Enzymatic Modification of Antioxidants
Towards Omega-3 Oil Protection

Zhiyong Yang

PhD Dissertation

July 2011

Department of Molecular Biology

Aarhus University, Denmark
Preface:

This dissertation entitled “Enzymatic Modification of Antioxidants Towards Omega-3 Oil Protection” is part of the requirements for obtaining a PhD degree at Aarhus University.

The work in this study was primarily performed at the Department of Molecular Biology, Aarhus University, under the supervision of Professor Xuebing Xu. The LC-MS analysis was performed in collaboration with the Associate Professor Marianne Glasius in the Department of Chemistry, Aarhus University.

The project was financed by the Strategic Food and Health Programme through the Danish Technological Research Council with co-financing from the University of Aarhus. The study was a sub-project of the large programme called Nutritious and Tasty n-3 PUFA Rich Food Products for a Slim and Healthy Population, which had involved a number of people. Special thanks go to my supervisor Professor Xuebing Xu for his general guidance, directing me toward a great field of study that I was not familiar with at the beginning, and encouraging and supporting me to participate in many international conferences. I would like to express my gratitude to all those who assisted, especially to the project leader, Dr. Charlotte Jacobsen, who has been supporting and providing guidance during my study. I also would like to thank Associate Professor Zheng Guo and Dr. Ling-Zhi Cheong for their valuable discussion time and advices. Vivian Feddern is thanked for joining the project as part of her visiting studies. Several people from the Department of Chemistry, Aarhus University, including Associate Professor Marianne Glasius, Grethe Vestergaard Jensen, Professor Jan Skov Partersen, are also thanked for their assistance in spectroscopic analysis.

I also wish to thank technician Flemming Lund Sørensen for his routing help. I also appreciate the assistance from Derya Kahveci, Seong-Chea Chua, Thomas Balle, Gündüz Güzel, Serguei Fedosov, Worawan Panpipat, as well as many current and former group members. I enjoyed a lot to stay as a family and many happy days together.

Last but not the least, I give my sincerely thank to my parents and sister for supporting and encouraging me to study in a country far away from home.

July 2011

Aarhus, Denmark

Zhiyong Yang
Table of Contents:

Table of Contents:.................................................................................................................................i
Summary:............................................................................................................................................iii
Sammenfatning: ...................................................................................................................................v
Abbreviations:....................................................................................................................................vii
Lists of Publications:.........................................................................................................................viii
Chapter 1: Introduction ........................................................................................................................1
  1.1 Background ................................................................................................................................1
  1.2. Project Outlines.........................................................................................................................3
Chapter 2: Health Benefit of Fish Oil and Oxidation Mechanism of Lipids .......................................5
  2.1. Fatty Acid Composition of Fish Oil..........................................................................................5
  2.2. Fish oil in Diet and Health ........................................................................................................5
  2.3. Enrichment of Foods with Omega-3 Fatty Acids .....................................................................6
      2.3.1 Mechanisms of Lipid Oxidation .........................................................................................6
      2.3.2. Lipid Oxidation in Food Enriched with Omega-3 Fatty Acids..........................................9
         2.3.2.1. Chemical Structure of Lipids ....................................................................................12
         2.3.2.2. Environmental Factors ..............................................................................................12
         2.3.2.3. Emulsified type and Charge Status ...........................................................................13
         2.3.2.4. Surface Character......................................................................................................14
         2.3.2.5. Droplet Characteristics..............................................................................................14
Chapter 3: Antioxidant Mechanism and Antioxidants from Natural Sources ...................................15
  3.1. Introduction.............................................................................................................................15
  3.2. Classification and Mechanism of Antioxidants ......................................................................15
      3.2.1. Primary Antioxidants .......................................................................................................16
      3.2.2. Secondary Antioxidants ...................................................................................................18
         3.2.2.1 Autoxidation Mechanisms of Metal and Chelators....................................................19
         3.2.2.2 Oxygen Scavengers and Reducing Agents ................................................................20
         3.2.2.3 Singlet Oxygen Quenchers.........................................................................................20
  3.3 Synthetic Antioxidants vs Natural Antioxidants......................................................................20
      3.3.1 Synthetic Antioxidants......................................................................................................20
      3.3.2. Natural Antioxidants........................................................................................................24
         3.3.2.1. Antioxidant Mechanisms of Tocopherols and Tocotrienols..................................... 24
         3.3.2.2. Antioxidant Mechanisms of Ascorbic Acid.............................................................. 25
         3.3.2.3. Antioxidant Mechanisms of Carotenoids..................................................................25
         3.3.2.4. Antioxidant Mechanisms of Phenolics/Polyphenolics..............................................26
         3.3.2.5. Antioxidant Mechanisms of Other Antioxidants from Natural Sources................... 28
  3.4 Application of Antioxidant in Food Products.......................................................................... 28
Chapter 4: Enzymatic Synthesis of Novel Antioxidants in Literature............................................... 31
  4.1. Enzymatic Synthesis of Lipophilic Antioxidants....................................................................31
  4.2. Synthesis of Lipophilic Phenolic Esters..................................................................................35
  4.3. Enzymatic Synthesis Lipophilic Ascorbic Esters ...................................................................36
Chapter 5: Experimental and Analytical Methodology .....................................................................37
  5.1. Enzymatic Synthesis of Lipophilic Fatty Alcohol Phenolic Esters ...........................................37
     5.1.1. Enzymatic Esterification of Fatty Alcohol Phenolic Esters in Organic Solvents (Paper I)
5.1.2. Enzymatic Esterification Octyl Phenolic Esters in ILs/octanol Binary System (Paper II) ................................................................. 39
5.2. Enzymatic Synthesis Esters of Phenolic with TAG by Transesterification ................................................................. 40
   5.2.1. Improved Enzymatic Production of Phenolated Glycerides through Alkyl Phenolate Intermediate (Paper III) ......................................................... 40
   5.2.2. Enzymatic Transesterification of EF with Fish Oils (Paper IV) ...................................................................................... 42
5.3. HPLC Analysis .................................................................................................................................................. 42
5.4. HPLC-MS Analysis ........................................................................................................................................... 43
5.5. Experiments Design and Data Analysis by RSM .............................................................................................. 43
5.6. Reaction Systems Characterization (Appendix 5) ................................................................................................. 44
   5.6.1. Measurement with Dynamic Light Scattering (DLS) ...................................................................................... 45
   5.6.2. Measurement with Small Angle X-ray Scattering (SAXS) ........................................................................ 46
Chapter 6: Overall Study Evaluations and Discussions ........................................................................... 47
   6.1. Enzymatic Esterification of Fatty Alcohol Phenolic Esters in Organic Solvents (Paper I) ............................ 47
   6.2. Enzymatic Esterification Octyl Phenolic Esters in IL/octanol Binary System (Paper II) ........................................... 51
   6.3. Improved Enzymatic Production of Phenolated Glycerides through Alkyl Phenolate Intermediate (Paper III) ........................................................................... 53
   6.4. Enzymatic Transesterification of EF with Fish Oils (Paper IV) ................................................................................ 57
   6.5. Characterization of Different Systems by DLS and SAXS (Appendix 5) ....................................................... 59
Chapter 7: Conclusions and Future Outlook ..................................................................................... 61
References: ............................................................................................................................................. 65
Appendix 1: Paper I ........................................................................................................................................ 75
Appendix 2: Paper II .................................................................................................................................... 89
Appendix 3: Paper III .................................................................................................................................. 103
Appendix 4: Paper IV .................................................................................................................................. 111
Appendix 5: Characterization of Reaction Systems Through DLS and SAXS ............................................. 131
Other Publications: .............................................................................................................................. 135
Summary:

This PhD dissertation entitled “Enzymatic Modification of Antioxidants Towards Omega-3 Oil Protection” is primarily focused on synthesizing novel antioxidants from natural sources for better protection of oxidation-prone omega 3 oil. Selected phenolic acids were conjugated with fatty alcohols in different chain lengths and triacylglycerol (TAG). Several synthesis strategies were evaluated.

Synthesis of lipophilic phenolic fatty alcohols esters were initially conducted in a binary organic solvent system, which was composed of hexane and butanone. Preliminary studies were conducted to identify the main parameters affecting yield of these novel lipophilized phenolic fatty alcohol esters in a binary solvent system. The parameters studied in these preliminary studies included volume ratio of hexane to butanone, chain lengths of fatty alcohols and types of phenolic acids (dihydrocaffeic acid (DHCA), ferulic acid, and caffeic acid). The optimal mixed volume ratio was found to be 65/35 (hexane/butanone, v/v), in which both substrates have reasonably good solubility without compromising the enzyme activity. Medium chain fatty alcohols were easier to be esterified with phenolic acids, and highest conversion was achieved when hexanol (C6) was used. It was possible to dissolve ferulic and caffeic acids in this reaction system, but the conversions of these two phenolic acids were much lower than DHCA. Based on the optimal mixed volume ratio from the preliminary experiments and using esterification of octanol and DHCA as model, effects of different reaction parameters (temperature, reaction time, enzyme load, substrate molar ratio) were evaluated according to the experimental designs generated by response surface methodology (RSM). Optimal reaction conditions were predicted by the model and conversion of DHCA was increased significantly to above 90%.

To further increase the productivity of lipophilic phenolic esters, another system using ionic liquid (IL) Methyltrioctylammonium Trifluoroacetate (tOMA·TFA) as an assisting solvent was developed. Several parameters including the effect of mixed volume ratio between tOMA·TFA and octanol, temperature, water removal techniques (molecular sieves or vacuum), and concentration of DHCA were studied. Conversion of DHCA increased slightly with increasing volume ratio of IL/octanol. There was a 10 to 20% higher conversion when the reaction was conducted at 80 °C and 70 °C respectively comparing to at 60 °C. Significant increase in conversion was observed when water was removed from the reaction. The conversion of DHCA was increased to 90% when molecular sieves were added, which is 30% higher than the reaction without molecular sieves. Moreover, the
initial reaction rate increased as the concentration of DHCA was increased. Compared to the reactions conducted in parallel experiments where rOMA·TFA was not added, system composed of IL/octanol increased both solubility of DHCA and productivity of lipophilic phenolic esters significantly. This binary system was also proved to be very efficient in the conversion of ferulic and caffeic acids. Conversions were very low in the system without rOMA·TFA.

Besides synthesis of amphiphilic phenolic esters with fatty alcohol, other novel antioxidants can also be produced by conjugating phenolic acids with TAG. Two individual studies were conducted to achieve this goal. In the first study, enzymatic transesterification was conducted between octyl dihydrocaffeate and tricaprylin. Octyl dihydrocaffeate was initially synthesized from esterification of octanol with DHCA and then used for subsequent incorporation into tricaprylin. Due to the good compatibility between octyl dihydrocaffeate and TAG, an improved volumetric productivity and high enzymatic activity were obtained within 72 hour. In the second study, ferulate was incorporated directly into the highly unsaturated fish oil through enzymatic transesterification of ethyl ferulate (EF) with fish oil. In both of these studies, significant increases in production of phenolic TAG were achieved after optimizing the reaction conditions with RSM.

Micro-emulsion systems have the potential to be efficient synthesis reaction systems for the present study. Three different systems, phenolic acids (DHCA and ferulic) in IL/octanol, rutin in IL/linoleic, and ferulic in IL/triolein, were developed and investigated. These systems were characterized using dynamic light scattering (DLS) and small angle X-ray scattering (SAXS). It was suspected that the first system was deduced to be a homogenous system because no particles were found in it. Particles with a size of 45 nm and above 1000 nm were found in the second and third system respectively. Nevertheless, the third system composed of phenolic and IL/triolein was not as stable emulsion system as the second system composed of rutin and IL/linoleic.

Overall, the study in this dissertation explored several different routes for synthesis of lipophilic antioxidants. Several novel antioxidants were synthesized though esterification of selected phenolic acids with different fatty alcohols and TAG. These antioxidants are amphiphilic molecules and can be used to prevent oxidation in lipid-based food products.
Sammenfatning:


Syntese af lipofile phenol-syrer med fede fede alkohol estere blev indledningsvist udført i binære organiske systemer bestående af hexane og butanone. Indledende studier er gennemført med henblik på at identificere de mest betydningsfulde parametre som kan påvirke udbyttet af nye lipophile phenol-syrer med fede alkoholer i et solvent system. Parametrerne der er analyseret i dette indledende studie inkluderer volumen ratio af hexane of butanone, kædelængde af fede alkoholer og tuper af phenol-syrer (dihydrocaffeid syre (DHCA), ferulic syre og caffëic syre). Den optimale proportion af volume ratio var bestemt til at være 65/35 (hexane/butanone, v/v), hvor systemet forbedrede både oploseligheden af phenol-syrer og aktiviteten af enzymer. Medium kædelængde alkoholer var mere tilbøjelige til at blive esterificeret af phenol-syrer, og den højeste omdannelse var opnået når hexanol (C6) blev anvendt. Det er muligt at oplose ferulic og caffëic syrer i dette reaktions system, men omdannelsen af disse to phenol-syrer var meget lavere relativt til DHCA. Baseret på den optimale volumen ratio fra de indledende eksperimenter og anvendelse af esterificering af octanol og DHCA som model, blev effekten af forskellige reaktions parametre (temperatur, reaktions tid, enzym mængde, substrat molær ratio) evalueret ifølge eksperimentelle designs genereret ved respons overflade metodologi (RSM). Optimale reaktions forhold blev bestemt ved denne model og omdannelsen af DHCA blev forhøjet signifikant med over 90%.

For at forhøje produktiviteten af lipophile phenol-syrer yderligere, blev der udviklet et andet system baseret på ionisk væske (IL), Methyltrioctyl ammonium Trifluoroacetate (tOMA-TFA). Adskillige parametre blev analyseret: effekt af volumen ratioer imellem tOMA-TFA og octanol, effekt af varierende temperatur, effekten af fjernelse af vand fra reaktionssystemet med molekylære filtre eller vakuum og effekten af koncentrationen af DHCA. Omdannelse af DHCA blev kun svagt forhøjet når volume ratio af IL/octanol blev forhøjet. Der var 10-20% højere omdannelse når reaktion var udført ved 80 °C and 70 °C henholdsvis, sammenlignet med 60 °C Signifikant forhøjelse af a omdannelse blev observeret når vand blev fjernet fra systemet. Omdannelsen af DHCA blev forhøjet med 90% efter at molekylære sier blev tilføjet, hvilket er 30% højere i forhold
til når molekylære sier ikke er til stede. Endvidere, den initielle reaktions rate blev højere i takt med at DHCA concentration blev højere. Sammenlignet med reaktions forholdene i parallele experimenter hvor tOMA·TFA ikke var tilsat, systemer bestående af IL/octanol forbedrede både opløselighed af DHCA og produktiviteten af lipophile fenol-syrer betydeligt. Dette binære system viste sig også yderst effektiv i omdannelse af ferulic og caffeic syrer. Omdannelserne var meget lave i systemer uden tOMA·TFA.

Udover syntese af amphiphile phenoid syrer med fede alkoholer, kan nye antioxidanter også produceres ved konjugering af phenol syrer med TAG. For at opnå dette mål blev to individuelle studier blev udført. I det første studie, blev der udført enzymatisk transesterificering mellem octyl dihydrocaffeate og triaprylin, hvor octyl dihydrocaffeate var syntetiseret fra esterificering af octanol og DHCA og det spillede en rolle som intermediat i dette reaktions system. På baggrund af kompatibiliteten af intermediatet med TAG, blev der opnået en optimeret volumetrisk produktivitet og høj enzymatisk aktivitet. I det andet studie, blev ferulate lokaliseret ved siden af umættede fedtsyrer ved enzymatisk transfereserificering af ethyl ferulate med fiskeolie. I begge disse studier, blev der anvendt RSM til at optimisere reaktionsforholdene for at opnå højere produktivitet af phenol TAG.

Mikroemulsions systemer har potentialet til at være effective syntese reactionssystemer for undersøgelserne i dette projekt. Tre forskellige systemer, phenolic (DHCA og ferulic) i IL/octanol, rutin I IL/linoleid og ferulic I IL/triolein, blev udviklet og analyseret. De fysiske egenskaber af disse systemer blev undersøgt med teknikkerne dynamisk lysspredning (DLS) og lav vinkel røntgen stråling spredning (SAXS). Det var forventet, at det første system var et homogent system fordi der ikke var nogen partikler i det. Partikler med en størrelse på 45 mm og over 1000 nm blev fundet i det andet og tredje system, henholdsvis. Men det tredje system bestående af fenol-syrer og IL/triolein var ikke stabilt som et emulsion system, sammenlignet med det andet system bestående af rutin og IL/linoleic.

Overordnet, er der i dette studie blevet udforsket flere forskellige router til syntese af lipophile antioxidanter. Forskellige modificerede antioxidanter blev syntetiseret ved esterificering af udvalgte phenol-syrer med forskellige fede alkoholer og TAG. Disse modificerede antioxidanter er amphifile molekyler, og de har potentielt mange anvendelsesmuligheder i industrien til effektiv bekæmpelse af fedt oxidering.
### Abbreviations:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHA</td>
<td>Butylated Hydroxyanisole</td>
</tr>
<tr>
<td>BHT</td>
<td>Butylated Hydroxytoluene</td>
</tr>
<tr>
<td>CCF</td>
<td>Central Composite Face Centred Design</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary Heart Disease</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic Acid</td>
</tr>
<tr>
<td>DHCA</td>
<td>Dihydrocaffeic Acid</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic Light Scattering</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EF</td>
<td>Ethyl Ferulate</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic Acid</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray Tandem</td>
</tr>
<tr>
<td>F1-MAG</td>
<td>Monoferuloyl-sn-glycerol</td>
</tr>
<tr>
<td>F2-DAG</td>
<td>Diferuloyl-sn-glycerol</td>
</tr>
<tr>
<td>F1-DAG</td>
<td>Monoferuloyl-monoglyceride</td>
</tr>
<tr>
<td>F1-TAG</td>
<td>Monoferuloyl-diglyceride</td>
</tr>
<tr>
<td>F2-TAG</td>
<td>Diferuloyl-monoglyceride</td>
</tr>
<tr>
<td>GC</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>HLB</td>
<td>Hydrophilicity/Lipophilicity Balance</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>IL</td>
<td>Ionic Liquid</td>
</tr>
<tr>
<td>MBI</td>
<td>Methylene Bridge Index</td>
</tr>
<tr>
<td>MRPs</td>
<td>Maillard Reaction Products</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>o/w</td>
<td>Oil-in-Water</td>
</tr>
<tr>
<td>PG</td>
<td>Propyl Gallate</td>
</tr>
<tr>
<td>PLs</td>
<td>Phospholipids</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated Fatty Acids</td>
</tr>
<tr>
<td>RSM</td>
<td>Response Surface Methodology</td>
</tr>
<tr>
<td>SAXS</td>
<td>Small Angle X-ray Scattering</td>
</tr>
<tr>
<td>SLs</td>
<td>Structure Lipids</td>
</tr>
<tr>
<td>TAG</td>
<td>Triacylglycerol</td>
</tr>
<tr>
<td>TBHQ</td>
<td>Tertiary Butylhydroxyquinone</td>
</tr>
<tr>
<td>THBP</td>
<td>Trihydroxybutyr Ophenone</td>
</tr>
<tr>
<td>TLC</td>
<td>thin-layer chromatography</td>
</tr>
<tr>
<td>tOMA-TFA</td>
<td>Methyl trioctylammonium Trifluoroacetate</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>w/o</td>
<td>Water-in-Oil</td>
</tr>
</tbody>
</table>
Lists of Publications:


Chapter 1: Introduction

1.1 Background

The main barrier against successful launch of foods containing n-3 long chain polyunsaturated fatty acids (PUFA) is the high susceptibility of these fatty acids to lipid oxidation, which is also a major concern to foods containing lipids. Oxidation of lipid will lead to the formation of unpleasant fishy off-flavours, loss of essential fatty acids, fat-soluble vitamins, and other bio-actives, formation of potentially toxic compounds, reactive free radicals and aldehydes (Shahidi & Zhong, 2010a). The latter compounds have been suggested to promote development of cardiovascular disease (Uchida, 2000).

One of the popular methods applied for delaying or preventing autoxidation in food containing PUFAs is adding antioxidants as an additive. The roles of antioxidant include increasing the shelf life of food, reducing waste of raw materials and nutrient, and widening the ranges of fats which can be used in food (Coppen, 1983). The addition of antioxidants is considered to be the most effective, convenient and economical strategy for stabilizing food and non-food commodities containing lipids (Wanasundara & Shahidi, 2005). There has been a broad range of substances identified as antioxidants with various mechanisms and efficiency. However, previous studies have shown that many of the commercial antioxidants are not efficient in preventing lipid oxidation in foods enriched with PUFAs (Jacobsen & Nielsen, 2007). Compounds with metal chelating properties, such as ethylenediaminetetraacetic acid (EDTA), are able to retard oxidations in multiphase food system such as mayonnaise and milk. However, EDTA is a synthetic compound which is not well accepted by the food industry and consumers. Other synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tertbutylhydroquinone (TBHQ) were widely used in foods before, but there is a suspicion that these compounds may possess weak carcinogenic effects if consumed at a high level in some animals. Therefore, they are not well accepted as food additives either and have been restricted in countries like Canada, European Economic Union, and Japan (Shahidi & Zhong, 2010b). As a result, antioxidants from natural sources with health benefits to human are becoming more attractive. There are many sources of antioxidants in nature. Phenolic acids are one of the most common and well known natural antioxidants which can be obtained from raw vegetable materials (Figueroa-Espinoza & Villeneuve, 2005). Compared to synthetic antioxidants which are believed to be
Chapter 1: Introduction

responsible for promoting carcinogenicity, plant phenols are of considerable interest from the viewpoint of dietary antioxidant supplementation and food preservation (Namiki, 1990; Halliwell et al., 1995a).

It has been said that the most active dietary antioxidants belong to the family of phenolic and polyphenolic compounds (Shahidi & Naczk, 1995). However, the application of phenolic acids as antioxidants in food and cosmetics industries is limited due to their relatively low solubility in aprotic media. Moreover, the efficacy of antioxidants seems to be influenced by their localization in the food system. Localization of antioxidant in food system is dependent on the polarity of the antioxidant and emulsifier used. Lipid oxidation in food emulsions is also dependent on its physical structure such as the charge properties of the interface surrounding the oil droplet. These factors are determined by the type of emulsifier used and the processing conditions such as the type of emulsification equipment used (Jacobsen et al., 2007). Therefore, it is necessary to develop new antioxidants based on natural sources with improved physical properties. These antioxidants should be designed to be located where they are needed and to have the right anti-oxidative properties required in the particular food system (e.g. free radical scavenging and metal chelating properties). One of the realistic ways to achieve this aim is to modify the structure of the original phenolic compounds to obtain amphiphilic molecules which are more readily to be incorporated in food matrices. Such amphiphilic molecules can be synthesized by conjugating the carboxylic acid function of the phenolic acids with hydrophobic compounds through chemical or enzymatic lipophilization. These new molecules are more likely to present at oil-water or oil-air interfaces in food matrices where oxidation is considered to occur frequently. Through their presence at the oil-water or oil-air interfaces in food matrices, these novel antioxidants are able to provide first line protection against oxidation (Figueroa-Espinoza & Villeneuve, 2005). In term of synthesis route for these novel antioxidants, chemical processes are less desirable as compared to enzymatic methods due to the heat sensitivity and oxidation susceptibility of phenolic acids. In addition, the use of high temperatures frequently causes a dark colour, burnt taste, and high energy consumption (Sun et al., 2008).

Compared to chemical methods, enzymatic methods allow the reaction to be carried out in mild conditions and thereby avoiding the risks associated with chemical methods. Besides, the price of enzymes for such purpose has been decreased greatly due to a large production in industry.
1.2. Project Outlines

The overall purpose of the project is to develop new surface active antioxidants from natural resources for prevention of lipid oxidation in foods enriched with omega-3 fatty acids. The study includes two parts:

1) Developing new antioxidants which are designed to prevent oxidation in fish oil enriched foods;

2) Developing omega-3 fatty acids-antioxidant by incorporating the antioxidants directly into the structure of the highly unsaturated fish oil next to omega-3 fatty acids.

The application of the products in food systems enriched with omega–3 fatty acids had been conducted in collaboration with the Technical University of Denmark, where the products from this work were used.

The present dissertation is organized into three parts:

The first part covers the theoretical background for this study (Chapter 1-4). Chapter 1 is the introduction, which gives the background, objectives, and main content of this dissertation. Chapter 2 presents the importance of fish oil in human health and explores the mechanisms of lipid oxidation, especially lipid oxidation in the food system enriched with omega-3 fatty acids. Chapter 3 reviews the antioxidants frequently applied in food system to prevent lipid oxidation. The classification and mechanisms of these antioxidants and the necessities to modify antioxidants from natural sources are carefully elucidated. Chapter 4 discusses the advantages of modifying antioxidants by enzymatic methods, and different possible strategies on modifying natural antioxidants.

The second part of this dissertation presents the experimental and analytical methodology (Chapter 5), a summary of obtained results and discussions (Chapter 6), conclusion and future expectations (Chapter 7).

The last part of this dissertation includes four research articles and a condensed description and discussion of substantial experimental work in the appendices, where the results of the experimental work are presented.
Chapter 2: Health Benefit of Fish Oil and Oxidation Mechanism of Lipids

2.1. Fatty Acid Composition of Fish Oil

Fish oil consists of approximately 95% TAG, 1% phospholipids (PLs) and the unsaponifiable matter such as cholesterol, hydrocarbons, fatty alcohols and fat soluble vitamins. Marine oils differ from oils of plant and animal origin in terms of fatty acid chain length and degree of unsaturation (Bockisch, 2010). Marine oil contains fatty acids with chain lengths up to 20 to 22 carbon atoms and double bonds at the omega-3 and -6 positions. It has been noted that at least 36 fatty acids have been identified in fish oil of menhaden origin. Around 8 to 10 kinds of PUFAs make up 85 to 90% of the total, while the remaining of fatty acids is present at levels of less than 2% (Table 2.1). Fish lipids differ between species and according to administered diet. Specifically, the fatty acid composition of fish oil has been related to the geographical location and season (Karam, 2007).

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Herring-type(^b)</th>
<th>Cod liver(^b)</th>
<th>Menhaden(^b)</th>
<th>Salmon(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0</td>
<td>7.4</td>
<td>6.7</td>
<td>9</td>
<td>2.9</td>
</tr>
<tr>
<td>C16:0</td>
<td>14.1</td>
<td>20.1</td>
<td>19.1</td>
<td>10.7</td>
</tr>
<tr>
<td>C18:0</td>
<td>2.2</td>
<td>9.6</td>
<td>3</td>
<td>3.6</td>
</tr>
<tr>
<td>C18:1 n-9</td>
<td>12.3</td>
<td>3.4</td>
<td>12.2</td>
<td>24.5</td>
</tr>
<tr>
<td>C18:2 n-6</td>
<td>2.4</td>
<td>0.7</td>
<td>3.3</td>
<td>5.2</td>
</tr>
<tr>
<td>C18:3 n-3</td>
<td>5.2</td>
<td>2.2</td>
<td>1.6</td>
<td>5.3</td>
</tr>
<tr>
<td>C20:1 n-9</td>
<td>4</td>
<td>7.2</td>
<td>2.1</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>C20:4 n-6</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>7.6</td>
</tr>
<tr>
<td>C20:5 n-3</td>
<td>&lt;1.0</td>
<td>11.5</td>
<td>&lt;1.0</td>
<td>4.5</td>
</tr>
<tr>
<td>C22:6 n-3</td>
<td>8</td>
<td>20.2</td>
<td>7.7</td>
<td>17</td>
</tr>
</tbody>
</table>

\(^a\)Relative percent (w/w) of fatty acid g/100 g sample.
\(^b\)Adapted from Willis et al.,1998.

2.2. Fish oil in Diet and Health

PUFA, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are important fatty acids for human health. They have attracted a great deal of attention due to their beneficial effect on human health in the past decade. The biological roles of these molecules were brought to
light by the observation of low rates of coronary heart disease (CHD) among the population of Japan and Greenland, where fish is frequently consumed. Based on studies conducted in the Netherlands, Sweden, and USA during the 1980s, diet rich in fish (a rich source of omega-3 fatty acids) was found to have inverse association with the risk of mortality from CHD. The beneficial effects of these essential bio-molecules have been proposed (Kromhout et al., 1985; Shekelle et al., 1985; Norell et al., 1986). Ever-since, there is growing interests in application of omega-3 lipids as a food additive in the USA and Europe (Sloan, 2006).

High contents of omega-3 PUFA were found in flaxseed oil, marine plankton, and fatty fish. The main components found in fatty fish and fish oil making them of high importance in terms of health and disease, are the presence of two essential fatty acid, EPA (C20:5 n-3) and DHA (C22:6 n-3). Recent increase in the public’s awareness and interest of the benefits of these two omega-3 PUFAs has lead the industry to expand the utilized marine oils, specifically fish oil, as a novel bio-ingredient and in the production of nutraceutical products (Shahidi & Wanasundara, 1998).

2.3. Enrichment of Foods with Omega-3 Fatty Acids

A major challenge in relation to the use of omega-3 PUFA is their susceptibility to lipid oxidation, which will give rise to the formation of undesirable fishy off-flavours, colour deterioration and loss of endogenous antioxidants. Moreover, the free radicals generated from lipid autoxidation can react with proteins and pigments, which together will lead to a decrease in nutritional quality and presence of toxic lipid oxidation products (Frankel, 2005). Lipid autoxidation has become an important issue in food industry in the past years. Therefore it is important to understand the mechanism of lipid oxidation which will aid in finding suitable solutions for preventing lipid autoxidations.

2.3.1 Mechanisms of Lipid Oxidation

There are many catalytic systems that can oxidize lipids. Lipid oxidation can be induced by various environmental factors, such as light, temperature, enzyme, metals, metalloproteins and microorganisms. Most of these reactions involve some type of free radical and/or oxygen species as an intermediate (Vercellotti et al., 1992). According to the induced factors, the oxidation processes
can be divided into autoxidation, photodoxidation, thermal or enzymatic oxidation. Among all these processes, autoxidation is the most common process which is the spontaneous reaction of lipids with atmospheric oxygen through a chain reaction of free radicals (Shahidi & Zhong, 2010a). It has been widely accepted that lipid oxidation proceeds through three stages, which are initiation, propagation and termination respectively. This series of reactions will lead to formation of a series of complex chemical changes as showing in Figure 2.1.

