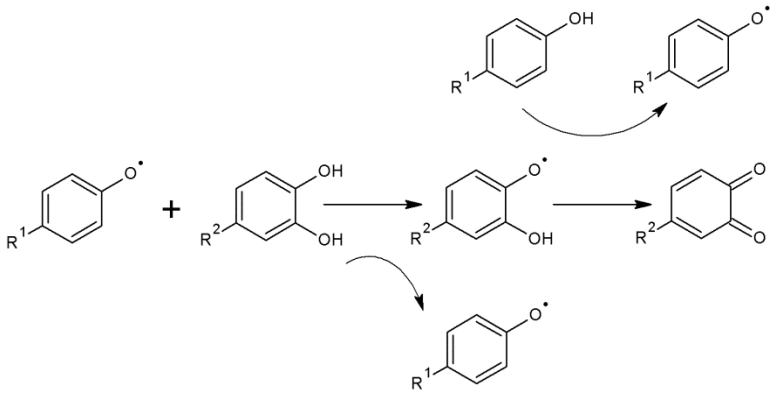


Figure 2.6 The regeneration of α-tocopherol (R^1) from α-tocopheroxyl radical by phenolic compounds (R^2) (Adapted from Pazos et al 2007. (*83*))

2.4.1.4 Effects of pH and microenvironment solvents on reduction potentials of antioxidants

In general, antioxidant properties in food are influenced by various parameters including the pH that can impact on the solubility of metal ions and antioxidants, and interactions with other food components. Therefore, it is not surprising that the impact of pH of lipid and antioxidant chemistry is not straight forward (*103, 111, 112*). (*113, 114*). (*112, 115*).

Thermodynamics of electron transfer reactions of antioxidants to regenerate other antioxidants can be explained on the basis of their electrochemical characters. Reduction potentials of antioxidants are strongly influenced by solvent properties such as non-polar, polar protic, and polar aprotic solvents, and pH values). In O/W emulsions, antioxidants are distributed into 3 different locations such as water (protic polar), the oil-water

interface, and lipids (non-polar) phases. Thus, actual antioxidant activity of the same antioxidant in these different environments would be not identical due to solvation effects. Electrochemical properties of a compound are different in liposomes and microemulsions (*116*). Laranjonha et al. speculated that the thermodynamically infeasible reactions of the regeneration of α-tocopherol by higher reduction potentials antioxidants such as caffeic acid could actually occur due to solvation effects altering the reduction potential of the antioxidants. For example, lipophilic antioxidants such as α-tocopherol are localized into non-polar environment of the membranes and emulsion droplets and thus may have higher reduction potential then the water soluble α-tocopherol homologue Trolox in water phases (*74*).

Direct observation of the effect of pH on electrochemistry of gallic acid and its esters was reported in 0.4 M Britton Robinson buffer with 0.3 M KCl by using differential pulse voltammetry (*117*). Electrochemical properties of gallic acid and its esters are pH dependent as illustrated in Figure 2.7. Results showed that the potential peak and peak current decreased with increasing the pH. At pH 2, gallic acid exhibited the highest electron-donating ability indicated by peak current, however, showed the highest potential peak.

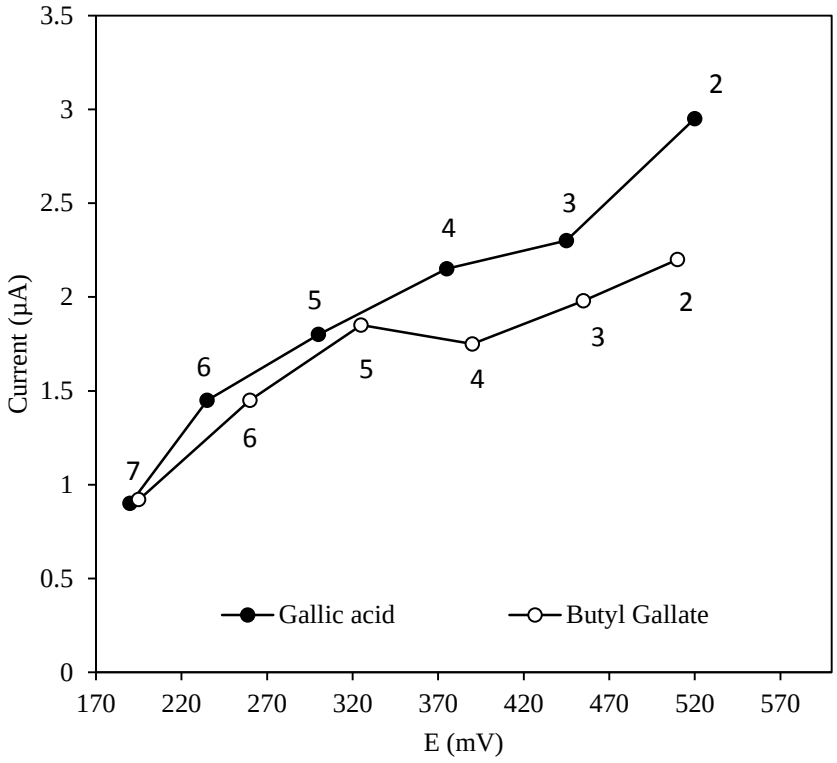


Figure 2.7 The pH-dependent (pH 2-7) differential pulse volammograms of gallic acid and butyl gallate. (Adapted from Gunckel et al. (1998) (*117*))

2.4.1.5 Effects of pH on regenerations of antioxidants

To estimate H-atom donating property of antioxidants including radical scavenging property and regeneration of antioxidants via electron transfer, understanding three radical scavenging mechanisms have been considered. Three main mechanisms have been proposed; 1) one step H-atom transfer (HAT) (Reaction 40), 2) stepwise electron-transfer-proton-transfer (ET-PT) (Reaction 41), and sequential proton-loss-electron-transfer (SPLET) (*118*) (Reaction 42) depending on the natural of radical

affinity toward electron or H-atom, and polarity of medium (Zhang and Ji 2006). HAT is favored in non-polar media because it does not involve charge separation while ET-PT and SPLET are preferred in polar media (*119*). However, it was suggested that HAT and SPLET are the two main mechanisms playing important roles in polar media such as methanol (*119, 120*).

$$RXH + RO\bullet \longrightarrow RX\bullet + ROH \quad (40)$$

$$RXH + RO\bullet \longrightarrow RXH\bullet^{+} + RO^{-} \qquad RX\bullet + ROH \quad (41)$$

$$RXH \longrightarrow RX^{-} + H^{+};$$

$$RX^{-} + RO\bullet \longrightarrow RX\bullet + RO^{-};$$

$$RO^{-} + H^{+} \longrightarrow ROH \quad (42)$$

Where, RXH and ROH represent an antioxidant and a free radical species. RX• and RO• are their radical forms. RX^{-} and RO^{-} are their dissociated forms.

Under biological and food relevant conditions, locations of antioxidants are varied from non-polar environments inside membranes, oil emulsion droplets, and micelles, and polar protic solutions such as water and alcohol. Thus, partial ionization of antioxidants in ionizing solvents influenced by different pH conditions would prefer SPLET process rather than HAT. It was proposed that experimental data of DPPH scavenging activity performed in alcohol solvent may provide an overestimation results. Rate constants of dissociated forms of phenolic antioxidants with DPPH• are very fast due to SPLET

process (*118*). Litwiniwnko and Ingold (*121*) reported that curcumin exhibited a rapid SPLET process in ionizing solvents (methanol and ethanol), but showed a slow HAT process in nonionizing solvents because non-dissociated form of curcumin. Addition of acetic acid decreased reaction rates due to elimination of SPELT leaving only HAT pathways (*120*).

Mukai and coworkers reported a series of kinetic studies on effects of pH on rate constants of the reaction of flavonoids and vitamin C with the ArO• radical and the 5,7-diPrToc• radical in solutions (*12, 122*). pH-dependent second-rate constants ((K_s) of EC, ECG, EGC, EGCG, MG, and vitamin C with the aroxyl radical in 5.0% Triton X-100 micelles at 25.0^{o}C were reported (Figure 2.8). Results showed that the rate constants increase with increasing pH. The rate constant of flavonoids exhibited much higher than that of vitamin C at pH 8-11. Under alkaline condition, SPLET process is favored. Examples of ionizations of dibasic epicatechin (EC) and ascorbic acid are demonstrated in Figure 2.9.

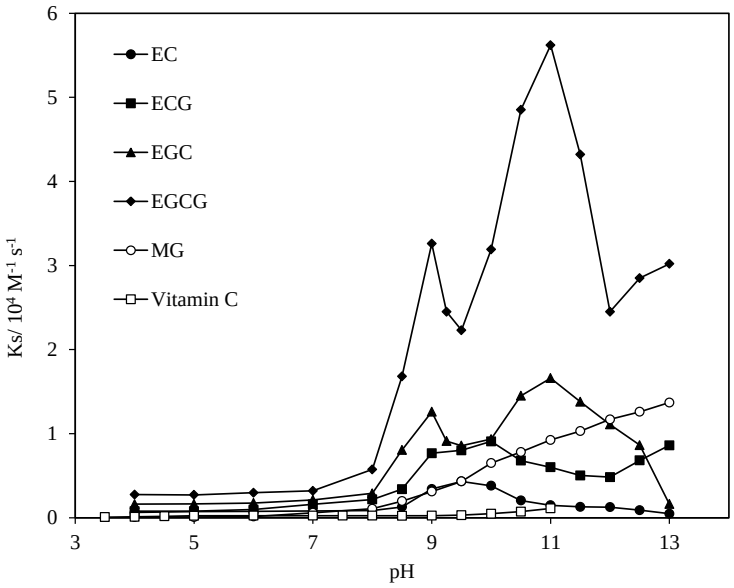


Figure 2.8 Effect of pH on second-order rate constants (K_s) of some green tea flavonoids and vitamin C with the aroxyl radical in 5.0% Triton X-100 micelles at 25.0^{o}C (Adapted fromMitani et al., 2008 (*122*))

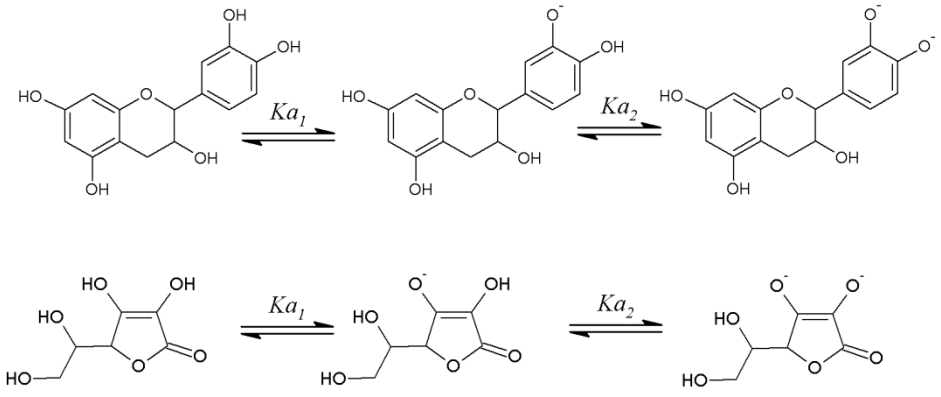


Figure 2.9 Different molecular forms of EC (EC-H_2, EC-H^-, and EC^{2-}) and ascorbic acid ((VO-H_2, VO-H^-, and VO^{2-}) as the effect of pH in aqueous solution (Adapted from Mitani et al., 2008 (*122*))

Relationships between mole fractions of various forms of epicatechin and pH is demonstrated according to the method originally described by Mukai et al., 1991 (*123*). The equilibrium of inonization of a dibasic antioxidant is expressed in equation 1. Three different forms of the antioxidant are undissociated form ($AO\text{-}H_2$) , monoaion ($AO\text{-}H^-$), and dianion (AO^{2-}). The analytical concentraction is sumation of concentrations of all forms (Equation 2).

$$AO\text{-}H_2 \underset{}{\overset{K_{a1}}{\rightleftharpoons}} AO\text{-}H^- \overset{K_{a2}}{\rightleftharpoons} AO^{2-} \qquad \text{(Eq.1)}$$

Where K_{a1} and K_{a2} are inonization constants.

$$C_a = [AO\text{-}H_2] + [AO\text{-}H^-] + [AO^{2-}] \qquad \text{(Eq.2)}$$

From equation 1, concentraction of each form can be expressed as

$$[AO\text{-}H^-] = \frac{[AO\text{-}H_2]\, K_{a1}}{[H_3O^+]} \qquad \text{(Eq.3)}$$

$$[AO^{2-}] = \frac{[AOH^-]\, K_{a2}}{[H_3O^+]} = \frac{[AO\text{-}H_2]\, K_{a1}K_{a2}}{[H_3O^+]^2} \qquad \text{(Eq.4)}$$

Mole fractions can be expressed by subtitutions of equation 3 and 4 into equation 2, yields equation 5, 6, and 7. Examples of the plots of mole fractions and second rate constants as a function of pH in the dibasic epicatechin and vitamin C are showed in Figure 2.10.

$$f = \frac{[AO\text{-}H_2]}{C_a} = \frac{[H_3O^+]^2}{[H_3O^+]^2 + [H_3O^+]K_{a1} + K_{a1}K_{a2}} \quad \text{(Eq.5)}$$

$$f = \frac{[AO\text{-}H^-]}{C_a} = \frac{[H_3O^+]^2 K_{a1}}{[H_3O^+]^2 + [H_3O^+]K_{a1} + K_{a1}K_{a2}} \quad \text{(Eq.6)}$$

$$f = \frac{[AO^{2-}]}{C_a} = \frac{K_{a1}K_{a2}}{[H_3O^+]^2 + [H_3O^+]K_{a1} + K_{a1}K_{a2}} \quad \text{(Eq.7)}$$

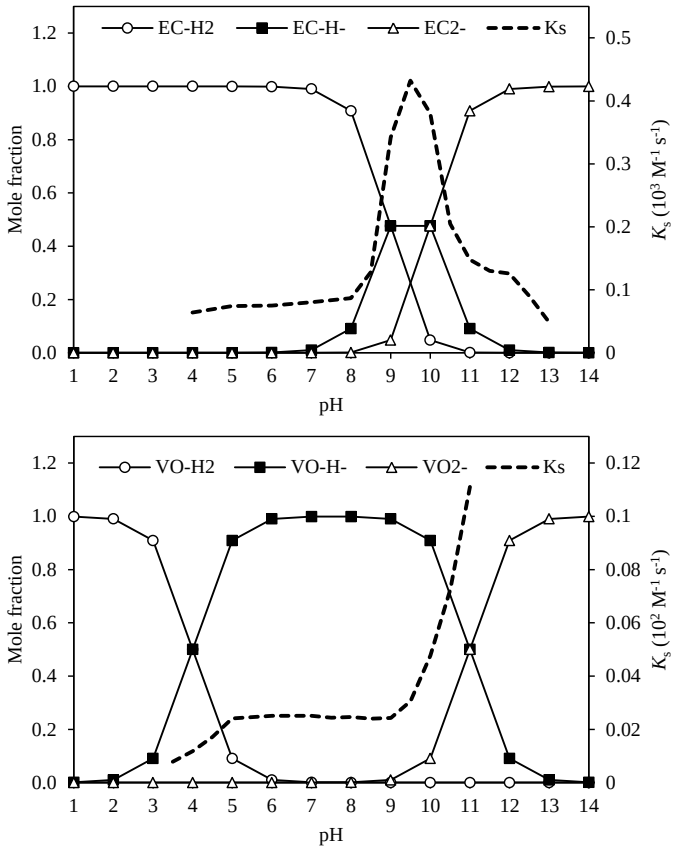


Figure 2.10. Examples of relationships between second-order rate constants (K_s) and mole fraction influenced by pH for EC and Vitamin C (Adapted from Mitani et al., 2008 (*122*))

As illustraction in Figure 2.10, the second rate constants srtongly depends on particular forms of deprotonated antioxidants. For example, monoanion of EC exhibits higher the second rate constant than dianoins and undissociated form, however, dianion of vitamin C is more reactive than monaion and undissociated form. Understanding structural relationships of polyphenolic compounds and their pH-dependent electron

donating abilities is important information for designing a suitable antioxidant combinations and synergistic mechanisms besides radical transfer to exert synergistic effects under particular pH. These relationships suggest that applications of synergistic antioxidant activity through radical transfer mechanisms may be limited under low pH conditions such as low-acid foods and breverages.

2.4.2 Combinations of free radical scavenging antioxidants

2.4.2.1 Synergistic antioxidant interactions between tocopherol homologs

Tocopherols (a group of compounds with varying vitamin E activity) are commonly found in 4 different forms (α-, β-, γ-, and δ-homologues). *In vivo*, the relative antioxidant activity of tocopherols is considered in the order of α > β > γ > δ., however, the reverse antioxidant activity of tocopherols (δ > γ > β > α) could be found in homogenous systems such as bulk oil. The reverse order of the antioxidant activity of tocopherols may be explained by the fact that α-tocopherol is highly reactive and could participate in other side reactions more than β-, γ-, and δ-tocopherols. Examples of these side reactions include thermal degradation, light decomposition, interactions with trace metals and oxygen (*25*). The most reactive form of tocopherols to be oxidized by air is α-tocopherol (*124*).

The activity of combinations of tocopherols has provided keen interest since interactions between tocopherols might improve antioxidant activity. α-Tocopherol has the lowest reduction potential followed by β-, γ, and δ-tocopherols (*125*).Thus, the

oxidative hierarchy of tocopherols should begin from α- to δ-tocopherols. Free radical exchanges between tocopherols were reported in homogenous solutions in an ESR study. According to the rate of consumption of tocopherols determined by HPLC, α-tocopherol was consumed rapidly before the other tocopherols. Then, after the depletion of α-tocopherol, β- then, γ-, and then δ-tocopherols were consumed, respectively (*126, 127*). The reactions among β-, γ-, and δ-tocopheroxyl radicals with α-tocopherol could create α-tocopheroxyl radicals and β-, γ-, and δ-tocopherols.

$$\beta\text{-TO}\bullet + \alpha\text{-TOH} \longrightarrow \beta\text{-TOH} + \alpha\text{-TO}\bullet \quad (16)$$

$$\gamma\text{-TO}\bullet + \alpha\text{-TOH} \longrightarrow \gamma\text{-TOH} + \alpha\text{-TO}\bullet \quad (17)$$

$$\delta\text{-TO}\bullet + \alpha\text{-TOH} \longrightarrow \delta\text{-TOH} + \alpha\text{-TO}\bullet \quad (18)$$

In homogenous solution, the possible free radical exchange mechanisms of the mixed tocopherol against lipid peroxyl radicals are showed in Figure 2.11.

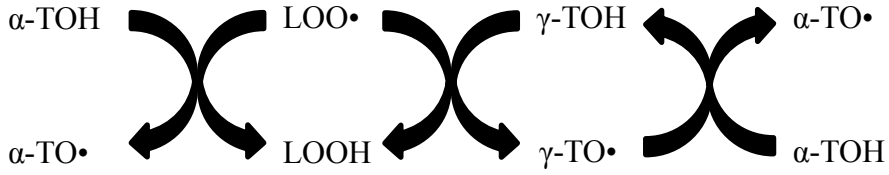


Figure 2.11 Free radical exchanges among the mixture of tocopherols and lipid peroxyl radicals in homogeneous systems (After Niki et al. (1986) (*126*))

Even though free radical exchange processes occurs among tocopherols, the overall antioxidant activity of the mixtures does not always produce strong synergistic

effects in sunflower oils (*128*). Mixed tocopherols (α-, γ- and δ-forms) also exhibited no better protection in lard oxidation than individual tocopherols.(*129*). These results suggest that free radical transfer interactions among antioxidants in the same location (homogenous system) may not exert synergistic antioxidant activity.

2.4.2.2 Antioxidant interactions of flavonoids

The interaction between α-tocopherol and ascorbic acid is the perfect example of free radical exchanges that leads to synergistic antioxidant activity. This system utilizes a weaker antioxidant (ascorbic acid) to regenerate a stronger antioxidant (α-tocopherol) that is more effective because it's hydrophobic and surface active properties allow it to partition into cell membranes to inhibit lipid oxidation at the oil-water interface. By using the same principle, several studies have focused on the use of natural flavonoids as other possible alternative antioxidants to regenerate α-tocopherol in homogenous (bulk oils and solvents) and heterogeneous systems (LDL, micelles, and emulsions). The best synergistic effect was found when the equal molar ratio of α-tocopherol and myricetin were added to bulk sunflower oil. However, the synergistic activity was lost at total antioxidant concentration higher than 100 μM (*130*). Other flavonoids including kaempferol, morin, quercetin and myrintin also showed interactions with α-tocopherol in human low density lipoprotein (*131*). Green tea polyphenols in combination with α-tocopherol and ascorbic acid showed synergistic antioxidant activity in SDS micelles. It was suggested that electrons are transferred from ascorbic acid to green tea polyphenols and then to α-tocopherol.(*74, 109*).

Kinetic studies of the regeneration of α-tocopherol by various phenolic antioxidants were reported in different environments as showed in Table 2.5. The secondary rate constants of regeneration by green tea polyphenols are approximately 4.5 x 10^1 - 10^3 $M^{-1}s^{-1}$. Among green tea polyphenols, epigallocatechin gallate (EGCG) exhibits the highest rate of α-tocopherol regeneration followed by epicatechin gallate (ECG), epigallocatechin (EGC), and epicatechin (EC) and gallic acid (GA). EC, ECG, EGC, and EGCG exhibited higher or similar α-tocopherol regeneration rates as ascorbic acid at pH 7-12 (*12*).

Table 2.5 The secondary rate constants of the regeneration of α-tocopherol by green tea flavonoids and other phenolics. (*10, 12, 132-135*)

Compounds	Rate constants	Systems
EGCG	1.91×10^2	SDS micelles, pH 7.4
	1.40×10^3	Ethanol
	3.64×10^3	Triton X-100 micelles, pH 7.0
ECG	1.31×10^2	SDS micelles, pH 7.4
	5.22×10^2	Ethanol
	1.68×10^3	Triton X-100 micelles, pH 7.0
EGC	1.11×10^2	SDS micelles, pH 7.4
	1.09×10^3	Ethanol
	2.42×10^3	Triton X-100 micelles, pH 7.0
EC	0.45×10^2	SDS micelles, pH 7.4
	1.52×10^2	Ethanol
	1.27×10^3	Triton X-100 micelles, pH 7.0
Gallic acid	0.43×10^2	SDS micelles, pH 7.4
Methyl gallate	4.69×10	Ethanol
	1.36×10^3	Triton X-100 micelles, pH 7.0
Rutin	1.03×10	Ethanol
	5.48×10^2	Triton X-100 micelles, pH 7.0
Quercetin	2.98×10^2	Ethanol
	3.73×10^5	Triton X-100 micelles, pH 7.0
Ubiquinol-10	3.64×10^4	Ethanol
	9.24×10^5	Triton X-100 micelles, pH 7.0
Caffeic acid	2.31×10^2	Ethanol
Chlorogenic acid	2.63×10^2	Ethanol
Rosmarinic acid	9.05×10^2	Ethanol
Isoeugenol	9.28×10^2	Ethanol
Ferulic acid	3.68	Ethanol
Methyl linoleate	1.26×10^{-2}	Ethanol
	2.40×10^{-2}	Triton X-100 micelles, pH 7.0

In addition, it is possible to observe this synergistic mechanism in a system containing three different antioxidants such as caffeic acid, vitamin C and α-tocopherol (*74*). In this system, caffeic acid acts as "an electron transfer bridge" between ascorbic acid and α-tocopherol because the ability of caffeic acid to localize closer to α-tocopherol at the oil-water interface compared to ascorbic acid (Figure 2.12). As discussed in the previous section, this type of synergistic mechanism may be influenced by various parameters such as homogenous and heterogeneous systems, solvents, microenvironment pH, and oxidation processes (with or without radical initiators).

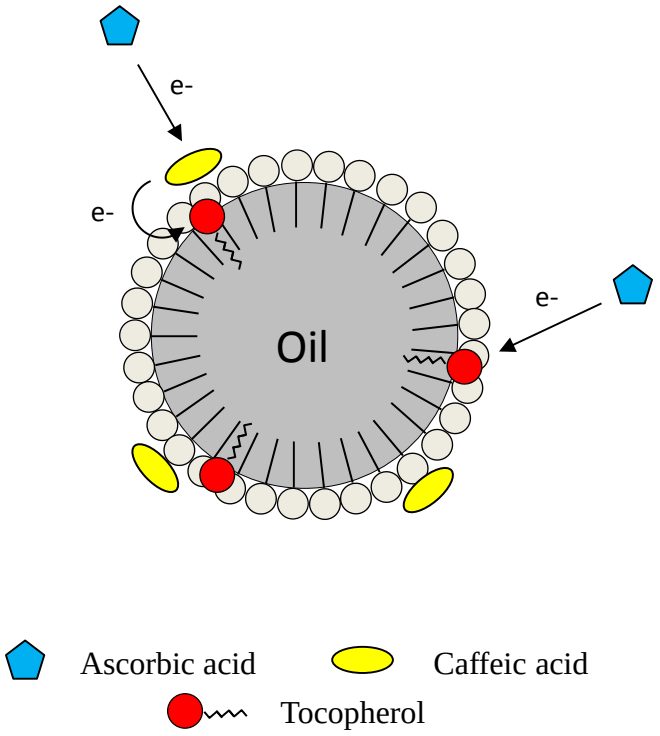


Figure 2.12 Antioxidant interaction mechanisms of ascorbic acid, caffeic acid, and α-tocopherol (Adapted from Laranjinh et al., 2001 (*74*))

2.4.2.3 Antioxidant interactions of carotenoids

Synergistic interactions between carotenoids (lycopene and β-carotene) and α-tocopherol have been reported by several investigators (*33, 136-140*). In general, β-carotene can donate electrons and hydrogens as shown in Reaction 19-20. The most well-known examples of these reactions are β-carotene radical (β-Car•) and β-carotene radical cation (β-CarH•$^{+}$).

$$\beta\text{-CarH} \rightleftharpoons \beta\text{-CarH}^{\bullet+} + e^{-} \rightleftharpoons \beta\text{-Car}^{\bullet} + e^{-} + H^{+} \quad (19)$$

$$\beta\text{-CarH}^{\bullet+} \rightleftharpoons \beta\text{-CarH}^{\bullet 2+} + e^{-} \quad (20)$$

Under low oxygen pressure, β-carotene inhibits lipid oxidation by donating hydrogen to lipid peroxyl radicals (Reaction 21), and then the resulting carbon center of β-carotene can react quickly and reversibly with oxygen, which itself is a biradical (Reaction 22). The reverse reaction depends on the concentration of oxygen.

$$\beta\text{-Car}{-}H + LOO^{\bullet} \longrightarrow \beta\text{-Car}^{\bullet} + LOOH \quad (21)$$

$$\beta\text{-Car}^{\bullet} + O_2 \rightleftharpoons \beta\text{-Car}{-}OO^{\bullet} \quad (22)$$

$$\beta\text{-Car} + LOO^{\bullet} \longrightarrow LOO{-}\beta\text{-Car}^{\bullet} \quad (23)$$

In addition to donating hydrogen to a lipid peroxyl radical, antioxidant action of β-carotene may be due to the addition reaction of a lipid peroxyl radical to the conjugated system of β-carotene forming lower energy resonance-stabilized, carbon centered radical (Reaction 23).

The ability of β-carotene act as a synergistic antioxidant may be due to the deeper location of β-carotene in lipophilic phase compared to other antioxidants (e.g. α-tocopherol, flavonoids, and ascorbic acid). This can be seen by its greater reactivity towards the lipophilic radical initiator (AMVN) compared to α-tocopherol and ascorbic acid (*141*). Recently, two opposite hypotheses of synergistic mechanisms between α-tocopherol and β-carotene have been proposed from two different experimental techniques.

The first hypothesis, the protection of β-carotene by α-tocopherol, is relied on the information from observations of decompositions of antioxidants and oxidation products initiated by AAPH and AMVN (*33*, *141*). Results indicated that the concentration of β-carotene is spared while decomposition of α-tocopherol is accelerated. According to their reduction potentials, reactions to protect the autoxidation of β-carotene (Reaction 24-25) are thermodynamic feasible where the standard reduction potentials of β-carotene, α-tocopherol, and ascorbic acid are approximately 700-100 mV (*142*), 480-500 mV, and 280 mV, respectively.

$$\beta\text{-Car}-\text{OO}\bullet + \text{TOH} \longrightarrow \beta\text{-Car}-\text{OOH} + \text{TO}\bullet \quad (24)$$

$$\beta\text{-Car}-\text{OO}\bullet + \text{AscH}_2 \longrightarrow \beta\text{-Car}-\text{OOH} + \text{AscH}\bullet + \text{H}^+ \quad (25)$$

Therefore, as shown in Figure 2.13, the synergistic antioxidant interactions of β-carotene and α-tocopherol may be due to minimizing the prooxidative effects of β-carotene. The reaction between β-carotene peroxyl radicals and ascorbic acid is thermodynamic feasible, however, it was suggested that it is kinetic infeasible due to different locations.

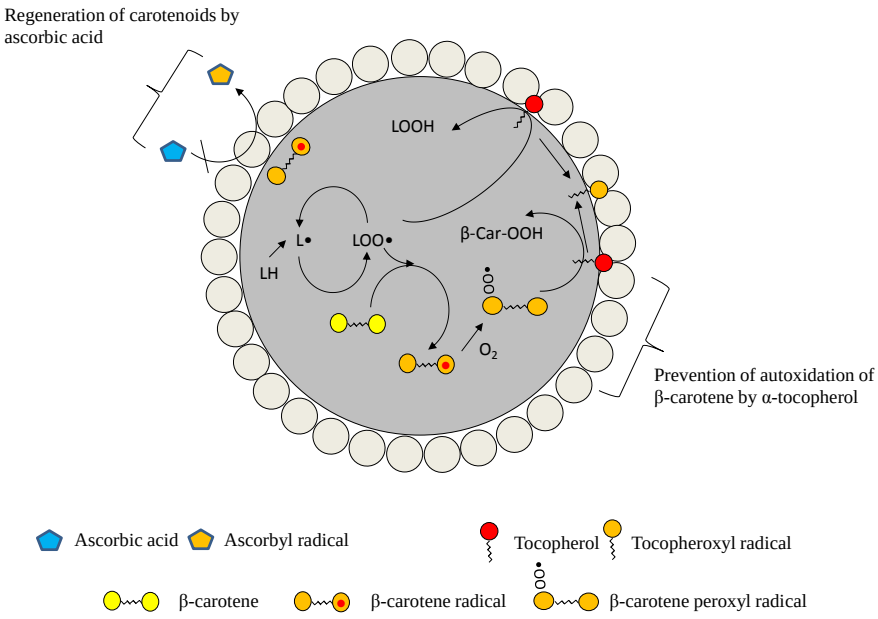


Figure 2.13 Probable antioxidant mechanisms of carotenoids with tocopherol and ascorbic acid based on the protection of β-carotene by α-tocopherol (Adapted from Niki et al. 1995 (*141*))

In contrast, the opposite hypothesis is protection of α-tocopherol by β-carotene as illustrated in Figure 2.14. From pulse radiolysis and laser flash photolysis studies, β-carotene can donate electrons to regenerate α-tocopherol, and then the resulting β-carotene radical cations can be recycled to β-carotene by ascorbic acid (Reaction 26-28). (*72*).

$$TO\bullet + \beta\text{-Car} + H^+ \longrightarrow TOH + \beta\text{-Car}\bullet^+ \quad (26)$$

$$\beta\text{-Car}\bullet^+ + AscH_2 \longrightarrow \beta\text{-Car} + AscH\bullet + H^+ \quad (27)$$

$$\beta\text{-Car}\bullet^+ + AscH^- \longrightarrow \beta\text{-Car} + Asc\bullet + H^+ \quad (28)$$

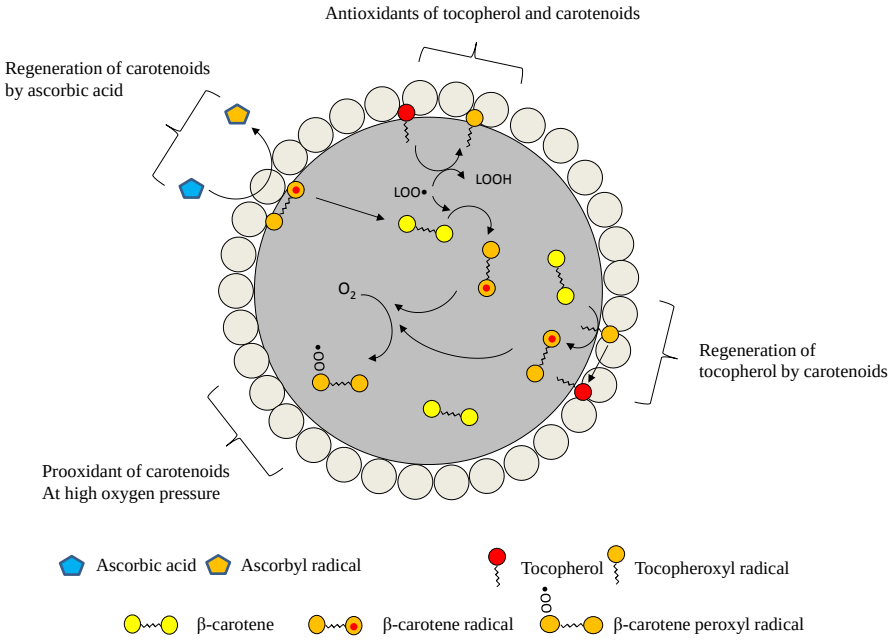


Figure 2.14 Probable antioxidant mechanisms of carotenoids with tocopherol and ascorbic acid based on the protection of α-tocopherol by β-carotene

2.4.2.4. Antioxidant interactions of phospholipids

Phospholipids are major components of biological membranes and are considered as one of the important minor components in refined oils. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS).and cardiolipin (CL) have been studied for their impact on the activity of α-tocopherol and ascorbic acid. The mechanisms of antioxidant synergy between phospholipids and α-tocopherol are complex because synergy can be due to the ability of phospholipids to form physical structures such as biological membranes, liposomal membranes, and reverse micelles in bulk oils (*14*), donate electron (*28, 143, 144*), and chelate metal ions

(*43, 45-47, 145-147*). These abilities are different among phospholipids due to their different chemical structures and properties.

Physical structures in bulk oils can be a site of lipid oxidation reactions (*15*). Chen and coworkers (*14, 52*) reported that reverse micelles formed by 1, 2-di-oleoyl-sn-glycero-3-phosphatidylcholine (DOPC) enhance oxidation of lipids and can alter the activity of α-tocopherol and Trolox. Koga and coworkers (*51*) suggested that the radical scavenging properties of vitamin E in bulk oils can be enhanced by phospholipids though formation physical structures which alter the mobility and location of α-tocopherol.

The ability of phospholipids to donate electron is due to their amino acid groups. The primary amine moiety of PE and PS is believed responsible for the electron donation rather than the tertiary amine moiety of PC and the sugar alcohol moiety of PI. In a lipid oxidation study, the antioxidant synergy between α-tocopherol and α-tocopheryl quinone was stronger with PE than PC and PS (*46, 148-151*).

It was suggested that synergistic effects can occur the regeneration of α-tocopherol by phospholipids (*152*). Equal or slightly synergistic effects between PC and tocopherols compared to PE were reported while PS and PI showed antagonistic effect (*46, 153, 154*). However, in some study, PE and PC can either accelerate or inhibit the oxidation depending on media and concentrations (*155*).

Radical transfer between α-tocopherol and ascorbic acid can be influenced by phospholipids. Lambelet and coworkers (*28*) reported PC and PI are not involved the radical transfer reaction between α-tocopheroxyl radicals and ascorbic acid. However, PE and PS can directly scavenge lipid peroxyl radicals resulting in formation of nitroxide

radicals on the phospholipid. Phospholipid nitroxide radicals can then react with lipid hydroperoxides (reaction 29-31)(*28, 156, 157*).

$$R_2\text{-NH} + R'\text{-OO}\bullet \longrightarrow R_2N\bullet + R'\text{-OOH} \quad (29)$$

$$R_2N\bullet + O_2 \longrightarrow R_2NOO\bullet \quad (30)$$

$$2\ R_2NOO\bullet \longrightarrow 2\ R_2NO\bullet + O_2 \quad (31)$$

Where R_2-NH, $R_2N\bullet$, $R_2NOO\bullet$, and $R_2NO\bullet$ represent primary or secondary amine, an aminyl radical, an N-peroxyl radical intermediate, and a nitroxide radical, respectively and R'OO• represents a lipid hydroperoxide

2.5 Mechanisms of antagonistic antioxidant interactions

2.5.1 Bipolar antioxidant behaviors: antioxidant vs. prooxidant activity

The polar paradox hypothesis attempts to explain differences in the activity of antioxidants through their ability to partition into different locations of food systems. However, the polar paradox ignores the fact that many of these antioxidants can exhibit prooxidant activity under particular conditions. Paradoxical behaviors of free radical scavengers and metal chelators can be explained by interactions with existent prooxidants in such a way that the antioxidant accelerates lipid oxidation.