**Initiation:**

\[
R_1 H + \text{initiator} \rightarrow R_1^+ + H^+
\]

*and/or*

\[
O_2 + \text{initiator} \rightarrow O_2^{..} \rightarrow \text{HOO} \cdot + R_1 H \rightarrow R_1^+ + H_2O_2
\]

**Propagation:**

\[
R_1^+ + O_2 \rightarrow R_1OO^+ \rightarrow R_2^+ \rightarrow R_2OO^+ \rightarrow R_3^+ \rightarrow R_3OO^+ \rightarrow \ldots
\]

- Heat, UV light, oxidizing metals, reducing metals.
- Products: \( R_1O^+ + 'OH \), \( R_2OO^+ + H^+ \), \( 'OH + R_3O^+ \), \( R_m^- \), \( R_y^- \), \( H_2O \), \( R_y^- \).

**Termination:**

\[
\{ \text{Non-radical products (R-R, ROR, ROOR, etc.)} \}
\]

![Figure 2.1. Lipid autoxidation pathways (Modified from Shahidi & Zhong, 2010a)](image)

Unsaturated fatty acids are particularly susceptible to oxidation as the hydrogen atoms next to the double bond are less stable due to lower C-H bond energy. Hence, they are easily attacked and replaced by free radicals and/or initiator at the initiation stage. Initiation stage is more complex than
the reaction scheme has shown, and still not fully understood so far. It was suggested before that triplet oxygen oxidation might be responsible for the lipid oxidation, but triplet oxygen oxidation cannot fully explain the initiation of lipid oxidation (Kim & Min, 1999). The oxidation at the initiation phase might be induced by the involving of singlet oxygen, because nonradical and electrophilic singlet oxygen can directly react with double bonds of food components without the formation of free radicals (Rawls & Van Santen, 1970). Compared to triplet oxygen oxidation, singlet oxygen oxidation is more significant because the oxidation rate of it is much greater and can be rapidly increased in very low temperature. Moreover, singlet oxygen oxidation produces new compounds which are not found in ordinary triple oxygen oxidation in foods (Kim & Min, 1999; Frankel et al., 1981; Bradley & Min, 1992).

Singlet oxygen can be formed chemically, enzymatically, photochemically (Choe & Min, 2005) during processing or marketing. As the singlet oxygen is formed, it will react directly with unsaturated fatty acids very quickly without formation of free radical intermediate and transform the unsaturated fatty acids into alkyl radical (R·) as shown in the Figure 2.1 (Adam, 1975). Triplet oxygen, on the other hand, cannot react with unsaturated fatty acids directly without a free radical intermediate because it is a diradical compound and can react only with radical compounds (Kim & Min, 1999).

Besides the oxidation induced by singlet oxygen, other factors, such as heat, light, radiation, and metalloprotein, may induce lipid oxidation as well, and they are all recognized as initiators. With presence of any of these initiators, the hydrogen atom on the carbon chain of lipid is lost and alkyl radicals are produced (Shahidi & Zhong, 2010b; Jacobsen & Nielsen, 2007).

The newly produced alkyl radicals may attack other lipid and lead to production of more free radicals. This process will repeat itself for many times until there are no more hydrogen sources available in the propagation stage. These radicals propagate in the presence of oxygen by a free radical chain mechanism to form peroxy radicals (ROO·), which can react with a new unsaturated lipid to form hydroperoxide (ROOH) and a new lipid radical, will subsequently propagate the chain reactions. The free radical chain reaction propagates until two free radicals join and for a non-radical product to terminate the chain reactions.
Lipid hydroperoxides are the primary products of autoxidation, which are tasteless and odourless chemicals (Jacobsen & Nielsen, 2007). Lipid hydroperoxides are not stable; they can be decomposed into alkoxy radicals (RO·) and hydroxyl radicals (OH·) at high temperature or in the presence of trace transition metals or light. The decomposed alkoxy radicals may further decompose to form a variety of non-volatile and volatile secondary oxidation products, which are responsible for the flavour deterioration (Jacobsen & Nielsen, 2007; Frankel, 1991). The secondary lipid oxidation products are mainly low-weight molecules and the composition of the molecules vary depending on the composition of fatty acids (Table 2.2).

### Table 2.2. Secondary oxidation products of fatty acid methyl esters by lipid oxidation (Modified from Shahidi & Zhong, 2010a)

<table>
<thead>
<tr>
<th>Aldehyde</th>
<th>Linoleic acid</th>
<th>Linolenic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octanal</td>
<td>Pentanal</td>
<td>Propanal</td>
</tr>
<tr>
<td>Nonanal</td>
<td>Hexanal</td>
<td>Butanal</td>
</tr>
<tr>
<td>2-Decenal</td>
<td>2-Octenal</td>
<td>2-Butenal</td>
</tr>
<tr>
<td>Decanal</td>
<td>2-Nonenal</td>
<td>2-Pentenal</td>
</tr>
<tr>
<td></td>
<td>2,4-Decadienal</td>
<td>2-Hexanal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3,6-Nonadienal</td>
</tr>
<tr>
<td>Carboxylic acid</td>
<td>Methyl heptanoate</td>
<td>Methyl heptanoate</td>
</tr>
<tr>
<td>Methyl octanoate</td>
<td>Methyl octanoate</td>
<td>Methyl heptanoate</td>
</tr>
<tr>
<td>Methyl 8-oxooctanoate</td>
<td>Methyl 8-oxooctanoate</td>
<td>Methyl heptanoate</td>
</tr>
<tr>
<td>Methyl 9-oxononanoate</td>
<td>Methyl 9-oxononanoate</td>
<td>Methyl heptanoate</td>
</tr>
<tr>
<td>Methyl 10-oxodecanoate</td>
<td>Methyl 10-oxodecanoate</td>
<td>Methyl heptanoate</td>
</tr>
<tr>
<td>Methyl 10-oxo-8-decenoate</td>
<td>Methyl 10-oxodecanoate</td>
<td>Methyl heptanoate</td>
</tr>
<tr>
<td>Methyl 11-oxo-9-undecenoate</td>
<td>Methyl 10-oxodecanoate</td>
<td>Methyl heptanoate</td>
</tr>
<tr>
<td>Alcohol</td>
<td>1-Heptane</td>
<td>1-Pentanol</td>
</tr>
<tr>
<td></td>
<td>1-Pentanol</td>
<td>1-Octene-3-ol</td>
</tr>
<tr>
<td>Hydrocarbon</td>
<td>Heptane</td>
<td>Pentane</td>
</tr>
<tr>
<td></td>
<td>Octane</td>
<td>Pentane</td>
</tr>
<tr>
<td></td>
<td>Octane</td>
<td>Pentane</td>
</tr>
</tbody>
</table>

### 2.3.2. Lipid Oxidation in Food Enriched with Omega-3 Fatty Acids

Lipid oxidation had been widely studied before in bulk oil, and the factors that affect the oxidation in this system are well understood (Halliwell et al., 1995b). However, many of the omega-3 enriched products on the market today are emulsified foods, and most of these are oil-in-water (o/w) emulsions. Although the oxidation mechanisms are the same, the factors that affect lipid oxidation of emulsion systems differ significantly from bulk oil systems. Lipid oxidation in emulsions is
generally acknowledged to include oxidation events in all the different phases of the systems, making the mechanisms of lipid oxidation in emulsions very different and more complex than those in bulk oil systems (Jacobsen & Nielsen, 2007). Unlike the bulk oil systems which have been studied for many years, interests in lipid oxidation on emulsion systems has only grown and become active in the past ten years (Coupland & McClements, 1996).

An emulsion consists of two immiscible liquids (such as oil and water), and one of them is dispersed into another in the form of small spherical droplets (Figure 2.2). If oil is dispersed in the water and formed oil droplets, then the system is called o/w emulsion, and vice versa. Conceptually, it is convenient to divide an emulsion into three distinct regions: the interior of the droplets, the continuous phase, and the interfacial region (Figure 2.3). The various molecules partition themselves into different regions according to their polarity and surface activity. Non-polar molecules tend to be located in the oil phase, polar molecules in the aqueous phase, and amphiphilic molecules at the inter-phase (Coupland & McClements, 1996). Certain type of molecules, such as hydroperoxides, pro-oxidants and antioxidants, which are normally presented in low concentration, may tend to partition into the interfacial region (McClements & Decker, 2000).

It was found that the environment where the molecules are located may have significant effect on its chemical reactivity or other properties in a system (Wedzicha, 1988). Beside the effect of the environment, the orientation of the lipid molecules in the interfacial region may also have an important effect on lipid oxidation in emulsion systems. Because of this, it will affect accessibility of lipid molecules to be attacked by water-soluble, reactive oxygen species (Coupland & McClements, 1996). Moreover, factors such as chemical structure of lipids, oxygen concentration, antioxidants, temperature, surface area, and water content, etc. may also have effects on lipid oxidation in an emulsion system. These factors have already been well studied before (Sun et al., 2011). Factors influencing the oxidation in the systems of o/w will be discussed in this dissertation. Additionally it has been suggested that oxidations in systems of water-in-oil (w/o) are as similar as in bulk oil because the surface of the oil phase is exposed directly to air (Coupland & McClements, 1996).
Figure 2.2. Photograph of hydrocarbon o/w emulsion. The dark regions are emulsion droplets and the light regions are the aqueous phase (Modified from McClements & Decker, 2000)

Figure 2.3. An o/w emulsion can be considered to consist of three phases: the oil droplet, the interfacial, and the aqueous phases. (Modified from McClements & Decker, 2000)
2.3.2.1. Chemical Structure of Lipids

As unsaturated fatty acids are more susceptible to oxidation than saturated fatty acids, food matrices containing rich content of unsaturated fatty acids are thus easier to be oxidized. Among the unsaturated fatty acids, the degree of unsaturation or methylene bridge index (MBI, the mean number of bisallylic methylene positions) determines the oxidation susceptibility of unsaturated fatty acid. Unsaturated fatty acids with higher MBI value are more susceptible to oxidation (Shahidi & Zhong, 2010a). The stereo-specific position distribution of fatty acids in TAG is believed to play a role in lipid oxidation. It was found that unsaturated fatty acids are more stable when they are located at the sn-2 position of TAG (Wijesundera, 2008; Shen & Wijesundera, 2009). This is probably due to the interaction of acyl chains within the same TAG molecule and possible steric hindrance for hydroperoxide formation (Shahidi & Zhong, 2010a). Moreover, fatty acids are oxidized faster in the free form than in their glyceryl esters, possibly due to greater ability to pick up trace metals from the environment (Nawar, 1997). However, the degree of unsaturation of fatty acids is perhaps less important in o/w emulsion systems than in w/o emulsion systems or bulk oil systems. There are studies found that the oxidative stability show trends of increasing as the degree of unsaturation increases when fatty acids dispersed in aqueous colloidal dispersions, and the reason for this phenomenon is still unknown (Miyashita et al., 1993; 1994).

The oxidative susceptibility is also related to the position of the double bond on an unsaturated fatty acid or the polarity of lipid molecules (McClements & Decker, 2000). In the micelles of emulsion systems, the fatty acids are orientated so that carboxyl group protrudes into the aqueous phase, while the hydrocarbon tail is located in the hydrophobic interior, therefore the lipid will be more stable when the double bonds are buried deeper or closer to the methyl end of fatty acids. On the other hand, the polarity of lipids determines the location of lipids in the micelles. The polar lipids tend to be located in the droplet surface and therefore more susceptible to lipid oxidation. Both position of the double bond and polarity of lipid molecules are thought to be important in o/w emulsion systems, but more studies need to be conducted to confirm it.

2.3.2.2. Environmental Factors

Although lipids occur naturally in various sources, procurement of commercial fats and oils requires extraction, refining operations, and packaging for storage. During these processes, lipids are
exposed to environmental factors, such as high temperature, light, atmospheric oxygen and moisture, and possibly worn metals, which may affect the oxidation rate of lipids (Shahidi & Zhong, 2010a). Moreover, it has been observed that lipids oxidation occurred faster in oil-in-water emulsions than in bulk oils (Frankel et al., 2002), which is partly because of high temperatures are required and oil are exposed to air during the mechanical processes for emulsification (Jacobsen & Nielsen, 2007). However, some of the oxidative factors can be removed during the refining processes as listed in Table 2.3.

<table>
<thead>
<tr>
<th>Processing</th>
<th>Components eliminated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degumming</td>
<td>Phospholipids</td>
</tr>
<tr>
<td>Refining</td>
<td>Free fatty acids, phospholipids, metal ions and soaps</td>
</tr>
<tr>
<td>Bleaching</td>
<td>Pigments, primary oxidation products</td>
</tr>
<tr>
<td>Fractionation</td>
<td>Waxes, solid triacylglycerol</td>
</tr>
<tr>
<td>Deodourization</td>
<td>Free fatty acids, secondary oxidation products, residual pigments, sterols, hydrocarbons, other volatiles</td>
</tr>
</tbody>
</table>

### 2.3.2.3. Emulsified type and Charge Status

To obtain physically stable emulsions, emulsifiers are required to be added into emulsions. Emulsifiers are surface-active molecules with amphiphilic properties, which can interact with the interface of emulsions and reduce surface tension. Emulsifiers can be either macromolecules or surfactant molecules, and they are able to influence lipid oxidation in different ways due to their ability to interact with other ingredients near the o/w interface. Some emulsifiers are protein, and the pH of emulsions is then adjusted below or above the pI of the protein in order to avoid coalescence of droplets. Adjusting the pH may then produce either negative or positive droplets (Jacobsen & Nielsen, 2007). It was reported that lipid oxidation rates were much higher for negatively charged droplets than positive droplets or uncharged droplets (Mancuso et al., 1999; Mei et al., 1998). Oxidative stabilities of different emulsifiers had been studied, and it was found the oxidative stabilities increase in the order: whey protein isolate > lecithin > mon-/diacylglycerols > Tween 20 > sucrose fatty acid ester (Fomuso & Akoh, 2002). Besides the charge droplets, pH can also influence lipid oxidation in other ways. Jacobsen et al. (1999; 2001c) reported lipid oxidation increased when the pH was decreased from 6.0 to 3.8. It is probably due to the fact that all food
products contain trace metals, which are potential oxidative initiator and their solubility generally increases with decreasing pH (Belitz & Grosch, 1999).

2.3.2.4. Surface Character

As shown in figure 2.3 above, an emulsion can be divided into three regions: oil phase, aqueous phase, and the interfacial region phase. Each of these “phases” may be chemically complex. The aqueous phase may contain ions, macromolecules (e.g., polysaccharides), salt, or amino acid, which may exert stabilizing or destabilizing effects, and they may even turn into oxidative initiators. The interfacial phase can be composed of proteins or of small emulsifiers or mixture of a number of these components. These components can enhance the stability of the emulsions as well as act as a physical barrier to prevent penetration and diffusion of pro-oxidant from the aqueous phase. There were studies that showed the lipid oxidation can be slowed in the emulsion containing droplets stabilized by surfactants with long polar head group or hydrophobic tail group (Sun et al., 2011).

2.3.2.5. Droplet Characteristics

The concentration and size of the emulsion droplets are one of the most important physical properties of emulsifications which can determine lipid oxidation (Coupland & McClements, 1996). Some studies reported that lipid oxidation rate decreased as the oil concentration increased (Jo & Ahn, 1999; Osborn & Akoh, 2004). Another study found high concentration of oil might retard lipid oxidation rate in the initial phase, but accelerated the rate in the propagation phase in the long term (Coupland et al., 1996). Moreover, the droplet sizes of emulsification are also related to lipid oxidation. In the emulsified systems, smaller droplet sizes are preferable because they can increase physical stability of the emulsions, but the rate of lipid oxidation will also increase at the same time because of the increase of the droplet surface, which increases the contact among the interface, oxygen, and radicals (Jacobsen & Nielsen, 2007; Coupland et al., 1996).
Chapter 3: Antioxidant Mechanism and Antioxidants from Natural Sources

3.1. Introduction

One of the most effective methods of preventing lipid oxidation is using antioxidants. Currently antioxidants are widely used in the food industry to prevent or delay lipid oxidation. The role of antioxidants is not to enhance or improve the quality of foods. Instead, application of antioxidants in food enriched with lipids can minimize rancid and prevent the formation of toxic oxidation products. Therefore the nutritional quality of food will be maintained for a longer time and the shelf life of a variety of lipid-containing food can be increased at the same time. There are several things that have to be considered when choosing proper antioxidants to be included in food product, such as inexpensive, nontoxic, effective at low concentrations, stable, and capable of surviving processing (carry-through effect); colour, flavour, and odour must be minimal (Reische et al., 1999). Besides extending shelf life and maintaining the quality of food products enriched with unsaturated lipids, antioxidants can also reduce raw material waste, reduce nutritional losses, and widen the range of fats that can be used in specific products (Coppen, 1983).

3.2. Classification and Mechanism of Antioxidants

In general, antioxidants are defined as molecules capable of significantly delaying or preventing oxidation when they are presented in low concentration compared to oxidant substrates (Halliwell & Gutteridge, 2007). However, based on the mechanisms of antioxidants, antioxidants are also defined as chemical compounds which can donate hydrogen radicals and reduce the primary radical chemical species, and are thus converted into oxidized antioxidant radicals (Madhavi et al., 1996b). Food antioxidants are usually classified as either primary or secondary antioxidants based on their functions (Jacobsen & Nielsen, 2007). Examples of primary antioxidants are hindered phenolic compounds such as tocopherols, BHA, BHT, and TBQH. Secondary antioxidants, which are also called synergistic antioxidants, can be classified into two sub-classifications: oxygen scavengers and chelators. Both of them may act as hydrogen donors to the phenoxy radical, thereby regenerating the primary antioxidant, or they may react with free oxygen and remove it in a closed system. Secondary antioxidants usually reduce the rate of autoxidation of lipids by processes such
as binding metal ions, absorbing ultraviolet (UV) radiation, decomposing hydroperoxides to nonradical products, scavenging oxygen, and deactivating singlet oxygen. That is to say, they usually require the presence of another minor component for effective action. Thus, they are also known as preventive antioxidants. Moreover, the secondary antioxidants often exert synergistic effects together with primary antioxidants. Typical examples of such kind of antioxidants are sequestering agents, metal ions, reducing agents (ascorbic acid), the tocopherols or other phenolics (Gordon, 1990; Madhavi et al., 1996b; Jacobsen & Nielsen, 2007). Moreover, there are antioxidants which exhibit more than one mechanism and are often referred to as multifunctional antioxidants (Reische et al., 1999). Figure 3.1 gives an overview of lipid oxidation and the interaction of antioxidants.

3.2.1. Primary Antioxidants

Primary antioxidants are free radical acceptors that delay or inhibit the initiation step or interrupt the propagation step of autoxidation. As shown in figure 3.1, initiation of autoxidation occurs when a hydrogen molecule is abstracted from an unsaturated fatty acid (RH) to form an alkyl radical (R·) (or lipid radical). This highly active radical then reacted with oxygen to form a peroxyl radical (ROO·). Following that, peroxyl radicals react with other fatty acids to form hydroperoxide (ROOH) and a new alkyl radical. This new alkyl radical will react with oxygen and repeat the above reaction. Therefore a few radicals will induce oxidation of a lot of lipids. On the other hand, hydroperoxide is an unstable molecule and can degrade to produce radicals that further accelerate propagation reaction. Hydroperoxide degradation will lead to undesirable odors, flavors and rancidity at late stages of oxidation (Reische et al., 1999).

These radicals can be removed by primary antioxidants (AH) through the following mechanisms. First, primary antioxidant donates hydrogen atoms to the lipid radicals, and lipid derivatives and antioxidant radicals are produced as a result (Equations 1 to 3). These later two molecules are more stable compared to other radicals. Like hydrogen donors, primary antioxidants have higher affinity to peroxyl radical than lipids (Reische et al., 1999; Porter, 1980).

\[
AH + ROO^\cdot \longrightarrow ROOH + A^\cdot 
\]

(1)

\[
AH + RO^\cdot \longrightarrow ROH + A^\cdot 
\]

(2)

\[
AH + R^\cdot \longrightarrow RH + A^\cdot 
\]

(3)
Figure 3.1. Overview of lipid oxidation and the interaction of antioxidants (Modified from Reische et al., 1999).
Therefore, antioxidants should be added into the food system before initiation of autoxidation and formation of free radicals, otherwise primary antioxidants may result in loss of function (Nawar, 1996).

The antioxidant radicals produced above have a very low activity, which means the reaction of antioxidants with oxygen or lipids is very slow. Therefore, the rate of propagation is reduced due to this low reactivity. There are two fates for these antioxidant radicals, either stabilized by delocalization of the unpaired electron around a phenol ring to form stable resonance hybrids or participating in termination reactions with peroxy (Equation 4), oxy (Equation 5), and other antioxidant radicals (Equation 6) (Reische et al., 1999).

\[
\begin{align*}
\text{ROO}^* + \text{A}^* & \rightarrow \text{ROOA} \quad (4) \\
\text{RO}^* + \text{A}^* & \rightarrow \text{ROA} \quad (5) \\
\text{A}^* + \text{A}^* & \rightarrow \text{AA} \quad (6)
\end{align*}
\]

The most common primary antioxidants used are synthetic antioxidants, for example, BHA, BHT, and TBQH. The applications of such antioxidant have been restricted recently due to heath concerns. A few naturally occurring primary antioxidants are more commonly used in foods, such as tocopherols and carotenoids (Reische et al., 1999). The application of antioxidants will be further discussed below.

### 3.2.2. Secondary Antioxidants

Unlike primary antioxidants, secondary antioxidants cannot convert free radicals into more stable products. Instead, they chelate prooxidant metals and deactivate them, replenish hydrogen to primary antioxidants, decompose hydroperoxides to nonradical species, deactivate singlet oxygen, absorb UV radiation, or act as oxygen scavengers (Belitz & Grosch, 1999; Minotti, 1993; Aust et al., 1985). Therefore the antioxidation mechanisms of secondary antioxidants are much more complex compared to primary antioxidants (Reische et al., 1999). Three kinds of secondary antioxidants are classified based on their anti-autoxidation mechanisms and introduced below.
3.2.2.1 Autoxidation Mechanisms of Metal and Chelators

Several heavy metals with two or more valence states can promote oxidation by acting as catalysts of free radical reactions. They act as catalyst of free radicals to promote oxidation. There are two mechanisms that have been proposed related to these kinds of antioxidants. The first proposal is that metal reacts directly with unsaturated lipids (Equation 7). But such reaction is probably not the major mechanism because of thermodynamic constraints, spin barriers, and an extremely slow reaction rate, which makes this reaction difficult to occur (Reische et al., 1999).

\[
RH + M^{(n-1)} \rightarrow M^{n+} + H^+ + R^* \quad (7)
\]

Another proposal, base on metal antioxidants, is that metal interacts with hydroperoxydies and promotes oxidation. It is thought that a metal–hydroperoxide complex forms and subsequently decomposes to produce free radicals. Metals enhance the rate of decomposition of hydroperoxides and the generation of free radicals. Two metal–hydroperoxide reactions are possible:

\[
ROOH + M^{(n+1)+} \rightarrow M^{n+} + H^+ + ROO^* \quad (8)
\]

\[
ROOH + M^{n+} \rightarrow M^{(n+1)+} + OH^- + RO^* \quad (9)
\]

The reaction shown in Equation 8 and 9 can be induced even by trace amounts of metals which promote electron transfer from lipids or hydroperoxides, because these two reactions are recycling and regenerate lower oxidation state of the metals (Belitz & Grosch, 1999). However, the reaction in equation 9 is less likely to happen than the reaction in equation 8. This is because metals in their lower oxidation states accelerate hydroperoxide degradation more than metals in their higher oxidation states (Gordon, 1990).

Chelating compounds can decrease prooxidant effect of metal by reducing metals redox potentials and stabilizing the oxidized of the metal through chelation with metals. Chelating compounds may also sterically hinder formation of the metal hydroperoxide complex. Examples of chelators are citric acid, phosphoric acid and EDTA, etc… The mechanism is that chelator forms a stable complex with metal and the autoxidation is therefore inhibited (Reische et al., 1999).
3.2.2.2 Oxygen Scavengers and Reducing Agents

Another type of secondary antioxidants such as ascorbic acid, ascorbyl palmitate, erythorbic acid, sodium erythorbate and sulfites can prevent oxidation by scavenging oxygen or acting as reductants. Oxygen scavenging is especially useful in products with headspace or dissolved oxygen, and reducing agents function by donating hydrogen atoms (Reische et al., 1999).

3.2.2.3 Singlet Oxygen Quenchers

Singlet oxygen is a high energy molecule responsible for photooxidation of unsaturated fats and the subsequent generation of hydroperoxides. Singlet oxygen quenchers deplete singlet oxygen of its excess energy and dissipate the energy in the form of heat. Carotenoids, including β-carotene, lycopene, and lutein are active singlet oxygen quenchers at low oxygen partial pressure (Reische et al., 1999).

3.3 Synthetic Antioxidants vs Natural Antioxidants

3.3.1 Synthetic Antioxidants

In the earlier stages of food industry development, synthetic antioxidants were widely use because of superior efficacy, low cost and high stability. Most of the synthetic antioxidants are compounds similar to phenol and contain one or two aromatic rings (Figure 3.2). Due to the difference in physical structure, the physical characteristics vary among synthetic antioxidants, which determine the selection of antioxidants in different food products. As summarized in Table 3.1, BHA and BHT are heat-stable antioxidants and they also have good solubility in both hydrophobic systems and hydrophilic systems. Therefore, they have the widest application in food products, especially in heat-process foods and oil-in-water emulsions. PG is a hydrophobic compound and is not stable at higher temperatures and its application is more limited than BHT and BHA. TBHQ is also a heat stable compound and is mainly used in frying processes (Reische et al., 1999).
Mechanisms of actions for both BHT and BHA are represented in the equations below. The representation of synthetic antioxidants in the food systems can be referred to as BHT and BHA. As mentioned above, BHT and BHA are primary antioxidants. They behave as a synthetic vitamin E and act as free radical scavengers. The autoxidation of unsaturated fatty acids are stopped by such antioxidants by converting peroxyl radicals to hydroperoxides (Equations 10 and 11) (Burton & Ingold, 1981).

\[
ROO^* + \text{ArOH} \rightarrow ROOH + \text{ArO}^* 
\]  
(10)

\[
ROO^* + \text{ArO}^* \rightarrow \text{nonradical products} 
\]  
(11)
### Table 3.1. Physical characters and application of some synthetic antioxidants (Modified from Reische et al., 1999)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular Weight</th>
<th>Appearance</th>
<th>Boiling Point (°C)</th>
<th>Melting point (°C)</th>
<th>Solubility</th>
<th>Functionality</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHA</td>
<td>180.25</td>
<td>Waxy solid</td>
<td>264 – 270</td>
<td>48 – 63</td>
<td>Insoluble in water, slightly soluble in glycerol and mineral oil, soluble in fats, alcohol, propylene glycol, petroleum, ether; glycercyld monooleate, paraffin</td>
<td>Good carry – through in baked and fried products, volatile, distillable, most effective in animal fats, use in packaging materials, synergistic with other antioxidants, slight phenol odor, less expensive than BHT, synergistic with other antioxidants, decomposes at frying temperatures.</td>
</tr>
<tr>
<td>BHT</td>
<td>220.356</td>
<td>White crystals</td>
<td>265</td>
<td>70</td>
<td>Insoluble in water, glycerol, and propylene glycol, slightly soluble in mineral oil, soluble in fats, paraffin, glycercyld monooleate, alcohol, petroleum, ether, most organic solvents</td>
<td>Less carry – through in baked and fried products than BHA, more sterically hindered than BHA, most effective in animal fats, slight phenol odor, poor carry – through properties, synergistic with other antioxidants, decomposes at frying temperatures, antioxidants.</td>
</tr>
<tr>
<td>PG</td>
<td>212.20</td>
<td>White crystals</td>
<td>Decomposes above 148</td>
<td>150</td>
<td>Slightly soluble in water, fats, mineral oil, glycercyld monooleate, soluble in alcohol, glycerol, propylene glycol</td>
<td>Decomposes at frying temperatures, stability of octyl and dodecyl forms greater than propyl, discolors in the presence of metals, always used in combination with a chelator, less soluble in fats than BHA and BHT, more effective in vegetable oils than BHA and BHT, poor carry – through in baking, but...</td>
</tr>
<tr>
<td>Antioxidant</td>
<td>Molecular Weight</td>
<td>Color</td>
<td>Solubility Details</td>
<td>Characteristics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>------------------</td>
<td>-------</td>
<td>-------------------</td>
<td>-----------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBHQ</td>
<td>166.22</td>
<td>White to tan crystals</td>
<td>Slightly soluble in water, moderately soluble in fats, propylene glycol, glyceryl monooleate, soluble in alcohol</td>
<td>Excellent antioxidant in vegetable oils, does not discolour in the presence of metals, little odor, poor carry-through in baking and frying</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trihydroxybutyrophenone (THBP)</td>
<td>196</td>
<td>Tan powder</td>
<td>Slightly soluble in water, moderately soluble in fats, propylene glycol, glyceryl monooleate, soluble in alcohol, propylene glycol, paraffin</td>
<td>Synergistic with other antioxidants, used in packaging materials, turns brown in the presence of metals, used extensively in animal rations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethoxyquin</td>
<td>217.31</td>
<td>Yellow liquid</td>
<td>Soluble in most organic solvents</td>
<td>Effective in pigment retention</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.3.2. Natural Antioxidants

Despite with the convenience of using synthetic antioxidants, there are increased concerns that such antioxidants are suspected in promoting carcinogenicity (Namiki, 1990). Due to such concerns, a trend toward the use of natural antioxidants in the food industry has been apparent for quite some time.

There have been a variety of studies on antioxidants from natural sources including ascorbic acid and tocopherols, carotenoids, flavonoids, amino acids, proteins, protein hydrolysates, Maillard reaction products (MRPs), phospholipids, sterols, and phenolic acids (Reische et al., 1999). It should be noted that not all the antioxidants from natural sources have been approved as safe for consumption. However, there would be fewer concerns if the antioxidants are from natural sources, such as fruit and vegetable which appear in people’s daily diets. Table 3.2 summarized some antioxidants that commonly exist in our daily diets. These compounds have been well known for years, not only because their antioxidant effects, but also because of medical effects.

<table>
<thead>
<tr>
<th>Antioxidants</th>
<th>Example</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tocopherols</td>
<td>α-, β-, γ- and δ-tocopherols</td>
<td>Seeds, cereal and legume grains, nuts, vegetable oils, etc.</td>
</tr>
<tr>
<td>Tocotrienols</td>
<td>α-, β-, γ- and δ-tocopherols</td>
<td>Palm oil, rice bran oil, etc.</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>Ascorbic acid, ascorbates</td>
<td>Fruits, vegetables, etc.</td>
</tr>
<tr>
<td>Carotenoids</td>
<td>β-carotene, lycopene, astaxanthin, fucoxanthin</td>
<td>Carrots, tomato, fish/shellfish, marine algae, etc</td>
</tr>
<tr>
<td>Phenolics/polyphenolics</td>
<td>Ferulic acid, quercetin, catechin, reverstrol, cyanidin</td>
<td>Fruits, vegetables, nuts, cereal, etc.</td>
</tr>
</tbody>
</table>

3.3.2.1. Antioxidant Mechanisms of Tocopherols and Tocotrienols

Both tocopherols and tocotrienols are isomers of vitamin E. Each contains four homologs in the vitamin E families. They are mostly present in soybean oil, other vegetable oils and deodorizer distillates. They are fat-soluble antioxidants that can effectively scavenge lipid peroxyl radicals and act as synergists with many other antioxidants. The mechanisms of scavenging lipid peroxyl radicals by α-tocopherol have been well studied. Lipid radicals are converted into hydroperoxides by α-tocopherol, and α-tocopherol turn into α-tocopheryl semiquinone radical (Equation 12). The α-
tocopheryl semiquinone radical can react further with another lipid radical and produce another hydroperoxide and methyltocopherylquinone (Equation 13) or react with another α-tocopheryl semiquinone radical and produce α-tocopherol dimer (Equation 14). The methyltocopherylquinone is unstable and will yield α-tocopherylquinone. The α-tocopheryl dimer will continue to produce antioxidant activity (Reische et al., 1999).

\[
\begin{align*}
\alpha\text{-tocopherol} + \text{ROO}^* & \rightarrow \text{ROOH} + \alpha\text{-tocopheryl semiquinone}^* \quad (12) \\
\alpha\text{-tocopheryl semiquinone}^* + \text{ROO}^* & \rightarrow \text{ROOH} + \text{methyltocopherylquinone} \quad (13) \\
\alpha\text{-tocopheryl semiquinone}^* + \alpha\text{-tocopheryl semiquinone}^* & \rightarrow \alpha\text{-tocopheryl dimer} \quad (14)
\end{align*}
\]

### 3.3.2.2. Antioxidant Mechanisms of Ascorbic Acid

L-ascorbic acid known as vitamin C is a water soluble antioxidant. Insufficient quantities of vitamin C will result in many physical diseases. Moreover, ascorbic acid is one of the natural antioxidants that can be used without limitation. Ascorbic acid can act either as a primary antioxidant by donating a hydrogen atom to a radical or as a secondary antioxidant by scavenging oxygen, shifting the redox potential of food systems to the reducing range, acting synergistically with chelators, and regenerating primary antioxidants (Madhavi et al., 1996a). Ascorbic acid is commonly used to regenerate tocopherol and acts as a reducing agent (equation 15) (Reische et al., 1999; Shahidi & Zhong, 2010b).

\[
\text{Tocopherol} + \text{Ascorbic acid} \rightarrow \text{tocopherol} + \text{dehydroascorbic acid} \quad (15)
\]

### 3.3.2.3. Antioxidant Mechanisms of Carotenoids

Carotenoids are yellow, orange, and red lipid soluble pigments that are widely found in fruits and vegetable. There are two classes of carotenoids namely carotenes and xanthopylls respectively. It is suspected that carotenoids can reduce the risk of several chronic diseases, but more studies are needed to confirm it (Vines et al., 2000). It is clear that certain carotenoids, such as β-carotene, are linked to vitamin A and have the highest vitamin A activities (Deshpande et al., 1996). Carotenoids
can be either primary antioxidant by trapping free radicals (Equations 16 and 17) or be a secondary antioxidant by quenching singlet oxygen (Equations 18 and 19).

\[
\begin{align*}
\text{Carotene} + \text{ROO}^\cdot & \rightarrow \text{Carotene}^* \quad \text{(16)} \\
\text{Carotene}^* + \text{ROO}^\cdot & \rightarrow \text{termination product} \quad \text{(17)} \\
\text{O}_2 + \beta\text{-carotene} & \rightarrow 3\beta\text{-carotene}^* + 3\text{O}_2 \quad \text{(18)} \\
3\beta\text{-carotene}^* & \rightarrow \beta\text{-carotene} + \text{heat} \quad \text{(19)}
\end{align*}
\]

### 3.3.2.4. Antioxidant Mechanisms of Phenolics/Polyphenolics

Compounds from the phenolic acid family are widely distributed in vegetable and fruits and most of them have medical benefits to human beings (Andlauer et al., 2000; Friedman & Jurgens, 2000; Hollman, 2001). The phenolic acid family is composed of the cinnamic (C6- C3) and the benzoic (C6-C1) acid derivatives (Figures 3.3 and 3.4), characterized by containing a benzene ring substituted with one or more hydroxyl or methoxy groups and carboxylic groups (Halliwell et al., 1995a).

![Chemical structures of the benzoic acid derivatives (A) and of the fatty alcohol esters of benzoic acid derivatives (B) (Modified from Figueroa-Espinoza & Villeneuve, 2005)](image)

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>R₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Hydroxybenzoic acid</td>
<td>H</td>
<td>H</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>H</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
</tr>
<tr>
<td>Gentisic acid</td>
<td>OH</td>
<td>H</td>
<td>H</td>
<td>OH</td>
</tr>
<tr>
<td>Isovanillic acid</td>
<td>H</td>
<td>OH</td>
<td>OCH₃</td>
<td>H</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>H</td>
<td>OH</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>OH</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Syringic acid</td>
<td>H</td>
<td>OCH₃</td>
<td>OH</td>
<td>OCH₃</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>H</td>
<td>OCH₃</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>Veratic acid</td>
<td>H</td>
<td>OCH₃</td>
<td>OCH₃</td>
<td>H</td>
</tr>
</tbody>
</table>

**Figure 3.3.** Chemical structures of the benzoic acid derivatives (A) and of the fatty alcohol esters of benzoic acid derivatives (B) (Modified from Figueroa-Espinoza & Villeneuve, 2005)
Phenolic acids are structurally related to flavonoids and serve as precursors of their biosynthesis. Hydroxyl groups, associated with phenolic compounds, are the most common, and often the most effective free radical scavengers in foods (Decker, 1998), as they may readily donate an electron or the hydrogen to intercept and convert free radicals to a more stable compound (Figueroa-Espinoza & Villeneuve, 2005). Most natural antioxidants, such as those found in plants, are phenolic in nature and they possess varying antioxidant activity. Phenolic antioxidants inhibit lipid oxidation by trapping the peroxyl radical as primary antioxidants and surperoxide anion scavengers (Rajalakshmi & Narasimhan, 1996). This could occur in one of two ways:

\[
\begin{align*}
PH + \text{ROO}^- & \rightarrow \text{ROOH} + \text{P}^+ \quad (20) \\
\text{P}^+ + \text{ROO}^- & \rightarrow \text{ROOP} \quad (21) \\
\text{P}^+ + \text{P}^+ & \rightarrow \text{PP} \quad (22)
\end{align*}
\]

Figure 3.4. Chemical structures of the cinnamic acid derivatives (A) and of the fatty alcohol esters of cinnamic acid derivatives with \( R_4 = H \) (B) and \( R_4 = C_7H_{11}O_5 \) (C) (Modified from Figueroa-Espinoza & Villeneuve, 2005)
PH represents phenolic acids. First, phenolic acids donate hydrogen to peroxyl radical generated by unsaturated fatty acids, converting the phenolic acids to phenolic radicals. Then, phenolic radicals can terminate radical reaction by reacting either with another peroxyl radical or with other phenolic radicals.

3.3.2.5. Antioxidant Mechanisms of Other Antioxidants from Natural Sources

Antioxidants can also be found in many other natural sources. In cellular systems, some enzymes are found to be able to remove either oxygen or oxidative species from biological environment, which is an important form of biological self-defence (Reische et al., 1999). Numerous peptides and proteins from vegetable oils, plants, microorganisms and animals have been found to have antioxidant activities. Most of them behave as primary antioxidants and show radical scavenging activities (Shahidi & Zhong, 2010b). MRPs are compounds arising as a result of cooking. MRPs have shown antioxidant effect in many food systems but the mechanism is still not clear (Reische et al., 1999). PLs are a class of lipids and major component of cell membrane. It was proposed that PLs can act as antioxidants in the mechanisms of synergism between PLs and tocopherols, chelation of pro-oxidant metals by the phosphate groups, formation of Maillard-type products, and action as an oxygen barrier between oil and air interfaces (Hidalgo et al., 2006). In addition, some polysaccharides also act as antioxidants as radical scavenging, reducing power, and metal ion chelation, and induction of gene expression of antioxidant enzymes based on in vitro and in vivo experiments (Shahidi & Zhong, 2010b).

3.4 Application of Antioxidant in Food Products

Most food products enriched with omega-3 fatty acids in the market are emulsified foods. Most of them are o/w emulsions which make the mechanisms of lipid oxidation in emulsions very different and more complex than those in bulk oil systems. Therefore, the efficacy of antioxidants is affected by many factors for the emulsified food products. In addition, inactivation of some type of antioxidants is observed in emulsified food. For example, polar antioxidants like ascorbic acid and ‘Trolox’ are more active in non-polar media than non-polar antioxidants, and vice versa (Jacobsen & Nielsen, 2007). Tocopherol, gallic acid and propyl gallate were found to be ineffective in food systems. This is probably due to their interaction with the emulsifier or due to the fact that these antioxidants are free radical scavengers which cannot prevent metal-catalysed oxidation at the oil-
water interface (Jacobsen et al., 1999; 2001a; 2001b). Effects of different antioxidants in food systems have been investigated (Table 3.3). And as shown in this table, the antioxidation effects in food systems depend very much on the choice of antioxidants and food system. Effects of incorporation of a mixture of antioxidants are currently being studied and it has been postulated to have better performance in food systems.

**Table 3.3.** Examples of antioxidants and their effects in different omega-3 enriched foods (Modified from Jacobsen et al., 2007)

<table>
<thead>
<tr>
<th>Antioxidants</th>
<th>Antioxidant mechanisma</th>
<th>Effect in milk</th>
<th>Effect in mayonnaise</th>
<th>Effect in dressing</th>
<th>Effect in meat patties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propyl gallate</td>
<td>Radical scavenger</td>
<td>-</td>
<td>Weakly prooxidative</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>Radical scavenger</td>
<td>-</td>
<td>Weakly prooxidative</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tocopherol mixture</td>
<td>Radical scavenger</td>
<td>Slightly prooxidative</td>
<td>Moderately antioxidant</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>γ-Tocopherol</td>
<td>Radical scavenger, ( \text{O}_2 ) scavenger, regeneration of tocopherol</td>
<td>-</td>
<td>Strongly prooxidative</td>
<td>-</td>
<td>Slightly antioxidative to prooxidative</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>Regeneration of tocopherol</td>
<td>Strongly antioxidative</td>
<td>Strongly prooxidative</td>
<td>None to weakly prooxidative</td>
<td>-</td>
</tr>
<tr>
<td>Ascorbyl palmitate</td>
<td>Metal chelator</td>
<td>-</td>
<td>None to moderately antioxidative</td>
<td>Strongly prooxidative</td>
<td>-</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>Metal chelator</td>
<td>-</td>
<td>-</td>
<td>Strongly antioxidative</td>
<td>-</td>
</tr>
<tr>
<td>EDTA</td>
<td>Metal chelator</td>
<td>Free radical scavenger, regeneration of tocopherol</td>
<td>Strongly prooxidative</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ascorbic acid, lecithin, tocopherol mixture, sodium erythorbate, sodium citrate, rosemary extract mixture</td>
<td>Free radical scavenger, metal chelator</td>
<td>-</td>
<td>Strongly prooxidative</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

aAble to reduce \( \text{Fe}^{3+} \) to \( \text{Fe}^{2+} \).

- The effect has not been investigated in the omega-3 enriched product.
Chapter 4: Enzymatic Synthesis of Novel Antioxidants in Literature

Antioxidants are frequently applied to prevent oxidation of food and cosmic products containing unsaturated fats and oils. The selection of the most effective antioxidants in a food system are often determined by the prospective properties of the antioxidants, the substrates being oxidized, the location of the antioxidants in the system and the effect of other components on the antioxidant activity (Frankel & Meyer, 2000).

However, most antioxidants from natural sources are hydrophilic compounds which limit their application in aprotic media due to their low solubility. Therefore, it is necessary to develop methods to change the character of natural antioxidants and increase their solubility in aprotic media. One of the realistic ways to achieve this aim is to modify or perform the functional properties of the original antioxidant compounds to obtain amphiphilicity. This can be achieved by manipulating the hydrophilicity/lipophilicity balance (HLB) of the naturally-occurring antioxidants (Shahidi & Zhong, 2010b). For example, carboxylic acid function of phenolic acids can be conjugated with hydrophilic compounds through chemical or enzymatic lipophilization which transform the phenolic acids into amphiphilic molecules and hopefully still retain their original functional properties. Eventually these new molecules will accumulate at o/w or w/o interfaces where oxidation is considered to occur frequently and increasing the oil protection due to their amphiphilic nature (Figueroa-Espinoza & Villeneuve, 2005).

4.1. Enzymatic Synthesis of Lipophilic Antioxidants

Amphiphilic compounds are conventionally prepared by chemical methods. However, chemical methods usually suffer from a number of disadvantages namely the use of less biocompatible chemicals and solvents, formation of by-products and low yields (Yan et al., 1999). Moreover, phenolic acids are heat sensitive and prone to oxidation. The use of high temperature frequently causes a dark colour, burnt taste and high energy consumption (Sun et al., 2008).

Compared to chemical methods, enzymatic methods allow the reaction to be carried out at mild condition and therefore avoiding the risks associated with chemical methods. A thermo-stable immobilized lipase B (EC 3.1.1.3) from Candida Antarctica is a very efficient enzyme. It is a well
characterized catalyst and applicable in diverse reaction (Anderson et al., 1998). This enzyme is adsorbed on a macroporous acrylic resin and presents some advantages. It can be used in batch and column reaction operations and in fixed-bed reactors; its optimum activity temperature is 40-60 °C; and it can be easily recovered from the reaction mix and recycled (Figueroa-Espinoza & Villeneuve, 2005). Furthermore, the catalyst has been found to tolerate a great variation in experimental conditions and catalyzes various organic reactions in immobilized form (Kirk & Christensen, 2002). Table 4.1 summarizes the enzymatic modification of natural antioxidants in recent studies where Candida Antarctica and other enzymes were applied.

| Table 4.1. Enzymatic modification of natural antioxidants from studies in the last ten years |
|-----------------------------------------------|---------------------|------------|----------|------------------|
| Antioxidants | Targeted Compounds | Conditions | Yield (%) | Reference |
| Chlorogenic (40 mM) | Octanol | Solvent free | 60 | Guyot et al., 2000 |
| | Dodecanol | 60 °C | 40 | |
| | Hexadecanol | 30 days | 40 | |
| | 9-octadecen-1-ol | | 40 | |
| Chlorogenic (30 mM) | Octanol | Solvent: 2-methyl-2-propanol | 75 | Guyot et al., 2000 |
| | Dodecanol | 60 °C | 70 | |
| | Hexadecanol | 30 days | 60 | |
| | 9-octadecen-1-ol | | 55 | |
| Ferulic (100 mM) | 1-octanol | Solvent: 2-methyl-2-propanol | 14 | Compton et al., 2000a |
| | | 60 °C, under nitrogen | | |
| | | 30 days | | |
| Ascorbic acid (50 mM) | EPA | Solvent: acetone | 48 | Watanabe et al., 2000 |
| | | 55 °C | | |
| | | 50 h | | |
| Ascorbic acid (100 mM) | retinol | Solvent: Hexane | 90 | Maugard et al., 2000 |
| | | 55 °C | | |
| | | 25 h | | |
| EF<sup>b</sup> | Triolein | Solvent: toluene | 73 | Compton et al., 2000a |
| | | 60 °C | | |
| | | 14 days | | |
| Ferulic (220 µM) | n-pentanol | Solvent: CTAB (100 mM)/hexane/pentanol (0.86 M) Water-in-oil microemulsion | 60 | Giuliani et al., 2001 |
| | | 40 °C, pH 6.0 | | |
| | | 8 h | | |
Table 4.1 (Continue)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent</th>
<th>Conditions</th>
<th>Yield (% w/w)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cinnamic (100 mM)</td>
<td>1-octanol</td>
<td>Solvent free</td>
<td>8-82</td>
<td>Stamatis et al., 2001</td>
</tr>
<tr>
<td>p-coumaric (100 mM)</td>
<td>45 °C</td>
<td>10-25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ferulic (100 mM)</td>
<td>12 days</td>
<td>3-30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-hydroxyphenylpropionic (100 mM)</td>
<td></td>
<td>29-97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl ferulate</td>
<td>Soybean oil</td>
<td>Packed-bed reactor</td>
<td>64</td>
<td>Laszlo et al., 2003</td>
</tr>
<tr>
<td></td>
<td>60 °C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>140 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cinnamic</td>
<td>1-propanol</td>
<td>Solvent: n-hexane/1-propanol/water microemulsions</td>
<td>Traces</td>
<td>Topakas et al., 2003</td>
</tr>
<tr>
<td>p-coumaric</td>
<td>30 °C</td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ferulic</td>
<td>9 days</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-hydroxyphenylpropionic</td>
<td></td>
<td>75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascorbic acid (57 mM)</td>
<td>Palmitic acid</td>
<td>Solvent: tert-amyl alcohol</td>
<td>86</td>
<td>Viklund et al., 2003</td>
</tr>
<tr>
<td></td>
<td>Oleic acid</td>
<td>60/65 °C</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascorbic acid (200 mM)</td>
<td>Dodecanoic acid</td>
<td>Pack-bed reactor</td>
<td>59</td>
<td>Watanabe et al., 2003</td>
</tr>
<tr>
<td></td>
<td>50 °C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascorbic acid (200 mM)</td>
<td>Palmitic acid</td>
<td>Solvent: Ionic liquids</td>
<td>81</td>
<td>Park et al., 2003</td>
</tr>
<tr>
<td></td>
<td>Oleic acid</td>
<td>60 °C</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>25 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gallic (50 mM)</td>
<td>1-propanol</td>
<td>Organic solvent</td>
<td>0-44</td>
<td>Yu et al., 2004</td>
</tr>
<tr>
<td></td>
<td>40 °C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHCAa (5 mM)</td>
<td>Linoleyl alcohol</td>
<td>Solvent: hexane/2-butanone</td>
<td>76</td>
<td>Sabally et al., 2005b</td>
</tr>
<tr>
<td>Ferulic (5 mM)</td>
<td>55 °C</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>16 days</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caffeic (4 mM)</td>
<td>triolein</td>
<td>Solvent: hexane/2-butanone</td>
<td>38</td>
<td>Karboune et al., 2005</td>
</tr>
<tr>
<td></td>
<td>55 °C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>16 days</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHCAa (5 mM)</td>
<td>Trilinolein</td>
<td>Solvent: hexane/2-butanone</td>
<td>66</td>
<td>Sabally et al., 2006a</td>
</tr>
<tr>
<td>Ferulic acid (5 mM)</td>
<td>55 °C</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14 days</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHCAa (5 mM)</td>
<td>Flaxseed oil</td>
<td>Solvent: hexane/2-butanone</td>
<td>83</td>
<td>Sabally et al., 2006b</td>
</tr>
<tr>
<td></td>
<td>55 °C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 days</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 4.1 (Contiune)

<table>
<thead>
<tr>
<th>Phenolic (50 mM):</th>
<th>Octanol</th>
<th>Solvent: Ionic liquids</th>
<th>Lipase</th>
<th>Katsoura et al., 2009</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferulic</td>
<td></td>
<td>60 °C 72 h</td>
<td>Lipase CalB/RML</td>
<td>56/59</td>
</tr>
<tr>
<td>Cinnamic</td>
<td></td>
<td></td>
<td>56/54</td>
<td></td>
</tr>
<tr>
<td>Coumaric</td>
<td></td>
<td></td>
<td>27/33</td>
<td></td>
</tr>
<tr>
<td>Caffeic</td>
<td></td>
<td></td>
<td>8/12</td>
<td></td>
</tr>
<tr>
<td>Sinapic</td>
<td></td>
<td></td>
<td>0.4/31</td>
<td></td>
</tr>
<tr>
<td>p-hydroxyphenyl acetic</td>
<td></td>
<td></td>
<td>63/13</td>
<td></td>
</tr>
<tr>
<td>p-hydroxyphenyl propionic</td>
<td></td>
<td></td>
<td>61/39</td>
<td></td>
</tr>
<tr>
<td>2,4-dihydroxyhydro cinnamic</td>
<td></td>
<td></td>
<td>23/28</td>
<td></td>
</tr>
<tr>
<td>3,4-dihydroxyhydro cinnamic</td>
<td></td>
<td></td>
<td>36/30</td>
<td></td>
</tr>
</tbody>
</table>

| p-hydroxyphenyl acetic (4 mM) | Triolein | Solvent: hexane/2-butanone | 62 | Safari et al., 2006 |
| p-coumaric (4 mM) | Fish oil | Solvent: hexane/2-butanone | 65 | Sabally et al., 2007 |
| Sinapic (4 mM) | Fish oil | Solvent: hexane/2-butanone | 10 | Sabally et al., 2007 |
| Ferulic (4 mM) | Fish oil | Solvent: hexane/2-butanone | 2 | Sabally et al., 2007 |
| 3,4-dihydroxybenzoic (4 mM) | Fish oil | Solvent: hexane/2-butanone | 2 | Sabally et al., 2007 |

| DHCA\(^a\) (5 mM) | Fish oil | Solvent: hexane/2-butanone | 46 | Safari et al., 2006 |
| Ethyl ferulare (167 mM) | Tributyrin | Solvent: toluene | 81 | Zheng et al., 2008a |
| EF\(^b\) (0.5 M) | tributyrin | Solvent: Hexane, toluene, 2-methyl-2-butanol | 94 | Zheng et al., 2008b |
| Ascorbic acid (200 mM) | Oleic acid | Solvent: Ionic liquids | 85 | Adamczak & Bornscheuer, 2009 |
| EF\(^b\) (167 mM) | Tributyrin | Selective organic solvents | 74 | Zheng et al., 2009a |
| EF\(^b\) (385 mM) | Triolein | Solvent free | 74 | Zheng et al., 2009b |
| EF\(^b\) (0.5 M) | Triolein | Solvent free | 57.9 | Xin et al., 2009 |

\(^a\)DHCA: Dihydrocaffeic acid; \(^b\)EF: Ethyl ferulate; \(^c\)CalB/RML: *Candida Antarctica* lipase B/Lipozyme RM IM.
4.2. Synthesis of Lipophilic Phenolic Esters

Several groups have been working on the esterification of phenolic acids with fatty alcohol or TAG by using Novozym 435 as a catalyst (Guyot et al., 2000; Sabally et al., 2006a; b; Compton et al., 2000b; 2006; Sun et al., 2009; Zheng et al., 2008a; b; Zheng et al., 2009a; b). Guyot et al. (2000) synthesized some phenolic esters by direct esterification of fatty alcohols. They reported that no esterification of caffeic acid occurred and only traces of esterified ferulic acid were obtained when n-butanol was used as a reaction media after 15 days of reaction. Higher esterification yields (55-75% depending on the chain lengths of fatty alcohols) were obtained when chlorogenic acid was used, and these yields are also higher than those obtained in a solvent-free medium. They further concluded that ferulic acid esterification was only possible with alcohols containing more than eight carbons in a low yield; esterification was possible in the cinamic series when the aromatic cycle was not parahydroxylated (cinnamic and 3,4-dimethoxycinnamic acids), and when the side chain was saturated (DHCA), parahydroxylation had no effect; the simultaneous presence of a double bond on the side chain conjugated with the cycle containing a p-hydroxyl group inhibits the Candida Antarctica lipase. On the other hand, studies found esterification efficient of phenolic acid can be significantly increased in ionic liquid system, which increase the solubility of phenolic acids greatly (Katsoura et al., 2009).

Structured lipids (SLs) refer to the TAG in which the composition and the distribution of fatty acids at the glycerol backbone are specified (Akoh & Yee, 1998). There has been an increased interest in the production of SLs that possess various enrichment levels of selected fatty acids for their benefits in clinical, nutritional and therapeutic purposes. Several strategies have been reported to synthesis SLs using different phenolic acids. Sabally et al. (2006a; b) reported a synthesize methods for acidolysis of DHCA with trilinolein, trilinoleinin or flaxseed oil by using a mixture solvent of hexane/2-butanone. Compton et al. (2000C; 2006) developed a method for quantification of ferulate lipids species and synthesized ferulate lipids using EF with triolein or vegetable oil through packed bed reactor. More recently, Sun et al. (2008; 2009) and Zheng et al. (2008a; b; 2009a; b) reported synthesis of EF with triolein either in solvent free condition or in selected solvent, and optimised reaction conditions by response surface modelling (RSM).
4.3. Enzymatic Synthesis Lipophilic Ascorbic Esters

Similar to phenolic acids, ascorbic acid is also an antioxidant from nature. Even though it had been proved to be effective in preventing oxidation in many systems, its hydrophilic property limits its application in cosmetics and in the presence of fats and oils. In the last ten years, there have been several studies aimed at synthesizing amphiphilic ascorbic molecules in different systems with different carbon chains of fatty acids, which resulted in various esterification efficient depending on type of fatty acids and systems (Yan et al., 1999; Maugard et al., 2000; Watanabe et al., 2000; 2003; Park et al., 2003; Viklund et al., 2003; Adamczak & Bornscheuer, 2009). It was found that such ascorbyl esters of fatty acids, especially ascorbyl palmitate and ascorbyl oleate, are very popular and active antioxidants (Adamczak & Bornscheuer, 2009).
Chapter 5: Experimental and Analytical Methodology

According to previous studies, the main barrier in synthesis of lipophilic phenolic esters is the low solubility of phenolic acids in most organic solvents, which resulted in long reaction time and low productivity. In the present study, selected phenolic acids (DHCA, ferulic, and caffeic acids) were conjugated either with fatty alcohols or TAG to produce lipophilic antioxidants. Several strategies for synthesis were evaluated and discussed in this thesis.

5.1. Enzymatic Synthesis of Lipophilic Fatty Alcohol Phenolic Esters

As mentioned above, HLB refers to the hydrophilicity/lipophilicity balance. The HLB is described by a number, which gives an indication of the overall affinity of an emulsifier for the oil and aqueous phases (Hiemenz & Rejogopolan, 1996). Each molecule can be assigned an HLB number according to the type of hydrophilic and lipophilic groups it contains or according to the experimental measurements of its cloud point. The HLB number indicates the solubility of a molecule in the oil and water phase, which means a molecule with a high HLB number has a high ratio of hydrophilic groups to lipophilic groups, and vice versa. Therefore, the molecules with high HLB number are more easily dissolved in the water phase, and those with low HLB number are more easily dissolved in the oil phase (McClements, 1999).

Conjugating phenolic acid with fatty alcohols in different carbon chains or different TAG can produce novel compounds with different HLB number because each HLB number varies according to chain length of fatty alcohol or fatty acids species on the backbone of TAG. The products can provide a better prevention toward lipid oxidation in specific emulsion systems in food, pharmacy and cosmic industry.

5.1.1. Enzymatic Esterification of Fatty Alcohol Phenolic Esters in Organic Solvents (Paper I)

It was said that there are several advantages to conduct enzymatic synthesis using lipase in non-aqueous organic solvents, such as increasing the solubility of non-polar substrates, shifting the
reaction to the direction of favor synthesis over hydrolysis, and recovering the reaction products easily by evaporation (Hsieh et al., 2006).

The enzymatic esterification of selected phenolic acids (DHCA, ferulic and caffeic acids) with different fatty alcohols was conducted in a binary organic solvent system which had been reported before (Sabally et al., 2005a) with some modifications. Fatty alcohols with different carbon chains from C4 to C18:1 were chosen for evaluation in this system. The solvent system is composed of hexane and 2-butanone. Hexane is a hydrophobic organic solvent, which can dissolve hydrophobic substrate (fatty alcohols or oils) and provide suitable reaction environment for enzyme Novozym 435. It was found that Novozym 435 performed better in a hydrophobic environment (Lue et al., 2005). 2-Butanone can provide suitable solubility to most of phenolic acids on the other hand.

Prior to the experiments, phenolic acids were dissolved in 2-butanone, and prepared as stock solution first. However, due to the differences of solubility of different phenolic acids in the solvent, the concentrations of DHCA, ferulic and caffeic acids in stock were 500 mM, 250 mM, and 50 mM respectively. The final concentrations of these three compounds in the reaction system were 50 mM (DHCA), 50 mM (ferulic acid), and 5 mM (caffeic acid) by diluting with hexane to a desired reaction volume ratio (hexane/2-butanone, v/v). The total reaction volume was 10 ml. Reactions conducted in bottles, which are equipped with a screw cap to prevent evaporation of the organic solvents. For the preparative experiments, which investigated the proper reaction volume between hexane and 2-butanone and effects of chain length of fatty alcohols in the enzymatic esterification, reactions were conducted at 60 ºC in the presence of 100 mg enzyme and 100 mg molecular sieves (3 Å). For the purpose of optimizing reaction parameters and increasing production of lipophilic phenolic esters, a three-lever four-factor central composite face centred design (CCF) with three star points was employed based on the esterification of DHCA with octanol. The variables and their levels selected for the study were reaction time (1, 4 and 7 days), temperature (40, 55 and 70 ºC), enzyme load (2, 11 and 20% relative to the total weight of substrates) and molar ratio of octanol/DHCA (1:1, 3:1 and 5:1). Each level was represented as -1, 0, and 1. The whole experiment was designed by software Modde 8.0 (Umetrics AB, Umeå, Sweden) based on above factors (the four variables) and response factor (conversion of DHCA). All the experiments were carried out in the binary system where hexane and butanone were mixed in the volume ratio of 65:35. 100 mg of molecular sieves was also added in each experiment. The reaction products were analyzed by high
performance liquid chromatography (HPLC) after removing any solid by centrifuge. For the details on the experimental designs please refer to Paper I in the appendices section.

5.1.2. Enzymatic Esterification Octyl Phenolic Esters in ILs/octanol Binary System (Paper II)

ILs are an interesting class of tuneable, designer solvents with essentially zero volatility (Eastoe \textit{et al.}, 2005). ILs can act as solvents for divers reactions and give different selectivity and reactivity compared with conventional organic solvents because the liquid is made of ions rather than molecules (Qiu & Texter, 2008). Over the past 10 years, ILs have continued to attract attention as a replacement for organic solvents for enzymatic transformation of various compounds (Katsoura \textit{et al.}, 2006).

Besides the advantages mentioned above, it was found ILs have very good solubility towards most of phenolic acids, which can improve the productivity of lipophilic phenolic esters greatly. In this part of study, Methyltrioctylammonium Trifluoroacetate (tOMA·TFA) was chosen as inter-media to increase the solubility of phenolic acids. It has been found tOMA·TFA is a strong solvent. It can increase the conversion of ascorbic acid by increasing its solubility (Chen \textit{et al.}, 2008). Therefore, it is expected that this ionic liquid can increase conversion to phenolic acids, which are similar compounds to ascorbic acid in physical properties.