2.5.1.1 Reduction of metals by free radical scavengers

Prooxidant activity of free radical scavengers is due to reduction of ferric ion (Fe^{3+}) to the more prooxidative ferrous ion (Fe^{2+}) (*25, 106, 107, 158*). This reaction decreases antioxidant activity by direct decomposition of antioxidants and also promotes lipid oxidation via Fe^{2+} (Reaction 32). This reaction is important because antioxidant consumption by metal ions can occur before the antioxidant can donate hydrogen to lipid radicals to inhibit lipid oxidation.

$$M^{(n+1)+} + AOH \longrightarrow M^{n+} + H^{+} + AO\bullet \qquad (32)$$

Another prooxidative mechanism is the ability of antioxidants to reduce iron to the more reactive Fe^{2+}, which can then rapidly promote hydroperoxide decomposition into free radicals (Reaction 2).

In general, most free radical scavengers can reduce metal ions. However, this prooxidant phenomenon mainly occurs with compounds that have in low reduction potentials such as ascorbic acid and propyl gallate (*13, 159-161*).

2.5.1.2 Reverse chain-braking reactions by free radical scavengers

In contrast to antioxidant reduction of metals at low concentrations to produce prooxidant actovity, α-tocopherol has been shown to have prooxidant activity at high concentractions(*128, 162*). The reactions (33-34) are commonly attributed to the prooxidtive mechanism of α-tocopherol. Due to the high stability of α-tocopheroxyl radicals t, the accumulation of high concentration of α-tocopheroxyl radicals may cause a

reverse in the reaction resulting increase the lipid peroxidation rate (*25, 163, 164*). A summary of the secondary rate constants for the reverse reaction of α-tocopherol radicals with free fatty acids and their hydroperoxides is shown in Table 2.6.

$$TO\bullet + LOOH \longrightarrow TOH + LOO\bullet \quad (33)$$

$$TO\bullet + LH \longrightarrow TOH + L\bullet \quad (34)$$

Table 2.6 The rate constants of the reverse reactions of α-tocopheroxyl radicals and various fatty acids at 25^{o}C (*165*) (*25*)

Lipid compounds	Rate constants (M^{-1} s^{-1})
Oleic acid	1.04×10^{-5}
Linoleic acid	1.82×10^{-2}
Linolenic acid	3.84×10^{-2}
Arachidonic acid	4.83×10^{-2}
Oleyl hydroperoxide	1.04×10^{-1}
Linoleyl hydroperoxide	2.42×10^{-1}
Arachidonyl hydroperoxide	3.65×10^{-1}

However, the role of these reverse prooxidative reactions has been criticized because of slow backward rate constants (*25*). Since the overall rate constants of biomolecular couplings of these reactions are actually just slow down, however the reactions could be still pulled forward in considerable fast rates to protect lipid peroxidation. Burton and his colleges (1985) suggested that the bimolecular couplings of

the reactions of α-tocopherol and lipid radicals is approximately 3 x 10^3 to 5 x 10^5 M^{-1} s^{-1} (*25, 166*).

Fuster and coworkers (*128*) reported that α-tocopherol did not exhibit prooxidant activity even at concentrations as high as 2000 ppm, unless oxidation was promoted by $FeSO_4$. They and others suggested that the prooxidant effect of α-tocopherol at high concentrations was due to reduction with metals (*25, 128*). Therefore, it is still unclear why only α-tocopherol exhibits prooxidant effect at high concentrations especially in light that this phenominon is not observed with other antioxidants.

2.5.1.3 Direct decomposition of hydroperoxides

The prooxidative activity of phenolic compounds could also be due to the direct decomposition of hydroperoxides to form alkoxyl (LO•) and antioxidant radicals (AO•) as shown in Reaction 35. This reaction involves hydrogen abstraction from AOH by LOOH and scission of the O-O bond in the hydroperoxide (*167*).

$$AOH + LOOH \rightleftharpoons [LOO{-}H\cdots HOA] \longrightarrow AO\bullet + LO\bullet + H_2O \qquad (35)$$

As shown in Table 2.7, rate constants of hydroperoxide decomposition reactions are relatively slow (10^{-3} to 10^{-5} M^{-1} s^{-1}) compared to the protective reaction of α-tocopherol (≈8 x 10^4 M^{-1} s^{-1}) (*60*) and other phenolics (Reaction 9). Very limited information on this reaction with common food antioxidants has been reported. However, Igarashi and coworkers (*168*) reported that α-tocopherol could not react with

hydroperoxide of methyl linoleate. Thus, this reaction may not contribute to prooxidant activity of all antioxidants.

Table 2.7 Rate constants of decomposition reaction of cumyl hydroperoxide by phenols in benzene at 413^{o}K (adapted from Denisov et al., 1997(*167*)

Phenols	Rate constants (M^{-1} s^{-1})
2,6-dimethoxyphenol	6.5×10^{-3}
4-methoxyphenol	4.3×10^{-3}
2,3-dihydroxynaphthalene	4.5×10^{-3}
2,4,6-tri-tert-butylphenol	7.8×10^{-5}
α-napthanol	6.7×10^{-4}

2.5.1.4 Increase of metal solubility

Metal chelators such as citric acid and EDTA inhibit lipid oxidation by inactivation of the prooxidant activity of metals by increasing reduction potentials, occupying all metal coordination sites, forming insoluble metal complexes and/or creating steric hindrance that inhibit metal and lipid interactions (*26*). However, it is widely accepted that EDTA and citric acid can also be prooxidative presumably by increasing solubility of metals and/or lower their reduction potentials Reduction potentials of Fe(II) are 110, 120, and 100 mV in aqueous, EDTA complex, and citric acid complex, respectively (*60*). EDTA has tendency to exhibit prooxidant activity when EDTA:iron ratios are ≤ 1. Under low concentrations of antioxidants, EDTA can exhibits stronger prooxidant effects because EDTA bound metals can still undergo reduction.

2.5.2 Other prooxidant hypotheses

2.5.2.1 Complex formation between antioxidants

In addition to free radical transfer between antioxidants, it is also suggested that the formation of stable a complex between antioxidants due to π-π stacking between the aromatic ring of phenolic acid and the B-ring of flavonol may influence on the overall electron donating capacity (*93, 169, 170*). However, the synergistic effect of the more stable complex of quercetin/rosmarinic acid does not explain why the less stable complex of quercetin/caffeic acid exhibits the antagonistic result. In addition, the mechanism behind antagonistic interaction due to the formation of hydrogen bonding between carbonyl and hydroxyl groups of antioxidants was proposed (*171*) as shown in Figure 2.15.

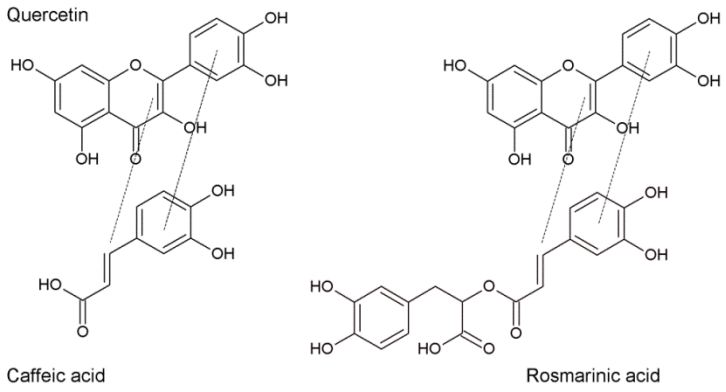


Figure 2.15 Possible interactions occurring in quercetin/caffeic acid complex and quercetin/rosmarinic acid complex (the original figure from Peyrat et al. (*93*))

2.5.2.2 Prooxidant activity of carotenoids

Under high oxygen pressure, prooxidative effects of β-carotene are due to the autoxidation of β-carotene peroxyl radical (Reaction 22) and the further oxygen addition of LOO−β-Car•. The end product of the reaction 36-37 yields a prooxidative lipid radical (L•), which can initiate propagation of lipid oxidation (*31, 142, 172*).

$$LOO{-}\beta\text{-}Car\bullet + O_2 \longrightarrow LOO{-}\beta\text{-}Car{-}OO\bullet \quad (36)$$

$$LOO{-}\beta\text{-}Car{-}OO\bullet + L'H \longrightarrow LOO{-}\beta\text{-}Car{-}OOH + L'\bullet \quad (37)$$

Antagonistic effects between β-carotene radical cation and flavonoids were reported (*173*). Results showed that β-carotene was not regenerated from β-carotene radical cation by an electron transfer reaction with flavonoids, but β-carotene radical cation undergone the addition of flavonoids to its conjugated system resulting strong antagonistic antioxidant interaction of the mixtures. Due to the complexity of antioxidant and prooxidant mechanisms of β-carotene, the combinations with other antioxidants may obtain unexpected results.

CHAPTER 3

EFFECTS OF CHITOSAN AND ROSMARINATES ON THE PHYSICAL AND OXIDATIVE STABILITY OF LIPOSOMES

3.1 Introduction

Liposomes are spherical, single or multiple layer vesicles that are spontaneously formed when phospholipids are dispersed in water. In recent years, liposomal encapsulation technologies have been extensively investigated in the food and agricultural industries as delivery systems to entrap and protect functional and unstable components such as antimicrobials, flavors, antioxidants and bioactive ingredients. Liposomes can entrap both hydrophobic and hydrophilic compounds within their structure, protect entrapped compounds from decomposition, and release the entrapped compounds at designated targets (*174, 175*). Commercially available phospholipid preparations, commonly referred to as lecithin, are isolated from natural sources such as chicken egg yolk and soybeans and are composed of mixtures of a variety of individual phospholipids. In the food industry, lecithins are a generally recognized as safe (GRAS) food ingredients that are biocompatible, biodegradable, and nontoxic (*176*). They are used as both emulsifiers and texture modifiers (*175, 177*). Phosphatidylcholine (PC) is the major phospholipids found in most lecithins (*178*).

One of the problems with liposomes in practical applications is their insufficient physical and chemical stability leading to changes in particle size distribution, turbidity, and ability to contain the encapsulated compounds. Aggregation, rupture, and coalescence of liposomes will change their size distribution. This destabilization is particularly prevalent when surface charges are reduced at low pH conditions and at high ionic strengths (*179*). The chemical stability of liposomes may also be problematic due to oxidation or hydrolysis of the fatty acids (*180, 181*).

Many lecithins are susceptible to lipid oxidation because the phospholipids in the lecithin may contain fatty acids that are highly unsaturated. Transition metals such as iron can accelerate the oxidation of liposomes by interacting with residual lipid hydroperoxides in the phospholipids to produce free radicals that promote oxidation (*182, 183*). In addition, the overall surface charge of liposomes manufactured from commercial lecithins is generally negative resulting in electrostatic attraction of transition metals thereby increasing metal-lipid interactions and further promoting oxidation (*184*). To minimize oxidative degradation of liposomes, several strategies have been reported including selecting high quality lecithins with low levels of hydroperoxides and transition metals (*185*), using phospholipids that are high in saturated fatty acids, (e.g. hydrogenated phospholipids; (*186*)), adding antioxidants (*187*), and modifying the liposomal surface charges (*183, 188, 189*).

Chitosan has been used successfully as a secondary layer on phospholipid stabilized oil-in-water emulsion droplets to increase physical stability (*190-192*). Modification of liposome surfaces by coating with chitosan has been demonstrated to

enhance the physical stability of liposomes against aggregation for up to 45 days (*193*). Electrostatic deposition of chitosan onto phospholipid-stabilized oil-in-water emulsion droplets has been shown to inhibit lipid oxidation presumably by producing a cationic interface that causes charge repulsion of iron thus minimizing lipid-metal interactions (*194*).

The objective of this research was to determine the impact of antioxidants and/or surface charge modifications on the chemical and physical stability of liposome. Surface charge modifications were accomplished by adsorbing a layer of chitosan on the surface of the liposomes using a layer-by-layer electrostatic deposition method. Chitosan was chosen as substrate for the electrostatic deposition because it is positively charged and thus can be electrostatically bound to negatively charged surfaces. The antioxidant tested in this study was a phenolic acid compound, rosmarinic acid. The antioxidant activity of surface active rosmarinic acid esters produced with aliphatic chains of varying length was also determined.

3.2 Materials and Methods

3.2.1 Materials

Soy lecithin (UltralecP) was kindly provided by Archer Daniels Midland (Decatur, Ill., U.S.A.). Sodium acetate trihydrate (99.1%) and glacial acetic acid ($C_2H_4O_2$) were purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). The chitosan used in this study were donated by Primex, Reykjavik, Iceland and had an average

molecular weight of 205.3 ± 2.0 kDa, a degree of deacetylation of 91.8%, and a viscosity of 45 cP in 1% acetic acid solution, according to the certificate of analysis supplied by the manufacturer. Sodium azide (NaN_3, 99.5%) was purchased from Sigma-Aldich (St. Louis, MO, USA). All other chemical were reagent grade. Double distilled and deionized water was used for the preparation of all solutions.

3.2.2 Synthesis of rosmarinate esters

The chemo-enzymatic esterification of rosmarinic acid to obtain rosmarinic esters was carried out following the procedure described by Lecomte et al. (*195*). Briefly, the chemical esterification of rosmarinic acid (56 µmol) was carried out in sealed brown flasks each containing 5 mL of alcohol (methanol, 123.44 mmol; n-butanol, 54.64 mmol; n-octanol, 31.905 mmol; n-dodecanol, 22.46 mmol; n-hexadecanol, 16.95 mmol; n-octadecanol, 15.09 mmol or n-eicosanol, 13.6 mmol). The reaction mixtures were stirred (orbital shaker, 250 rpm, 55-70 °C) prior to the addition of the catalyst; the strongly acidic sulfonic resin Amberlite® IR-120H (5% w/w - total weight of both substrates) that had been previously dried at 110°C for 48h. The water generated during the reaction was removed by adding 3 Angström, 4-8 mesh molecular sieves (40 mg/mL, Aldrich, St. Louis, MO, USA) to the medium. Samples (20 µL) were regularly withdrawn from the reaction medium then mixed with 980 µL of methanol, filtered (0.45 µm syringe filter Millex®-FH, Millipore Corporation Bedfork, MA, USA), and finally analyzed by reverse phase HPLC with UV detection at 328 nm (*195*). After complete (4-21 days) conversion of rosmarinic acid into the corresponding ester, the latter was purified in a two steps

procedure. First, a liquid-liquid extraction using hexane and acetonitrile was performed to remove the excess alcohol. Then, the remaining traces of the alcohol and rosmarinic acid were eliminated by flash chromatography on a CombiFlash® Companion® system (Teledyne Isco Inc, Lincoln, NE, USA). Separation was carried out on a silica column using an elution gradient of hexane and ether (20% to 100% in 35 min). The yield of purified esters, obtained as pale yellow to yellow amorphous powders, was calculated from calibration curves previously established with pure compounds. Pure esters and rosmarinic acid were then fully characterized by ESI-MS, 1H-NMR and 13C-NMR as previously described by Lecomte et al. (*195*).

3.2.3 Preparation of liposomes

Multilayer liposomes were prepared according to the method described by Laye et al. (*193*) with slight modifications. A lecithin stock solution (1%, w/v) was freshly prepared in 0.1 M acetate buffer (pH 3.0 + 0.1). The lecithin solution was mixed for 2 min with a handheld high-speed blender at maximum speed (Bio homogenizer, Model M133.1281-0, Biospec product Inc, Barlesville, OK). Chitosan stock solution (1 %, w/v) was prepared with the same buffer and stirred overnight followed by filtration through Miracloth (Calbiochem, USA) and a hydrophobic PTFE 5.0 μm Millipore filter (Millex-LS; Danvers, MA). Stock solutions were stored at 5^{o}C and used within 24 h. In experiments using rosmarinic acid esters, the esters were first dissolved in methanol and then mixed with the lecithin stock solution followed by mixing with a handheld high-

speed blender as described above. Samples without antioxidants contained an equal amount of methanol as the antioxidant treated samples.

Liposomes were prepared by passing the lecithin stock solution three times through a high-pressure homogenizer at 9000 psi (Model 110L, Microfluidizer, Microfluidics, Newton, Mass., U.S.A.). To produce chitosan-coated liposomes, the homogenized liposomes added to an equal volume of chitosan solution under constant stirring (700 rpm for 2 min) to obtain a final concentration of 0.5% (w/v) lecithin and 0.2% (w/v) chitosan. In order to decrease bridging flocculation of the liposomes by chitosan, the chitosan-coated liposome solutions were passed 3 times through a high-pressure homogenizer at 9000 psi. To inhibit microbial growth during the study, all liposome solutions were mixed with 0.04% (w/v) NaN_3 and stirred for 2 min. To conduct stability studies, one milliliter of coated or uncoated liposome solutions were transferred to 10 mL headspace vials, sealed with poly(tetrafluoroethylene) butyl rubber septa and stored at 50^{o}C in the dark. In experiments with ethylenediaminetetraacetic acid (EDTA), EDTA (100 μM) was added after liposome preparation.

3.2.4 Liposomal charge and size measurements

The electrical charge and size of liposomes were measured by dynamic light scattering measurements (Zetasizer Nano-ZS, Model ZEN3600, Malvern Instruments, Worchester, U.K.), and expressed as ζ -potential and z-average mean diameter, respectively. Samples were diluted approximately 10-fold with the same buffer, mixed,

and immediately transferred into plastic cuvettes for size determination or capillary cells for ζ –potential determination (DTS1060, Malvern Instruments, Worchester, U.K.).

3.2.5 Measurements of lipid hydroperoxides

Lipid hydroperoxide formation in liposome solutions was determined according to an adapted method as described by Alamed et al. (*2*). Liposome solutions (0.3 mL) were mixed with 5 mL of isooctane/2-propanol (3:1 v/v) and vortexed (10 s, 3 times). After centrifugation at 1000g for 2 min, 200 μL of the organic solvent phase was mixed with 2.8 mL of methanol/1-butanol (2:1). Hydroperoxide detection was started by addition of 15 μL of 3.94 M ammonium thiocyanate and 15 μL of ferrous iron solution (prepared by adding equal amounts of 0.132 M $BaCl_2$ and 0.144 M FeSO4). After 20 min of incubation at room temperature, the absorbance was measured at 510 nm using a UV-VIS spectrophotometer (GENESYS 20, Thermo Spectronic). Hydroperoxide concentrations were determined using a standard curve prepared from hydrogen peroxide.

3.2.6 Measurement of hexanal

Headspace hexanal was determined according to the method described by Pignoli et al. (*196*) with some modification using a Shimadzu GC-2014 gas chromatograph (GC) equipped with an AOC-5000 Auto-injector (Shimadzu, Tokyo, Japan). A 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/Carboxen/PDMS)stable flex SPME fiber (Supelco, Bellefonte, PA, USA) was inserted through the septum into the

vial and exposed to the sample headspace for 15 min at 55°C. The SPME fiber was desorbed at 250^{o}C for 3min in the GC detector at a split ratio of 1:7. The chromatographic separation of volatile aldehydes was performed on a fused-silica capillary column (30m×0.32mm i.d. ×1μm) coated with 100% poly(dimethylsiloxane) (Equity-1, Supelco, Bellefonte, PA, USA). The temperature of the oven, injector and flame ionization detector were 65^{o}C, 250°C and 250^{o}C, respectively. Sample run time was 10 min. Concentrations were determined using a standard curve made from hexanal.

3.2.7 Determination of antioxidant partitioning

For the determination of the physical location of rosmarinic acid and its esters in the liposome suspensions, liposome solutions were centrifuged at 146,550g (40,000 rpm) for 1h at 4^{o}C using a Sorvall A-1256 rotor with a high speed centrifuge (Sorvall Ultra 80, Waltham, MA USA). The supernatant was carefully collected with a pipette, and the amounts of rosmarinic acid esters in the supernatants were determined at 333 nm using a UV-VIS scanning spectrophotometer (UV-2010PC) with a quartz cell. The concentrations of rosmarinic acid esters were calculated using a standard curve made from rosmarinic acid dissolved in methanol.

3.2.8 Statistics

All analysis was performed on triplicate samples. Oxidation lag phases were defined as the first data point significantly greater than the 0 time value. In all cases,

comparisons of the means were performed using Duncan's multiple range tests. A significance level of $p < 0.05$ was defined as being statistically different. All calculations were performed using SPSS17 (http://www.spss.com; SPSS Inc., Chicago, IL).

3.3 Results and Discussion

Liposomes are susceptible to oxidative degradation limiting their utilization in foods. Therefore, this study was conducted to find methods to reduce lipid oxidation in liposomes using antioxidant technologies including electrostatic deposition of chitosan onto the surface of liposomes and use of surface active antioxidants. While electrostatic deposition of chitosan onto the surface of liposomes did alter the physical properties of the liposomes (see discussion below), none of the antioxidants tested had any impact on either liposome size or charge (data not shown).

3.3.1 Effect of chitosan and EDTA on liposomal stability

A secondary chitosan layer coated on liposomes led to higher physical and chemical stability of liposomes during incubation at pH 3.0 and 55^{o}C. Figure 3.1a shows that immediately after preparation (0 h), chitosan coated liposomes were significantly larger in diameter (205.1 nm) than uncoated liposomes (87.8 nm). Increase in liposome size in the presence of chitosan could be due to the thicker interface of the coated liposomes as well as bridging flocculation by the chitosan polymer. Electrostatic deposition of chitosan onto the liposomes increased the charge of liposomes from -37.8

mV for the uncoated liposomes to + 66.3 mV for the coated liposomes (Figure 3.1b). Change in charge is due to the electrostatic deposition of the positively charged chitosan onto the negatively charged phospholipid. The observed increase in size and charge of the liposomes after coating was in agreement with other studies (*190, 193, 197*). During storage, the particle diameter of the coated liposomes only increased slightly (approximately 1.02-fold) while the uncoated liposomes increased approximately 1.5-fold by at the end of the storage study (although none of the liposome systems exhibited sedimentation over the course of the oxidation studies). The higher physical stability of the coated liposome could be due to an increased charge density and a thicker outer layer which could decrease liposome coalescence and aggregation during storage.

Lipid oxidation was significantly inhibited by coating the liposomes with chitosan as indicated by lipid hydroperoxides and hexanal determination (Figure 3.2a and b, respectively). Without coating, lipid oxidation of liposomes occurred rapidly with the lag phase of both lipid hydroperoxide and hexanal formation being $\leq$ 1 day. Coating with chitosan extended the lag phase to 3 and 4 days for lipid hydroperoxide and hexanal formation, respectively. Lipid oxidation was strongly inhibited by EDTA indicating that transition metals were major prooxidants in the liposome system. The fact that transition metals were important prooxidants suggests that inhibition of lipid oxidation by the chitosan coating was due to the formation of a cationic layer on the surface of the liposomes that can electrostatically repel transition metals away from the lipid-water interface (*194, 198-200*).

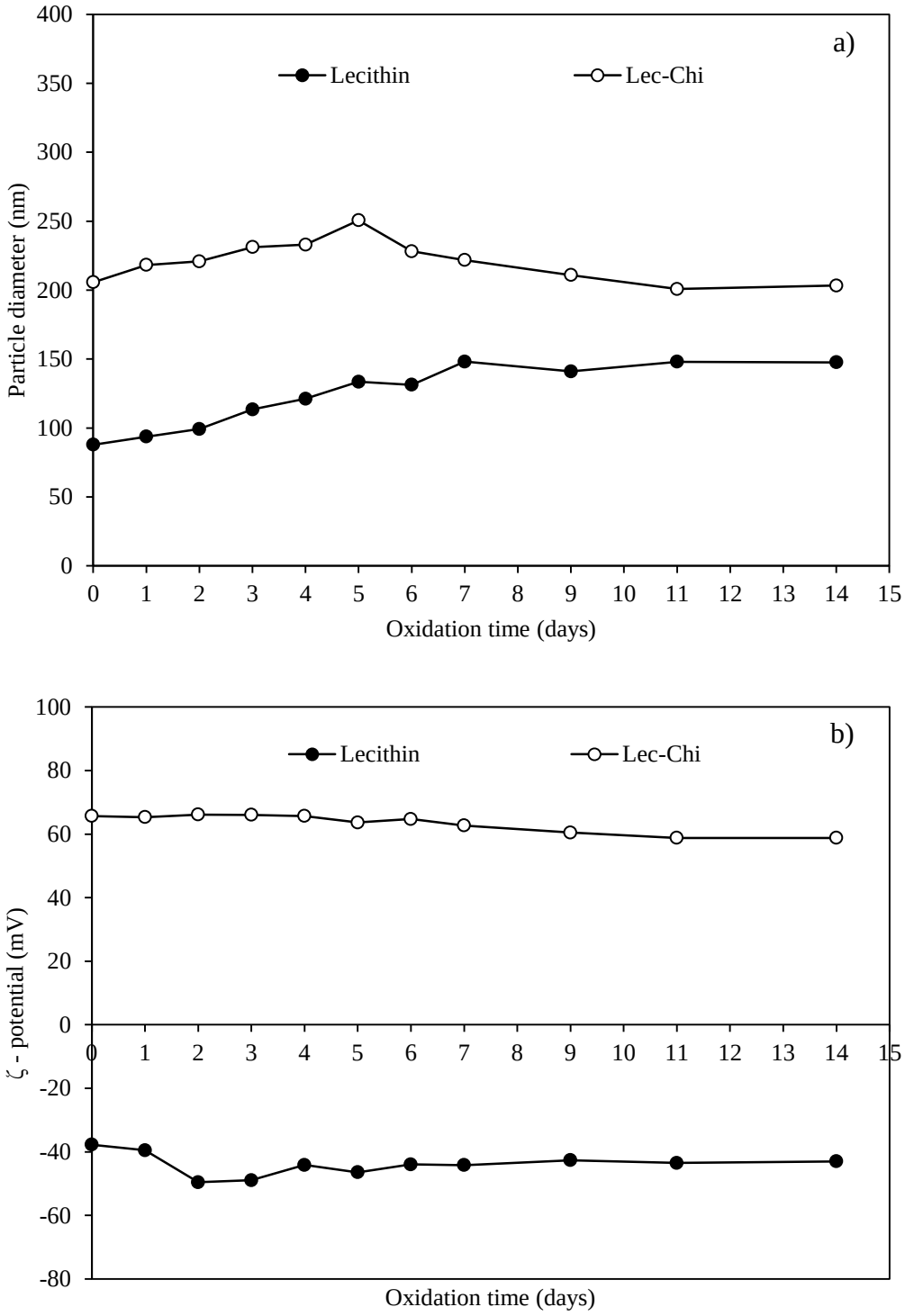


Figure 3.1 Physical stability of liposomes (pH 3.0 and 55^{o}C) during storage as determined by particle diameter (a) and droplet charge (b)

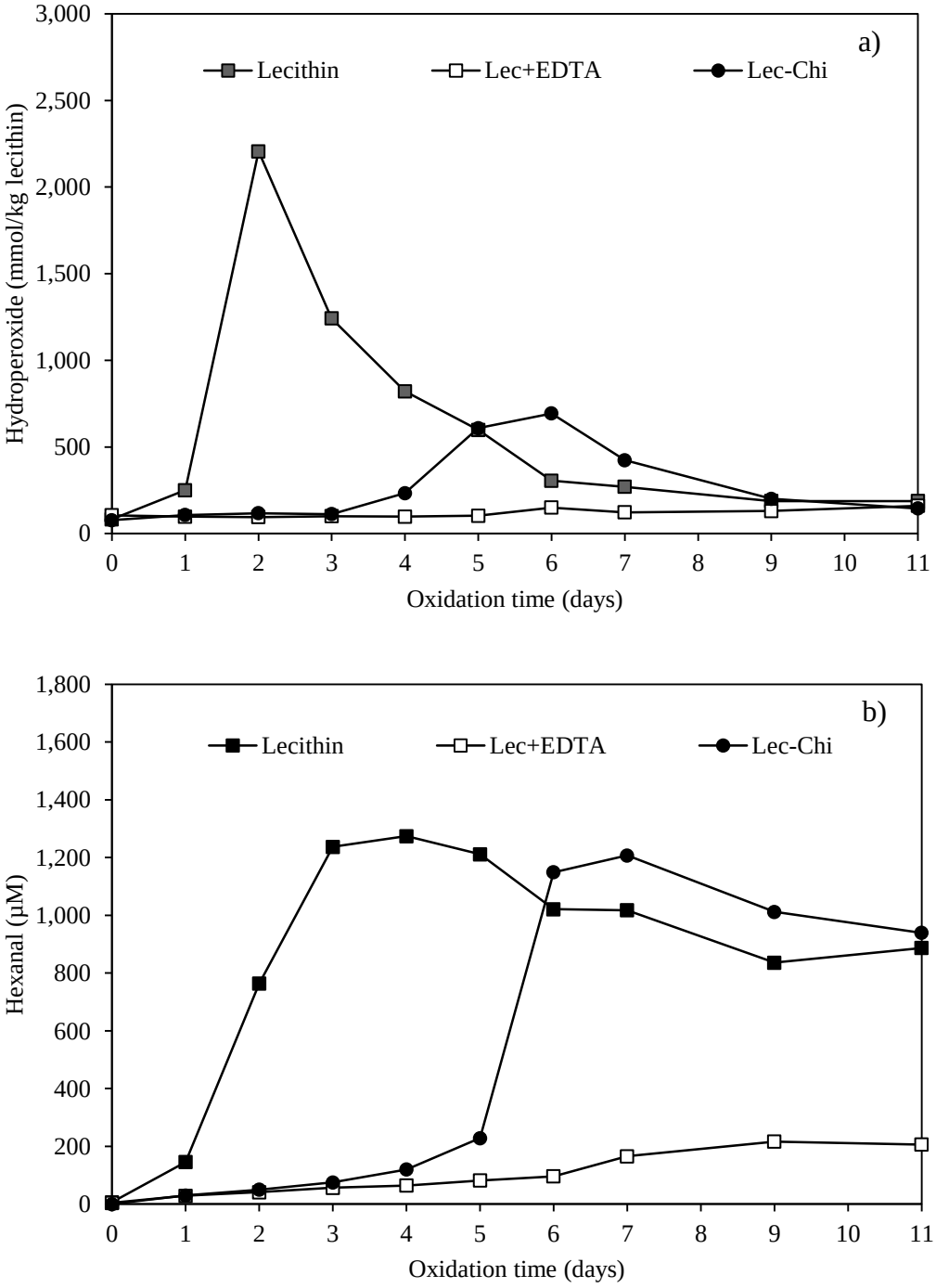


Figure 3.2 Oxidative stability of uncoated (lecithin with and without EDTA) and chitosan coated (lec-chi) liposomes during storage (pH 3.0 and 55^{o}C) as determined by formation of hydroperoxides (a) and hexanal (b)

3.3.2 Effect of rosmarinic acid ester on the oxidative stability of liposomes

Initial studies were conducted to determine if rosmarinic acid esters could increase the oxidative stability of uncoated and chitosan coated liposomes. Octadecyl rosmarinate was chosen for these initial studies since phenolic esters with 18 carbon chains have been found to inhibit lipid oxidation in oil-in-water emulsions (*16*). As shown in Figure 3.3a and b, octadecyl rosmarinate (40 µM) did not increase the oxidative stability of uncoated liposomes as determined by both lipid hydroperoxide and hexanal formation. However, the combination of octadecyl rosmarinate and chitosan coating very effectively inhibited oxidation with the lag phase of lipid hydroperoxide and hexanal formation increasing to 10 and 14 days, respectively, compared to a lag phase of 4 days for hydroperoxides and 7 days for hexanal for the chitosan coating alone. The synergistic antioxidant activity of octadecyl rosmarinate and chitosan coating could be due to the ability of chitosan to decrease the reactivity of transition metals by providing a cationic barrier that decreases metal-lipid hydroperoxide interactions. By decreasing metal-lipid hydroperoxide interactions, fewer free radicals would be generated thus sparing the octadecyl rosmarinate from rapid degradation as seen in the uncoated liposomes. Thus, in the chitosan-coated liposomes, the octadecyl rosmarinate concentrations would remain higher for a longer period of time and thus be more effective at inhibiting free radical promoted lipid oxidation.