For a standard reaction system, 1 mmol DHCA was dissolved in 0.5 µl tOMA·TFA first, and then mixed with 2 ml of octanol. Reactions were initiated by adding 100 mg Novozym 435 at 80 °C with or without molecular sieves (3 Å). To investigate the effect of mixing volume between ILs and octanol, volume of octanol was increased from 2 ml to 8 ml, and the volume ratio of IL/octanol was changed from 1:4 to 1:16 correspondingly. To investigate the effect of temperature, the reaction temperature was varied from 60 to 80 °C in the system where the mixing volume ratio between IL/octanol was 1:4. Above reactions were conducted without adding any molecular sieves. For the purpose of studying effect of removing water from the reaction, both molecular sieves and vacuum were applied to compare with the reaction without adding molecular sieves or applying vacuum in the system where IL/octanol was mixed in the ratio of 1:4 (v/v). For the reactions where molecular sieves were added, to one batch was added 100 mg molecular sieves and to another batch was also added 100 mg molecular sieves, in addition 50 mg more were added each day until reactions were stopped. For the reactions applied with vacuum, two batches were also set up: one batch was
conducted under 800 mbar, another batch was conducted under 400 mbar. Effect of DHCA concentration on conversion was studied by varying amount of DHCA from 1 mmol to 4 mmol in the system where IL/octanol was mixed in the ratio of 1:4 (v/v). Parallel reactions, by changing concentration of DHCA, were also conducted at the same time in the system where no ILs was added. At last, esterifications of ferulic and caffèic acids with octanol were studied in the system where IL/octanol was mixed in the volume ratio of 1:4. Parallel experiments without ILs were also conducted with these two phenolic acids under the same conditions. All products from the reactions were analyzed by HPLC and HPLC- electrospray tandem (ESI)-mass spectrometry (MS). For details of the experiments, please refer to Paper II in the appendices.

5.2. Enzymatic Synthesis Esters of Phenolic with TAG by Transesterification

As mentioned above, SLs refer to the TAG in which the composition and the distribution of fatty acids at the glycerol backbone are specified (Xu, 2000). There is an increasing interest in production of SLs because of its potential of nutritional and functional benefits have been reported (Akoh & Huang, 1995; Akoh et al., 1995; Halldorsson et al., 2003). Incorporation of phenolic acids into TAG can produce novel SLs. Using enzyme with the specificity of sn-1,3, it is possible to designate phenolic acids on the positions of sn-1 or sn-3 on the backbone of glycerol, which will produce SLs with rich contents of PUFAs because most of PUFAs locate at position sn-2 on the backbone of glycerol in fish oils. Such novel molecules may offer numerous combined beneficial properties of both PUFAs and phenolic compounds, which mean they can both be used as nutritional ingredients and novel antioxidants (Karboune et al., 2008).

5.2.1. Improved Enzymatic Production of Phenolated Glycerides through Alkyl Phenolate Intermediate (Paper III)

In this work, a two-step approach for enzymatic synthesis of phenolated glycerides with DHCA as a model penolic acid was set up. An intermediate product octyl dihydrocaffeate was first synthesized by lipase catalyzed esterification of DHCA with octanol according to the method described in section 5.1.1 in large scale. DHCA was firstly dissolved in butanone as 500 mM stock solution, and then was diluted by hexane with the volume ratio of 65/35 (hexane/butanone, v/v) and final DHCA concentration was 50mM. Octanol was added into the binary solvent mixture afterward at a
concentration of 150 mM. Around 200 ml substrate solution was added to a 500 ml flask and thermostated at 60 °C by waterbath with magnetic stirring at 300 rpm. The reaction was initiated by adding 2 g Novozym 435 and 2 g activated molecular sieves (3Å). The reaction was conducted for 7 days to reach maximal bioconversion, and then the reaction was terminated through filtration for removing enzyme and molecular sieves. The resulting product was re-dissolved in hexane and washed with salt water (0.5 M NaCl) 5 times to remove the remaining DHCA. The obtained octyl dihydrocaffeate was found to be around 97% purity (containing 3% DHCA) according to HPLC analysis. After purification, product octyl dihydrocaffeate was used as an intermediate to react with tricaprylin and produce SLs incorporated with phenolic acids.

Interesterification of octyl dihydrocaffeate with tricaprylin was conducted in a jacketed reactor thermostated by circulated water. For a typical reaction, 0.154 g octyl dihydrocaffeate (0.5 mmol) was mixed with 0.705 g tricaprylin (1 mmol). Interesterification reaction was initiated by adding 10% of immobilized enzyme (on the mass basis of octyl dihydrocaffeate) at 60 °C and stirred (300 rpm) by a magnetic plate. Sample aliquots from the reaction mixture were periodically withdrawn and diluted 100 times by methanol, then subjected to HPLC analysis after centrifuged (12,000 rpm for 10 min) to remove solid impurity.

To examine the effects of substrate form and solvent property and compare with above solvent-free transesterification of octyl dihydrocaffeate with tricaprylin, two other types of reactions: solvent-free direct transesterification of DHCA with tricaprylin and hexane mediated transesterification of octyl dihydrocaffeate with tricaprylin were conducted under other identical conditions, respectively. These two systems were set up as described below: for the former system, 0.5 mmol DHCA was reacted with 1 mmol tricaprylin and for the latter system 0.5 mmol octyl dihydrocaffeate with 1 mmol tricaprylin in 10 ml hexane, with the same agitation rate, reaction temperature and enzyme dosage as for the solvent-free transesterification of octyl dihydrocaffeate with tricaprylin.

Similar to the experiments set up in section 5.1.1, a three-lever four-factor CCF with three star points generated by software Modde 8.0 was employed. Four factors were chosen for investigation: temperature (40, 55 and 60 °C), molar ratio of tricaprylin/octyl dihydrocaffeate (1/1, 3/1, 5/1), reaction time (0.5, 24.25 and 48 h) and enzyme load (50, 150 and 250 mg). The 3 levels of each parameter, in an increasing order, were coded as -1, 0 and 1, respectively. Details of experiments are described in Paper III.
5.2.2. Enzymatic Transesterification of EF with Fish Oils (Paper IV)

As mentioned above, incorporating phenolic acid into oil enriched with PUFAs can result in a novel molecule with both nutrimental and antioxidant benefits. In this study, fatty acids on the backbone of glycerol of fish oils were replace by ferulate through enzymatic transesterification between EF and fish oil. One of the greatest challenges in synthesis phenolic fish oil is the multiple fatty acid profiles of fish oil, which makes it difficult to quantify the reaction products. Base on previous study (Compton et al., 2006), a qualification method was first developed by comparing products from trasesterification of EF and triolein and identifying by HPLC and HPLC-ESI-MS. For a standard reaction, transesterification of EF with fish oil was conducted in the molar ratio of 1:2 at 60 °C catalyzed by Novozym 435. Effect of glycerol on production of ferulate fish oil was investigated by various amount of glycerol from 0 to 5 mmol in reactions. RSM was used to study the effects of reaction time, temperature, enzyme load, and substrate amount ratio. A three-level and four-factor CCF was employed in this study. The variables and their levels selected for the study were reaction time (1, 3 and 5 days), temperature (40, 55 and 70 ºC), enzyme load (2, 11 and 20; wt% based on the total weight of substrates) and amount ratio between fish oil and EF (1, 3 and 5). Details of experimental designs please refer to Paper IV.

5.3. HPLC Analysis

There are several methods which can be applied for the analysis of phenolic acids, such as thin-layer chromatography (TLC), gas chromatography (GC), HPLC, capillary electrophoretic and capillary electrochromatographic methods, and spectrophotometric detection (Stalikas, 2007). Among these methods, HPLC is the most common method that dominated the separation and characterization of phenolic compounds because it enables us to acquire spectra for each peak and calculate the absorbance maximum within a specified wavelength (Amarowicz & Weidner, 2001). Phenolic acids and their derivatives have UV absorption in the range of 200 to 360 nm due to their benzoic acid carbon framework (Robbins, 2003), which make it convenient to identify them by a HPLC instrument with UV detector.

Reaction products were regularly withdrawn. For the samples from production of fatty alcohol esters (Paper I and Paper II), withdrawn samples were diluted 100 times with methanol. For the samples from production of phenolic TAG (Paper III and Paper IV), withdrawn samples were
diluted with acetone first and then further diluted with methanol. All samples were then subjected to 
HPLC analysis after removing impurities by centrifugation. Different HPLC gradient were 
developed to quantify reaction products. For details of the HPLC methods please refer to the papers 
in the appendices.

5.4. HPLC-MS Analysis

HPLC can help to identify or quantify products by separating the components from the reaction 
mixture. But the chromatography of HPLC cannot provide detailed information on the components 
of a mixture without being compared to the retention time of reference materials even though each 
component is able to be separated completely. Even with the help of reference materials, it is still 
possible to make errors on identification because some components may have the same or similar 
retention time under identical experiment conditions. In such case, one cannot identify two 
components with absolute certainty (Ardrey, 2010). In this study, it is more difficult to get reference 
materials to help with identification because the products from the experiments are new products 
and not available in market.

MS is an analytical technique which can help researchers to measure mass of a component over 
charge ratios of molecules (m/z). The combinations of chromatography and MS have attracted 
much interest over the last forty years or so. It is an ideal tool for analysis of samples with a high 
degree of complexity as in e.g. biological samples and environmental samples. The advantage of 
using MS include high selectivity, high sensitivity, structure elucidation, and easy to manipulate. 
Therefore, technique that combines HPLC with MS can provide information with a high degree a 
confidence.

HPLC-ESI-MS was conducted using the identical separating conditions of HPLC analysis to further 
identify the synthesized products. Please refer to the papers in the appendix for the conditions of 
HPLC-ESI-MS.

5.5. Experiments Design and Data Analysis by RSM

RSM is a collection of statistical design and numerical optimization techniques used to optimize 
process and product designs (Myers et al., 2004). This methodology is based on the fit of a
polynomial equation to the experimental data and well applied when a response or set of responses of interest are influenced by several variables to achieve the goal of optimizing the level of these variables and attaining the best system performance (Bezerra et al., 2008). Optimization using RSM is very useful for multivariate systems when there are several factors studied. Advantages of using RSM include providing a statistical mathematical model between responses and variables, combined with software to make it easier to generate plots response surfaces and contour plots to characterize the shape of the surface and to locate the optimum, and performing evaluation on the significance of variables (Xu, 2002). When RSM was applied in the study, the software Modde 8.0 (Umetrics AB, Umeå, Sweden) was used to analyze the main effects and interactions base on a three-level four-factor experiment.

5.6. Reaction Systems Characterization (Appendix 5)

Frequently, reagent incompatibility is a major problem when synthesizing a new molecule by using reaction species in different physical properties. There are different ways to solve the problem of incompatibility between the reaction species, and one of the most common approaches is to find a solvent or a solvent combination capable of dissolving both the lipophilic and hydrophilic species, such as the binary solvent system described in Paper I. However, this solution is not useful sometime because many polar solvents are toxic, high cost, and suffering from problems in removing (Holmberg, 2007).

In recent year, there is a growing interest in using micro-emulsions as reaction systems. Micro-emulsions are excellent solvents both for hydrophobic and hydrophilic compounds because they are thermodynamically stable mixtures of hydrophobic and hydrophilic media. One of the obvious advantages of using micro-emulsions, as reaction media, is that they can solubilise a very broad spectrum of substances in one single formulation and therefore can overcome reagent incompatibility, enhance reaction rate, and induce region selectivity (Holmberg, 2007).

Micro-emulsions are normally composed of two phases. The first phase is a liquid phase which disperses in a second phase in form of droplets (Sole et al., 2006). It is macroscopically homogeneous but microscopically heterogeneous. Therefore, they are transparent or slightly opaque in appearance (Holmberg, 2007). If droplet sizes are in the range of 20-500 nm then the emulsion
systems can be claimed as nano-emulsions (Fernandez et al., 2004), and can also claim to be nano-reactor if such systems are used as reaction media.

rOMA·TFA is a water like molecule. A nonaqueous ILs/oil micro-emulsions can be formed when rOMA·TFA is mixed with oil or fatty alcohols. Because of strong solubility of this ionic liquid towards phenolic acids, such system may improve the productivity of lipipholic phenolic esters.

In this part of the study, three different reaction systems were developed, which are phenolic acids (DHCA and ferulic acid) in IL/octanol, rutin in IL/linoleic, ferulic acid in IL/triolein respectively. Due to the low solubility of phenolic acids and rutin in hydrophobic media, it is possible that emulsions similar to w/o microemulsion will be formed in these systems. To investigate and explore the inner properties of these systems, the below listed techniques were applied.

5.6.1. Measurement with Dynamic Light Scattering (DLS)

DLS is also known as Photon Correlation Spectroscopy. This technique is one of the most popular methods used to determine the size of particles. The principle of this technique is base on wavelength of a monochromatic light, such as a laser, which will be changed when the light hits the moving spherical particles in a solution (Sartor, 2011). This change is related to the size of the particle and can be reflected by the below equation:

\[
D = \frac{K_B T}{6 \pi \eta a}
\]

where D is the diffusion constant, a is the radius of the particles in samples, \( K_B \) is the Boltzmann constant, T is the temperature in Kelvin degrees, and \( \eta \) is the viscosity of the solvent. Base on this equation, one can calculate particle sizes (a) after measuring the diffusion constant (D) by DLS instrument.

Please refer to Appendix 5 for the details of the measurement.
5.6.2. Measurement with Small Angle X-ray Scattering (SAXS)

Besides DLS, there are also many other methods which can be used to characterize porosity and pore size distribution. Among these techniques, small angling X-ray scattering is one of the powerful tools, which permit detection of a wide range of pore size in a single run. SAXS is a technique that allows the study of structural features of colloids size (Kratry, 1982). The basic principle of SAXS is to scatter X-ray photons elastically off molecules in solution and to record the scattering intensity as a function of the scattering angle (Lipfert & Doniach, 2007). This technique was discovered at the beginning of 20th century based on a series of discoveries, and it has quickly become a powerful tool which can give direct insight into the structure of the materials that caused the scattering. Today, this technique together with scattering from neutrons and light is used by scientists in many different disciplines to study a vast range of materials ranging from polymers to proteins (Polymer and Nanomaterials Characterization Homepage, 2011).

For the details of SAXS measurement please refer to Appendix 5.
Chapter 6: Overall Study Evaluations and Discussions

This chapter is divided into five sections, which gives a brief description and discussion of the main findings obtained from the experiments on enzymatic synthesis fatty alcohol phenolic esters in organic solvents system and ILs intermedia system, enzymatic synthesis phenolic tricaprylin, enzymatic synthesis ferulate fish oils, and characterization of several systems with DLS and SAXS techniques respectively.

6.1. Enzymatic Esterification of Fatty Alcohol Phenolic Esters in Organic Solvents (Paper I)

Enzymatic esterifications of selected phenolic acids with fatty alcohols were conducted in a binary organic solvent system using a method previously reported with some modification (Sabally et al., 2005a). To evaluate the effect of chain lengths of fatty alcohols on esterification in this binary organic solvent system, fatty alcohols with different chain length (from C4 to C18:1) were selected to conjugate with DHCA. The results showed that higher conversion of DHCA was achieved when conjugated with medium chain fatty alcohols (hexanol > dodecanol > octanol > decanol). Relatively lower conversion of DHCA was found when short chain (butanol) and long chain (octadecenol) fatty alcohol was applied.

Novozym 435 was used as a catalyst in this study. It was reported that this enzyme has better activity in a hydrophobic environment (Buisman et al., 1998). However, phenolic acids do not have good solubility in hydrophobic organic solvents. Therefore, hexane which has a low polarity index (0.1) was mixed with 2-butanone which has a higher polarity index (4.7) to facilitate solubility of phenolic acids in order to achieve higher conversion. Solvents ratio (hexane/butanone) was varied from 85/15 to 45/55 to determine how it influenced the enzyme activity. Very low conversion was obtained when hexane and butanone were mixed in the volume ratio of 85/15 (v/v). The reaction rate of Novozym 435 was increased to almost 30 times when hexane and butanone were mixed in the volume ratio of 75/25 (v/v). Increasing volume of 2-butanone has helped to improve the mass transfer between substrates and overall reaction rate. The conversion rates continued to increase when the proportion of hexane was further decreased to 65%. Nevertheless, enzyme activity and conversion of DHCA gradually decreased as the proportion of hexane in the solvent system was
decreased after this level (65%). These results confirmed that both polarity of the solvent systems and solubility of phenolic acids play important role in increasing yield of lipophilic phenolic esters.

Several medium chain fatty alcohols (hexanol, octanol, and dodecanol) were chosen to conjugate with ferulic and caffeic acids, respectively. However, no significant improvement on conversion of these two phenolic acids was observed. The highest conversions of ferulic acid and caffeic acid with hexanol were only 5% and 20%, respectively. In addition, the conversions were even lower when reacted with longer fatty alcohols namely octanol and dodecanol.

Using esterification of octanol with DHCA as a model and optimum solvent volume ratio (hexane/butanone, 65/35), RSM was applied to optimize the temperature, enzyme load, reaction time, and substrate molar ratio (octanol/DHCA) for maximal conversion of DHCA. A set of 27 experiments were suggested and a secondary polynomial model was generated by RSM.

The relationship between each response factor can be clearly interpreted from the response contour plots (Figure 6.1) which were constructed based on the second polynomial model generated by RSM.

Figure 6.1A depicts the mutual effects of temperature and substrate molar ratio (octanol/DHCA) on bioconversion by fixing the reaction duration at four days and enzyme load at 150 mg. As shown in this figure, temperature plays a more important role on the bioconversion of ester product than substrate molar ratio (octanol/DHCA). When reaction temperature was below 60 °C, the bioconversion increased up to only 10% by increasing the substrate molar ratio (octanol/DHCA) to above four. However, there were mutually positive effects when reaction temperature was increased above 60 °C. It was predicted that the highest bioconversion of ester product can reach 99.6% if the temperature was increased to 70°C and substrate molar ratio (octanol/DHCA) above four under the reaction conditions mentioned above.

Figure 6.1B shows the mutual interaction of reaction time and temperature on bioconversion of ester product when the substrate molar ratio (octanol/DHCA) was fixed at three and enzyme load was fixed at 150 mg. It was observed that there was a linear relationship between reaction time and bioconversion. The bioconversion increased with reaction time. On the other hand, temperature had no effect on the bioconversion if the reaction was carried out for a shorter period less than five days.
The maximal bioconversion (around 99%) was predicted to be achieved when reaction temperature was increased above 65 °C after five days of reaction.

Figure 6.1C depicts the mutual effect of temperature and enzyme load on bioconversion of ester product whereby the reaction duration was fixed at four days and substrate molar ratio was fixed at (octanol/DHCA) of 3. The pattern of this graph is quite similar as that of Figure 2B. It indicates that there is a linear relationship between enzyme load and bioconversion. When the enzyme load is less than 160 mg, the bioconversion cannot be increased further if reaction temperature is less than 45 °C. Another conclusion that can be drawn from this graph is temperature has no effect on bioconversion if enzyme load is low (less than 160 mg), but a mutually positive effect of enzyme load and temperature was found when enzyme load was increased to above 160 mg in the reaction system. In short, the maximal bioconversion can be obtained when the reaction temperature was increased above 65 °C and more than 200 mg of enzyme was supplied at the same time.

Figure 6.1D denotes effects of substrate molar ratio (octanol/DHCA) and reaction time on bioconversion when enzyme load was fixed at 150 mg and temperature was fixed at 55 °C. Similar to the pattern of Figure 2B and 2C, the bioconversion of ester product cannot be increased if reaction time was less than four days. Bioconversion can be slightly increased by both increasing substrate molar ratio and reaction time afterwards. It was predicted that maximal bioconversion (about 89%) can be achieved when substrate molar ratio was four after six days of reaction.

Figure 6.1E shows effects of enzyme load and reaction time on bioconversion of ester product at reaction temperature of 55 °C and substrate molar ratio of 3. These two factors have a mutually positive effect on the bioconversion. Bioconversion can be increased with both enzyme load and reaction time. It is worth noting that increasing only one of these two factors resulted in only minor increment in the bioconversion. It was predicted above 80% of bioconversion can be achieved when enzyme load was increased to 200 mg after four days of reaction.

According to the experiment and model, the optimum conditions suggested by the model were a reaction temperature 60 °C, reaction time of 7 days, enzyme load of 100 mg, and substrate molar ratio at 4.34 (octanol/DHCA). Based on these optimum conditions, 92.475 conversion of DHCA was achieved. There was an agreement between prediction value and experimental value which indicated the validity of the model.
Figure 6.1. Response contour plots between any two parameters for bioconversion of octanol dihydrocaffoylated: (A) mutual effects of temperature and substrate molar ratio (octanol/DHCA) at 150 mg of enzyme load and four days of reaction; (B) mutual effects of reaction time and temperature at 150 mg of enzyme load and 3/1 of substrate molar ratio (octanol/DHCA); (C) mutual effects of temperature and enzyme load at four days of reaction and 3/1 of substrate molar ratio (octanol/DHCA); (D) mutual effects of substrate molar ratio (octanol/DHCA) and reaction time at 55°C and 150 mg of enzyme load; (E) mutual effects of enzyme load and reaction time at 55°C and 3/1 of substrate molar ratio (octanol/DHCA).
6.2. Enzymatic Esterification Octyl Phenolic Esters in IL/octanol Binary System (Paper II)

In this study, tOMA·TFA was applied as assisting solvent to improve solubility of phenolic acids. tOMA·TFA was firstly mixed with octanol in different volume ratios ranging from 1:4 to 1:16 (IL/octetanol). The conversion of DHCA was firstly increased from 60% to 70% by increasing the volume ratio from 1:4 to 1:12, and then decreased slightly with further increment of volume ratio to 1:16.

As Novozym 435 is a thermostable enzyme, it is possible to conduct the reactions in a relatively high temperature. The preliminary experiments were conducted at 80 °C with 60% yield in 5 days. Enzyme was found to be still stable after 5 days of reaction. Approximately 70% conversion rate was achieved in 5 days when the reactions were conducted at 70 °C as compared to only 50% in 5 days when reactions were conducted at 60 °C.

Water is a by-product of the reaction, which may have negative effect on production of desired products. Water was removed either by molecular sieves or vacuum. There is significant improvement on conversion when water was removed by either of these two methods. There was no difference in conversion when water was removed at different vacuum pressure (800 mbar or 400 mbar). Addition of molecular sieves to remove water had better performance than using vacuum. Stepwise addition of molecular sieves (50 mg fresh molecular sieves each day) throughout the reaction showed improvement in terms of conversion.

The effects of concentration of DHCA on enzyme activity were also studied by varying the concentration of DHCA from 0.4 M to 1.6 M. The reactions were conducted in a system composed of IL/octanol in the ratio of 1:4 (v/v). It was observed the reaction rate increased as the concentration of DHCA was increased. Parallel experiments were also conducted in octanol without IL with fixed DHCA concentration of 0.4M. The initial reaction rate was 178 mM h⁻¹ (g enzyme)⁻¹ in the system without IL and concentration of DHCA was 0.4 M. This reaction rate was much higher than the parallel reaction which was also conducted with 0.4 M of DHCA in IL/octanol system. This indicates tOMA·TFA may have an inhibitive effect on enzyme. However, when concentration of DHCA was increased to 1.6 M, the reaction rate was 361 mM h⁻¹ (g enzyme)⁻¹ in
the system composed of IL/octanol. This reaction rate is almost 70% higher than the parallel reaction under same concentration of DHCA without IL. These results indicate tOMA-TFA contributed to increase conversion of DHCA by increasing solubility of DHCA. In short, at low concentration of DHCA, IL has an inhibiting effect on enzyme which led to lower reaction kinetics. At high concentration of DHCA, IL helps in improving the solubility of DHCA which led to higher reaction rates.

IL-based reaction systems were further conducted with other antioxidants namely ferulic and caffeic acids to evaluate the positive effects of IL. Parallel reactions without IL were also conducted for comparison. Significant improvement on conversion of these two phenolic acids in a system of IL/octanol was observed. Around 86% of conversion (ferulic acids) was achieved in system of IL/octanol in 16 days, but only 58% of conversion on was achieved in system without tOMA-TFA. Similarly, conversion of caffeic acid in the system of IL/octanol was 60% in 12 days, which is 46% higher than the parallel reaction without tOMA-TFA.

Compared to previous studies (Table 6.1), the reaction system developed in this study was much more efficient with significant improvements both in terms of conversions and productivities.

<table>
<thead>
<tr>
<th>Phenolic acid</th>
<th>Concentration (mM)</th>
<th>Solvent</th>
<th>Enzyme</th>
<th>Reaction time (Day)</th>
<th>Conversion (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHCA</td>
<td>400</td>
<td>IL/Octanol</td>
<td>Novozym 435</td>
<td>8</td>
<td>94</td>
<td>This study</td>
</tr>
<tr>
<td>Ferulic</td>
<td>400</td>
<td>Ditto</td>
<td>Ditto</td>
<td>16</td>
<td>84</td>
<td>This study</td>
</tr>
<tr>
<td>Caffeic</td>
<td>400</td>
<td>Ditto</td>
<td>Ditto</td>
<td>12</td>
<td>60</td>
<td>This study</td>
</tr>
<tr>
<td>DHCA</td>
<td>73</td>
<td>Octanol</td>
<td>Ditto</td>
<td>8</td>
<td>80</td>
<td>Guyot et al., 1997</td>
</tr>
<tr>
<td>Ferulic</td>
<td>73</td>
<td>Ditto</td>
<td>Ditto</td>
<td>15</td>
<td>Trace</td>
<td>Guyot et al., 1997</td>
</tr>
<tr>
<td>Caffeic</td>
<td>100</td>
<td>Ditto</td>
<td>Ditto</td>
<td>12</td>
<td>Trace</td>
<td>Stamatis et al., 1999</td>
</tr>
<tr>
<td>Ferulic</td>
<td>100</td>
<td>Ditto</td>
<td>Lipozyme RMIM</td>
<td>12</td>
<td>30</td>
<td>Stamatis et al., 1999</td>
</tr>
<tr>
<td>Caffeic</td>
<td>50</td>
<td>[bmim]PF6</td>
<td>Ditto</td>
<td>3</td>
<td>11.6</td>
<td>Katsoura et al., 2009</td>
</tr>
<tr>
<td>Ferulic</td>
<td>50</td>
<td>Ditto</td>
<td>Ditto</td>
<td>3</td>
<td>34.9</td>
<td>Katsoura et al., 2009</td>
</tr>
</tbody>
</table>
6.3. Improved Enzymatic Production of Phenolated Glycerides through Alkyl Phenolate Intermediate (Paper III)

The major products of transesterification between octyl dihydrocaffeate and tricaprylin are: mono-DHCA dicaprylin which contains one DHCA moiety and two caprylic acids on the glycerol backbone; di-DHCA monocaprylin which contains two DHCA moieties and one caprylic acid on the glycerol backbone; mono-DHCA monocaprylin which contains one DHCA moiety and one caprylic acid on the glycerol backbone; mono-DHCA acylglycerol which contain one DHCA moiety only on the glycerol backbone; di-DHCA acylglycerol which contains two DHCA moieties on the glycerol backbone; and DHCA (Scheme 6.1). These products were separated by HPLC method and identified by HPLC-ESI-MS described in Paper III.

In preliminary experiments, around 70% of octyl dihydrocaffeate were converted into products within 72 hours. Under the same reaction conditions, two parallel reaction systems were set up. In the first reaction system, dihydrocaffoylated glycerides were synthesized in a solvent-free system through esterification of DHCA with tricaprylin. In the second reaction, dihydrocaffoylated glycerides were synthesized through transesterification of octyl dihydrocaffeate with tricaprylin in hexane. Only 3.2% conversion of DHCA was achieved in the solvent-free system after 72 hours meanwhile 20% conversion of octyl dihydrocaffeate was achieved in the hexane system after 72 hours. Both of these systems were less efficient than the transesterification of octyl dihydrocaffeate with tricaprylin without solvent described in Paper III.

Novozym 435 was found to be a more effective enzyme in this reaction system than enzyme Lipozyme TL IM and Lipozyme RM IM. The enzyme activity of Novozym 435 was 9.57 µmol/g/min, which is almost 20 times higher than Lipozyme TL IM (0.57 µmol/g/min) and Lipozyme RM IM (0.44 µmol/g/min).

Different parameters were studied for optimization of conversion of octyl dihydrocaffeate and production of dihydrocaffoylated glycerides. Effects of temperature, enzyme load (on the base of octyl dihydrocaffeate), reaction time, and molar ratio between tricaprylin and octyl dihydrocaffeate were evaluated as major variables.
Scheme 6.1. Possible products from transesterification between octyl dihydrocaffeate and tricaprylin. A: mono-DHCA dicaprylin; B: di-DHCA monocaprylin; C: mono-DHCA monocaprylin; D: mono-DHCA acylglycerol; E: di-DHCA acylglycerol; F: dihydrocaffeic acid (DHCA). (The potential isomers not shown)
Figures 6.2A and 6.3A reveals the interaction effect of enzyme load and temperature on bioconversion of octyl dihydrocaffeate and yield of dihydrocaffoylated glycerides with reaction time fixed at 24.25 h and substrate molar ratio fixed at 3. When the enzyme load was increased to 14% and temperature was increased to 60 °C, bioconversion and yield was increased to 70.6% and 67.1% respectively. Further increment of reaction temperature and enzyme load leaded to 80.4% of bioconversion and 76.6% yield of dihydrocaffoylated glycerides. Moreover, this plot also indicates that positive effect of these two variables is mutually interactive. When one of two variables remained unchanged, there was only a slight increase of bioconversion and yield by increasing another variable.

Figures 6.2B and 6.3B depicts mutual effects of enzyme load and substrate molar ratio (tricaprylin/octyl dihydrocaffeate) on bioconversion and yield with temperature fixed at 55 °C and reaction time fixed at 24.25 h. It was found that 80.4% of bioconversion and 79.5% of yield were obtained when enzyme load was increased to 16% and substrate molar ratio was increased to 5. However, there was no significant increase in bioconversion and yield with further increase in the enzyme load.

Figures 6.2C and 6.3C shows the effect of reaction time and substrate molar ratio (tricaprylin/ octyl dihydrocaffeate) on bioconversion and yield at 55 °C and 11% of enzyme load. This plot indicates at low substrates molar ratio, longer reaction time is important in determining the bioconversion. The highest bioconversion (88.2%) and yield (83.9%) can be achieved when substrate molar ratio was 5 after 40 h reaction.

An optimized condition was generated by the model with reaction temperature at 65.2 °C, reaction time of 43.6 h, enzyme load of 19.97% (based on substrate weight) and substrate molar ratio at 4.97 (tricaprylin/octyl dihydrocaffeate). At the optimized conditions, a validation reaction was conducted. 86.47% bioconversion of octyl dihydrocaffeate and around 78.07% yield of dihydrocaffoylated glycerides were achieved. The experiment values generally agreed with the model predicted values indicating the model is valid and adequate.
Chapter 6: Over Study Evaluation and Discussions

Figure 6.2. Response contour plots between two parameters for bioconversion of octyl dihydrocaffeate: (A) mutual effects of temperature and enzyme load at 24.25 h and 3 of substrate molar ratio (tricaprylin/octyl dihydrocaffeate); (B) mutual effects of enzyme load and substrate molar ratio (tricaprylin/octyl dihydrocaffeate) at 55 °C and 24.25 h; (C) mutual effects of reaction time and substrate molar ratio (tricaprylin/octyl dihydrocaffeate) at 55 °C and 11% of enzyme load.

Figure 6.3. Response contour plots between two parameters for yield of dihydrocaffoylated glycerides: (A) mutual effects of temperature and enzyme load at 24.25 h and 3 of substrate molar ratio (tricaprylin/octyl dihydrocaffeate); (B) mutual effects of enzyme load and substrate molar ratio (tricaprylin/octyl dihydrocaffeate) at 55 °C and 24.25 h; (C) mutual effects of reaction time and substrate molar ratio (tricaprylin/octyl dihydrocaffeate) at 55 °C and 11% of enzyme load.
6.4. Enzymatic Transesterification of EF with Fish Oils (Paper IV)

The products of transesterification between EF and fish oils are quite complicated due to the complex fatty acids profile of fish oil (Scheme 6.2). A HPLC method was first developed to identify products from transesterification of EF with fish oil by comparing products from transesterification of EF and triolein. The reaction products were then classified into two major groups: feruloyl-sn-glycerol (F1-MAG and F2-DAG) which do not contain fatty acids of fish oil, while the second group is the feruloyl fish oil (F1-TAG, F1-DAG and F2-TAG) which contains one or two fatty acids of fish oil on the backbone of glycerol. The former group is products from transesterification of EF with glycerol and therefore are treated as by-products.

\[
\text{Scheme 6.2. Scheme of possible product on transesterification of EF with triolein or fish oil. R= oleic acid or fatty acids of fish oil. Feruloyl acylglycerol species were defined according to amount of feruloyl moieties or fatty acids on the backbone of glycerol: F1-MAG contains one feruloyl moieties on the back bone of glycerol; F2-DAG contains two feruloyl moieties on the back bone of glycerol; F1-DAG contains one feruloyl moieties and one oleic acid or one fatty acid of fish oil on the backbone of glycerol; F2-TAG contains two feruloyl moieties and one oleic acids or one fatty acids of fish oil on the backbone of glycerol; F1-TAG contains one feruloyl moiety and two oleic acids or two fatty acids of fish oil on the back bone of glycerol. FA and EF are abbreviation for ferulate acid and ethyl ferulate respectively.} \]
Glycerol is necessary to improve conversion of EF and production of feruloyl fish oil. Nevertheless, excess amount of glycerol did not seem to further increase the conversion of EF. Glycerol has an important effect on formation of species of products. The formation of F1-TAG decreased with the increase of glycerol in the reaction, meanwhile, F1-MAG showed the opposite trends. Small amount of glycerol have positive effect on production of F2-DAG and F2-TAG/F1-DAG. Excess of glycerol will have an adverse effect on production of feruloyl fish oil.

A four-factor and three-level CCF was chosen to investigate the effect of four variables (temperature, reaction time, amount ratio of fish oil/EF, and enzyme load) on conversion of EF, production of feruloyl-sn-glycerol, and production of feruloyl fish oil.

RSM showed that temperature, reaction time, amount ratio of fish oil/EF and enzyme load had a positive effect on conversion of EF, production of feruloyl-sn-glycerol, and feruloyl fish oil. In general, higher temperature was preferred as it increased bioconversion of EF and production of feruloyl fish oils. However, high temperature also had a significant effect on increasing formation of feruloyl-sn-glycerols, which are undesired by-products in this reaction. Therefore, it is better to conduct reaction in relatively lower temperatures to minimize formation of by-product, and optimize other conditions to increase bioconversion of EF and production of feruloyl fish oils. The substrate amount ratio had the similar effect as temperature. In most cases, bioconversion of EF and production of feruloyl-sn-glycerol and feruloyl fish oils were increased by increasing the substrate amount ratio. In terms of interaction between substrate amount ratio and enzyme load, higher formation of feruloyl-sn-glycerol was observed in lower value of substrate amount ratio, and vice versa. Prolonging reaction time had a positive effect on formation of feruloyl-sn-glycerol, but had a negative effect on bioconversion of EF and formation of feruloyl fish oils. Higher enzyme loads increased the bioconversion of EF and formation of feruloyl fish oils and feruloyl-sn-glycerol. There was no significant effect on bioconversion of EF and formation of feruloyl fish oils by changing other conditions if the enzyme load was too low. Therefore, it is preferable to keep enzyme loads at a certain level and minimize formation of by-products by changing other conditions. According to the analysis of the model, it is possible to increase bioconversion of EF and formation of feruloyl fish oil and minimize formation of feruloyl-sn-glycerol at the same time base on the following conditions: reaction temperature, 70 °C; enzyme load, 4.3%; amount ratio, 4.7 (fish oils/EF); reaction time, 5 days.
6.5. Characterization of Different Systems by DLS and SAXS (Appendix 5)

The characteristics of phenolic acids in IL/octanol, rutin in IL/linoleic acid and ferulic acid in IL/triolein were investigated using DLS and SAXS. Spherical droplets were not detected in the system of phenolic acid in IL/octanol either with DLS (Table 6.2) or SAXS. Considering that phenolic acids can be dissolved in rOMA-TFA in high amounts and in octanol to some extent, this system was concluded as a homogenous system.

Rutin has very low solubility in linoleic acid, but it can be dissolved in rOMA-TFA. After rutin was dissolved in IL and mixed with linoleic alcohol, a stable and opaque homogeneous system was obtained. Nano-sizes spherical droplets were detected both by DLS and SAXS. According to the results obtained from DLS (Table 6.2), spherical droplets with the sizes of around 38-82 nm were detected in the system of rutin in IL/linoleic.

Table 6.2. DLS results for different systems.

<table>
<thead>
<tr>
<th>Systems</th>
<th>(^a\text{Rg (nm)})</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHCA in IL/octanol</td>
<td>n/a</td>
</tr>
<tr>
<td>Ferulic in IL/octanol</td>
<td>n/a</td>
</tr>
<tr>
<td>Rutin in IL/linoleic</td>
<td>38 - 82</td>
</tr>
<tr>
<td>Ferulic in IL/triolein</td>
<td>914 - 1253</td>
</tr>
</tbody>
</table>

\(^a\text{Rg: Radius of gyration, refered to the size of particle}\)

These results were further confirmed by SAXS (Figure 6.4), which observed small droplets in the sizes of around 40 nm (Peak 1). At the same time, SAXS also indicated presence of larger particles with a size of around 100 nm in this system. Nevertheless, these signals were rather weak (Peak 2). There was no further evidence which can prove the existence of larger particles in the system, but particles with smaller sizes probably exist since it was detected both by DLS and SAXS. There was a small difference between the results on the size of smaller particles obtained from DLS and SAXS. However, they are not in conflict with each other because the droplet sizes detected by DLS are normally a bit larger than those detected by SAXS due to the difference in instruments.

Ferulic acid can be dissolved in rOMA-TFA in high concentration. However, the ionic liquid is not miscible with triolein. The emulsion prepared in this system was not stable. The emulsion can only be formed when the system was stirred at high speed (above 500 rpm) under high temperature.
When the stirring was stopped and the system was cooled down, they will separate into two phases again after a while. Surfactant is necessary in this system to stabilize the emulsion, which can be an area to be studied in the future. Using DLS to detect the emulsion formed in this system, particles with sizes of above 1000 nm were observed (Table 6.2). Nevertheless, no sign was detected with SAXS, probably because the sizes of the particles are too big to be detected with this instrument.

Based on the above experiments, it can be concluded that there was no particle in the system of phenolic acids in IL/octanol and it was probably a homogeneous system. Stable emulsion can be formed in the system of rutin in IL/linoleic and small spherical droplets with size of around 40 nm were detected both with DLS and SAXS. OMA·TFA did not mix well with triolein, and the system of ferulic acid in IL/triolein was not a stable emulsion system unless it was stirred under high speed at a high temperature. When the emulsion was formed with the assistance of surfactant, the size of the emulsion droplet was found to be around 1000 nm. Furthermore, the system of phenolic in IL/octanol had been proved to be an very efficient system on synthesis of lipophilic phenolic esters by increasing the solubility of phenolic acids (Paper II), it is expected that the later two systems will have good performance on synthesis lipophilic rutin ester and lipophilic ferulic TAG as well. The third system of ferulic in IL/triolein which did not form a stable emulsion system can be further investigate in terms of ways to stabilize the emulsions.

For the details of the experiments please refer to Appendix 5.
Chapter 7: Conclusions and Future Outlook

Present dissertation provides better understanding of strategies concerning enzymatic synthesis of novel antioxidants towards oils enriched with PUFAs. Different lipophilic phenolic antioxidants including phenolic fatty alcohols and phenolic TAG were synthesis via several routes namely solvent or solvent free systems. The main achievements are summarized below.

Different lipophilic phenolic antioxidants were synthesized by conjugating selected phenolic acids with fatty alcohols with various carbon chains from C4 to C18:1 in a binary organic solvent system. Higher conversion of phenolic acids was achieved when phenolic acids were reacted with medium chain fatty alcohols. The volume ratios of hexane and 2-butanone were varied from 85/15 to 45/55 (v/v). The optimal volume ratio was found to be 65/35, where conversion of DHCA and the enzyme activity were higher than other conditions. Ferulic and caffeic acids were conjugated with several medium chain fatty alcohols in this binary organic solvent system, but the conversions of these two phenolic acids were relatively lower than that of DHCA. Using synthesis of octyl dihydrocaffeate by conjugating octanol and DHCA as model, effects of several variables on conversion of DHCA were studied. Optimal conditions were predicted by RSM model, and relative higher conversion of DHCA was achieved based on the predicted conditions.

The solubility of phenolic acids can be increased greatly in \( \text{tOMA-TFA} \) as a reaction media. The effect of volume ratio between \( \text{tOMA-TFA} \) and octanol on conversion of DHCA was firstly studied. Increasing volume ratio of \( \text{tOMA-TFA} \) and octanol from 1:4 to 1:12 slightly increased conversion of DHCA. Higher conversion of DHCA was achieved when reaction was conducted in a higher temperature. Novozym 435 was stable even when reactions were conducted at 80 °C. Removing water from reactions had a significant effect on increasing conversion of DHCA. Adding molecular sieves was found to be a more efficient water removal technique as compared to usage of vacuum. The initial reaction rate can be increased by increasing concentration of DHCA. However, the initial reaction rate was higher in the reaction system without \( \text{tOMA-TFA} \) when the concentration of DHCA was low (0.4 M). Nevertheless, even a higher initial reaction rate was achieved in the system using IL as reaction media when the concentration of DHCA was high (1.2 M or 1.6 M).
Relatively higher conversion of ferulic and caffeic acids were also achieved in the system using IL using IL as reaction media than solvent free system

Using octyl dihydrocaffeate as intermediate, lipophilic phenolic TAG was firstly synthesis by interesterification between octyl dihydrocaffeate and tricaprylin. This system was very efficient as compared to either direct reaction of DHCA with tricaprylin in solvent free system or interesterification of octyl dihydrocaffeate with tricaprylin in organic solvent system. The activity of Novozym 435 was higher than Lipozyme TL IM and Lipozyme RM IM in this system. Optimal conditions were predicted by RSM through evaluating several variables.

A HPLC chromatography method was developed for quantifying products of EF with fish oil. Glycerol was added in this system to create an intermediate reaction product (insert structure name) which helped to increase the overall reaction rate. Effect of glycerol on bioconversion of EF and production of feruloyl triglyceride species was investigated by varying the amount of glycerol from 0 mmol to 5 mmol in reactions. Adding a little glycerol could increase the bioconversion of EF and production of feruloyl triglyceride species. Excess amount of glycerol in the reaction did not contribute towards bioconversion of EF and desired products of feruloyl fish oils. Higher reaction temperature was unpreferable as it might increase formation of by-products even though formation of feruloyl fish oils could be increased at the same time. Enzyme load is another important factor that can increase bioconversion of EF and formation of feruloyl fish oils. Nevertheless, similar to effect of temperature, too high amount of enzyme load will increase formation of by-products. A higher amount substrate ratio is desired as it can increase formation of feruloyl fish oils and minimize formation of by-products at the same time. Longer reaction time should be avoided as it decreased formation of feruloyl fish oil, and productivity.

The characteristics of three systems phenolic acids in IL/octanol, rutin in IL/linoleic, and ferulic acid in IL/triolein were explored and investigated through techniques of DLS and SAXS. Phenolic in IL/octanol system appear to be a homogenous system. Nano-particles were found in the system of rutin in IL/linoleic with the sizes of around 40 nm. Ferulic in IL/triolein formed unstable emulsion system with droplet size of above 1000 nm.
In general, several strategies on synthesis of amphiphile phenolic antioxidants were explored and relatively high productivities were achieved compared to previously published studies. Enzymatic method was proved to be an efficient way to modify these natural antioxidants into amphiphile molecules with high efficiency and specificity. Further investigation should be carried out concerning the development of an economical industrial scale with good yield of reaction product. These amphiphile molecules are expected to have better solubility in any oil-base food system while retaining their original antioxidant properties, and therefore can provide better protection from lipid oxidation. Therefore research on the functional, application, and toxicological of these modified amphiphile antioxidants should also be conducted in the future. If these novel molecules can be proven to be non-toxic and efficient in preventing lipid oxidation, they can be applied widely in food, cosmetic, and pharmaceutical industries. Moreover, micro-emulsions systems can be a very promising reaction system on increasing both solubility of reaction materials and productivity of desired products. Nevertheless, problem may persist in the form of purification of the final desirable product. In short, production of antioxidant conjugates has great potential for application in various industries. Thorough investigation of the reaction condition especially in terms of economic feasibility at industrial scale will help in successful launch of such products.
References:


Appendix 1: Paper I

Title: Enzymatic Lipophilization of Phenolic Acids through Esterification with Fatty Alcohols in Organic Solvents

Authors: Zhiyong Yang, Zheng Guo, Xuebing Xu*

Journal: Food Chemistry

(Manuscript Submitted)
Enzymatic Lipophilization of Phenolic Acids through Esterification with Fatty Alcohols in Organic Solvents

Zhiyong Yang · Zheng Guo · Xuebing Xu*
Department of Molecular Biology, Aarhus University,
Gustav Wieds Vej 10, 8000 Aarhus C, Denmark

Abstract

In this study, we investigated and optimized the synthesis of lipophilized esters between selected phenolic acids and fatty alcohols in a binary solvent system, which is composed of hexane and butanone. The effect of different proportion of hexane and butanone was firstly studied by changing the volume ratio from 85:15 to 45:55. It was found that the conversion strongly depended on the proportion of hexane and butanone in the reaction system. Following the effect of carbons of fatty alcohol chains on esterification performance with dihydrocaffeic acid (DHCA) was evaluated by choosing different fatty alcohols from C4 to C18. The conversion of DHCA was significantly affected by the carbons of fatty alcohol chains. Roughly 95% conversion was achieved within 3 days when DHCA was esterified with hexanol (C6), while only 56% and 44% conversion were achieved when esterified with 1-butanol and octadecenol respectively. However, the conversions of ferulic and caffeic acids under the same conditions were much lower than DHCA. The optimal mixture ratio of hexane to butanone was found to be 65:35. Using octanol and DHCA as model, the reaction parameters, such as temperature, enzyme load, reaction time and substrate molar ratio, were optimized with response surface modelling (RSM). The optimum conditions are finalized as: temperature 60 °C, reaction time 7 days, enzyme load 100 mg, and substrate molar ratio 4.34 (octanol/DHCA).

Keywords: Phenolic acids · Fatty alcohols · Novozym 435 · RSM

*Corresponding author: Xuebing Xu, Department of Molecular Biology, Aarhus University, Gustav Wieds Vej 10, 8000 Aarhus C, Denmark
Tel: +45-89425089, Fax: +45-86123178, Email address: xu@mb.au.dk
1. Introduction

Phenolic acids are secondary plant metabolites, and they are widely dispersed throughout the plant kingdom, such as beverage, fruits, vegetables, and miscellaneous minor commodities (Crozier, Jaganath, & Clifford, 2009). There were continuing research interests on phenolic acids in recent years due to their potential biological properties (Figueroa-Espinoza & Villeneuve, 2005). Phenolic acids are antioxidants with redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers (Pietta, 2000). Among dietary antioxidants, phenolic acids are by far the most abundant in most of the diets (Stalikas, 2010). More importantly, many phenolic compounds have been reported to have anticancer or anticarcinogenic/antimutagenic, antiatherosclerotic, antibacterial, antiviral, and anti-inflammatory activities to a greater or lesser extent (Huang, Cai, & Zhang, 2010). Therefore phenolic acids are potential candidates as ingredients in food, health, cosmetics, and pharmaceutical industries.

One of major barrier in application of phenolic acids is their low solubility and stability in oil-based formulae, which limited their application in oil based food processing and cosmetic industries (Figueroa-Espinoza & Villeneuve, 2005). A practical thinking on expanding application of phenolic acid is to modify their hydrophilic property and enhance their hydrophobic property. Normally, this aim can be achieved through enzymatic lipophilization by esterifying the carboxylic acid group of phenolic acids with an aliphatic molecule (such as fatty alcohols) to produce an amphiphilic molecule, which should keep its original functional properties (Ha, Nihei, & Kubo, 2004; Nihei, Nihei, & Kubo, 2004).

There have been several reports on esterification of phenolic acids with fatty alcohols, either in solvent free or in organic solvents (Guyot, Bosquete, Pina, & Graille, 1997; Guyot et al., 2000; Stamatis, Sereti, & Kolisis, 1999 & 2001; Sabally, Karboune, Yebaoh, & Kermasha, 2005, a & b; Katsoura et al., 2009; Vosmann, Weitkamp, & Weber, 2006). According to these studies, lipase catalyzed esterification in organic solvents is still more preferable than in the solvent free system because organic solvents can increase the solubility of phenolic acid and shift the reaction equilibrium to favour synthesis reactions (Sabally et al., 2005a; Halling, 1994). However, lipase catalyzed esterification of phenolic acids is still difficult due to prolonged reaction time and low productivity (Figueroa-Espinoza and Villeneuve, 2005). To increase the solubility and conversion of phenolic acids, Sabally et al. (2005a) developed a binary solvent system. Such binary solvent can improve solubility of phenolic acid and remain the maxima activity of catalyzed enzyme (Novozym 435), which required hydrophobic environment. This binary solvent system had been proved to be
efficient on esterification of DHCA with long carbon chain fatty alcohol (linolenyl alcohol) and triglyceride (Sabally et al., 2005a; Sabally, Karboune, St-Louis, Kermasha, 2006). However, the effect of esterification of DHCA and other phenolic acids with short or medium carbon length of fatty alcohols in this system has not been evaluated. Moreover, the previous studies were conducted with rather low concentration of DHCA which resulted in low productivity. This is not practical for industrial interests.

In the present study, we investigated the esterification of selected phenolic acids with different fatty alcohols using the binary solvent system. The effects of carbon number of fatty alcohols and the type of phenolic acid on esterification reaction in such system were evaluated. The aim of this study is to improve the conversion of phenolic acid and increase productivity of target product in the binary solvent system practically. To assist this operation, response surface methodology (RSM) was applied for the purpose to optimize the selected conditions with respect to the optimal conversion.

2. Materials and Methods

2.1. Materials

Dihydrocaffeic acid (DHCA), fatty alcohol (n-butanol, hexanol, octanol, deconanol, dodecanol) with 99% purity and octadecenol with 85% purity were purchased from Sigma Aldrich (Brøndby, Denmark). Catalysis enzyme Novozym 435 from Candida antarctica lipase B was obtained from Novozymes A/S (Bagsværd, Denmark). Organic solvents of analytical and HPLC grades were purchased from Fisher Scientific Inc. (Slangerup, Denmark).

2.2. Enzyme Esterification of DHCA with Fatty Alcohol in Organic Solvents

The synthesis method of phenolic acids with fatty alcohols was modified from a reported method introduced by Sabally et al., (2005a). The esterification reaction of DHCA with fatty alcohols was conducted in a 50 ml bottle, which is equipped with a tightly screw cap to prevent evaporation of solvent. A standard reaction was composed of 0.5 mmol DHCA and 1.5 mmol fatty alcohol in 10 ml volume of binary organic solvent mixture. DHCA was prepared as 500 mM of fresh solution in butanone prior adding into hexane. However, due to the problem of solubility, ferulic acid was prepared as 250 mM and caffeic acid was 50 mM. The final concentration of DHCA, ferulic acid, and caffeic acid in reaction systems were 50 mM, 50 mM, and 5 mM respectively, and they were mixed with fatty alcohols in the molar ratio of 1:3. The reaction was initiated by adding 100 mg of Novozym 435 and 100 mg of molecular sieves (3 Å). The reaction bottles were incubated at 60 °C.
for up to 5-6 days and the reaction mixture were stirred at 300 rpm. These reaction conditions were also applied to other reactions unless otherwise specified. Samples were withdrawn periodically and diluted in 100 times with methanol. The solution was analyzed by HPLC after removing any solid by centrifugation. In all cases, a single product was detected by HPLC analysis with UV detector in the range from 200 to 325 nm. HPLC analysis and LC-MS were conducted in a similar way as described before (Yang, Feddern, Glasius, Guo, & Xu, 2011).

2.3. RSM Optimization

Response surface methodology (RSM) combined with three level and four factors CCF design (a centre composite design) was used to investigate the relationship between variables and conversion. The variables and their levels selected for the study were reaction time (1, 4 and 7 days), temperature (40, 55 and 70 °C), enzyme load (2, 11 and 20% relative to the total weight of substrates) and molar ratio of octanol/DHCA (1:1, 3:1 and 5:1). Each level was represented as -1, 0, and 1. The whole experiment was designed by software Modde 8.0 (Umetrics AB, Umeå, Sweden) based on above factors (the four variables) and response factor (conversion of DHCA) (Table 1). All the experiments were carried out in the binary system where hexane and butanone were mixed in the volume ratio of 65:35. 100 mg of molecular sieves was also added in each experiment.

2.4. Statistical Analysis

The mathematical relationship among variables with each response can be calculated by the quadratic polynomial equation:

\[ Y = \beta_0 + \sum_{i=1}^{4} \beta_i x_i + \sum_{i=1}^{4} \beta_{ii} x_i^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{4} \beta_{ij} x_i x_j \]  

(1)

where \( \beta_0, \beta_i, \beta_{ii} \) and \( \beta_{ij} \) are regression coefficients (\( \beta_0 \) is a constant term, \( \beta_i \) is a linear effect term, \( \beta_{ii} \) is a squared effect term, and \( \beta_{ij} \) is an interaction effect term) and \( Y \) is the predicted response value.
Table 1: Experiment design and results of the predicted and experimental responses.

<table>
<thead>
<tr>
<th>Trials</th>
<th>Temperature (°C)</th>
<th>Molar Ratio (Octonl/DHCA)</th>
<th>Time (Day)</th>
<th>Enzyme Load (%)</th>
<th>Conversion</th>
<th>Prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40 (-1)</td>
<td>1 (-1)</td>
<td>1 (-1)</td>
<td>50 (-1)</td>
<td>8</td>
<td>3.58</td>
</tr>
<tr>
<td>2*</td>
<td>70 (1)</td>
<td>1 (-1)</td>
<td>1 (-1)</td>
<td>50 (-1)</td>
<td>4</td>
<td>-22.72</td>
</tr>
<tr>
<td>3</td>
<td>40 (-1)</td>
<td>5 (1)</td>
<td>1 (-1)</td>
<td>50 (-1)</td>
<td>3</td>
<td>5.91</td>
</tr>
<tr>
<td>4</td>
<td>70 (1)</td>
<td>5 (1)</td>
<td>1 (-1)</td>
<td>50 (-1)</td>
<td>9</td>
<td>6.79</td>
</tr>
<tr>
<td>5*</td>
<td>40 (-1)</td>
<td>1 (-1)</td>
<td>7 (1)</td>
<td>50 (-1)</td>
<td>39</td>
<td>0.68</td>
</tr>
<tr>
<td>6</td>
<td>70 (1)</td>
<td>1 (-1)</td>
<td>7 (1)</td>
<td>50 (-1)</td>
<td>18</td>
<td>19.52</td>
</tr>
<tr>
<td>7</td>
<td>40 (-1)</td>
<td>5 (1)</td>
<td>7 (1)</td>
<td>50 (-1)</td>
<td>36</td>
<td>31.49</td>
</tr>
<tr>
<td>8</td>
<td>70 (1)</td>
<td>5 (1)</td>
<td>7 (1)</td>
<td>50 (-1)</td>
<td>76</td>
<td>77.50</td>
</tr>
<tr>
<td>9</td>
<td>40 (-1)</td>
<td>1 (-1)</td>
<td>1 (-1)</td>
<td>250 (1)</td>
<td>15</td>
<td>16.41</td>
</tr>
<tr>
<td>10</td>
<td>70 (1)</td>
<td>1 (-1)</td>
<td>1 (-1)</td>
<td>250 (1)</td>
<td>47</td>
<td>47.09</td>
</tr>
<tr>
<td>11</td>
<td>40 (-1)</td>
<td>5 (1)</td>
<td>1 (-1)</td>
<td>250 (1)</td>
<td>14</td>
<td>8.06</td>
</tr>
<tr>
<td>12</td>
<td>70 (1)</td>
<td>5 (1)</td>
<td>1 (-1)</td>
<td>250 (1)</td>
<td>63</td>
<td>65.91</td>
</tr>
<tr>
<td>13</td>
<td>40 (-1)</td>
<td>1 (-1)</td>
<td>7 (1)</td>
<td>250 (1)</td>
<td>67</td>
<td>64.79</td>
</tr>
<tr>
<td>14*</td>
<td>70 (1)</td>
<td>1 (-1)</td>
<td>7 (1)</td>
<td>250 (1)</td>
<td>92</td>
<td>140.60</td>
</tr>
<tr>
<td>15</td>
<td>40 (-1)</td>
<td>5 (1)</td>
<td>7 (1)</td>
<td>250 (1)</td>
<td>82</td>
<td>84.91</td>
</tr>
<tr>
<td>16*</td>
<td>70 (1)</td>
<td>5 (1)</td>
<td>7 (1)</td>
<td>250 (1)</td>
<td>94</td>
<td>187.90</td>
</tr>
<tr>
<td>17</td>
<td>40 (-1)</td>
<td>3 (0)</td>
<td>4 (0)</td>
<td>150 (1)</td>
<td>37</td>
<td>46.84</td>
</tr>
<tr>
<td>18</td>
<td>70 (1)</td>
<td>3 (0)</td>
<td>4 (0)</td>
<td>150 (0)</td>
<td>89</td>
<td>85.19</td>
</tr>
<tr>
<td>19</td>
<td>55 (0)</td>
<td>1 (-1)</td>
<td>4 (0)</td>
<td>150 (0)</td>
<td>50</td>
<td>53.61</td>
</tr>
<tr>
<td>20</td>
<td>55 (0)</td>
<td>5 (1)</td>
<td>4 (0)</td>
<td>150 (0)</td>
<td>76</td>
<td>78.42</td>
</tr>
<tr>
<td>21</td>
<td>55 (0)</td>
<td>3 (0)</td>
<td>1 (-1)</td>
<td>150 (0)</td>
<td>20</td>
<td>25.24</td>
</tr>
<tr>
<td>22</td>
<td>55 (0)</td>
<td>3 (0)</td>
<td>7 (1)</td>
<td>150 (0)</td>
<td>84</td>
<td>84.79</td>
</tr>
<tr>
<td>23</td>
<td>55 (0)</td>
<td>3 (0)</td>
<td>4 (0)</td>
<td>50 (-1)</td>
<td>21</td>
<td>26.21</td>
</tr>
<tr>
<td>24</td>
<td>55 (0)</td>
<td>3 (0)</td>
<td>4 (0)</td>
<td>250 (1)</td>
<td>87</td>
<td>87.82</td>
</tr>
<tr>
<td>25</td>
<td>55 (0)</td>
<td>3 (0)</td>
<td>4 (0)</td>
<td>150 (0)</td>
<td>68</td>
<td>65.97</td>
</tr>
<tr>
<td>26</td>
<td>55 (0)</td>
<td>3 (0)</td>
<td>4 (0)</td>
<td>150 (0)</td>
<td>79</td>
<td>65.97</td>
</tr>
<tr>
<td>27</td>
<td>55 (0)</td>
<td>3 (0)</td>
<td>4 (0)</td>
<td>150 (0)</td>
<td>69</td>
<td>65.97</td>
</tr>
</tbody>
</table>

*These experiment trials were removed from modelling because of disagreement between actual value and prediction value and they do not follow normality distribution in the whole data set.

3. Results and Discussions

3.1. Effect of Solvent Mixture Ratio between Hexane and Butanone

The solvent mixture between hexane and butanone was varied from 85:15 to 45:55 (v/v) for the purpose of investigating the optimal reaction systems. Several short carbon chain, medium carbon chain and long carbon chain fatty alcohol (butanol, octanol, dodecanol and octadecenol) were chosen for evaluation by esterifying with DHCA in these systems. The total volume of solvent mixture was 10 ml, and the final concentration of DHCA and fatty alcohols were 50 mM and 150 mM respectively. As shown in Table 2, the initial rates of reactions between DHCA and these fatty
alcohols were quite low, when the system was composed mainly with hexane but smaller proportion butanone (hexane/butanone, 85:15, v/v), the decrease of hexane proportion from 85% to 65% increased the conversion of DHCA significantly. After this level, the conversion of DHCA decreased again but still higher than the system where hexane and butanone were mixed as 85:15. However, very low conversion was observed when the esterification reaction was conducted either in hexane or butanone alone (data not shown).

Table 2: Esterification of DHCA with several fatty alcohols in the binary solvent systems with different proportion of hexane and butanone in six days.