The impact of increasing concentrations of the octadecyl rosmarinate on the oxidative stability of the chitosan coated liposomes was also determined. The lag phase of lipid hydroperoxide formation in chitosan coated liposomes was 10, 10 and 12 days for

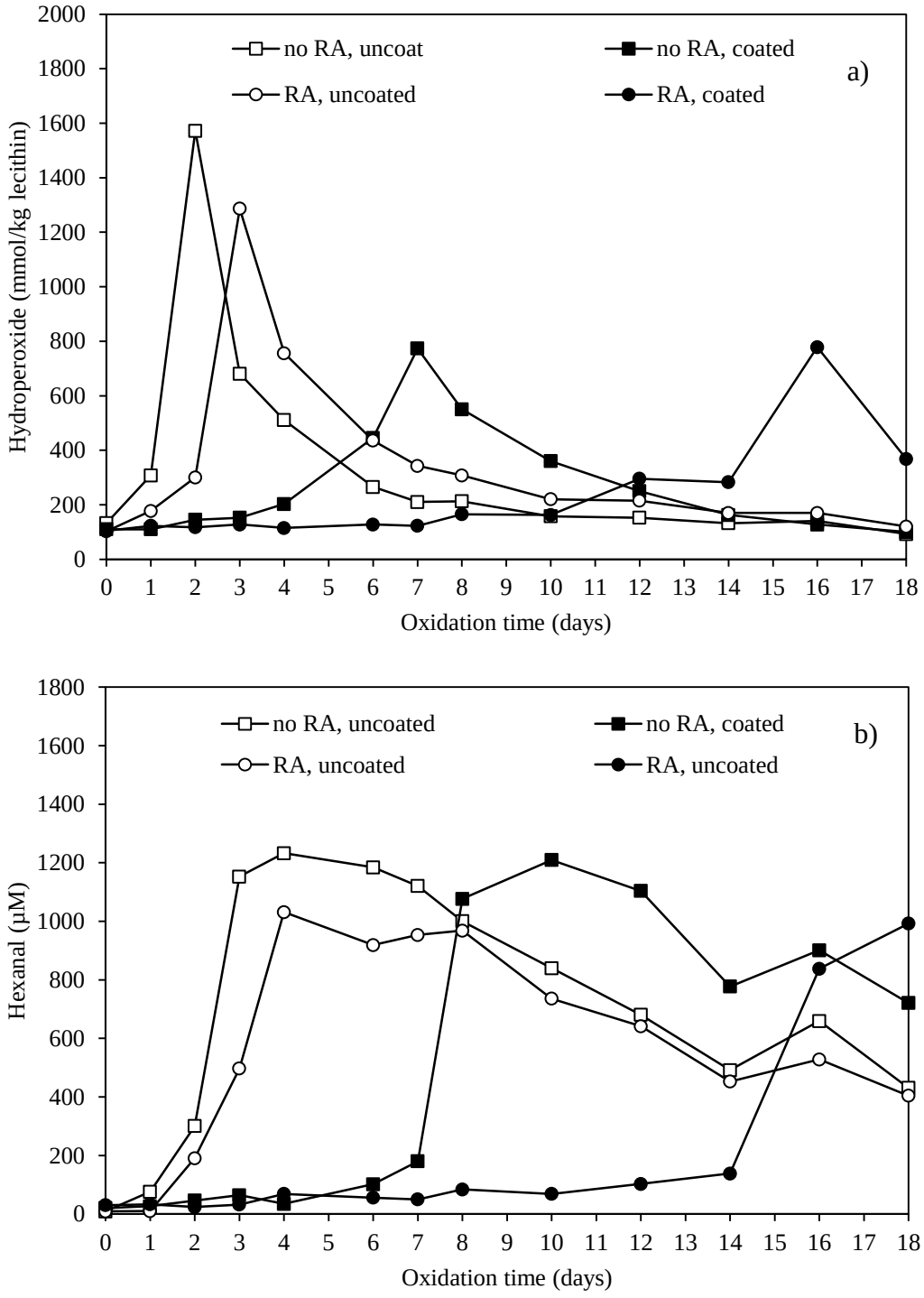


Figure 3.3 Oxidative stability of uncoated and chitosan coated liposomes in the presence and absence of 40 μM octadecyl rosmarinic acid ester (RA) during storage at 55^{o}C at pH 3.0. Lipid oxidation was monitored by measuring hydroperoxide (a) and hexanal (b)

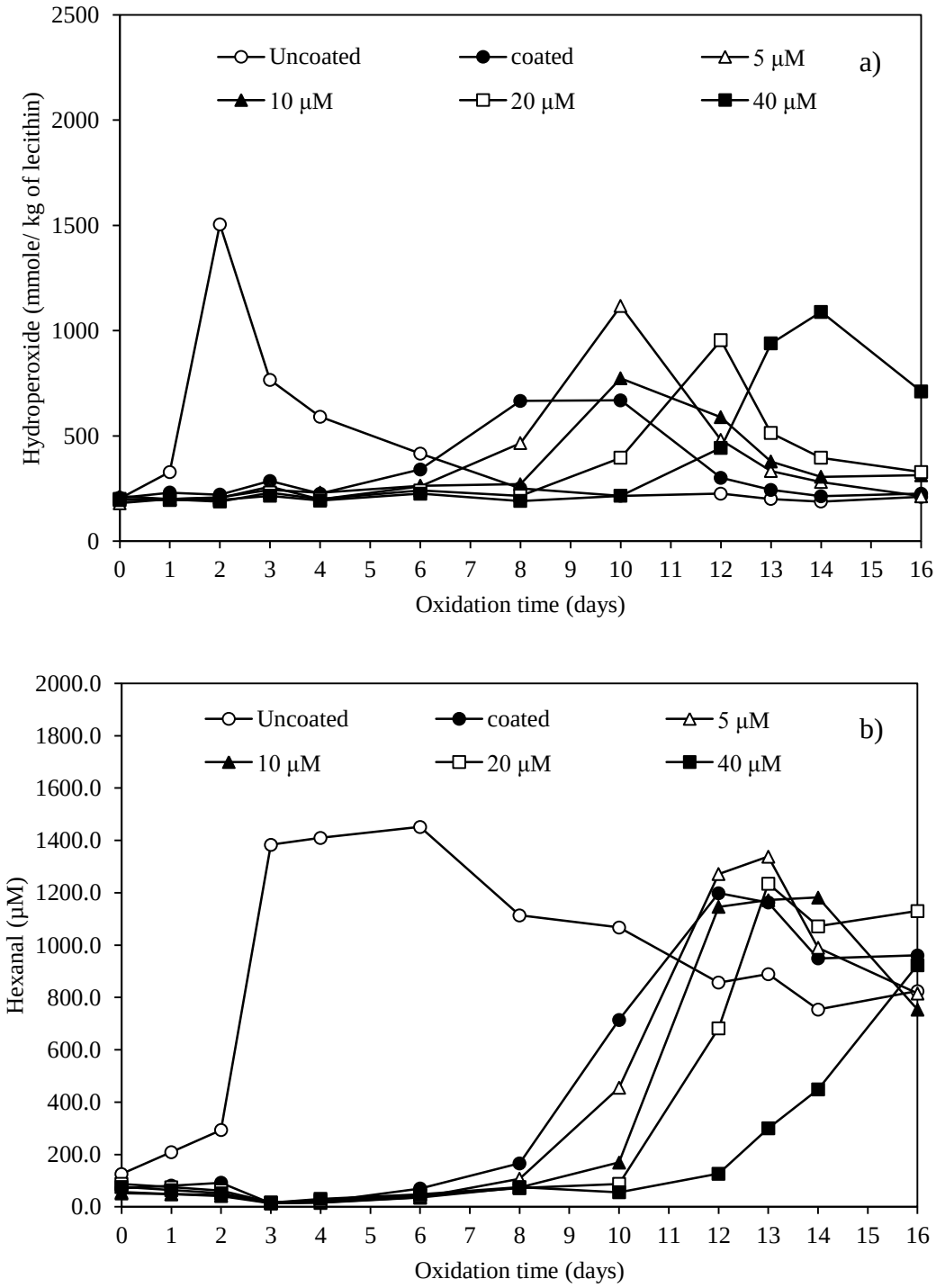


Figure 3.4 Influence of various concentrations of octadecyl rosmarinic acid ester on oxidative stability of chitosan coated liposomes during storage at 55^{o}C at pH 3.0. Lipid oxidation was monitored by measuring hydroperoxide (a) and hexanal (b)

10, 20 and 40 μM octadecyl rosmarinate, respectively (Figure 3.4a). Hexanal formation showed a similar trend with increasing octadecyl rosmarinate concentrations increasing the lag phase in chitosan coated liposomes to 8, 8 and 10 days for 10, 20 and 40 μM, respectively (Figure 3.4b). Addition of 5 μM octadecyl rosmarinate did not inhibit either lipid hydroperoxide or hexanal formation in chitosan coated liposomes.

3.3.3 Effect of rosmarinic acid esters hydrocarbon length on the oxidative stability of coated liposomes

The effects of the hydrocarbon chain length of rosmarinic acid esters on the ability of the rosmarinate esters to inhibit lipid oxidation in the coated liposomes were determined at a molar antioxidant concentration of 40 μM. Figure 3.5a and b show a non-linear relationship between the chain length and antioxidative activity of rosmarinate esters. For lipid hydroperoxide formation, the lag phases were 18, 21, 18, 18, 14 days in chitosan coated liposomes for rosmarinic acid, butyl rosmarinate, dodecyl rosmarinate, octadecyl rosmarinate, and eicosyl rosmarinate, respectively. A similar trend was observed for hexanal formation with lag phases in the chitosan coated liposomes of 17, 21, 18, 16 and 10, respectively. Overall, butyl rosmarinate was the most effective while eicosyl rosmarinate was the least effective. Addition of dodecyl or octadecyl hydrocarbons onto the rosmarinic acid did not improve antioxidant activity compared to rosmarinic acid alone.

This non-linear relationship between antioxidant polarity and antioxidant activity has also been reported in other esters of antioxidants in various systems. Takahashi et al.

(*201*) showed nonlinear effects of length of fatty acid ester side chains of L-ascorbic acid on their antioxidative activity in liposomal membranes. In addition, fatty acid esters of gallic acid exhibited non linear relation between chain length and antioxidative activity in SDS, partially hydrolyzed lecithin, and Brij 58 stabilized oil-in-water emulsions (*161*). Various explanations have been proposed in order to explain this phenomenon. In the case of gallate esters, their lower antioxidative activity with increasing number and length of hydrocarbon chain may be explained by reduction of the intramembrane and intermembrane mobilities of antioxidant esters due to an increase in hydrophobic interaction with the surfactant or phospholipid membrane (*161, 201*). Results suggested that rosmarinic acid required at least 4 carbons on the side chain in order to obtain enough hydrophobicity to penetrate into the palisade layer of the liposomal membrane. Interestingly, the same was true for gallate esters in partially hydrolyzed lecithin stabilized oil-in-water emulsions as reported by Stockman et al (*161*). Even though there were differences in the studied systems (emulsions vs. liposomes), both systems had phospholipids in the interfacial region bordering the aqueous phase. The observed decrease in the antioxidative activity of eicosyl rosmarinate has also been reported for chlorogenic acid esterified to long chain hydrocarbons (≥ C18) in oil-in-water emulsions (*16*).

The antioxidant polar paradox hypothesis states that non-polar antioxidants are more effective in lipid dispersion since they are more highly retained in the lipid phase where oxidation is most prevalent (*4, 202*). To determine if there was a correlation between antioxidant location and antioxidant activity in the chitosan coated liposomes, the concentration of the rosmarinic acid derivatives in the aqueous phase of the liposome

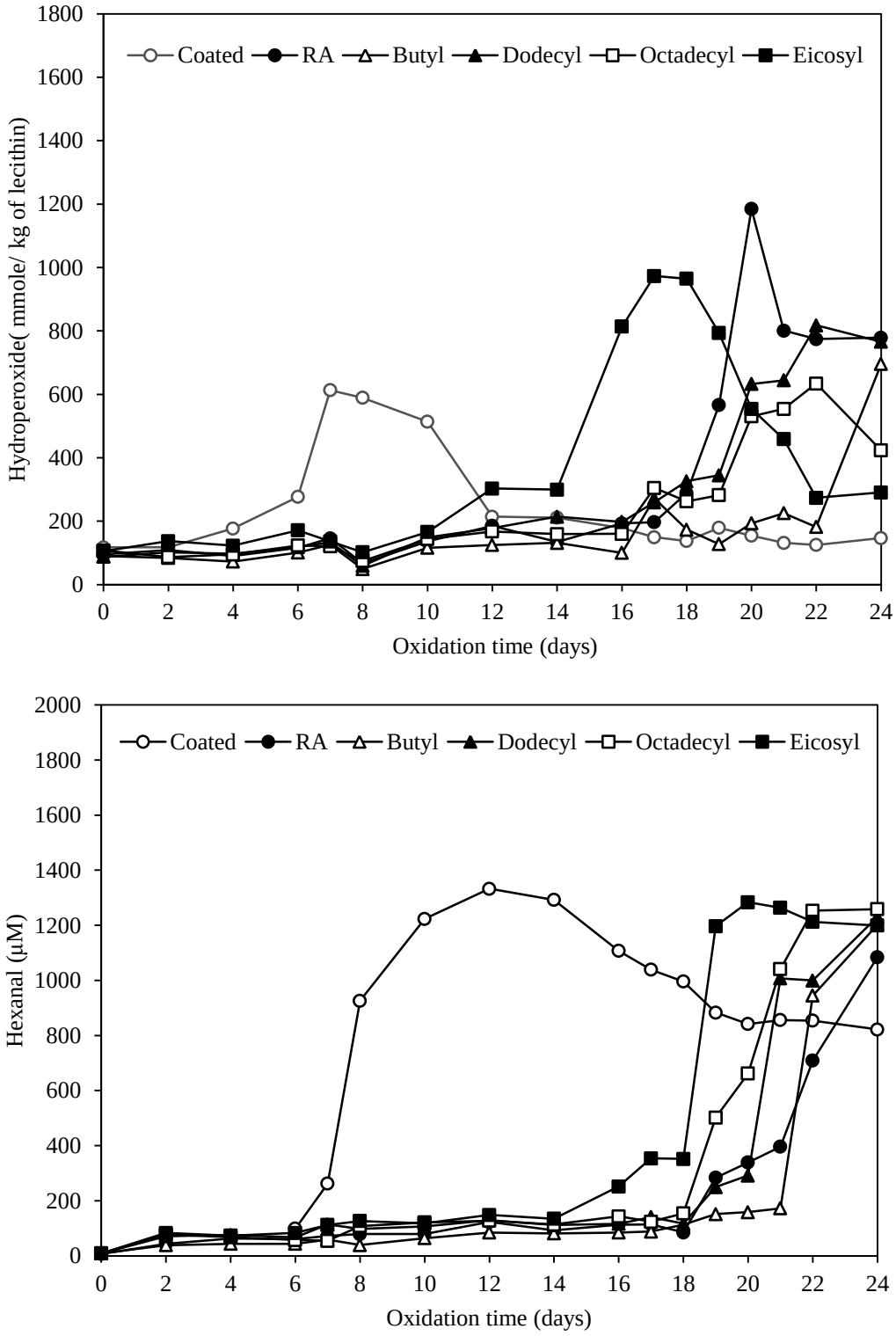


Figure 3.5 Influence of different aliphatic side chains on rosmarinic acid esters on oxidative stability of chitosan coated liposomes during storage (pH 3.0 and 55^{o}C). Lipid oxidation was monitored by measuring hydroperoxide (a) and hexanal (b)

system was determined (Figure 3.6). Overall, partitioning into the liposomes increased as the hydrocarbon chain length on the rosmarinic acid increased from 0 to 20 carbons. Rosmarinic acid showed a high affinity for the lipid phase in 10% o/w emulsions where 83% of the antioxidant partitioned into the oil phase compared to 30% of gallic acid in the oil phase (*203*). While statistically significant differences could be seen between the different rosmarinic acid esters, it should be noted that these differences were very small (< 2 μM). Such small differences in aqueous phase antioxidant concentrations suggest that the overall partitioning of the antioxidants into the liposomes is not responsible for differences in antioxidant activity. However, it is possible that the size of the hydrocarbon chain could impact the orientation and depth of the antioxidant in the lipid bi-layer which could impact its ability to scavenge free radicals (*161*).

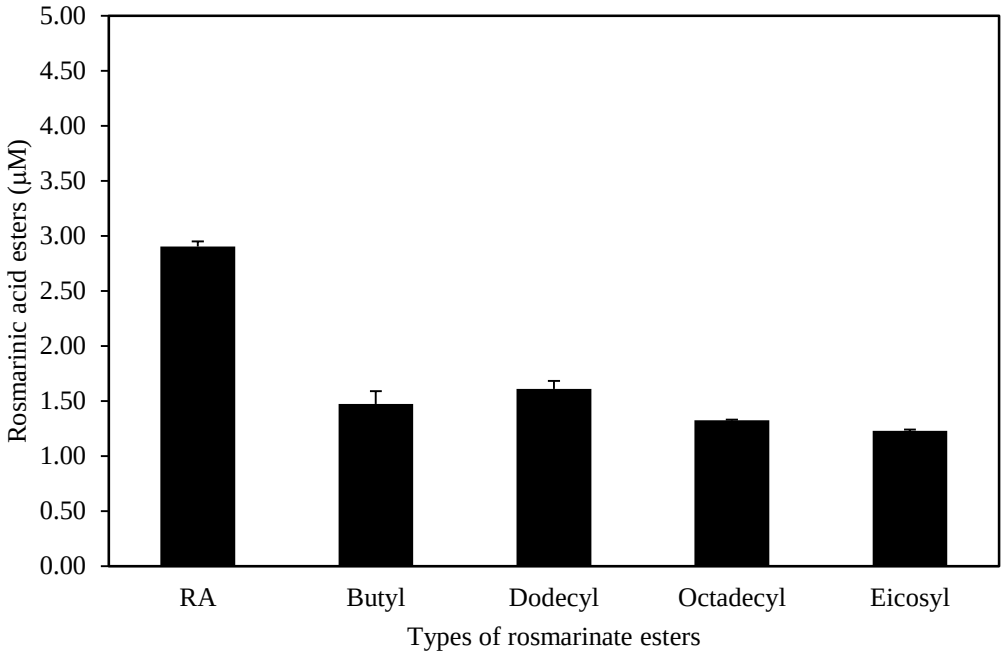


Figure 3.6 Concentration of rosmarinic acid and its esters in the aqueous phase of chitosan-coated liposomes

CHAPTER 4

AN INVESTIGATION OF THE VERSATILE ANTIOXIDANT MECHANISMS OF ACTION OF ROSMARINATE ALKYL ESTERS IN OIL-IN-WATER EMULSIONS

4.1 Introduction

Lipid oxidation in food and biological systems has been a concern in various fields of science because it is related to both food quality deterioration and health complications such as cardiovascular diseases and cancers. In the food industry, the use of free radical scavenging antioxidants is one of the main strategies to delay the occurrence of rancidity by inhibiting the initiation and propagation steps of lipid oxidation. Because of the complexity of the lipid oxidation process, the selection of antioxidants for various applications based on their intrinsic chemical properties, including free radical scavenging rate and stoichiometry of electron transfer, has proven to be inefficient for predicting antioxidant activity in real food systems (*2*).

Ideally, free radical scavengers should be located in the microenvironments where lipid radicals are generated for maximum effectiveness. With regard to this matter, the polar paradox hypothesis was developed in an attempt to predict the antioxidant activity of compounds based on their polarity in different lipid media (*3*, *202*). Accordingly, nonpolar antioxidants are more effective than their polar homologues in oil-in-water

emulsion. This hypothesis was later utilized by Frankel et al. (*4*) to explain how the physical location of free radical scavengers impacts their antioxidant activity in heterogeneous systems. Even though a number of studies seemingly confirmed the antioxidant polar paradox theory (*3, 4*). Several recent publications have shown cases where the polar paradox theory does not accurately predict antioxidant behavior.

A series of recent papers examined the activity of antioxidants (chlorogenic acid, rosmarinic acid, hydroxytyrosol, dehydrocaffeic acid and rutin) whose polarity was modified by esterification to alkyl chains of varying length (1-20 carbons) (*16-19*). In these studies, a nonlinear relationship of antioxidant effectiveness in oil-in-water emulsions as a function of polarity was observed presenting a challenge to the antioxidant polar paradox hypothesis. From a general perspective, as the alkyl chain length of the alkyl group was increased up to a medium chain length (typically 8-12 carbons), antioxidant activity increased in oil-in-water emulsions as would have been predicted by the polar paradox hypothesis. However, antioxidant activity then sharply declined with a further increase in the size of the alkyl chain even though this would have made the antioxidant even more nonpolar. One possible explanation of this cut-off effect is that the sudden decrease in the antioxidant activity with longer alkyl chains was due to their increased hydrophobicity which led to their partitioning into the oil phase rather than at the emulsion droplet interface. In addition, it has been suggested that the more nonpolar antioxidants might form mixed micelles with emulsifiers used to prepare the emulsions resulting in their migration away from the emulsion droplet (*16, 17, 20*). Unfortunately, there is limited information on how the length of the alkyl chain of esterified phenolics impacts their partitioning inside emulsion droplets or in mixed micelles.

In this research, we aimed to study the influence of esterification of rosmarinic acid on its ability to inhibit lipid oxidation in oil-water emulsions in relation to its free radical scavenging capacity, antioxidant activity in emulsions and interfacial partitioning behavior. Differences in stoichiometry of free radical scavenging of rosmarinic acid esters have been previously reported (*195*). To avoid bias due to differences in the ability of rosmarinic acid esters to transfer electrons or hydrogen atom to free radicals, oxidation studies were performed at equal DPPH scavenging activity. Influence of excess surfactants on antioxidant partitioning and antioxidant activity in oil-in-water emulsions was also performed to obtain useful information on how the various rosmarinic acid alkyl esters partitioned in co-micelles and emulsion droplets. In addition, the microenvironments and distribution of rosmarinic acid esters on the emulsion droplets were evaluated by front-face fluorescence and by measuring interactions with hexadecylbenzenediazonium tetrafluoroborate (16-ArN_2BF_4).

4.2 Materials and methods

Soybean oil was purchased from a local grocery market in Amherst, MA. Ethylenediaminetetraacetic acid (EDTA) disodium salt was purchased from Chempure ™ Ultra (Houston, Texas). Brij®35, acetonitrile, methanol, hydrochloric acid were obtained from Fisher Scientific (Pittsburgh, PA). Rosmarinic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH•), $FeSO_4$, Tween 20™, BaCl2, phosphoric acid, sodium phosphate mono and dibasic and N-(1-naphthyl) ethylenediamine dihydrochloride (NED) were purchased from Sigma-Aldrich (St. Louis, MO). 4-hexadecylbenzenediazonium tetrafluoroborate (16-

ArN_2BF_4) was prepared from commercial 4-hexadecylaniline (Aldrich, 97%) by diazotization according to (10, 11). Miglyol 812 (medium chain triglycerides; MCT) was purchased from Sasol (Witten, Germany). Double-distilled and deionized water was used for the preparation of all solutions.

4.2.1 Synthesis of Rosmarinate Esters

The chemoenzymatic esterification of rosmarinic acid to obtain rosmarinate esters was performed as the same method in the section 3.2.2.

4.2.2 DPPH Scavenging Activity

The free radical scavenging activity of rosmarinate esters was determined using the modified DPPH• free radical method as previously described by Alamed and coworkers (*2*) with some modifications. Stock solution (50 μL) of the test compounds in methanol were mixed with 1.5 mL of 50 μM methanolic DPPH• solution to make the final antioxidant concentrations of 10-100 μM. Loss of DPPH• after 1 hr was measured at 515 nm using an Ultrospec 3000 pro UV/visible spectrophotometer (Biochrom Ltd., Cambridge, England). The exact DPPH• concentration at the completion of the reaction was determined using a DPPH• standard curve. The free radical scavenging activity of rosmarinate esters were compared with the activity of α-tocopherol in methanol.

4.2.3 Emulsion Preparation

Stripped soybean oil was prepared according to the method of Waraho et al. (*204*). The effectiveness of stripping was monitored by measuring the removal of tocopherols by HPLC (13). Oil-in-water (O/W) emulsions were prepared using 1.0% (wt) stripped soybean oil in a 10 mM phosphate buffer solution (pH 7.0). Tween 20 was used as an emulsifier at a 1:10 emulsifier/oil ratio. Stripped soybean oil, Tween 20, and phosphate buffer were added to a beaker, and a coarse emulsion was made by blending with a hand-held homogenizer (M133/1281-0, Biospec Products, Inc., Bartlesville, OK) for 2 min. The coarse emulsion was then homogenized with a microfluidizer (Microfluidics, Newton, MA) at a pressure of 9 kbar for three passes.

After the O/W emulsion was prepared, rosmarinic acid and its esters with various chain lengths (4, 8, 12, 18, and 20 carbons) in methanol were added to the emulsion at a final concentration of 30 μM and stirred for 1 h at room temperature. Samples without addition of the antioxidant were used as control samples. The emulsions (0.5 mL) were transferred into 10 mL GC vials and sealed with (tetrafluoroethylene) butyl rubber septa, and then stored at 25^{o}C in the dark. Three vials of each treatment were taken every day to determine lipid hydroperoxides and hexanal formation.

In some studies, emulsions were washed to remove aqueous phase surfactants as previously described by Faraji and coworkers (*205*) with some modifications. In short, emulsions were centrifuged at 38,518 g (17,000 rpm) for 1 h at 4 ^{o}C using a Fiberlite® F40L-8x100 rotor with a high-speed centrifuge (Thermo scientific WX Ultra 80, Asheville, NC). After the centrifugation, the bottom suspension (phosphate buffer) was

carefully removed using a needle and syringe, and then the same volume of the fresh phosphate buffer was used to re-disperse the creamed emulsion droplet layer by vortexing. This washing procedure was performed a total of three times. The lipid content of the final washed emulsion was determined by the modified Bligh and Dyer method (*206*) and then phosphate buffer was used to adjusted the lipid content back to 1% (w/w).

4.2.4 Measurements of Lipid Hydroperoxides

Lipid hydroperoxide formation in emulsion solutions was determined according to the method described by Panya and coworkers (*207*) with some modifications. Emulsion solutions (0.2 mL) were mixed with 1.5 mL of isooctane/2-propanol (3:1 v/v) and vortexed (10 s, three times). After centrifugation at 1000 g for 2 min, 30 μL of the organic solvent phase was mixed with 1.5 mL of methanol/1-butanol (2:1). Hydroperoxide detection was started by the addition of 7.5 μL of 3.94M ammonium thiocyanate and 7.5 μL of ferrous iron solution (prepared by adding equal amounts of 0.132 M BaCl2 and 0.144 M FeSO4). After 20 min of incubation at room temperature, the absorbance was measured at 510 nm using a UV-Vis spectrophotometer (Genesys 20, Thermo Spectronic). Hydroperoxide concentrations were determined using a standard curve prepared from hydrogen peroxide.

4.2.5 Measurement of Hexanal

Measurement of headspace hexanal was determined as the same method in the section 3.2.6

4.2.6 Determination of Antioxidant Partitioning

Determination of the physical location of rosmarinic acid and its esters in the emulsions were performed according to the procedure described by Panya and coworkers (*207*). Regular O/W emulsions and washed emulsions with added surfactants (0, 0.1, 0.5, 1.0, and 2.5%; w/w) were prepared with 10 mM phosphate buffer including 200 µM ETDA to minimize oxidation during analysis. Rosmarinic acid and its alkyl esters in methanol were added to the emulsion at a final concentration of 100 µM followed by stirring at room temperature for 1 h. The emulsions were centrifuged at 162102 g (46,000 rpm) for 1 h at 4^{o}C using a PTI F65L-6x13.5 rotor with a high-speed centrifuge (Thermo scientific WX Ultra 80, Asheville, NC). The aqueous phase was carefully collected with a pipette and the amounts of rosmarinic acid esters in the aqueous phase were determined directly by HPLC using a modified method described by Lecomte and coworkers (*207*).

Briefly, HPLC determination of rosmarinic acid and its esters was carried out with a Hypersil gold C18 reversed phase column (250 mm x 4.6 mm, 5 µm) equipped with a Hypersil gold guard column (10 mm x 4 mm, 5 µm) (Thermo scientific, USA) using a LC-10ATvp HPLC system (Shimadzu, USA). Peak integration was performed using Shimadzu EZstart (Version 7.2). Gradient elution was performed using methanol

and 3 mM phosphoric acid at1 mL/min at 40^{o}C (column temperature), in linear gradients from 0/100 (v/v) to 100/0 (v/v) for 5 min, then 100/0 (v/v) for 10 min, back to 0/100 (v/v) in 5 min, and hold at 0/100 (v/v) for 5 min. Rosmarinic acid and its alkyl esters [(R4 (4 carbons) – R20 (20 carbons)] were detected with a photodiode array detector (SPD-M10Avp, Shimadzu, USA) at 328 nm. The concentrations of rosmarinic acid esters were calculated using a standard curve made from each rosmarinic acid ester dissolved in methanol.

4.2.7 Front-face fluorescence measurements

Front-face fluorescence of rosmarinic acid and its alkyl esters in O/W emulsions was determined by steady-state emission measurements recorded with a PTI spectrofluorometer (PTI, Ontario, Canada). Washed emulsions were prepared as above, but phosphate buffer including 200 μM EDTA was used in order to minimize oxidation and Brij 35 was employed instead of Tween 20.Brij 35 was used because Tween 20 contained fluorescent components that interfered with the fluorescence signal of the rosmarinic acid derivatives. A 10% (w/w) Brij 35 solution in 10 mM phosphate buffer with 200 μM EDTA was added into the washed emulsions to obtain surfactant concentrations of 0, 0.1, 0.5, 1.0, and 2.5% (w/w). All antioxidants were used at the concentration of 30 μM in the final emulsions. Samples (1.5 mL) were transferred into triangular suprasil cuvettes. The samples were held at 30^{o}C and stirred with a 3 mm magnetic stirring bar (Fisher scientific, USA). Emission was observed at 90^{o} to the incident beam, that is, 22.5^{o} with respect to the illuminated cell surface. The emissions of

the rosmarinic acid and its esters were scanned from 370 to 470 nm at the excitation wavelength of 323 nm. Spectral bandwidth for both excitation and emission slits was 2.0 nm, integration time was 1 s, and the wavelength increment was 2.5 nm. The intensity of the spectra was determined as the emission signal intensity (counts per second) measured by means of a photomultiplies.

4.2.8 Determination of Interfacial Rosmarinate Esters

The existence of rosmarinic acid esters in the interface of emulsion droplets and surfactant micelles was determined using 4-hexadecylbenzenediazonium ions, 16-ArN_2^+. Medium chain triglycerides (1% w/w) oil-in-water emulsions stabilized with 0.1% (w/w) Tween 20 were prepared as described above. Freshly prepared emulsion (1 mL) was transferred into test tubes and 20 µL of the stock solution (30 mM) of rosmarinate esters in methanol (R4, R12, and R20) were added by vortexing for 1 min and placing in a sonicating water bath for 30 min at 25 ^oC. The reaction between rosmarinate esters and 16-ArN_2^+ was measured as described by Sánchez-Paz and coworkers (*101*). In brief, 10 µL of the 16-ArN_2^+ stock solution in acetonitrile (0.017 M) was added to rosmarinic acid containing emulsions at specific time intervals and then the reaction mixtures (40 µL) were transferred into 1 mL of a 0.01 M ethanolic solution of NED at 25^oC. Final concentrations of rosmarinate esters, 16-ArN_2^+, and NED were 30 µM, 170 µM and 0.01 M respectively. The reaction mixtures were incubated for 20 min. The NED azo dye formation was determined spectrophotometrically at 572 nm using an Ultrospec 3000 pro UV/visible spectrophotometer (Biochrom Ltd., Cambridge, England). Rate constants

were obtained from slope of the consumption of 16-ArN_2^+ during the first 5 min of the reaction in the presence of low surfactant (0%) concentrations and during the first 12 hr in the presence of high surfactant (2.5%) concentrations. Results were presented as secondary rate constants.

4.2.9 Statistical Analysis

All analyses were performed on triplicate samples. Oxidation lag phases were defined as the first data point significantly greater than the 0 time value. In all cases, comparisons of the means were performed using Duncan's multiple-range tests. A significance level of $p<0.05$ was defined as being statistically different. All calculations were performed using SPSS17 (http://www.spss.com; SPSS Inc., Chicago, IL).

4.3 Results and Discussion

4.3.1 Influence of esterification on the DPPH scavenging activity of rosmarinic acid

DPPH scavenging activity of the rosmarinate esters was performed in this study so subsequent lipid oxidation studies in oil-in-water emulsions could be performed at antioxidant concentrations representing equal free radical scavenging activity (Figure 4.1). The rosmarinate esters exhibited approximately 10-14 times more DPPH scavenging activity than α-tocopherol (data not shown). A non-linear behavior of DPPH scavenging activity by rosmarinate esters was observed with maximum DPPH scavenging activity with the octyl and dodecyl rosmarinic acid esters (R8-R12). There was not a statistical

difference ($p>0.05$) between the octyl- and dodecyl rosmarinic acid esters. This trend was also observed by Lecomte and coworkers (*195*) who found that dodecyl rosmarinate had the greatest DPPH scavenging activity of all the esters tested (4-20 carbons). A similar non-linear trend was also reported by Lopez-Giraldo and corkers (*208*) who found that butyl, and octyl chlorogenate esters had higher DPPH scavenging activity than chlorogenic acid itself and its esters with alkyl chains longer than 12 carbons.

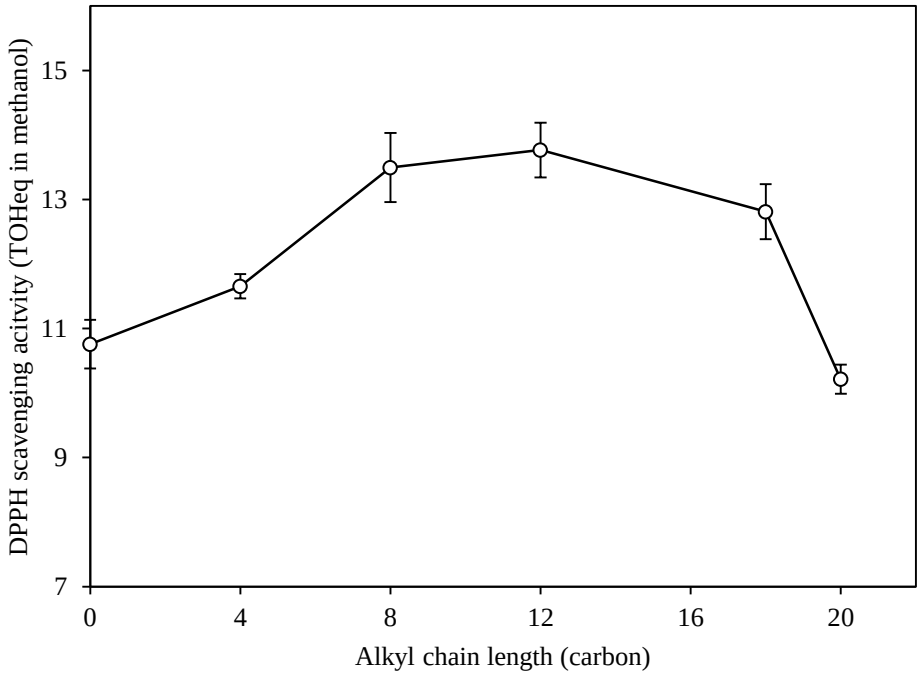


Figure 4.1 DPPH scavenging activity of the rosmarinic acid and its alkyl esters in methanol. Data represents means (n=3) ± standard deviations

4.3.2 Effects of alkyl chain lengths of rosmarinates on oxidation stability of stripped soybean oil-in water (O/W) emulsions

Due to the observed non-linear DPPH scavenging activity of the rosmarinic acid esters, the ability of the rosmarinic acid esters to inhibit lipid oxidation in oil-in-water

emulsions was tested at both equal molar concentrations (Figure 4.2) and equal DPPH scavenging activity concentrations (Figure 4.3). For the equal DPPH scavenging activity experiments, the concentrations of the esters were normalized based on the DPPH scavenging activity of the R12 ester which had the highest level of activity. Hydroperoxide and hexanal formation in the O/W emulsions were investigated during storage at 25 ^{o}C in the dark. All forms of the rosmarinic acid were able to inhibit the formation of hydroperoxides and hexanal compared to the control (Figure 4.2 and 4.3).

Results indicated there were no major differences in the trend of each ester to inhibit hydroperoxide or hexanal formation when determined on an equal molar or DPPH scavenging activity basis. Differences in the ability of the different esters to inhibit hexanal formation will be discussed below since volatile lipid oxidation products such as hexanal are more strongly related to rancidity development than lipid hydroperoxides. R4 was slightly better than R8 and R12 in increasing hexanal lag times at both equal molar (Figure 4.2B) and DPPH scavenging activity (Figure 4.3B). The R20 ester was consistently the worst of the antioxidants and R18 tended to be slightly better than R0 (unesterified) rosmarinic acid. A similar decrease in antioxidant activity was observed when the alkyl chain lengths of the rosmarinate esters were increased above 8 carbon chain lengths in tung oil-in-water emulsions (*17, 207*) while in chitosan coated liposomes the R4 ester exhibited the best antioxidant activity, although the octyl rosmarinate was not evaluated in this study, (*207*).

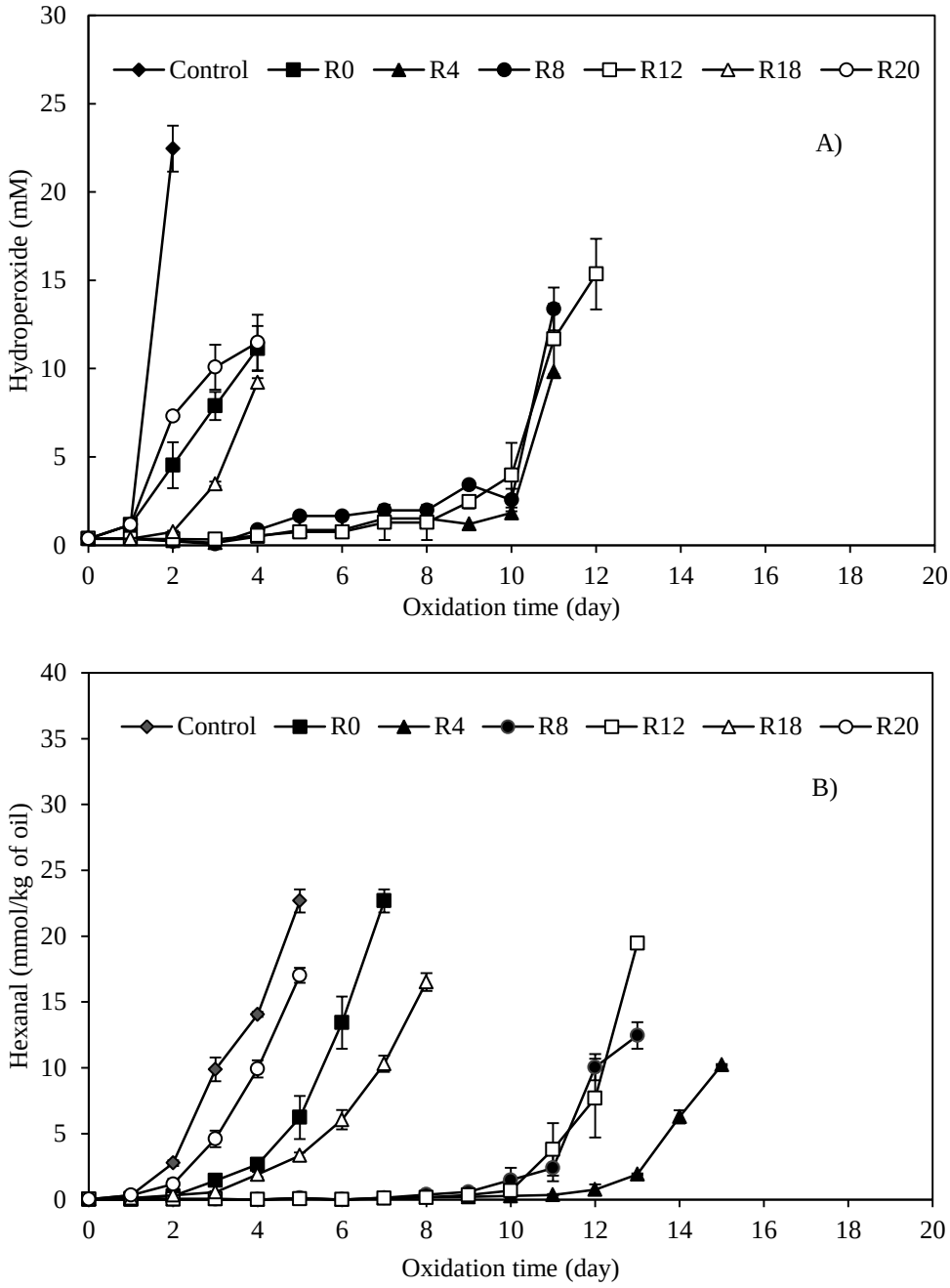


Figure 4.2 Hydroperoxide (A) and hexanal (B) formation in 1% stripped soybean oil-Tween 20 emulsions at 25^{o}C in the presence of rosmarinic acid and its alkyl esters at equivalent molarities (30μM). Data represents means (n=3) ± standard deviations.