<table>
<thead>
<tr>
<th>Hexane/butanone (v/v)</th>
<th>Fatty alcohols</th>
<th>1-butanol</th>
<th>Octanol</th>
<th>Dodecanol</th>
<th>Octadecenol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial rate conversion</td>
<td>Initial rate conversion</td>
<td>Initial rate conversion</td>
<td>Initial rate conversion</td>
<td>Initial rate conversion</td>
</tr>
<tr>
<td>85:15</td>
<td>0.05±0.04 5.30±1.43</td>
<td>0.01±0.01 4.60±2.01</td>
<td>0.02±0.00 7.20±1.52</td>
<td>0.01±0.00 2.22±0.55</td>
<td></td>
</tr>
<tr>
<td>75:25</td>
<td>0.29±0.02 68.53±1.33</td>
<td>0.31±0.02 72.05±0.36</td>
<td>0.33±0.05 74.58±24.85</td>
<td>0.19±0.02 57.60±6.43</td>
<td></td>
</tr>
<tr>
<td>65:35</td>
<td>0.80±0.01 96.72±0.86</td>
<td>0.66±0.01 85.50±2.89</td>
<td>0.50±0.02 92.70±0.53</td>
<td>0.39±0.01 86.21±0.74</td>
<td></td>
</tr>
<tr>
<td>55:45</td>
<td>0.67±0.02 94.86±1.09</td>
<td>0.53±0.07 82.10±0.14</td>
<td>0.46±0.00 87.42±2.23</td>
<td>0.36±0.00 80.96±0.60</td>
<td></td>
</tr>
<tr>
<td>45:55</td>
<td>0.41±0.05 86.42±0.83</td>
<td>0.34±0.01 76.97±3.18</td>
<td>0.31±0.05 73.81±0.59</td>
<td>0.26±0.05 68.33±1.40</td>
<td></td>
</tr>
</tbody>
</table>

*aStandard deviations were calculated from duplicated experiments
bInitial rate was calculated as mM of product h⁻¹

It has been reported that Novozym 435 was a good candidate as biocatalyst for esterification of phenolic acid with medium or high chain lengths of fatty alcohol (Stamatis et al., 1999). The enzyme has better performance in lipophilic environment (Buisman et al., 1998). However, phenolic acids have very low solubility in lipophilic solvents, which is the major hindrance of interaction between phenolic acid and biocatalysts. In order to improve the solubility of DHCA and maintain activity of Novozym 435 at the same time, Sabally et al. (2005a) proposed to apply binary solvent system of hexane/butanone for synthesis of DHCA with linoleyl alcohol. They found that higher esterification yield was obtained in the reaction system where hexane and butanone were mixed in the volume ratio of 85:15, and the esterification yield decreased further corresponding to increase the volume ratio of butanone in the binary system. However, the highest conversion was found in the binary solvent systems where hexane and butanone were mixed in volume ratio of 65:35 in this study. It should be noted that the concentration of DHCA was 50 mM in this study, which is 10 times higher than the previous study. The early reported system was simply cloudy for the current highly concentrated substrate.
3.2. Esterification of Fatty Alcohols with DHCA

The effect of carbons of fatty alcohol chains on esterification with DHCA was studied by using different fatty alcohols with carbons varied from C4 to C18 (Fig. 1). It was observed that the highest conversion of DHCA was achieved when reacted with hexanol, which reached reaction equilibrium in 3 days and with around 95% conversion of DHCA. Followed was dodecanol, where the reaction reached 70% conversion of DHCA in 5 days. Similar conversion rate was observed among butanol, octanol, and decanol, where the conversion of DHCA with these three fatty alcohols were 57%, 65% and 61% respectively after 5 days, and the reactions still had the trends to increase. On the other hand, esterification of DHCA with the longest carbon chain fatty alcohol (octadecenol) resulted in the lowest conversion. The conversions of DHCA with octadecenol in 5 days were 44% only, which are much lower than using other medium chain fatty alcohols as substrate. These results are generally agreed with previous report, which indicate that the length of fatty alcohol chains plays an important role (Guyot et al., 1997).

![Fig. 1. Esterification of DHCA with various fatty alcohols in different carbon chain organic solvent mixtures (hexane/butanone, 75:25, v/v): n-butanol (◇), hexanol (▲), octadecanol (○), dodecanol (◆) and oleyl octadecenol (■).]
3.3. Esterification of Fatty Alcohols with Ferulic acid and Caffeic acid

As observed in above results, the highest conversion was achieved when DHCA was esterified with hexanol, octanol and dodecanol. These three fatty alcohols were chosen for further evaluation of reactions with ferulic and caffeic acids. It was observed that the solubility of caffeic acid in butanone is not as high as DHCA and ferulic acid. Thus the concentrations of caffeic acid in the reaction system were reduced to 5 mM, while other reaction conditions were as same as when DHCA was esterified with different fatty alcohols above. As can be seen in Fig. 2, it was possible to esterify these two phenolic acids with fatty alcohols in the solvent system, but the conversions of these two phenolic acids were not as high as DHCA. The highest conversions of ferulic acid (Fig.2A.) and caffeic acid (Fig.2B.) with hexanol were only 5% and 20% respectively, and the conversions were even lower when reacted with octanol and dodecanol. The reasons that ferulic acid and caffeic acid are more difficult to be esterified with fatty alcohols than DHCA had been discussed in several previous reports (Stamatis et al., 1999; Guyot et al., 1997; Buisman et al., 1998; Figueroa-Espinoza and Villeneuve, 2005). A recent study (Katsoura et al., 2009) reported that the conversion of ferulic acid could be increased to 38.1% and 34.9% when was esterified with hexanol and octanol respectively within 72 h, and the reactions were catalyzed by immobilized RM IM in ionic liquid [bmim]PF6. The study indicates that both enzyme and reaction system can be important factors for improving conversion of phenolic acids.

3.4. RSM Optimization

In a previous study it denoted that the antioxidant activity of those lipophilized compounds increase with the increase of lipophilicity property (Katsoura et al., 2009). However, in our recently study we also found that octyl dihydrocaffeate has better antioxidant property than oleyl dihydrocaffeate in the oil/water emulsion system, which is probably due to long chain lipophilized dihydrocaffeate form micelles in aqueous phase instead of being located in oil phase (data not shown). Therefore DHCA and octanol were used as the model system where response surface methodology (RSM) was employed to evaluate the relationship between a set of controllable experimental factors. As observed from the above results, the highest conversion was achieved when 50 mM of DHCA was dissolved in the binary solvent mixture where hexane and butanone were mixed in 65:35 (v/v). Therefore this mixture ratio was chosen as reaction system for evaluation by RSM. Results from the experimental design were presented on Table 1.
Coefficients of a full model were evaluated by regression analysis and tested for their significance. The insignificant coefficients were eliminated stepwise on the basis of the $p$ value after the

**Fig. 2.** Esterification of ferulic acid (A) and caffeic acid (B) with hexanol (◇), octanol (□) and dodecanol (Δ) in binary organic solvent mixtures (hexane/butanone, 75:25, v/v).

Coefficients of a full model were evaluated by regression analysis and tested for their significance. The insignificant coefficients were eliminated stepwise on the basis of the $p$ value after the
coefficients were tested (Table 3). As indicated in Table 3, the independent variables ($x_1$, $x_2$, $x_3$ and $x_4$), quadratic terms ($x_3^2$ and $x_4^2$) and interactions ($x_1x_2$, $x_1x_3$, $x_1x_4$, $x_2x_3$, $x_3x_4$) were significant ($p<0.05$). On the other hand, the quadratic terms $x_1^2$, $x_2^2$ and interaction terms $x_2x_4$ did not produce a significant effect on the conversion within the designed intervals ($p>0.05$) and, therefore, were removed from the original model. Thus the final second polynomial model was generated as:

$$Bioconversion(\%) = 68.36 + 16.46x_1 + 12.11x_2 + 29.33x_3 + 29.56x_4 - 14.53x_3^2 - 12.53x_4^2 + 7.37x_1x_2 + 9.88x_1x_3 + 13.75x_1x_4 + 6.56x_2x_3 + 11.19x_3x_4$$

(2)

where $x_i$ is the coded value of each factor.

**Table 3: Regression coefficients and significance ($p<0.05$) after backward elimination for conversion of DHCA.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient</th>
<th>$p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>68.36</td>
<td>2.09E-08</td>
</tr>
<tr>
<td>$x_1$</td>
<td>16.46</td>
<td>0.000251</td>
</tr>
<tr>
<td>$x_2$</td>
<td>12.118</td>
<td>0.000264</td>
</tr>
<tr>
<td>$x_3$</td>
<td>29.33</td>
<td>2.72E-06</td>
</tr>
<tr>
<td>$x_4$</td>
<td>29.56</td>
<td>5.11E-06</td>
</tr>
<tr>
<td>$x_1x_1$</td>
<td>7.83</td>
<td>0.16084</td>
</tr>
<tr>
<td>$x_2x_2$</td>
<td>-3.53</td>
<td>0.418449</td>
</tr>
<tr>
<td>$x_3x_3$</td>
<td>-14.53</td>
<td>0.009529</td>
</tr>
<tr>
<td>$x_4x_4$</td>
<td>-12.53</td>
<td>0.018614</td>
</tr>
<tr>
<td>$x_1x_2$</td>
<td>7.37</td>
<td>0.007932</td>
</tr>
<tr>
<td>$x_1x_3$</td>
<td>9.88</td>
<td>0.007062</td>
</tr>
<tr>
<td>$x_1x_4$</td>
<td>13.75</td>
<td>0.000651</td>
</tr>
<tr>
<td>$x_2x_3$</td>
<td>6.56</td>
<td>0.013669</td>
</tr>
<tr>
<td>$x_2x_4$</td>
<td>-2.32</td>
<td>0.276677</td>
</tr>
<tr>
<td>$x_3x_4$</td>
<td>11.19</td>
<td>0.002565</td>
</tr>
</tbody>
</table>

$x_1$: temperature (°C); $x_2$: substrate molar ratio (octanol/DHCA);
$x_3$: reaction time (day); $x_4$: enzyme load (mg)

According to the ANOVA analysis, the coefficient of determination ($R^2$) of the model was 0.987, which indicates that the model is suitable to represent the real relationship among the selected reaction parameters. At the same time, the probabilities for the regression of the model were found to be significant ($p<0.0001$) while the lack of fit of the model was insignificant ($p=0.563$), which indicate that the model was statistically prominent and adequate to explain most of the variability for conversion of DHCA.

Base on the secondary polynomial model generated by RSM, optimum conditions for conversion of DHCA was predicted within the experimental range studies. Three sets of experiments were conducted base on the predicted conditions, and the experimental values are presented together on
Table 4 together with the predicted values. The results show the experimental values are generally agreed with the predicted values, which indicated the model is a solid and adequate model. The highest yield was found on run 3, where the observed conversion is 92.47% under the optimized conditions: temperature 60 °C, reaction time 7 days, enzyme load 100 mg, and substrate molar ratio 4.34 (octanol/DHCA).

Table 4: Optimum conditions predicted by the model for the conversion of DHCA.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Run 1</th>
<th>Run 2</th>
<th>Run 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction time (day)</td>
<td>46</td>
<td>51</td>
<td>60</td>
</tr>
<tr>
<td>Enzyme load (mg)</td>
<td>7</td>
<td>6.6</td>
<td>7</td>
</tr>
<tr>
<td>Molar ratio (octanol/DHCA)</td>
<td>206</td>
<td>244</td>
<td>100</td>
</tr>
<tr>
<td>Conversion (%)</td>
<td>Predicted 70.42</td>
<td>92.16</td>
<td>91.05</td>
</tr>
<tr>
<td>Experiment±SD*</td>
<td>73.66±1.62</td>
<td>88.69±1.14</td>
<td>92.47±2.48</td>
</tr>
</tbody>
</table>

* Standard deviation of duplicated determination from different experiments

4. Conclusion
This work demonstrates the esterification of selected phenolic acids with fatty alcohols from short chain to long chain in a binary solvent system. The effect of solvent system was firstly studied by varying the proportion of hexane and butanone from 85:15 to 45:55 (v/v). The results show that highest conversion of DHCA was achieved when hexane/butanone was mixed in the ratio of 65:35 (v/v). The effect of fatty alcohol chain length was further studied by chosen fatty alcohol from C4 to C18. It was found that the medium chain fatty alcohols were easier to esterify with phenolic acids. Around 95% conversion of DHCA was achieved within 3 days when it was esterified with hexanol, while other fatty alcohols took longer time to reach reaction equilibrium under same conditions. It is also possible to esterify ferulic acid and caffeic acid with fatty alcohols in the system, but the yields are much lower comparing to DHCA. In order to optimize the reaction conditions and increase esterification yield, RSM was employed to investigate the mutual effect among factors of temperature, reaction time, enzyme load, and substrate molar ratio. DHCA and octanol were used as model for evaluation. A prediction model was achieved and it generated an optimized reaction conditions. Base on the optimized condition, the conversion of DHCA was increased greatly.

Acknowledgments
This work was supported by the Strategic Food and Health Program (FoSu) as well as grant from the Graduate School of Science, Aarhus University.
References


Appendix 2: Paper II

Title: Ionic liquid-assisted solublization for improved enzymatic esterification of phenolic acids

Authors: Zhiyong Yang, Zheng Guo, Xuebing Xu*

Journal: Journal of the American Oil Chemists’ Society

(Manuscript Submitted)
Ionic liquid-assisted solublization for improved enzymatic esterification of phenolic acids

Zhiyong Yang · Zheng Guo · Xuebing Xu*

Department of Molecular Biology, Aarhus University, Gustav Wieds Vej 10, 8000 Aarhus C, Denmark

Abstract

Phenolic acids are very difficult to dissolve in most of solvent, which lead to low yield and long reaction time for production of lipophilic phenolic derivatives according to previous studies. This work is focused on increasing production of lipophilic phenolic derivatives catalyzed by Novozym 435 in a binary system, which is composed of ionic liquid rOMA·TFA (trioctylmethylammonium Trifluoroacetate) and octanol. Ionic liquid rOMA·TFA has great solubility towards most of phenolic acid. The strategy of increasing the solubility of phenolic in ionic liquid rOMA·TFA was proved to be an efficient way for increasing conversion of phenolic acids. The mixture ratio between rOMA·TFA and octanol was varied from 1:4 to 1:16 (v/v), it was found that the highest conversion of dihydrocaffeic acid (DHCA) was achieved when rOMA·TFA and octanol was mixed as 1:12 (v/v). It was also found that conversion of DHCA at 70 °C was slightly higher than at 60 °C and 80 °C. Removing water by molecular sieves and vacuum has significant improvement on the conversion. Even though the conversion was much higher when the concentration of DHCA was 0.4 M, the highest reaction rate was observed when the concentration of DHCA was 1.6 M, which was also higher than the parallel experiment where no ionic liquid was applied for increasing solubility of DHCA. Relative high conversion of ferulic and caffeic acids was achieved when similar reactions were conducted with these two phenolic acids, and the conversion in the system containing ionic liquid was significant higher than the system without ionic liquid.

Keywords Phenolic acids · Octanol · Ionic liquid · Novozym 435 · Esterification

*Corresponding author: Xuebing Xu, Department of Molecular Biology, Aarhus University, Gustav Wieds Vej 10, 8000 Aarhus C, Denmark
Tel: +45-89425089, Fax: +45-86123178, Email: xu@mb.au.dk
**Introduction**

Phenolic acids are biologically active antioxidants, which find a variety of applications in pharmaceutical, cosmetic and food industries (1-3). However, most of phenolic acids are hydrophilic compounds and can mainly render their functional properties or health effects in aqueous environments or water compartments (4). Therefore, to apply natural phenolic acids in lipophilic formulation and process, the lipophilization is needed where enzymatic esterification with alcohols has proven to be promising approaches (5-8).

However, there exists a technical dilemma for creating an efficient enzymatic reaction system: phenolic acids have low solubility in most of organic solvents (such as hexane) where the enzyme is active; while in those solvents (such as DMSO) phenolic acids has good solubility but the enzyme is deactivated or shows low activity. Ionic liquids (ILs) are an interesting class of tuneable, designer solvents with essentially zero volatility, which is claimed as “green” media (9). Ionic liquids can act as solvents for diverse reactions and give different selectivity and reactivity compared with conventional organic solvents because the liquid are made of ions rather than molecules (10). In the past 10 years, ionic liquids had been continued to attract attention as a replacement for organic solvents for enzymatic transformation of various compounds (12). Katsoura *et al.* (13) investigated enzymatic esterification of several phenolic acids with short and medium chain fatty alcohol in several ionic liquids, and found out the enzymatic esterifications were very efficient in ionic liquids. More recently, Chen *et al.* (14) reported synthesis of oleyl ferulate in ionic liquid/iso-octane binary system, which improved productivity was obtained.

In a previous work, we have demonstrated the superior performance of trioctylmethylammonium trifluoroacetate (tOMA.TFA) to other types of ILs in improving the conversion and volumetric productivity of ascorbic acids (15). It is anticipated this ionic liquid may be applicable to the reaction system for enzymatic conversion of phenolic acids with preferable performance. In the current study, we investigated tOMA·TFA mediated enzymatic esterification of dihydrocaffeic acid (DHCA) with octanol (Scheme 1), where octanol is selected as a model alcohol because octyl phenolates show better antioxidation property according to our results (data not shown). Effects of dosage of the IL (denoted as ratio of tOMA·TFA/octanol), substrate concentration, reaction temperature, the methods of water removal, etc were examined. To envisage its general applicability, this system was also applied to enzymatic transformation of ferulic and caffeic acids, where significant enhancement in conversion and volumetric productivity was observed in comparison with the absence of the ILs.
**Materials and Methods**

**Materials**

Ionic liquid tOMA·TFA (trioctylmethylammonium Trifluoroacetate) of 99.77% purity was purchased from Merck KGaA (Darmstadt, Germany). Novozym 435 was obtained from Novozymes A/S (Bagsværd, Denmark). Selected phenolic acids and Octanol were bought from Sigma-Aldrich or Fluka with the highest available purity. All other organic solvents of analytical and HPLC grades were purchased from Fisher Scientific Inc. (Slangerup, Denmark).

Enzymatic esterification of octyl phenolic acid esters

All reactions were conducted in 50 ml volume of jacked reactors. For the preliminary experiments, 1 mmol of DHCA was dissolved in 0.5 ml of ionic liquid tOMA·TFA first and then diluted with 2 ml of octanol. In the case of investigating the effect of volume ratio between ionic liquid tOMA·TFA and octanol, the amount of octanol was increased from 2 ml to 8 ml and the volume ratio between ionic liquid tOMA·TFA and octanol was then increased from 1:4 to 1:16 correspondingly. Reactions were initiated by adding 100 mg of Novozym 435 under 80 °C temperature and 300 rpm with or without same amount of molecular sieves (3 Å). Reactions were conducted up to 6 days and samples were withdrawn in regular time for HPLC analysis.
Enzymatic esterifications of ferulic and caffeic acids with octanol were conducted in the similar manner as above. The final concentration of these two phenolic acids was 0.4 M and the mixture volume ratio of ILs/octanol was 1:4. Reactions were conducted at 80 °C with the presence 100 mg of enzyme and molecular sieves respectively.

To the reaction without ILs, the selected phenolic acids were just dissolved in 2.5 ml octanol directly to achieve desire concentration by heating. Other conditions were as same as their parallel reactions correspondingly.

Analysis

The initial reaction rate was calculated from the linearly part within 24 hour. Each experiment was conducted in two replicates. In all cases, a single product was detected by HPLC analysis with UV detector in the range from 200 to 325 nm and the responses at 280 nm (DHCA and caffeic acid) and or 325 nm (ferulic acid) were used for result evaluation. HPLC analysis and LC-MS were conducted in a similar way as described before (16).

Results and Discussions

rOMA·TFA dosage and enzyme activity

Firstly, the effect of reaction volume was evaluated. 1 mmol of DHCA was dissolved in 0.5 ml ionic liquid rOMA·TFA and then further diluted with octanol from 2 ml to 8 ml to give a volume ratio of 1:4 to 1:16 (v/v) between ionic liquid rOMA·TFA and octanol. Te reactions were catalyzed with 100 mg Novozym 435 at 80 °C and 300 rpm without presence of molecular sieves. As observed in Fig.1, the conversion of DHCA was around 60% in 120 h when the volume ratio of ionic liquid rOMA·TFA to octanol was 1:4 (v/v), and there was almost no too much change on the conversion when the volume ratio was increased to 1:8. When the volume ratio was increased to 1:12 (v/v), the conversion was increased to around 70%. However, further increased reaction volume to 1:16 does not seem to can further increase the conversion of DHCA. On the other hand, if the volume ratio between ionic liquid and octanol was too low (below 1:2), no conversion of DHCA was observed (data not shown), which is probably due to the inhibition effect of the ionic liquid rOMA·TFA to the catalyzed enzyme Novozym 435.
Appendix 2: Paper II

Effect of temperature

It has been reported that Novozym 435 is a heat-tolerant, immobilized enzyme with a maximum activity at 70-80 °C according the Novozym 435 product sheet, and the suggested enzyme should be used at 40-60 °C for the sake of stability (17). However, a previous study conducted esterification of various phenolic acids with long-chain fatty alcohol catalyzed by Novozym 435 and other immobilized lipases at 80 °C, and it showed the enzymes were still stable under this moderate temperature and a high conversion rate was achieved (7). In this work, preliminary experiments were conducted under 80 °C and catalyzed by Novozym 435 without adding molecular sieves. The conversion of DHCA in 5 days under these conditions was above 60% (Fig. 2). However, when reaction was carried out at 70 °C, there was 10% increase compared to at 80 °C. It is interesting to see the conversion of DHCA was only above 50% when reaction was conducted at 60 °C, which is almost 10% and 20% lower than previous conditions.

Fig. 1. Effect of reaction volume ratio of TMA-TFA/octanol (v/v) on conversion of DHCA (○, 1:4; ◇, 1:8; △, 1:12; □, 1:16). Reactions were conducted at 80 °C and catalyzed by 100 mg of Novozym 435 without molecular sieves or vacuum. Concentration of DHCA was 0.4 M.
The reaction between DHCA and octanol is esterification reaction. During the esterification reaction, water was produced as by product. Although Novozym 435 needs certain amount of water to maintain its catalytic activity, too much water will not be benefit to the esterification reaction toward the production of target product. Besides, more water in the reaction system might lead to the aggregation of the enzyme, which would have a negative effect on the reaction rate (18). In Fig. 3, the effect of removing water by molecular sieves or vacuum was investigated. As indicates in the figure, when vacuum was applied to remove water from the reaction, conversion of DHCA was increased 20% compared to the reaction without vacuum or molecular sieves under the same conditions. However, there was almost no difference on conversion when vacuum was decreased from 800 mbar to 400 mbar. The effect of adding molecular sieves (100 mg) seems to have better performance than the effect of vacuum. About 86% of conversion was achieved in 5 days under this condition, and the conversion can be further increased up to 90% when the reaction system was supplied 20 mg/ml of molecular sieves each days. However, the initial reaction rates were quite close for the reactions removing water by above methods and they are almost twice higher comparing to the reaction without adding molecular sieves or applying vacuum to remove water.
Effect of substrate concentration

The effect of increasing concentration of DHCA on conversion was also studied by varying concentration of DHCA from 0.4 M to 1.6 M. However, it was noticed that DHCA can only be totally dissolved in higher concentration when it was heated above 60 °C, and 1.6 M is almost the maximum concentration that DHCA can be dissolved by the system. It was noticed using ionic liquid rOMA·TFA as assistant solvent to increase solubility of DHCA can increase the conversion of DHCA greatly, especially when the concentration of DHCA was high. When the concentration of DHCA was increased to 1.2 M, the conversion of DHCA in 5 days was 62% when ionic liquid rOMA·TFA was added in the reaction system, which is about 8% higher than the system where ionic liquid rOMA·TFA was not used as assistant solvent (Fig. 4A).
According to Michaelis–Menten kinetic model of a single-substrate reaction, the reaction rate is increasing as the increasing of substrate before enzyme is saturated and reached maximum reaction rate. This model can be used to explain what we observed in Fig. 4B, which denotes the relationship of reaction rate with the concentration of DHCA. As indicates in this figure, the reaction rate was 68 mM h\(^{-1}\) (g enzyme\(^{-1}\)) when the concentration of DHCA was 0.4 M and reaction was conducted in binary solvent system of ionic liquid \(t\)OMA·TFA /octanol, and the initial reaction rate was gradually increased to 244 mM h\(^{-1}\) (g enzyme\(^{-1}\)) when concentration of dihydrocaffeic acid was increased to
1.6 M. In general, this study is agreed with the previous report which studied the relationship of concentration and reaction rate by using p-coumaric acid and cinnamic acid (6).

Interestingly, the initial reaction rate was 178 mM h\(^{-1}\) (g enzyme\(^{-1}\)) when reaction was conducted in the reaction system without ionic liquid and the concentration of DHCA was 0.4 M, then the reaction rate decreased to 127 mM h\(^{-1}\) (g enzyme\(^{-1}\)) when the concentration was brought to 0.8 M. Afterwards, the initial reaction rate was increased again and reached 212 mM h\(^{-1}\) (g enzyme\(^{-1}\)) when concentration was increased to 1.6 M. The possible explanation to this phenomenon is, solubility of DHCA in octanol is lower than in ionic liquid tOMA·TFA, and the enzyme activity may be inhibited by mass-transfer when the concentration of DHCA was increased to the level beyond the solubility of DHCA in octanol. On the other hand, adding ionic liquid tOMA·TFA as co-solvent may increase the solubility of DHCA, but it also make the reaction system to be less hydrophobic than octanol at the same time, because ionic liquid tOMA·TFA are amphiphile molecule as shown in Scheme 1, and it has been said that Novozym 435 has better performance in hydrophobic environment (19). Moreover, ionic liquid tOMA·TFA may also increases the viscosity of the system as ionic liquid tOMA·TFA is quite sticky. Viscosity of the reaction medium may control the enzyme activity by affecting the mass-transfer limitations and lead to lower reaction rate (20). These may explain why the initial reaction rate was much higher when the concentration of DHCA was 0.4 M in the system without ionic liquid tOMA·TFA than the parallel reactions. Nevertheless, adding ionic liquid tOMA·TFA as assistant solvent can increase solubility of DHCA greatly and result in higher initial reaction rate, which will lead to higher productivity of desired products as observed in the systems containing ionic liquid tOMA·TFA with higher concentration of DHCA (1.2 M or 1.6 M).

Esterification of other selected phenolic acids

In previous studies (5-8, 21-23), different phenolic acids from cinnamic serials have been used for esterification in various media. It was found esterification was possible for the phenolic acids which aromatic cycle was not parahydroxylated and the side chain was saturated. Ferulic acid was only possible to be esterified with fatty alcohol more than eight carbons, but in low yields (3). In a recently study (13), various cinnamic acid derivatives were chosen for conjugation with short and media chains (C1-C10) of fatty alcohols in different ionic liquids. They achieved relative high yield in short time, but the yield of esterification of ferulic acid and caffeic acid with octanol were still relative low.
In this study, ferulic acid and caffeic acid were also chosen for evaluation in this binary solvent system. Reactions were conducted either with or without presence of ionic liquid rOMA-TFA, and the effect of ionic liquid rOMA-TFA is more significant to these two cinnamic acids. As shown in Fig. 5, the conversion of ferulic acid can reach 86% after 16 days of reaction when there is ionic liquid rOMA-TFA presented, but only 58% was achieved if ionic liquid rOMA-TFA was not added into the reaction system. More interestingly, the conversion of caffeic acid reached 60% after 12 days of reaction when ionic liquid was added in the reaction system as co-solvent, but only 14% was achieved when there was no ionic liquid in the reaction system. These results indicate that conversion of these two cinnamic acids can be significant increase by increasing their solubility with ionic liquid rOMA-TFA.

Fig. 5. Conversion of ferulic acid (A) and caffeic acid (B) in the reaction system with ionic liquid rOMA-TFA (◇) or without ionic liquid rOMA-TFA (□). Ionic liquid rOMA-TFA and octanol was mixed in the ratio of 1:4 (v/v). Concentration of these two phenolic acids were both 0.4 M. Reactions were catalyzed with 100 mg of enzyme with the presence of 100 mg molecular sieves at 80 °C.
Conclusion

This work demonstrates a binary solvent system composed of ionic liquid/octanol for synthesis of lipophilic cinnamic derivative. It was found that the solubility of selected phenolic acids (dihydrocaffeic acid, ferulic acid, and caffeic acid) can be greatly improved by dissolved in ionic liquid rOMA·TFA. Several factors have been evaluated for increasing conversion of DHCA. Both increasing the volume mix ratio (rOMA·TFA/octanol, v/v) and temperature to some extent can increase the conversion rate. Molecular sieve is a good drying material for removing water from the reaction system and increasing conversion greatly. The conversion of DHCA decreased as the concentration was increased. However, the reaction rate was much higher when the concentration of DHCA was increased, which resulted in higher productivity. The binary system was also efficient on synthesis other lipophilic phenolic derivatives by using ferulic and caffeic acids. Conversions of both ferulic and caffeic acids were higher than previous studies. Parallel experiments were also conducted without adding ionic liquid rOMA·TFA as co-solvent to compare above experiments where ionic liquid were used as co-solvent. The results showed using ionic liquid rOMA·TFA as co-solvent to increase the solubility of phenolic acids can significantly increase the conversion.

Acknowledgments

This work was supported by the Strategic Food and Health Program (FoSu) as well as grant from the Graduate School of Science, Aarhus University.
References


Appendix 3: Paper III

Title: Improved enzymatic production of phenolated acylglycerols through alkyl phenolate intermediates

Authors: Zhiyong Yang, Vivian Feddern, Marianne Glasius, Zheng Guo, Xuebing Xu

Journal: Biotechnology Letter

Volume: 33

Page: 673-679
Improved enzymatic production of phenolated acylglycerols through alkyl phenolate intermediates

Zhiyong Yang · Vivian Feddern · Marianne Glasius · Zheng Guo · Xuebing Xu

Received: 23 October 2010/Accepted: 17 November 2010/Published online: 1 December 2010
© Springer Science+Business Media B.V. 2010

Abstract A novel approach is reported for the synthesis of dihydrocaffeoylated glycerols that consists of two steps: enzymatic synthesis of octyl dihydrocaffeate (as a synthetic intermediate) from octanol and dihydrocaffeic acid, and enzymatic interesterification of triacylglycerols with octyl dihydrocaffeate. Due to the good compatibility of the intermediate with triacylglycerols, an improved volumetric productivity [147 mol h⁻¹(kg Novozym 435)⁻¹] and high enzyme specific activity [up to 9.6 μmol⁻¹ min⁻¹(g Novozym 435)⁻¹] have been obtained.

Keywords Alkyl phenolate · Dihydrocaffeic acid · Lipase · Phenolic acids · Interesterification

Introduction

Phenolic acids are antioxidants that are widely distributed in fruits, vegetables, spices, and aromatic herbs. However, their applications in oil-based food processing and cosmetic industries are limited due to their low solubility in hydrophobic media (Buismann et al. 1998; Figueroa-Espinoza and Villeneuve 2005). To improve the lipophilicity of phenolic acids, an alternative method is the incorporation of phenolic acids into triacylglycerols through enzymatic reactions (Compton et al. 2000, 2006; Sabally et al. 2005, 2006, 2007; Laszlo and Compton 2006). However, direct transesterification of phenolic acids with triacylglycerols is generally suffered from long reaction time and low efficiency (Figueroa-Espinoza and Villeneuve 2005; Compton et al. 2000). Thus, many efforts, for example, through medium engineering to improve solubility of phenolic acids (Sabally et al. 2006; Lue et al. 2005), and through chemo-enzymatic approach (Sun et al. 2009), have been made to improve the efficiency of related reactions.