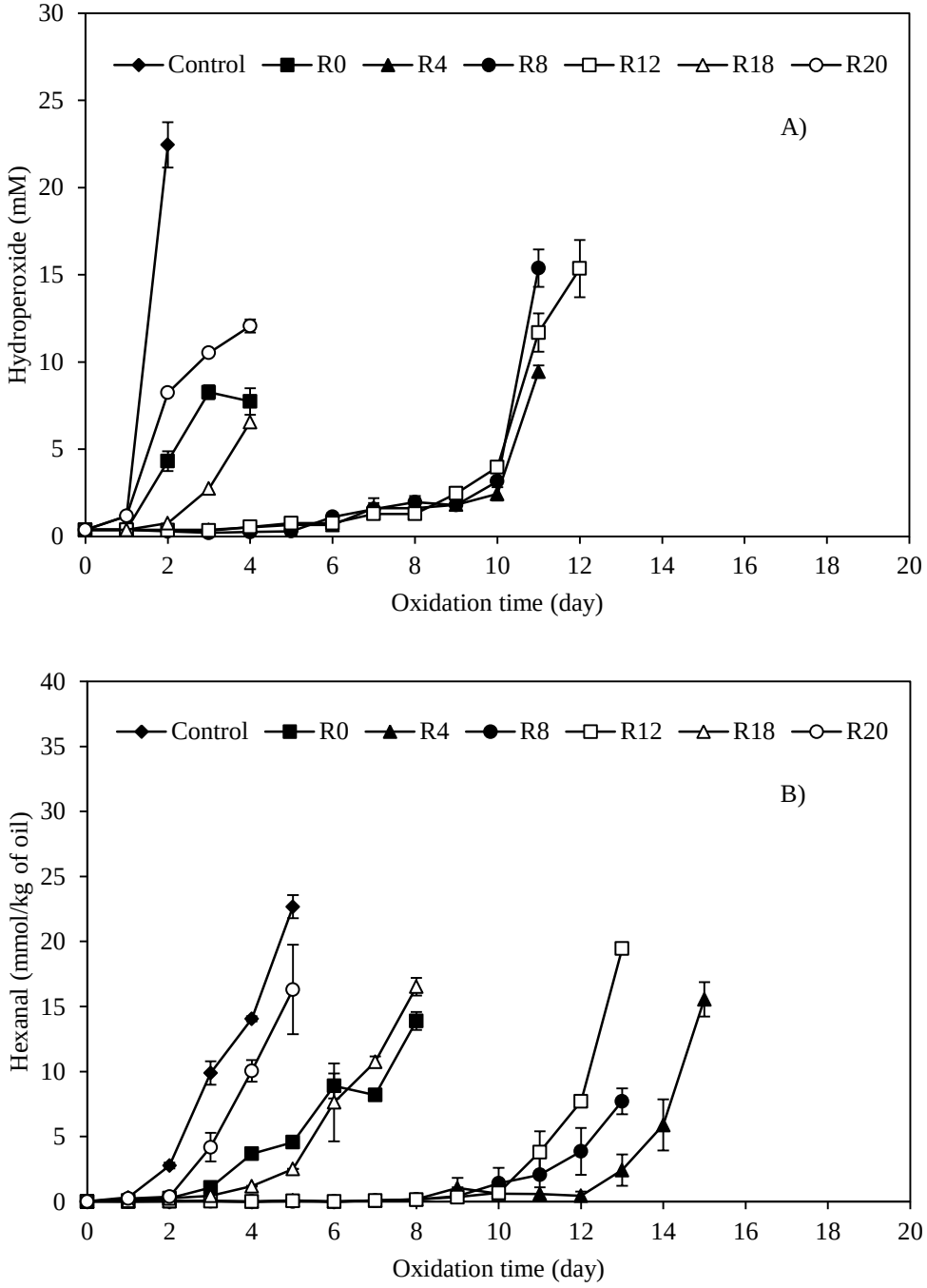


Figure 4.3 Hydroperoxide (A) and hexanal (B) formation in 1% stripped soybean oil-Tween 20 emulsions at 25^{o}C in the presence of rosmarinic acid and its alkyl esters at equivalent DPPH radical scavenging activity. Data represents means (n=3) ± standard deviations.

4.3.3 Effect of Tween 20 micelles on the physical location and antioxidant activity of the rosmarinic acid esters in stripped soybean O/W emulsions

The antioxidant polar paradox hypothesis states that nonpolar antioxidants are more effective than polar antioxidants in oil-in-water emulsions presumably due to the greater retention of the nonpolar antioxidants in the interface (*4*). However, the above results with the rosmarinic acid esters as well as the previous work of others (*16-19, 207*) does not support the concept of the antioxidant polar paradox hypothesis for oil-in-water emulsions because the most nonpolar antioxidants (R18 and R20) had lower antioxidant activity than their more polar homologues (R0, R4, R8 and R12). Our results thus confirm the cut-off hypothesis put forward by Laguerre et al. (*16*). One possible reason for this nonlinear influence of the alkyl chain length could be the ability of the esters to partition into the aqueous phase of the emulsion either by forming micelles by themselves or via mixed micelles with Tween 20 not absorbed at the emulsion droplet surface (*17*). To better understand how the antioxidant activity and physical location of the rosmarinic acid esters is influenced by surfactant micelles, emulsions were first washed to remove Tween 20 not absorbed to the emulsion droplet interface. Surfactant micelles were then re-introduced into the emulsions by adding 0, 0.1, 0.5, 1.0, and 2.5% Tween 20 to the washed emulsions. Since Tween 20 has a low critical micelle concentration [<0.1 mM at 21^{o}C (17)], the majority of added Tween 20 would exist as surfactant micelles in the aqueous phase of the emulsion. In these experiments, R4, R12, and R20 esters were utilized to test antioxidant activity and partitioning. As illustrated in Figure 4.4A and B, increasing the surfactant and thus aqueous phase micelle concentrations did not impact the lag time of hydroperoxide and hexanal formation in the control (no added

antioxidant) emulsions. This suggests that the additional Tween 20 did not impact oxidation chemistry directly through mechanisms such as free radical scavenging. In emulsions containing R4 (Figure 4.5A and B) and R12 (Figure 4.6A and B) increasing Tween 20 micelles concentrations had no effect or decreased the lag phase for both hydroperoxide and hexanal formation. Surprisingly, the antioxidant activity of R20 increased with increasing concentrations of Tween 20 micelles as determined by both lipid hydroperoxides and headspace hexanal (Figure 4.7A and B). The lag time of hexanal formation in the presence of R20 was improved from 4 to10 days in emulsions containing 0% and 2.5% Tween 20, respectively. At 2.5% Tween 20, the differences among the lag time of all the rosmarinate esters had become similar with lag phases for hexanal formation of 11, 12 and 10 days for R4, R12 and R20, respectively (*16*). Richards et al (*209*) also reported that surfactant micelles from Brij 700) could increase the antioxidant activity of TBHQ in salmon oil-in-water emulsions.

To try to understand why the Tween 20 micelles had such varying effects on the antioxidant activity of the different rosmarinic acid alkyl esters, the impact of micelle concentration on antioxidant partitioning into the aqueous phase was determined (Figure 4.8). In emulsions with no added Tween 20, the amount of R4, R12 and R20 in the aqueous phase was similar ranging from 4.2 to 8.3%. Increasing added Tween 20 to 0.1% resulted in an increase in the aqueous phase concentration of all the rosmarinic acid esters but most dramatically with R20 whose aqueous phase concentration increased over 7.5 fold. The equilibrium distributions of all the rosmarinate esters became saturated at 1% Tween 20. At all added Tween 20 concentrations, R12 exhibited the highest

association with the emulsion droplets followed by R4 and R20, with aqueous phase concentrations

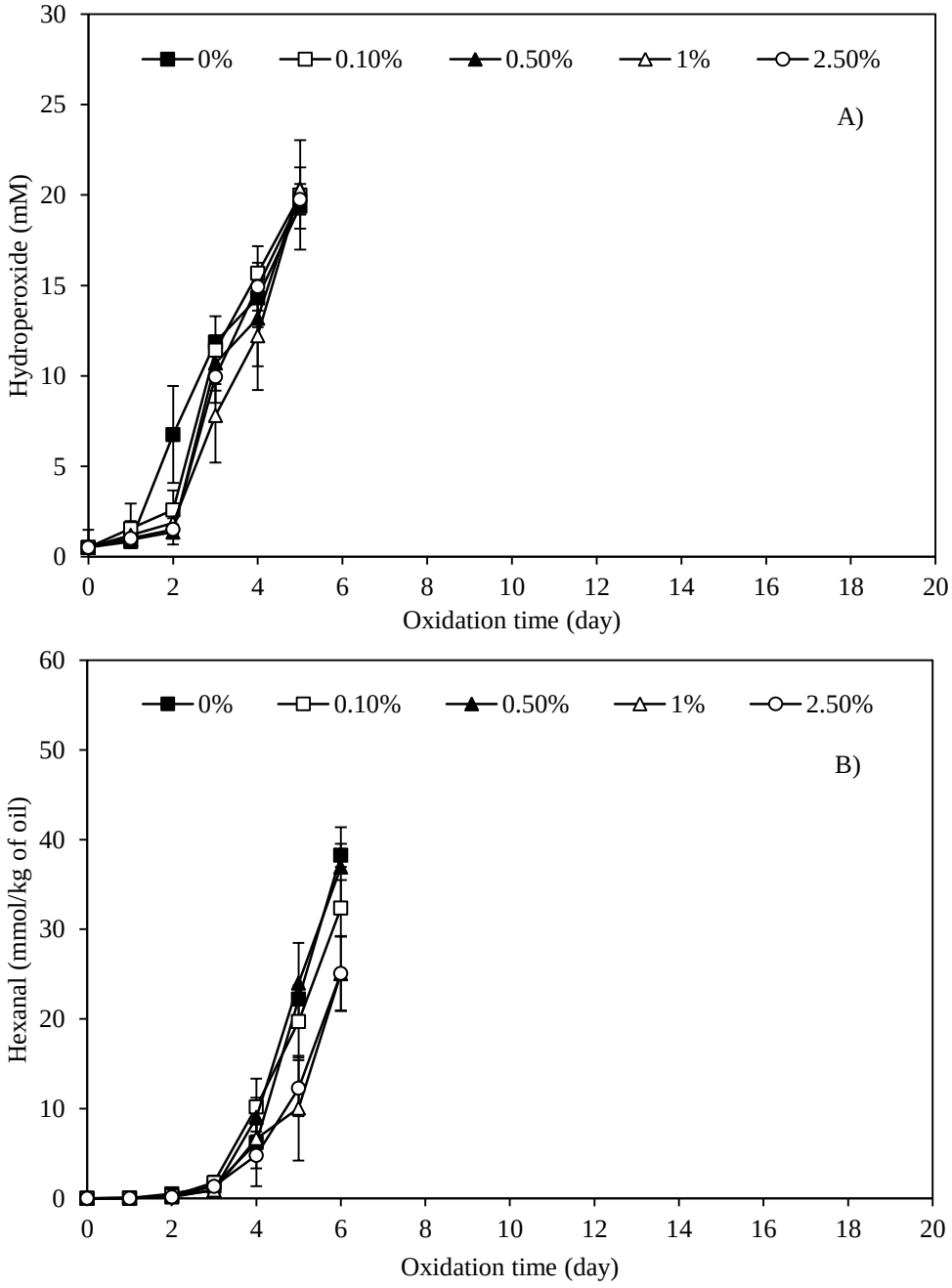


Figure 4.4 Hydroperoxide (A) and hexanal (B) formation in 1% stripped soybean oil-Tween 20 emulsions at 25^{o}C as a function of increasing Tween 20 concentrations (0.1, 0.5, 1.0, and 2.5% w/w). Data represents means (n=3) ± standard deviations.

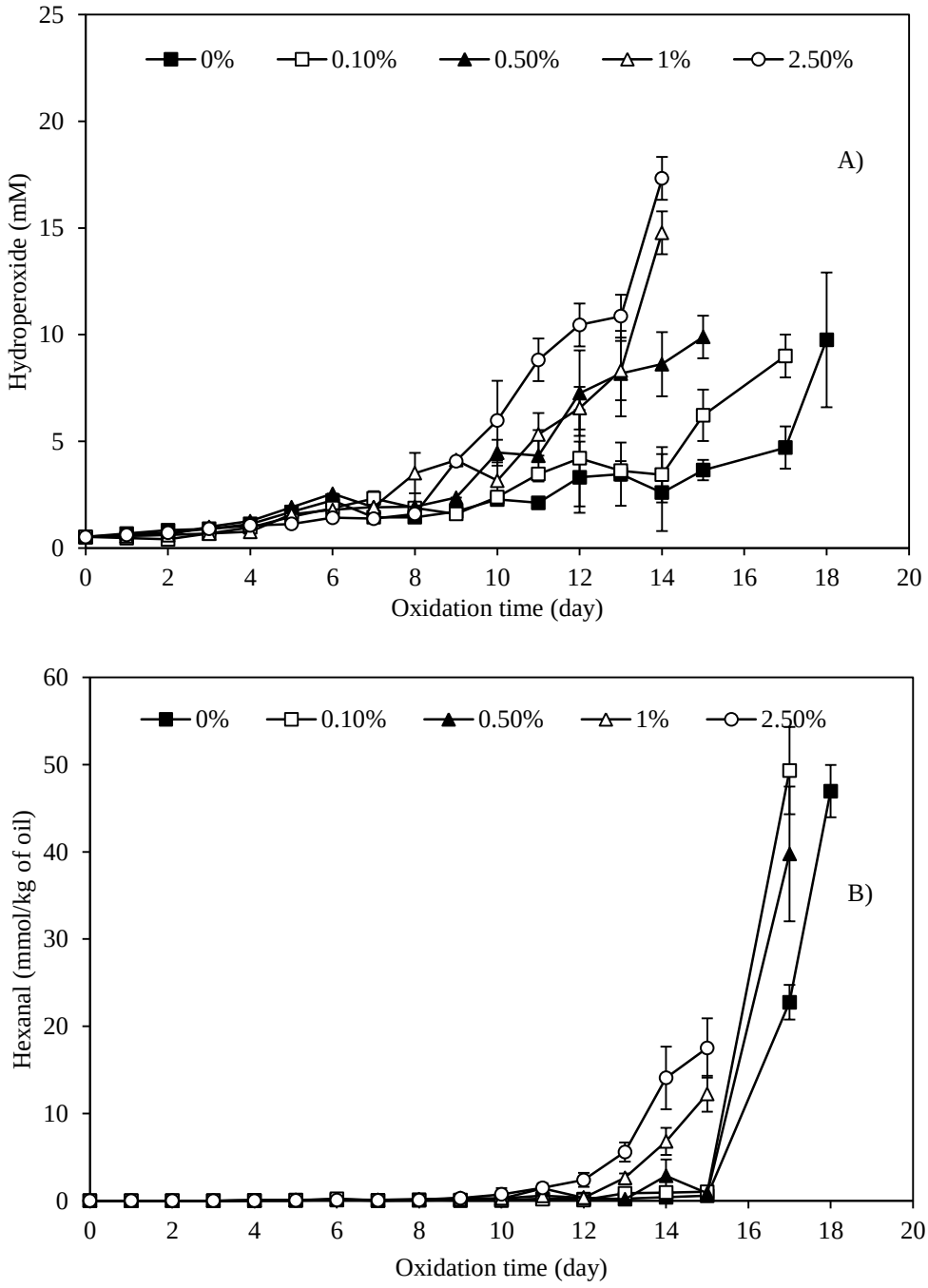


Figure 4.5 Hydroperoxide (A) and hexanal (B) formation in 1% stripped soybean oil-Tween 20 emulsions at 25^{o}C as a function of increasing Tween 20 concentrations (0.1, 0.5, 1.0, and 2.5% w/w) in the presence of butyl rosmarinate ester (R4). Data represents means (n=3) ± standard deviations.

of 52, 64, and 75%, respectively. The partitioning patterns for R4 and R12 could help explain why Tween 20 micelles decreased their antioxidant activity (Figures 4.5-4.6) as increasing surfactant micelle concentration increased the partitioning of the antioxidants into the aqueous phase and thus presumably prevented them from scavenging lipid radicals in the emulsion droplet. Unexpectedly, while R20 was the most prone to be removed from the emulsion droplets by the surfactant micelles, its antioxidant activity improved. Effect of surfactants on the partitioning esterified antioxidants has also been reported in sunflower oil emulsions with Brij 35 as the emulsifier however the reason for this phenomenon was not discussed in detail (*16*, *17*).

4.3.4 Measuring the chemical microenvironments of the rosmarinate esters in O/W emulsions by Front-Face Fluorescence.

In order to better understand the location of the rosmarinic acid alkyl esters in emulsions systems, their fluorescent properties were evaluated. As shown in Figure 4.9, the fluorescence emission spectra of R4 rosmarinate varied as a function of solvent polarities. For example, R4 exhibited relatively strong fluorescence intensity in isopropanol but had very low fluorescence emission in10 mM phosphate buffer. In hexadecane there was no fluorescence peak observed for R4. The highest level of R4 fluorescence was observed in Brij 35 micelles (Brij 35 was used instead of Tween 20 since Tween 20 contained fluorescent compounds that interfered with the signal of R4). Similar trends were observed with the R12 and R20 esters (data not shown). These data

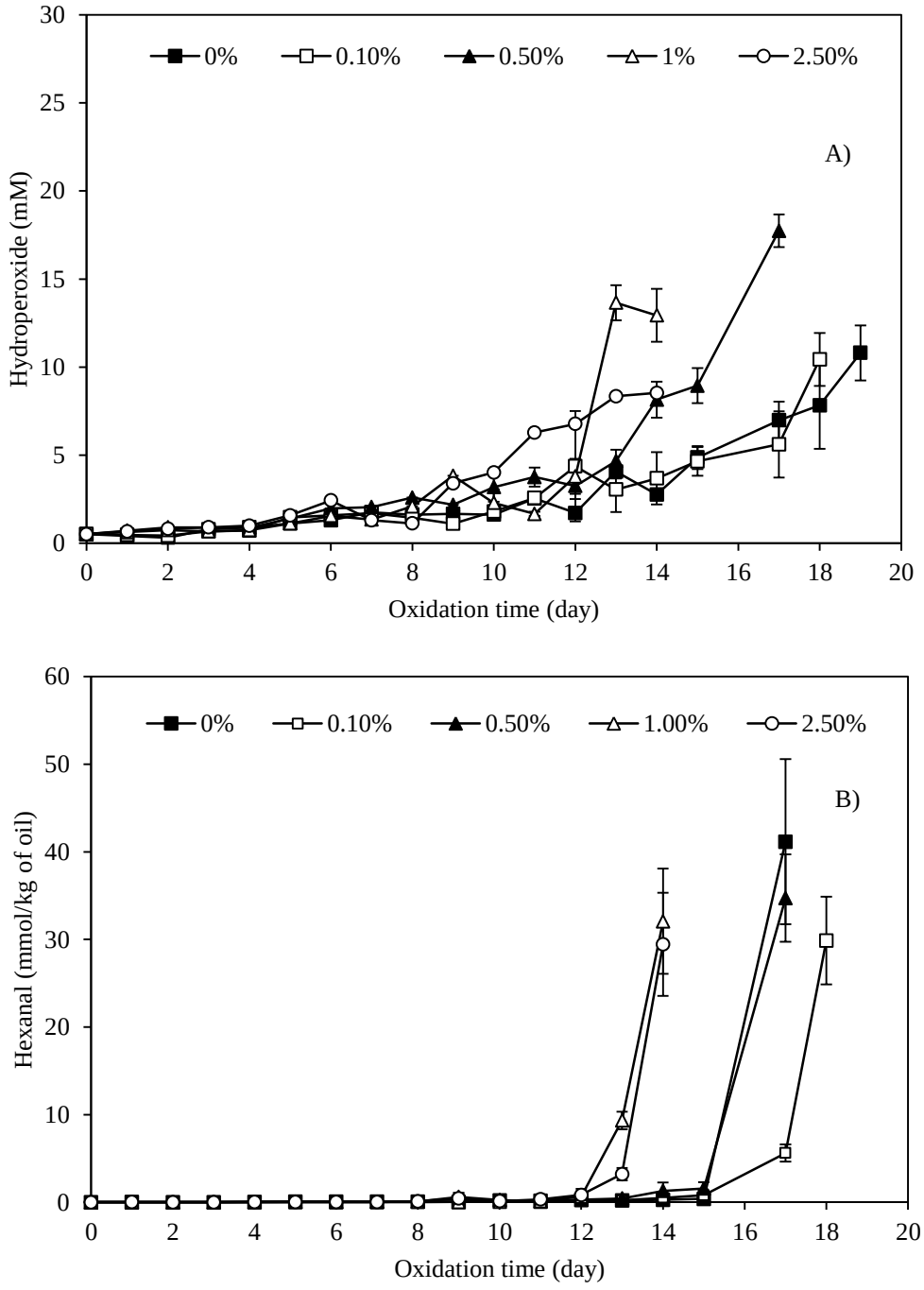


Figure 4.6 Hydroperoxide (A) and hexanal (B) formation in 1% stripped soybean oil-Tween 20 emulsions at 25^{o}C as a function of increasing Tween 20 concentrations (0.1, 0.5, 1.0, and 2.5% w/w) in the presence of dodecyl rosmarinate ester (R12). Data represents means (n=3) ± standard deviations.

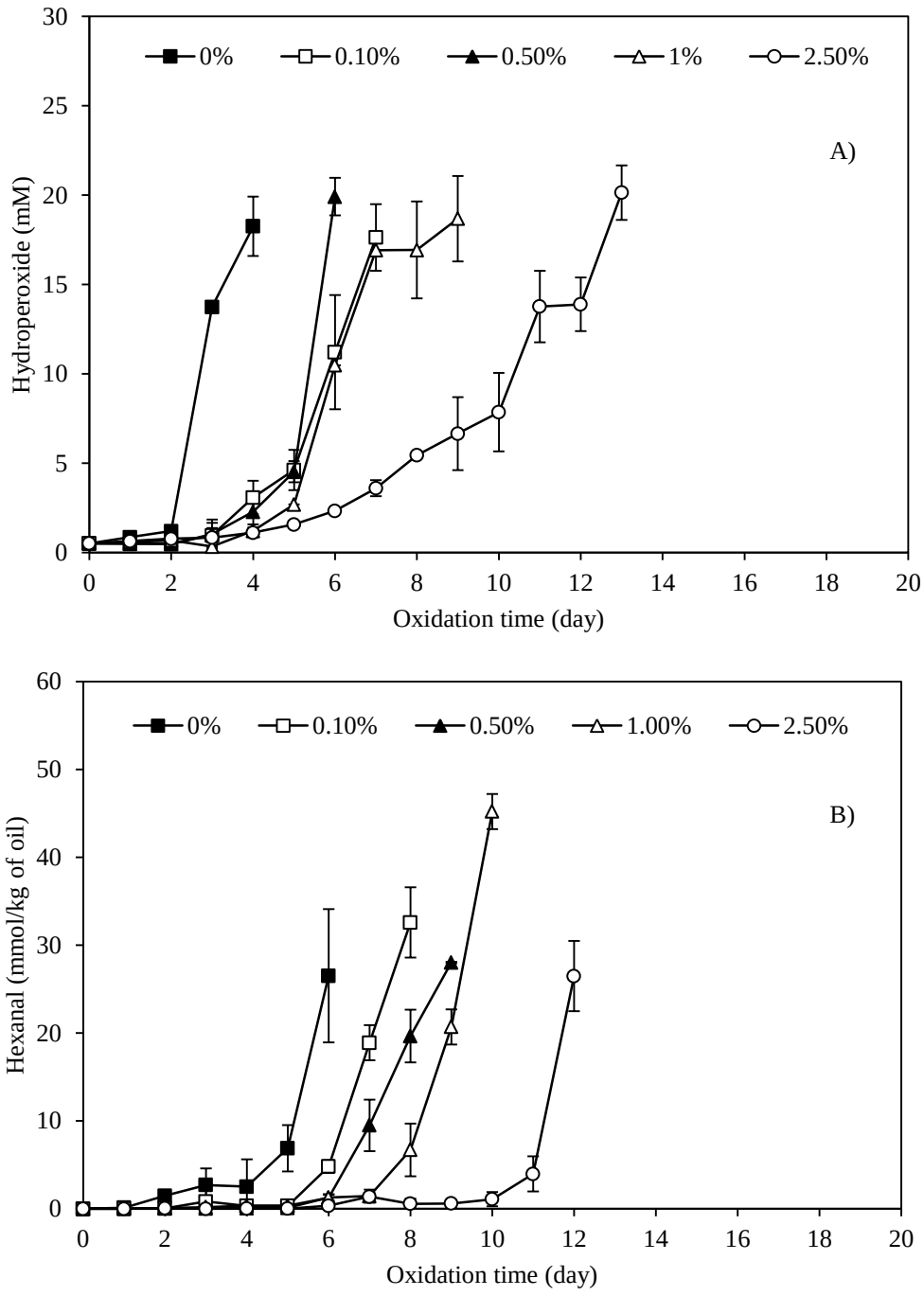


Figure 4.7 Hydroperoxide (A) and hexanal (B) formation in 1% stripped soybean oil-Tween 20 emulsions at 25^{o}C as a function of increasing Tween 20 concentrations (0.1, 0.5, 1.0, and 2.5% w/w) in the presence of eicosyl rosmarinate ester (R20). Data represents means (n=3) ± standard deviations.

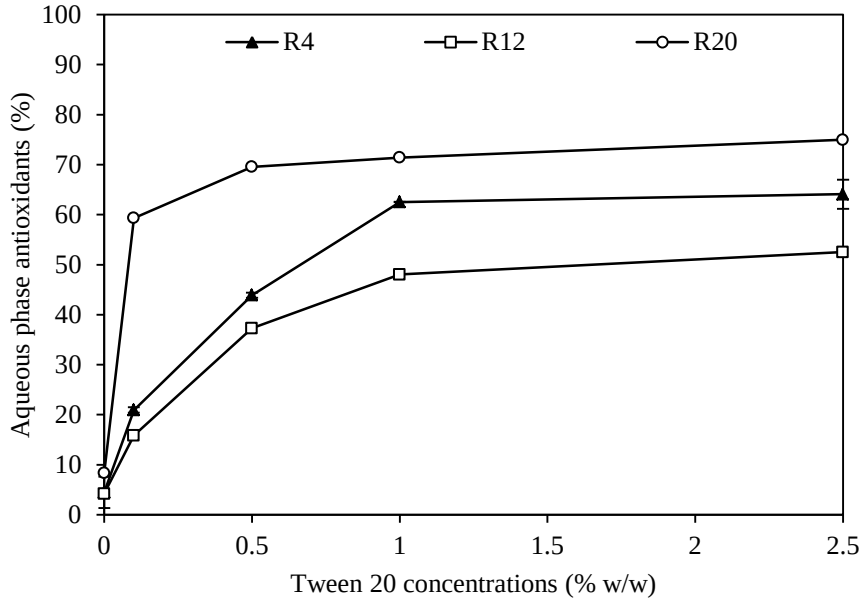


Figure 4.8 The effect of surfactant concentrations on the antioxidant partitioning of butyl (R4), dodecyl (R12) and eicosyl (R20) rosmarinate esters (100 μM) into aqueous phase of in oil-in-water emulsions. Data represents means (n=3) ± standard deviations. Error bars lie within data point.

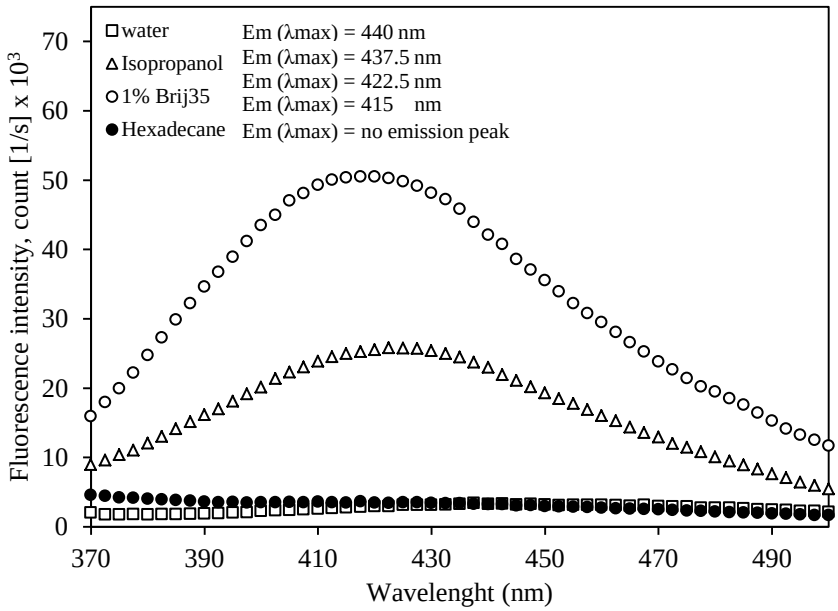


Figure 4.9 Fluorescence emission scans of butyl rosmarinate ester (30 μM) in various solvents with different polarities

suggested that the fluorescence properties of the rosmarinic acid esters could be used as a probe to help understand their physical location in emulsion and surfactant micelles.

The fluorescence intensity of the C4, C12 and C20 esters in washed oil-in-water emulsion to which excess Brij 35 was added is shown in Figure 4.10. R20 exhibited larger increases in fluorescence intensity than R4 and R12 as surfactant micelles concentrations were increased. The increase in R20 fluorescence could be due either to its greater association with the surfactant micelles or the fact that R20 becomes more dilute in the emulsion system such that less self-quenching between R20 molecules was occurring. For example, if the R20 was concentrating in the lipid core of the emulsion droplet, its localized concentration would be high and thus self-quenching would be greater. When the R20 migrated into the surfactant micelles, they become more dilute in the system and less self-quenching occurs thus a greater net fluorescence is observed. In either case, this data confirms the antioxidant partitioning data (Figure 4.8) where R20 partitioning into surfactant micelles in the aqueous phase of the emulsions was greater than R4 and R12.

4.3.5 Evidence of rosmarinate esters at oil-water interfaces using a surface active probe

Increases in fluorescence intensity (Figure 4.10) suggest that R20 becomes more highly associated with surfactant micelles. However, as discussed above this also could be due to dilution of the R20 throughout the emulsion system resulting in less self-quenching. To gain further insights on whether the surfactant micelles increased the

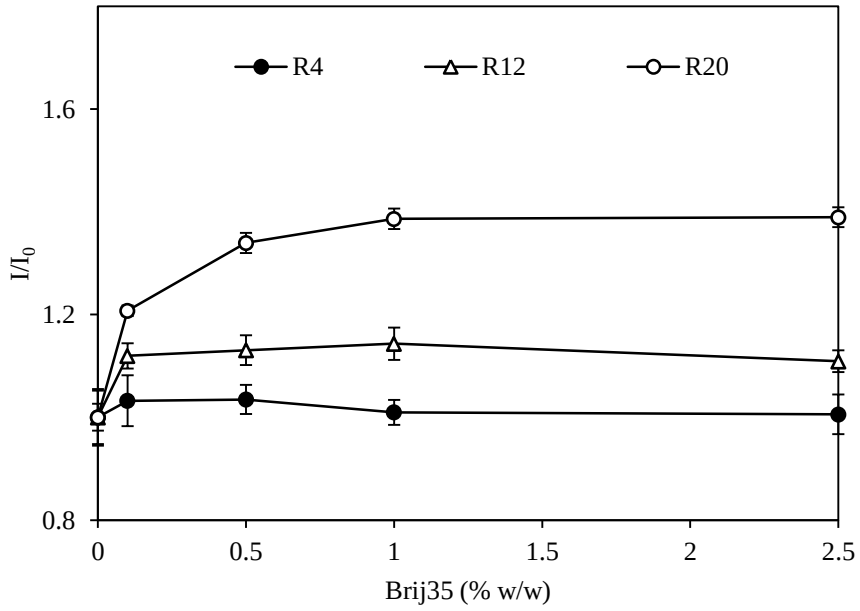


Figure 4.10 Front-face fluorescence measurement (λ_{ex} 323, λ_{em} 420) of rosmarinic acid and its butyl (R4), dodecyl (R12) and eicosyl (R20) esters in washed O/W emulsions with varying Brij concentrations. Data represents means (n=3) ± standard deviations.

ability of rosmarinic acid esters to concentrate at the lipid-water interface, the ability of R4, R12 and R20 to interact with 4-hexadecylbenzenediazonium ions (16-ArN_2^+) was determined. These interactions are important because 16-ArN_2^+ is a surface active probe with a water soluble cationic headgroup and a water insoluble hexadecyl tail that concentrates at the interface of oil-in-water emulsions and thus provides an indication of interfacial antioxidant concentrations (*101*).

Reactions between rosmarinate esters and 16-ArN_2^+ were determined in a washed O/W emulsions with 1% (w/w) medium chain triglycerides and 0.1% (w/w) Tween 20 in

a 3 mM HCl solution with and without an additional 2.5% (w/w) Tween 20. The rate constants between the rosmarinic acid esters and 16-ArN_2^+ are shown in Table 4.1.

Table 4.1 Initial secondary rate constants of 16-ArN_2^+ (170 μM) consumption in the presence of rosmarinate esters (30 μM) in washed oil-in-water emulsions with and without 2.5% Tween 20.

Rosmarinate esters	k_{obs} at 0% Tween 20 (M^{-1} s^{-1})	k_{obs} at 2.5% Tween 20 (M^{-1} s^{-1})
Butyl rosmarinate (R4)	221.7±13.2[a]	0.12±0.008[a]
Dodecyl rosmarinate (R12)	224.3±21.3[a]	0.11±0.014[a]
Eicosyl rosmarinate (R20)	124.0±8.6[b]	0.12±0.010[a]

Means (± SD) with different superscript letters (a and b) in the same column indicate significant differences ($P<0.05$).

In the washed emulsions, the rate constants for R4 and R12 were statistically similar. However, the rate constant for R20 was 45% lower than R4 and R12. These results indicate that the concentration of R20 at the interface of the oil-in-water emulsion was much less than R4 and R12. When 2.5% Tween 20 was added to the emulsions to increase surfactant micelle concentrations, interactions between the esters and 16-ArN_2^+ were much lower and all three esters became statistically similar. Interaction rates were most likely slower because the additional Tween 20 caused a dilution of both the 16-ArN_2^+ and the rosmarinic acid esters into the micelles. However, in the presence of Tween micelles the rate constant for R20 was similar to R4 and R12 unlike in the absence

of Tween micelles where R20 was much less reactive. This is possibly because the Tween micelles could increase the association of R20 with the interfacial layer at concentrations similar to R4 and R20. This could occur if the Tween 20 micelles were able to solubilize R20 out of the emulsion droplet core. These data again support the partitioning (Figure 4.8) and fluorescence (Figure 4.10) data which also show that surfactant micelles increase the concentration of R20 at the water interface.