This work reports a two-step approach for enzymatic synthesis of phenolated glycerols with dihydrocaffeic acid (DHCA) as a model phenolic acid. An intermediate product, octyl dihydrocaffeate, was first synthesized by lipase-catalyzed esterification of DHCA with octanol. Then, the target products were synthesized through enzymatic interesterification between octyl dihydrocaffeate and triacylglycerol. To develop this approach was from the following considerations: (1) octyl
dihydrocaffeate is supposed to have better compatibility with triacylglycerols than DHCA or short-chain alcohol ester like ethyl dihydrocaffeate; (2) Good compatibility of octyl dihydrocaffeate with triacylglycerols allows a solvent-free reaction to occur; hence a faster reaction and a better productivity could be expected. Response surface methodology (RSM) was employed for reaction optimisation based on a preliminary parameter study. The validation reactions were conducted based on the model optimized reaction conditions. For comparison with the protocol developed in this work, the reactions in other solvent and solvent-free systems were also conducted.

 Materials and methods

 Materials

 Dihydrocaffeic acid, tricaprylin (>98%) and octanol (>99%) were purchased from Sigma-Aldrich Co. Novozym 435 (Candida antarctica lipase B), Lipzyme RM IM (Rhizomucor miehei) and Lipzyme TL IM (Thermomyces lanuginosus) were obtained from Novozyme A/S ( Bagsvaerd, Denmark). All other solvents were of analytical or HPLC grades.

 Preparing octyl dihydrocaffeate

 Octyl dihydrocaffeate was synthesized according to the method of Sabally et al. (2005) with some modifications. The reaction was terminated by removing enzyme through filtration and the unreacted octanol were removed through vacuum evaporation at 70°C. The resulting product was re-dissolved in hexane and washed five times with 0.5 M NaCl to remove the remaining DHCA. The obtained octyl dihydrocaffeate was ∼97% pure (containing 3% DHCA) according to HPLC analysis.

 Interesteralification

 Interesteralification of octyl dihydrocaffeate with tricaprylin was conducted in a 100 ml thermostat reactor. For a typical reaction, 0.154 g octyl dihydrocaffeate (0.5 mmol) was mixed with 0.705 g tricaprylin (1 mmol). Interesteralification was initiated by adding 10% (w/v) immobilized enzyme (on the mass basis of octyl dihydrocaffeate) at 60°C and stirred magnetically (300 rpm). Sample aliquots from reaction mixture were withdrawn and diluted 100 times by methanol, then subjected to HPLC analysis after removing solid impurity by centrifugation (∼10000 rpm for 10 min).

 For comparison, other two reaction systems: solvent-free direct transesterification of DHCA with tricaprylin and hexane-mediated interesterification of octyl dihydrocaffeate with tricaprylin were conducted under other identical conditions, respectively. Namely, for the former system 0.5 mmol DHCA was reacted with 1 mmol tricaprylin and for the latter system 0.5 mmol octyl dihydrocaffeate with 1 mmol tricaprylin in 10 ml hexane.

 HPLC analysis

 HPLC analysis was performed on a RP C18-column (250 × 4.6 mm, 5 μm). 10 μl samples were eluted by 90% solvent A (methanol) and 10% solvent B (water with 0.75% acetic acid) for 16 min at 1 ml/min. Detection was at 284 nm.

 The conversion of octyl dihydrocaffeate was calculated based on the deduction of the area percentage of octyl dihydrocaffeate on the basis of all components concerning dihydrocaffeoyl moiety (including DHCA) in reaction mixture. The yield of individual dihydrocaffeoylated glycerol was calculated as the area percentage of the compound in the total areas of the components concerning dihydrocaffeoyl moieties. The enzyme activity was calculated from the initial reaction rate based on the conversion of octyl dihydrocaffeate versus reaction time. The unit of enzymatic activity was defined as μmol octyl dihydrocaffeate consumed per min catalyzed by per g immobilized enzyme preparation.

 HPLC–ESI–MS analysis

 HPLC–ESI–MS analyses were performed with an electrospray ionisation (ESI) coupled to a quadrupole time-of-flight mass spectrometer (Bruker microOTOF-Q, Bremen, Germany). The column used and elution conditions for HPLC were the same as for HPLC analysis. Ionisation was performed in the negative mode with 81 N/min at 0.8 bar nebuliser pressure and 190°C. Scan range was from 50 to 1200 m/z.
Experimental design by RSM

Response surface methodology was employed to optimize parameters of lipase-catalyzed interesterification between octyl dihydrocaffeate and tricaprylin. The software Model 8.0, Umetrics (Umeå, Sweden) was used to design the reaction sets and fit the experimental data. Four factors were chosen for investigation with three-level setting: temperature (40, 55 and 60°C), molar ratio of tricaprylin/octyl dihydrocaffeate (1/1, 3/1, 5/1), reaction time (0.5, 24.25 and 48 h) and enzyme load (50, 150 and 250 mg). Central-Composite-Face-centred (CCF) design combined with RSM was used to fit full second-order polynomial model.

Total 27 reactions based on RSM designed parameters were conducted, and the experimental results (conversion of octyl dihydrocaffeate and yield of dihydrocaffoylated glycerols) were then used for model fitting and regression analysis to generate the 2 s polynomial models (the equations not shown). Two sets of optimized parameters could be generated based on the RSM model prediction. Two experimental reactions were thus conducted using the model optimized conditions to validate the model predictions.

All the reactions in this work were conducted in two replicates, and the means ± standard deviations of the data from two replicates (within 95% confidence limitation) were used for evaluation of the results.

Results and discussion

Establishment of reaction protocol

The first step was to synthesize high purity intermediate product—octyl dihydrocaffeate. This was implemented successfully by Novozym 435-catalyzed esterification of DHCA with octanol in 2-butanol/hexane (1:3; v:v) binary solvent system according to the method of Sabally et al. (2006). HPLC analysis showed the purity of the resulting product was 97% with minor unreacted DHCA. The octyl dihydrocaffeate was then used for interesterification with tricaprylin for synthesis of dihydrocaffoylated glycerols in a solvent free system.

Scheme 1 shows that the possible products from interesterification of octyl dihydrocaffeate with tricaprylin. Based on HPLC analysis results, four major peaks were detected in the reaction mixture, and their structures were further identified by HPLC ESI–MS analysis. The first peak (eluted after 3.14 min) corresponded to products of mono- and di-DHCA glycerol (Compound D and E in Scheme 1) with molecular ion peaks at m/z 255 and 419 ([M–H]−), respectively. The first peak also contained DHCA (181, [M–H]−) (Compound F in Scheme 1), which was in lower abundance than other two products identified. It probably came from the substrate which contains approx. 3% DHCA. The second peak (3.57 min) was identified as di- and mono-DHCA monocaprylin (Compound B and C in Scheme 1) with molecular ion peaks at m/z 345 and 381 ([M–H]−), respectively. Analysis of the ion of the third peak (4.13 min) indicated it was octyl dihydrocaffeate (m/z, 293 [M–H]−). Similarly, MS analysis of the last peak (5.21 min) showed it corresponded to mono-DHCA dicaprylin with molecular ion peaks at m/z 507 ([M–H]−) (Compound A in Scheme 1). Thus, through HPLC and HPLC–MS analysis, the reaction protocol was established and the structures of desired products were confirmed.

Comparison of different reaction systems

For comparison of the efficiencies of different reaction systems, we also conducted direct transesterification of DHCA with tricaprylin in a solvent free system, and interesterification of octyl dihydrocaffeate with tricaprylin in hexane which the same intermediate was used but performed in solvent instead of solvent-free system (Fig. 1). In terms of conversion, for direct transesterification DHCA with tricaprylin only 3.2% conversion of DHCA could be achieved after 72 h. In contrast, for solvent-free interesterification of octyl dihydrocaffeate with tricaprylin around 60% conversion of octyl dihydrocaffeate could be reached at 24 h and at 72 h the conversion added up to over 70%. However, further dilution of octyl dihydrocaffeate-tricaprylin by hexane did not improve the conversion of octyl dihydrocaffeate; instead it was decreased (14% at 24 h). Poor mixing (or low solubility of DHCA in tricaprylin) will result in a less effective dynamic molecular interaction, which might account for low reaction of DHCA with tricaprylin (Fig. 1). Introducing hexane
Scheme 1 Possible products from interesterification between octyl dihydrocaffeate and tricaprylin. A mono-DHCA dicaprylin; B di-DHCA monocuprylin; C mono-DHCA monocuprylin; D mono-DHCA acylglycerol; E di-DHCA acylglycerol; F dihydrocaffeic acid (DHCA). (The potential isomers not shown.)

to the octyl dihydrocaffeate-tricaprylin system generated a negative effect, contradicting to a possible benefit from solvent introduction—improving mass transfer. The reason for this is not clear; however, the results seem suggest that, compared to solvent-free system, hexane-mediated system showed lower enzyme activity and thus resulted in a lower conversion of octyl dihydrocaffeate (Table 1).

To quantify the efficiency of these three systems further, we calculated the individual enzyme specific
As indicated in Table 1, the saliva-free, octyl dihydrocaffeate-tricaprilin intermediate system was superior to the other two systems in terms of volumetric productivity (15 and 34 times higher, respectively). It also can be estimated that the system developed in this work is also much higher than that developed in previous work [Shahly et al., 2006].

Table 1: Effects of enzymes and substrates on conversion of octyl dihydrocaffeate and yield of dihydrocaffeoylated glycerides

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme</th>
<th>Solvent</th>
<th>Enzyme A activity (μmol g⁻¹ min⁻¹)</th>
<th>Conversion (%)</th>
<th>Yield (%)</th>
<th>Volumetric productivity (mol h⁻¹ (kg enzyme)⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octyl dihydrocaffeate/tricaprilin (1/2)</td>
<td>Lipoyzyme RMIM</td>
<td>No</td>
<td>0.44 ± 0.02</td>
<td>14.7 ± 2.4</td>
<td>8.8 ± 2.02</td>
<td>21.3 ± 4.12</td>
</tr>
<tr>
<td>Octyl dihydrocaffeate/tricaprilin (1/2)</td>
<td>Lipoyzyme TILM</td>
<td>No</td>
<td>0.57 ± 0.02</td>
<td>12.8 ± 0.5</td>
<td>6.9 ± 0.09</td>
<td>12.2 ± 3.99</td>
</tr>
<tr>
<td>Octyl dihydrocaffeate/tricaprilin (1/2)</td>
<td>Novozym 345</td>
<td>No</td>
<td>9.6 ± 0.06</td>
<td>72.6 ± 0.61</td>
<td>35.6 ± 0.35</td>
<td>147 ± 2.14</td>
</tr>
<tr>
<td>DHC/A/tricaprilin (1/2)</td>
<td>Novozym 345</td>
<td>No</td>
<td>0.05 ± 0.02</td>
<td>3.22 ± 1.8</td>
<td>1.11 ± 0.97</td>
<td>9.1 ± 4.37</td>
</tr>
<tr>
<td>Octyl dihydrocaffeate/tricaprilin (1/2)</td>
<td>Novozym 435</td>
<td>Hexane</td>
<td>2.3 ± 0.01</td>
<td>24.3 ± 3.40</td>
<td>9.2 ± 1.2</td>
<td>4.9 ± 0.11</td>
</tr>
</tbody>
</table>

* Product 1 represents yield of di-DHCA/mono-DHCA monocaprylin
* Product 2 represents yield of mono-DHCA dicaprylin
* Volumetric productivity was calculated as conversion of octyl dihydrocaffeate or DHCA to dihydrocaffeoylated glycerides based on the reaction in 48 h
Fig. 2 Time course of Novozym 435 catalyzed interesterification of octyl dihydrocaffeate with tricaprylin. Open square yield of di-DHCA/mono-DHCA monocaprylin; Open triangle yield of mono-DHCA dicaprylin. Open diamond yield of mono-DHCA/di-DHCA acylglycerol. Open circle percentage of octyl dihydrocaffeate

Reaction time course and effects of lipase species

Figure 2 displays a typical time course of Novozym 435 catalyzed interesterification of octyl dihydrocaffeate with tricaprylin in a solvent-free system. As depicted, three groups of phenolic derivatives from octyl dihydrocaffeate are generally converted in the concentration di-DHCA/mono-DHCA monocaprylin > mono-DHCA dicaprylin > mono-DHCA/di-DHCA glycerol. This indicated that the reaction simultaneously proceeds in different stages of the interesterification. Less mono-DHCA/di-DHCA glycerol might be a natural result with excessive tricaprylin and little water presence. As can be seen, this solvent-free system was intrinsically fast: within 24 h 60% of octyl dihydrocaffeate converted to products. After another 24 h the reaction conversion reached 70%, thereafter the increases of products levelled off, indicating the reaction came close to the equilibrium of the reaction. Compared with the system previously reported (Sabally et al. 2006; Safari et al. 2006), this system is faster since most of the other systems needed 4–5 days to reach equilibrium. Moreover, this result also presented an important hint for the level setting of reaction time for the following RSM optimization.

In order to determine the appropriate biocatalyst, three commercially available immobilized enzymes were examined for their capacity to catalyze interesterification of octyl dihydrocaffeate with tricaprylin (Table 1). In terms of specific activity, Lipozyme TL IM (0.57 μmol g⁻¹ min⁻¹) was not so different from Lipozyme RM IM (0.44 μmol g⁻¹ min⁻¹), whereas Novozym 435 was significantly higher (9.57 μmol g⁻¹ min⁻¹). This fact again proved that Candida antarctica lipase B (Novozym 435) was a robust lipase for synthesis application as observed in many different systems (Compton et al. 2000, 2006; Sabally et al. 2005, 2006; Guyot et al. 1997). Therefore, Novozym 435 was chosen as a biocatalyst for RSM optimization.

RSM optimization and experimental validation

RSM was applied to investigate the effects of temperature, reaction time, enzyme load, and substrate molar ratio on the conversion of octyl dihydrocaffeate and yield of dihydrocaffeoylated glycerides (the details of statistical analysis not shown). Based on RSM model prediction, two parameter settings could be generated with expected high yield and conversion (Table 2).

Two experiments were thus conducted based on the predicted optimum conditions to validate the RSM model predictions (Table 2). As shown in Table 2, the experimental results generally agreed with the predicted values, which confirmed the validity and adequacy of the model prediction. Moreover, high substrate conversion and yield of desired dihydrocaffeoylated glycerides in the validation tests were achieved in a relatively shorter reaction time (24–44 h), indicating an intrinsic advantage over the reported systems (Sabally et al. 2006).

<table>
<thead>
<tr>
<th>Table 2 Optimum conditions predicted by RSM model and the results from validation reactions</th>
<th>Run 1</th>
<th>Run 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>70</td>
<td>65.2</td>
</tr>
<tr>
<td>Reaction time (hour)</td>
<td>24.25</td>
<td>43.6</td>
</tr>
<tr>
<td>Enzyme load (%)</td>
<td>14.6</td>
<td>19.9</td>
</tr>
<tr>
<td>Molar ratio (tricaprylin/ octyl dihydrocaffeate)</td>
<td>3</td>
<td>4.97</td>
</tr>
<tr>
<td>Conversion (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Predicted</td>
<td>81.33</td>
<td>91.91</td>
</tr>
<tr>
<td>Experimental</td>
<td>79.8 ± 0.37</td>
<td>86.5 ± 0.21</td>
</tr>
<tr>
<td>Yield (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Predicted</td>
<td>76.61</td>
<td>89.86</td>
</tr>
<tr>
<td>Experimental</td>
<td>76.5 ± 0.22</td>
<td>78 ± 1.75</td>
</tr>
</tbody>
</table>

* Experimental data are means ± standard deviations of two replicates
Conclusions

A novel route for enzymatic synthesis of dihydrocaffeoylated glycerides with octyl dihydrocaffeate as a synthetic intermediate has been established. The results demonstrated that the new approach has distinct advantages over reported solvent systems, namely, faster reaction rate and higher volumetric productivity. Optimized reaction conditions were generated through RSM optimization. In two validation reactions based on optimized conditions, further improved conversion of octyl dihydrocaffeate and yield of dihydrocaffeoylated glycerols were obtained.

Acknowledgments Financial support from the Strategic Food and Health Program (FoSu) and the Grant from the Graduate School of Science, Aarhus University are gratefully acknowledged.

References

Appendix 4: Paper IV

Title: Enzymatic Transesterification of Ethyl Ferulate with Fish Oil and Reaction Optimization by Response Surface Methodology

Authors: Zhiyong Yang, Marianne Glasius, Xuebing Xu*

Journal: Food Technology & Biotechnology (accepted, March 2011)
Enzymatic Transesterification of Ethyl Ferulate with Fish Oil and Reaction Optimization by Response Surface Methodology

Zhiyong Yang\textsuperscript{1}, Marianne Glasius\textsuperscript{2}, Xuebing Xu\textsuperscript{*}

\textsuperscript{1}Department of Molecular Biology, Aarhus University, Gustav Wieds Vej 10, 8000 Aarhus C, Denmark
\textsuperscript{2}Department of Chemistry, Aarhus University, Langelandsgade 140, 8000 Aarhus C, Denmark

Summary

The enzymatic transesterification of ethyl ferulate (EF) with cod liver fish oil was investigated with Novozym 435 as catalyst under solvent-free conditions. The purpose of the study is to evaluate the synthesis system for production of feruloyl fish oil in industry. The modified HPLC method was firstly set up to characterise the reaction products together with HPLC-ESI-MS. The adding of glycerol to the system on the profile of feruloyl acylglycerol species was investigated in terms of transesterification performance. The bioconversion rate of EF can be significantly increased with increased formation of feruloyl fish oil products as well when appropriate amount of glycerol was present in the reaction. Therefore, the addition of equivalent molar amount of glycerol to EF was decided for the practical optimization of the system. The mutual effects of temperature (40 to 70 °C), reaction time (1 to 5 days), enzyme load (2 to 20\%) and substrate amount ratio of fish oil/EF (1 to 5) were thus studied with assistance of response surface methodology (RSM) for the purpose of maximizing the formation towards feruloyl fish oil. The models were well fitted and verified. The optimized conditions were found to be: temperature, 70 °C; enzyme load, 4.3\%; substrate ratio, 4.7; and reaction time, 5 days. Under these conditions, the maximum conversion of EF could reach 92.4\%, and formation of feruloyl fish oil could reach 80.4 \%. But the formation of by product was minimized to 11.4\% only.

Key words: Transesterification, ethyl ferulate, fish oil, HPLC and HPLC-ESI-MS, Feruloyl acylglycerol species, RSM

\*Corresponding author: Xuebing Xu, Department of Molecular Biology, Aarhus University, Gustav Wieds Vej 10, 8000 Aarhus C, Denmark. Tel: +45-89425089, Fax: +45-86123178, Email: xu@mb.au.dk
Introduction

Interests in long chain omega-3 fatty acids (PUFA) were increased over the past 20 years due to their potential health benefits. It has been found that omega-3 fatty acids are essential for normal growth and development. They also play an important role in the prevention and treatment of coronary artery disease, hypertension, diabetes, arthritis, other inflammatory and autoimmune disorders and cancer (1, 2). However, omega-3 fatty acids, particularly EPA (C20:5 n-3) and DHA (C22:6 n-3), are very easy to be oxidized due to rich contents of unsaturated double bonds. Oxidized fish oil or omega-3 fatty acids will give rise to a serial of problems because they will develop undesirable fishy off-flavours, colour deterioration and loss of endogenous antioxidants. Moreover, the free radicals generated from lipid autoxidation can react with proteins and pigments, which together will lead to decreased nutritional quality and presence of toxic lipid oxidation products (3).

For the purpose to prevent or delay lipid oxidation and increase the application of fish oil in industry, one of the popular methods is to add antioxidants. The phenolic acid family is naturally available and good antioxidants as reported (4-6). Ferulic acid (E-3-(4-hydroxy-3-methoxy-phenyl) prop-2-enoic acid) is a common polyphenolic compound abundant in vegetables. Beside the potent antioxidant property, ferulic acid was also found to have medical effects on age-related diseases such as neurodegenerative disorders, cardiovascular diseases, diabetes and cancer (7). However, the application of ferulic acids as antioxidants is limited due to the relatively low solubility in aprotic media. One of realistic ways to improve its solubility is to modify the functional properties of the original phenolic compounds to obtain an amphiphilic molecule, which is normally achieved by conjugating the carboxylic acid function with fatty alcohol or triglycerides through chemical or enzymatic lipophilization. The new amphiphilic molecule will still keep its original functional properties as antioxidant because the hydroxyl groups associated with phenolic compounds may readily donate an electron or proton to intercept and convert free radicals to a more stable compound (6). On the other hand, the new amphiphilic molecule contains both hydrophobic head (fatty acid moiety) and hydrophilic tail (phenolic moiety) possesses surface activity. It can be located in oil-water interfaces or water-oil interfaces, where the antioxidants can function in a different way in particular emulsion systems. Ideally we look forward to a better protection/encapsulation through surface active antioxidants. Research work is certainly going on to explore the concept as lipid oxidation is most frequently occurred in the interface area of the emulsion systems (6, 8). Initial results have shown certain positive aspects for the lipophilized antioxidants in emulsion systems (9-10).
There have been previously many studies on synthesis of feruloyl ester using different approaches (11-18). The studies showed that, in general, Novozym 435 from Candida antarctica is an efficient enzyme for such synthesis. Sabally et al. reported the transesterification of dihydrocaffeic acid with two oils in organic solvents, but the reaction was long and the yields of structure lipids were also low (19, 20). Thus, a practically mature system is not readily available for the synthesis of conjugated products from ferulic acid and fish oil for the purpose to obtain a product with high content of feruloyl fish oil.

Therefore in the present study, a systematic approach was conducted aiming for practical synthesis of those transesterified products from ethyl ferulate and fish oil. The addition of glycerol to the system was firstly evaluated thoroughly for the purpose to increase the reaction efficiency. The decided system was further optimized with the assistance of response surface methodology (RSM). The target was to maximize the formation of feruloyl fish oil.

Materials and methods

Materials

Immobilized lipase from Candida antarctica (Novozym 435) was obtained from Novo Nordisk (Bagsværd, Denmark). EF (ethyl 4-hydroxy-3-methoxy cinnamate) was purchased from SYNTTHON Chemicals GmbH & Co. KG, Germany (Cas-No. 4046-02-0) with 98% purity. Fish oil (Cod liver oil) was a gift donated by Tine Ingredient (TINA BA, Oslo, Norway). Triolein with 90% purity was purchased from Dr. Frischer GmbH (Bremen, Germany). Organic solvents of analytical and HPLC grades were purchased from Sigma-Aldrich, Germany.

Transesterification reaction of EF with fish oil

Reactions were conducted in 50 ml jacketed reactor and protected from light. A thermostat water bath was used to maintain the reaction temperature at 60 °C. 2 mmol of fish oil was mixed with 1 mmol of EF. For the purpose of investigating effect of glycerol on transesterification reaction, glycerol was supplied from 0 to 5 mmol. The reaction was started by adding 10% of Novozym 435 on the basis of substrates mass and stirred (300 rpm) by a magnetic plate. Water produced during the reaction was removed by vacuum at 5 mbars with a vacuum pump. Samples of the reaction products were withdrawn at various intervals. 10 µl reaction mixtures was dissolved in 90 µl acetone and then further diluted 10 times by methanol. Samples were centrifuged and subjected to HPLC analysis afterwards. The same reaction conditions were also applied for synthesis of feruloyl mono- and di-oleoyl-glycerols, where 2 mmol triolein was mixed with 1 mmol EF with 1 mmol of glycerol added to increase the transesterification rate. The reaction was
continued for up to 5 days and the reaction mixture was subjected to LC-TOF-MS analysis using the same analysis method of normal HPLC analysis.

**HPLC analysis**

The transesterification reaction was monitored by HPLC analysis, using a procedure modified from Sun *et al.* (21) and Compton *et al.* (11). The analysis of reaction components was performed with a HPLC system purchased from Thermo Fisher Scientific (Copenhagen, Denmark), which equipped with a RP C18 column (250×4.6 mm, 5 µm), a LC pump, an autosampler, and a PDA detector. Data were analysed by equipped program ChromQuest 5.0. Elution was conducted with solvent A (methanol) and solvent B (contain 0.75% of acetic acid) at a flow rate of 1 ml/min. The elution gradient was initiated with 50% solvent A, and increased to 100% in 10 min, which was maintained for 10 min before reverse to 90% in another 5 min. The gradient was brought back to initial condition in 5 min. Injection volume of sample was 10 µl and feruloyl acylglycerol species were detected under 325 nm of UV adsorption. The total bioconversion of EF was calculated by comparing ratio of EF among the total feruloyl acylglycerol species (including EF and FA) based on peak area with its ratio before reaction. Formation of feruloyl acylglycerol species were determined by comparing the total peak area of each species with total peak areas of all feruloyl acylglycerol species. Duplicate analyses were conducted and standard deviations were below 6%.

**HPLC-ESI-MS analysis**

HPLC-ESI-MS analyses were performed with elution conditions being identical to those described previously (19-21). Mass spectroscopy was conducted using an electrospray ionization (ESI) coupled to a quadrupole time-of-flight mass spectrometer (TOF-MS, Bruker micrOTOF-Q, Bremen, Germany). Ionization was performed in the negative mode with an 8 L/min nitrogen flow, 0.8 bar nebulizer pressure and a temperature of 190 ºC. Scan range was from 50–1,200 m/z.

**Experiment designs**

Response surface methodology (RSM) was used to study the effects of reaction time, temperature, enzyme load, and substrate amount ratio. A three-level and four-factor CCF design (a central composite design) was employed in this study. The variables and their levels selected for the study were reaction time (1, 3 and 5 days), temperature (40, 55 and 70 ºC), enzyme load (2, 11 and 20 wt% based on the total weight of substrates) and amount ratio between fish oil and EF (1, 3 and 5). Table 1 shows the independent factors (x_i), levels, and experimental design. Modde 8.0 (Umetrics AB, Umeå, Sweden) was used for assistance.
### Table 1. Experimental designs and correspondent observed as well as predicted responses.

<table>
<thead>
<tr>
<th>Trials</th>
<th>Temperature (°C)</th>
<th>Enzyme Load (%)</th>
<th>Amount Ratio (Fish oil/EF)</th>
<th>Time (Day)</th>
<th>Bioconversion of EF (%)</th>
<th>Observed (%)</th>
<th>Predicted (%)</th>
<th>Formation of feruloyl-sn-glycerol (%)</th>
<th>Observed (%)</th>
<th>Predicted (%)</th>
<th>Formation of feruloyl fish oil (%)</th>
<th>Observed (%)</th>
<th>Predicted (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40 (-1)</td>
<td>2 (-1)</td>
<td>1 (-1)</td>
<td>1 (-1)</td>
<td>24.61</td>
<td>21.03</td>
<td></td>
<td>15.17</td>
<td>16.19</td>
<td></td>
<td>9.22</td>
<td>4.73</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>70 (1)</td>
<td>2 (-1)</td>
<td>1 (-1)</td>
<td>1 (-1)</td>
<td>47.61</td>
<td>47.00</td>
<td></td>
<td>25.67</td>
<td>26.03</td>
<td></td>
<td>21.94</td>
<td>20.84</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>40 (-1)</td>
<td>20 (1)</td>
<td>1 (-1)</td>
<td>1 (-1)</td>
<td>66.38</td>
<td>80.08</td>
<td></td>
<td>44.97</td>
<td>39.95</td>
<td></td>
<td>21.40</td>
<td>40.11</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>70 (1)</td>
<td>20 (1)</td>
<td>1 (-1)</td>
<td>1 (-1)</td>
<td>74.53</td>
<td>74.05</td>
<td></td>
<td>38.70</td>
<td>38.39</td>
<td></td>
<td>35.80</td>
<td>35.69</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>40 (-1)</td>
<td>2 (-1)</td>
<td>5 (1)</td>
<td>1 (-1)</td>
<td>8.62</td>
<td>-41.46</td>
<td></td>
<td>0.31</td>
<td>-14.33</td>
<td></td>
<td>8.30</td>
<td>-26.83</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>70 (1)</td>
<td>2 (-1)</td>
<td>5 (1)</td>
<td>1 (-1)</td>
<td>41.87</td>
<td>41.14</td>
<td></td>
<td>7.10</td>
<td>6.66</td>
<td></td>
<td>34.70</td>
<td>34.49</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>40 (-1)</td>
<td>20 (1)</td>
<td>5 (1)</td>
<td>1 (-1)</td>
<td>40.95</td>
<td>41.02</td>
<td></td>
<td>1.70</td>
<td>1.58</td>
<td></td>
<td>39.24</td>
<td>39.35</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>70 (1)</td>
<td>20 (1)</td>
<td>5 (1)</td>
<td>1 (-1)</td>
<td>91.62</td>
<td>91.62</td>
<td></td>
<td>11.17</td>
<td>11.17</td>
<td></td>
<td>80.15</td>
<td>80.15</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>40 (-1)</td>
<td>2 (-1)</td>
<td>1 (-1)</td>
<td>5 (1)</td>
<td>57.30</td>
<td>61.16</td>
<td></td>
<td>46.91</td>
<td>30.23</td>
<td></td>
<td>10.25</td>
<td>30.77</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>70 (1)</td>
<td>2 (-1)</td>
<td>1 (-1)</td>
<td>5 (1)</td>
<td>51.59</td>
<td>51.23</td>
<td></td>
<td>23.15</td>
<td>23.34</td>
<td></td>
<td>28.37</td>
<td>27.91</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>40 (-1)</td>
<td>20 (1)</td>
<td>1 (-1)</td>
<td>5 (1)</td>
<td>87.10</td>
<td>87.54</td>
<td></td>
<td>46.11</td>
<td>46.62</td>
<td></td>
<td>40.96</td>
<td>40.82</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>70 (1)</td>
<td>20 (1)</td>
<td>1 (-1)</td>
<td>5 (1)</td>
<td>93.16</td>
<td>45.61</td>
<td></td>
<td>46.04</td>
<td>28.34</td>
<td></td>
<td>46.97</td>
<td>17.43</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>40 (-1)</td>
<td>2 (-1)</td>
<td>5 (1)</td>
<td>5 (1)</td>
<td>37.52</td>
<td>37.71</td>
<td></td>
<td>7.70</td>
<td>8.07</td>
<td></td>
<td>29.75</td>
<td>29.52</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>70 (1)</td>
<td>2 (-1)</td>
<td>5 (1)</td>
<td>5 (1)</td>
<td>84.41</td>
<td>84.41</td>
<td></td>
<td>12.33</td>
<td>12.33</td>
<td></td>
<td>71.88</td>
<td>71.88</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>40 (-1)</td>
<td>20 (1)</td>
<td>5 (1)</td>
<td>5 (1)</td>
<td>86.92</td>
<td>87.53</td>
<td></td>
<td>16.97</td>
<td>16.61</td>
<td></td>
<td>69.27</td>
<td>70.37</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>70 (1)</td>
<td>20 (1)</td>
<td>5 (1)</td>
<td>5 (1)</td>
<td>98.93</td>
<td>102.22</td>
<td></td>
<td>10.43</td>
<td>9.48</td>
<td></td>
<td>88.06</td>
<td>92.21</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>40 (-1)</td>
<td>11 (0)</td>
<td>3 (0)</td>
<td>3 (0)</td>
<td>74.18</td>
<td>76.44</td>
<td></td>
<td>21.00</td>
<td>19.59</td>
<td></td>
<td>53.01</td>
<td>56.66</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>70 (1)</td>
<td>11 (0)</td>
<td>3 (0)</td>
<td>3 (0)</td>
<td>97.89</td>
<td>96.78</td>
<td></td>
<td>19.79</td>
<td>20.94</td>
<td></td>
<td>77.90</td>
<td>75.64</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>55 (0)</td>
<td>2 (-1)</td>
<td>3 (0)</td>
<td>3 (0)</td>
<td>47.88</td>
<td>52.96</td>
<td></td>
<td>13.24</td>
<td>11.74</td>
<td></td>
<td>34.52</td>
<td>41.01</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>55 (0)</td>
<td>20 (1)</td>
<td>3 (0)</td>
<td>3 (0)</td>
<td>95.33</td>
<td>91.40</td>
<td></td>
<td>20.95</td>
<td>22.19</td>
<td></td>
<td>73.97</td>
<td>68.87</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>55 (0)</td>
<td>11 (0)</td>
<td>1 (-1)</td>
<td>3 (0)</td>
<td>82.5</td>
<td>87.08</td>
<td></td>
<td>42.66</td>
<td>40.89</td>
<td></td>
<td>39.72</td>
<td>46.03</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>55 (0)</td>
<td>11 (0)</td>
<td>5 (1)</td>
<td>3 (0)</td>
<td>87.58</td>
<td>84.15</td>
<td></td>
<td>14.69</td>
<td>16.20</td>
<td></td>
<td>72.56</td>
<td>67.64</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>55 (0)</td>
<td>11 (0)</td>
<td>3 (0)</td>
<td>1 (-1)</td>
<td>59.87</td>
<td>65.19</td>
<td></td>
<td>16.59</td>
<td>16.08</td>
<td></td>
<td>43.23</td>
<td>49.03</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>55 (0)</td>
<td>11 (0)</td>
<td>3 (0)</td>
<td>5 (1)</td>
<td>94.73</td>
<td>90.56</td>
<td></td>
<td>22.00</td>
<td>22.25</td>
<td></td>
<td>72.49</td>
<td>68.08</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>55 (0)</td>
<td>11 (0)</td>
<td>3 (0)</td>
<td>3 (0)</td>
<td>86.40</td>
<td>88.43</td>
<td></td>
<td>21.26</td>
<td>22.05</td>
<td></td>
<td>65.04</td>
<td>66.13</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>55 (0)</td>
<td>11 (0)</td>
<td>3 (0)</td>
<td>3 (0)</td>
<td>90.70</td>
<td>88.43</td>
<td></td>
<td>22.19</td>
<td>22.05</td>
<td></td>
<td>68.05</td>
<td>66.13</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>55 (0)</td>
<td>11 (0)</td>
<td>3 (0)</td>
<td>3 (0)</td>
<td>91.64</td>
<td>88.43</td>
<td></td>
<td>21.91</td>
<td>22.05</td>
<td></td>
<td>69.47</td>
<td>66.13</td>
<td></td>
</tr>
</tbody>
</table>

---

*a 0.184 g (2 mmol) of glycerol was supplied to each trials.  
*b these runs were eliminated during model fitting.
Statistical analysis

The mathematical relationship among variables with each response can be calculated by the quadratic polynomial equation:

\[ Y = \beta_0 + \sum_{i=1}^{4} \beta_i x_i + \sum_{i=1}^{4} \beta_{ii} x_i^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{4} \beta_{ij} x_i x_j \] (1)

where \( \beta_0, \beta_i, \beta_{ii} \) and \( \beta_{ij} \) are regression coefficients (\( \beta_0 \) is a constant term, \( \beta_i \) is a liner effect term, \( \beta_{ii} \) is a squared effect term, and \( \beta_{ij} \) is an interaction effect term) and \( Y \) is the predicted response value.