In summary, Figure 4.11 illustrates a possible mechanism for how Tween 20 micelles had such a different impact on the antioxidant activity of rosmarinic acid esters of varying polarity in oil-in-water emulsions. Fluorescence spectra suggest that the association of R4 and R12 with surfactant micelles was not increased by the presence of surfactant micelles (Figure 4.10). This suggests that these more polar rosmarinic acid esters will primarily localize at the emulsifier-water interface in both washed emulsions with low concentrations of surfactant micelles as well as with emulsions with excess surfactant micelles. However, addition of surfactant micelles did remove some of the R4 and R12 from the emulsion droplets as shown by the increase aqueous phase R4 and R12 concentrations (Figure 4.8). Increased partitioning of R4 and R12 into the aqueous phase by the surfactant micelles could help explain why their antioxidant activity was decreased in the oil-in-water emulsions in the presence of excess surfactant micelles (Figure 4.5 and 4.6).

The R20 ester behaved very differently than R4 and R12. The concentration of R20 at the emulsion droplet interface in washed emulsions was lower than R4 and R12 as determined by the surface active probe, 16-ArN_2^+. However, R20 very readily associated

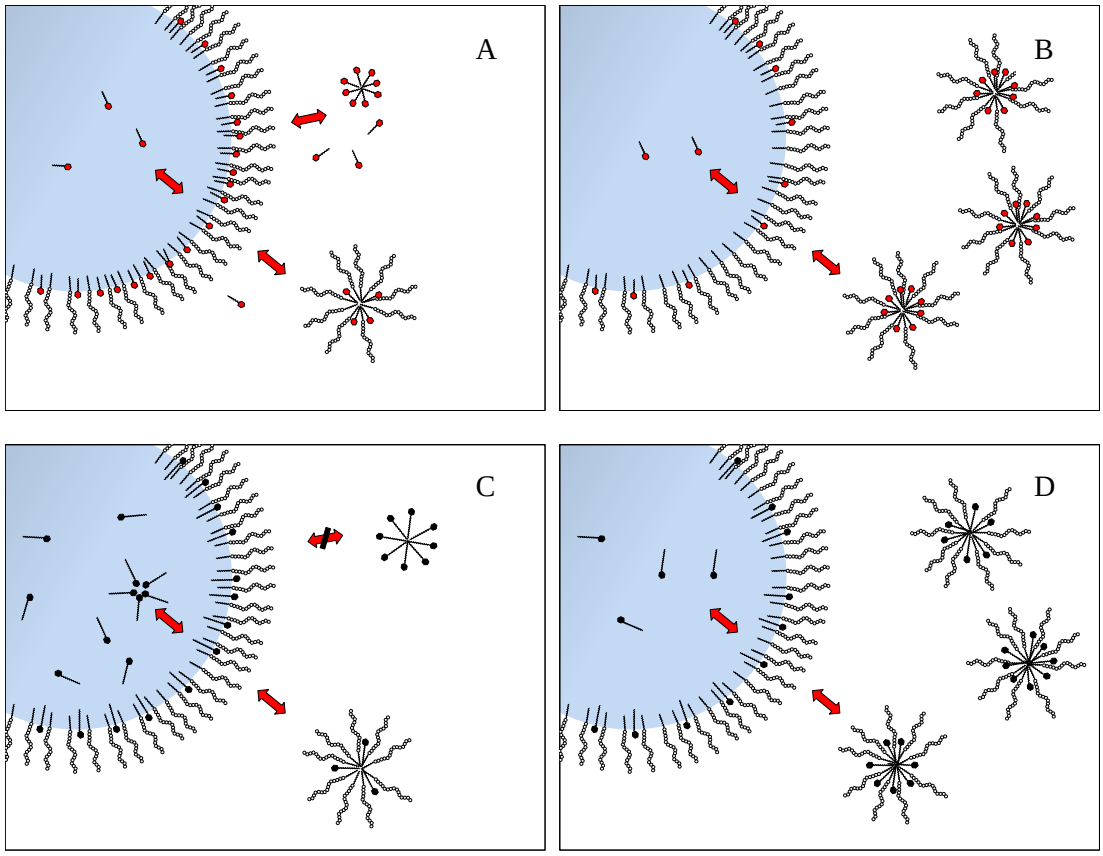


Figure 4.11 Schematic demonstration of the distribution of the rosmarinate esters in the interface region of O/W emulsions and in non-ionized surfactant micelles. In the O/W emulsions the rosmarinate esters can exist as monomers, micelles (or reverse micelles) or co-micelles. A and B show medium chain rosmarinate esters (4-12 carbons) in low and high surfactant concentration. In this case, the rosmarinate esters are mainly at the oil-water interface of the emulsion droplet. C and D show long chain rosmarinate ester (20 carbons) in low and high surfactant concentration. In this case, a higher proportion of the rosmarinate ester is in the interior and/or aggregated outside of the emulsion droplet in the absence of excess surfactant micelles.

with Tween 20 micelles as could be seen by low levels of Tween 20 increasing its aqueous phase concentration in the oil-in-water emulsions (Figure 4.8). This increase in aqueous phase concentrations is presumably due to its solubilization from the emulsion droplet into surfactant micelles as supported by its increasing fluorescence emission (Figure 4.10). Unlike R4 and R12, surfactant micelles increased the antioxidant activity of R20 in the oil-in-water emulsion (Figure 4.7).

Lipid oxidation chemistry in oil-in-water emulsions is thought to occur at the emulsion droplet interface since the oxidation substrate, lipid hydroperoxides, are surface active and thus can migrate to the emulsion droplet interface where they are decomposed into free radicals by prooxidants such as transition metals (*23*, *200*). Therefore, one possible explanation for the differences in the antioxidant activity of the rosmarinic acid esters is that in the absence of excess Tween 20 and thus surfactant micelles, a portion of the more nonpolar R20 localizes in the emulsion droplet core instead of the emulsion droplet interface. This would decrease its interfacial concentrations and thus its ability to scavenge free radicals produced from the decomposition of interfacial lipid hydroperoxides. Surfactant micelles could promote the migration of R20 out of the emulsion droplet core by forming Tween 20-R20 co-micelles. As aqueous phase surfactant micelles readily exchange with emulsion droplets, they could form a reservoir of R20 allowing it to replace R20 at the emulsion droplet interface after it is consumed by scavenging free radicals in the emulsion droplet.

This phenomenon may not be limited to rosmarinic acid alkyl esters. Tocopherols are also very non-polar antioxidants that have very low water solubility and surface

activity (*15*). In studies by Cho et al. (2002), Nuchi et al. (2002) and Richard et al. (2002) it was found that surfactant micelles inhibited lipid oxidation to oil-in-water emulsions (*209-211*). Unlike the present study these studies were conducted with non-stripped corn oil which would contain tocopherols (surfactant micelles did not alter lipid oxidation rates in this study which used tocopherol free oil, Figure 4.4). This suggests that it may be useful to determine if the ability of surfactant micelles to inhibit lipid oxidation in these studies was due to the ability of surfactant micelles to solubilize tocopherols out of the emulsion droplet core in a manner similar to what we have observed for the R20 ester of rosmarinic acid. This could help provide more insight into how non-polar antioxidant such as tocopherol could be made more effective in oil-in-water emulsions.

CHAPTER 5

COMPLEX MECHANISMS OF ANTIOXIDANT INTERACTIONS BETWEEN ROSMARINATE ESTERS AND α-TOCOPHEROL IN STRIPPED SOYBEAN OIL-IN-WATER EMULSIONS

5.1 Introduction

Under current limitations of approved antioxidants for food applications, it is often challenging for food scientists to maintain the oxidative stability of processed foods. To try to solve this problem, several strategies have been attempted to improve antioxidant performance. One interesting strategy is to use combinations of antioxidants to produce synergistic interactions via free radical transfer mechanisms. For example, the regenerations of oxidized α-tocopherol by ascorbic acid, flavonoids, carotenoids, phospholipids, amino acids and peptides and has been reported (*27-33*). These regeneration reactions have been postulated to produce synergistic antioxidant interactions.

Several models to study interactions between antioxidant combinations include the oxygen radical absorbance capacity (ORAC) (*212, 213*), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging (*136, 214-217*), ferric reducing antioxidant power (FRAP) (*215*), and homogeneous solutions of peroxidizing methyl linoleate (*218*). Unfortunately, these models often produce inconsistent results. It was reported that the

combinations between α-tocopherol and flavonoids exhibit synergistic, additive, and antagonistic effects in the ORAC model (*212, 213*). However, Hiramoto and coworkers (*217*) reported that synergistic antioxidant interactions were observed only when α-tocopherol was combined with ascorbic acid, but not with other water soluble antioxidants in the DPPH model.

The free radical scavenging properties of antioxidants in homogenous model systems (e.g. DPPH and ORAC) might not correlate to foods since food are heterogeneous (*2*) and thus may have physical attributes that can impact antioxidant interactions. For example, the existence of both lipid and aqueous phase would affect antioxidant partitioning and thus interactions between oil and water soluble antioxidants at the oil-water interface. Fukazawa and coworkers reported that interactions between α-tocopherol and ascorbic acid in liposomal membranes are influenced by physical barriers and surface charge of the membranes (Fukuzawa, Ikebata et al. 1993). Currently there are no systematic methods to be able to predict how combinations of antioxidants can inhibit oxidation synergistically in real food systems.

In this research, we hypothesized that antioxidants partitioning at different locations in oil-in-water (O/W) emulsions may influence the ability of antioxidants to interaction. Rosmarinic acid and its esters are excellent tools to study interactions with α-tocopherol because their distributions and locations in O/W emulsions can be varied without impacting on their reactive hydroxyl groups (*195*). In the current study, several methods were utilized to study α-tocopherol-rosmarinate interactions. Electrochemical properties of rosmarinic acid and its esters and α-tocopherol were investigated by cyclic

voltammetry (CV) to explain thermodynamic reactions between antioxidants. Direct observations of interactions between the rosmarinates and α-tocopherol were observed by a fluorescence quenching technique. The efficiency of tocopheroxyl radical regeneration by the rosmarinates was studied by electron paramagnetic resonance (EPR) in homogeneous (ethanol) and heterogeneous (Tween 20 micelles) systems. Finally, the sparing effects of antioxidant interactions between the rosmarinates and α-tocopherol during the oxidation of oil-in-water emulsions were investigated by determining the rates of antioxidant decomposition by high-performance liquid chromatography (HPLC). Through these studies, important information can be obtained to better predict when synergistic antioxidant interactions can occur in oil-in-water emulsions.

5.2 Materials and methods

5.2.1 Chemicals and Materials

Soybean oil was purchased from a local grocery market in Amherst, MA. Ethylenediaminetetraacetic acid (EDTA) disodium salt was purchased from Chempure ™ Ultra (Houston, Texas). Acetic acid, acetonitrile, methanol, hydrochloric acid were obtained from Fisher Scientific (Pittsburgh, PA). Chelex® 100, rosmarinic acid, caffeic acid, 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$), $FeSO_4$, Tween 20™ (Mw ≈1228), $BaCl_2$, $Bu_4 NPF_6$, phosphoric acid, sodium phosphate mono and dibasic, 2,2,6,6-tetramethylpiperidinoxyl (TEMPO) radical were purchased from Sigma-Aldrich (St. Louis, MO). α-Tocopherol quinone was purchased from Tokyo chemical industry,

Tokya, Japan. Double-distilled and deionized water was used for the preparation of all solutions.

5.2.2 Synthesis of Rosmarinate Esters

The chemoenzymatic esterification of rosmarinic acid to obtain rosmarinate esters was carried out following the procedure described by Lecomte and coworkers (*195*). Briefly, the chemical esterification of rosmarinic acid (56 μmol) was conducted in sealed brown flasks each containing 5 mL of alcohol (methanol, 123.4 mmol; n-butanol, 54.6 mmol; n-octanol, 31.9 mmol; n-dodecanol, 22.5 mmol; n-hexadecanol, 17.0 mmol; n-octadecanol, 15.1 mmol or n-eicosanol, 13.6 mmol). The reaction mixtures were stirred (orbital shaker, 250 rpm, 55-70 ^{o}C) prior to the addition of the catalyst; the strongly acidic sulfonic resin Amberlite IR-120H (5% w/w, total weight of both substrates) that had been previously dried at 110 ^{o}C for 48 h. The water generated during the reaction was removed by adding 3 A°, 4-8 mesh molecular sieves (40 mg/mL, Aldrich, St. Louis,MO) to the medium. Samples (20 μL) were regularly withdrawn from the reaction medium and then mixed with 980 μL of methanol, filtered (0.45 μm syringe filter Millex-FH, Millipore Corp., Bedford, MA), and analyzed by reverse phase HPLC with UV detection at 328 nm (*195*). After complete (4-21 days) conversion of rosmarinic acid into the corresponding ester, the latter was purified in a two-step procedure. First, a liquid-liquid extraction using hexane and acetonitrile was performed to remove the excess alcohol. Then, the remaining traces of the alcohol and rosmarinic acid were eliminated by flash chromatography on a CombiFlash Companion system (Teledyne Isco Inc., Lincoln,

NE). Separation was carried out on a silica column using an elution gradient of hexane and ether (20-100% in 35 min). The yield of purified esters, obtained as pale yellow to yellow amorphous powders, was calculated from calibration curves previously established with pure compounds. Pure esters and rosmarinic acid were then fully characterized by ESI-MS, 1H NMR, and ^{13}C NMR as previously described by Lecomte et al. (*195*).

5.2.3 Emulsion Preparation

Stripped soybean oil was prepared according to the method of Waraho et al. (*204*). The effectiveness of stripping was monitored by measuring the removal of tocopherols by HPLC (*219*). No tocopherols could be detected in the stripped oils. Oil-in-water (O/W) emulsions were prepared using 1.0% (wt) stripped soybean oil in a 10 mM phosphate buffer solution (pH 7.0). Tween 20 was used as an emulsifier at a 1:10 emulsifier/oil ratio. Stripped soybean oil, Tween 20, and phosphate buffer were added to a beaker, and a coarse emulsion was made by blending with a hand-held homogenizer (M133/1281-0, Biospec Products, Inc., Bartlesville, OK) for 2 min. The coarse emulsion was then homogenized with a microfluidizer (Microfluidics, Newton, MA) at a pressure of 9 kbar for three passes.

After the O/W emulsion was prepared, rosmarinic acid and its esters with various chain lengths (4, 8, 12, 18, and 20 carbons) in methanol were added to the emulsion at a final concentration of 30 μM and stirred for 1 h at room temperature. Samples with methanol but without addition of the antioxidant were used as control samples. The

emulsions (0.5 mL) were transferred into 10 mL GC vials and sealed with (tetrafluoroethylene) butyl rubber septa, and then stored at 25^{o}C in the dark. Three vials of each treatment were taken every day to determine lipid hydroperoxides and hexanal formation.

In some studies, emulsions were washed to remove aqueous phase surfactants as previously described by Faraji and coworkers (*205*) with some modifications. In short, emulsions were centrifuged at 38,518 g (17,000 rpm) for 1 h at 4 ^{o}C using a Fiberlite® F40L-8 x 100 rotor with a high-speed centrifuge (Thermo scientific WX Ultra 80, Asheville, NC). After the centrifugation, the bottom layer (phosphate buffer) was carefully removed using a needle and syringe, and then the same volume of the fresh phosphate buffer was used to re-disperse the creamed emulsion droplet layer by vortexing. This washing procedure was performed a total of three times. The lipid content of the final washed emulsion was determined by the modified Bligh and Dyer method (*206*) and then phosphate buffer was used to adjusted the lipid content back to 1% (w/w). In some experiments Tween 20 was added back into the washed emulsions (0, 0.1, 0.5, 1.0, and 2.5%; w/w) so that a known amount of surfactant would be in the continuous phase.

5.2.4 Measurements of particle size of emulsions

The size of the emulsion droplets were measured by a dynamic light scattering (Zetasizer Nano-ZS, Model ZEN3600, Malvern Instruments, Worchester, U.K.), and expressed as z-average mean diameter. Samples were diluted 50 times with the same

buffer as the emulsion, mixed, and immediately measured by transferring the solution into 3 ml plastic cuvettes for determining the size. Measurements were performed on three replicates and repeated 3 times on each samples at room temperature. The emulsion droplets size ranged from 173.3 ± 11.7 nm and there was no significant change in droplet size of each emulsion over the course of study (data not shown). In addition, there was no visual observation of creaming during storage in all treatments

5.2.5 Measurements of Lipid Hydroperoxides

Lipid hydroperoxide formation in emulsion solutions was determined according to the method described by Panya and coworkers (*207*) with some modifications. Emulsion solutions (0.2 mL) were mixed with 1.5 mL of isooctane/2-propanol (3:1 v/v) and vortexed (10 s, three times). After centrifugation at 1000 g for 2 min, 30 μL of the organic solvent phase was mixed with 1.5 mL of methanol/1-butanol (2:1). Hydroperoxide detection was started by the addition of 7.5 μL of 3.94M ammonium thiocyanate and 7.5 μL of ferrous iron solution (prepared by adding equal amounts of 0.132 M $BaCl_2$ and 0.144 M $FeSO_4$). After 20 min of incubation at room temperature, the absorbance was measured at 510 nm using a UV-Vis spectrophotometer (Genesys 20, Thermo Spectronic). Hydroperoxide concentrations were determined using a standard curve prepared from hydrogen peroxide.

5.2.6 Measurements of Hexanal

Headspace hexanal was determined according to the method described by Panya and coworkers (*207*) with some modification using a Shimadzu GC-2014 gas chromatograph (GC) equipped with an AOC-5000 autoinjector (Shimadzu, Tokyo, Japan). A 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/Carboxen/PDMS) stable flex solid phase microextraction (SPME) fiber (Supelco, Bellefonte, PA) was inserted through the vial septum and exposed to the sample headspace for 8 min at 55 ^{o}C. The SPME fiber was desorbed at 250 ^{o}C for 3 min in the GC detector at a split ratio of 1:7. The chromatographic separation of volatile aldehydes was performed on a fused-silica capillary column (30 m x 0.32 mm i.d. x 1 μm) coated with 100% poly(dimethylsiloxane) (Equity-1, Supelco). The temperatures of the oven, injector, and flame ionization detector were 65, 250, and 250 ^{o}C respectively. Sample run time was 10 min. Concentrations were calculated by using a standard curve made from the above emulsions containing known hexanal concentrations and 200 μM EDTA.

5.2.7 Calculation of antioxidant interaction indexes of rosmarinate esters and α-tocopherol combinations

Interaction indexes of rosmarinate esters with α-tocopherol were calculated based on the oxidation lag times of lipid hydroperoxides and hexanal formation. Lag times were determined as the first data point that was statistically ($p \leq 0.05$) greater than time zero. Briefly, the oxidation lag times of individual antioxidants were used to estimate the expected oxidation lag times of its combination. Interaction indexes were calculated from

the ratio between the obtained oxidation lag times of the combination and the expected oxidation lag time of the combination with the following equation:

$$\text{Interaction Index} = \frac{\text{Observed lag time of the combination}}{\text{Expected lag time of the combination}}$$

$$= \frac{Lag\ time\ (control\ A+B) - Lag\ time\ (A+B)}{[Lagtime\ (control\ A) - Lag\ time\ (A)] + [Lagtime\ (control\ B) - Lag\ time\ (B)]}$$

Where, A and B represent α-tocopherol and rosmarinates, respectively. Controls of A, B and A+B represent the lag time of individuals and combinations without adding antioxidants. Interaction indexes were expressed as synergistic (> 1), additive (≈ 1) and antagonistic (< 1) antioxidant effects

5.2.8 Determination of Antioxidant Partitioning

Determination of the physical location of rosmarinic acid and its esters in the emulsions were performed according to the procedure described by Panya and coworkers (*207*). EDTA (200 μM) was added to regular emulsions and washed O/W emulsions with added surfactants (0, 0.1, 0.5, 1.0, and 2.5%; w/w) to minimize oxidation during analysis. Rosmarinic acid and its alkyl esters in methanol were added to the emulsion at a final concentration of 100 μM followed by stirring at room temperature for 1 h. The emulsions were centrifuged at 162102 g (46,000 rpm) for 1 h at 4^{o}C using a PTI F65L-6x13.5 rotor with a high-speed centrifuge (Thermo Scientific WX Ultra 80, Asheville, NC). The continuous phase was carefully collected with a pipette and the amount of aqueous phase rosmarinic acid esters was determined directly by HPLC using a modified method

described by Lecomte and coworkers (*195*). Briefly, HPLC determination of rosmarinic acid and its esters was carried out with a Hypersil gold C18 reversed phase column (250 mm x 4.6 mm, 5 μm) equipped with a Hypersil gold guard column (10 mm x 4 mm, 5 μm) (Thermo Scientific, USA) using a LC-10ATvp HPLC system (Shimadzu, USA). Peak integration was performed using Shimadzu EZstart (Version 7.2). Gradient elution was performed using methanol and 3 mM phosphoric acid at1 mL/min at 40^{o}C (column temperature), in linear gradients from 0/100 (v/v) to 100/0 (v/v) for 5 min, then 100/0 (v/v) for 10 min, back to 0/100 (v/v) for 5 min and hold at 0/100 (v/v) for 5 min. Rosmarinic acid and its alkyl esters [(R4 (4 carbons) – R20 (20 carbons)] were detected with a photodiode array detector (SPD-M10Avp, Shimadzu, USA) at 328 nm. α-Tocopherol was detected at 295 nm. The concentrations of rosmarinic acid esters and α-tocopherol were calculated using a standard curve made from each antioxidant dissolved in methanol.

5.2.9 Determination of Antioxidant Decomposition during Oxidation Studies

Determination of the decomposition of α-tocopherol and rosmarinic acid and its esters in the emulsions during storage were performed according to the procedure described by Panya and coworkers (*207*). A 200 μL sample of O/W emulsions was transferred into 1.5 mL eppendorf tubes containing 50 μL of 200 μM EDTA to inhibit further lipid oxidation. Samples were frozen at -80 ^{o}C until freezing drying. The freezing drying condition was operated at -10 ^{o}C for 16 h, then increased the temperature to 5 ^{o}C for 4 h. Dried emulsions were stored at -80 ^{o}C until analysis. Antioxidants in dried

emulsions were extracted immediately prior to analysis by adding 200 µL of methanol. The mixtures were vortexed for 2 min, sonicated in ultrasonic bath for 2 min, and then centrifuged at 1000g for 5 min.

The clear methanolic solutions were carefully collected with a pipette and antioxidant concentrations were determined directly by HPLC using a modified method described by Fujimoto and Masuda (*220*). Briefly, HPLC determination of rosmarinic acid and its esters was carried out with a Hypersil gold C18 reversed phase column (250 mm x 4.6 mm, 5 µm) equipped with a Hypersil gold guard column (10 mm x 4 mm, 5 µm) (Thermo scientific, USA) using a LC-10ATvp HPLC system (Shimadzu, USA). Peak integration was performed using Shimadzu EZstart (Version 7.2). Gradient elution was performed using acetonitrile and 1% acetic acid at1 mL/min in linear gradients from 5/95 (v/v) to 100/0 (v/v) for 40 min, then 100/0 (v/v) for 10 min and then back to 5/95 (v/v) for 5 min. Rosmarinic acid and dodecyl rosmarinate ester (12 carbons) were detected with a photodiode array detector (SPD-M10Avp, Shimadzu, USA) at 328 nm. The oxidation products of the rosmarinates were detected at 280 and 328 nm. α-Tocopherol and α-tocopheryl quinone were detected at 295 and 265 nm, respectively. The concentrations of antioxidants (rosmarinic acid and its ester, α-tocopherol, and α-tocopheryl quinone) were calculated using a standard curve made from the standard antioxidants dissolved in methanol.

5.2.10 Front-Face Fluorescence Quenching Measurements

Front-face fluorescence quenching between α-tocopherol and rosmarinic acid, and its alkyl esters in O/W emulsions was determined by steady-state emission measurements recorded with a PTI spectrofluorometer (PTI, Ontario, Canada). Stripped soybean oil (1 %; w/w) emulsions were prepared with 0.1 % (w/w) Tween 20 in 10 mM phosphate buffer (pH 7) with 200 µM EDTA to minimize oxidation . A final concentration of 100 µM of α-tocopherol was added to the O/W emulsions from the stock solution of α-tocopherol in methanol. The mixtures were stirred at room temperature for 1 hour. Then, rosmarinate esters in methanol were transferred into the emulsions containing α-tocopherol at concentrations of 0, 5, 10, 25, 50, and 100 µM.

After vortexing for 2 min, the final emulsions (1.5 mL) were transferred into triangular suprasil cuvettes. The samples were held at 30 ^{o}C and stirred with a 3 mm magnetic stirring bar (Fisher scientific, USA). Emission was observed at 90^{o} to the incident beam, that is, 22.5^{o} with respect to the illuminated cell surface. The emission of α-tocopherol was measured at 320 nm using an excitation wavelength of 295 nm. Spectral bandwidth for both excitation and emission slits was 2.0 nm, integration time was 1 s, and the wavelength increment was 2.5 nm. The intensity of the spectra (I) of α-tocopherol after addition of the rosmarinate derivatives was determined as the emission signal intensity (counts per second) measured by means of a photomultiplier. The intensity ratio (I_o/I) where I_o is the fluorescence intensity of α-tocopherol, was plotted versus the concentrations of rosmarinate esters. The slope of this line was used to determine the quenching constant of the different rosmarinates.

5.2.11 Cyclic Voltammetry (CV) Measurements

Cyclic voltammetry was performed according to the method described by Wilson and coworkers (Wilson, Lin et al. 2006). Rosmarinic acid and its esters (2 mM) were freshly dissolved in acetonitrile and evaluated with a BASi Model C-3 cell stand using a planar 1 mm diameter glassy carbon working electrode, Ag/AgCl reference electrode, and a Pt wire auxiliary electrode. Samples were scanned at 100 mV s^{-1} at 25^{o}C in acetonitrile with 0.5 M Bu_4 NPF_6 as a salt bridge in 50 mM phosphate buffer, pH 7 or in 1% Tween 20 in 50 mM phosphate buffer, pH 7.

5.2.12 Measuring the Efficiency of the Regeneration of α-Tocopherol by Rosmarinic Acid and Its Esters by Electron Paramagnetic Resonance (EPR) Spectroscopy

The efficiency of rosmarinates to regenerate α-tocopherol from α-tocopheroxyl radicals was determined in two different systems; a homogeneous ethanolic solution and a heterogeneous Tween 20 micelle solution. The experiment procedure was adapted from the method described by Pazos and coworkers (*110*) with some modifications.

For experiments in homogeneous environments, stock solutions of α-tocopherol (2 mM), DPPH radical (0.5 mM), and rosmarinates were prepared freshly with N_2 saturated ethanol. In heterogeneous environments, a stock solution of α-tocopherol (2 mM) was prepared in 100 mM Tween 20 in N_2 saturated 50 mM phosphate buffer solution at pH 7. Two mL of the stock solution of α-tocopherol (ethanolic or Tween 20 solutions) was transferred into a 4 mL vial which was purged with N_2. Then, 50 uL of the

DPPH solution was transferred into the stock solution of α-tocopherol, and mixed immediately at room temperature. After reacting for 20 sec to form α-tocopheroxyl radicals, 50 µL of the stock solutions of rosmarinates was added and mixed. The final concentrations of α-tocopherol and DPPH were 1.9 and 0.01 mM, respectively. The final concentrations rosmarinates were ranged from 2.5 to 20 µM. All solutions were transferred into an EPR spectrometer via a 5 mL syringe. EPR spectra were recorded 1 min after the reaction with DPPH.

The experiments were performed using a Bruker ELEXSYS E-500 EPR Spectrometer (Bruker, Germany) equipped with an X-band microwave bridge and an ER 4122-SHQE high sensitivity single cavity. Samples were injected into the cavity through with Aqua-X flow-through cell. EPR parameters were at the following setting: microwave power, 10 dB; sweep width, 100 G; sweep time, 20.9 s; modulation amplitude, 3 G; time constant, 81.92 m s; receiver gain, 80 dB. All samples were handled under N_2 sealed environment at room temperature.

The Mn (II) marker attached with the Aqua-X-flow cell was used to determine the relative signal intensity of the α-tocopheroxyl radical (the peak-to-peak ratio between α-tocopheroxyl radical and the marker). Concentrations of α-tocopheroxyl radical were quantitated by comparing the double-integrated areas of α-tocopheroxyl radical to known concentration of TEMPO radical. The integration of the signal was performed by using Bruker Xepr software. Efficiencies of the regeneration of α-tocopherol by each rosmarinates were estimated from the slopes of α-tocopheroxyl radical reduction at various concentrations of rosmarinates.

5.2.13 Statistical Analysis

All analyses were performed on triplicate samples. Oxidation lag phases were defined as the first data point significantly greater than the 0 time value. In all cases, comparisons of the means were performed using Duncan's multiple-range tests. A significance level of $p<0.05$ was defined as being statistically different. All calculations were performed using SPSS17 (http://www.spss.com; SPSS Inc., Chicago, IL).

5.3 Results and discussion

5.3.1 Antioxidant Activities of Rosmarinic Acid Esters and α-Tocopherol Combinations in Stripped Soybean O/W Emulsions

The ability of rosmarinate esters and α-tocopherol to synergistically inhibit lipid oxidation in the O/W emulsions was tested with rosmarinic acid (R0) and its different esters (R4, R12, and R20). This system was used since the different forms of rosmarinate could primarily exist in either the aqueous phase or emulsion droplet and thus could interact differently with α-tocopherol (T) which would mainly associate with the emulsions droplet. Hydroperoxide and hexanal formation in the O/W emulsions were determined during storage at 25 ^{o}C in the dark. All forms of the rosmarinates (30 μM) were able to inhibit the formation of hydroperoxides and hexanal compared to the control (Figure 5.1) which is consistent with the previous reports. For example, the R4 ester was slightly better than R12 at increasing hexanal lag times and R0 was slightly better than R20 which is the worst antioxidant (*221*).

All the combinations of the rosmarinic acid esters (30 μM) with α-tocopherol (30 μM) exhibited better antioxidant activity compared to the individual compounds. This was not entirely unexpected since the total antioxidant concentrations were higher. As shown in Figure 5.2, it was noted that the combinations of α-tocopherol and butyl rosmarinate ester (T-R4), and α-tocopherol and dodecyl rosmarinate ester (T-R12) exhibited similar increases in the lag phase of lipid hydroperoxides and hexanal formation as the sum of the individual antioxidants (Figure 5.1). Surprisingly, the combination of α-tocopherol and rosmarinic acid (T-R0) showed significant increases in

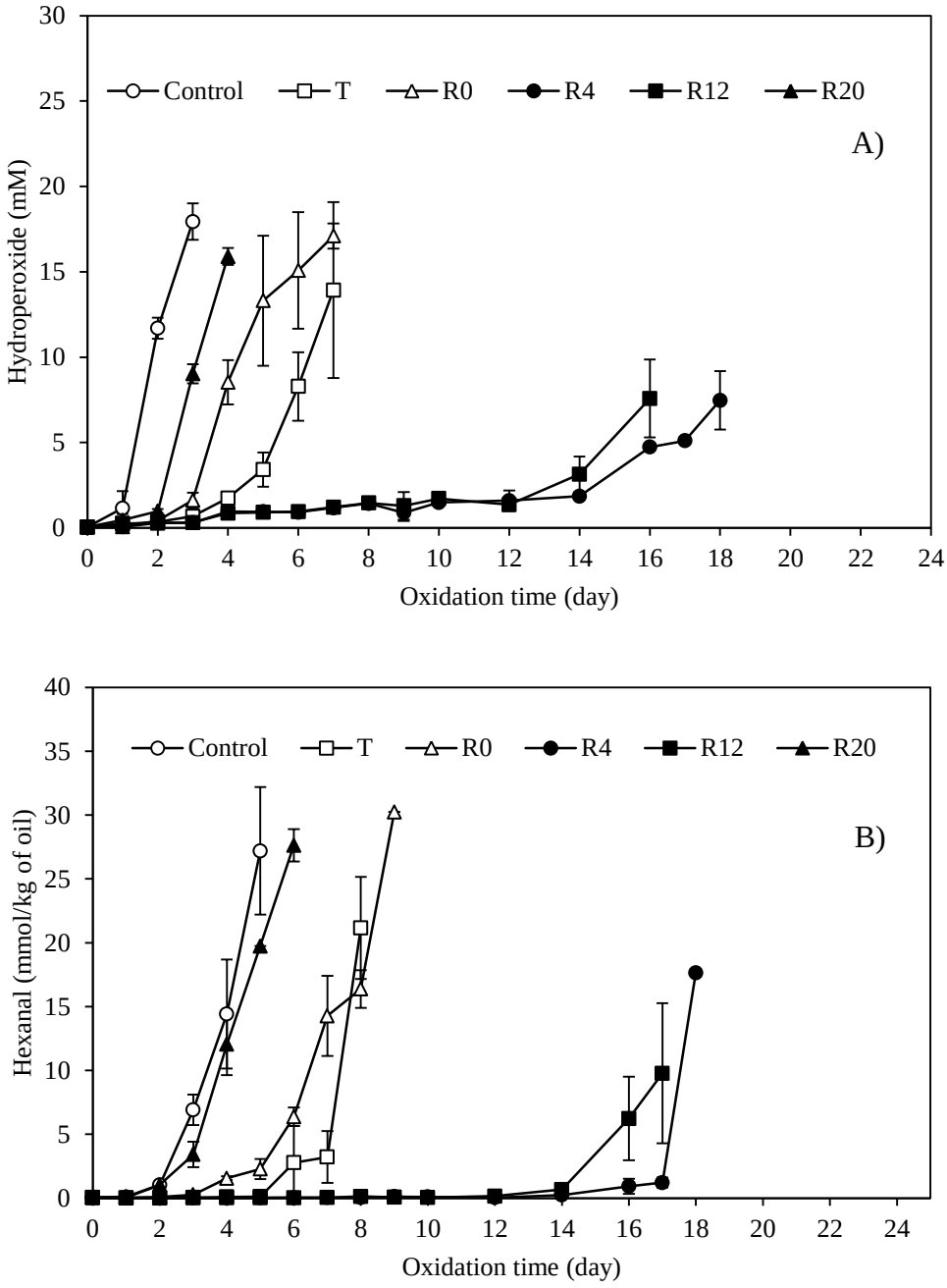


Figure 5.1 Hydroperoxide (A) and hexanal (B) formation in 1% stripped soybean oil-Tween 20 emulsions at 25^{o}C in the presence of individual of rosmarinic acid and its alkyl esters and α-tocopherol (30μM). Data points and error bars represent means (n=3) ± standard deviations.

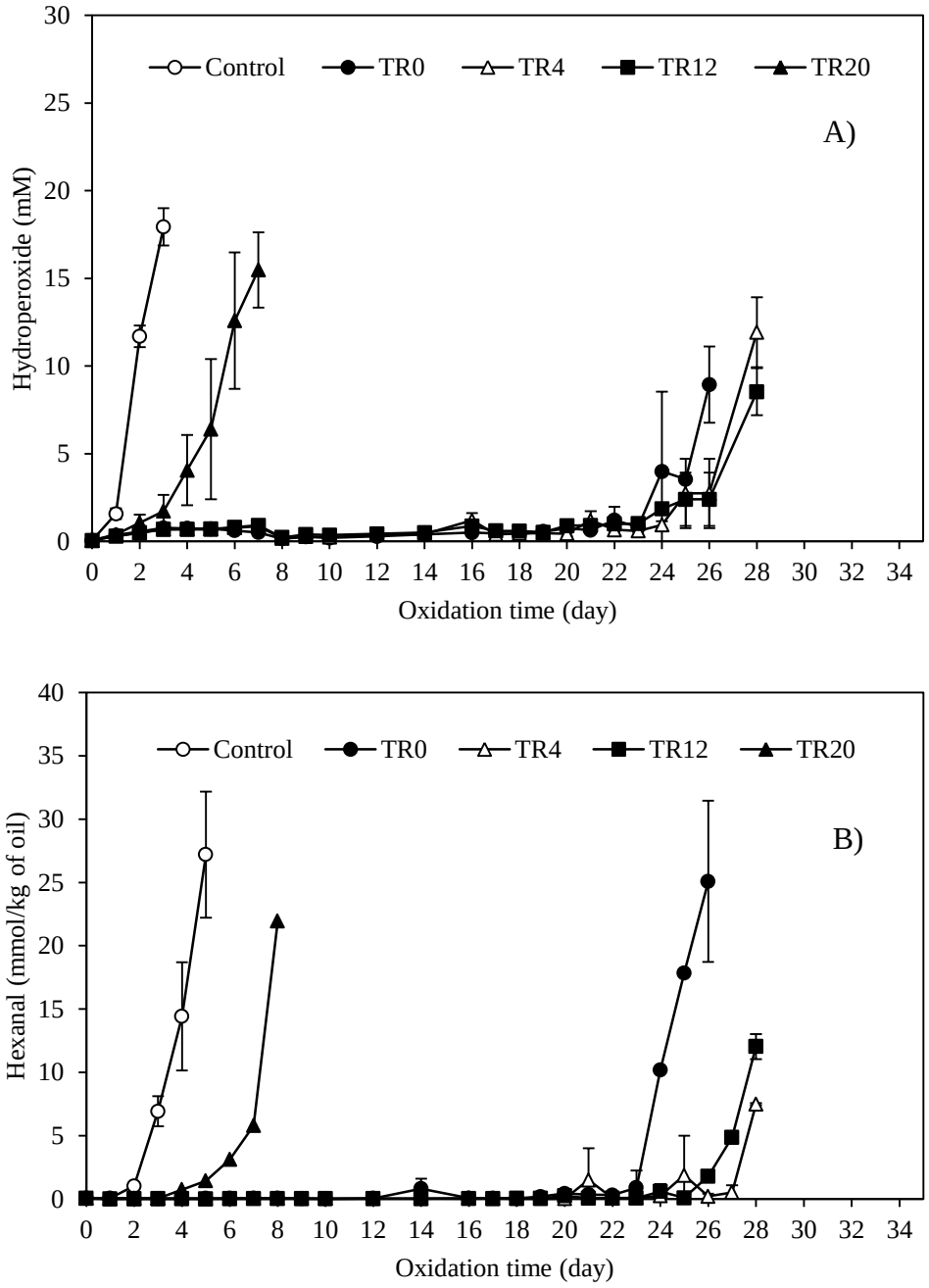


Figure 5.2 Hydroperoxide (A) and hexanal (B) formation in 1% stripped soybean oil-Tween 20 emulsions at 25^{o}C in the presence of combinations of rosmarinic acid and its alkyl esters with α-tocopherol (30μM+30 μM). Data points and error bars represent means (n=3) ± standard deviations.

the lag phase of lipid hydroperoxides and hexanal formation compared to the individual antioxidants. The lag times of hexanal formation and lipid hydroperoxides for the T-R0 combination ended up being similar to the lag times of the T-R4 and T-R12 combinations.

To quantitate the effects of the antioxidant combinations, the lag times for the formation of lipids hydroperoxides and hexanal for both individual and combined antioxidants were used to calculate the interaction index. As illustrated in Figure 5.3, R0 had the strongest antioxidant synergy with α-tocopherol producing interaction indexes for lipid hydroperoxides and hexanal formation of 4 to 5, while the interaction indexes of α-tocopherol and R4 or R12 were approximately 1.5 to 2 meaning that the synergistic effects were smaller. In contrast, R20 showed significant antagonistic effect with α-tocopherol exhibiting an interaction index of 0.3 to 0.6.

5.3.2 Partitioning of the Rosmarinic Acid Esters and α-Tocopherol in O/W Emulsions

A factor that could be important for the observed variations in synergistic antioxidant interactions would be differences in the physical location of the rosmarinic acid esters which would impact their ability to interact with α-tocopherol. The aqueous phase of O/W emulsions was collected to determine whether the antioxidants partitioned into the aqueous or emulsion droplet phases. Results showed that rosmarinic acid (R0) had the lowest association with emulsion droplets partitioning at approximately 90% in aqueous phase, while butyl-(R4) and dodecyl-(R12) rosmarinate esters and α-tocopherol

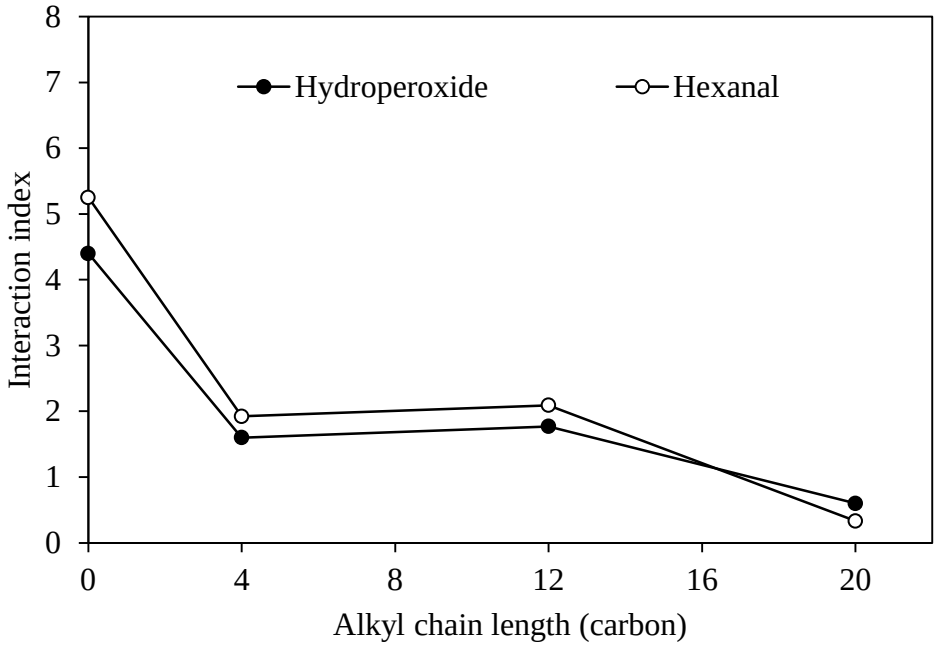


Figure 5.3 Interaction indexes of the combinations of rosmarinic acid and its esters with α-tocopherol in 1% stripped soybean oil-Tween 20 emulsions at 25^{o}C. Data were calculated from oxidation lag time obtained from hydroperoxide and hexanal formation.

were highly associated with the emulsion droplets indicated by low concentrations (2-9%) in aqueous phase (Figure 5.4). The antioxidant partitioning of R4, R12 and R20 were consistent with our previous report (*221*). There was slight precipitation observed in the continuous phase of emulsions containing R20. According to our previous study, R20 may form poorly soluble self-aggregates or co-micelles in the aqueous phase.

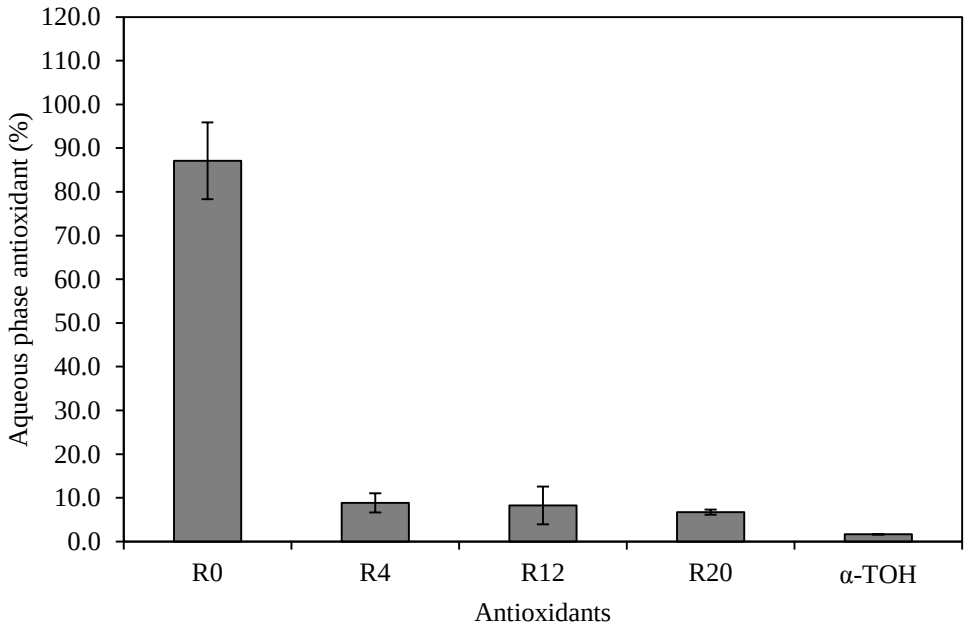


Figure 5.4 The antioxidant partitioning of rosmarinic acid (R0), butyl- (R4), dodecyl- (R12) and eicosyl- (R20) rosmarinate esters and α-tocopherol (100 μM) into aqueous phase of in oil-in-water emulsions. Data points and error bars represent means (n=3) ± standard deviations.

5.3.3 Interactions between Antioxidants in O/W Emulsions as Determined by Front-Face Fluorescence Quenching Measurements

Just because antioxidants partition into the continuous phase or the emulsion droplet does not mean they will interact to produce synergistic or antagonist interactions. Direct observations of the rosmarinates interacting with α-tocopherol in O/W emulsions can be determined by fluorescence quenching of α-tocopherol by the rosmarinates. Although the exact quenching mechanisms between these antioxidants have not been reported, one potential mechanism might be Förster resonance energy transfer (FRET)

because the emission wavelength of α-tocopherol (325 nm) overlaps with the excitation wavelength (323 nm) of the rosmarinates. Therefore, closer the proximity between α-tocopherol and the rosmarinates would be expected to produce greater quenching.

In the O/W emulsions, the fluorescence intensity of α-tocopherol was decreased in the presence of the rosmarinates. As illustrated in Figure 5.5, R0 was more effective at quenching the α-tocopherol fluorescence in O/W emulsion compared to the rosmarinic acid esters (R4 to R12) as shown by R0's higher quenching constant. Results suggested that the more water soluble rosmarinic acid (R0) was able to interact with α-tocopherol on the emulsion droplet surfaces. The lower the quenching constants of rosmarinic acid esters suggest that slow lateral diffusion on the droplet surface would limit their ability to interact with α-tocopherol.

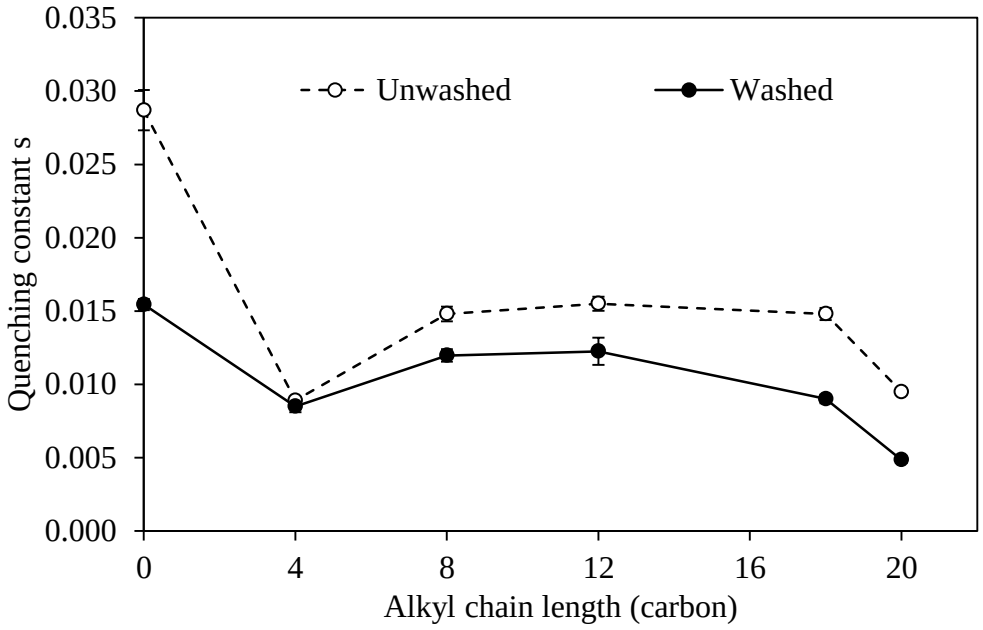


Figure 5.5 Fluorescence quenching of α-tocopherol (λ_{em}= 325 nm) by the rosmarinic acid esters in O/W emulsions with/without influences of surfactant micelles

When emulsions are produced, excess surfactant that is not absorbed onto the emulsion droplet surface partitions into the aqueous phase forms micelles. These micelles can alter the partitioning of antioxidants into the continuous phase by solubilizing the antioxidants into the micelles. To eliminate the influence of surfactant micelles, the excess surfactants in O/W emulsions were removed by a washing process (*221*). Removal of the surfactant micelles decreased the ability of all the rosmarinic acid derivatives to quench the fluorescence of α-tocopherol with the exception of the R4 ester. This decrease was most dramatic for rosmarinic acid (R0) such that its quenching constant became similar to the rosmarinic acid esters. This suggests that the removal of the surfactant micelles decreased the partitioning of α-tocopherol into the aqueous phase which decreased the ability of α-tocopherol and water-soluble rosmarinic acid to interact. The α-tocopherol quenching constants of the other rosmarinic esters also decreased suggesting that the micelles also decreased their ability to interact with α-tocopherol. Since all of the antioxidants are surface active, the decrease in interfacial area caused by the removal of the micelles would be expected to decrease the partitioning of the antioxidants in the interface of the emulsion droplets and micelles and thus decrease antioxidant interactions especially if some of the antioxidant was forced into the interior of the emulsion droplet.

5.3.4 Electrochemical Properties of the Rosmarinic Acid Esters and α-Tocopherol

One possible reason for the observed variations in antioxidant activity for combinations of rosmarinic acid esters and α-tocopherol could be due to the regeneration

of one antioxidant by the other. The hierarchy of antioxidant regeneration by electron transfer can be estimated by their oxidation-reduction potentials. Generally, an antioxidant with lower reduction potential is thermodynamically preferred to give electron to an antioxidant with higher reduction potential (*60*). Cyclic voltammetry can be a useful method for studying the reduction potential of antioxidant compounds (*117, 222-225*). In this study, cyclic voltammetry was performed in order to investigate the influence of esterification on the electrochemical properties of rosmarinic acid and α-tocopherol in various solutions.

Results showed that cyclic voltammograms of the rosmarinate esters and α-tocopherol exhibited one anodic and one cathodic peak (data not shown) in acetonitrile. This is also true for other phenolic compounds (*226, 227*). As shown in Table 5.1, all the rosmarinate esters showed a similar oxidation peak (E_{Pa}^{ox}) at approximately 1115-1137 mV (vs. Ag/AgCl) in acetonitrile solution which was significantly higher than α-tocopherol (634.9 mV). Electrochemical parameters of the rosmarinate esters did not show the non-linear behaviors that where observed when their activity was tested with the DPPH assay or in lipid oxidation studies (*16, 17, 221, 228*).

Oxidation peak potentials of rosmarinic acid and α-tocopherol were further studied in 50 mM phosphate buffer, pH 7.0 and 1% Tween 20 in 50 mM phosphate buffer solutions. Trolox was used instead of α-tocopherol in these experiments due to solubility limitations of α-tocopherol in aqueous solutions. Results showed that rosmarinic acid reduction potential decreased in the phosphate buffer however, the rosmarinic acid still had higher reduction potentials than Trolox. This was also true in the presence of Tween

micelles where the rosmarinic acid reduction potential decreased compared to acetonitrile but was still greater than α-tocopherol. These results suggested that α-tocopherol is thermodynamically preferred to donate electrons to rosmarinic acid.

Table 5.1 Electrochemical parameters of tested antioxidants in acetonitrile, 50 mM phosphate buffer (pH 7), and 1% Tween 20 in 50 mM phosphate buffer (pH 7) solutions at 25^{o}C obtained from cyclic voltammetry (CV)

Antioxidants	Oxidation peak potential (mV vs. Ag/AgCl)
R0 (ACN)	1115.1±10.7
R4 (ACN)	1132.9±7.1
R8 (ACN)	1126.0±8.2
R12 (ACN)	1133.9±5.6
R18 (ACN)	1135.3±8.4
R20 (ACN)	1137.4±1.6
α-TOH (ACN)	634.9±0.85
R0 (PBS, pH 7)	359.0±19.2
Trolox (PBS, pH 7)	275.0±12.0
R0 (Tw20, pH7)	394.3±16.9
α-TOH (Tw20, pH 7)	263.3±2.3
Trolox (Tw20, pH 7)	298.7±18.5

5.3.5 Regeneration Efficiency of the Rosmarinic Acid Esters to Reduce α-Tocopheroxyl Radical in Homogeneous and Heterogeneous Systems

The ability of rosmarinic acid and its esters and α-tocopherol to regenerate each other's radicals can be determined by electron paramagnetic resonance. To determine these interactions, antioxidant radicals were produced by exposing the antioxidant to DPPH radicals. However, rosmarinic acid and its esters were not able to produce stable radicals that could be observed by electron paramagnetic resonance (EPR) using this method so the following studies focused on the fate of α-tocopherol radicals in the presence of rosmarinic acid and its esters. Experiments were performed in both ethanol and 100 mM Tween 20 solutions in order to observe the ability of the different alkyl chain lengths of rosmarinic acid to impact the efficiency of α-tocopherol regeneration in homogenous and heterogeneous systems. Overall, the efficiency of rosmarinic acid and its esters to reduce α-tocopherol radical was very low as predicted by electron reduction potential (Table 5.1). The range of α-tocopherol radical regeneration efficiencies by rosmarinic acid and its esters ranged from 0.08 to 0.55 moles α-tocopherol radicals reduced/ mole of phenolics.

As shown in Figure 5.6, esterification of rosmarinic acid increased electron-donating ability towards α-tocopheroxyl radicals compared to rosmarinic acid in ethanolic solutions. Esterification has also been observed to increase the DPPH scavenging activity of rosmarinic acid esters (*195, 221*) and chlorogenic acid esters (*208*) in methanolic solution. Lecomte and coworkers (*195*) reported that dodecyl rosmarinate (R12) had the greatest DPPH scavenging activity of all the esters tested (4-20 carbons),

while Lopez-Giraldo and corkers (2009) showed that butyl- and octyl chlorogenate esters had higher DPPH scavenging activity than chlorogenic acid itself and its esters with alkyl chains longer than 12 carbons. In this study, R4 had the highest the α-tocopheroxyl radical scavenging efficiency compared to other esters in ethanol solution.

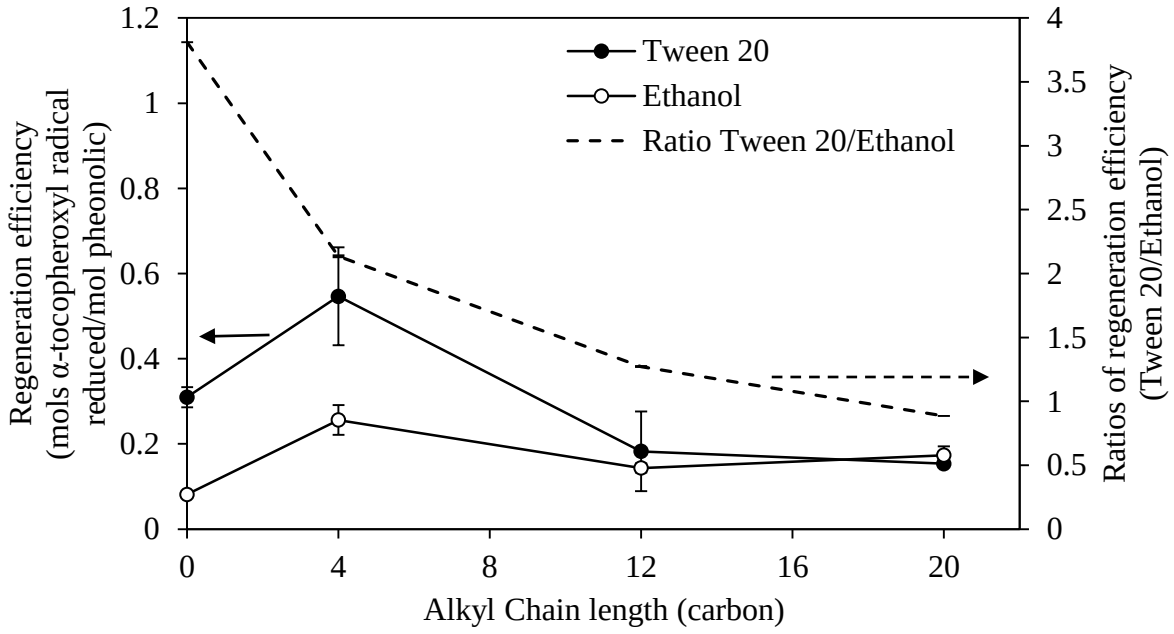


Figure 5.6 Regeneration efficiencies of the rosmarinic acid esters to reduced α-tocopheroxyl radicals in homogenous (ethanol) and heterogeneous (Tween 20) micelle solutions. Dash line indicates ratios of regeneration efficiency of Tween 20 and ethanol. Data points and error bars represent means (n=5) ± standard deviations.

To investigate the influence of physical structure on α-tocopherol radical regeneration by the rosmarinates, measurements were also performed in 50 mM

phosphate buffer solution with surfactant micelles produced from 100 mM Tween 20, pH 7.0. The ratio of scavenging regeneration efficiency in Tween 20 versus ethanol for R0 and R4 increased by 381 and 214 %, respectively while R12 and R20 where essentially the same in ethanol and Tween 20. The results for R0 are similar to those observed for R0 quenching of fluorescence again suggesting that the high partitioning of R0 in the aqueous phase allowed it to interact with α-tocopherol radicals at the Tween 20 micelle interface. The fact that R4 was effective at interacting with α-tocopheroxyl radicals but did not alter α-tocopherol fluorescence could be due to the lack of emulsion droplets in the EPR study. The presence of emulsion droplets in the fluorescence study could result in R4 partitioning in the emulsion droplet in a manner where it did not readily interact with α-tocopherol whereas R4 would interact with α-tocopheroxyl radicals in surfactant micelles.

5.3.6 Effects of Various Concentrations of the Rosmarinic Acid Esters on Antioxidant Interactions with α-Tocopherol

Studies on α-tocopherol fluorescence quenching and α-tocopherol radical regeneration efficiency indicated that of all the rosmarinates, R0 interacted with α-tocopherol more than the rosmarinic acid esters. This suggests that the ability of α-tocopherol to greatly increase the antioxidant activity of R0 could be due to their interactions. To further investigate the potential interaction between the rosmarinates and α-tocopherol, decomposition of the antioxidants was determined during storage and compared to formation of the lipid oxidation product, hexanal in the O/W emulsion.

The decomposition of R0, R12 and α-tocopherol in the O/W and subsequent hexanal formation are shown in (Figure 5.7A-C). Results showed that α-tocopherol concentrations decreased in a linear fashion (Figure 7A). α-Tocopherol concentrations were approximately 8-12 μM when the lag phase of hexanal formation ended. The concentration of R0 and R12 also decreased in a linear fashion during storage of the O/W emulsions (Figure 5.7B and C). However, both R0 and R12 were completely depleted prior to formation of hexanal.

Decomposition of the rosmarinates and α-tocopherol during the storage of the O/W emulsions when the antioxidants were added in combination is shown in Figure 5.8 and 9. In this study, α-tocopherol concentration was constant at 30 μM while the rosmarinates ranged from 15 to 60 μM. As was previously observed in Figures 5.3, the combination of α-tocopherol and R0 produced synergistic antioxidant activity while α-tocopherol and R12 showed an additive effect.

The decomposition of R0 and R12 was very similar in the presence of α-tocopherol (Figure 5.8A and 5.9 A). For example, the time at which approximately 50% of R0 and R12 was lost was about 9 days. The similarity of R0 and R12 depletion in the presence of α-tocopherol suggests that the increase in the antioxidant activity of R0 by α-tocopherol was not due to α-tocopherol regenerating R0 and keeping R0 concentrations higher. Conversely, α-tocopherol depletion was much faster in the presence of R0 than R12 (Figure 5.8B and Figure 5.9B). For example at 30 days of storage, α-tocopherol concentrations were less than 5 μM in the presence of R0 (60 μM) compared to 11 μM in the presence of R12 (60 μM). In addition, the lag times of tocopheryl quinone (TQ)

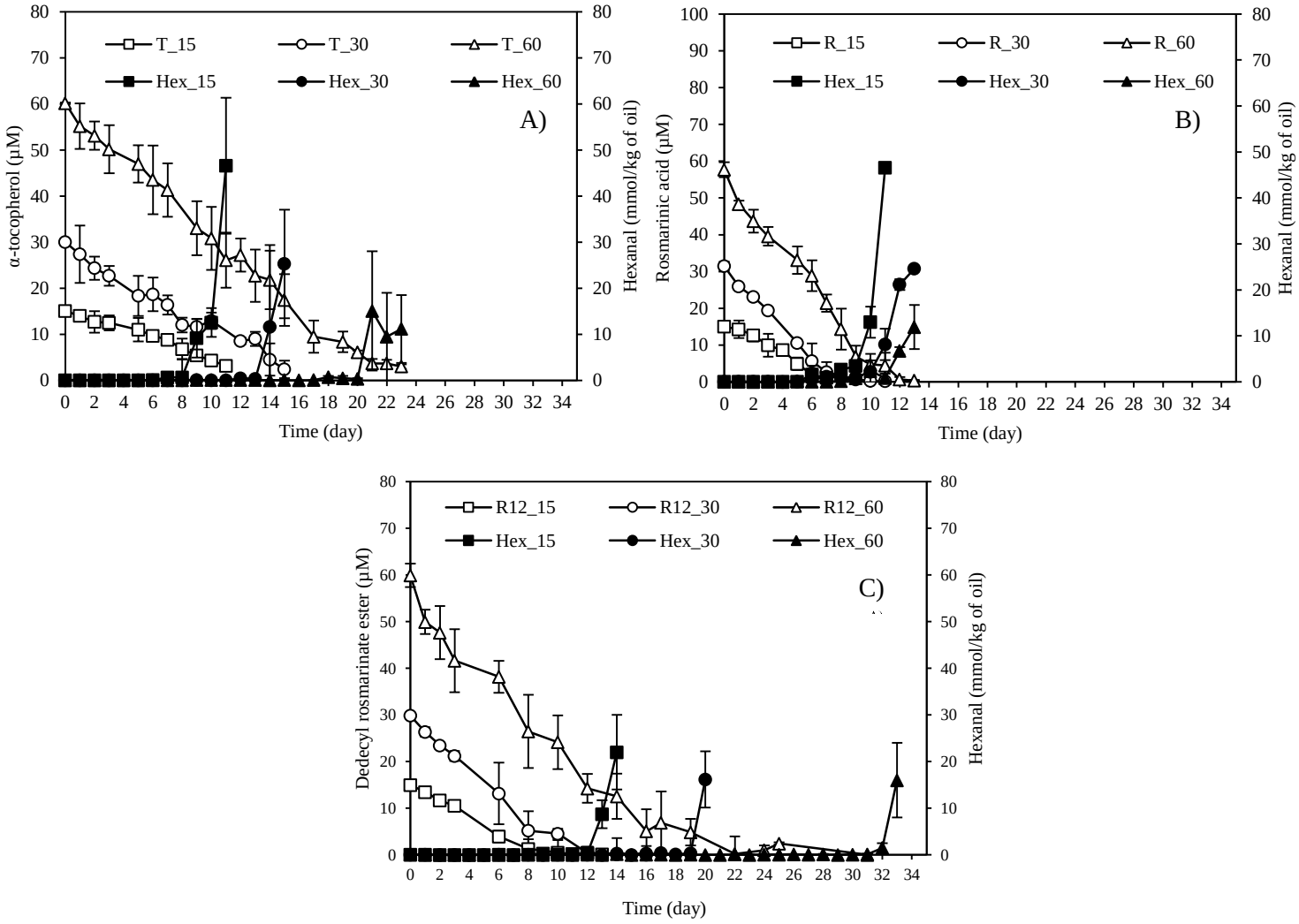


Figure 5.7 Effects of different concentrations of antioxidants (15, 30 and 60 μM) on decompositions of α-tocopherol (A), rosmarinic acid (B) and dodecyl rosmarinate ester (C), and hexanal formation during the oxidation of O/W emulsions at 25°C

formation were different between R0 and R12 samples (Figure 5.8B and 5.9B). For example, tocopheryl quinone was detected in the R0 samples when R0 was almost depleted whereas tocopheryl quinine was detected much earlier during storage in the

presence of R12 with the lag phase for TQ formation being independent of R12 concentration.

The observation that R0 increased the lag phase for TQ formation again suggests that the two antioxidants were interacting. It's also interesting to note that R0 and α-tocopherol samples that had similar oxidative stability to R12 and α-tocopherol samples even though α-tocopherol concentrations were lower in the R0 samples. This suggests that other antioxidative compounds might exist in the emulsions since it has been reported that some antioxidants can produce other antioxidative compounds via their oxidative degradation (*220, 229*).

Besides chemical changes in α-tocopherol, α-tocopheryl quinone and the rosmarinates, the major oxidation products generated from R0 and R12 were analyzed to get a better understanding of the dynamics of the antioxidant mechanisms in the O/W emulsions. Only one major breakdown product of R0 was observed during storage while two major breakdown products were observed for R12. From LC-MS analysis, the products at HPLC retention times of 10.5 (in R0 and R12) and 35.9 (in R12) min are caffeic acid [m/z = 179.2 $(M-H)^-$] and a rosmarinic acid quinone formed on the 2-oxyphenylpropanonyl moiety [m/z = 525.06 $(M-H)^-$]. The structures of major antioxidant oxidation products were showed in Figure 10. The quinone was previously reported that the major oxidation product of rosmarinic acid as determined in 2,20-Azobis(isobutyronitrile)(AIBN)-induced ethyl linoleate and DPPH model systems (*220*).

However, unlike the above single phase model systems using ethyl linoleate and alcohols, our results indicated that the antioxidant product of rosmarinic acid in O/W

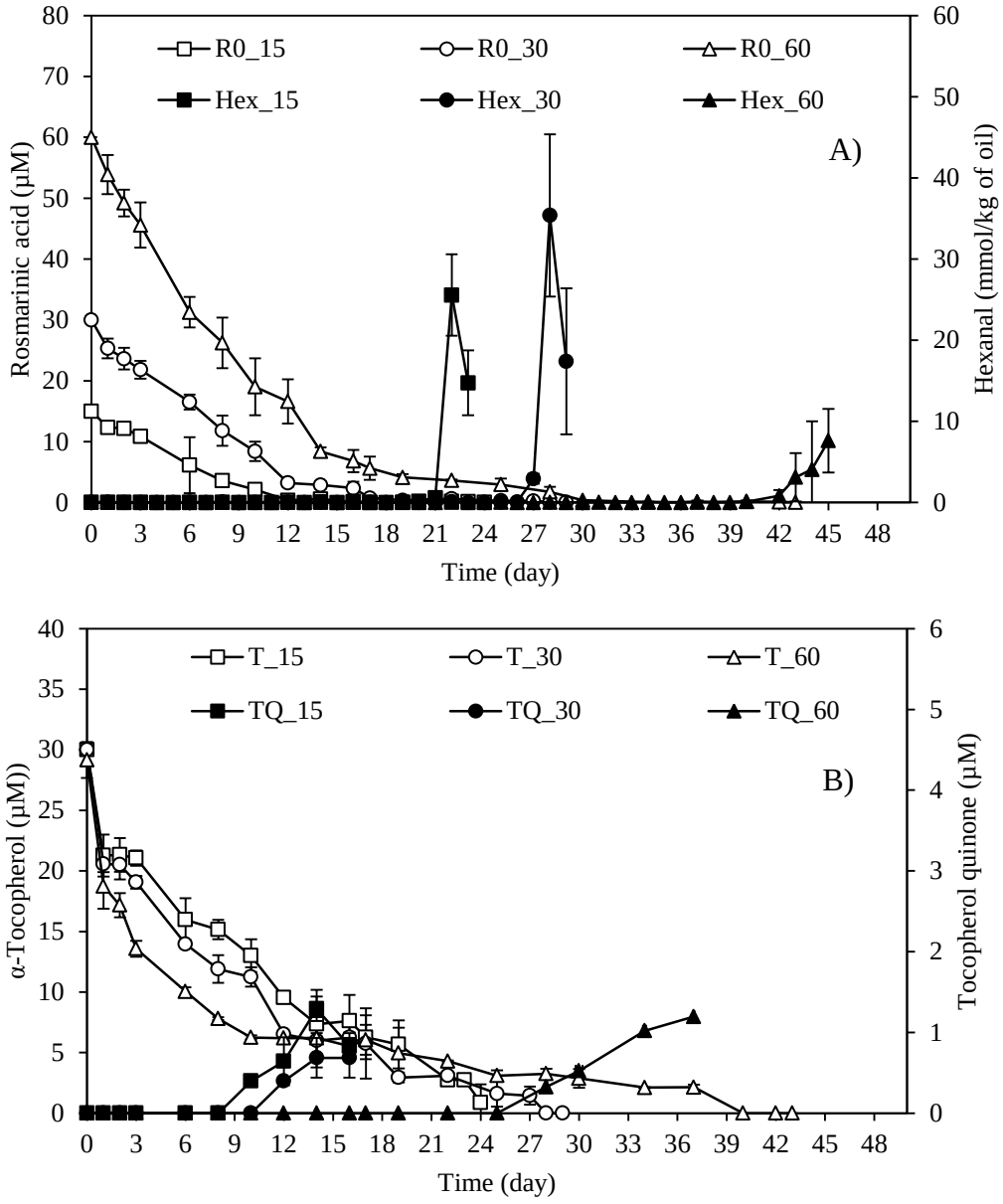


Figure 5.8 Effects of various concentrations (15, 30 and 60 µM) of rosmarinic acid (R0) on decompositions of rosmarinic acid and hexanal formation (A), and decompositions of α-tocopherol and tocopheroxyl quinone (TQ) formation (B) in 1% stripped soybean oil-Tween 20 emulsions at 25^{o}C in the combination with α-tocopherol (30µM). Data points and error bars represent means (n = 3) ± standard deviations.

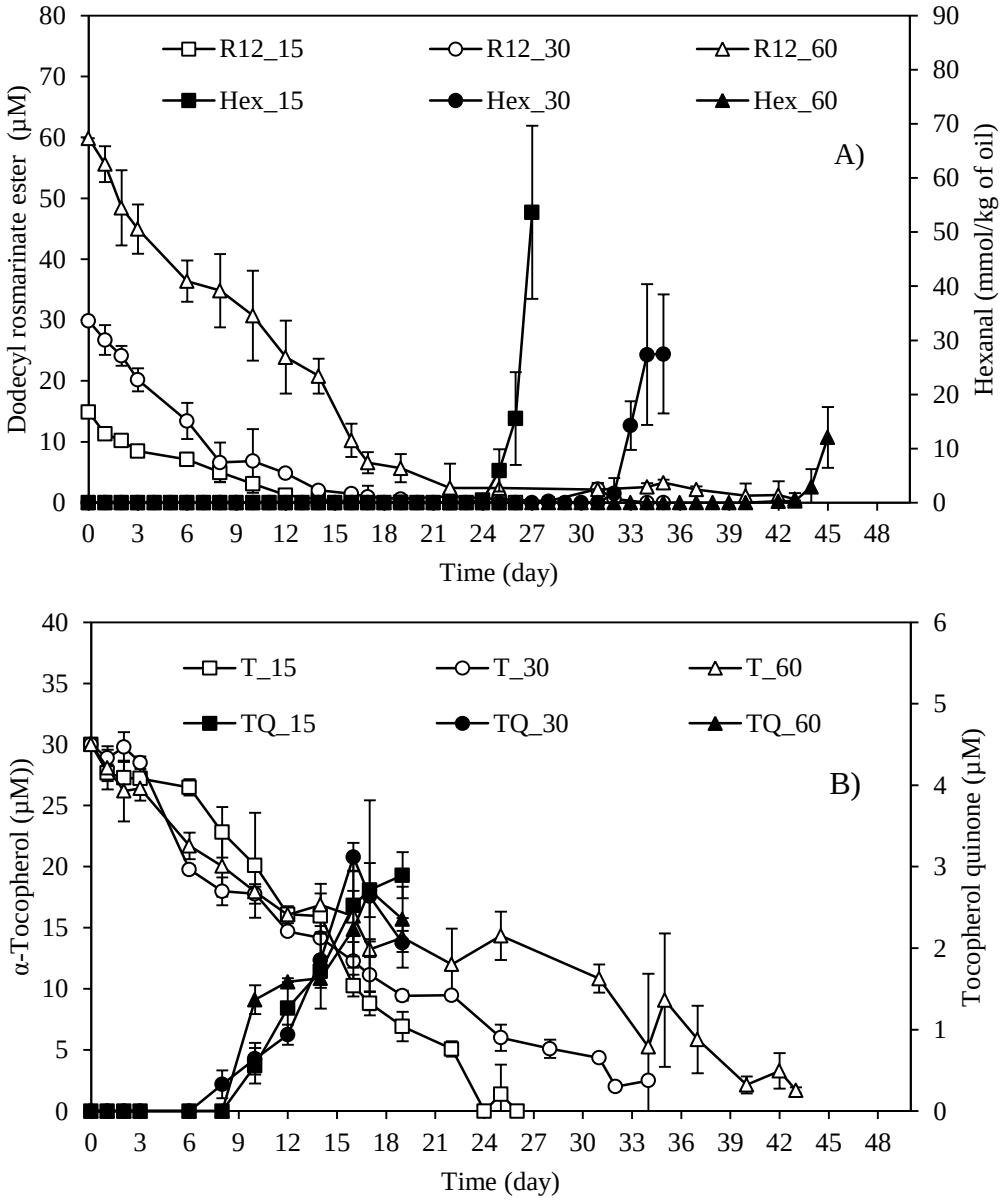
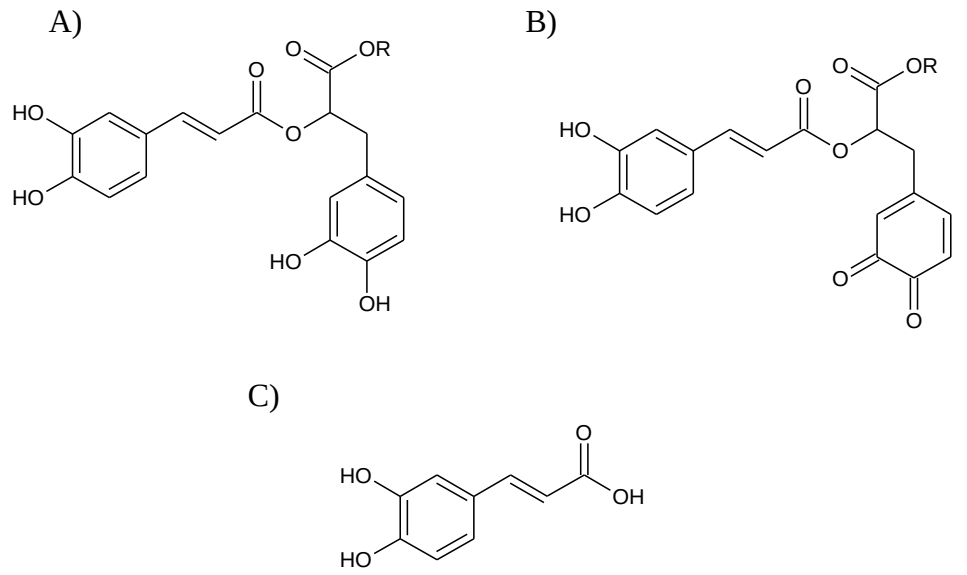


Figure 5.9 Effects of various concentrations (15, 30 and 60 µM) of dodecyl rosmarinate ester (R12) on decompositions of dodecyl rosmarinate ester and hexanal formation (A), and decompositions of α-tocopherol and tocopheroxyl quinone (TQ) formation (B) in 1% stripped soybean oil-Tween 20 emulsions at 25^{o}C in the combination with α-tocopherol (30µM). Data points and error bars represent means (n = 3) ± standard deviations.



R = H = Rosmarinic acid

R= $C_{12}H_{25}$ = Dodecyl rosmarinate

Figure 5.10 Structures of main antioxidant products observed during oxidation. A) represents reduced form of rosmarinic acid and dodecyl rosmarinate. B) represents the quinone formed on the 2-oxyphenylpropanonyl moiety of dodecyl rosmarinate (the quinone of rosmarinic acid was not detected). C) represents caffeic acid observed in the oxidation od rosmarinic acid and dodecyl rosmarinate.

emulsions was mainly caffeic acid (Figure 5.11). This could be because the rosmarinate quinone originating from R0 was unstable in O/W emulsions and was fragmented to caffeic acid. It was reported that rosmarinic acid can be decomposed into caffeic acid via a McLafferty rearrangement (γ-H rearrangement with β-cleavage in the electron ionization) in mass spectrometry analysis (*229*). Caffeic acid has also been found to be one of the metabolites of rosmarinic acid in rats (*230*). Hydrolysis of rosmarinic acid by

esterases to caffeic acid and 3,4-didydroxyphenyllactic acid *in vitro* was reported, however the hydrolysis was not observed in a gastrointestinal model with lipase and pancreatic enzymes (*231*). The exact mechanism for caffeic acid formation in the O/W emulsions is not known.

In both the absence and presence of α-tocopherol, caffeic acid was produced from both R0 and R12 (Figure 5.11). More caffeic acid was produced from R0 in the presence of α-tocopherol (Figure 5.11A) suggesting that α-tocopherol could be involved in the formation of caffeic acid. α-Tocopherol was not found to increase production of caffeic acid from R12 (Figure 5.11B).

In all treatments, caffeic acid was detected at the beginning of the oxidation process and then decreased until at the end of the oxidation lag times (Figure 5.11A and B). Except for R0 alone, the hexanal lag times ended before all caffeic acid was depleted. Caffeic acid has the ability to scavenge free radicals (*65*) and Chen and Ho (*232*) (1997) reported that caffeic acid had better antioxidant activity in O/W emulsions than rosmarinic acid and α-tocopherol. In this study, the degradation of caffeic acid prior to the end of the lag phase also suggests that it is being preferentially oxidized prior to the fatty acids and thus is acting as an antioxidant. Therefore the formation of caffeic acid from R0 in the presence of α-tocopherol could explain why this combination had much better antioxidant activity that the individual antioxidants since the caffeic acid would provide an additional antioxidant to slow down oxidation (Figure 5.9A and 5.10A).

The quinone of dodecyl rosmarinate ester was observed in the samples with R12 but not R0 suggesting that the esterification of rosmarinic acid with an alkyl chain can

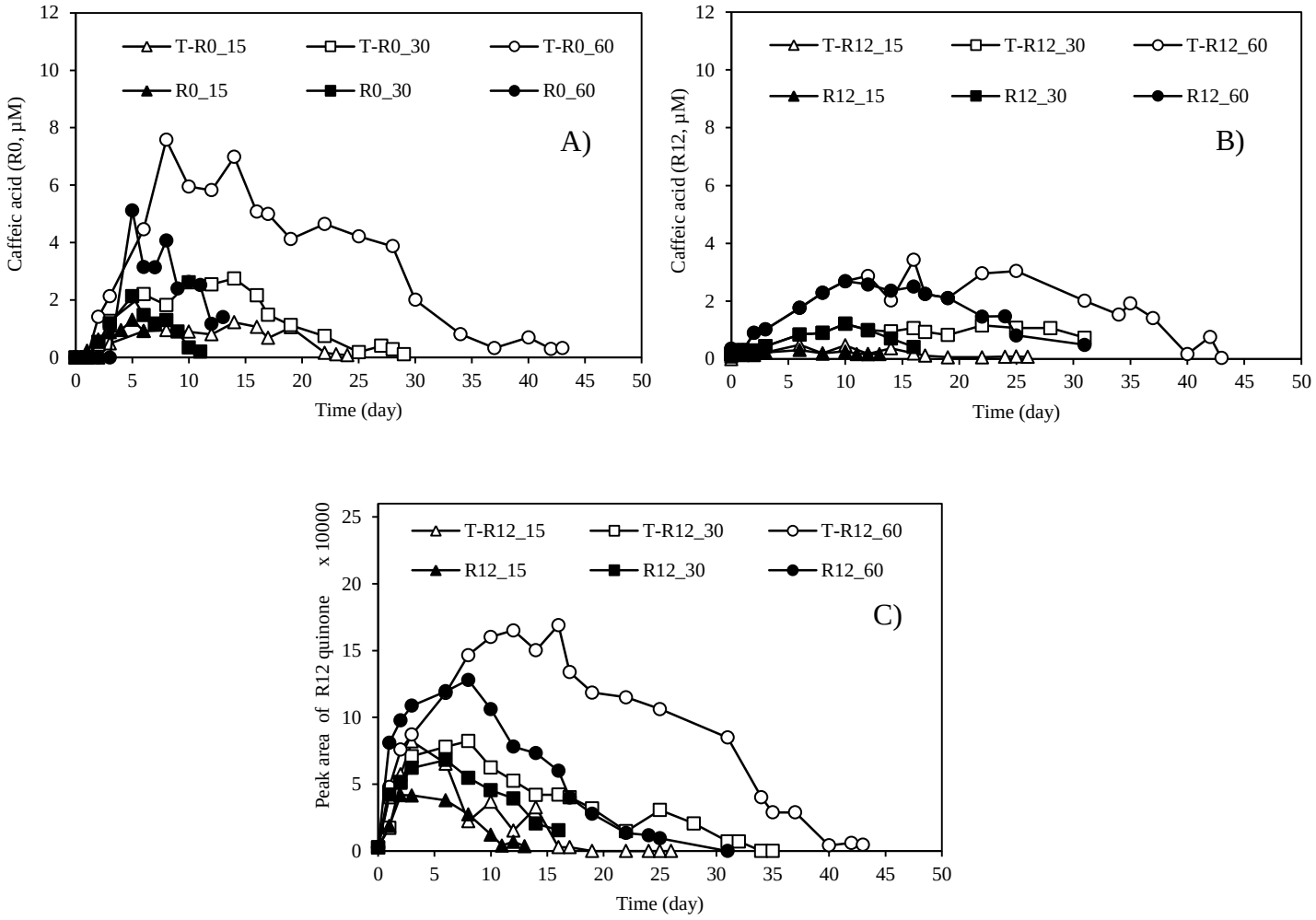


Figure 5.11 Accumulation (A_{330}) of caffeic acid in R0 (A) and R12 (B), and the quinone in R12 (C) observed in the individual and the combinations with α-tocopherol at different concentrations during the oxidation in O/W emulsions

increase the stability of the quinone (Figure 5.11C). However, this could also be due to R12 reacting less with α-tocopherol than R0 as shown by the fluorescence and EPR data (Figures 5.5 and 5.6) thus decreasing the conversion of the quinone to caffeic acid.

In conclusion, R0 was physically able to interact with α-tocopherol in surfactant micelles and O/W emulsions. We hypothesize that the observed synergistic antioxidant activity of the combination of R0 and α-tocopherol were not due to the regeneration of α-tocopherol by rosmarinic acid due to the thermodynamic infeasibility of this reaction and the fact that α-tocopherol degradation rates in O/W emulsions were not decreased by rosmarinic acid. In addition, the regeneration of the rosmarinate radical by α-tocoherol was also unlikely since is reaction was slow and α-tocopherol did not alter R0 degradation rates. Instead α-tocopherol and R0 interactions produced an increased in antioxidant activity by promoting the conversion of rosmarinic acid to caffeic acid thus providing a third molecule that could inhibit lipid oxidation and increased the oxidative stability of the O/W emulsion.

CHAPTER 6

CONCLUSION

To improve performance of antioxidants in O/W emulsions, many investigated strategies have underlined the importance of understanding principles of antioxidant mechanisms and how they interact with other antioxidants and food matrixes. This research has demonstrated 3 main strategies for improving antioxidant activity in oil-in-water (O/W) emulsions, which are 1) modifications of interfacial properties of emulsion droplets, 2) increasing antioxidant partitioning at the interface, and 3) combinations of antioxidants.

The interfacial property of negatively charged liposomes was modified by chitosan. Chitosan coated liposomes have positively charged surfaces which can repulse prooxidative metal ions from the interface. Using octadecyl rosmarinate esters (40 μM) alone was not efficient enough to inhibit lipid oxidation of negatively charged liposomes at 55 ^{o}C. After coating with chitosan, octadecyl rosmarinate ester has higher antioxidant performance in positively charged liposomes. This phenomenon highlights how antioxidants can be prone to be directly oxidized by iron or can be consumed by iron generated free radicals in food systems. Effectiveness of antioxidant performance can be improved synergistically by controlling prooxidative metal ions by addition of metal chelators (EDTA) and modification of interfacial property.

Esterification of rosmarinic acid with various alkyl chain lengths has improved its antioxidant activity due to increase partitioning of antioxidants at the interface of liposomes and O/W emulsions, so antioxidants can effectively inhibit lipid oxidation. Interestingly, there were no differences in the partitioning behavior or the antioxidant activity from butyl- (R4) to dodecyl-(R12) rosmarinate esters in liposomes and O/W emulsions. This finding was different from previously reports in other model studies which lipid oxidation was artificially accelerated by other free radial species. The cut-off effect was observed when alkyl chain lengths were longer than 18 carbons as these antioxidants had reduced activity.

The observed versatile antioxidant activity of rosmarinate esters has emphasized the significant or interactions between antioxidant and food matrix (excess surfactant). Overall alterations in antioxidant activity could be partially explained by dynamic changes in antioxidant locations between the interface of emulsion droplets and micelles. In general, antioxidant activity of surface active antioxidants (R4-R12) decreased due to increased partitioning into the aqueous phase by excess surfactants micelles thus diluting the antioxidant concentrations in the emulsion droplet and droplet interface. However, the antioxidant activity of eicosyl rosmarinate (R20) has improved by surfactant micelles due to increases in its concentration at the interface of emulsion droplets and micelles. It was noted that increases in alkyl chain length longer than 18 carbons results in abnormal antioxidant behaviors and locations which can be both located in oil interior of the emulsion droplets and micellar structures in the water phase.

Another potential strategy to improve antioxidant activity is combinations of antioxidants. It has been well known that combination between free radical scavengers and metal chelators may results in strong synergistic antioxidant effect as can combinations of antioxidants that can promote antioxidant regeneration by free radical transfer mechanisms. Results suggested that locations of antioxidants have a large influence on their ability to interact at the O/W interface. Rosmarinic acid (water soluble) exhibited more interactions with α-tocopherol at the interface than any of the more nonpolar ester forms. Antioxidant synergy was observed only between rosmarinic acid and α-tocopherol while surface active antioxidants (R4-R12) exhibited additive effect with α-tocopherol. Eicosyl rosmarinate ester (R20) was an exception to this trend as it exhibited antagonistic effect with α-tocopherol.

Synergistic antioxidant mechanism between rosmarinic acid and α-tocopherol could not be explained by electron transfer mechanism. Although direct observations of regeneration of α-tocopherol by rosmarinate esters were determined by the EPR study, the HPLC analysis of oxidative decompositions products of antioxidants suggested that α-tocopherol could not be spared by rosmarinic acid. Based on the cyclic voltammetry analysis, thermodynamic reaction between rosmarinate esters and α-tocopherol is not an important mechanism. It was pointed out that different oxidation mechanisms of rosmarinic acid and dodecyl rosmarinate ester were influenced by esterification. Adding an alkyl chain to the structure of rosmarinic acid has improved the stability of the quinone form of dodecyl rosmarinate ester while the main antioxidant oxidation products of rosmarinic acid was caffeic acid. Disappearance of rosmarinic acid and increase in caffeic acid were believed to be the key of shifting antioxidant/prooxidant balance of

rosmarinic acid as indicated by reduction of the oxidation rate of α-tocopherol and improved oxidative stability of the emulsion.

This research demonstrated complex mechanisms of antioxidant interactions between rosmarinate esters and α-tocopherol in O/W emulsions. Since most antioxidants have reduction potential higher than α-tocopherol, this knowledge might apply to other natural antioxidants to explain antioxidant interactions in O/W emulsions. To obtained synergistic antioxidant effects, the ratios and concentration of antioxidants have to be optimized. For example if the concentration of rosmarinic acid higher than 60 μM, the concentration of α-tocopherol might reduce dramatically in short period of time. Caffeic acid alone from the oxidation of rosmarinic acid could not effective enough to inhibit lipid oxidation. In this case, the combination might result in additive or antagonistic effect.

More interestingly, the additive effect of surface active antioxidants (e.g. dodecyl rosmarinate ester and α-tocopherol) is more predictable and less dependent on concentrations and ratios used. Overall, combining surface active antioxidant to obtained additive effect might be a better strategy when choosing combinations of antioxidants to use in food systems.

BIBLIOGRAPHY

1. Inverson, F., Phenolic antioxidants: health protection branch studies on butylated hydroxyanisole. *Cancer Lett.* **1995,** 93, 49-54.

2. Alamed, J.; Chaiyasit, W.; McClements, D. J.; Decker, E. A., Relationships between Free Radical Scavenging and Antioxidant Activity in Foods. *Journal of agricultural and food chemistry* **2009,** 57, (7), 2969-2976.

3. Porter, W. L. B., E. D.; Drolet, A. M., 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* **1989,** 1989, (37), 615-624.

4. Frankel, E. N.; Huang, S. W.; Kanner, J.; German, J. B., Interfacial phenomena in the evaluation of antioxidants - bulk oils vs emulsions. *Journal of agricultural and food chemistry* **1994,** 42, (5), 1054-1059.

5. Alvin C. Chanl, K. T., Tina RaynorP, eter R. Ganz, and ChingK . Chow, Regeneration of vitamin E in human platelets. *The journal of biological chemistry* **1991,** 266, (26), 17290-17295.

6. Mukai, K.; Kikuchi, S.; Urano, S., Stopped-flow kinetic-study of the regeneration reaction of tocopheroxyl radical by reduced ubiquinone-10 in solution. *Biochimica et biophysica acta* **1990,** 1035, (1), 77-82.

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

8. Thomas, C. E.; McLean, L. R.; Parker, R. A.; Ohlweiler, D. F., Ascorbate and phenolic antioxidant interactions in prevention of liposomal oxidation. *Lipids* **1992,** 27, (7), 543-550.

9. Han, R. M.; Chen, C. H.; Tian, Y. X.; Zhang, J. P.; Skibsted, L. H., Fast regeneration of carotenoids from radical cations by isoflavonoid dianions: Importance of the carotenoid keto group for electron transfer. *Journal of Physical Chemistry A* **2010,** 114, (1), 126-132.

10. Zhou, B.; Wu, L. M.; Yang, L.; Liu, Z. L., Evidence for alpha-tocopherol regeneration reaction of green tea polyphenols in SDS micelles. *Free radical biology & medicine* **2005,** 38, (1), 78-84.

11. Zhu, Q. Y.; Huang, Y.; Tsang, D.; Chen, Z.-Y., Regeneration of α-Tocopherol in Human Low-Density Lipoprotein by Green Tea Catechin. *Journal of agricultural and food chemistry* **1999,** 47, (5), 2020-2025.

12. Mukai, K.; Mitani, S.; Ohara, K.; Nagaoka, S., Structure-activity relationship of the tocopherol-regeneration reaction by catechins. *Free radical biology & medicine* **2005,** 38, (9), 1243-56.

13. Mahoney, J. R. a. G., E., Role of α–tocopherol, ascorbic aicd, and citric and EDTA as oxidants in model systems. *Journal of Food Science* **1986,** 51, (5), 1293-1296.

14. Chen, B. C.; Han, A.; McClements, D. J.; Decker, E. A., Physical Structures in Soybean Oil and Their Impact on Lipid Oxidation. *Journal of agricultural and food chemistry* **2010,** 58, (22), 11993-11999.

15. Chaiyasit, W.; Elias, R. J.; McClements, D. J.; Decker, E. A., Role of physical structures in bulk oils on lipid oxidation. *Critical reviews in food science and nutrition* **2007,** 47, (3), 299-317.

16. Laguerre, M.; Giraldo, L. J.; Lecomte, J.; Figueroa-Espinoza, M. C.; Barea, B.; Weiss, J.; Decker, E. A.; Villeneuve, P., Chain length affects antioxidant properties of chlorogenate esters in emulsion: the cutoff theory behind the polar paradox. *Journal of agricultural and food chemistry* **2009,** 57, (23), 11335-42.

17. Laguerre, M.; Lopez Giraldo, L. J.; Lecomte, J.; Figueroa-Espinoza, M. C.; Barea, B.; Weiss, J.; Decker, E. A.; Villeneuve, P., 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* **2010,** 58, (5), 2869-76.

18. Medina, I.; Lois, S.; Alcantara, D.; Lucas, R.; Morales, J. C., Effect of lipophilization of hydroxytyrosol on its antioxidant activity in fish oils and fish oil-in-water emulsions. *Journal of agricultural and food chemistry* **2009,** 57, (20), 9773-9779.

19. Sorensen, A. D. M. d. D., S. Petersen, L. K., Nielsen, N. S., Yang, Z., Xu, X., Jacobsen, C. In *Effect of lipophilization of dihydrocaffeic acid on its antioxidative properties in fish oil enriched emulsion.*, Proceedings of the 8th Euro Fed Lipid Congress, Munich, Germany, Nov 21-24, 2010; Munich, Germany, 2010.

20. Lucas, R.; Comelles, F.; Alcántara, D.; Maldonado, O. S.; Curcuroze, M.; Parra, J. L.; Morales, J. C., Surface-active properties of lipophilic antioxidants tyrosol and hydroxytyrosol fatty acid esters: A potential explanation for the nonlinear hypothesis of the antioxidant activity in oil-in-water emulsions. *Journal of agricultural and food chemistry* **2010,** 58, (13), 8021-8026.

21. McClements, D. J. a. D., E. A. , Lipids. In *Fennema's Food chemistry*, 4 ed.; Damodaran, S., Parkin, K. L. and Fennema, O. R., Ed. CRC Press: FL, 2008; p 1144.

22. Min, D. B.; Li, T. L.; Lee, H. O., Effects of processing steps on the contents of minor compounds and oxidation of soybean oil. In *Process-Induced Chemical Changes in Food*, Shahidi, F.; Ho, C. T.; vanChuyen, N., Eds. 1998; Vol. 434, pp 161-180.

23. Waraho, T.; McClements, D. J.; Decker, E. A., Mechanisms of lipid oxidation in food dispersions. *Trends in Food Science & Technology* **2011,** 22, (1), 3-13.

24. Fan, X., Control of irradiation-induced lipid oxidation and volatile sulfur compounds using antioxidants in raw meat and ready-to-eat meat products. In *Antioxidant Measurement and Applications*, American Chemical Society: 2007; Vol. 956, pp 401-418.

25. Kamal Eldin, A.; Appelqvist, L. A., The chemistry and antioxidant properties of tocopherols and tocotrienols. *Lipids* **1996,** 31, (7), 671-701.

26. Decker, E. A., Chen, B., Panya, A. and Elias, R.J., Antioxidant mechanisms. In *Oxidation in foods and beverages and antioxidant applications, Volume 1. Understanding mechanisms of oxidation and antioxidant activity*, Decker, E. A., Elias, R.J. and McClements, D.J., Ed. Woodhead Publishing: London, UK, 2010; Vol. 1, p 408.

27. Gardner, H. W.; Kleiman, R.; Weisleder, D.; Inglett, G. E., Cysteine adds to lipid hydroperoxide. *Lipids* **1977,** 12, (8), 655-660.

28. Lambelet, P. S., F. and Loliger, J, Radical exchange reactions between vitamin E and vitamin C and phospholipids in autoxidizing polyunsaturated lipids. *Free Rad. Res* **1994,** 20, (1), 1-10.

29. Sims, R. J.; Fioriti, J. A., Methional as an antioxidant for vegetable-oils. *Journal of the American Oil Chemists Society* **1977,** 54, (1), 4-7.

30. Terao, J.; Yamauchi, R.; Murakami, H.; Matsushita, S., Inhibitory effects of tocopherols and beta-carotene on singlet oxygen-initiated photo-oxidation of methyl linoleate and soybean oil. *Journal of Food Processing and Preservation* **1980,** 4, (1-2), 79-93.

31. Burton, G. W.; Ingold, K. U., Beta-carotene - An unusual type of lipid antioxidant. *Science* **1984,** 224, (4649), 569-573.

32. Kennedy, T. A.; Liebler, D. C., Peroxyl radical scavenging by beta-carotene in lipid bilayers - effect of oxygen partial-pressure. *Journal of Biological Chemistry* **1992,** 267, (7), 4658-4663.

33. Palozza, P.; Krinsky, N. I., Beta-carotene and alpha-tocopherol are synergistic antioxidants. *Archives of Biochemistry and Biophysics* **1992,** 297, (1), 184-187.

34. Let, M. B.; Jacobsen, C.; Meyer, A. S., Ascorbyl palmitate, gamma-tocopherol, and EDTA affect lipid oxidation in fish oil enriched salad dressing differently. *Journal of agricultural and food chemistry* **2007,** 55, (6), 2369-2375.

35. Mancuso, J. R.; McClements, D. J.; Decker, E. A., The effects of surfactant type, pH, and chelators on the oxidation of salmon oil-in-water emulsions. *Journal of agricultural and food chemistry* **1999,** 47, (10), 4112-4116.

36. Malesev, D.; Kuntic, V., Investigation of metal-flavonoid chelates and the determination of flavonoids via metal-flavonoid complexing reactions. *Journal of the Serbian Chemical Society* **2007,** 72, (10), 921-939.

37. Perron, N. R.; Brumaghim, J. L., A review of the antioxidant mechanisms of polyphenol compounds related to iron binding. *Cell Biochemistry and Biophysics* **2009,** 53, (2), 75-100.

38. Mira, L.; Fernandez, M. T.; Santos, M.; Rocha, R.; Florencio, M. H.; Jennings, K. R., Interactions of flavonoids with iron and copper ions: A mechanism for their antioxidant activity. *Free Radical Research* **2002,** 36, (11), 1199-1208.

39. Yee, J. J.; Shipe, W. F., Using enzymatic proteolysis to reduce copper-protein catalysis of lipid oxidation. *Journal of Food Science* **1981,** 46, (3), 966-&.

40. Riisom, T.; Sims, R. J.; Fioriti, J. A., Effect of amino-acids on the autoxidation of safflower oil in emulsions. *Journal of the American Oil Chemists Society* **1980,** 57, (10), 354-359.

41. Bishov, S. J.; Henick, A. S., Antioxidant effect of protein hydrolyzates in a freeze-dried model system. *Journal of Food Science* **1972,** 37, (6), 873-&.

42. Yamagushi, N. a. F., M., Browning reaction products from reducing sugars and amino acids. XV. Comparison of antioxidant activity of melanoidin with that of tocopherol homologues and the synergistic effect of melanoidin with tocopherols. *Japan J. Food Sci. Technol* **1974,** 21, 13-18.

43. Evans, C. D.; Cooney, P. M.; Scholfield, C. R.; Dutton, H. J., Soybean lecithin and its fractions as metal-inactivating agents. *Journal of the American Oil Chemists Society* **1954,** 31, (7), 295-297.

44. Hildebrand, D. H.; Terao, J.; Kito, M., Phospholipids plus tocopherols increase soybean oil stability. *Journal of the American Oil Chemists Society* **1984,** 61, (3), 552-555.

45. Hudson, B. J. F. a. M., S.E.O., Synergism between phospholipids and naturally-occurring antioxidants in leaf lipids. *J. Sci. Food. Agric.* **1981,** 32, 208-210.

46. Hudson, B. J. F. a. G., M., Phospholipids as antioxidant sysnergists for tocopherols in the autoxidation of edible oils. *Lebensm.-Wiss. u.-Technol* **1984,** 17, 191-194.

47. Dziedzic, S. Z.; Hudson, B. J. F., Phosphatidyl ethanolamine as a synergist for primary antioxidants in edible oils. *Journal of the American Oil Chemists Society* **1984,** 61, (6), 1042-1045.

48. Bazin, B.; Cillard, J.; Koskas, J. P.; Cillard, P., Arachidonic-acid autoxidation in an aqueous-media effect of alpha-tocopherol, cysteine and nucleic-acids. *Journal of the American Oil Chemists Society* **1984,** 61, (7), 1212-1215.

49. Hamzawi, L. F., Role of phospholipids and alpha-tocopherol as natural antioxidants in buffalo butterfat. *Milchwissenschaft-Milk Science International* **1990,** 45, (2), 95-97.

50. Pokorny, J., Major factors affecting the autoxidation of lipids. In *Autoxidation of unsaturated lipids*, Chan, H. W. S., Ed. Academic Press: London, 1987; pp 141-206.

51. Koga, T. a. T., J, Phospholipids increase radical-scavenging activity of vitamin E in a bulk oil model system. *Journal of agricultural and food chemistry* **1995,** 43, 1450-1454.

52. Chen, B. C.; Han, A.; Laguerre, M.; McClements, D. J.; Decker, E. A., Role of reverse micelles on lipid oxidation in bulk oils: impact of phospholipids on antioxidant activity of alpha-tocopherol and Trolox. *Food & Function* **2011,** 2, (6), 302-309.

53. Slater, T. F., Biochemical studies on liver injury. In *Biochemical mechanisms of liver injury*, Slater, T. F., Ed. Acadamic press: London, 1978; pp 745-810.

54. Tappel, A. L., Will antioxidant nutrients slow aging processes? *Geriatrics* **1968,** 23, (10), 97-&.

55. Packer, J. E.; Slater, T. F.; Willson, R. L., Direct observation of a free-radical interaction between vitamin-E and vitamin-C. *Nature* **1979,** 278, (5706), 737-738.

56. Niki, E. T., J., Tanimura, R., and Kamiya, Y., Regeneration of vitamin E from alpha-Chromanoxyl radical by glutathione and vitamin C. *Chemistry Letters* **1982**, 789-792.

57. Scarpa, M.; Rigo, A.; Maiorino, M.; Ursini, F.; Gregolin, C., Formation of alpha-tocopherol radical and recycling of alpha-tocopherol by ascorbate during peroxidation of phosphatidylcholine liposomes - an electron-paramagnetic resonance study. *Biochimica et biophysica acta* **1984,** 801, (2), 215-219.

58. Nagaoka, S.; Kakiuchi, T.; Ohara, K.; Mukai, K., Kinetics of the reaction by which natural vitamin E is regenerated by vitamin C. *Chemistry and physics of lipids* **2007,** 146, (1), 26-32.

59. Bisby, R. H.; Parker, A. W., Reaction of ascorbate with the alpha-tocopheroxyl radical in micellar and bilayer-membrane systems. *Archives of Biochemistry and Biophysics* **1995,** 317, (1), 170-178.

60. Buettner, G. R., The pecking order of free-radicals and antioxidants - lipid-peroxidation, alpha-tocopherol, and ascorbate. *Archives of Biochemistry and Biophysics* **1993,** 300, (2), 535-543.

61. Sharma, M. K.; Buettner, G. R., Interaction of vitamin-C and vitamin-E during free-radical stress in plasma - an ESR study. *Free Radical Biology and Medicine* **1993,** 14, (6), 649-653.

62. Jovanovic, S. V.; Simic, M. G., Antioxidants in nutrition. In *Reactive Oxygen Species: From Radiation to Molecular Biology - a Festschrift in Honor of Daniel L Gilbert*, Chiueh, C. C., Ed. 2000; Vol. 899, pp 326-334.

63. Jovanovic, S. V.; Steenken, S.; Hara, Y.; Simic, M. G., Reduction potentials of flavonoid and model phenoxyl radicals. Which ring in flavonoids is responsible for antioxidant activity? *Journal of the Chemical Society-Perkin Transactions 2* **1996**, (11), 2497-2504.

64. Jovanovic, S. V.; Steenken, S.; Tosic, M.; Marjanovic, B.; Simic, M. G., Flavonoids as antioxidants. *Journal of the American Chemical Society* **1994,** 116, (11), 4846-4851.

65. Foley, S.; Navaratnam, S.; McGarvey, D. J.; Land, E. J.; Truscott, T. G.; Rice-Evans, C. A., Singlet oxygen quenching and the redox properties of hydroxycinnamic acids. *Free Radical Biology and Medicine* **1999,** 26, (9-10), 1202-1208.

66. Luczaj, W.; Skrzydlewska, E., Antioxidative properties of black tea. *Preventive medicine* **2005,** 40, (6), 910-8.

67. Rice-Evans, C. a. P., L., *Flavonoides in health and disease*. CRC Press: FL, 2003.

68. Jovanovic, S. V.; Hara, Y.; Steenken, S.; Simic, M. G., Antioxidant potential of gallocatechins - A pulse-radiolysis and laser photolysis study. *Journal of the American Chemical Society* **1995,** 117, (39), 9881-9888.

69. Jovanovic, S. V.; Hara, Y.; Steenken, S.; Simic, M. G., Antioxidant potential of theaflavins. A pulse radiolysis study. *Journal of the American Chemical Society* **1997,** 119, (23), 5337-5343.

70. Amorati, R.; Ferroni, F.; Lucarini, M.; Pedulli, G. F.; Valgimigi, L., A quantitative approach to the recycling of alpha-tocopherol by coantioxidants. *J Org Chem* **2002,** 67, 9295-9303.

71. Mukai, K.; Daifuku, K.; Okabe, K.; Tanigaki, T.; Inoue, K., Structure-activity relationship in the quenching reaction of singlet oxygen by tocopherol (vitamin E) derivatives and related phenols. Finding of linear correlation between the rates of quenching of singlet oxygen and scavenging of peroxyl and phenoxyl radicals in solution. *The Journal of Organic Chemistry* **1991,** 56, (13), 4188-4192.

72. Bohm, F.; Edge, R.; Land, E. J.; McGarvey, D. J.; Truscott, T. G., Carotenoids enhance vitamin E antioxidant efficiency. *Journal of the American Chemical Society* **1997,** 119, (3), 621-622.

73. Iglesias, J.; Pazos, M.; Andersen, M. L.; Skibsted, L. H.; Medina, I., Caffeic acid as antioxidant in fish muscle: mechanism of synergism with endogenous ascorbic acid and alpha-tocopherol. *Journal of agricultural and food chemistry* **2009,** 57, (2), 675-81.

74. Laranjinha, J., Redox cycles of caffeic acid with alpha-tocopherol and ascorbate. *Flavonoids and Other Polyphenols* **2001,** 335, 282-295.

75. Luo, Y.-R., Introduction. In *Comprehensive Handbook of Chemical Bond Energies*, CRC Press: 2007; pp 1-11.

76. Berkowitz, J.; Ellison, G. B.; Gutman, D., Three methods to measure RH bond energies. *J Phys Chem* **1994,** 98, 2744-2765.

77. Menon, A. S.; Wood, G. P. F.; Moran, D.; Radom, L., Bond dissociation energies and radical stabilization energies: An assessment of contemporary theoretical procedures. *Journal of Physical Chemistry A* **2007,** 111, (51), 13638-13644.

78. Zhu, Q. Y.; Zhang, X. M.; Fry, A. J., Bond dissociation energies of antioxidants. *Polymer Degradation and Stability* **1997,** 57, (1), 43-50.

79. Takeuchi, Y.; Okuno, K.; Yoshioka, H., Characteristics of the OH radical scavenging activity of tea catechins. *Journal of Radioanalytical and Nuclear Chemistry* **2007,** 272, (3), 455-459.

80. Amic, D.; Lucic, B.; Kovacevic, G.; Trinajstic, N., Bond dissociation enthalpies calculated by the PM3 method confirm activity cliffs in radical scavenging of flavonoids. *Molecular diversity* **2009,** 13, (1), 27-36.

81. Tikhonov, I.; Roginsky, V.; Pliss, E., The chain-breaking antioxidant activity of phenolic compounds with different numbers of O-H groups as determined during the oxidation of styrene. *International Journal of Chemical Kinetics* **2009,** 41, (2), 92-100.

82. Foti, M. C.; Daquino, C.; Geraci, C., Electron-transfer reaction of cinnamic acids and their methyl esters with the DPPH center dot radical in alcoholic solutions. *Journal of Organic Chemistry* **2004,** 69, (7), 2309-2314.

83. Pazos, M.; Andersen, M. L.; Medina, I.; Skibsted, L. H., Efficiency of natural phenolic compounds regenerating alpha-tocopherol from alpha-tocopheroxyl radical. *Journal of agricultural and food chemistry* **2007,** 55, (9), 3661-6.

84. Fujisawa, S.; Ishihara, M.; Atoshiko, T.; Kadoma, Y., A quantitative approach to the free radical interaction between alpha-tocopherol or ascorbate and flavonoids. *In vivo* **2006,** 20, 445-452.

85. Denisova, T. G.; Denisov, E. T., Dissociation energies of O-H bonds in natural antioxidants. *Russian Chemical Bulletin* **2008,** 57, (9), 1858-1866.

86. Wright, J. S., Predicting the antioxidant activity of curcumin and curcuminoids. *Journal of Molecular Structure-Theochem* **2002,** 591, 207-217.

87. Wang, Y. N.; Lau, K. C.; Lam, W. W. Y.; Man, W. L.; Leung, C. F.; Lau, T. C., Kinetics and mechanism of the oxidation of ascorbic acid in aqueous solutions by a trans-dioxoruthenium(VI) complex. *Inorganic Chemistry* **2009,** 48, (1), 400-406.

88. Mortensen, A.; Skibsted, L. H., Reactivity of beta-carotene towards peroxyl radicals studied by laser flash and steady-state photolysis. *Febs Letters* **1998,** 426, (3), 392-396.

89. Foti, M. C., Antioxidant properties of phenols. *Journal of Pharmacy and Pharmacology* **2007,** 59, (12), 1673-1685.

90. Luo, Y. R., *Comprehensive handbook of chemical bond energies*. CRC Press: Boca Raton, FL, 2007.

91. Lucarini, M.; Mugnaini, V.; Pedulli, G. F., Bond dissociation enthalpies of polyphenols: The importance of cooperative effects. *Journal of Organic Chemistry* **2002,** 67, (3), 928-931.

92. Denisov, E. T. a. D. T. G., *Handbook of antioxidants: bond dissociation energies, rate constants, activation energies and enthalpies of reactions*. CRC Press: Boca Raton, FL, 2000.

93. Peyrat-Maillard, M. N.; Cuvelier, M. E.; Berset, C., 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* **2003,** 80, (10), 1007-1012.

94. Sim, W. L.; Han, M. Y.; Huang, D., Quantification of antioxidant capacity in a microemulsion system: synergistic effects of chlorogenic acid with alpha-tocopherol. *Journal of agricultural and food chemistry* **2009,** 57, (9), 3409-14.

95. Hamilton, R. J.; Kalu, C.; McNeill, G. P.; Padley, F. B.; Pierce, J. H., Effects of tocopherols, ascorbyl palmitate, and lecithin on autoxidation of fish oil. *Journal of the American Oil Chemists Society* **1998,** 75, (7), 813-822.

96. Fukuzawa, K.; Ikebata, W.; Sohmi, K., Location, antioxidant and recycling dynamics of alpha-tocopherol in liposome membranes. *Journal of Nutritional Science and Vitaminology* **1993,** 39, S9-S22.

97. Fukuzawa, K., Dynamics of lipid peroxidation and antioxidion of alpha-tocopherol in membranes. *Journal of Nutritional Science and Vitaminology* **2008,** 54, (4), 273-285.

98. Bellemare, F.; Fragata, M., Polarity studies on the head group of single-layered phosphatidylcholine-alpha-tocopherol vesicles. *Journal of colloid and interface science* **1980,** 77, (1), 243-252.

99. Urano, S.; Matsuo, M.; Sakanaka, T.; Uemura, I.; Koyama, M.; Kumadaki, I.; Fukuzawa, K., Mobility and molecular-orientation of vitamin-eE in liposomal membranes as determined by F-19 NMR and fluorescence polarization techniques. *Archives of Biochemistry and Biophysics* **1993,** 303, (1), 10-14.

100. Gunaseelan, K.; Romsted, L. S.; Gallego, M. J.; Gonzalez-Romero, E.; Bravo-Diaz, C., Determining alpha-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* **2006,** 123-126, 303-11.

101. Sánchez-Paz, V.; Pastoriza-Gallego, M. J.; Losada-Barreiro, S.; Bravo-Díaz, C.; Gunaseelan, K.; Romsted, L. S., Quantitative determination of α-tocopherol distribution in a tributyrin/Brij 30/water model food emulsion. *Journal of colloid and interface science* **2008,** 320, (1), 1-8.

102. Romsted, L. S.; Zhang, J. B., Kinetic method for determining antioxidant distributions in model food emulsions: Distribution constants of t-butylhydroquinone in mixtures of octane, water, and a nonionic emulsifier. *Journal of agricultural and food chemistry* **2002,** 50, (11), 3328-3336.

103. Huang, S. W.; Frankel, E. N.; Schwarz, K.; German, J. B., Effect of pH on antioxidant activity of alpha-tocopherol and Trolox in oil-in-water emulsions. *Journal of agricultural and food chemistry* **1996,** 44, (9), 2496-2502.

104. Heins, A.; Sokolowski, T.; Stockmann, H.; Schwarz, K., Investigating the location of propyl gallate at surfaces and its chemical microenvironment by H-1 NMR. *Lipids* **2007,** 42, (6), 561-572.

105. Heins, A.; McPhail, D.; Sokolowski, T.; Stöckmann, H.; Schwarz, K., The Location of Phenolic Antioxidants and Radicals at Interfaces Determines Their Activity. *Lipids* **2007,** 42, (6), 573-582.

106. Minotti, G.; Aust, S. D., Redox cycling of iron and lipid-peroxidation. *Lipids* **1992,** 27, (3), 219-226.

107. Schaich, K. M., Metals and lipid oxidation - contemporary-issues. *Lipids* **1992,** 27, (3), 209-218.

108. Foti, M.; Piattelli, M.; Baratta, M. T.; Ruberto, G., Flavonoids, coumarins, and cinnamic acids as antioxidants in a micellar system. Structure-activity relationship. *Journal of agricultural and food chemistry* **1996,** 44, (2), 497-501.

109. Dai, F.; Chen, W. F.; Zhou, B., Antioxidant synergism of green tea polyphenols with alpha-tocopherol and L-ascorbic acid in SDS micelles. *Biochimie* **2008,** 90, (10), 1499-505.

110. Pazos, M.; Torres, J. L.; Andersen, M. L.; Skibsted, L. H.; Medina, I., Galloylated polyphenols efficiently reduce alpha-tocopherol radicals in a phospholipid model system composed of sodium dodecyl sulfate (SDS) micelles. *Journal of agricultural and food chemistry* **2009,** 57, (11), 5042-8.

111. Almajano, M. P.; Carbo, R.; Delgado, M. E.; Gordon, M. H., Effect of pH on the antimicrobial activity and oxidative stability of oil-in-water emulsions containing caffeic acid. *Journal of Food Science* **2007,** 72, (5), C258-63.

112. Paiva-Martins, F.; Gordon, M. H., Effects of pH and ferric ions on the antioxidant activity of olive polyphenols in oil-in-water emulsions. *Journal of the American Oil Chemists Society* **2002,** 79, (6), 571-576.

113. O'Brien, P. J., Intracellular mechanisms for the decomposition of a lipid peroxide. I. Decomposition of a lipid peroxide by metal ions, heme compounds, and nucleophiles. *Can. J. Biochem* **1969,** 47, (5), 485-192.

114. Nishiike, T.; Kondo, S.; Yamamoto, T.; Shigeeda, A.; Yamamoto, Y.; Takamura, H.; Matoba, T., Effects of metal ions and pH on the stability of linoleic acid hydroperoxide in the water phase. *Bioscience Biotechnology and Biochemistry* **1997,** 61, (12), 1973-1976.

115. Yamauchi, R.; Tatsumi, Y.; Asano, M.; Kato, K.; Ueno, Y., Effect of metal-salts and fructose on the autoxidation of methyl linoleate in emulsions. *Agricultural and Biological Chemistry* **1988,** 52, (3), 849-850.

116. Roy, R. C.; Das, D. K.; Das, B., Electrochemistry of Fe(4)S(4)(SCH(2)CH(2)COO(-))(4) (6-) in liposomes and microemulsions. *Indian Journal of Chemistry Section a-Inorganic Bio-Inorganic Physical Theoretical & Analytical Chemistry* **2007,** 46, (8), 1252-1256.

117. Gunckel, S.; Santander, P.; Cordano, G.; Ferreira, J.; Munoz, S.; Nunez-Vergara, L. J.; Squella, J. A., Antioxidant activity of gallates: an electrochemical study in aqueous media. *Chemico-biological interactions* **1998,** 114, (1-2), 45-59.

118. Litwinienko, G.; Ingold, K. U., Abnormal solvent effects on hydrogen atom abstractions. 1. The reactions of phenols with 2,2-diphenyl-1-picrylhydrazyl (dpph(center dot)) in alcohols. *Journal of Organic Chemistry* **2003,** 68, (9), 3433-3438.

119. Zhang, H. Y.; Ji, H. F., How vitamin E scavenges DPPH radicals in polar protic media. *New Journal of Chemistry* **2006,** 30, (4), 503-504.

120. Musialik, M.; Litwinienko, G., Scavenging of DPPH• radicals by vitamin E is accelerated by Its partial ionization: The role of sequential proton loss electron transfer. *Organic Letters* **2005,** 7, (22), 4951-4954.

121. Litwinienko, G.; Ingold, K. U., Abnormal solvent effects on hydrogen atom abstraction. 2.Resolution of the curcumin antioxidant controversy. The role of sequential proton loss electron transfer. *The Journal of Organic Chemistry* **2004,** 69, (18), 5888-5896.

122. Mitani, S.; Ouchi, A.; Watanabe, E.; Kanesaki, Y.; Nagaoka, S. I.; Mukai, K., Stopped-flow kinetic study of the aroxyl radical-scavenging action of catechins and vitamin C in ethanol and micellar solutions. *Journal of agricultural and food chemistry* **2008,** 56, (12), 4406-4417.

123. Mukai, K.; Nishimura, M.; Kikuchi, S., Stopped-flow investigation of the reaction of vitamin-c with tocopheroxyl radical in aqueous triton x-100 micellar solutions - the structure-activity relationship of the regeneration reaction of tocopherol by vitamin-c. *Journal of Biological Chemistry* **1991,** 266, (1), 274-278.

124. Cillard, J.; Cillard, P., Behavior of alpha-tocopherol, gamma-tocopherol and delta-tocopherol with linoleic-acid in aqueous-media. *Journal of the American Oil Chemists Society* **1980,** 57, (1), 39-42.

125. Kim, H. J. a. M., D.B. , Chemistry of Lipid Oxidation. In *Food lipids: chemistry, nutrition, and biotechnology*, third ed.; Casimir C. Akoh and Min, D. B., Ed. CRC Press: Florida, 2008; p 928.

126. Niki, E.; Tsuchiya, J.; Yoshikawa, Y.; Yamamoto, Y.; Kamiya, Y., Oxidation of lipids .13. Antioxidant activities of alpha-tocopherols, beta-tocopherols, gamma-tocopherols, and delta-tocopherols. *Bulletin of the Chemical Society of Japan* **1986,** 59, (2), 497-501.

127. Ha, K. H.; Igarashi, O., The oxidation-products from 2 kinds of tocopherols coexisting in autoxidation system of methyl linoleate. *Journal of Nutritional Science and Vitaminology* **1990,** 36, (4), 411-421.

128. Fuster, M. D.; Lampi, A. M.; Hopia, A.; Kamal-Eldin, A., Effects of alpha- and gamma-tocopherols on the autooxidation of purified sunflower triacylglycerols. *Lipids* **1998,** 33, (7), 715-722.

129. Parkhurs.Rm; Skinner, W. A.; Sturm, P. A., Effect of various concentrations of tocopherols and tocopherol mixtures on oxidative stability of a sample of lard. *Journal of the American Oil Chemists Society* **1968,** 45, (10), 641-&.

130. Marinova, E.; Toneva, A.; Yanishlieva, N., Synergistic antioxidant effect of α-tocopherol and myricetin on the autoxidation of triacylglycerols of sunflower oil. *Food Chemistry* **2008,** 106, (2), 628-633.

131. Zhu, Q. Y.; Huang, Y.; Chen, Z. Y., Interaction between flavonoids and alpha-tocopherol in human low density lipoprotein. *Journal of Nutritional Biochemistry* **2000,** 11, (1), 14-21.

132. Mukai, K.; Oka, W.; Watanabe, K.; Egawa, Y.; Nagaoka, S.; Terao, J., Kinetic study of free-radical-scavenging action of flavonoids in homogeneous and aqueous Triton X-100 micellar solutions. *Journal of Physical Chemistry A* **1997,** 101, (20), 3746-3753.

133. Rieker, A. a. S. K., Die beteiligung von phenylresten an der aroxylmesomerie. *Liebigs Ann. Chem* **1965**, (78), 689.

134. Terao, J.; Matsushita, S., The peroxidizing effect of alpha-tocopherol on autoxidation of methyl linoleate in bulk phase. *Lipids* **1986,** 21, (4), 255-260.

135. Ohara, K.; Ichimura, Y.; Tsukamoto, K.; Ogata, M.; Nagaoka, S.; Mukai, K., Kinetic study on the free radical-scavenging and vitamin E-regenerating actions of caffeic acid and its related compounds. *Bulletin of the Chemical Society of Japan* **2006,** 79, (10), 1501-1508.

136. Liu, D. H.; Shi, J.; Ibarra, A. C.; Kakuda, Y.; Xue, S. J., The scavenging capacity and synergistic effects of lycopene, vitamin E, vitamin C, and beta-carotene mixtures on the DPPH free radical. *Lwt-Food Science and Technology* **2008,** 41, (7), 1344-1349.

137. Palozza, P.; Krinsky, N. I., Both alpha-tocopherol and beta-carotene inhibit radical-initiated peroxidation of microsomal lipids. *Faseb Journal* **1991,** 5, (5), A1324-A1324.

138. Shi, J.; Maguer, M. L., Lycopene in tomatoes: chemical and physical properties affected by food processing. *Critical reviews in food science and nutrition* **2000,** 40, (1), 1-42.

139. Shixian, Q.; Da, Y.; Kakuda, Y.; Shi, J.; Mittal, G.; Yeung, D.; Jiang, Y., Synergistic anti-oxidative effects of lycopene with other bioactive compounds. *Food Reviews International* **2005,** 21, (3), 295-311.

140. Fuhrman, B.; Volkova, N.; Rosenblat, M.; Aviram, M., Lycopene synergistically Inhibits LDL oxidation in combination with vitamin E, glabridin, rosmarinic acid, carnosic acid, or garlic. *Antioxidants & Redox Signaling* **2000,** 2, (3), 491-506.

141. Niki, E.; Noguchi, N.; Tsuchihashi, H.; Gotoh, N., Interaction among vitamin-C, vitamin-E, and beta-carotene. *American Journal of Clinical Nutrition* **1995,** 62, (6), S1322-S1326.

142. Choe, E.; Min, D. B., Mechanisms of Antioxidants in the Oxidation of Foods. *Comprehensive Reviews in Food Science and Food Safety* **2009,** 8, (4), 345-358.

143. Saito, H.; Ishihara, K., Antioxidant activity and active sites of phospholipids as antioxidants. *Journal of the American Oil Chemists Society* **1997,** 74, (12), 1531-1536.

144. Loliger, J.; Lambelet, P.; Savoy, M. C.; Ducret, F., Radical exchange-reactions between autoxidizing lipids, vitamin-E and vitamin-C in binary lipid water-systems. *Fette Seifen Anstrichmittel* **1986,** 88, (14), 584-588.

145. Brandt, P.; Hollstei.E; Franzke, C., Prooxidative and antioxidative effect of phosphatides (review). *Lebensmittel Industrie* **1973,** 20, (1), 31-33.

146. Ho, Y. P.; Huang, P. C.; Deng, K. H., Metal ion complexes in the structural analysis of phospholipids by electrospray ionization tandem mass spectrometry. *Rapid Communications in Mass Spectrometry* **2003,** 17, (2), 114-121.

147. Hendrick.Hs; Fullingt.Jg, Stabilities of metal complexes of phospholipids - ca(2) mg(2) and ni(2) complexes of phosphatidylserine and triphosphoinositide. *Biochemistry* **1965,** 4, (8), 1599-&.

148. Weng, X. C.; Gordon, M. H., Antioxidant synergy between phosphatidyl ethanolamine and alpha-tocopherylquinone. *Food Chemistry* **1993,** 48, (2), 165-168.

149. Ohshima, T.; Fujita, Y.; Koizumi, C., Oxidative stability of sardine and mackerel lipids with reference to synergism between phospholipids and alpha-tocopherol. *Journal of the American Oil Chemists Society* **1993,** 70, (3), 269-276.

150. Khan, M. A. a. S., F., Tocopherols and phospholipids enhance the oxidative stability of borage and evening primrose triacylglycerols. *Journal of food lipids* **2000,** 7, (3), 143-150.

151. Bandarra, N.; Campos, R.; Batista, I.; Nunes, M.; Empis, J., Antioxidant synergy of α-tocopherol and phospholipids. *Journal of the American Oil Chemists' Society* **1999,** 76, (8), 905-913.

152. Segawa, T., Hara, S. and Totani, Y, Antioxidant behavior of phospholipids for polyunsaturated fatty acids of fish oil. II; Synergistic effect of phospholipids for tocopherol. *J. Jpn. Oil Chem. Soc* **1994,** 43, 515-519.

153. King, M. F.; Boyd, L. C.; Sheldon, B. W., Antioxidant properties of individual phospholipids in a salmon oil model system. *Journal of the American Oil Chemists Society* **1992,** 69, (6), 545-551.

154. Judde, A.; Villeneuve, P.; Rossignol-Castera, A.; Le Guillou, A., Antioxidant effect of soy lecithins on vegetable oil stability and their synergism with tocopherols. *Journal of the American Oil Chemists Society* **2003,** 80, (12), 1209-1215.

155. Chen, Z. Y. a. N., W. W., Prooxidative and antioxidative effects of phospholipids on milk fat. *Journal of american oil chemists society* **1991,** 68, (12), 938-940.

156. Roberts, J. R.; Ingold, K. U., Kinetic applications of electron-paramagnetic resonance spectroscopy .10. reactions of some alkylamino radicals in solution. *Journal of the American Chemical Society* **1973,** 95, (10), 3228-3235.

157. Faucitano, A.; Buttafava, A.; Martinotti, F.; Bortolus, P., 1st Electron-spin resonance identification of a nitrogen peroxy radical as intermediate in the photooxidation of 2,2,6,6-tetramethylpiperidine derivatives. *Journal of Physical Chemistry* **1984,** 88, (6), 1187-1190.

158. Bascetta, E.; Gunstone, F. D.; Walton, J. C., Electron-spin resonance study of the role of vitamin-e and vitamin-c in the inhibition of fatty-acid oxidation in a model membrane. *Chemistry and physics of lipids* **1983,** 33, (2), 207-210.

159. Chang, Y. C.; Almy, E. A.; Blamer, G. A.; Gray, J. I.; Frost, J. W.; Strasburg, G. M., Antioxidant activity of 3-dehydroshikimic acid in liposomes, emulsions, and bulk oil. *Journal of agricultural and food chemistry* **2003,** 51, (9), 2753-2757.

160. Huang, S. W.; Frankel, E. N., Antioxidant activity of tea catechins in different lipid systems. *Journal of agricultural and food chemistry* **1997,** 45, (8), 3033-3038.

161. Stockmann, H.; Schwarz, K.; Huynh-Ba, T., The influence of various emulsifiers on the partitioning and antioxidant activity of hydroxybenzoic acids and their derivatives in oil-in-water emulsions. *Journal of the American Oil Chemists Society* **2000,** 77, (5), 535-542.

162. Huang, S.-W.; Frankel, E. N.; German, J. B., Effects of Individual Tocopherols and Tocopherol Mixtures on the Oxidative Stability of Corn Oil Triglycerides. *Journal of agricultural and food chemistry* **1995,** 43, (9), 2345-2350.

163. Mukai, K.; Sawada, K.; Kohno, Y.; Terao, J., Kinetic-study of the prooxidant effect of tocopherol - hydrogen abstraction from lipid hydroperoxides by tocopheroxyls in solution. *Lipids* **1993,** 28, (8), 747-752.

164. Mukai, K.; Noborio, S.; Nagaoka, S., Why is the order reversed? Peroxylp-scavenging activity and fats-and-oils a protecting activity of vitamin E. *International Journal of Chemical Kinetics* **2005,** 37, (10), 605-610.

165. Mukai, K.; Okauchi, Y., Kinetic-study of the reaction between tocopheroxyl radical and unsaturated fatty-acid esters in benzene. *Lipids* **1989,** 24, (11), 936-939.

166. Burton, G. W.; Doba, T.; Gabe, E. J.; Hughes, L.; Lee, F. L.; Prasad, L.; Ingold, K. U., Autoxidation of biological molecules .4. Maximizing the antioxidant activity of phenols. *Journal of the American Chemical Society* **1985,** 107, (24), 7053-7065.

167. Denisov, E. T.; Khudyakov, I. V., Mechanisms of action and reactivities of the free-radicals of inhibitors. *Chemical Reviews* **1987,** 87, (6), 1313-1357.

168. Igarashi, O.; Matsukawa, H.; Inagaki, C., Reactivity of alpha-tocopherol with hydroperoxide of methyl linoleate. *Journal of Nutritional Science and Vitaminology* **1976,** 22, (3), 267-270.

169. Jung, D. M.; de Ropp, J. S.; Ebeler, S. E., Study of interactions between food phenolics and aromatic flavors using one- and two-dimensional H-1 NMR spectroscopy. *Journal of agricultural and food chemistry* **2000,** 48, (2), 407-412.

170. Maccarone, E.; Maccarrone, A.; Rapisarda, P., Stabilization of anthocyanins of blood orange fruit juice. *Journal of Food Science* **1985,** 50, (4), 901-904.

171. Meyer, A. S.; Heinonen, M.; Frankel, E. N., Antioxidant interactions of catechin, cyanidin, caffeic acid, quercetin, and ellagic acid on human LDL oxidation. *Food Chemistry* **1998,** 61, (1-2), 71-75.

172. Burton, G. W., Antioxidant action of carotenoids. *Journal of Nutrition* **1989,** 119, (1), 109-111.

173. Song, L. L.; Liang, R.; Li, D. D.; Xing, Y. D.; Han, R. M.; Zhang, J. P.; Skibsted, L. H., Beta-carotene radical cation addition to green tea polyphenols. mechanism of antioxidant antagonism in peroxidizing liposomes. *Journal of agricultural and food chemistry* **2011,** 59, (23), 12643-12651.

174. Benech, R. O.; Kheadr, E. E.; Laridi, R.; Lacroix, C.; Fliss, I., Inhibition of *Listeria innocua* in cheddar cheese by addition of nisin Z in liposomes or by in situ production in mixed culture. *Applied and Environmental Microbiology* **2002,** 68, (8), 3683-3690.

175. Shehata, T.; Ogawara, K.; Higaki, K.; Kimura, T., Prolongation of residence time of liposome by surface-modification with mixture of hydrophilic polymers. *International Journal of Pharmaceutics* **2008,** 359, (1-2), 272-279.

176. Administration., U. S. F. a. D. Food ingredients and package: Generally Recognized as Safe (GRAS). http://www.fda.gov/food/foodingredientspackaging/default.htm

177. Nara, E.; Miyashita, K.; Ota, T., Oxidative stability of liposomes prepared from soybean PC, chicken egg PC, and salmon egg PC. *Bioscience Biotechnology and Biochemistry* **1997,** 61, (10), 1736-1738.

178. Taylor, T. M.; Davidson, P. M.; Bruce, B. D.; Weiss, J., Liposomal nanocapsules in food science and agriculture. *Critical reviews in food science and nutrition* **2005,** 45, (7-8), 587-605.

179. Gregoria.G, Drug entrapment in liposomes. *Febs Letters* **1973,** 36, (3), 292-296.

180. Grit, M.; Crommelin, J. A., Chemical-stability of liposomes - Implications for their physical stability. *Chemistry and physics of lipids* **1993,** 64, (1-3), 3-18.

181. Thompson, A. K.; Haisman, D.; Singh, H., Physical stability of liposomes prepared from milk fat globule membrane and soya phospholipids. *Journal of agricultural and food chemistry* **2006,** 54, (17), 6390-6397.

182. Mozuraityte, R.; Rustad, T.; Storro, I., The role of iron in peroxidation of polyunsaturated fatty acids in liposomes. *Journal of agricultural and food chemistry* **2008,** 56, (2), 537-543.

183. Tampo, Y.; Yonaha, M., Effects of membrane charges and hydroperoxides on Fe(II)-supported lipid peroxidation in liposomes. *Lipids* **1996,** 31, (10), 1029-1038.

184. Fukuzawa, K.; Tadokoro, T.; Kishikawa, K.; Mukai, K.; Gebicki, J. M., Site-specific induction of lipid-peroxidation by iron in charged micelles. *Archives of Biochemistry and Biophysics* **1988,** 260, (1), 146-152.

185. Miyashita, K.; Nara, E.; Ota, T., Comparative-study on the oxidative stability of phosphatidylcholines from salmon egg and soybean in an aqueous-solution. *Bioscience Biotechnology and Biochemistry* **1994,** 58, (10), 1772-1775.

186. Samuni, A. M.; Lipman, A.; Barenholz, Y., Damage to liposomal lipids: protection by antioxidants and cholesterol-mediated dehydration. *Chemistry and physics of lipids* **2000,** 105, (2), 121-134.

187. Nacka, F.; Cansell, M.; Entressangles, B., In vitro behavior of marine lipid-based liposomes. Influence of pH, temperature, bile salts, and phospholipase A2. *Lipids* **2001,** 36, (1), 35-42.

188. Kogure, K.; Fukuzawa, K.; Kawano, H.; Terada, H., Spermine accelerates iron-induced lipid-peroxidation in mitochondria by modification of membrane-surface charge. *Free Radical Biology and Medicine* **1993,** 14, (5), 501-507.

189. Yoshida, K.; Terao, J.; Suzuki, T.; Takama, K., Inhibitory effect of phosphatidylserine on iron-dependent lipid-peroxidation. *Biochemical and biophysical research communications* **1991,** 179, (2), 1077-1081.

190. Chen, R. H.; Win, H. P.; Fang, H. J., Vesicle size, size distribution, stability, and rheological properties of liposomes coated with water-soluble chitosans of different molecular weights and concentrations. *Journal of liposome research* **2001,** 11, (2-3), 211-228.

191. Ogawa, S.; Decker, E. A.; McClements, D. J., Production and characterization of O/W emulsions containing cationic droplets stabilized by lecithin-chitosan membranes. *Journal of agricultural and food chemistry* **2003,** 51, (9), 2806-12.

192. Aoki, T.; Decker, E. A.; McClements, D. J., Influence of environmental stresses on stability of O/W emulsions containing droplets stabilized by multilayered membranes produced by a layer-by-layer electrostatic deposition technique. *Food Hydrocolloids* **2005,** 19, (2), 209-220.

193. Laye, C.; McClements, D. J.; Weiss, J., Formation of biopolymer-coated liposomes by electrostatic deposition of chitosan. *Journal of Food Science* **2008,** 73, (5), N7-N15.

194. Shaw, L. A.; McClements, D. J.; Decker, E. A., Spray-dried multilayered emulsions as a delivery method for omega-3 fatty acids into food systems. *Journal of agricultural and food chemistry* **2007,** 55, (8), 3112-3119.

195. Lecomte, J.; Giraldo, L. J. L.; Laguerre, M.; Baréa, B.; Villeneuve, P., Synthesis, characterization and free radical scavenging properties of rosmarinic acid fatty esters. *Journal of the American Oil Chemists' Society* **2010,** 87, (6), 615-620.

196. Pignoli, G.; Bou, R.; Rodriguez-Estrada, M. T.; Decker, E. A., Suitability of saturated aldehydes as lipid oxidation markers in washed turkey meat. *Meat science* **2009**.

197. Henriksen, I.; Smistad, G.; Karlsen, J., Interactions between liposomes and chitosan. *International Journal of Pharmaceutics* **1994,** 101, (3), 227-236.

198. Mei, L. Y.; McClements, D. J.; Decker, E. A., Lipid oxidation in emulsions as affected by charge status of antioxidants and emulsion droplets. *Journal of agricultural and food chemistry* **1999,** 47, (6), 2267-2273.

199. Kulmyrzaev, A.; Sivestre, M. P. C.; McClements, D. J., Rheology and stability of whey protein stabilized emulsions with high CaCl2 concentrations. *Food Research International* **2000,** 33, (1), 21-25.

200. McClements, D. J.; Decker, E. A., Lipid oxidation in oil-in-water emulsions: Impact of molecular environment on chemical reactions in heterogeneous food systems. *Journal of Food Science* **2000,** 65, (8), 1270-1282.

201. Takahashi, M.; Komuro, E.; Niki, E.; Tanaka, K., Action of fatty-acid esters of l-ascorbic-acid as antioxidants in phosphatidylcholine liposomal membranes. *Bulletin of the Chemical Society of Japan* **1992,** 65, (3), 679-684.

202. Porter, W. L., Paradoxical behavior of antioxidants in food and biological-systems. *Toxicology and Industrial Health* **1993,** 9, (1-2), 93-122.

203. Huang, S. W.; Frankel, E. N.; Aeschbach, R.; German, J. B., Partition of selected antioxidants in corn oil-water model systems. *Journal of agricultural and food chemistry* **1997,** 45, (6), 1991-1994.

204. Waraho, T.; Cardenia, V.; Rodriguez-Estrada, M. T.; McClements, D. J.; Decker, E. A., Prooxidant mechanisms of free fatty acids in stripped soybean oil-in-water emulsions. *Journal of agricultural and food chemistry* **2009,** 57, (15), 7112-7.

205. Faraji, H.; McClements, D. J.; Decker, E. A., Role of continuous phase protein on the oxidative stability of fish oil-in-water emulsions. *Journal of agricultural and food chemistry* **2004,** 52, (14), 4558-4564.

206. Bligh, E. G.; Dyer, W. J., A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **1959,** 37, (8), 911-917.

207. Panya, A.; Laguerre, M.; Lecomte, J.; Villeneuve, P.; Weiss, J.; McClements, D. J.; Decker, E. A., Effects of chitosan and rosmarinate esters on the physical and oxidative stability of liposomes. *Journal of agricultural and food chemistry* **2010,** 58, (9), 5679-5684.

208. Lopez-Giraldo, L. J.; Laguerre, M.; Lecomte, J.; Figueroa-Espinoza, M. C.; Barea, B.; Weiss, J.; Decker, E. A.; Villeneuve, P., Kinetic and stoichiometry of the reaction of chlorogenic acid and its alkyl esters against the DPPH radical. *Journal of agricultural and food chemistry* **2009,** 57, (3), 863-870.

209. Richards, M. P.; Chaiyasit, W.; McClements, D. J.; Decker, E. A., Ability of surfactant micelles to alter the partitioning of phenolic antioxidants in oil-in-water emulsions. *Journal of agricultural and food chemistry* **2002,** 50, (5), 1254-1259.

210. Nuchi, C. D.; Hernandez, P.; McClements, D. J.; Decker, E. A., Ability of lipid hydroperoxides to partition into surfactant micelles and alter lipid oxidation rates in emulsions. *Journal of agricultural and food chemistry* **2002,** 50, (19), 5445-5449.

211. Cho, Y. J.; McClements, D. J.; Decker, E. A., Ability of surfactant micelles to alter the physical location and reactivity of iron in oil-in-water emulsion. *Journal of agricultural and food chemistry* **2002,** 50, (20), 5704-5710.

212. Parker, T. L.; Miller, S. A.; Myers, L. E.; Miguez, F. E.; Engeseth, N. J., Evaluation of synergistic antioxidant potential of complex mixtures using oxygen radical absorbance capacity (ORAC) and electron paramagnetic resonance (EPR). *Journal of agricultural and food chemistry* **2010,** 58, (1), 209-17.

213. Freeman, B. L.; Eggett, D. L.; Parker, T. L., Synergistic and Antagonistic Interactions of Phenolic Compounds Found in Navel Oranges. *Journal of Food Science* **2010,** 75, (6), C570-C576.

214. Gonzalez, E. A.; Nazareno, M. A., Antiradical action of flavonoid-ascorbate mixtures. *Lwt-Food Science and Technology* **2011,** 44, (2), 558-564.

215. Hidalgo, M.; Sanchez-Moreno, C.; de Pascual-Teresa, S., Flavonoid-flavonoid interaction and its effect on their antioxidant activity. *Food Chemistry* **2010,** 121, (3), 691-696.

216. Chen, J.; Shi, J.; Macnaughton, L.; Kakuda, Y.; Xue, S. J.; Ma, Y.; Zhang, M.; Jiang, Y., The scavenging capacity of combinations of lycopene, B-carotene, vitamin e, and vitamin c on the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). *Journal of Food Biochemistry* **2009,** 33, (2), 232-245.

217. Hiramoto, K.; Miura, Y.; Ohuki, G.; Kato, T.; Kikigawa, K., Are water-soluble natural antioxidants: synergistic in combination with alpha-tocopherol. *Journal of oleo science* **2002,** 51, (9), 569-576.

218. Pedrielli, P.; Skibsted, L. H., 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 chemistry* **2002,** 50, (24), 7138-7144.

219. Boon, C. S.; Xu, Z.; Yue, X.; McClements, D. J.; Weiss, J.; Decker, E. A., Factors affecting lycopene oxidation in oil-in-water emulsions. *Journal of agricultural and food chemistry* **2008,** 56, (4), 1408-1414.

220. Fujimoto, A.; Masuda, T., Antioxidation mechanism of rosmarinic acid, identification of an unstable quinone derivative by the addition of odourless thiol. *Food Chemistry* **2012,** 132, (2), 901-906.

221. Panya, A.; Laguerre, M.; Bayrasy, C.; Lecomte, J.; Villeneuve, P.; McClements, D. J.; Decker, E. A., An Investigation of the Versatile Antioxidant Mechanisms of Action of Rosmarinate Alkyl Esters in Oil-in-Water Emulsions. *Journal of agricultural and food chemistry* **2012,** 60, (10), 2692-2700.

222. Hotta, H.; Ueda, M.; Nagano, S.; Tsujino, Y.; Koyama, J.; Osakai, T., Mechanistic study of the oxidation of caffeic acid by digital simulation of cyclic voltammograms. *Analytical biochemistry* **2002,** 303, (1), 66-72.

223. Yang, B.; Kotani, A.; Arai, K.; Kusu, F., Estimation of the antioxidant activities of flavonoids from their oxidation potentials. *Analytical Sciences* **2001,** 17, (5), 599-604.

224. Kilmartin, P. A.; Zou, H. L.; Waterhouse, A. L., A cyclic voltammetry method suitable for characterizing antioxidant properties of wine and wine phenolics. *Journal of agricultural and food chemistry* **2001,** 49, (4), 1957-1965.

225. Firuzi, O.; Lacanna, A.; Petrucci, R.; Marrosu, G.; Saso, L., Evaluation of the antioxidant activity of flavonoids by "ferric reducing antioxidant power" assay and cyclic voltammetry. *Biochimica Et Biophysica Acta-General Subjects* **2005,** 1721, (1-3), 174-184.

226. Simic, A.; Manojlovic, D.; Segan, D.; Todorovic, M., Electrochemical Behavior and antioxidant and prooxidant activity of natural phenolics. *Molecules* **2007,** 12, (10), 2327-2340.

227. Born, M.; Carrupt, P. A.; Zini, R.; Bree, F.; Tillement, J. P.; Hostettmann, K.; Testa, B., Electrochemical behaviour and antioxidant activity of some natural polyphenols. *Helvetica Chimica Acta* **1996,** 79, (4), 1147-1158.

228. Shahidi, F.; Zhong, Y., Revisiting the Polar Paradox Theory: A Critical Overview. *Journal of agricultural and food chemistry* **2011,** 59, (8), 3499-3504.

229. Kikuzaki, H.; Nakatani, N., Structure of a new antioxidative phenolic acid from oregano (origanum-vulgare L). *Agricultural and Biological Chemistry* **1989,** 53, (2), 519-524.

230. Nakazawa, T.; Ohsawa, K., Metabolism of rosmarinic acid in rats. *Journal of Natural Products* **1998,** 61, (8), 993-996.

231. Bel-Rhlid, R.; Crespy, V.; Page-Zoerkler, N.; Nagy, K.; Raab, T.; Hansen, C. E., Hydrolysis of Rosmarinic Acid from Rosemary Extract with Esterases and Lactobacillus johnsonii in Vitro and in a Gastrointestinal Model. *Journal of agricultural and food chemistry* **2009,** 57, (17), 7700-7705.

232. Chen, J. H.; Ho, C. T., Antioxidant activities of caffeic acid and its related hydroxycinnamic acid compounds. *Journal of agricultural and food chemistry* **1997,** 45, (7), 2374-2378.