The models were fitted with multiple regressions with backward elimination.

Result and discussion

Identification of feruloyl acylglycerol species

Transesterification of EF with triacylglycerols results in a mixture of feruloyl acylglycerol species due to the multiple fatty acids composition of cod liver fish oil (Fig. 1.). In order to simplify the analysis, transesterification of EF with triolein was firstly investigated following the method used by Compton et al. (11). Using a similar analysis method, reaction products from transesterification of EF with triolein were qualified by HPLC and LC-MS. Comparing to the HPLC method used by Compton et al., in which a tertiary solvent system was applied to achieve the separation of the different feruloyl acylglycerol species (Fig. 1.), we developed a binary solvent system using methanol/water (contained 0.75% acetic acid) gradient based on a modified method developed by Sun et al. (21). The similar results were achieved (Fig. 2A). The chromatogram in Fig. 2A was obtained for reaction products from transesterification of EF with triolein, which was carried out by mixing the substrate in the ratio of 2/1/1 of triolein/EF/glycerol. The elution sequence is listed in Table 2 and each peak was identified by HPLC-ESI-MS according to the corresponding mass. The elution order of the chromatography is in agreement with the previous study (11).

<table>
<thead>
<tr>
<th>Species</th>
<th>( R_f ) (min)</th>
<th>MW</th>
<th>Major ion (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1-MAG</td>
<td>4.2</td>
<td>268</td>
<td>267 [M-H]</td>
</tr>
<tr>
<td>FA</td>
<td>4.5</td>
<td>194</td>
<td>193 [M-H]</td>
</tr>
<tr>
<td>F2-DAG</td>
<td>7.6</td>
<td>444.4</td>
<td>443 [M-H]</td>
</tr>
<tr>
<td>EF</td>
<td>8.3</td>
<td>222</td>
<td>221 [M-H]</td>
</tr>
<tr>
<td>F1-DAG</td>
<td>15.2</td>
<td>532</td>
<td>531 [M-H]</td>
</tr>
<tr>
<td>F2-TAG</td>
<td>15.8</td>
<td>708</td>
<td>707 [M-H]</td>
</tr>
<tr>
<td>F1-TAG</td>
<td>22.5</td>
<td>797.2</td>
<td>796 [M-H]</td>
</tr>
</tbody>
</table>
According to the present sequence, the first eluted peak (Fig. 2A) was identified as monoferuloyl-sn-glycerol (F1-MAG), and the next peak as ferulic acid (FA). The third and fourth peaks were found to be diferuloyl-sn-glycerol (F2-DAG) and EF, respectively. Monoferuloyl-monoolein (F1-DAG) is an ester product which contains one feruloyl moiety and one oleic acid on the backbone of glycerol and the eluted peak of this product was found to be adjacent to the peak of diferuloyl-monoolein (F2-TAG) which contains two feruloyl moieties and one oleic acid on the backbone of glycerol. The last eluted peak was identified as monoferuloyl-diolein (F1-TAG).

**Fig. 1.** Scheme of possible product on transesterification of EF with triolein or fish oil. R= oleic acid or fatty acids of fish oil. Feruloyl acylglycerol species were defined according to amount of feruloyl moieties or fatty acids on the backbone of glycerol: F1-MAG contains one feruloyl moiety on the backbone of glycerol; F2-DAG contains two feruloyl moieties on the backbone of glycerol; F1-DAG contains one feruloyl moiety and one oleic acid or one fatty acid of fish oil on the backbone of glycerol; F2-TAG contains two feruloyl moieties and one oleic acid or one fatty acid of fish oil on the backbone of glycerol; F1-TAG contains one feruloyl moiety and two oleic acids or two fatty acids of fish oil on the backbone of glycerol. FA and EF are abbreviation for ferulate acid and ethyl ferulate respectively.
The present sequence in Fig. 2A was then used to identify the ester products from the transesterification of EF with fish oil. The transesterification of fish oil/EF/glycerol resulted in a series of complex mixtures (Fig. 2B) due to multiple fatty acids composition in fish oil, which make the classification of each peak in Fig. 2B is difficult and ambiguous. However, as suggested by

Fig. 2. Analytical reversed-phase HPLC chromatograms on products from transesterification of EF/triolein/glycerol (A) or EF/fish oil/glycerol (B) (2/1/1). The reactions were catalyzed by 10 % of Novozym 435 (on the base of substrate) at 60 °C and stirred by 300 rpm for 5 days. See Fig. 1. for acronym definitions.

The present sequence in Fig. 2A was then used to identify the ester products from the transesterification of EF with fish oil. The transesterification of fish oil/EF/glycerol resulted in a series of complex mixtures (Fig. 2B) due to multiple fatty acids composition in fish oil, which make the classification of each peak in Fig. 2B is difficult and ambiguous. However, as suggested by
Compton et al. 2006 (11), we assumed those feruoyl acyglycerol with similar structure as to feruloy oleylglycerol will be eluted at similar times, and they were classified into different groups based on the number of feruloyl moieties and fatty acids on the backbone of glycerol following the classification in Fig. 2A.

Influence of glycerol on formation of feruloyl acylglycerol species

It has been reported that glycerol can increase the bioconversion rate of EF and formation of feruloyl acylglycerol species (11, 12, 22). However, too much glycerol will have negative effect on the transesterification reaction on the other hand. The reason could be that the glycerol would extract the water layer from the enzyme and the water layer outside the enzyme is essential to maintain its activity (23). Therefore we are interested in studying how glycerol will affect the transesterification of EF with oil. For this purpose, glycerol was added from 0 to 5 mmol into reaction mixture, which was consisted of 2 mmol of cod liver fish oil and 1 mmol of EF.

The results (Fig. 3) show that the effect of glycerol on bioconversion of EF was significant. When no glycerol was present in the reaction, the maximum conversion of EF was only 67%. On the other hand, when small amount of glycerol (1 mmol) was supplied, conversion of EF reached above 80% within 50 h of reaction and finally reached almost 99% when the reaction was terminated at 216 h (8 days). However, continue to increase the amount of glycerol in reaction did not seem to contribute for increasing the conversion rate.

Bioconversion of Ethyl Ferulate

![Graph showing bioconversion of Ethyl Ferulate over reaction time](image)

**Fig. 3.** Effect of glycerol on bioconversion of EF. Reactions were catalyzed by 10 % of Novozym 435 (on the base of substrate) at 60 °C, stirred at 300 rpm and under five mbars for 216 h. Amount ratio of fish oil/EF/glycerol was: 2/1/0 (Δ), 2/1/1 (○), 2/1/2 (□), 2/1/3 (○), 2/1/5 (*).
Considering the formation of different feruloyl acylglycerol species (Fig. 4.), glycerol had a positive effect in general except for the production of F1-TAG (Fig. 4D), where F1-TAG content decreased from 42% to 9% following the increase of glycerol from 0 to 5 mmol in reaction. When there was no glycerol supplied, the highest production of F1-MAG (Fig. 4A), F2-DAG (Fig. 4B) and F2-TAG/F1-DAG (Fig. 4C) were only 6%, 2% and 23%, respectively, which were much lower than when 1 mmol of glycerol was added. In Fig. 4A, it also shows that production of F1-MAG increased following the increase of glycerol. However, too much of glycerol seems to have adverse effect for the formation of F2-DAG and F2-TAG/F1-DAG (Fig. 4B and 4C), especially when glycerol was increased to 5 mmol.

**Fig. 4.** Effect of glycerol on formation of feruloyl acylglycerol species. Reaction conditions and corresponding simples were as the same as in Fig. 3.
In general, yields of feruloyl acylglycerol species can be classified into two groups according to whether containing fatty acids of fish oil on the backbone of glycerol or not. The first group is the feruloyl-sn-glycerol (F1-MAG and F2-DAG) which do not contain fatty acids of fish oil, while the second group is the feruloyl fish oil (F1-TAG, F1-DAG and F2-TAG) which contains one or two fatty acids of fish oil on the backbone of glycerol. The first group is basically from the transesterification of EF with glycerol and therefore can be treated as by-products in the reaction. On the other hand, the second group contains desired products because these products have hydrophobic heads (fatty acid moieties) and hydrophilic tails (feruloyl moieties). The production of two groups of products is summarized in Table 3. There is a linear formation of the first group (feruloyl-sn-glycerol) and the amount of glycerol added. The total production of this group increased from 8% to 52%, following the increase of glycerol in reaction from 0 to 5 mmol. On the other hand, the highest yield of feruloyl fish oil (77%) was found when the amount ratio of fish oil/EF/glycerol was 2/1/1. However, the yield of feruloyl fish oil decreased to about 65% when amount ratio of substrate was changed to 2/1/2, which was almost equal to the yield when there was no glycerol supplied in the reaction, and the yield of this product decreased further if more glycerol was added.

<table>
<thead>
<tr>
<th>Amount Ratio</th>
<th>Feruloyl-sn-glycerol</th>
<th>Feruloyl fish oil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F1-MAG (%)</td>
<td>F2-DAG (%)</td>
</tr>
<tr>
<td>2/1/0</td>
<td>6.00±0.61*</td>
<td>2.00±0.03*</td>
</tr>
<tr>
<td>2/1/1</td>
<td>10.81±2.14*</td>
<td>10.64±0.66*</td>
</tr>
<tr>
<td>2/1/2</td>
<td>24.09±0.78*</td>
<td>9.70±0.01*</td>
</tr>
<tr>
<td>2/1/3</td>
<td>32.54±0.72*</td>
<td>8.11±0.18*</td>
</tr>
<tr>
<td>2/1/5</td>
<td>46.82±0.02*</td>
<td>5.75±0.04*</td>
</tr>
</tbody>
</table>

*Relative standard deviation was calculated from the duplicate results from different experiment.

RSM model fitting

Response surface methodology (RSM) is a collection of statistical design and numerical optimization techniques used to optimize process and product designs (24). This methodology is based on the fit of a polynomial equation to the experimental data and well applied when a response or set of responses of interest are influenced by several variables to achieve the goal of optimizing the level of these variables and attaining the best system performance (25). In this study, a four-
factor and three-level central composite face centred design (CCF) was chosen to investigate the effect of four variables (temperature, reaction time, amount ratio of fish oil/EF, and enzyme load). The whole experiment design is shown in Table 1, which includes the experimental results and predicted values. For each factor, the conventional level was set at zero as a coded level and the quadric model (Equation 1) was chosen to fit the experiment data. In addition, the significant level to each term in the selected model was set as 0.05.

Among the different trials of the experiment designs (Table 1), the greatest bioconversion of EF and the formation of feruloyl fish oil were achieved in experiment 11, while the highest content of feruloyl-sn-glycerol was found in experiment 9. The effect of the four variables as well as their interaction was evaluated based on the coefficients of the full model by regression analysis and tested for their significance on the basis of the \( p \) value. The predicted coefficient values of each variable which has significant effect on bioconversion of EF are present in Table 4. The independent variables (\( x_1, x_2 \) and \( x_4 \)), interactions (\( x_1x_2, x_1x_3, x_1x_4, x_2x_3, x_2x_4 \) and \( x_3x_4 \)), and quadratic terms (\( x_2^2 \) and \( x_4^2 \)) are the most significant factors affecting the bioconversion of EF (with \( p < 0.05 \)). The quadratic term \( x_1^2 \) and \( x_2^2 \) were excluded from the original model by backward elimination since they did not show significant effect. Variable \( x_3 \) was kept in the model despite its insignificant effect (\( p=0.32 \)) because it showed significant interaction with other independent variables.

**Table 4.** Regression coefficients and significance (\( p<0.05 \)) after backward elimination for bioconversion of EF and formation of feruloyl-sn-glycerol and feruloyl fish oil

<table>
<thead>
<tr>
<th>Variable</th>
<th>Bioconversion of EF</th>
<th>Formation towards feruloyl glycerol</th>
<th>Formation towards feruloyl fish oil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coefficient</td>
<td>( p )-value</td>
<td>Coefficient</td>
</tr>
<tr>
<td>Constant</td>
<td>88.43</td>
<td>3.05909e-007</td>
<td>22.05</td>
</tr>
<tr>
<td>Temp ( x_1 )</td>
<td>10.17</td>
<td>0.000188904</td>
<td>0.68</td>
</tr>
<tr>
<td>Enzy ( x_2 )</td>
<td>19.22</td>
<td>0.000236505</td>
<td>5.23</td>
</tr>
<tr>
<td>Mol ( x_3 )</td>
<td>-1.47</td>
<td>0.000116675</td>
<td>-12.34</td>
</tr>
<tr>
<td>Tim ( x_4 )</td>
<td>12.68</td>
<td>2.11735e-005</td>
<td>3.09</td>
</tr>
<tr>
<td>Temp*Temp ( x_1x_1 )</td>
<td>-1.82</td>
<td>0.0057857</td>
<td>-1.78</td>
</tr>
<tr>
<td>Enzy*Enzy ( x_2x_2 )</td>
<td>-16.24</td>
<td>0.0154269</td>
<td>-5.08</td>
</tr>
<tr>
<td>Mol*Mol ( x_3x_3 )</td>
<td>-2.81</td>
<td>0.000145708</td>
<td>6.50</td>
</tr>
<tr>
<td>Tim*Tim ( x_4x_4 )</td>
<td>-10.55</td>
<td>0.000282499</td>
<td>-2.88</td>
</tr>
<tr>
<td>Temp*Enzy ( x_1x_2 )</td>
<td>-8.00</td>
<td>0.00073279</td>
<td>-2.85</td>
</tr>
<tr>
<td>Temp*Mol ( x_1x_3 )</td>
<td>14.16</td>
<td>0.00687478</td>
<td>2.79</td>
</tr>
<tr>
<td>Temp*Tim ( x_1x_4 )</td>
<td>-8.98</td>
<td>0.101996</td>
<td>-4.18</td>
</tr>
<tr>
<td>Enzy*Mol ( x_2x_3 )</td>
<td>5.86</td>
<td>0.0225241</td>
<td>-1.96</td>
</tr>
<tr>
<td>Enzy*Tim ( x_2x_4 )</td>
<td>-8.17</td>
<td>0.759813</td>
<td>-1.84</td>
</tr>
<tr>
<td>Mol*Tim ( x_3x_4 )</td>
<td>9.76</td>
<td>0.0485351</td>
<td>2.09</td>
</tr>
</tbody>
</table>

Abbreviations: Temp, temperature; Enzy, enzyme load; Mol, substrate amount ratio of fish oil/EF; and Tim, reaction time.
Similarly, independent variables ($x_2$, $x_3$ and $x_4$), quadratic terms ($x_2^2$, $x_3^2$ and $x_4^2$), and interaction variables ($x_1x_2$, $x_1x_3$, $x_1x_4$, $x_2x_3$, $x_2x_4$ and $x_3x_4$) were the significant variables for the formation of F1-MAG and F2-DAG. The independent variables ($x_1$, $x_2$, $x_3$ and $x_4$), quadratic terms ($x_2^2$ and $x_3^2$), and interaction variables ($x_1x_3$, $x_2x_3$ and $x_3x_4$) were the significant variables for the formation of F1-TAG and F2-TAG. Other insignificant variables are excluded from the prediction models except those have significant interaction effect with other variables.

The three models were further analyzed by ANOVA. The analysis shows that the coefficient of determination ($R^2$) of the three models for the bioconversion of EF and formation of feruloyl-sn-glycerol and feruloyl fish oil were 0.96, 0.983, and 0.933, respectively, which indicate that these models were suitable to represent the real relationships among the selected reaction parameters. ANOVA analysis also shows that the probabilities for regression of the model were significant ($p < 0.0001$) and the lack of fits was insignificant ($p > 0.05$). This means that these models were statistically good and the models had no lack of fit at 95% level of significance. Therefore, these models were successfully established.

**Reaction optimization**

Fig. 5-7 are contour plots which predict the mutual effect among temperature, enzyme load, substrate amount ratio of fish oil/EF and reaction time on bioconversion of EF (Fig. 5) and formation of feruloyl-sn-glycerol (Fig. 6) and feruloyl fish oil (Fig. 7). As from the former studies, 2 mmol glycerol was added to increase the conversion rate.

Fig. 5A represents the effects of temperature and enzyme load and their mutual interaction on the reaction conversion at 3 days and substrate amount ratio (fish oil/EF) of 3. Both temperature and enzyme load had positive effect on the bioconversion of EF. The optimal temperature was found to be above 50 °C, where the bioconversion of EF could reach above 90% when the enzyme load was increased to 16%. But the same high conversion could also be achieved at lower enzyme load when temperature was increased further. The positive effect of temperature on conversion of EF is in agreement with previous finding (26). At the same time, a slight decrease of conversion occurred if the enzyme load is increased above 17% at 50 °C.

Fig. 5B shows mutual relationships between substrate amount ratio and temperature where the enzyme load was 11% and reaction time was 3 days. The conversion rate decreased as the substrate amount ratio increased at low temperature (below 55 °C). This situation was not the same case when the reaction temperature was higher, where the bioconversion of EF could reach above 90% when the reaction temperature was above 60 °C. Such phenomenon is probably due to the viscosity of fish oil, where higher amount of substrate ratio inhibits the mass contact of substrate
with enzyme, but higher temperature can reduce the viscosity of fish oil and, consequently, lead to the increase of the bioconversion of EF.

In Fig. 5C, the bioconversion of EF increases following the reaction time on the first four days, but then there is slight decrease after five days. The effect of reaction time on bioconversion of EF with fish oil in this study agrees with the previous reports (9, 27), where they speculated that it could be due to the hydrolysis products in longer reaction time.

The effect of factors on the formation of feruloyl-sn-glycerol can be evaluated in the similar approach. Fig. 6A describes the mutual effects between reaction time and temperature, where the amount ratio of fish oil/EF was 3 and enzyme load was 11%. Both reaction time and temperature had positive effect on the formation of feruloyl-sn-glycerol. But when the temperature was increased above 60 °C, there was a slightly decrease. Therefore, low temperature and shorter reaction time is preferred if we expect to reduce the formation of feruloyl-sn-glycerol.
Fig. 6B presents the mutual effect of substrate amount ratio of fish oil/EF and enzyme load, where the reaction temperature was 55 °C and reaction time was 3 days. The formation of feruloyl-sn-glycerol could reach 39.6% when the enzyme load was above 10%. On the other hand, formation of feruloyl-sn-glycerol reduced as the increase of substrate amount ratio of fish oil/EF. Therefore, feruloyl-sn-glycerol can be minimized through increase of substrate amount ratio of fish oil/EF and decrease of enzyme load.

The formation of feruloyl fish oil is the key intention for the reaction. The evaluation of the factor effects as well as their interactions is present in Fig. 7. Fig. 7A shows mutual effects of temperature and amount ratio on formation of feruloyl fish oil, where enzyme load was 11% and reaction time was 3 days. Temperature had no effect on formation of the product when the substrate amount ratio of fish oil/EF was below 2. However, when the substrate amount ratio was further increased, temperature had positive effect following the increase of substrate amount ratio.

Fig. 7B shows the interactive effects of substrate amount ratio of fish oil/EF and enzyme load on the formation of feruloyl fish oil, where temperature was 55 °C and reaction time was 3 days. There was no obvious effect of substrate amount ratio of fish oil/EF on formation when enzyme load was lower than 9%. However, the formation could be increased following the increase of enzyme load beyond 14 % and substrate amount ratio of fish oil/EF beyond 3.5.

Fig. 7C shows the interactive effects of substrate amount ratio of fish oil/EF and reaction time on the formation of feruloyl fish oil, where temperature was 55 °C and enzyme load was 11%. There was no obvious effect on the formation of the products either through increase of substrate amount ratio of fish oil/EF or reaction time. However, the feruloyl fish oil could be increased through increase of the substrate amount ratio of fish oil/EF and the prolongation of reaction time.
Optimal conditions for a lower formation of by-product and higher bioconversion of EF as well as formation of feruloyl fish oil were predicted within the experiment design ranges (Table 5). Two sets of predicted conditions were generated. The predicted values from the models were found to be reasonably in agreement with the experimental values (observed), which confirmed the validity and adequacy of the predicted models.

Table 5. Optimum conditions generated and verification of the models

<table>
<thead>
<tr>
<th></th>
<th>run 1</th>
<th>run 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>Enzyme load (%)</td>
<td>4.3</td>
<td>14.5</td>
</tr>
<tr>
<td>Amount ratio (fish oil/EF)</td>
<td>4.7</td>
<td>2.7</td>
</tr>
<tr>
<td>Reaction time (days)</td>
<td>5</td>
<td>4.9</td>
</tr>
<tr>
<td>Bioconversion of EF (%)</td>
<td>Predicted</td>
<td>92.3</td>
</tr>
<tr>
<td></td>
<td>Observed &amp; SD*</td>
<td>92.4±0.0</td>
</tr>
<tr>
<td>Formation of By-products (%)</td>
<td>Predicted</td>
<td>13.5</td>
</tr>
<tr>
<td></td>
<td>Observed &amp; SD*</td>
<td>11.4±0.6</td>
</tr>
<tr>
<td>Formation of feruloyl fish oil (%)</td>
<td>Predicted</td>
<td>78.6</td>
</tr>
<tr>
<td></td>
<td>Observed &amp; SD*</td>
<td>80.4±0.4</td>
</tr>
</tbody>
</table>

*Relative standard deviation was calculated from duplicated results from different experiment.
Under the predicted conditions, the bioconversion of EF and selectivity towards desired product were greatly improved, except the reaction time, which was still 5 days and could not be decreased further. Nevertheless, these reaction conditions are still preferable compared to previous studies (11-17, 19-23, 26, 27), where most of the reactions took place within more than 6 to 10 days with lower yield of phenolic lipids than in this study.

Conclusions

The effect of glycerol on bioconversion of EF and formations of feruloyl-sn-glycerol and feruloyl fish oil was investigated. The results show that the amount ratio of fish oil/EF/glycerol of 2/1/1 was the best condition. Under this condition, the bioconversion of EF was almost completely converted and feruloyl fish oil reached 77% in formation, while the formation to by-product (feruloyl-sn-glycerol) was only 21.4%. Under these concerns, three quadric models were generated using response surface methodology for the study of the interactive effect of temperature, enzyme load, substrate amount ratio of fish oil/EF and reaction time. Three responses were decided on bioconversion of EF, as well as formation of feruloyl-sn-glycerol and feruloyl fish oil. Two sets of optimal conditions were eventually established. According to the optimum conditions, the bioconversion of EF and formation of feruloyl fish oil could exceed 92% and 80% respectively, while the by-product (feruloyl-sn-glycerol) was less than 12%.

Acknowledgement

The financial support from the Strategic Food and Health Program (FøSu) as well as grant from the Graduate School of Science, Aarhus University is appreciated.
References


Appendix 5: Characterization of Reaction Systems Through DLS and SAXS

1. Objects:
It has been proved that ionic liquid tOMA·TFA is a good reaction media for synthesis of novel antioxidants from natural sources (Paper II; Chen, 2008; 2011). However, it is not clear how ionic liquid aids in increasing the production. In this section, several systems were prepared namely phenolic in IL/octanol, rutin in IL/linoleic, and ferulic in IL/triolein, respectively. Ionic liquid tOMA·TFA is an amphiphilic molecule; meanwhile, octanol, linoleic, and triolein are hydrophobic. Therefore, these systems are probably emulsion systems containing spherical particles (Sheme 1). To confirm this hypothesis, the properties of these systems were investigated by techniques of DLS and SAXS.

Scheme 1. Illustration of systems containing phenolic in ILs/octanol, rutin in ILs/linoleic, ferulic in ILs/triolein
2. Materials and Methods:
Antioxidant compounds (phenolic, rutin) were dissolved in ionic liquid tOMA·TFA firstly by heating and then by mixing with hydrophobic solvents (octanol, linoleic, or triolein) under 1000 rpm. 1 ml sample was then taken out into a 5 ml glass tube and measured with DLS and SAXS, respectively. Both measurements were conducted at the Department of Chemistry, Aarhus University.

The measurements of DLS were performed on a commercially available instrument (ALV, Langen, Germany) consisting of an ALV/CGS-8F goniometer equipped with an ALV-6010/EPP multi-tau digital correlator. The instrument is equipped with an ALV-static and dynamic enhancer with fiber-splitting at 632.8 nm for operation in the pseudo-cross correlation mode using two avalanche photo diodes as the detection unit. A He–Nediode laser (JDS Uniphase) with an output power of 25 mW and operating at a wavelength of 632.8 nm was used as the light source. The incident light was vertically polarized with respect to the scattering plane and the light intensity was regulated with a software controlled ALV/8-steps beam attenuator. The measurements were performed at 40 °C (temperature) under angle of 90 °C. The measurements of SAXS were performed at 40 °C (temperature) according to the method described elsewhere (Petersen, 2004). It is important to keep all the sample and instrument to be dust-free.

3. Results:
All the systems were firstly measured by DLS, and the results are shown in Table 1.

<table>
<thead>
<tr>
<th>Systems</th>
<th>aRg (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHCA in IL/octanol</td>
<td>n/a</td>
</tr>
<tr>
<td>Ferulic in IL/octanol</td>
<td>n/a</td>
</tr>
<tr>
<td>Rutin in IL/linoleic</td>
<td>38 - 82</td>
</tr>
<tr>
<td>Ferulic in IL/triolein</td>
<td>914 - 1253</td>
</tr>
</tbody>
</table>

*aRg: Radius of gyration, refered to the size of particle

As showed in Table 1, there was no detection of any droplets in the system of phenolic (DHCA and ferulic) in IL/octanol. The droplets found in systems of rutin in IL/linoleic and ferulic in ILs/triolein was found to be in the range of 38 – 82 nm and 914 – 1253 nm, respectively.

To further confirmed the sizes of droplet in the system of rutin in IL/linoleic and ferulic in IL/triolein, SAXS was applied. Fig. 1 is the result of measurement from the system of rutin in IL/octanol. As shown in Fig. 1, there is sign of particles in the sizes of 450 Å (=45 nm) was
observed (the first peak). However, there was also sign of particles with large size (around 1000 Å) as indicates in Fig. 1 (the second peak). In agreement with the results obtained from DLS in which larger particles were not observed, it can be concluded that the particle sizes in the system of rutin in ILs/linoleic is approximately 45 nm. The particles in system of ferulic in ILs/triolein are probably too big thus beyond the measurement range (<500nm). As a result, no signal is obtained from the measurement.

Concluded above results, the system of phenolic in IL/octanol is probably a homogenous system as no droplet was observed in this system. Rutin in IL/linoleic is a stable emulsion system which contains droplets in the size of around 45 nm. Ferulic in IL/triolein is not a stable emulsion system, the sizes of droplets in this system is around 1000 nm when it was stired at high speed.

References:


Appendix 5: Characterization of Reaction System Through DLS and SAXS
Other Publications:


