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APPLICATION OF ENZYMATIC REACTIONS IN STRUCTURING AND OXIDATIVE STABILITY IMPROVEMENT OF OILS AND FATS

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MAGs	Monoacylglycerols
DAGs	Diacylglycerols
TAGs	Triacylglycerols
FFAs	Free fatty acids
SFAs	Saturated fatty acids
MUFA	Monounsaturated fatty acids
PUFA	Polyunsaturated fatty acids
RSM	Response surface methodology
IV	Iodine value
sn	Stereospecific numbering
POO	Palm olein oil
RBO	Rice bran oil
PSO	Pumpkin seed oil
SFO	Sunflower oil
IP	Induction period
DSC	Differential scanning calorimetry
O/W	Oil-in-water
TLC	Thin-layer chromatography

1. INTRODUCTION

In the first section of the thesis, the application of enzymatic reactions was used to solidify liquid vegetable oils containing mostly triacylglycerols (TAGs) into solid fats containing partial acylglycerols. This was done by enriching them with monoacylglycerols (MAGs) and diacylglycerols (DAGs) while keeping free fatty acids (FFAs) as low as possible and selectively removing saturated fatty acids (SFAs) from vegetable oils.

Solidifying liquid vegetable oils without compromising their nutritional properties has always posed a challenge for food scientists. Traditional methods such as partial hydrogenation, fractionation, physical blending with hard fats, and interesterification have been used for solidification. In this context, partial hydrogenation has been recently restricted due to the formation of trans fatty acids (Arellano *et al.,* 2015; Bhattacharya, 2023; Gibon & Kellens, 2014; O'Brien, 2008; Talbot, 2015). However, innovative techniques have emerged in the last two decades, including oleogelation using lipidic or biopolymeric oleogelators, bi-phasic structured systems, and glycerolysis, offering successful alternatives for structuring liquid oils. These methods allow for the use of fats with lower SFAs while creating technologically functional fats, making them highly valuable in the field of food science (Gibon & Kellens, 2014; Nicholson & Marangoni, 2020; O'Brien, 2008; Potter, 1986; Robinson & Mattil, 1959). In this context, methods allowing the use of lower SFAs while developing technologically functional fat are of great importance.

Conventional lipidic oleogelators, namely MAGs, DAGs, and FFAs, have the ability to structure liquid oils and exhibit higher melting temperatures compared to their TAGs counterparts. Traditionally, these oleogelators are dispersed directly into the liquid oils at temperatures above their melting points, and upon cooling, they form structured gels (Subroto, 2020). Additionally, due to their surface-active properties, they can also contribute to the

development of structured bi-phasic systems. These MAGs and DAGs are widely used in the food industry, particularly in bakeries, margarine, dairy, and confectionary sections, making them extensively consumed and relatively expensive (Talbot, 2015). Partial acylglycerols and FFAs can be produced either chemically or enzymatically, adding to their versatility and potential applications (Mardani *et al.,* 2015).

Lipase-catalyzed reactions present promising alternatives to high-temperature chemical processes for producing heat-sensitive MAGs and DAGs enriched with polyunsaturated fatty acids. Various methodologies for enzymatic MAGs and DAGs production in oil mixtures have been explored in the past, including esterification of FFAs with glycerol, glycerolysis of oils and glycerol, and partial hydrolysis of oils (Zhang *et al*., 2022). Among these methods, glycerolysis reactions have found extensive use in food applications for creating partial acylglycerols. Nicholson and Marangoni (2020, 2021, 2022) recently demonstrated the direct structuring of different non-solid edible oils using enzymatic glycerolysis, eliminating the need to produce separate MAGs and then add them to vegetable oils. Alternatively, partial hydrolysis of oils can serve as a structuring strategy, where vegetable oils are partially hydrolyzed to produce a mixture of partial acylglycerols and fatty acids that contribute to oil structuring. Previous applications of partial hydrolysis of vegetable oils have mostly focused on designing DAGs-enriched oils, which have been associated with numerous beneficial health outcomes (Subroto, 2020; Zhang *et al*., 2022). Furthermore, enzymatic hydrolysis reactions offer selective options using regioselective or fatty acid-specific lipases, enabling enrichment or removal of specific fatty acids. The specificity of lipases, particularly their fatty acid- and regio-selectivity, opens up possibilities for altering oils and fats in various processes. These lipases have frequently been employed for enriching poly omega-3 unsaturated fatty acids (Chen *et al*., 2023; Yang *et al*., 2021). This approach can lead to improved nutritional properties by converting vegetable oils into acylglycerols with higher unsaturated fatty acid content, which

is crucial for formulating functional food products. Additionally, these modified oils can serve as healthier alternatives to shortenings and margarine in food formulations (Ahmadi & Marangoni, 2009; Harvey, 1937; Macias-Rodriguez & Marangoni, 2016b; Nor Aini & Miskandar, 2007; Ramli *et al.,* 2008).

With this in mind, our study aimed to structure vegetable oils by harnessing the acylglycerol formation ability and selectivity of lipases. Specifically, we sought to selectively remove SFAs from vegetable oils and enrich them with MAGs and DAGs. The outcomes of the hydrolysis reactions resulted in two target structured products: a crude hydrolyzed fat containing FFAs, MAGs, DAGs, and TAGs, and a deacidified hydrolysate with removed FFAs. Both of these products hold potential as specialty fats in the food industry.

Additionally, the application of enzymatic reactions was used to synthesis alkyl rosmarinates using ethyl rosmarinate as a model after investigating the impact of the oil-based food matrix on the antioxidant properties of rosmarinic acid derivatives.

Rosmarinic acid, named after rosemary, is a good antioxidant and one of the most abundant phenolic acids that appears naturally in free or esterified form in many herbs, such as rosemary, sage, basil, and mint. Rosmarinic acid has different polar groups in its structure and therefore exhibits good solubility in aqueous media (Guan *et al.,* 2022). However, its hydrophilic nature may negatively influence its efficiency in protecting some complex oil-based food systems. This is due to the fact that lower solubility of phenolic acids can become problematic for the homogeneity of the fat-based products, their accessibility where the oxidation reaction happens, and hence their ease of application in different oil-based food systems (Decker *et al.,* 2017; Farooq *et al.,* 2021; Laguerre, Sørensen, *et al.,* 2013). Therefore, comprehending the efficacy of rosmarinic acid derivatives with different level of hydrophobicity in suppressing oil

oxidation in a particular food matrix holds importance in development of enhanced antioxidant compounds and the formulation of functional products.

The effectiveness of a particular antioxidant in oil-based food systems is significantly dictated by its chemical structure and concentration. Moreover, these properties are interconnected with the characteristics of the oil-based food matrix (Ghelichi *et al.,* 2023). In this context, the type of structuring agent or emulsifier utilized appears to have a significant impact, and there is a likelihood that particular interactions between the structuring agent or the emulsifier and antioxidants contribute to the additive or synergistic effect that influences their antioxidant activity (Sørensen *et al.,* 2017). Robust interactions occurring between the structuring agent or emulsifier and the antioxidant might position the antioxidant in closer proximity to the interphase. This proximity has the potential to enhance its protective efficacy within the food system where the oxidation reactions happen. Conversely, diminished interactions could result in a comparatively dispersed placement of the antioxidant within the food system, consequently adversely impacting its antioxidant activity. In this context, the hydrophobicity of the antioxidant can change its interaction in the oil-based food matrix (Laguerre, Bayrasy, *et al.,* 2013; Laguerre *et al.,* 2015; Laguerre, Sørensen, *et al.,* 2013).

The longstanding theory that has sought to predict the efficacy of antioxidants in various oil matrices is the “polar paradox”. This theory posits that polar antioxidants tend to be more efficient in bulk oils, whereas nonpolar antioxidants are expected to outperform polar antioxidants in emulsified systems (Laguerre *et al.,* 2015). Building upon this, the conception of interfacial oxidation was introduced, suggesting that these distinctions may arise due to the preference of polar antioxidants in the air-oil interface in bulk oils because of their limited solubility in oil. Conversely, lipophilic antioxidants are more inclined to position themselves at the oil-water interphase in emulsions. Contrasting viewpoints suggest that the polar-paradox

theory may have overlooked certain variables, whereas the cutoff effect suggests a non-linear association (resembling a parabolic trend) between hydrophobicity and antioxidant capacity. Consequently, antioxidant capacity can increase up to a certain threshold as the hydrophobic chain length is extended (Mardani *et al.,* 2022).

In the context of emulsions, various researchers have noted that enhancing hydrophobic properties does not consistently yield improved antioxidant efficacy. Expectedly, an observed parabolic, or cut-off effect on antioxidant capability emerges once extending the length of the homologous series of lipophilic alkyl esters derived from phenolic acids (Ghelichi *et al.,* 2023; Sørensen *et al.,* 2017). This outcome challenges the anticipated trend set by the polar paradox, as medium-sized alkyl chains demonstrated the highest antioxidant capacity within emulsions. Additionally, the use of oleogels or oleogel-based emulsions, using lipidic or biopolymeric oleogelators, have emerged as a means of structuring oil within food formulations in recent years, in comparison to traditional methods of blending or using monoacylglycerol as a structuring agent (Silva *et al.,* 2021). This structural transformation is achieved through the incorporation of a gelators, acting as a structuring agent, into the liquid oil phase. The adoption of oleogels serves to rectify quality issues, notably by curbing or mitigating concerns such as fat migration, which can be substantial in the context of fat bloom, as well as by reducing the saturated fatty acid content in related products (Naeli *et al.,* 2020). However, although unsaturation levels reduce considerably which causes a considerable reduction in oxidative stability, no emphasis has been placed on improving their antioxidant activity. In this regard, understanding the intricate relationship between the structuring agent and its influence on antioxidant activity in relation to the hydrophobicity of antioxidants can lead to the formulation of enhanced food products with extended shelf life and improved oxidative stability.

Moreover, the presence of natural rosmarinate esters in plants are naturally trivial and the procedures for their isolation and refinement are normally difficult, calling for lipophilization techniques (Guan *et al.,* 2022; Wu *et al.,* 2019). Therefore, the synthesis of alkyl rosmarinates is necessary which can be done by structurally modifying rosmarinic acid. In this regard, structural modifications via esterification with aliphatic molecules like long-chain fatty acids and alcohols or any other proper substrate can be used to improve the solubility or bioavailability of these compounds (Laguerre *et al.,* 2015; Mardani *et al.,* 2022). Esterification of rosmarinic acid esters can be done by chemical or enzymatic reactions. Among these approaches, chemical esterification has been successfully reported for the production of alkyl rosmarinates (Lecomte *et al.,* 2010). However, enzymatic ones are believed environmentally friendly and need lower purification or downstream handling (Schär & Nyström, 2015; Yang *et al.,* 2012). Consequently, a practical approach to broaden the utility of phenolic acids involves enzymatically altering their hydrophilic characteristics and augmenting their hydrophobicity. Typically, this can be accomplished via enzymatic lipophilization, entailing the esterification of the carboxylic acid group of phenolic acids with an aliphatic compound, like fatty alcohols while retaining its initial functional characteristics (Peng *et al.,* 2023).

Therefore, in this study, the antioxidant activity of alkyl rosmarinates (methyl and ethyl rosmarinates) were compared with rosmarinic acid with radical scavenging activity (*in vitro*), and in food systems, including bulk oil system, structured oil with monoacylglyceride, oleogel, O/W emulsion, and gelled O/W emulsion under accelerated oxidation condition at 35 °C during one month. Additionally, the lipophilization of rosmarinic acid with ethanol was done with Lipozyme 435 (Novozyme) as a model and optimized by considering reaction conditions, including time, temperature, enzyme-to-substrate ratio, and the concentrations of rosmarinic acid and alcohols.

2. AIMS

2.1. First section (application of enzymatic reactions to solidify liquid vegetable oils)

1. Screening enzymes for the ability of enzymes to selectively enrich oils with MAGs (preferably) and DAGs and simultaneously remove SFAs.
2. Optimization of reactions for the selected enzyme for enriching them with MAGs and DAGs.
3. Expanding the application to vegetable oils with different degrees of saturation.
4. Deacidification of the obtained products.
5. Assessment of the properties of the obtained structured fats.

2.2. Second section (application of enzymatic reaction to synthesis alkyl rosmarinates after assessment of their antioxidant activity in oil-based food systems)

1. Studying the antioxidant activity of alkyl rosmarinates (methyl and ethyl rosmarinates) compared with rosmarinic acid through radical scavenging activity (*in vitro*), and in food systems, including bulk oil system, structured oil with MAGs, oleogel, O/W emulsion, and gelled O/W emulsion under accelerated oxidation condition at 35 °C during one month.
2. Examining the "Polar Paradox Hypothesis" and "cut-off Effect" by testing the antioxidant effects of the modified antioxidants in both oil systems and emulsions.
3. Enzymatic synthesis of alkyl rosmarinates using ethyl rosmarinate as a model.
4. Optimizing the reaction conditions to obtain the highest yield of the ethyl rosmarinate.

3. LITERATURE REVIEW

3.1.TAGs structure, fatty acids, and fatty acid positioning on TAGs

Understanding the nomenclature of fatty acids, the structure of TAGs, and the positioning of fatty acids on TAG molecules is crucial for comprehending lipase regiospecificity. This section provides an overview of these key concepts.

TAGs (triacylglycerols, also known as triglycerides), are the primary constituents of natural fats and oils. They consist of a glycerol molecule esterified with three fatty acid chains. TAGs exhibit structural diversity based on the types of fatty acids esterified to the glycerol backbone. The arrangement of these fatty acids on the glycerol molecule plays a crucial role in determining the physical and chemical properties of TAGs. Fatty acids can be esterified to the glycerol backbone in different positions, leading to various TAG molecular species. The position of fatty acids on the glycerol molecule is denoted by sn-1, sn-2, and sn-3 positions, where "sn" stands for the stereospecific numbering (sn) system. Lipases exhibit regiospecificity, meaning they preferentially hydrolyze fatty acids from specific positions on the glycerol backbone of TAG molecules (Gunstone, 2009). Figure 1 shows the structure of TAGs.

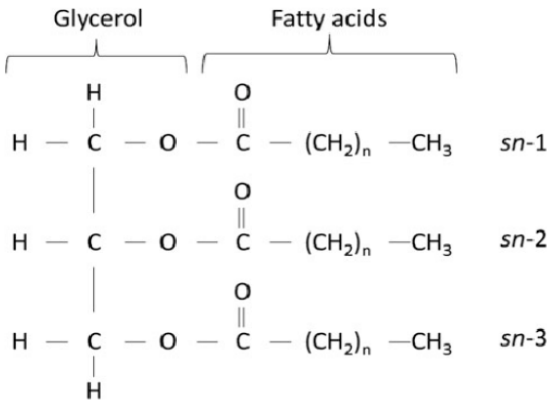

Figure 1. Structure of triacylglycerols.
(Gunstone, 2009).

Understanding the positioning of fatty acids on TAGs is crucial for interpreting the regiospecificity of lipases. For example, lipases with sn-1,3 regiospecificity hydrolyze fatty acids preferentially from the sn-1 and sn-3 positions of TAG molecules, while lipases with sn-2 regiospecificity target the sn-2 position. This knowledge is important for designing enzymatic processes tailored to specific applications, such as the production of structured lipids with desired fatty acid compositions and properties (Gunstone, 2009).

Fatty acids, integral constituents of lipids, play a pivotal role in determining the nutritional and organoleptic properties of food. Chemically, fatty acids are comprised of long aliphatic chains terminating in a carboxyl group, and they vary based on chain length and degree of saturation—classified into SFAs, MUFAs, and PUFAs. SFAs, typically found in animal fats and tropical oils, lack double bonds, rendering them solid at ambient temperature due to their ability to pack closely together. In contrast, unsaturated fatty acids, prevalent in plant oils and marine sources, contain one or more cis-configured double bonds that introduce bends in the hydrocarbon chain, preventing tight packing and thus remaining liquid at room temperature (Gunstone, 2009). The geometric configuration of these double bonds is crucial, as the cis configuration maintains membrane fluidity and functionality. However, during industrial hydrogenation, trans fatty acids can form, which have been associated with adverse cardiovascular effects. The compositional profile and balance of fatty acids in dietary lipids are critical for influencing food texture, flavor, and overall nutritional quality, underscoring the necessity for a detailed understanding of their chemistry to optimize food processing and promote healthful consumption (Gunstone, 2009). Therefore, fatty acid nomenclatures with their structures are also shown in Table 1.

Table 1. Fatty acid nomenclatures with their structures.

Common Name	IUPAC Name	Structure	Omega	Double Bonds
Butyric Acid	Butanoic Acid	$CH_3(CH_2)_2COOH$	-	C4:0
Caproic Acid	Hexanoic Acid	$CH_3(CH_2)_4COOH$	-	C6:0
Lauric Acid	Dodecanoic Acid	$CH_3(CH_2)_{10}COOH$	-	C12:0
Myristic Acid	Tetradecanoic Acid	$CH_3(CH_2)_{12}COOH$	-	C14:0
Palmitic Acid	Hexadecanoic Acid	$CH_3(CH_2)_{14}COOH$	-	C16:0
Stearic Acid	Octadecanoic Acid	$CH_3(CH_2)_{16}COOH$	-	C18:0
Oleic Acid	(9Z)-Octadec-9-enoic Acid	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$	ω-9	C18:1 (n-9)
Linoleic Acid	(9Z,12Z)-Octadeca-9,12-dienoic Acid	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$	ω-6	C18:2 (n-6)
α-Linolenic Acid	(9Z,12Z,15Z)-Octadeca-9,12,15-trienoic Acid	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_7COOH$	ω-3	C18:3 (n-3)
Arachidonic Acid	(5Z,8Z,11Z,14Z)-Eicosa-5,8,11,14-tetraenoic Acid	$CH_3(CH_2)_4CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_3COOH$	ω-6	C20:4 (n-6)
Eicosapentaenoic Acid (EPA)	(5Z,8Z,11Z,14Z,17Z)-Eicosa-5,8,11,14,17-pentaenoic Acid	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_3COOH$	ω-3	C20:5 (n-3)
Docosahexaenoic Acid (DHA)	(4Z,7Z,10Z,13Z,16Z,19Z)-Docosa-4,7,10,13,16,19-hexaenoic Acid	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_3COOH$	ω-3	C22:6 (n-3)

3.2.Historical Background and definition of lipases

Enzymes, as proteins, play a vital role in catalyzing various chemical and biochemical reactions inside or outside cells. These highly specific natural catalysts exhibit remarkable efficiency under diverse environmental conditions, such as temperature, pressure, and pH, facilitating high conversion rates (Bornscheuer *et al.,* 2012; Xia *et al.,* 2019). The discovery of lipase dates back to 1856 when Claude Bernard identified it as an enzyme in pancreatic juice responsible for hydrolyzing insoluble oil droplets into soluble products. Subsequently, lipase production was observed in various bacteria, including *Bacillus prodigiosus, B. pyocyaneus,* and *B. fluorescens* in 1901, and currently, species such as *Serratia marcescens, Pseudomonas aeruginosa,* and *Pseudomonas fluorescens* are known for their large-scale lipase production. In 1994, the first commercial recombinant lipase, Lipolase, was industrially produced from the

fungus *Thermomyces lanuginosus* and expressed in *Aspergillus oryzae*. Traditionally, lipase was obtained from animal pancreas and used as digestive supplements in crude or purified form. Over time, lipase has found extensive applications as a biocatalyst for synthesizing novel chemical compounds (Baena *et al.,* 2022; Chandel *et al.,* 2022; de Andrade Silva *et al.,* 2023; Mehta *et al.,* 2021).

Lipases (EC 3.1.1.3) are part of the hydrolases family and are known as TAGs acyl hydrolase. These enzymes act on carboxylic ester bonds and belong to the class of serine hydrolases, not requiring any cofactor. Naturally, lipases hydrolyze TAGs into diglycerides, monoglycerides, fatty acids, and glycerol. The hydrolysis of ester bonds at the interface, catalyzed by lipases, occurs between an insoluble substrate phase and an aqueous phase, where the enzymes remain soluble under natural conditions (Choi *et al*., 2015; Kumar *et al*., 2022; Sorour *et al*., 2017). Lipases enable various conversion reactions, including esterification, transesterification, interesterification, acidolysis, alcoholysis, and aminolysis (Figure 2).

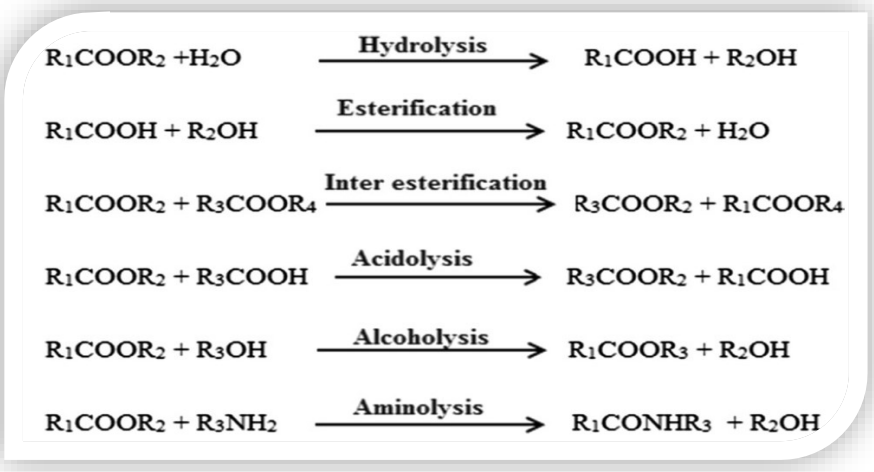

Figure 2. Various reactions done by lipases.

R is used to represent different possible alkyl groups (Gunstone, 2009).

In this context, in the first section of the thesis, the application of enzymatic hydrolysis is used to enrich oils with MAGs and DAGs while selectively removing SFAs and. In the second section, the application of esterification reaction is used to investigate the impact of different chain lengths of fatty alcohols on the antioxidant activity of rosmarinic acid.

3.3.Sources of Lipases

These versatile enzymes are widely distributed in animals, plants, and microorganisms, with microbial lipases being of particular commercial importance due to their ease of cultivation and genetic manipulation, resulting in higher yields. Microbial lipases are not only more stable than their plant and animal counterparts but also offer a safer and more stable production process for industrial and research applications. Microbes are abundant in nature, thriving in diverse environments, such as the Dead Sea, Antarctica, alkaline lakes, hot springs, volcanic vents, and contaminated soils, which provides extraordinary potential for lipase production with specific features. The diverse microbial resources, coupled with their remarkable adaptation abilities to inhospitable atmospheres, make them ideal candidates for lipase production with tailored functionalities (Bourlieu *et al.,* 2009; Iwasaki & Yamane, 2000; P. A. Lopes *et al.,* 2019; Moazeni *et al.,* 2019; Quezada & Hernandez, 2011).

The microbial world offers a wide array of lipase sources, and various microorganisms have been explored for their lipolytic capabilities. Notably, fungi and bacteria are major contributors to the production of lipases with varied characteristics. Fungal lipases have been studied since the 1950s and are preferred for their thermal and pH stability, substrate specificity, and activity in organic solvents, making them advantageous over bacterial lipases. Key filamentous fungi, including *Rhizopus, Aspergillus, Penicillium, Mucor, Ashbya, Geotrichum, Beauveria, Humicola, Rhizomucor, Fusarium, Acremonium, Alternaria, Eurotium,* and *Ophiostoma*, have been extensively studied for their lipase production. *Candida rugosa, Candida antarctica,*

Trichosporon lanuginosus, Rhizomucor miehei, Pseudomonas, Mucor, and *Geotrichum* are among the important fungal species contributing to commercial lipase production (Agyei *et al.,* 2019; Gunathilake, Akanbi, Van Vuong, *et al.,* 2022; Jala & Ganesh Kumar, 2018; D. B. Lopes *et al.,* 2017; Lortie, 1997; Speranza *et al.,* 2019; Walsh, 2007).

Bacterial lipases have also been widely explored and well-studied compared to plant and animal sources. Bacterial lipases are typically glycoproteins, although some extracellular forms can be lipoproteins. Certain polysaccharides have been observed to affect the enzyme production in bacteria. Bacterial lipases are known for their thermostable properties, and while most are constitutive and nonspecific in substrate specificity, some exhibit remarkable thermal stability. Key bacterial genera utilized for lipase production include *Pseudomonas, Bacillus, Achromobacter, Alcaligenes, Arthrobacter, Chromobacterium,* and *Staphylococcus* (Carvalho, 2011; Kim *et al*., 2023; Kontkanen *et al*., 2011; Lai *et al*., 2012; X. Xu *et al*., 2007; Zam, 2020). In addition to microorganisms, plant and animal sources are also potential contributors to lipase diversity. Plants, especially their seeds containing large amounts of TAGs, serve as reservoirs for lipases. During seed germination, lipase activity increases as TAGs are converted to soluble sugars to supply energy to growing tissues. Animal lipases play a crucial role in lipid digestion within biological systems (Ang *et al.,* 2019; Domínguez de María *et al.,* 2006; Moussavou Mounguengui *et al.,* 2013; Rodrigues *et al.,* 2019; Seth *et al.,* 2014; Speranza & Macedo, 2012; Stergiou *et al.,* 2013; Wei *et al.,* 2020).

Overall, the abundance and diverse adaptations of microorganisms make them promising sources for the production of lipases with specific functionalities. The commercial availability and varied characteristics of microbial lipases open up a wide range of applications, including food, detergent, pulp and paper, leather industries, and environmental management. As researchers continue to explore and exploit the potential of microbial lipases, these enzymes

will continue to play a vital role in biotechnological applications (Baadhe *et al*., 2014; Barriuso *et al*., 2016; Bornscheuer, 1995; Mustranta *et al*., 1993; Subileau *et al*., 2018; Villeneuve, 2007).

3.4.General characteristics of lipases

Lipases typically have molecular weights ranging from 19 to 60 kDa and exist as monomeric proteins. Lipases demonstrate pH-dependent activities, with stability observed at neutral pH 7.0 or within the range of pH 4.0 to 8.0. Different lipases exhibit specificity for specific acylglycerol substrates, resulting in either the release of fatty acids from all three positions of glycerol in one group of lipases or the regio-specific discharge of fatty acids from the 1, 3 positions of acylglycerols in another group (Choi *et al*., 2015; L. R. Kumar *et al*., 2022; Quezada & Hernandez, 2011; Sorour *et al*., 2017). Under certain experimental conditions, lipases can even reverse reactions, leading to esterification and interesterification in the absence of water. Calcium serves as a stimulating divalent cation for lipase activity, while various other cations and compounds like Co^{2+}, Ni^{2+}, Hg^{2+}, and Sn^{2+} strongly inhibit their activities. Temperature stability profiles indicate that lipases have greater stability at lower temperatures (Choi *et al*., 2015; Kumar *et al*., 2022; Quezada & Hernandez, 2011; Sorour *et al*., 2017). By utilizing organic media with low water activity, enzymes can display exceptional specificity, and altering solvent properties can transform an enzyme's specificity due to the influence of soft structures and delicate interactions. This property allows lipases to be employed in extracting optically pure esters and alcohols, making them valuable tools for various industrial applications (Choi *et al*., 2015; Kumar *et al*., 2022; Quezada & Hernandez, 2011; Sorour *et al*., 2017).

3.5.Specificities of lipases

3.5.1. Regioselective (regiospecific) lipases

TAGs are composed of a glycerol backbone, which is a three-carbon alcohol, chemically known as propane-1,2,3-triol. Each of the three hydroxyl (-OH) groups on the glycerol molecule is esterified with a fatty acid, forming an ester bond. The fatty acids attached to the glycerol can vary in chain length and degree of saturation, influencing the physical properties of the TAGs. Regioselective lipases possess the valuable ability to direct reactions towards favorable outcomes while minimizing undesired side reactions by selecting one of the carbons in the glycerol backbone of the TAGs. This property holds significant importance for chemical and pharmaceutical industries, particularly in the production of structured glycerides in different industries, such as cocoa-butter substitutes (Barriuso *et al.,* 2016; Fadnavis & Koteshwar, 1997; Janssen *et al.,* 1996; Yahya *et al.,* 1998). In this context, regiospecific lipases can be further classified into two categories:

(a) 1,3 regiospecific lipases: These lipases catalyze the hydrolysis of TAGs at carbons on first and third positions (side carbons) of the glycerol backbone, resulting in the generation of FFAs, 2-MAGs, and 1,2 or 2,3 DAGs. Notably, immobilized 1,3-specific lipases from *R. delemar* (PPRhDL) and *R. miehei* (Lipozyme) have been effectively utilized in acidolysis reactions, enabling the synthesis of long-chain fatty acid esters from walnut oil and caprylic acid. Additionally, immobilized 1,3-specific *R. oryzae* lipase, expressed in *Pichia pastoris*, has been employed for biodiesel production. A 1,3-regiospecific lipase has been identified from *Streptomyces violascens* (ATCC 27968), which selectively hydrolyzed EPA from the sn-1/3 position (stereospecific numbering) of codfish oil, enriching the final hydrolyzed product with 3.24-fold of EPA compared to the initial levels, in contrast to the 1.98-fold enrichment of DHA located at the sn-2 position (Barriuso *et al.,* 2016; Fadnavis & Koteshwar, 1997; Janssen *et al.,* 1996; Yahya *et al.,* 1998).

(b) 2-regiospecific lipases: These lipases display the unique ability to selectively remove fatty acids from the 2nd position of the glycerol backbone of a TAGs molecule, leading to the specific formation of 1,3-DAGs. Although sn-2 specificity is rare, it has been attributed to a lipase derived from *Geotrichum candidum*, which exhibits the capacity to hydrolyze oleic and linoleic acids specifically from the sn-2 position of TAGs (Barriuso *et al.,* 2016; Fadnavis & Koteshwar, 1997; Janssen *et al.,* 1996; Yahya *et al.,* 1998).

3.5.2. Substrate specific lipases

Substrate-specific lipases play a crucial role in various reactions, selectively targeting specific substrates from mixtures to facilitate desired product synthesis. These substrates typically include fatty acids and alcohols. Recent studies emphasize the significance of substrate specificity and enzyme stability to effectively harness lipases for various industrial processes. Substrate-specific lipases can be broadly categorized as follows:

(a) Fatty acid specific lipases: These lipases exhibit specificity towards fatty acids based on their chain length. Some lipases are particularly effective with short-chain fatty acids, while others show preference for medium or long-chain fatty acids. For instance, the lipase from *Bacillus cereus* demonstrates specificity for short (C4:0) to medium chain fatty acids (C12:0), whereas the lipase from *Pseudomonas sp.* exhibits specificity for fatty acids based on the position of the double bond, with α-linoleic acid being the most preferred, followed by stearic acid, oleic acid, β-linoleic acid, and conjugated linoleic acid. Various lipases from *Penicillium citrinum, A. niger, A. oryzae, Bacillus coughing, Geotrichum candidum,* and *C. lypolytica* show strong specificity for short-chain fatty acid esters. Additionally, a recombinant lipase from *Streptomyces violascens* (OUC-Lipase 6) has been found to preferentially hydrolyze EPA from the glyceride backbone of codfish oil compared to DHA (Barriuso *et al.,* 2016; Fadnavis & Koteshwar, 1997; Janssen *et al.,* 1996; Yahya *et al.,* 1998).

(b) Alcohol specific lipases: Another form of substrate specificity in lipases is observed towards the alcohol moiety of the substrate. The type of alcohol present influences the affinity of the lipase towards the substrate. Microbial lipases exhibit wide substrate specificity, enabling them to catalyze the synthesis of esters from primary, secondary, and tertiary alcohols with aromatic, aliphatic, and allylic compounds. Successful synthesis of esters from primary and secondary alcohols has been achieved using lipases, but biotransformation of tertiary alcohols remains challenging due to steric hindrance. However, certain lipases like *Candida antarctica* lipase have demonstrated enantioselective transesterification of tertiary alcohols, albeit at a lower rate. A novel halophilic, alkalithermostable lipase (LipR2) from *Alkalispirillum sp*. NM-R002 has been used for the synthesis of levulinic acid esters of different alcohols, showing preference for short-chain alcohols and providing higher ester yields for ethanol and 1-butanol. *Candida rugosa* lipase has been utilized for synthesizing biolubricants from soybean oil products and alcohols, with higher productivity achieved for NPG-based biolubricant than TMP-based biolubricant. Researchers have also employed lipase engineering through semi-rational design to enhance the biotransformation rate of tertiary alcohols. Furthermore, mutated and designed lipases have been employed to improve methanol tolerance in biodiesel/biofuel production (Barriuso *et al.,* 2016; Fadnavis & Koteshwar, 1997; Janssen *et al.,* 1996; Yahya *et al.,* 1998).

3.5.3. Enantioselective lipases

Enantioselective lipases possess the remarkable ability to selectively hydrolyze one of the isomers in a racemate, distinguishing enantiomers within a racemic mixture. These lipases play a significant role in various processes, such as the transesterification of secondary alcohols for pharmaceutical products, hydrolysis of menthol benzoate for cosmetic/food products, and hydrolysis of glycidic acid methyl ester for medical/health care products. Notably, enantiospecific lipases find applications in synthesizing isomeric compounds that function

optimally only under specific configurations. Recent discoveries in this field include the acylation of quercetin with ferulic acid using *Rhizopus (R.) oryzae* lipase for synthesizing flavonoid derivatives, as well as the production of acacetin and resveratrol 3,5-diO-beta-glucopyranoside using *C. antarctica* lipase B (Novozym 435) and *Burkholderia cepacia* lipase (Amano PS-IM), respectively (Balcão *et al.,* 1996; Chandel *et al.,* 2022; Gunathilake, Akanbi, Bucher, *et al.,* 2022; Mehta *et al.,* 2021).

3.5.4. Non-specific lipases

The lipases in this category are highly versatile and exhibit robustness in their ability to interact with various substrates, exemplified by the broad range of applications demonstrated by *Mucor meihei* lipases, ranging from the cosmetic industry to biodiesel production. Typically, they catalyze the hydrolysis of TAGs, breaking them down into FFAs and glycerol, with MAGs and DAGs forming as intermediates. Additionally, under microaqueous conditions, they can reverse the reaction, leading to the formation of glycerides from glycerol and fatty acids.

3.6.Applications of lipases in food industry

Lipases are versatile enzymes that have found extensive applications in various industrial sectors, including the food industry. In the context of fats and oils, lipases play a crucial role in modifying the properties of lipids, enabling the production of tailored fats with enhanced nutritional, sensory, and functional characteristics. This thesis highlights the significant applications of lipases in the food industry, specifically focusing on their role in the manufacturing of fats from vegetable oils and designing antioxidant for the final product by using an example of rosmarinic acid and its derivatives (Agyei *et al.,* 2019; Bourlieu *et al.,* 2009; Jala & Ganesh Kumar, 2018; Lortie, 1997; Quezada & Hernandez, 2011; Speranza *et al.,* 2019).

Here are some general applications of lipases in the food industry:

Modification of Oils and Fats: Fats and oils are essential components of various food products, and their properties significantly influence the quality and appeal of these products. Lipases offer a powerful tool to modify lipids by altering the position of fatty acid chains within the glyceride backbone and substituting specific fatty acids with others. By employing lipases, less expensive and less suitable lipids can be transformed into higher value fats, opening up new possibilities for the food industry. Enzyme-catalyzed esterification and inter-esterification reactions are employed to produce value-added products, such as specialty fats and partial glycerides, utilizing positional and fatty acid-specific lipases (Gunathilake, Akanbi, Van Vuong, *et al*., 2022; Lopes *et al*., 2017; Xu *et al*., 2007).

Esterification and Transesterification: Lipases play a key role in various enzymatic reactions involving oils and fats. Esterification reactions are employed to synthesize esters of short-chain fatty acids and alcohols, which are widely used as antioxidants, flavor and fragrance compounds in the food industry. Additionally, lipases are involved in transesterification reactions to produce structured lipids that have enhanced nutritional profiles, such as cocoa butter substitutes, low-caloric TAGs, and oils enriched with PUFAs (Khan *et al.,* 2021; Lortie, 1997; Milisavljević *et al.,* 2014; Mustranta *et al.,* 1993; Shin *et al.,* 2020; Stergiou *et al.,* 2013; Topakas *et al.,* 2003).

Enzymatic Degumming and Phospholipid Removal: Lipases are also employed in the physical refining of vegetable oils, where they act as environmentally friendly alternatives for enzymatic degumming. Selective microbial phospholipases, such as *Lecitase Novo*, have been utilized to efficiently remove phospholipids from vegetable oils, improving the economy of the degumming process (Choi *et al.,* 2015).

Production of Cocoa Butter Analogs: Cocoa butter is a crucial ingredient in chocolate and confectionary products. However, its high cost has driven the development of cocoa butter analogs using lipase-catalyzed transesterification. By exploiting sn-1,3 specific lipases from various microbial sources, low-cost fats like palm-oil fractions can be enriched to produce cocoa butter-type TAGs, mimicking the properties of cocoa butter (Biswas *et al.,* 2018; Gutiérrez-Macías *et al.,* 2021; Joseph *et al.,* 2021; Kadivar *et al.,* 2016; S. Kim *et al.,* 2014; Mohamed, 2015; Naik & Kumar, 2014; Osborn & Akoh, 2002; Shukla Vijai, 2005; Verstringe *et al.,* 2012; Zarringhalami *et al.,* 2010).

In conclusion, lipases have emerged as valuable biocatalysts with diverse applications in the food industry, especially in the production and modification of oils and fats. Their ability to selectively modify fatty acids and their position in glycerides has enabled the creation of specialty fats with enhanced functionality, nutritional value, and sensory attributes. As research continues to explore new microbial sources and optimize enzymatic processes, lipases are expected to play an increasingly significant role in improving the efficiency and sustainability of food processing, leading to innovative products that cater to evolving consumer demands.

3.6.1. Application of enzymatic hydrolysis as a tool for manufacturing structured fat

MAGs and DAGs find widespread utilization across various sectors of the food industry. These products can be synthesized through catalytic processes involving glycerol, oils, or specific fatty acids (achieved via techniques like interesterification or direct esterification). From a physicochemical perspective, mono- and diglycerides of fatty acids exhibit attributes of emulsifiers due to their inherent molecular polar duality. In essence, the hydrophilic component is embodied by glycerin, while the non-polar/lipophilic aspect is formed by the aliphatic chains. This unique configuration imparts these molecules with intriguing characteristics, including

the capacity for stabilizing emulsions and foams, as well as forming inclusion complexes (Saberi, Kee, *et al.,* 2011; Saberi, Lai, *et al.,* 2011).

In recent times, consumers have significantly heightened their attention towards the products they consume. Their primary expectations revolve around natural offerings, devoid of preservatives and additives, leading to growing skepticism towards industrial goods. This has given rise to the clean-label trend, compelling manufacturers to substitute synthetic additives with natural alternatives, while maintaining the same level of functionality proves to be a complex endeavor. To align with the clean-label strategy and incorporate MAGs and DAGs into food products, some producers have adopted an enzymatic approach utilizing lipases. Enzymes exhibit remarkable selectivity and engage in specific interactions with reactants through catalytic processes. Lipases, for instance, selectively act upon TAGs, catalyzing hydrolysis reactions that yield a range of products including diglycerides (comprising two positional isomers: 1,2- and 1,3- diglycerides), monoglycerides (with two isomers: 1- and 2-monoglycerides), FFAs, and glycerol. Except for glycerol, which is hydrophilic, all the molecules have lipophilic properties and thus are preferably found in the oil phase (Chen *et al*., 2014; Shakerardekani *et al*., 2013; Subroto, 2020).

Enzymatic hydrolysis, particularly when involving a lipase, can exhibit effectiveness in the presence of water, but it necessitates oil as a vital reactant. To facilitate the enzymatic reaction, it becomes imperative to bring these two phases into contact within an emulsion configuration (oil-in-water, O/W). Consequently, the sole substrates engaged in the reaction are fat/oil and water, simplifying the optimization process for these reactions as well (Mardani *et al.,* 2015). Figure 3 shows the reaction scheme for the hydrolysis of TAGs into FFAs, MAGs, DAGs, and glycerol.

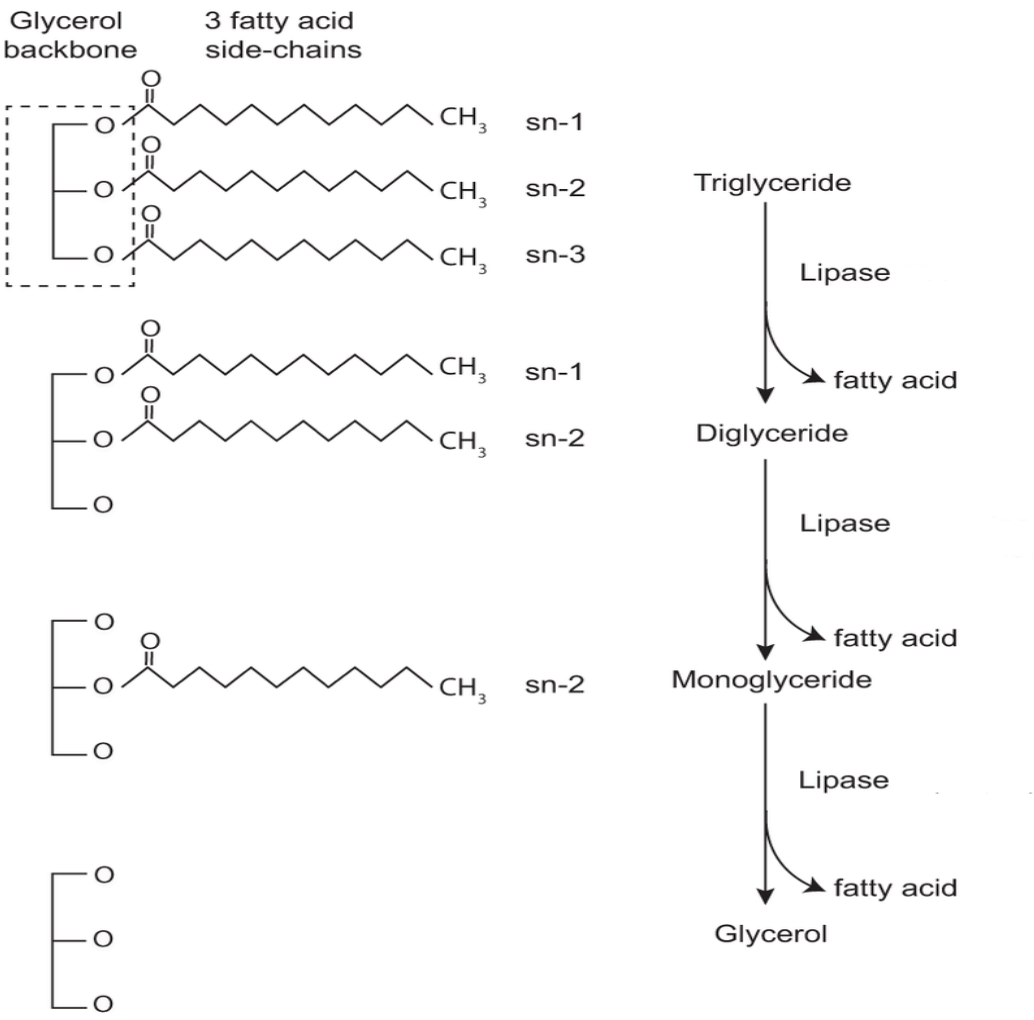

Figure 3. Reaction scheme for the hydrolysis of TAGs.
(Gunstone, 2009).

The hydrolysis of TAGs involves breaking the ester bonds linking the glycerol backbone to its three fatty acid chains through enzymatic catalysis by lipases. Initially, TAG undergo hydrolysis, where a water molecule cleaves one ester bond, releasing a FFA and converting TAG to DAG. This process continues as DAGs undergoes further hydrolysis, producing another FFA and resulting in MAG. Finally, MAG is hydrolyzed to release the third fatty acid and yield glycerol. Each step involves the addition of a water molecule and the action of lipases, systematically breaking down TAG into glycerol and FFAs (Figure 3).

In this context, enzymatic hydrolysis reactions involving lipases present a considerable avenue for generating structured fats possessing targeted desired fatty acid compositions. This strategy holds the potential to enhance nutritional attributes by transforming vegetable oils into acylglycerols enriched with greater unsaturated fatty acid content, a pivotal aspect for developing functional food products. Furthermore, these tailored oils can serve as more health-conscious substitutes for shortenings and margarine in diverse food formulations (Bornscheuer, 1995; Chen *et al*., 2014; Subroto, 2020). With this perspective in mind, our study was conducted to focus on the acylglycerol formation capability and selectivity of lipases in order to engineer the structure of vegetable oils by turning liquid vegetable oils into solids and reducing their saturated fatty acids.

3.6.2. Application of enzymatic esterification in changing hydrophobicity of antioxidants

The solubility of polar antioxidants in hydrophilic phases is higher, resulting in poor mixture homogeneity and functionality in emulsion systems (Farooq *et al.,* 2021). To enhance their applicability in lipid-rich food products, grafting hydrophobic moieties into their structure has been explored through chemical, enzymatic, or chemoenzymatic esterification, while retaining the original properties of the parent molecule as a radical scavenger (Decker *et al.,* 2017; López Giraldo *et al.,* 2007). Consequently, the product of this lipophilization process can be referred to as a lipophilized antioxidant, lipophenol, or phenolipid in the case of phenolic compounds. Traditional chemical reactions are commonly employed to produce phenolic esters, but they often necessitate harsh pH and temperature conditions. Due to the relative instability of phenolic acids in alkali solutions, chemical esterification usually relies on strong acid catalysts (González-Sabín *et al.,* 2011). While this method of lipophilization is fast and straightforward, it lacks selectivity, leading to poor yields and generating toxic solvents and numerous side products. As a result, additional purification steps are required to separate the unwanted

byproducts (Xu *et al*., 2006). Consequently, the chemical lipophilization of antioxidants may not always be a practical, economical, environmentally friendly, and viable option for synthesizing novel antioxidants.

Additionally, enzymes have become widely employed in various industries, including cosmetic, pharmaceutical, and food manufacturing, to obtain valuable lipophilized antioxidants (May, 2019). Enzymatic reactions are highly selective, environmentally friendly, and result in fewer side reactions. Unlike chemical reactions, enzymatic methods are conducted under mild conditions, preserving the structural integrity of the involved molecules (Xu *et al*., 2006). This makes enzymatic reactions particularly advantageous when dealing with edible oils and emulsions, as they do not generate potentially toxic substances or cause flavor reversion. Furthermore, enzyme separation can be achieved through simple unit operations such as filtration and membrane separation after completing the process (May, 2019). As a result, the advantages of enzymatic antioxidant modification have garnered increasing interest over the last two decades.

3.7.Role of hydrophobicity on antioxidant activity

Over the past two decades, there has been ongoing debate regarding the impact of hydrophobicity on antioxidant activity. The question of whether increasing hydrophobicity leads to a more effective antioxidant has been extensively discussed. Despite significant research on antioxidants, predicting their mechanism of action still poses challenges. In this context, the significance of active groups in determining the potency of antioxidants, specifically in donating hydrogen atoms to hydroperoxyl radicals, is widely acknowledged and has direct effect on antioxidant activity. Nevertheless, the radical scavenging activity of antioxidants is not solely governed by the presence or absence of active groups. An equally critical factor is the accessibility of active antiradical agents at the reaction site, enabling them

to effectively delay oxidative reactions (Laguerre, Bayrasy, *et al.,* 2013; Laguerre *et al.,* 2015; Laguerre, Sørensen, *et al.,* 2013). This aspect becomes particularly crucial in compartmentalized systems like emulsions at the interface of lipids and water (Farooq *et al.* 2021). As a result, the mobility and position of these compounds can also significantly influence the ability of antioxidants to prevent or delay oxidation reactions. In this regard, the hydrophobicity of antioxidants plays a predominant role in their mobility (Decker *et al.,* 2017; López Giraldo *et al.,* 2007).

Therefore, the influence of hydrophobicity on antioxidant efficiency has given rise to contrasting theories about their behavior in different systems. In essence, lipophilization could be a double-edged sword, as it may either enhance or diminish antioxidant activity. On one hand, there has been a consensus among scientists that hydrophobicity enhances antioxidant activity, supported by numerous publications without a clear explanation (Laguerre *et al.,* 2015). On the other hand, the polar-paradox concept proposes that lipophilic antioxidants exhibit higher antioxidant capacity than their hydrophilic counterparts in dispersed lipids, such as emulsions and active surfaces of oil and air. For almost two decades, the polar-paradox phenomenon has been widely accepted in lipophilization and extensively used to predict antioxidant activity in food systems. However, different contrasting publications over the years proved that the polar paradox may not encompass all variables involved in oxidation reactions and overlooks some essential factors (Laguerre, Bayrasy, *et al.,* 2013; Laguerre *et al.,* 2015; Laguerre, Sørensen, *et al.,* 2013).

More recently, the cut-off effect has emerged as a new proposal in the field. According to this phenomenon, there exists a non-linear relationship, resembling a parabolic-like trend, between hydrophobicity and antioxidant capacity (Decker *et al.,* 2017). As a result, the antioxidant capacity shows an increase up to a certain threshold with the elongation of the hydrophobic chain length. In light of the cut-off concept, the design of new antioxidants takes into account

the critical chain length, ensuring efficient targeting of active oxidation sites. In other words, lipophilization of moieties that are either too short or too long can diminish antioxidant activity (Decker *et al.,* 2017; Laguerre, Bayrasy, *et al.,* 2013; Laguerre, Sørensen, *et al.,* 2013). This dependence on hydrophobicity may also vary slightly when antioxidants are employed in different food systems with different level of hydrophobicity or different ingredients, including different emulsifiers or oleogelators. In this context, the cut-off phenomenon has helped clarify some previously observed discrepancies and has paved the way for the synthesis of more relevant and highly effective novel antioxidants for a particular food system (Crauste *et al.,* 2016).

However, to date, no single concept can fully elucidate the relationship between hydrophobicity and antioxidant activity in existing biological media. The biological characteristics and antioxidant activity of these modified antioxidants are determined by several factors, including their reducing capability (number and type of active groups), the position of the active moiety in the parent antioxidant, diffusion, location, and partitioning in different phases of dispersed systems (Mardani *et al.,* 2022). Even in the case of pure frying oils, oxidation predominantly occurs at the interface of the oil and the air (Aladedunye & Gruczynska, 2019; Gruczynska *et al.,* 2015). Therefore, it is crucial to adjust the degree of hydrophobicity, ensuring that antioxidants are present at the active site and not overly solubilized in the oil due to excessive hydrophobicity. Consequently, the key to developing novel antioxidants that enhance the existing ones lies in designing a suitable lipophilic moiety with an appropriate critical chain length for a specific medium while retaining the reactive moiety of the parent antioxidant (Mardani *et al*., 2022).

In the case of lipophilization of antioxidants, Table 2 provides a comprehensive summary of advancements in the enzymatic lipophilization of bioactive compounds over the past decade, presenting both successful and unsuccessful examples. Efforts to enhance the antioxidant

activity of specific phenolic compounds have yielded both successful and unsuccessful outcomes, as depicted in Table 2. Lipophilized antioxidants synthesized through these efforts generally exhibit thermal stability, which is advantageous compared to natural polar antioxidants known for their thermal sensitivity (Rashmi & Negi, 2020). However, a noteworthy limitation in most studies is the lack of comprehensive comparison between the effects of lipophilization in different systems, such as bulk oil and emulsions. These differences are attributed to the improved diffusion and efficacy of lipophilized antioxidants in emulsions, allowing them to better access and protect active oxidation sites compared to oil-based systems (Laguerre, Bayrasy, *et al.,* 2013).

Table 2. Examples of enzymatic lipophilization of bioactive compounds with high antioxidative potentials

Acyl Acceptor (AA)	Acyl Donor (AD)	Medium	Enzyme	Optimized reaction condition						Biological effect	Reference
				AA/AD	V (mL)	En (%)	T (˚C)	Ti (h)	Y (%)		
(-)-pigallocatechin gallate (EGCG) derivatives	Vinyl esters	Acetone	Lipase DF-Amano	1	20	6	50	96	80	Antioxidant↑	(C. Jiang *et al.,* 2021)
		2-propanol, acetonitrile	Lipozyme RM IM	5	10	-	50	10	83	Antioxidant↑	(Zhu *et al.,* 2020)
		Ionic liquids	Novozym® 435	90	5	5	70	10	98	Lipophilicity↑	(Zhu *et al.,* 2021)
		Acetonitrile	Lipozyme RM IM	1	20	2	40	8	84	Antioxidant↑	(Zhu *et al.,* 2014)
Anthocyanin derivatives	Fatty acids	tert-amyl alcohol	Novozym® 435	10	2	3	60	12	96	Antioxidant↑	(Xiao *et al*., 2021)
		2-methyl-2-butanol	C. antarctica B	-	15	2	60	12	47	Stability↑	(Guimarães *et al.,* 2018)
		Acetone, acetonitrile, tert-butanol	Novozym® 435	10	10	1	60	72	73	Antioxidant↓ Stability↑	(Yang *et al*., 2018)
		2-methyl-2-butanol	C. antarctica B	100	2.5	2	60	48	-	Antioxidant↑	(Cruz *et al.,* 2016)
	Fatty acid methyl ester	tert-amyl alcohol	Lipozyme 435	150	-	2	60	24	7	Antioxidant↑	(Zhang *et al*., 2021)
Rutin derivatives	Fatty acids	Acetone, 2-Methyl-2-butanol	Novozym® 435	4	300	-	50	96	30	Antioxidant↓ (in oil) Antioxidant↑ (in emulsion)	(Mbatia *et al.,* 2011) (Vaisali *et al.,* 2017)
			C. antarctica B	4	5	-	55	96	66	-	(Viskupicova *et al*., 2010; Viskupicova *et al*., 2015)
			C. antarctica B	-	-	-	60	168	62	Antioxidant↔ Lipophilicity↑	
			Novozym® 435	4			50	72	90	-	(Zheng *et al*., 2013)
	Fatty acid esters	Acetone, 2-Methyl-2-butanol	Novozym® 435	10	5	3	65	72	94	Antioxidant↓ (in oil system)	(Hadj Salem *et al.,* 2011)
Silybin derivatives	Fatty Acids	Acetone	Novozym® 435	15	10	0.5	50	96	50	-	(Theodosiou *et al.,* 2011)
		Acetonitrile							37		
		2-methyl-2-butanol,							31		
		2-methyl-2-propanol							14		
	Vinyl esters	Acetonitrile	Novozym® 435	2.7		1.2	45	72	66	Antioxidant↑	(Vavříková *et al.,* 2014)
Naringin derivatives	Vinyl esters	Aceton	Novozym® 435	10	150	-	50	48	100	Antifungal↑	(Salas *et al.,* 2011)
		Acetone, t-butanol, acetonitrile, THF	Novozym® 435, Lipozyme RM IM	10	3	-	50	6	-	Lipophilicity↑ Antiradical ↔	(Céliz & Daz, 2011)
		methyl-butanol/DMSO	Lipozyme TL IM	7	4	-	50	0.5	90	-	(Luo *et al.,* 2013)
	Fatty acids	Acetone,2-methyl-2-butanol	Novozym® 435	4	-	-	50	72	90	-	(Zheng *et al*., 2013)
	Castor oil	Acetone	C. antarctica B	3	25	-	50	120	24	-	(Almeida *et al.,* 2012)

Acyl Acceptor (AA)	Acyl Donor (AD)	Medium	Enzyme	Optimized reaction condition						Biological effect	Reference
				AA/ AD	V (mL)	En (%)	T (˚C)	Ti (h)	Y (%)		
Coumaric acid	Alcohols	Solvent-free	B. licheniformis	1	-	1	55	8	69	-	(Sharma *et al.,* 2014)
Caffeic acid derivatives	Alcohols	BMIM TFSI	Novozym® 435	-	0.5	6	75	36	97	-	(Wang *et al*., 2015)
			Novozym® 435	-	0.5	6	75	9	99.8	-	(Wang *et al*., 2015)
		Isooctane	Novozym® 435	71	-	-	70	9.6	93	-	(Chen *et al*. 2011)
			Novozym® 435	78	2	-	75	55	90.3	-	(Chen *et al*., 2010)
		[bmim] [OTf]	Novozym® 435-	40	-	-	60	2.5	99.5	-	(Wang *et al*., 2013)
		Solvent free	Novozym® 435	10	-	6	60	36	97	Antioxidant ↔	(Xu *et al*., 2018)
	Soybean oil	Solvent free	Novozym 435, Lipozyme RMIM, Lipozyme TLIM	6	-	25	85	60	73	-	(Sun *et al*., 2018)
Ferulic acid derivatives	Alcohols	Acetone and *t*-butanol	Bacillus AKL 13.	-	-	-	50	96	-	-	(Sankar & Achary, 2017)
		Ionic Liquid	Novozym® 435	4	10	-	60	96	-	-	(Chen *et al*. 2011)
		Dehydrated DMSO	Steapsin	1	1	-	45	6	-	-	(Kumar & Kanwar, 2011)
		n-hexane	Lipozyme RM IM	10^{-3}	2.95	-	70	72	92	-	(Schär & Nyström, 2015)
	Flaxseed oil	Super critical CO2	Novozym® 435	6	10	15	80	27	58	-	(Ciftci & Saldaña, 2012)
Ethyl ferulate derivatives	Castor oil	Solvent-free	Novozym® 435	1	-	20	90	72	100	-	(Sun *et al.,* 2013)
			Novozym® 435	1	-	20	90	72	100	-	(Sun *et al.,* 2014)
	Tributyrin	Toluene	Novozym® 435	3	9	-	50	120	-	Antioxidant↑	(Zheng *et al*., 2010)
	Fish oil	Solvent-free	Novozym® 435	4.7	-	4.3	70	120	92	-	(Yang *et al*., 2012)
	Triolein	Solvent-free	Novozym® 435	3	3	4	55	62	92	-	(Yu *et al*., 2010)
	Glycerol	Solvent-free	Novozym® 435	10	-	6	60	36	97	Antioxidant ↔	(Xu *et al*., 2018)
Vanillic acid derivatives	Fatty acids	Acetone	Novozym® 435	1.5	25	-	50	48	60	Antioxidant (oil)↓ Antioxidant (emulsion)↑	(Mbatia *et al.,* 2011)
		Acetonitrile	Novozym® 435	2	2	2	50	72	54	Antioxidant ↔	(Roby *et al.,* 2015)
		Solvent-free	Novozym® 435	-	-	2	50	72	100		
Cinnamic acid derivatives	Alcohols	Hexane	S. Xyloses	160	-	-	52	6	90	Antioxidant↑	(Bouaziz *et al.,* 2010)
Vitamin E derivatives	Ethyl ferulate	Toluene	Novozym®435, other lipases	6	5	-	60	72	18	-	(Xin *et al.,* 2011)
		Isooctane							3		
		n-hexane							7		
		Tertiary butyl alcohol							0		
		Solvent-free	Solvent-free	5					25		
		Solvent-free	Novozym 435	1/5	5	-	60	72	25		

Acyl Acceptor (AA)	Acyl Donor (AD)	Medium	Enzyme	Optimized reaction condition						Biological effect	Reference
				AA/ AD	V (mL)	En (%)	T (˚C)	Ti (h)	Y (%)		
	Succinic anhydride	Organic solvents	Candida rugosa (modified)	4	5	0.6	55	15	63	-	(Jiaojiao *et al.,* 2021)
		tert-butanol and DMSO (2/3)	Succinyl-Novozym® 435	5	5	-	40	48	94	-	(Yin *et al.,* 2011)
		Solvent-free	Novozym435, other lipases	5	5	1	55	18	47	-	(Jiang *et al*., 2013)
Vitamin A derivatives	Lactic acid	n-Hexane	Recombinant	9	10	-	30	7	87	-	(Liu *et al*., 2012)
Vitamin C derivatives	Fatty acids	Acetone	Novozym® 435	4	10	0.3	60	72	-	Antioxidant↑	(Ćorović *et al.,* 2018)
		tert-butanol	Novozyme 435,	3	50	-	55	4	86	Antioxidant↓	(Jiang *et al*., 2016)
	Various plant oils	t-butanol-MeTHF	Novozyme 435, other enzymes	3	2	-	50	24	90	Antioxidant↓	(Hu *et al.,* 2016)
	Lard, Vegetable oils	Acetone, t-butanol	Novozym® 435	8	10	1	70	24	86	Antioxidant↑	(Ćorović *et al.,* 2020)
	Ethyl palmitate	2-methyl-2-butanol	Lipozyme TLIM	300	5	2.5	40	120	20	Antioxidant↑	(Reyes-Duarte *et al.,* 2011)
	Palmitic acid	2-methyl-2-butanol	Lipozyme TLIM	300	5	2.5	40	120	20	Antioxidant↑	
	Vinyl palmitate	2-methyl-2-butanol	Lipozyme TLIM	300	5	2.5	40	120	100	-	
	Tripalmitin	2-methyl-2-butanol	Lipozyme TLIM	300	5	2.5	40	120	50	-	
	Triolein	2-methyl-2-butanol	Lipozyme TLIM,	300	5	2.5	40	120	84	Antioxidant↑	
	Olive oil	2-methyl-2-butanol	Novozym® 435	300	5	2.5	40	120	33	-	
	Olive oil	t-Amyl alcohol	Thermomyces L., Candida A., Rhizomucor M.,	1	5	-	45	140	84	-	(Moreno-Perez *et al.,* 2013)
	Vinyl acetate	Acetone	Lipozyme TL IM, Novozym® 435	-	3	-	40	4	99	-	(Zhang *et al*., 2012)
	Carboxylic acid	Acetone	Novozyme 435	8	-	0.2	60	-	-	Antioxidant↑	(Stojanović *et al.,* 2013)
Kojic acid	Fatty acids	Acetonitrile	Lipozyme RM IM				50	42	35	-	(Lajis *et al*. 2012)
		Solvent-free	Lipozyme TL IM	1		7.8	80		87	Lipophilicity↑	(El-Boulifi *et al.,* 2014)
Lipoic acid	Tyrosol, tyramine	Ionic solvents	C. antarctica B	1:5	1	-	60	72	99	-	(Papadopoulou *et al.,* 2013)
Lutein	Saturated fatty acid vinyl esters	MTBE, toluene, acetone	Novozym® 435, other lipases	10	10	4	45	16	88	Antioxidant↑ Stability↑	(Tan *et al.,* 2021)
Hydroxy benzoic acid derivatives	Glycerol	t-butanol/acetonitrile	Novozym® 435	2.2	3		36	48	77	Antioxidant ↔	(Kharrat *et al.,* 2017)
	Octadecanol	2-methyl-2-butanol	C. antarctica B	9/5	10	10	55	24	-	Stability↑	(Aladedunye *et al.,* 2015)

Acyl Acceptor (AA)	Acyl Donor (AD)	Medium	Enzyme	Optimized reaction condition						Biological effect	Reference
				AA/ AD	V (mL)	En (%)	T (˚C)	Ti (h)	Y (%)		
Chlorogenic acid derivatives	Vinyl esters	MTBE	Lipozyme RM	10	10	-	55	7 d	-	Antioxidant ↔	(Wang *et al*., 2021)
β-Arbutin derivatives	Vinyl esters	THF-isopropyl ether	P. Expansum	11	2	-	50	96	99	-	(Yang *et al*., 2020)
		Anhydrous THF	P. Expansum	25	2	-	35	1	99	-	(Yang, Li, Li, *et al*., 2010)
	Lipoic acid Ferulic acid	t-Butanol	C. antarctica B	5	30	-	55	168	57	-	(Ishihara *et al.,* 2010)
Prunin derivatives	Vinyl esters	Aceton or solvent-free	Novozym® 435	5	3	-	50		<10	-	(Céliz *et al.,* 2012)
		Acetone, t-butanol, acetonitrile, THF	Novozym® 435, Lipozyme RM IM	10	3	-	50	6	-	Lipophilicity↑ Antiradical↔	(Céliz & Daz, 2011)
		Aceton	Novozym® 435	10	150	-	50	48	100	Antifungal↑	(Salas *et al.,* 2011)

↑ (increased activity), ↔ (no change in activity), ↓ (decreased activity), AA/AD (ratio of acyl acceptor to acyl donor), V (volume of the reaction), En (enzyme load), T (temperature), Ti (time), Y (yield of the reaction)

3.8.Optimization of enzymatic reactions

Employing reaction engineering techniques can undeniably enhance the effectiveness of lipases for a specific application. Nonetheless, in most cases, determining the optimal reaction conditions remains a laborious process that involves trial and error. Although hydrolysis reactions are simple and only need water (Mardani *et al.,* 2015), the utilization of diverse acyl donors in lipase-catalyzed (trans-)esterification reactions is important, and alcohols are a common choice due to their ability to facilitate swift and irreversible reactions (Mardani *et al.,* 2022).

In enzyme-catalyzed reactions, the optimization of reaction conditions between a lipophilic substrate and a hydrophilic one presents a crucial challenge in achieving a reasonable kinetic rate and high production yield within a minimal timeframe, given their significant polarity differences (Farooq *et al.,* 2021; Vaisali *et al.,* 2017). Several factors play a role in this optimization process, including the type and amount of acyl acceptor and acyl donor, the enzyme used, the solvent (or solvent-free) system, the reaction time, the water activity of the system, and the intensity of temperature and agitation. Additionally, in the case of esterification reactions, the use of molecular sieves to adsorb water should be considered in the optimization process (Bouaziz *et al.,* 2010; Kharrat *et al.,* 2017). In the case of hydrolysis reaction water is needed to initiate the reaction whereas in the esterification reaction different substrates such as fatty alcohols or fatty acids can be used. The aim of optimization is to increase the yield while minimizing substrate consumption and ultimately waste production. To achieve this, response surface methodology (RSM), a standard statistical technique, is commonly employed in optimization studies. It helps to evaluate the interaction between all the reaction parameters to achieve the highest conversion yield and the most economical and practical production (Kharrat *et al.,* 2017). Table 2 also compares the optimized reaction conditions for esterification reactions involving antioxidants, including used solvents and conversion yields

of esters for different antioxidant derivatives. In the case of hydrolysis reactions, no studies have been done in optimization of reaction conditions to maximize MAGs and DAGs productions.

In the following sections, each parameter affecting the efficiency of enzymatic reactions to reach an optimized yield will be discussed.

3.8.1. Substrates

In general, ester synthesis involves the acylation of various alcohols using a range of organic acids. Typical esterification reactions entail heating a mixture of carboxylic acids and an excess of corresponding alcohols in the presence of a catalyst (Choi *et al*., 2015; Kumar *et al*., 2022). The rate of esterification and the extent of equilibrium are determined by the molecular structures and the type of functional groups linked to the alcohols or carboxylic acids (Agyei *et al.,* 2019; Lortie, 1997; Walsh, 2007). Primary alcohols exhibit faster and more complete esterification reactions, with methanol being known for providing the highest yield and fastest reactions. Ethyl, propyl, and butyl alcohols demonstrate similar reactivity and conversion rates compared to each other (Khan *et al.,* 2021). On the other hand, secondary and tertiary alcohols participate in esterification reactions at a slower pace, resulting in lower ester yields. The reduced reaction rates for these alcohols are attributed to the bulkiness or steric hindrance on their hydroxyl groups. This hindrance decreases the surface area accessible for nucleophilic attack by the alcohols on the carbonyl carbons of carboxylic acids, influencing the reaction rates. Additionally, the presence of branched chains further lowers the reaction rates. However, branched-chain substrates could offer higher conversions compared to normal straight-chain acids (Bornscheuer, 1995; Bornscheuer *et al.,* 2012; Khan *et al.,* 2021).

As indicated in Table 2, various substrates such as alcohols, fatty alcohols, long-chain or short-chain fatty acids, anhydrides, fatty acid esters, TAGs, and their derivatives can be utilized.

Although, in most studies conducted in the last decade, transesterification reactions have emerged as a more practical and flexible choice, many researchers have successfully employed alcohols in one-step enzymatic esterification of simple phenolic acids to produce phenolic esters with enhanced antioxidant capacity (Kumar & Kanwar, 2011; Sankar & Achary, 2017; Schär & Nyström, 2015). The type of acyl donor can significantly impact both the yield and mechanism of the reaction. When FFAs are employed for one-step esterification with antioxidants, water is generated, potentially leading to the reversal of the reaction toward hydrolysis (Mardani *et al.,* 2022). To address this issue, molecular sieves have been widely utilized in related studies during the last decade to remove water. However, it is important to note that molecular sieves can have a detrimental effect on the support of immobilized enzymes and do not provide control over water activity (Laguerre, Sørensen, *et al*., 2013; Subileau *et al*., 2018). As demonstrated in Table 2, alternative acyl donors, such as methyl, ethyl, or vinyl esters, can also be utilized. Transesterification reactions using these donors produce alcohols, such as ethanol and methanol, which can be easily removed under reduced pressure. Vinyl esters, being irreversible acyl donors, have been repeatedly employed by different researchers. The vinyl alcohol produced during the reaction is subsequently transformed into acetaldehyde, which can be evaporated at low temperatures (Ishihara *et al.,* 2010; Laguerre, Bayrasy, *et al.,* 2013; Tan *et al.,* 2021).

The concentration or mole ratio of substrates, which refers to the proportion of acyl donors and acyl acceptors, plays a crucial role in enzymatic reactions. Properly selecting this ratio can lead to cost reduction and alleviate difficulties associated with downstream processing of the final products. In general, a higher mole ratio tends to result in greater acyl incorporation and shorter production time for lipophilized antioxidants (Subileau *et al*., 2018). However, careful optimization of the ratio is necessary to achieve maximum conversion yield while minimizing downstream processing requirements. As esterification is a reversible reaction, maintaining the

equilibrium shift in favor of synthesizing lipophilized products is essential. Having an excess of one of the substrates typically favors the synthesis of the desired reaction over hydrolysis. Hydrophobic substrates have been commonly used in excess, as indicated by optimization reactions conducted in the last decade. Some rare exceptions are shown in Table 2 (Schär & Nyström, 2015). Additionally, significant differences in the ratios of the substrates are observed in the literature for esterification reactions, as demonstrated in Table 2.

In the case of hydrolysis reactions of oils and fats, given the substantial molecular weight disparity between reactants, the process is often conducted with a substantial excess of water. However, a significant water load can lead to increased energy consumption, primarily during downstream separation stages, while potentially introducing additional mass transfer constraints due to limited miscibility of reactants (Baena *et al.,* 2022). Additionally, in the context of enzymatic hydrolysis, water assumes a critical role in configuring enzyme structures and, consequently, their activity. Although a water layer formed by hydrogen bonding is pivotal in maintaining the enzyme's three-dimensional structure, excessive water can lead to multilayer adsorption, forming a thick aqueous film. This film impairs the solubility of immiscible substrates such as TAGs, diminishes accessibility for non-polar and large molecules to active sites, and heightens the enzymes' susceptibility to denaturation (Baena *et al.,* 2022; Choi *et al.,* 2015; Quezada & Hernandez, 2011). Moreover, in enzymatic processes, the water content dictates the requisite amount of buffer in the reaction medium, as buffer components dissolve in the aqueous phase. The chemical equilibrium constant for TAGs hydrolysis is approximately 0.45 at 225°C, imposing an upper limit on the achievable reaction conversion, which typically remains below 90%. Strategies to manipulate this equilibrium include using a molar excess of water (> 3:1, water to TAGs) or selectively removing glycerol or fatty acids from the reaction mixture. Last but not least, as the aim in our study was to partially hydrolyze oils by aiming to maximize the proportion of mainly monoacylglycerol and partly DAGs to FFAs (The highest

value of (MAGs+DAGs)/FFAs), the amount of water is critical in determining the rate of reaction while keeping hydrolytic reactions as low as possible (Baena *et al*., 2022; Iwasaki & Yamane, 2000; Lopes *et al*., 2019).

3.8.2. Solvent

Although in the first section of the thesis and in the hydrolysis of oils and fats, solvents were not used and liquid oil acted as a proper media for enzymatic reactions, using solvent was necessary for the esterification reaction involving rosmarinic acid. Therefore, the importance of using organic solvents is explained in this section.

Phenolic and polyphenolic compounds exhibit a wide range of polarity, prompting the recommendation for optimizing reaction conditions with different solvents to assess the most suitable solvent system for the selected compound. The goal is to choose a solvent that provides the highest yield, optimal enzyme efficiency, and favorable reaction kinetics (Farooq *et al.,* 2021). Alternatively, another approach involves conducting the synthesis in solvent-free systems by using an excessive amount of substrates, which serves as both the substrate and solvent. Solvent-free systems offer advantages, such as improved safety, reduced solvent recovery costs, and fewer purification steps. However, in cases where achieving higher yields of phenolic esters is challenging due to the high viscosity of the medium and a high conversion rate to byproducts (*e.g.,* FFAs and glycerol), the choice of solvent becomes crucial for the success of the reaction (Farooq *et al.,* 2021; Mardani *et al.,* 2022)

Organic solvents are favored in enzymatic esterification due to their ability to enhance the solubility of hydrophobic compounds, resulting in stronger interactions between enzymes and substrates. However, these solvents have notable drawbacks, such as high cost, flammability, and toxicity, making them less desirable in the food industry unless no viable alternatives exist (Subileau *et al*., 2018). Recent research reports suggest that the use of organic solvents in

enzymatic esterification can indeed improve the yield of enzymatic reactions (Mardani *et al.,* 2022). Furthermore, it has been observed that organic solvents can enhance the thermal resistance of enzymes, as the absence of water restricts enzyme unfolding at higher reaction temperatures. A variety of common solvents have been employed in enzymatic esterification over the last decades, including butanol, n-pentane, cyclohexane, tert-butanol, 2-propanol, acetonitrile, 2-methyl-2-butanol, 2-methyl-2-propanol, hexane, tetrahydrofuran, dimethyl sulfoxide, isopropyl ether, toluene, diethyl ether, and acetone. Binary solvent systems involving these solvents have also been utilized to optimize the system's polarity and increase the solubility of antioxidants. Nonetheless, it is crucial to carefully consider safety aspects and comply with current regulations regarding the use of solvents in these processes (Johny *et al.,* 2019; Laguerre *et al.,* 2015; Luo *et al.,* 2013; Mardani *et al.,* 2022).

The logP concept is often used to select appropriate solvent, but the influence of the solvent on activity, stability and enantioselectivity is still rather unpredictable. In addition to the conventional organic solvents, other solvent systems such as deep eutectic solvents, ionic solvents, and supercritical CO_2 have been explored in the field of enzymatic esterification (Ciftci & Saldaña, 2012; Papadopoulou *et al.,* 2013; J. Wang *et al.,* 2013). Ionic solvents, in particular, offer several advantages as they are thermally stable, non-toxic, and non-volatile. Moreover, the hydrophilicity/hydrophobicity ratio of ionic solvents can be adjusted, facilitating higher conversion yields by providing more substrates to the enzymes. However, the adoption of this medium is limited due to its higher cost, making it a viable option primarily for synthesizing high-added value products (Papadopoulou *et al.,* 2013).

In conclusion, when selecting a solvent for enzymatic esterification, two crucial aspects to consider are the equilibrium position of the desired reaction and enzyme activity (Yu *et al*., 2004). Previous studies have extensively explored the influence of solvent concentration on the

production yield of various esters derived from different phenolic antioxidants (Jiang *et al*., 2013; Vaisali *et al*., 2017; Yang *et al*., 2020). Solvent polarity is a key factor that significantly impacts the production of polyphenol esters in synthetic reactions, as evidenced by the partition coefficient between different reaction components (Farooq *et al.,* 2021). Intermediate polarity solvents are often preferred based on similar investigations, as they maintain the essential water content needed to prevent enzyme deactivation (Stamatis *et al.,* 2001). This preference is justified by the fact that enzymes tend to be less flexible in low-water environments. Other important criteria for selecting suitable solvents include cost, inertness, waste disposal, viscosity, density, flammability, and surface tension (Mardani *et al.,* 2022).

3.8.3. Enzymes (type, specificity, and concentration)

The exploration of enzymes as bio-based catalysts within the oleochemical sector has gotten significant attention due to their established key functions in various metabolic reactions and processes. Notably, lipases, among the most prominent industrial enzymes, have been extensively investigated (Kumar *et al*., 2022; Sorour *et al*., 2017). These enzymes play a central role in the catalytic hydrolysis of TAGs. In addition to hydrolysis, lipases exhibit esterification catalytic capabilities as well. The hydrolysis of vegetable oils holds particular significance, as it yields FFAs pivotal for the synthesis of diverse non-food and food items like soaps, detergents, healthcare products, emulsifiers, and nutraceuticals. In contrast to the conventional non-enzymatic hydrolysis of vegetable oils requiring high temperatures (250°C) and pressures (50 bar), lipase-catalyzed hydrolysis offers advantages due to milder reaction conditions and selectivity, allowing for targeted enrichment of specific fatty acids (Iwasaki & Yamane, 2000; Moazeni *et al.,* 2019). Partial hydrolysis of vegetable oils can also facilitate the production of DAG oils, known to confer various health benefits. DAGs have digestibility and energy values similar to TAGs, but their unique metabolism offers significant nutritional benefits. Unlike TAGs, DAGs are metabolized in a way that enhances fat oxidation and reduces fat

accumulation in the body, making them a valuable component in weight management and cardiovascular health. This efficient metabolism can also lead to improved blood lipid profiles, lowering triglycerides and potentially increasing good cholesterol levels. Thus, incorporating DAG oil into the diet can provide a range of health benefits beyond those of traditional TAG oils. Additionally, the utility of MAGs in structuring vegetable oils is well-recognized and widely practiced in industry, achievable through the partial hydrolysis of oils (Agyei *et al.,* 2019; Lortie, 1997; Speranza *et al.,* 2019).

On the other hand, phenolic and polyphenolic compounds are commonly lipophilized using lipases, primarily derived from *Candida sp., Pseudomonas sp., Mucor sp., or Rhizopus sp*. However, specific commercial lipases, such as Novozym® 435, Lipozyme RM IM, Lipozyme TL IM, and *C. Antarctica B*, have predominantly been employed in these enzymatic reactions. Among these, Novozyme 435 has emerged as the preferred choice for most enzymatic reactions in this field over the last decade. This CALB lipase, immobilized on a hydrophobic carrier (acrylic resin), demonstrates stability across a broad pH range, particularly in alkaline conditions. The freshly produced enzyme has a declared activity of 10,000 PLU/g or Propyl Laurate Unit per gram. It exhibits optimal performance at temperatures ranging from 30 to 60°C and displays specificity toward esters and alcohols (Domínguez de María *et al*., 2006; Mardani *et al*., 2022; Ortiz *et al*., 2019; Subileau *et al*., 2018).

In general, as the concentration of the enzyme increases, the reaction equilibrium tends to shift toward synthesis until it reaches a constant conversion yield, after which it starts to decrease. This behavior is attributed to steric hindrance caused by an excessive enzyme load (Chandel *et al*., 2022; Andrade Silva *et al*., 2023; Villeneuve, 2007). To ensure economic efficiency and avoid steric hindrance, it is essential to optimize the addition of enzymes. Numerous studies have focused on optimizing enzyme concentration for various purposes (Bouaziz *et al.,* 2010;

Kharrat *et al.,* 2017). The specificity or selectivity of the lipase can also significantly influence the reactions, particularly in the synthesis of specific phenolic esters. Thus, selecting the most suitable catalyst requires careful consideration of the lipase's characteristics. Customizing the enzyme to enhance features like reuse, thermostability, catalytic activity, and selectivity is the goal. In recent decades, various conditioning methods have been developed, including immobilization, chemical modification, and molecular engineering. Immobilization on support is a commonly used approach for lipophilization reactions. Immobilized lipases offer greater stability compared to free enzymes and can be easily recovered after completing the reactions (Bornscheuer, 1995; Bornscheuer *et al.,* 2012).

3.8.4. Role of temperature

In synthetic reactions, it is crucial to conduct them at the optimal temperature, as higher temperatures can lead to protein denaturation, while lower temperatures may reduce the conversion yield. Thermal stability has also been found to be influenced by the reactants used in enzymatic reactions, as some of them can remove accessible water from around the enzymes. Numerous studies have examined and optimized the impact of temperature on the production of phenol esters (Bornscheuer, 1995; Bornscheuer *et al.,* 2012; Bouaziz *et al.,* 2010; Kharrat *et al.,* 2017; Mardani *et al.,* 2022). For Novozyme 435, different researchers have reported optimized temperatures ranging from 30 to 70°C for different applications (Table 2).

3.8.5. Agitation rate

The lack of compatibility between reactants and the ensuing constraints on mass transfer represent significant drawbacks encountered during the hydrolysis of vegetable oils. Within customary reaction conditions, TAGs and water do not readily mix, and several investigations have indicated that insufficient miscibility between water and oil leads to restricted mass transfer between these distinct phases, serving as the rate-limiting kinetic step during

hydrolysis. This characteristic is typically evident in the sigmoidal pattern observed in kinetic profiles, where the reaction rate is relatively slow in the initial and final stages but accelerates during intermediate phases. This behavior can be attributed to the dynamic evolution of the reactive environment and the intermediate glycerides' surfactant properties. Initially, the formation of diglycerides and monoglycerides assists in emulsifying the otherwise immiscible reactants, augmenting mass transfer and subsequently enhancing the reaction rate. However, a contrary effect emerges once glycerol is generated, as it amplifies the size of dispersed phase droplets, diminishing contact area and thereby impeding mass transfer and reaction rate. While elevated temperatures might address these issues, potential drawbacks such as polyunsaturated fatty acid degradation and the occurrence of side reactions like oxidation or enzyme inactivation can arise. This underscores the mounting significance of agitation rate as a potential solution (Bornscheuer, 1995; Bornscheuer *et al.,* 2012; Bouaziz *et al.,* 2010; Kharrat *et al.,* 2017; Mardani *et al.,* 2022). In previous studies, researchers have reported that increasing agitation up to a certain degree can lead to improved enzyme activity. This enhancement is attributed to the relationship between the diffusion rate of the substrate and the degree of agitation during the synthesis of phenolic esters or hydrolysis reactions. The presence of a glycerol layer around the lipase can limit mass transfer and reduce the accessibility of the substrate, resulting in lower ester production levels (Agyei *et al.,* 2019; Lortie, 1997; Speranza *et al.,* 2019). Similarly, a straightforward approach to mitigate the constraints posed by mass transfer limitations, without resorting to adding a solvent for phase homogenization or an emulsifying agent, involves enhancing the overall available interfacial area by inducing turbulence through mechanical agitation. Nonetheless, operating at high stirring rates could lead to enzyme denaturation due to excessive shear forces. As an alternative, the introduction of turbulence can be achieved through intensified methods such as cavitation, ultrasound, and microwaves. These techniques contribute to amplifying the interfacial area and creating

localized zones of elevated temperature for brief periods, thereby accelerating reaction rates without subjecting the system to shear-related stresses. These unique attributes render intensified technologies appealing for potential industrial adoption (Bornscheuer, 1995; Bornscheuer *et al.,* 2012; Bouaziz *et al.,* 2010; Kharrat *et al.,* 2017; Mardani *et al.,* 2022).

3.8.6. Molecular sieves

Molecular sieves are porous materials commonly used to remove water and other small molecules in various chemical reactions, including esterification. The most frequently used types include zeolites, activated alumina, and silica gel. Zeolites, which are crystalline aluminosilicates, have a highly uniform pore structure that makes them ideal for selectively adsorbing water molecules, thus driving esterification reactions to completion by shifting the equilibrium towards ester formation. Activated alumina, a form of aluminum oxide, is another effective desiccant that can adsorb water and other impurities, enhancing the efficiency and yield of esterification processes. Silica gel, composed of silicon dioxide, is well-known for its high surface area and pore volume, making it excellent at trapping moisture and facilitating the removal of water during esterification. These molecular sieves are crucial in both laboratory and industrial settings to achieve higher reaction efficiency and purity of the final ester products (Laguerre, Bayrasy, *et al.,* 2013; Laguerre *et al.,* 2015; Laguerre, Sørensen, *et al.,* 2013).

In hydrolytic reactions, as the water is considered a substrate, the molecular sieves are not needed. However, during the lipophilization of phenolic acids, the main product formed is phenolic esters, accompanied by the generation of water as a byproduct. Consequently, under unfavorable conditions, the esterification reaction may undergo simultaneous hydrolysis. In other words, higher water levels can shift the reaction equilibrium towards hydrolysis. Traditionally, in chemical catalysis, water removal has been achieved through evaporation under reduced pressure. However, in enzyme catalysis reactions for synthesizing lipophilized

antioxidants, the reduction of water can be achieved by incorporating molecular sieves (Mardani *et al.,* 2022). In recent studies, the use of molecular sieves in the reaction has been prevalent in the past decade to enhance the conversion yield. Nevertheless, it is essential to note that excessive amounts of molecular sieves may strip away the crucial water surrounding the enzymes, leading to a reduction in enzyme activity (Laguerre, Bayrasy, *et al.,* 2013; Laguerre *et al.,* 2015; Laguerre, Sørensen, *et al.,* 2013). Water activity is a frequently examined factor in lipase-catalyzed reactions. This parameter holds significance not only in preserving the enzyme's catalytic efficacy within a particular solvent but also in exerting a considerable impact on acyl migration. Therefore, achieving high product yields hinges on precise control of water activity. In the first section of the thesis, the water is a key substrate for the hydrolysis reaction, whereas in the second section the water has to be constantly removed to increase the tendency of the enzymes for the esterification reactions as opposed to hydrolytic reactions (Baena *et al.,* 2022).

4. MATERIALS AND METHODS

4.1.Main materials

Novozymes, Denmark, generously provided three commercial enzymes: Lipozyme 435 (from *Candida antarctica*, 10 PLU/mg or Propyl Laurate Unit per gram, 45 °C, and non-specific), Lipozyme TL IM (from *Thermomyces lanuginosus*, 250 IUN/g or Interesterification Unit per gram, 60 °C, and 1,3 specific), and Lipozyme RM IM (from *Rhizmucor miehei*, 275 IUN/g, 40 °C, and sn-1,3 specific). Additionally, Amano, UK, kindly provided two lipases: Lipase AY "Amano" 30SD (from *Candida cylindracea*, 30 U/mg, 50 °C, non-specific) and Lipase DF "Amano" 15 (from *Rhizopus oryzae*, 150 U/mg, 37 °C, and 1,3 specific). All reagents, standards, and solvents used in the study were of analytical or HPLC grades. Refined oils were sourced from local stores. Butylated hydroxytoluene (BHT) was obtained from Sigma-Aldrich. Ethylcellulose (50 centipoise) was obtained from Roth in Germany. Monoacylglycerol was acquired from Danisco in Hungary. Molecular sieves (3 Å) was purchased from Sigma-Aldrich. All standards, reagents, and remaining chemicals were of HPLC or analytical grade and were purchased from either Sigma-Aldrich or Merck.

4.2. Methods - section 1 (application of enzymatic reactions to solidify vegetable oils)

4.2.1. Screening of lipases

Various lipases were assessed for their ability to synthesize MAGs through partial hydrolysis in palm olein. The reaction conditions were customized based on the supplier-declared optimum temperature and in the range of pH for each enzyme. After conducting pre-trials, a weight ratio of 0.05:1 (water/palm olein), pH of 7, and the enzymes' respective optimum temperatures were established before commencing the reactions. Due to disparities in the declared activities between Novozymes and Amano products, making direct comparisons was not feasible as they used different units of measurement. Consequently, for the enzyme screening process, a lipase dosage of 50 U/g of oil was used for Amano enzymes, while 5%

per weight of oil was employed for Novozymes enzymes (500 PLU/g for Novozym 435, 12.5 IUN/g for Lipozyme RM IM, and 13.75 IUN/g for Lipozyme TL IM). The acylglycerol phase obtained from the hydrolysate was isolated through alkali deacidification, following the described procedure below. This phase was then utilized for measuring enzyme selectivity towards specific fatty acids or assessing the potential reduction of SFAs in the acylglycerol phase. Throughout the reaction, samples were withdrawn at regular intervals for analysis, as detailed in the subsequent sections.

4.2.2. Hydrolysis reactions and optimization of partial hydrolysis

For this section, lipase DF "Amano" 15 was chosen based on the results obtained in the previous section. The hydrolysis of oils was carried out under various conditions using a central composite rotatable design, which incorporated four factors to maximize the production of monoacylglycerol and partly DAGs while minimizing the release of FFAs. The factors and their respective quantities included the water level (2-10 wt% of oil mass), enzyme load (10-90 units per gram of oil), temperature (22-54 °C), and time (1-5 hours). Design Expert 13 software from Minneapolis, Minnesota, USA, was utilized to create 30 experiments based on the central composite rotatable design. The responses observed in the experiments were focused on acylglycerol compositions, with particular emphasis on the degree of hydrolysis (level of FFAs), as well as the contents of corresponding MAGs and DAGs.

A desired pre-heated amount of water was added to the oil, followed by stirring at 200 rpm using a magnetic stirrer and maintaining the desired temperature. The reaction was initiated by adding the required amount of lipase. After the specified duration of time, the water phase containing the lipase was separated from the reaction mixtures through centrifugation at 1610 g for 5 minutes. Before centrifugation, the mixture was heated to 80°C for 5 minutes to disrupt any emulsion, especially those formed with palm olein oil and rice bran oil hydrolysates due

to the stirring during the reaction. The resulting hydrolysate was then filtered and stored at -80°C for immediate analyses or for further processing steps.

4.2.3. Determination of acylglycerol composition

In summary, 5-10 mg of fat was dissolved in 1 mL of isooctane, and then thin-layer chromatography (TLC) was performed following a previously described method with some modifications (Bakala-N'Goma *et al.,* 2022). Next, 20 µL of the diluted sample was applied to a silica gel 60 TLC plate with glass support, measuring 20 cm × 20 cm. The plates were eluted twice using n-heptane: diethyl ether: formic acid (in a volume ratio of 55:45:2) and then dried at room temperature between runs. For staining, a solution of copper acetate-phosphoric acid was used, which involved mixing a saturated copper acetate solution with 85% phosphoric acid in a 1:1 volume ratio. After staining, the TLC plates were dried under a fume hood for 10 minutes and then heated at 180°C in an oven for 15 minutes. The densitometry evaluation of the stained lipids on the TLC plates was conducted using ImageJ software (version 1.54i).

4.2.4. Alkali deacidification of hydrolysates

The deacidification of vegetable oils was conducted following a modified version of the titrimetric method described by Liu *et al.* (2023). For the process, 10 grams of hydrolysates were neutralized using a 12 g/100 mL sodium hydroxide solution as part of the alkali deacidification. Due to the presence of a high content of MAGs and DAGs (acting as emulsifiers) in the hydrolysates, a demulsifier (1% KCl) was also added. The reaction was carried out at 60°C for 10 minutes on a magnetic stirrer set at 50 rpm, continuing until no further soap formation occurred. Following neutralization, the mixture of soap stock and oil was mixed with 200 mL of hexane and stirred for 10 minutes. The resulting oil-hexane mixture was then mixed with hot water, allowing it to settle into two layers, after which the water layer was discarded. To ensure the complete removal of any remaining fatty acid soaps and alkali,

the oil was washed with water three times until no trace of alkali or soap was present (Liu *et al*., 2023). Finally, the deacidified oils underwent vacuum removal of hexane at 40°C (Buchi Rotavapor R-210). The deacidification efficiency was assessed using a formula that involved determining the FFA content as a percentage of palmitic acid (for palm olein oil) and oleic acid (for other oils) through the AOCS Official Method Ca 5a-40 (AOCS, 1998, 2017, 2023).

$$\text{Deacidification efficiency (\%)} = 100 - \left(\frac{\text{Final FFA based on acid value} \times 100}{\text{Initial FFA based on acid value}} \right)$$

(Equation 1)

4.2.5. Evaluating the changes in the properties of crude the hydrolysates and the deacidified hydrolysates

4.2.5.1.Fatty acid composition

The preparation of fatty acid methyl esters (FAMEs) followed the guidelines outlined in ISO 12966-2:2017. The analysis of the FAMEs was performed using an Agilent 6890 GC-FID system and a Phenomenex Zebron ZB-FAME column (60 m, 0.25 mm, 0.20 μm) with a cyanopropyl stationary phase. Hydrogen gas was used as the mobile phase at a flow rate of 1.2 mL/min, following a previously described method (Tormási & Abrankó, 2021).

4.2.5.2. Iodine Value

The iodine value (IV) was measured according to the AOCS method Cd 1c-85 (AOCS, 1998).

4.2.5.3. Lipase selectivity

The enrichment number of each acyl moiety was calculated to investigate the enrichment or depletion of specific fatty acids in the acylglycerol section. The enrichment number of each fatty acid in the acylglycerol phase is determined using the following equation:

$$\text{Enrichment number of fatty acid in acylglycerol phase} = \frac{\text{Percentage of the fatty acid in the acylglycerol phase}}{\text{Percentage of the fatty acid in the initial oil}}$$

(Equation 2)

4.2.5.4. Differential scanning calorimetry

Differential scanning calorimetry (DSC 3500 Sirius, Netzsch) was employed to study the crystallization and melting behavior of the samples. Between 8-12 mg of samples were put in aluminum crucibles, and then closed. Samples initially reached 100 °C (15 min), prior to studying their crystallization behaviors by reaching −70 °C (5 °C min^{-1}), and afterward melting behaviors by heating to 80 °C (5 °C min^{-1}) (Nicholson & Marangoni, 2021).

4.2.5.5. Rheological studies

Different rheological measurements, including strain sweeps, frequency sweeps, and temperature sweeps, were conducted to study the viscoelastic behaviors of fats using a rheometer (Physica MCR 301, Anton-Paar, GmbH, Graz, Austria) equipped with parallel measuring plates PP50 (50 mm in diameter and a 1000 μm gap) (Naeli *et al*., 2022). The sample temperatures were controlled using the Peltier system. The Anton Paar Rheoplus software (RheoCompass 1.30 31) was employed to establish different factors for the experiments. To describe the rheological parameters essential for understanding deformation behaviors, the two-plates model was utilized. Strain sweep tests were performed at a strain amplitude ranging from 0.01% to 1000% and a constant frequency of 1 Hz at a temperature of 20 °C to determine the linear viscoelastic range. Frequency sweep tests were carried out with a frequency ramp of 0.1 to 50 Hz (at a constant strain of 0.02% in the linear viscoelastic range) at a temperature of 20°C. Temperature sweep tests were conducted with a constant frequency and strain of 1 Hz and 0.5%, respectively, over temperatures ranging from 10°C to 80°C, with an increase rate of 0.08°C per minute (Naeli *et al*., 2022).

4.2.5.6. Textural analysis

The textural analysis of the samples for the target product was performed at room temperature (20 °C) using a Stable Micro System TA-XT 2i universal device with the TTC Spreadability

Rig (HDP/SR) attachment equipped with the male/female cone. Firmness values were determined as the force (in grams) required for the penetration of the male cone into the female one, causing the fat to be pushed out over a distance of 15 mm. The test was conducted at a speed of 3 mm/s, with a time duration of 5 seconds, and using a load cell of 5 kg (Naeli *et al.,* 2022).

4.2.5.7. Oxidative stability of hydrolyzed samples

The oxidation induction period was assessed using a Rancimat machine (Model 743, Metrohm Herisau, Switzerland) with 3 grams of the sample at 120°C and an airflow rate of 20 L/h (Mardani *et al*., 2021).

4.3. Method - section 2 (application of enzymatic reaction to synthesis alkyl rosmarinates after assessment of their antioxidant activity)

4.3.1. *In vitro*-radical scavenging activity measurements

4.3.1.1. DPPH scavenging activity

This technique was done based on the method explained earlier with some modifications (Mardani *et al.,* 2021). In summary, DPPH solution was created at the concentration of 0.2 mmol/L and then left in darkness at room temperature. Next, 100 µL samples were added to 96-well microplate and 100 µL ethanolic DPPH solution was added to each well. The absorbance was calculated at 515 nm. IC_{50} values were determined as the concentration to scavenge 50 % of free radicals. The initial concentrations of analyzed compounds were 50 mmol/L.

The inhibition percentage was determined as:

$$\text{Inhibition percentage} = \left(1 - \left(\frac{\text{Abs samples} - \text{Abs control}}{\text{Abs blank}}\right)\right) * 100$$

(Equation 3)

4.3.1.2.Ferric reducing antioxidant power (FRAP)

The ferric reducing ability was calculated based on the method illustrated by (Mardani *et al.,* 2021). The FRAP solution was freshly made and kept at 37 °C for 1 h prior to the measurements. 10 µL sample solutions were included into a 96-well microplate, 100 µL FRAP solution and 100 µL distilled water were added to each well. The absorbance was determined at 593 nm. Ferrous sulfate ($FeSO_4$) was used as the standard, and the results were expressed in micromoles of ferrous sulfate equivalents (μmol Fe^{2+}/100 g). The initial concentrations for analyzed compounds were 50 mmol/L.

4.3.1.3.ABTS scavenging activity

The ABTS scavenging activity was calculated based on the method illustrated by (Aihaiti *et al.,* 2022). 7 mM ABTS stock solution mixing with 2.45 mM potassium persulfate was kept in a dark room at room temperature for 16 hours. To achieve an absorbance of 0.7 ± 0.02 at 734 nm, the ABTS solution was diluted with ethanol. In a 96-well microplate, 16 µL of the sample solution and 184 µL of ethanolic ABTS solution were combined and incubated in darkness at room temperature for 30 minutes. The absorbance was subsequently measured at 734 nm. The inhibition percentage of the ABTS value was calculated using the following formula:

$$\text{Inhibition percentage} = \left(1 - \left(\frac{\text{Abs samples} - \text{Abs control}}{\text{Abs blank}}\right)\right) * 100$$

(Equation 4)

IC_{50} values were calculated as the effective concentration to scavenge 50% of free radicals. The initial concentrations for analyzed compounds were 50 mmol/L.

4.3.2. Antioxidant activity measurements in oil-based food systems

4.3.2.1.Preparation of stripped sunflower oil and bulk oil samples

Sunflower oil employed in this project was removed from its endogenous antioxidants in order to avoid affecting the oxidative strength of the samples. Stripped sunflower oil was prepared based on a simplified technique described earlier by Oh and Shahidi (2018). Briefly, 100 g of sunflower oil was mixed with 1 liter of hexane and subjected to activated silicic acid (150 g) and charcoal (25 g). The solvent used for elution was subsequently evaporated using a rotary evaporator at 40 °C and pure oil was obtained.

In the case of bulk oil samples, rosmarinic acid and alkyl rosmarinates were added to the purified oil at 1.31 mmol/L oil (Oh & Shahidi, 2018).

4.3.2.2.Production of structured fat with MAGs

Briefly, MAGs were precisely measured and evenly distributed in stripped sunflower oil at 15% wt% using a magnetic stirrer based on the method previously explained with some modifications (Naderi *et al.,* 2016, 2018). Then, rosmarinic acid and alkyl rosmarinates were added to 1 g of prepared oil at 1.31 mmol/L. The blend was warmed to 60 °C and maintained at this temperature for 5 minutes under magnetic stirring. Following this, it was stored in a refrigerator at 4 °C overnight before undergoing further analysis.

4.3.2.3.Production of ethyl cellulose oleogel

In summary, 7% ethyl cellulose was used in stripped sunflower oil based on the method previously explained with some modifications (Naeli *et al.,* 2020, 2022). The oleogel was created by homogenizing using an Ultra-Turrax dispersing tool at 12000 rpm at 90 °C for 5 minutes. Then, rosmarinic acid and alkyl rosmarinates were added to 1 g of prepared oil at 1.31 mmol/L. The resulting samples were then stored at 4 °C overnight before subsequent analysis.

4.3.2.4.Preparation of sunflower oil O/W emulsion

The emulsion phase inversion technique was employed to create an O/W emulsion based on the procedure described by Keramat *et al*. (2023) with slight modifications. Briefly, a potassium phosphate buffer solution (40 mmol/L, pH 7) was slowly added to stripped oil containing Tween 80. The oil-to-water ratio stood at 1 to 10, while the ratio of oil to Tween 80 was 2 to 1. Initially, a magnetic stirrer set at 750 rpm was used to blend Tween 80 with stripped oil for 30 minutes. Following this step, the aqueous phase was introduced gradually into the oil phase while maintaining ongoing stirring of the system (Keramat *et al.,* 2023). Rosmarinic acid and alkyl rosmarinates were added to 1 g of prepared emulsion while shaking for 5 minutes at 1.31 mmol/L.

4.3.2.5.Production of emulsion gel

The O/W emulsion, stabilized using Tween 80 as previously described, was employed in the creation of an emulsion gel based on kappa-carrageenan based on the procedure described earlier (Keramat *et al*. 2023). Potassium chloride was incorporated into the aqueous phase at a concentration of 1.25% (w/w). Initially, the emulsions were heated to 80°C for a duration of 5 minutes using a magnetic stirrer. Following that, kappa-carrageenan (2% w/w) was introduced and stirred for 10 minutes to evenly distribute it within the O/W emulsion. Rosmarinic acid and alkyl rosmarinates were added to 1 g of prepared emulsion gel while shaking for 5 minutes at 1.31 mmol/L. Afterward, the samples were brought to a temperature of 25°C and stored in a fridge for 24 hours to enable the gel network to develop (Keramat *et al.,* 2023).

4.3.2.6.Monitoring oxidation of food systems

The vials containing bulk oil, structured fat with MAGs, oleogel, emulsion gel and non-gelled emulsion were stored in a forced air incubator at 35 °C. Samples were drawn on 0, 10, 20, and 30 days for assessments. In all cases, controls including samples with no added antioxidant and

samples having BHT at the upper limit concentration of 200 ppm (0.9076 mM) were employed in the study. Conjugated dienes and p-anisidine values were utilized for observing the oxidative stability of samples based on the AOCS Method, Ti 1a-64 (Conjugated dienes, 1980), and Method Cd 18-90 (p-anisidine values, 1990) (AOCS, 2017, 2023). To extract the oil from emulsions, 1 g of either emulsion gel or non-gelled emulsion samples was combined with 1.5 mL of a mixture containing hexane and methanol in a 3:2 ratio (v/v). The samples underwent three rounds of 10-second vertexing. Afterward, they were subjected to centrifugation at 6000 rpm for 2 minutes. Following centrifugation, the upper phase was used for monitoring oxidative products after removal of solvent under vacuum at 60 °C.

4.3.3. Enzymatic synthesis

4.3.3.1. Enzymatic synthesis and the optimization of reaction conditions

The reaction of rosmarinic acids with ethanol was done based on the report by Lecomte *et al*. (2010) with modifications for enzymatic reactions based on trial and error (Lecomte *et al.,* 2010; López Giraldo *et al.,* 2007). In summary, for a typical ethyl rosmarinate reaction, 1 mg of rosmarinic acid and 3 mL of n-hexane were mixed in a 10 ml tight screw-cap container. Additionally, 3 mg of molecular sieves (3 Å) was added to each reaction for absorption of water produced during esterification. Based on the model, the desired amount of ethanol (1-5 mmol/mmol rosmarinic acid) and enzyme (4-16 % relative to the total weight of substrates) was put into the reaction system which initiated the reaction at 150 rpm. Blank reactions were prepared under similar conditions with no enzyme, where no product could be identified. The reactions were done in different conditions based on RSM to yield the highest conversion of rosmarinate esters as the response. A Randomized Box-Behnken design with 27 runs was used for the optimization of reaction conditions with RSM to assess the connection among a defined group of adjustable experimental parameters in the enzymatic production of ethyl rosmarinate. The variables used were time (1–5 days), temperature (40-70 °C), enzyme-to-substrate ratio

(4-16 % compared to the overall substrate weight), and the molar ratio of alcohol to rosmarinic acid (1-5).

The conversion of rosmarinic acid to ethyl rosmarinate was determined when considering the product peak's area percentage in relation to the combined areas of both substrate and product peaks which was detected by HPLC-UV analysis. Additionally, the presence of ethyl rosmarinate was confirmed by HPLC-DAD-ESI-qTOFMS.

4.3.3.2.Conversion yield and detection and of ethyl rosmarinate

After completion of the reaction, samples were diluted 10 times with methanol to stop the reaction. The solutions were then diluted 10 times with the HPLC mobile phase (solvent A was 0.1% acetic acid in water and solvent B was acetonitrile and the solvent ratio was 50:50) and analyzed by HPLC after filtration through a 0.22 μm PTFE filter into an HPLC vial. A reverse phase XBridge C18 column (3.5 μm, 2.1 × 50 mm, Waters Corp., Milford, MA, USA) was used for the optimization of ethyl rosmarinate reactions with a UV detector at 290 nm. Isocratic elution at room temperature was used for the separation of the rosmarinic acid and ethyl rosmarinate. The flow was set to 0.6 mL/min. Commercially available rosmarinic acid and ethyl rosmarinate were used for the determination of retention times and method developments. Finally, compounds inside the reaction mixtures were purified with the Agilent 1100 HPLC G1364C fraction collector (automatic time-based separation of each corresponding peak) and their presence was again confirmed in pure form with HPLC-UV. In all reactions, two main peaks were produced as the rosmarinic acid and ethyl rosmarinate which was additionally confirmed in the next step.

HPLC system with a diode array detector (DAD) was coupled to an Agilent (Santa Clara, CA USA) 6530 quadrupole – time-of-flight (q-TOF) hybrid mass spectrometer, equipped with a dual spray ESI source based on the method described by Abrankó *et al*. (2015). In summary,

the q-TOFMS was utilized with the subsequent operational parameters: a capillary voltage of 4,000 V, nebulizer pressure of 40 psig, drying gas flow rate of 13 l/min, and gas temperature of 350°C. For these experiments, the fragmentor voltage was automatically triggered between 160 V and 210 V. The lower value aimed to create mild conditions to minimize in-source fragmentation, while the higher value was intended to promote in-source fragmentation. Throughout the chromatographic run, full-scan mass spectra within the m/z range of 50-1100 were consistently recorded at a scanning speed of 1.5 spectra per second. The instrument automatically conducted internal mass calibration by utilizing an automated calibrant delivery system. This system introduced a combination of the chromatograph's outlet flow and a low-flow calibrating solution (approximately 10 µl/min). The calibrating solution contained the internal reference masses of HP-921 [hexakis-(1H,1H,3H-tetrafluoro-pentoxy)-phosphazene] and purine. The protonated molecules of purine ([C5H5N4]+ at m/z 121.050873) and HP-0921 ([C18H19O6N3P3F24]+ at m/z 922.009798) served as the reference masses. Concurrently, the DAD (Diode Array Detector) collected data within the 200-800 nm range, with 2-nm intervals, at an acquisition speed of 0.5 spectra per second (Abrankó *et al.,* 2015).

4.4.Statistical analysis

Significant levels were based on the confidence level of 95% ($P < 0.05$). Results were analyzed by applying a two-way analysis of variance (ANOVA) using IBM SPSS-25 software. Tukey's post hoc test was used when homogeneity of variances was assumed to be valid. In cases where homogeneity of variances was not met, the Games-Howell post hoc test was utilized.

5. RESULTS AND DISCUSSIONS

5.1.Section 1 (application of enzymatic reactions to solidify liquid vegetable oils)

5.1.1. Screening of lipase for maximizing monoacylglycerol formation

Five types of enzymes were tested to assess their capacity for producing monoacylglycerol in palm olein through partial hydrolysis while minimizing FFAs in the samples were taken and prepared at 0.5, 2, and 5 hours (Table 3 and Figure 4).

Table 3. Lipid composition obtained in the hydrolysis-catalyzed palm olein by different lipases.

	Lipozyme 435	Lipozyme TL IM	Lipozyme RM IM	Lipase AY Amano 30SD	Lipase DF Amano 15
0 h (Starting palm olein)					
FFA	0.02±0.00				
MAG	0.00±0.00				
DAG	7.40±3.52				
TAG	92.6±3.60				
SFAs	46.25±0.15				
IV	55.15±0.20				
0.5 h hydrolysis Hydrolysates:					
FFA	1.20±0.60 ^{aA}	14.42±1.41^{aB}	14.22±2.86 ^{aB}	16.07±0.05 ^{aB}	21.61±0.02 ^{aC}
MAG	0.10±0.09 ^{aA}	0.00±0.00 ^{aA}	0.82±0.05 ^{aA}	0.67±0.62 ^{aA}	5.64±0.36 ^{aB}
DAG	9.66±0.51^{bA}	10.53±1.06 ^{aA}	14.69±1.59 ^{aB}	31.57±1.50 ^{cD}	20.14±0.95 ^{aC}
TAG	89.04±1.34^{cD}	75.06±1.47 ^{cC}	70.27±2.1 ^{cB}	51.69±0.88 ^{cA}	52.60±1.30 ^{cA}
AG fraction:					
SFAs	47.97±0.39 ^{aB}	44.93±0.50 ^{bAB}	45.93±0.10 ^{aAB}	43.38±0.13 ^{cAB}	42.96±0.28 ^{bA}
IV	53.84±0.43^{cA}	57.07±0.24 ^{bAB}	55.30±0.21 ^{cAB}	58.79±0.46 ^{aB}	59.07±0.39 ^{bC}
2 h hydrolysis Hydrolysates:					
FFA	12.30±1.20 ^{bA}	55.45±1.18 ^{bC}	54.86±1.33 ^{bC}	63.58±2.43 ^{bD}	29.29±1.62 ^{bB}
MAG	0.50±0.45 ^{bA}	1.85±0.37 ^{bA}	1.42±0.09 ^{bA}	1.56±0.24 ^{bA}	7.83±0.14 ^{bB}
DAG	7.86±1.67 ^{aA}	19.81±2.15 ^{cB}	17.15±1.54 ^{cB}	20.12±1.29 ^{bB}	29.09±0.56 ^{cC}
TAG	79.35±1.85 ^{bD}	22.89±1.6 ^{bB}	26.57±2.29 ^{bB}	14.74±1.48 ^{bA}	33.80±1.31 ^{bC}
AG fraction:					
SFAs	49.25±0.45 ^{bBC}	44.78±0.17 ^{aB}	50.21±0.06 ^{cC}	39.16±0.06 ^{aA}	39.19±0.11 ^{aA}
IV	52.50±0.52 ^{bA}	57.52±0.55 ^{cB}	51.53±0.13 ^{aA}	63.69±0.06 ^{cC}	63.71±0.08 ^{cC}
5 h hydrolysis Hydrolysates:					
FFA	16.47±0.92 ^{cA}	61.47±1.44 ^{cD}	56.48±1.48 ^{cC}	72.27±2.39 ^{cE}	34.76±2.00 ^{cB}
MAG	0.64±0.13 ^{cA}	2.35±0.33 ^{cA}	1.68±0.32 ^{cA}	1.68±1.07 ^{cA}	9.75±0.75 ^{cB}
DAG	20.16±1.15 ^{cB}	15.71±1.02 ^{bA}	16.32±1.49 ^{bA}	13.31±1.13 ^{aA}	26.83±1.50 ^{bC}
TAG	62.73±2.40 ^{aD}	20.47±2.35 ^{aB}	25.53±1.51 ^{aC}	12.75±1.34 ^{aA}	28.66±1.26 ^{aC}
AG fraction:					
SFAs	50.55±0.17 ^{cC}	48.26±0.36 ^{cBC}	49.43±0.19 ^{bBC}	42.76±0.10 ^{bA}	45.46±0.02 ^{cAB}
IV	49.92±0.20 ^{aA}	53.35±0.41 ^{aAB}	52.02±0.14 ^{bAB}	59.83±0.14 ^{bC}	56.05±0.02 ^{aBC}

A-E, Means with different letters in each row are significantly different (P<0.05). a-c, Means with different letters in each column are significantly different in comparison to their corresponding responses at different times of 0.5, 2, and 5 hours (P<0.05). Monoacylglycerols (MAGs), diacylglycerols (DAGs), triacylglycerols (TAGs) free fatty acids (FFAs). Optimum temperatures were 50 ℃ for Lipase AY, 37 ℃ for Lipase DF, 45 ℃ for Novozyme 435, 40 ℃ for lipozyme RM IM, and ℃ for Lipozyme TL IM.

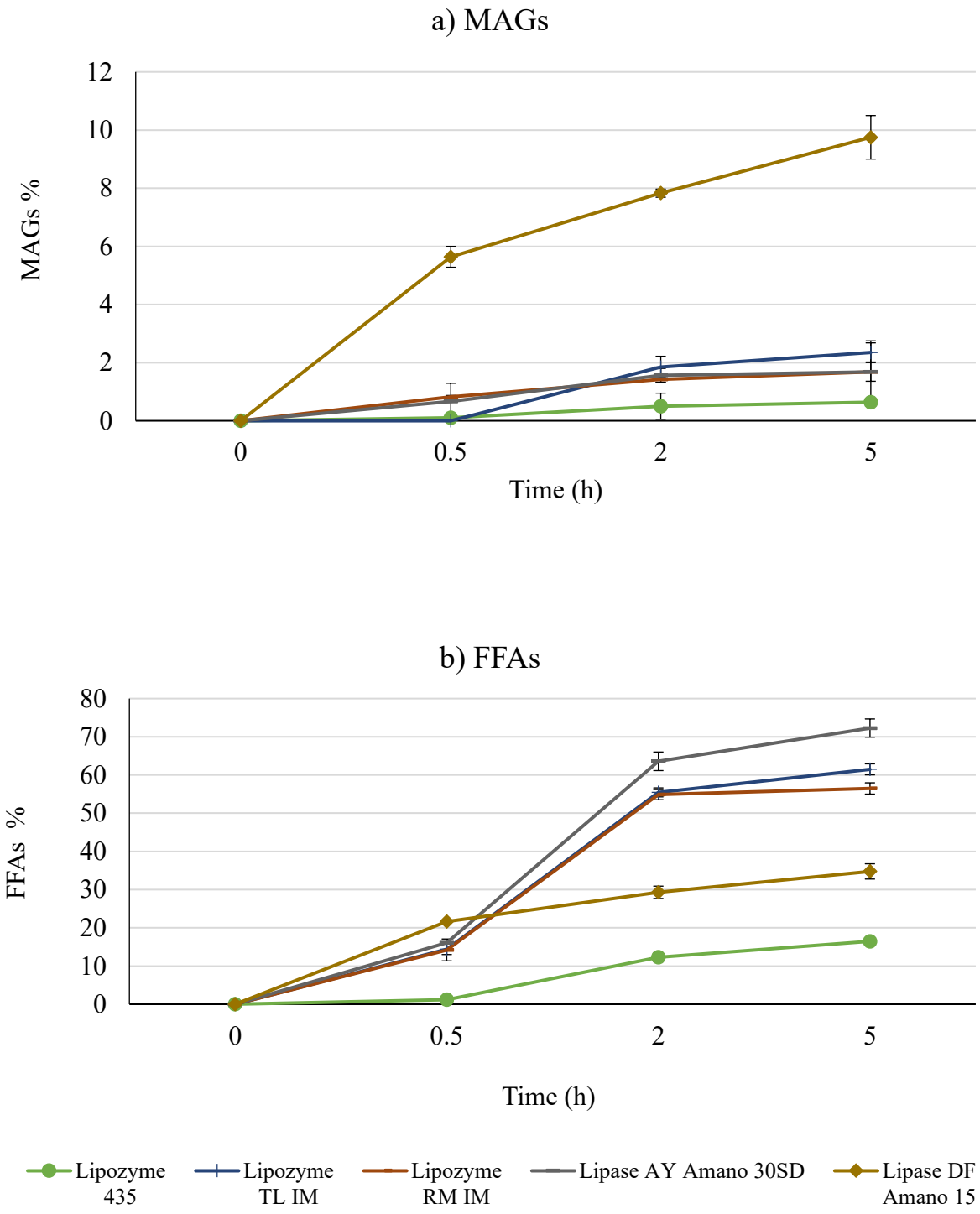

Figure 4. MAGs and FFAs produced by different lipases at different times.

Lipozyme 435 exhibited the lowest hydrolysis activity for palm olein, yielding only 16.47% FFAs (0.64% monoacylglycerol) after 5 hours, previously reaching 12.3% FFAs (0.5% monoacylglycerol) after 2 hours. Similar findings have been reported in other studies involving

Lipozyme 435 for the hydrolysis of algal oil (Yang *et al*., 2021) and linseed oil (Chen *et al.,* 2014). In another investigation, Kiatsimkul *et al.* (2006) studied the hydrolysis reactions of eight lipases and found that *Penicillium camembertii* lipases were ineffective for TAGs substrates (Kiatsimkul *et al.,* 2006). Conversely, Lipozyme TL IM, Lipozyme RM IM, and Lipase AY Amano 30SD displayed comparable hydrolysis activity for palm olein. For example, Lipozyme TLM showed a slight increase in monoacylglycerol levels, reaching a maximum of 2.35% after 5 h of reaction, while the FFAs content increased significantly to 61.47%. Similar results for Lipozyme TLM and Lipozyme RM IM were observed in our prior study on the efficiency of some commercial enzymes in the hydrolysis of palm olein for producing FFAs and DAG oil (Mardani *et al.,* 2015).

The highest hydrolytic activity and the least efficiency for monoacylglycerol purification were observed for Lipase AY Amano 30SD, resulting in 72.27% FFAs and 1.68% monoacylglycerol. Similar monoacylglycerol production outcomes were also achieved for Lipase AY Amano 30SD in enzymatic hydrolysis of tuna oil (Chen *et al.,* 2014; Z. Yang *et al.,* 2021). On the other hand, Lipase DF demonstrated remarkable efficacy in monoacylglycerol preparation through hydrolysis, with 5.61% monoacylglycerol (21.61% FFAs) after only 0.5 h of reaction. Subsequently, the monoacylglycerol level slightly increased, reaching a maximum of 8.76% after 5 h of reaction (34.76% FFAs). The DAGs level began to decrease gradually after 2 h of reaction, reaching 29.09% and then declining further to 26.83%, indicating partial hydrolysis of DAGs. Until now, selective hydrolysis of vegetable oils has primarily been employed for enriching DAGs to produce DAG oils. In a study by Li *et al*. (2021) on the production of DAG oil, four different lipases were evaluated for the preparation of DAGs through partial hydrolysis. Similar to our findings, their results indicated that Lipase DF "Amano" 15 was the most efficient lipase among the four tested. Under optimal conditions, Li

et al. (2021) achieved over 30% of DAGs and approximately 10% of monoacylglycerol (Li *et al.,* 2021).

Furthermore, the selectivity of different enzymes towards various fatty acids was investigated after the separation of FFAs. Table 3 also presents the selectivity of different lipases for different fatty acids. Lipase AY Amano 30SD exhibited higher selectivity for hydrolyzing SFAs from oils, resulting in a reduction of SFAs to 39.16 %. The selectivity of AY Amano 30SD for SFAs has been previously reported in various studies (Chen *et al*., 2014; Yang *et al*., 2021). This selectivity appeared to decrease after 2 hours, indicating the occurrence of esterification reactions as well as hydrolysis reactions in longer reaction times. Similarly, Lipase DF also reduced the overall SFAs after 2 hours, reaching an IV of 63.71 (39.19% saturated fatty acids). On the other hand, Lipozyme 435, TL IM, and RM IM showed no selectivity towards any specific fatty acid. In fact, SFAs slightly increased after 5 hours, possibly due to the increase in acyl migration of SFAs to the sn-2 position and the high potential of these enzymes for esterification reactions leading to acyl migration reactions, involving the movement of SFAs from sn-1,3 positions to the sn-2 position or external bonds to the central one of the glycerol backbone (Ai *et al.,* 2023; Zhou *et al.,* 2021).

Up to now, the selective hydrolysis of vegetable oils has primarily been used for the enrichment of omega-3 oils and the production of DAG oils. For instance, Kiatsimkul *et al.* (2006) investigated the hydrolysis reactions of a group of eight lipases, aiming to selectively eliminate SFAs from epoxidized soybean oil. While the lipase from *Aspergillus niger* exhibited a preference for hydrolyzing SFAs in soybean oil, this selectivity was not observed in the case of epoxidized soybean oil. In contrast, the lipase from *Candida rugosa* displayed enhanced selectivity towards SFAs in epoxidized soybean oil. Additionally, *Burkholderia cepacia* lipase showed selectivity for both palmitic acid and stearic acid but did not exhibit selectivity towards the epoxy acyl moieties (Kiatsimkul *et al.,* 2006). In another study, Chen *et al.* (2023)

conducted a two-step enzymatic hydrolysis of tuna oil to release MUFAs and SFAs from TAGs, leading to acylglycerols rich in omega-3 PUFAs and lower SFAs. In the first step, Lipase AY "Amano" 400SD was employed to primarily hydrolyze MUFAs from the tuna oil (resulting in a slight reduction of SFAs). For the second step, *Candida antarctica* lipase A (CAL-A) was utilized to mainly eliminate SFAs from the acylglycerols obtained from the hydrolysate of the first step (Chen *et al.,* 2023).

In conclusion, considering the MAGs content and partly reducing the SFAs in the acylglycerol phase, which is known to be nutritionally desirable for structured fats, Lipase DF “Amano” 15 was selected for the optimization studies and extending the experiments to other vegetable oils.

5.1.2. Modeling and optimization of selective partial hydrolysis

RSM was utilized to model and optimize the reaction conditions with four factors. The investigation focused on the main effects and interactions between contributing factors, including water level (2-10 wt% of oil mass), enzyme load (10-90 units per gr of oil), temperature (22-54 °C), and time (1-5 h), to assess their influence on the MAGs in hydrolyzed oil. The resulting acylglycerol compositions, including FFAs, MAGs, DAGs, and TAGs, are presented in Table 4.

Table 4. Central composite design and responses for the partial hydrolysis of palm olein.

Run	Enzyme (U/g)	Water (%)	Temp (°C)	Time (h)	TAG (%)	DAG (%)	MAG (%)	FFA (%)
1	50	6	22	3	52.18±1.12	21.57±3.12	5.53±3.05	20.73±2.93
2	50	6	38	5	26.40±0.96	27.34±1.23	10.38±1.61	35.89±0.60
3	30	8	46	4	31.43±1.58	28.14±2.14	9.33±0.67	31.11±1.46
4*	70	8	30	2	48.15±5.12	28.27±2.65	3.24±2.86	20.34±4.33
5	30	4	46	4	32.53±0.54	28.49±1.65	9.07±3.14	29.92±1.53
6	50	10	38	3	27.95±0.34	27.79±1.12	9.38±2.85	34.89±0.24
7	30	4	30	4	55.06±0.83	22.30±2.25	4.87±2.03	17.78±2.54
8*	70	8	30	4	59.73±5.54	15.96±3.56	4.33±2.12	19.98±4.15
9	70	4	46	4	34.10±1.70	26.35±2.12	9.18±1.08	30.39±1.45
10*	50	6	38	3	28.86±2.31	27.30±2.34	9.76±1.50	34.08±1.99
11	50	6	38	3	32.41±2.67	30.10±2.76	8.57±1.46	28.92±2.27
12	50	6	38	3	31.31±1.78	27.20±3.15	9.63±1.86	31.86±1.73
13	50	2	38	3	51.98±2.88	23.27±0.56	5.52±1.80	19.22±1.42
14	70	4	30	2	52.07±2.76	25.50±0.83	5.10±2.41	17.33±2.08
15	50	6	38	3	28.32±2.19	27.96±2.15	10.23±2.39	33.49±1.61
16	70	4	30	4	52.49±1.34	23.17±2.12	5.36±2.84	18.98±1.60
17*	50	6	54	3	51.28±6.14	24.13±4.15	3.00±0.63	21.6±5.24
18	50	6	38	3	29.55±3.24	26.51±0.38	10.22±2.17	33.72±2.70
19*	10	6	38	3	41.82±2.29	32.55±1.50	5.08±2.35	20.55±2.61
20	30	8	30	2	45.15±1.56	26.50±2.15	6.30±1.96	22.05±2.95
21	70	8	46	2	30.35±1.20	31.39±1.13	8.63±1.67	29.63±3.02
22	70	8	46	4	29.43±2.94	26.97±0.84	9.73±3.84	33.87±3.88
23	30	8	46	2	35.95±3.16	28.45±1.12	8.01±2.84	27.60±3.44
24	50	6	38	3	32.62±2.95	26.14±2.18	9.58±2.96	31.66±1.86
25	90	6	38	3	27.03±0.72	26.17±1.12	11.18±2.66	35.63±3.16
26	50	6	38	1	34.15±2.45	28.53±2.51	8.47±3.15	28.86±1.21
27	30	4	30	2	57.91±2.34	21.65±0.54	4.20±0.84	16.24±3.44
28	70	4	46	2	32.78±2.98	30.82±3.14	8.42±±1.34	27.98±3.14
29	30	8	30	4	31.85±1.64	27.10±3.05	9.56±1.24	31.50±1.62
30	30	4	46	2	34.17±1.09	30.22±0.92	8.09±2.33	27.52±2.10

Wa =Water content (wt% of oil mass); En = Enzyme load; (wt% of oil mass); Te = Reaction temperature (°C); Ti = Reaction time (h). Monoacylglycerols (MAGs), diacylglycerols (DAGs), triacylglycerols (TAGs) free fatty acids (FFAs). *Note that outlier including rows 4,8,10, 17, and 19 are removed from the model. The data shown in green recorded MAGs/FFAs higher than 0.30.

The data were fitted to the model to explain the dependent variables as a function of independent variables. The analysis of variance for the best-fitting optimized model, which was established by optimizing acylglycerol compositions or the proportion of lipid classes after hydrolysis reactions, is presented in Table 5.

Table 5. The analysis of variance (ANOVA) of the modeled responses.

Response	Best model	p-value	Lack of fit	R^2	Adequate precision
FFA (%)	= -101.40 + 0.07En + 12.50Wa + 3.92Te + 1.78Ti – 0.16 Wa*Te – 0.37 Wa^2 – 0.03 Te^2 (Equation 5)	< 0.0001	NS	0.89	14.26
MAG (%)	= -36.14 + 0.02En + 4.49Wa + 1.36Te + 0.55Ti – 0.06 Wa*Te – 0.15 Wa^2 – 0.01 Te^2 (Equation 6)	< 0.0001	NS	0.90	15.06
DAG (%)	=15.69 + 0.01 En + 0.42Wa + 0.28Te – 0.65Ti (Equation 7)	< 0.0001	NS	0.68	10.94
TAG (%)	=253.23 – 0.30 En –18.67Wa – 7.10 Te + 2.39Ti – 0.04 En*Wa + 0.01 En* Te +0.33 Wa* Te – 0.7 Wa*Ti + 0.60 Wa^2 + 0.05 Te^2 (Equation 8)	< 0.0001	NS	0.96	18.41
MAG/FFA	=0.30+0.0065Te – 0.0054 En*Wa – 0.0048 En*Ti – 0.0048 Wa*Te – 0.0057 Wa^2 – 0.0053 Te^2 (Equation 9)	< 0.0001	NS	0.78	11.76
DAG/FFA	=0.98 – 0.113 Wa – 0.086 Ti + 0.089 Wa*Te (Equation 10)	< 0.0001	NS	0.54	10.00

Wa =Water content (wt% of oil mass); En = Enzyme load; (wt% of oil mass); Te = Reaction temperature (°C); Ti = Reaction time (h). Monoacylglycerols (MAGs), diacylglycerols (DAGs), triacylglycerols (TAGs) free fatty acids (FFAs). Note that outlier including rows 4,8,10, 17, and 19 are removed from the model.

Drawing from the work by Nicholson and Marangoni (2021) on making structured fats with the glycerolysis of different vegetable oils, the presence of higher amounts of DAGs and MAGs is considered desirable for structuring vegetable oils. In the context of partial hydrolysis, it is advantageous to have increased MAGs and DAGs while keeping FFAs as low as possible (Nicholson & Marangoni, 2020, 2021, 2022). As a result, the model's desirability also prioritized maximizing DAGs, but with three times less importance than MAGs, while minimizing FFAs release (Table 6). All the models were found to be significant at $p < 0.0001$, with a non-significant lack of fit ($p > 0.05$). Additionally, the proportion of MAGs/FFAs and DAGs/FFAs is also presented and modeled. The R-squared (R^2) values and adequate precisions were found to be high for all models, indicating a good fit of the data.

Table 6. Two different conditions predicted and tested by the model with high desirability.

Parameters	Desirability Importance		Run 1	Run 2
Enzyme (U/g of oil)	In range		70	40.00
Water (wt % of oil)	In range		5.11	5.68
Temperature (°C)	In range		45	45
Time (h)	In range		2	3.05
Run desirability			0.605	0.589
FFA (%)	Minimize (+)	Predicted	29.58	29.09
		Detected	28.91±2.64	31.76±1.50
		Confidence intervals (95 %)	25.06-36.21	25.59-36.43
MAG (%)	Maximize (+++)	Predicted	8.98	8.78
		Detected	8.96±0.54	9.27±1.17
		Confidence intervals (95 %)	7.59-11.05	7.66-11.02
DAG (%)	Maximize (+)	Predicted	27.69	27.94
		Detected	29.64±3.07	27.76±2.15
		Confidence intervals (95 %)	25.34-32.90	24.92-32.25
TAG (%)	None	Predicted	33.21	33.57
		Detected	32.49±2.34	31.21±1.62
		Confidence intervals (95 %)	25.88-37.90	24.50-36.14
MAG/FFA	None	Predicted	0.309	0.302
		Detected	0.31±0.01	0.29±0.02
		Confidence intervals (95 %)	0.29-0.33	0.28-0.32
DAG/FFA	None	Predicted	0.94	0.96
		Detected	1.02±0.02	0.87±0.01
		Confidence intervals (95 %)	0.77-1.40	0.68-1.29

Monoacylglycerols (MAGs), diacylglycerols (DAGs), triacylglycerols (TAGs) free fatty acids (FFAs).

5.1.3. Effect of reaction parameters

5.1.3.1. Effect of lipase content

During lipase-catalyzed reactions, the choice of enzymes not only influences the reaction yield and rate but also impacts the economic aspect of the reactions. Figure 5 illustrates the proportion of MAGs to FFAs as a function of different factors, including water, enzyme, temperature, and time.

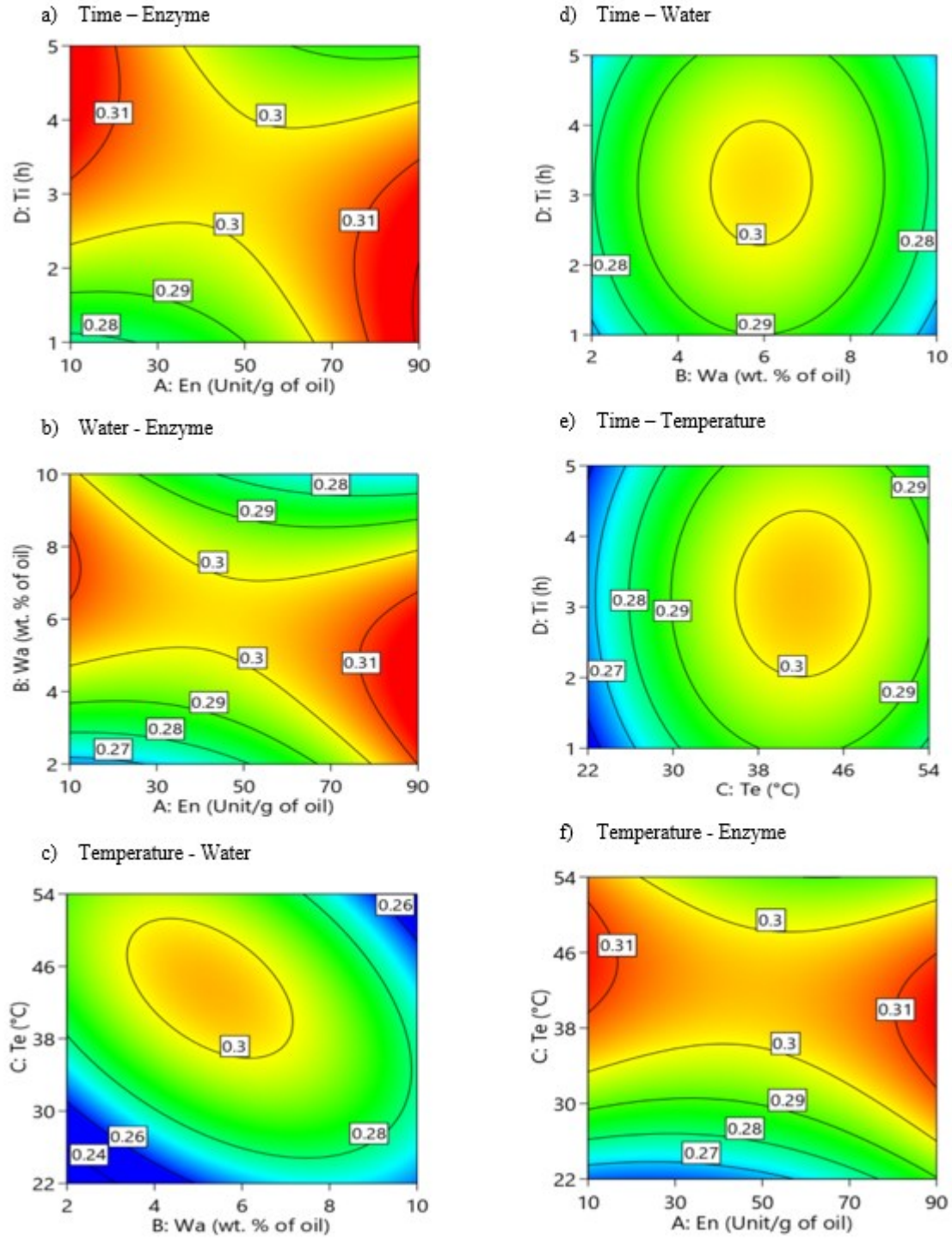

Figure 5. Effect of reaction parameters and their interactions on the rate of MAGs/FFAs.

Wa =Water content (wt% of oil mass); En = Enzyme load; (wt% of oil mass); Te = Reaction temperature (°C); Ti = Reaction time (h).

The hydrolytic activity of the lipase, represented by the degree of hydrolysis or FFAs percentage, increased with higher lipase levels, and greater lipase content resulted in shorter

reaction times to reach equilibrium (Figure 5-a, -b, and -c). Based on the time-enzyme interaction graph for MAGs/FFAs (Figure 5-c), a higher MAGs/FFAs content could be achieved at lower times when a higher enzyme content was used or at longer times when a lower enzyme was employed. Additionally, around the center points of enzyme and time (i.e., 2.5 hours with 60 U/g), a favorable MAGs/FFAs content was attained. Specifically, when 90 U/g of the enzyme was used, the highest MAGs/FFAs ratio of 0.31 with 11.18% MAGs was achieved. Conversely, with only 10 U/g enzyme, a lower hydrolytic activity of 20.55% FFAs (5.08% MAGs) and a MAGs/FFAs ratio of 0.25 were observed, indicating that a lower enzyme level leads to reaching equilibrium at longer times and with a lower MAGs/FFAs ratio (Figure 5-c). According to the desirability model, further increasing the enzyme content to more than 70 U/g did not seem to cause a significant difference ($p > 0.05$) in the proportion of MAGs/FFAs. Considering these findings and being mindful of the reaction's economy, a lipase content of 70 U/g was preferred for the subsequent experiments.

5.1.3.2.Effect of water content

Water plays a crucial role in lipase-catalyzed oil hydrolysis, serving as both a substrate and a determinant of enzyme distribution at the oil-water interface, thereby influencing the reaction rates. As expected, the hydrolysis rate increased with higher water levels. While the rate of MAGs production also increased with the increase in water content (similar to FFAs), indicating its direct impact on the hydrolysis rate, the effect of water on the proportion of MAGs/FFAs was found to be different. Beyond a water content of approximately 4-6% in the reactions, the MAGs/FFAs ratio decreased, as observed through its interactions with enzyme (Figure 5.a), temperature (Figure 5.d), and time (Figure 5.e).

At the center point of water addition (6 wt. %), the maximum MAGs level of 11.18% was recorded at a reaction time of 2.5 h, 38 °C, and an enzyme load of 90 U/g of oil (row 25). At the center points of other reaction conditions (except time), the MAGs level slightly decreased

after 2.5 hours when the reaction time was extended to 4.5 h (row 30), whereas the FFAs content increased, suggesting partial hydrolysis of MAGs. When water addition was at the maximum level of 10 wt. % (center points of other factors), the MAGs content (9.38%) and MAGs/FFAs ratio (0,263) decreased, while the FFAs content increased (35.63%). Finally, when water addition was at the minimum level of 2 wt % (center points of other factors), the hydrolytic reaction of the lipase was very low (19.22% FFAs, with 5.53% MAGs).

In conclusion, although a higher addition of water benefited the hydrolysis reactions, leading to reaching equilibrium and a slow increase in FFAs and MAGs, a reduction in the MAGs/FFAs ratio was observed after around 4-6% water content, indicating that part of MAGs was hydrolyzed with the increase in the water level.

5.1.3.3.Effect of temperature

The temperature of the reactions can significantly influence both the hydrolytic activity and thermostability of enzymes. As specified by the enzyme supplier, lipase DF should exhibit thermostability within a temperature range below 50 °C, with the highest activity observed between 35 and 43 °C, a finding that aligns with the results of this study as shown in Figure 5.-b, -d, and -f. At a reaction temperature of 22 °C, one of the lowest hydrolytic activities of the lipase (20.73% FFAs) was recorded. Temperatures below 34 °C were observed to decrease the hydrolytic activity of the lipase (manifesting as low FFAs%), resulting in a much lower MAGs level and MAGs/FFAs ratio. On the other hand, higher reaction temperatures led to higher reaction rates, but excessively high temperatures slightly reduced hydrolytic activity, MAGs level, and MAGs/FFAs ratio. For instance, when the temperature was maintained at 54 °C, the lowest MAGs yield of 0.22 was achieved (corresponding to 3.66% MAGs with 16.74% hydrolysis). The relatively lower rates observed at higher temperatures could be attributed to lower retention of lipase activity or a decline in their selectivity.

5.1.3.4.Effect of time

The reaction time has a significant impact on the degree of hydrolysis. As expected, both the FFAs and MAGs content increased as the reaction continued and reached equilibrium around 2 hours (based on other factors). After 2 hours and reaching equilibrium, the FFAs% showed a slight increase while MAGs, DAGs, and TAGs decreased, suggesting that part of the MAGs was hydrolyzed to FFAs, resulting in a reduction of the MAGs/FFAs ratio (Figure 5.-c, -e, and -f).

To further investigate the effect of time at optimum conditions and to validate the model, two sets of tests were performed based on the predicted conditions by the model with the highest possible desirability within the experimental range. The desirability criteria included maximizing MAGs and DAGs content while minimizing FFAs%. MAGs content was given three times more desirability importance than high DAGs and low FFAs%. As a result, the software provided desirability values for the reactions, and two predicted reactions were re-done in the lab (Table 6). Run 1 represented the highest desirability value according to the chosen criteria within the experimental range (Table 6). Additionally, considering the cost of enzymes, run 2 was selected as the second-highest desirable value, as it consumed a lower amount of enzyme. This step demonstrated that, based on the model, the reaction could be achieved with similar outcomes using a lower amount of enzyme and longer time, confirming Figure 5.-c of the model. The predicted values closely resembled the experimental values (within their 95% confidence level), as shown in Table 6.

In conclusion, the reaction conditions for the next stage were chosen based on the optimized tested model as follows: a water level of 5.11%, 70 U/g enzyme, and 45 °C, based on a maximum desirability of 0.605. The acylglycerol classes were monitored until 10 hours.

5.1.4. Selective partial hydrolysis of different vegetable oils

The optimized conditions achieved for palm olein were extended to other vegetable oils with varying degrees of SFAs. Table 7 presents the initial fatty acid and acylglycerol composition of the vegetable oils used in this study.

Table 7. Fatty acid and acylglycerol compositions of untreated vegetable oils used in this study.

Parameters	SFO	PSO	RBO	POO
Fatty acid composition (%):				
Palmitic acid (C16:0)	6.24±0.00 ^{a}	11.85±0.00 ^{b}	21.05±0.06 ^{c}	39.89±0.13 ^{d}
Stearic acid (C18:0)	3.69±0.01 ^{b}	6.03±0.01 ^{d}	2.64±0.01 ^{a}	4.51±0.02 ^{c}
Oleic acid (C18:1n-9c)	27.95±0.02 ^{a}	45.82±0.13 ^{d}	39.64±0.10 ^{b}	42.86±0.15 ^{c}
Linoleic acid (C18:2n-6c)	60.38±0.03 ^{d}	34.59±0.09 ^{c}	31.23±0.09 ^{b}	10.16±0.04 ^{a}
Linolenic acid (C18:3n-3c)	0.05±0.00 ^{a}	0.16±0.01 ^{b}	1.13±0.02 ^{c}	0.17±0.01 ^{b}
SFAs	11.34±0.05 ^{a}	18.87±0.01 ^{b}	25.98±0.07 ^{c}	46.25±0.15 ^{d}
MUFAs	28.22±0.03 ^{a}	46.13±0.13 ^{d}	40.40±0.06 ^{b}	43.25±0.15 ^{c}
PUFAs	60.43±0.03 ^{d}	35.00±0.13 ^{c}	33.61±0.11 ^{b}	10.51±0.04 ^{a}
Total trans	0.01±0.01 ^{a}	0.02±0.01 ^{a}	0.06±0.04 ^{b}	0.05±0.01 ^{b}
IV	128.95±0.06 ^{d}	100.00±0.29 ^{c}	91.68±0.21 ^{b}	55.15±0.20 ^{a}
Acylglycerol composition (%):				
FFA	0.00±0.00 ^{a}	2.82±0.52 ^{c}	3.45±1.68 ^{d}	0.02±0.00 ^{b}
MAG	0.00±0.00 ^{a}	0.00±0.00 ^{a}	0.05±0.02 ^{b}	0.00±0.00 ^{a}
DAG	2.90±0.54 ^{a}	13.93±1.15 ^{c}	20.49±2.89 ^{d}	7.40±3.52 ^{b}
TAG	97.1±2.54 ^{d}	82.8±2.20 ^{b}	75.57±3.17 ^{a}	92.6±3.60 ^{c}

SFO: sunflower oil, PSO: pumpkin seed oil, RBO: rice bran oil, POO: palm olein oil. Monoacylglycerols (MAGs), diacylglycerols (DAGs), triacylglycerols (TAGs) free fatty acids (FFAs). a-d, Means with different letters in each row are significantly different ($P<0.05$).

Figure 6 presents the acylglycerol composition of different vegetable oils at a water level of 5.5%, 70 U/g enzyme, and 45 °C for 10 hours. As predicted by the model, the reaction reached equilibrium after only two hours during lipase-catalyzed hydrolysis. As can be seen, the established model exhibited good fitness when applied to these other oils as well.

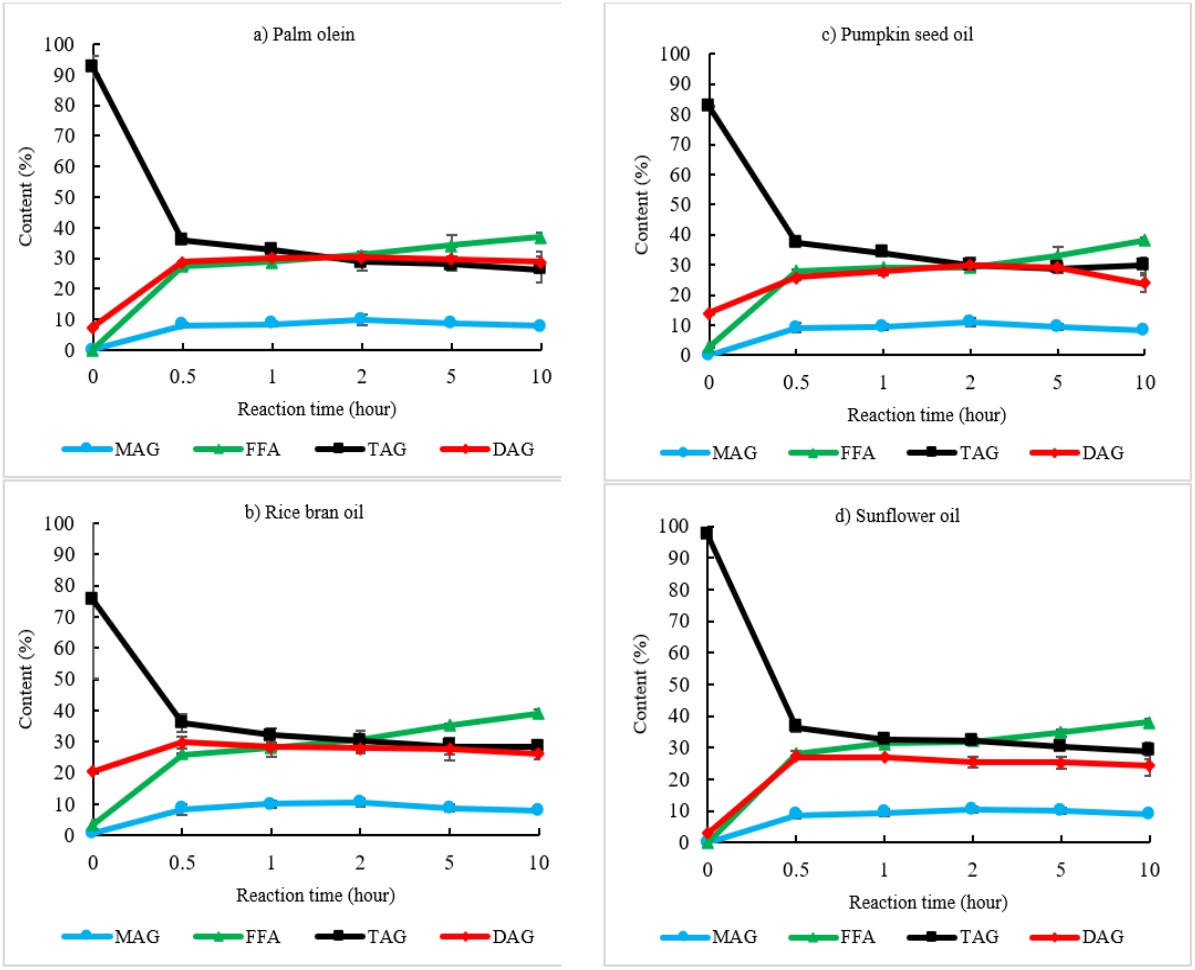

Figure 6. Extending and testing the model to four different vegetable oils for 10 hours.

Monoacylglycerols (MAGs), diacylglycerols (DAGs), triacylglycerols (TAGs) free fatty acids (FFAs).

In general, a sharp change was observed in the first half-hour of the reaction for all acylglycerol classes. As time passed, the level of TAGs slightly and continuously decreased, while the level of FFAs slightly and continuously increased. Additionally, DAGs and MAGs gradually increased and started to decrease after two hours (except in the case of sunflower oil, where DAGs showed a slight increase until 5 hours and then decrease). In conclusion, the high proportion of MAGs/FFAs and DAGs/FFAs was observed when the reaction reached equilibrium, representing the peak of hydrolysis where MAGs and DAGs reached high levels. Beyond this point, MAGs and DAGs started to undergo hydrolysis as well (Figure 7). A similar

trend has been observed with Lipase DF in the production of DAG oil from soybean oil (Li *et al.,* 2021).

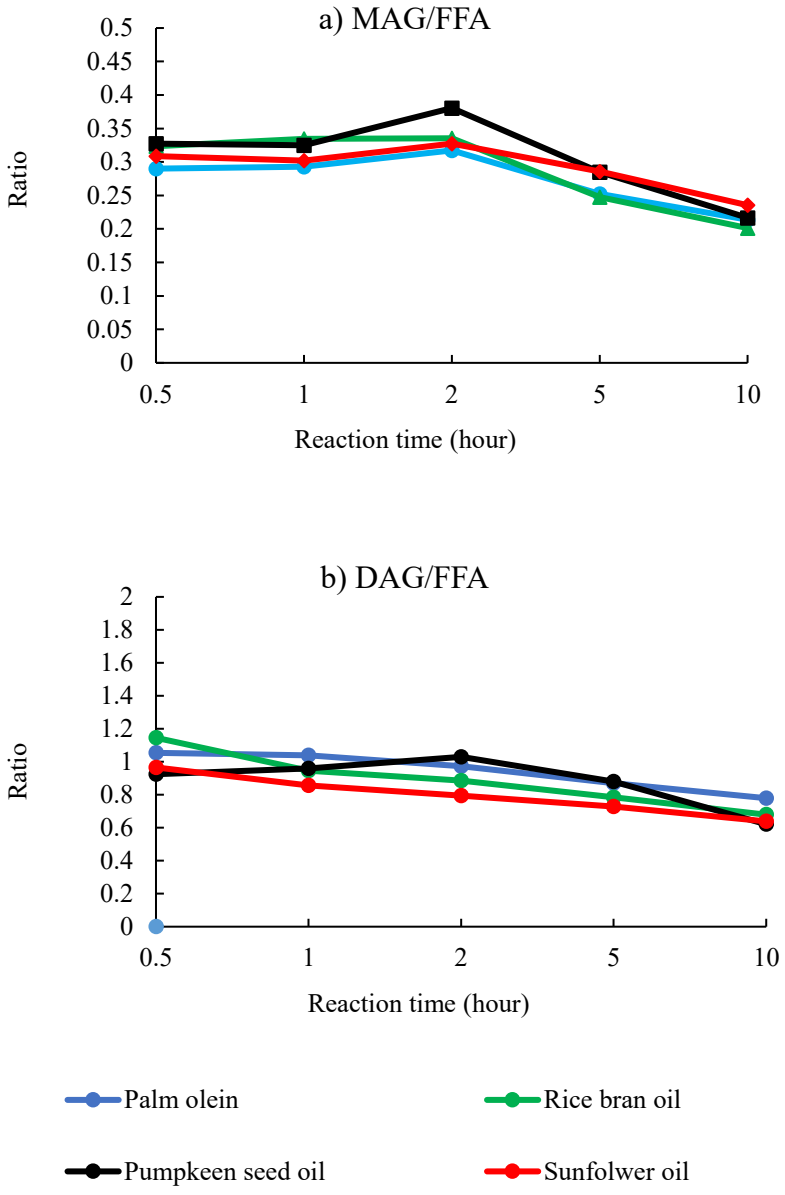

Figure 7. Changes in amount of MAG/FFA (a) and DAG/FFA (b)

The production of solid fats with vegetable oils benefits from higher amounts of MAGs and DAGs, while keeping FFAs as low as possible. Lower FFAs content is advantageous in selective partial hydrolysis of vegetable oils to reduce by-product production. Monie *et al*. (2021) reported that a total substitution of rapeseed oil (113 ± 10 mg KOH/g at 24 hours hydrolysis) led to a negative impact on sponge cake structure compared to partially hydrolyzed rapeseed oil, such as a decrease in volume and elastic crumbs (Monié *et al.,* 2021). Therefore, after 2 hours, samples were taken for further analysis of their thermal, rheological, and textural properties.

5.1.5. Properties of structured fats obtained from selective partial hydrolysis

5.1.5.1. Acylglycerol composition

The structured fats obtained in this study consist of a mixture of MAGs, DAGs, TAGs, and FFAs (Table 8). For food applications, these fats can be utilized as either crude hydrolysates or deacidified hydrolysates. In the production of solid fats using vegetable oils, it is generally preferred to have higher amounts of MAGs and DAGs while keeping FFAs at low levels. However, crude hydrolysates of vegetable oils containing significant FFAs content can serve as specialty fats in certain applications. In fact, FFAs are sometimes used as ingredients in food formulas, and these crude hydrolysates can act as a source of FFAs for such purposes. For example, a crude hydrolysate of rapeseed oil has been demonstrated to enhance the quality of bakery products, particularly in improving the softness of sponge cakes (Monié *et al.,* 2021). Additionally, the crude hydrolysate can be deacidified to produce a structured fat with reduced FFAs content. Therefore, this study offers two products as a result of hydrolysis reactions, one being a specialty fat containing FFAs and the other a deacidified fat (Bhattacharya, 2023; O'Brien, 2009).

The removal of FFAs from hydrolysates was attempted using the alkali deacidification method. During the neutralization process, emulsions can form. To minimize oil loss and liberate the

oil trapped in soapstock (O/W emulsion), demulsification was employed, involving the use of KCl and heating at 60 °C. However, alkali deacidification led to excessive loss of MAGs and DAGs due to entrapment and emulsification of these lipids with the soapstock, attributed to the high FFAs content. Consequently, alkali deacidification was not deemed effective for the separation of FFAs from highly hydrolyzed oils/fats with high acid value. The theoretical corresponding acylglycerol composition, assuming the total removal of FFAs without any loss of lipids, is presented in Table 8 to estimate the potential acylglycerol content.

Based on the theoretical calculation, the final products at 2 hours would have a corresponding acylglycerol composition of approximately 14.31-15.61% MAGs, 37.26-43.89% DAG, and 41.82-47.37% TAGs. Notably, the theoretical maximum content of MAGs, DAGs, and TAGs is achieved at 2 hours, validating the model's applicability for different vegetable oils with varying levels of saturated fatty acids.

Table 8. Acylglycerol composition of treated oils and deacidified products after 2 h of hydrolysis.

Parameters		SFO	PSO	RBO	POO
Hydrolyzed product					
	FFA (%)	31.94±0.40 ^{b}	29.09±0.94 ^{a}	31.39±0.67 ^{b}	31.08±1.22 ^{ab}
	MAG (%)	10.46±1.11 ^{a}	11.07±0.79 ^{a}	10.53±1.35 ^{a}	9.86±1.73 ^{a}
	DAG (%)	25.36±1.78 ^{a}	29.96±2.40 ^{b}	27.82±1.60 ^{ab}	30.25±0.20 ^{b}
	TAG (%)	32.24±0.87 ^{a}	29.89±1.67 ^{a}	30.26±3.08 ^{a}	28.82±2.74 ^{a}
Deacidified product *					
	FFA (%)	3.54±0.73 ^{a} (0.00)	3.43±0.86 ^{a} (0.00)	3.50±0.44 ^{a} (0.00)	4.90±0.12 ^{a} (0.00)
	MAG (%)	5.71±1.14 ^{a} (15.37)	4.95±1.35 ^{a} (15.61)	4.20±1.92 ^{a} (15.34)	4.70±1.60 ^{a} (14.31)
	DAG (%)	40.67±3.43 ^{a} (37.26)	39.86±2.19 ^{a} (42.25)	42.27±2.57 ^{a} (40.55)	37.78±3.04 ^{a} (43.89)
	TAG (%)	50.08±3.17 ^{a} (47.37)	51.76±2.08 ^{a} (42.15)	50.03±2.38 ^{a} (44.10)	52.62±3.10 ^{a} (41.82)

SFO: sunflower oil, PSO: pumpkin seed oil, RBO: rice bran oil, POO: palm olein oil. * Data within the parenthesis represent theoretical acylglycerol contents assuming the complete removal of FFAs without any acylglycerol losses. a-d, Means with different letters in each row are significantly different ($P<0.05$). Monoacylglycerols (MAGs), diacylglycerols (DAGs), triacylglycerols (TAGs) free fatty acids (FFAs).

5.1.5.2.Fatty acid composition and selectivity

Figure 8 illustrates the enrichment numbers of each acyl moiety in the acylglycerol phase while Table 9 presents the fatty acid composition of both the crude and deacidified hydrolysates. A higher enrichment number indicates a lower enzyme selectivity for hydrolyzing that specific acyl moiety. An enrichment number of 1 suggests no selectivity, while a value higher than 1 indicates lower selectivity.

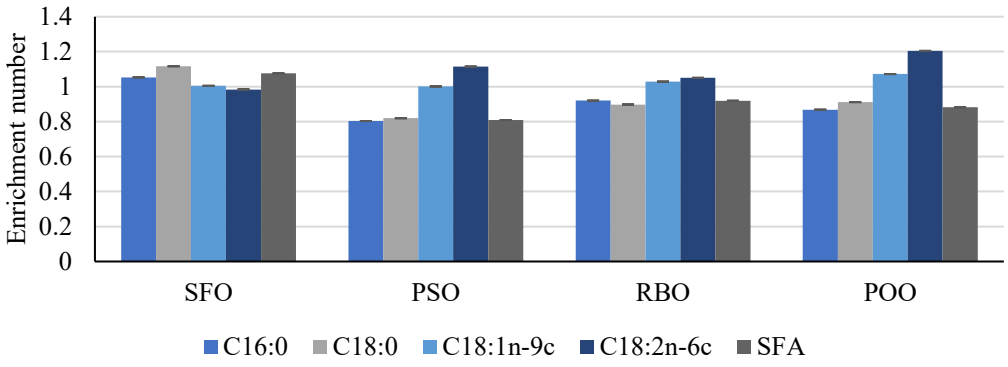

Figure 8. Enrichment number of fatty acid in acylglycerol phase of different vegetable oils using Lipase DF. SFO: sunflower oil, PSO: pumpkin seed oil, RBO: rice bran oil, POO: palm olein oil.

In all samples, with the exception of sunflower oil which has the lowest SFAs content, lipase DF exhibited higher rates of cleavage for SFAs (both palmitic and stearic acids), indicating the highest lipase selectivity. Unexpectedly, pumpkin seed oil exhibited the lowest enrichment number for SFAs, despite having a lower saturated fatty acid content compared to palm olein oil and rice bran oil. This can likely be attributed to the specific arrangement of saturated fatty acids on the glycerol backbone. The structural configuration of these fatty acids plays a significant role in their interaction with enzymes. In this case, Lipase DF Amano 15 shows selectivity for the fatty acids positioned on the sn-1 and sn-3 positions of the glycerol backbone, which might be a contributing factor to the observed results. Following this exception, palm olein oil presented the next lowest enrichment number for saturated fatty acids. This was somewhat expected given its fatty acid composition and the enzyme's selectivity profile. Rice bran oil followed palm olein oil in terms of the enrichment number, exhibiting a slightly higher

value. Finally, sunflower oil showed the highest enrichment number for saturated fatty acids among the oils tested. This sequence underscores the impact of both the fatty acid composition and their positional distribution on the glycerol backbone in determining the effectiveness of the lipase-mediated enrichment process.

Table 9. Fatty acid composition and oxidative stability of treated oils and deacidified products.

Parameters	SFO	PSO	RBO	POO
Fatty acid composition*:				
SFA (%)				
Hydrolyzed product	11.34±0.05 [a]	18.87±0.01 [b]	25.89±0.07 [c]	46.25±0.15 [d]
Deacidified product	12.20±0.04 [a]	15.27±0.05 [b]	23.89±0.05 [c]	40.78±0.29 [d]
MUFA (%)				
Hydrolyzed product	28.22±0.03 [a]	46.13±0.13 [d]	40.40±0.04 [b]	43.25±0.15 [c]
Deacidified product	28.34±0.01 [a]	46.12±0.03 [c]	41.67±0.09 [b]	46.38±0.35 [c]
PUFA (%)				
Hydrolyzed product	60.43±0.03 [d]	35.00±0.05 [c]	33.61±0.06 [b]	10.51±0.04 [a]
Deacidified product	59.46±0.04 [d]	38.68±0.07 [c]	34.45±0.11 [b]	12.68±0.08 [a]
IV				
Hydrolyzed product	128.95±0.06 [d]	100.00±0.29 [c]	91.68±0.21 [b]	55.15±0.20 [a]
Deacidified product	127.37±0.10 [d]	106.48±0.02 [c]	95.68±0.24 [b]	61.56±0.46 [a]
IP120 °C (h):				
Untreated oil	1.53±0.03 [a]	4.88±0.06 [b]	6.82±0.05 [c]	11.68±0.00 [d]
Hydrolyzed product	0.90±0.06 [a]	2.58±0.08 [b]	5.44±0.29 [c]	10.39±0.28 [d]
Deacidified product	1.92±0.05 [a]	4.94±0.18 [b]	8.55±0.10 [c]	13.33±0.36 [d]

SFO: sunflower oil, PSO: pumpkin seed oil, RBO: rice bran oil, POO: palm olein oil. *Untreated oils and treated samples without deacidification expectedly recorded the same fatty acid compositions. a-d, Means with different letters in each row are significantly different ($P<0.05$). Iodine value (IV), saturated fatty acids (SFAs), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA).

Conversely, linoleic acid displayed the highest enrichment number, meaning all unsaturated fatty acids increased in all oils (except for sunflower oil). Among the vegetable oils used in this study, palm olein exhibited the highest enrichment of unsaturated fatty acids. This can be attributed to the higher level of SFAs in palm olein compared to the other oils and the use of sn-1 and sn-3 specific enzymes during their production.

The following sections will investigate the impact of these changes in fatty acid composition on the properties of the structured fats.

5.1.5.3.Oxidative stability

The oxidative stability of the structured hydrolyzed fats was evaluated before and after deacidification and compared to that of the initial oils (Table 10). The presence of FFAs in the oil samples resulted in lower oxidative stability. This phenomenon is consistent with findings from a study conducted by Wang *et al*. (2010), which demonstrated a prooxidant impact of FFAs in various vegetable oils, irrespective of their lipidic substrate or level of saturated fatty acids. The degree of this effect was found to be related to the concentration of FFAs, but a consistent relationship between FFAs concentration and induction time (*i.e.,* higher FFAs concentration leading to higher prooxidant impact) was not observed (Wang *et al*., 2010).

However, the oxidative stability of all samples improved after the removal of FFAs, despite the decrease in saturated fatty acids, compared to both the initial oils and the hydrolyzed oils/fats. This improvement could be attributed to the increase in MAGs ratio, which significantly slowed down the oxidative processes in all cases. This phenomenon aligns with the findings of another study, which showed that the presence of MAGs significantly decelerated oxidative processes, especially as oxidation continued (Gomes *et al.,* 2010).

Table 10. Oxidative stability of treated oils and deacidified products after 2 h of hydrolysis

Parameters		SFO	PSO	RBO	POO
IV					
	Hydrolyzed product	128.95±0.06 ^{d}	100.00±0.29 ^{c}	91.68±0.21 ^{b}	55.15±0.20 ^{a}
	Deacidified product	127.37±0.10 ^{d}	106.48±0.02 ^{c}	95.68±0.24 ^{b}	61.56±0.46 ^{a}
IP120 °C (h):					
	Untreated oil	1.53±0.03 ^{a}	4.88±0.06 ^{b}	6.82±0.05 ^{c}	11.68±0.00 ^{d}
	Hydrolyzed product	0.90±0.06 ^{a}	2.58±0.08 ^{b}	5.44±0.29 ^{c}	10.39±0.28 ^{d}
	Deacidified product	1.92±0.05 ^{a}	4.94±0.18 ^{b}	8.55±0.10 ^{c}	13.33±0.36 ^{d}

SFO: sunflower oil, PSO: pumpkin seed oil, RBO: rice bran oil, POO: palm olein oil. a-d, Means with different letters in each row are significantly different ($P<0.05$). Iodine value (IV), Induction period (IP).

The literature presents conflicting findings regarding the prooxidant effects of MAGs and DAGs. For example, a study by Chen *et al.* (2014) reported inconsistent results regarding the influence of MAGs and DAGs on the oxidative stability of soybean oil. They investigated different levels of DAGs and MAGs and their effect on lipid oxidation during accelerated

oxidation. Their results indicated that the addition of MAGs and DAGs in minor concentrations did not alter the oxidation pathway and did not impact the lag phase of oxidation (Chen *et al*., 2014).

Similarly, Mistry and Min (1988) investigated the impact of MAGs and DAGs on the oxidative stability of soybean oil's bulk lipid oxidation. They assessed the effects of different levels of monostearin, distearin, monolinolein, or dilinolein on the oxidative stability of soybean oil. Their study found that DAGs had a prooxidative effect on the stability of the oil (Mistry & Min, 1988).

The varying behavior of MAGs and DAGs on the oxidative stability of vegetable oils in the literature can be attributed to factors such as the fatty acid composition of the oil matter to which they are added, the fatty acid composition of MAGs and DAGs, the presence of other bioactive compounds, emulsifiers, or additional ingredients, and the type of measurements used for oxidative stability assessments.

5.1.5.4.Crystallization and melting behaviors

Figure 9 illustrates the differences in crystallization and melting curves for various target products before and after the hydrolysis reactions. The thermograms revealed significant changes in crystallization and melting behaviors for each of the samples. In all cases, noticeable shifts or broadening of the bulk crystallization or melting peaks were observed, and at least one new peak emerged at higher temperatures. The appearance of these new peaks at higher temperatures can be attributed to the presence of MAGs and DAGs, which have higher crystallization and melting points compared to their TAGs counterparts. These MAGs and DAGs, formed during the partial hydrolysis reactions, are primarily responsible for the new peaks at elevated temperatures.

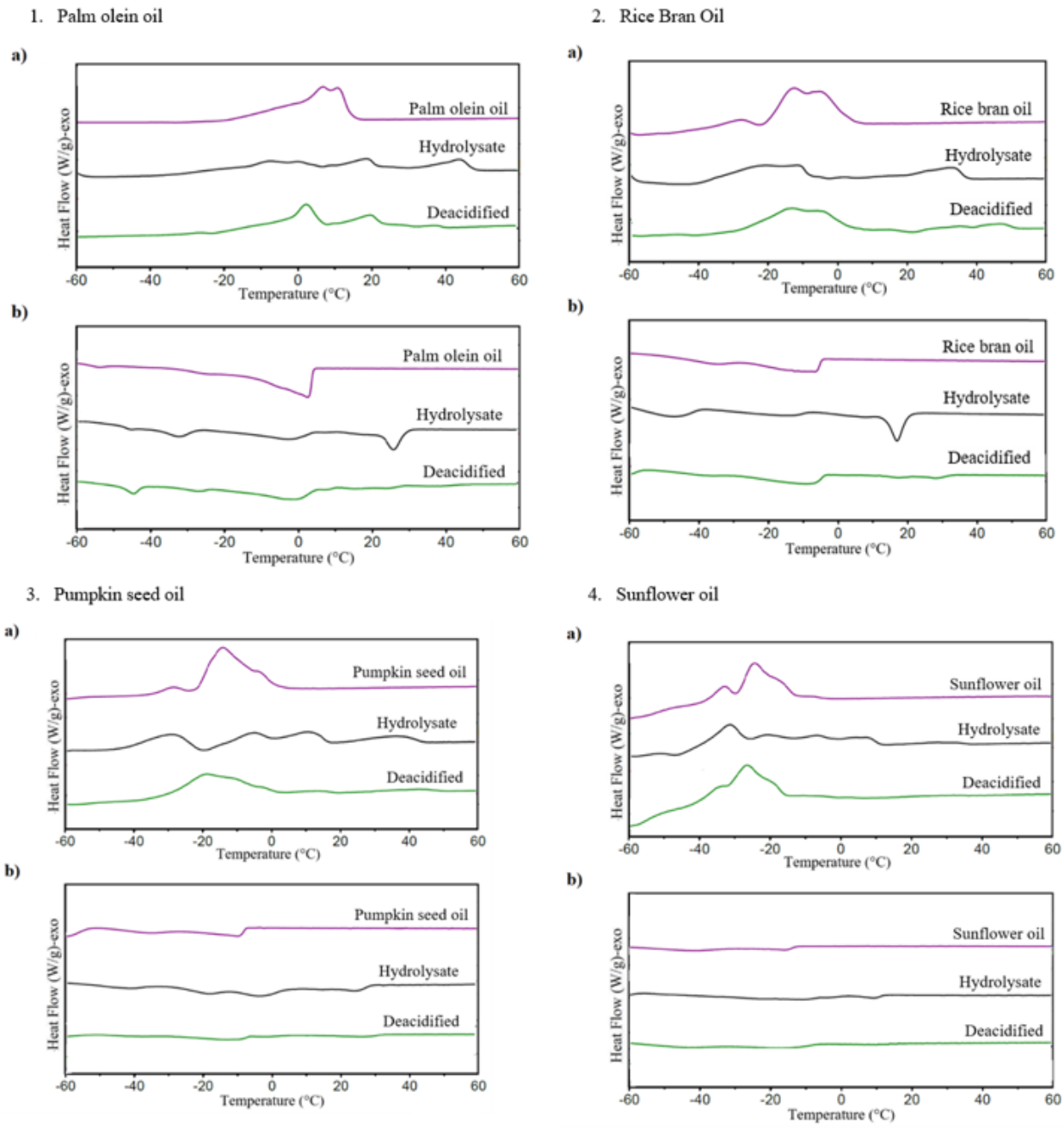

Figure 9. Crystallization (a) and melting (b) behaviors of vegetable oils before and after deacidification.

Specifically, the newly formed acylglycerols, particularly MAGs and DAGs, led to crystallization peaks with an onset occurring approximately 20 to 30 °C higher than that of the unaltered vegetable oils. Among the hydrolysis products, palm olein exhibited the highest maximum crystallization onset temperatures, followed by rice bran oil and pumpkin seed oil hydrolyzed samples. For instance, in the case of palm olein, a new crystallization peak was

generated at higher temperatures, between 35-45 °C. This can be attributed to the selective release of saturated fatty acids, such as palmitic acid and stearic acid, from the oil by Lipase DF.

Similarly, the hydrolysis products of rice bran oil, pumpkin seed oil, and sunflower oil also showed new crystallization peaks at higher temperatures, around 25-40 °C, 20-45 °C, and 0-10 °C, respectively. Additionally, there were shifts or widening in the bulk crystallization or melting peaks for all samples, with a tendency towards higher temperatures. Interestingly, despite the use of different types of vegetable oils, a consistent trend in the change of behaviors was observed.

The thermal behavior of the rice bran oil hydrolysis samples resembled that of the pumpkin seed oil hydrolysis product, although the high-temperature peaks of pumpkin seed oil were slightly shifted to lower temperatures, likely due to a slightly lower saturated fatty acid level. On the other hand, the hydrolysis products of sunflower oil showed crystallization peaks at lower temperatures compared to other vegetable oils, which could be attributed to its lower saturated fatty acid content, the lowest among the oils studied. Additionally, an extra peak appeared at lower temperatures, possibly due to the presence of unsaturated FFAs or highly unsaturated acylglycerols (Humphrey & Narine, 2004; Moorthy, 2018; Narine & Humphrey, 2004).

The data obtained in this study exhibited some similarities to the fats obtained in the study conducted by Nicholson and Marangoni (2021) through glycerolysis of different vegetable oils. However, the changes observed in this study were less pronounced due to the presence of much lower levels of MAGs. Figure 9 also illustrates the thermal properties of the obtained fats after the alkali deacidification reaction. As expected, the crystallization peaks were shifted to lower temperatures, resembling the initial oils, with some small peaks remaining at higher

temperatures. This shift can be attributed to the loss of partial lipids, particularly DAGs and MAGs, during deacidification (Nicholson & Marangoni, 2020, 2021, 2022).

Furthermore, except for deacidified sunflower oil, a loss of SFAs was observed in all vegetable oils after deacidification, which could also partly explain the lower temperature peaks in different deacidified vegetable oils. This finding further supports the notion that alkali refining through saponification reaction, although effective in reducing FFAs, is not an ideal method due to the high loss of partial acylglycerols (Liu *et al*., 2023). Consequently, alkali refining should only be applied to crude oils with low FFAs content to minimize the loss of partial acylglycerols.

Overall, the study reveals important insights into the effects of hydrolysis and deacidification on the thermal properties of the obtained fats, highlighting the significance of MAGs and DAGs in the crystallization behavior of the structured fats (Liu *et al*., 2023).

5.1.5.5.Rheological and textural properties

5.1.5.5.1. Strain sweep analysis

At small or linear reversible deformations, fats exhibit viscoelastic solid behavior, with G' and G" values remaining relatively constant. However, as the deformation increases beyond a critical point, fats undergo nonlinear and irreversible deformations, behaving as viscoelastoplastic materials. Beyond this critical point, G' and G" values start to decline with the strain growth, and the fats exhibit rich nonlinear behavior, including softening and thixotropy, which is important to consider in all processing and end-use applications involving nonlinear flows (Garcia-Macias *et al.,* 2012; Macias-Rodriguez, 2019; Macias-Rodriguez *et al.,* 2018; Macias-Rodriguez & Marangoni, 2016b, 2016a, 2018). Figure 10 displays the rheological behaviors of various treated oils.

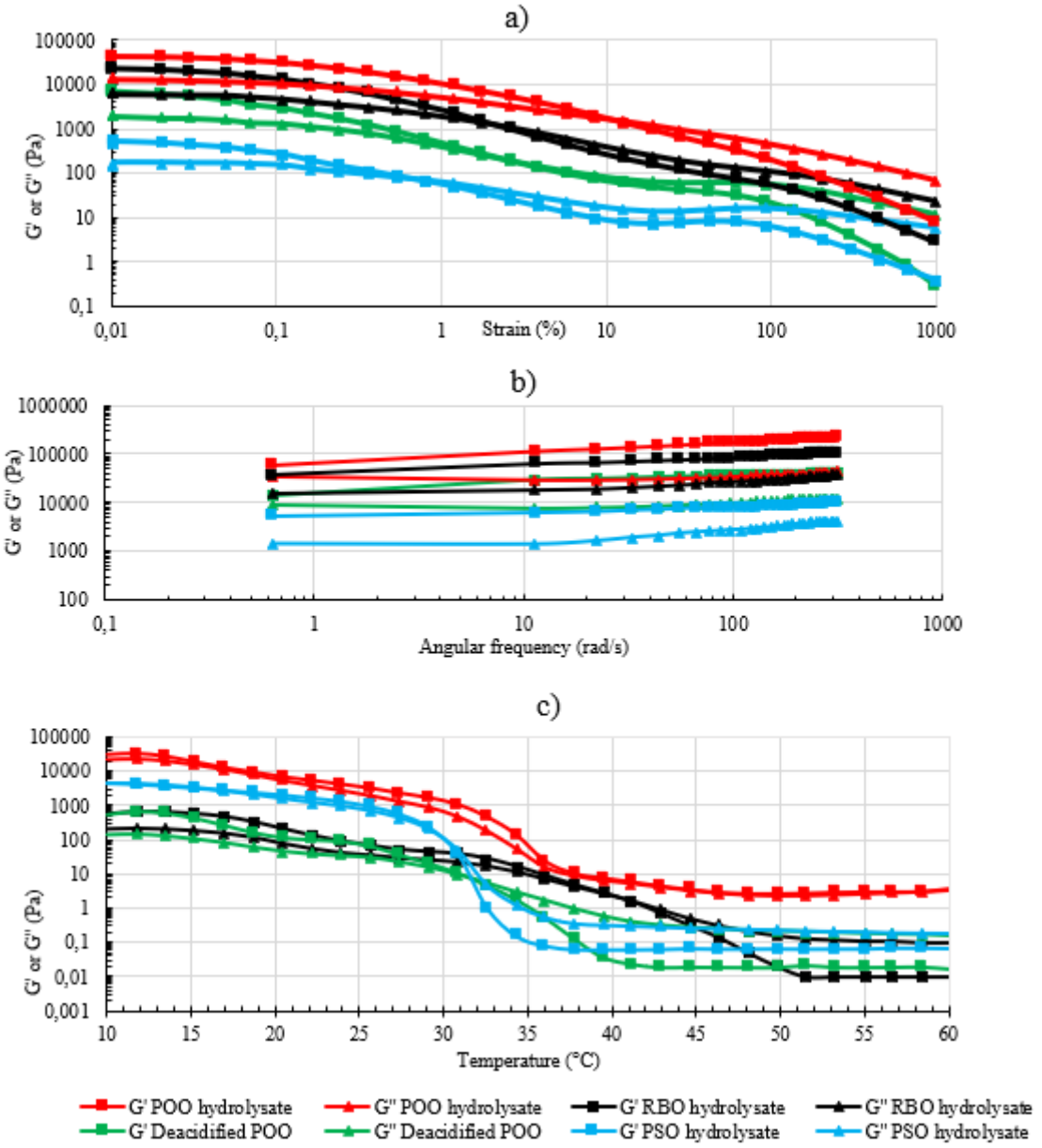

Figure 10. Strain sweep (a), frequency sweep (b) and temperature sweep (c) of samples.

Note that sunflower oil samples, and saponified PSO was too weak to measure the rheological values. (PSO: pumpkin seed oil, RBO: rice bran oil, POO: palm olein oil).

The behaviors of hydrolysis systems for pumpkin seed oil and rice bran oil show close similarities, which can be attributed to their similar SFAs contents and acylglycerol compositions. In Figure 10.a, the G' values were higher than the G" values before the crossover points in all target products, indicating their solid-like behavior in the linear viscoelastic region. However, at strains between 1% to 10%, all target products exhibited a decline in G' and G", with pumpkin seed oil showing this behavior at around 1% strain, palm olein at approximately 10% strain, and rice bran oil sample falling in between. Moreover, there was a sharp drop in G' at high strains, indicating the fats' great spreadability properties (Naeli *et al.,* 2022).

It is worth mentioning that treated sunflower oil, deacidified PSO, and deacidified RBO samples were too weak to measure rheological values. Table 11 presents the G'LVR and γLVR values, as well as the flow points (G'=G"), which were compared for the different samples. The γLVR values of the fats were similar across all samples, indicating consistent viscoelastic behaviors and structural properties at comparable strain values. This suggests that these fats have the potential to maintain their structures as solids and exhibit similar endurance under strain sweeps. Additionally, the G'LVR values of the fats showed an increase with the rise of SFAs in the oils. However, after alkali deacidification for all vegetable oils, the G'LVR values decreased. This observation aligns with the acylglycerol compositions, which revealed a loss of MAGs and a reduction in SFAs in the deacidified samples.

Table 11. Rheological and textural properties of treated oils and deacidified products after 2 h of hydrolysis.

Parameters		SFO	PSO	RBO	POO
Rheological properties:					
Strain sweep:					
	Hydrolyzed product				
	γ_{LVR} (%)	NS	0.04±0.03 [a]	0.01±0.00 [a]	0.03±0.02 [a]
	G'_{LVR} (Pa)	NS	443.76±274.24 [a]	23226.00±552.84 [b]	41363.71±1964.74 [c]
	G'=G" (Pa)	NS	99.71±38.45 [a]	1112.53±82.22 [b]	2028.07±118.80 [c]
	$\gamma_{crosspoint}$ (%)	NS	0.66±0.22 [a]	4.33±1.39 [b]	14.67±2.14 [c]
	Deacidified product				
	γ_{LVR} (%)	NS	NS	NS	0.01±0.00
	G'_{LVR} (Pa)	NS	NS	NS	6918.07±420.74
	G'=G" (Pa)	NS	NS	NS	235.63±12.32
	$\gamma_{crosspoint}$ (%)	NS	NS	NS	5.65±0.06
Frequency sweep:					
(Power Law model: $G'=a\omega^b$)					
	Hydrolyzed product				
	a (Pa.s)	NS	4743.47±198.60 [a]	39976±8322.16 [b]	42131.33±8322.16 [b]
	b	NS	0.12±0.02 [a]	0.16±0.05 [ab]	0.27±0.08 [b]
	R^2	NS	0.90±0.01 [a]	0.93±0.03 [a]	0.95±0.04 [a]
	Deacidified product				
	a (Pa.s)	NS	NS	NS	17541.00±3267.02
	b	NS	NS	NS	0.16±0.04
	R^2	NS	NS	NS	0.94±0.06
Hardness (N):					
	Hydrolyzed product	NS	3.13±0.45 [a]	6.65±0.64 [b]	8.48±0.32 [c]
	Deacidified product	NS	NS	NS	5.22±0.97

SFO: sunflower oil, PSO: pumpkin seed oil, RBO: rice bran oil, POO: palm olein oil. NS: Not studied (note that the NS fats were too weak to measure the firmness and rheological values. a-d, Means with different letters in each row are significantly different ($P<0.05$).

5.1.5.5.2. Frequency sweep analysis

Frequency sweeps are used to study the time-dependent rheological characteristics of fats under non-destructive deformation ranges. In this test, high frequencies simulate fast motions on short time scales, while low frequencies mimic slow motions over longer time scales or during resting. Figure 10b illustrates the frequency sweep rheograms of the target products. During the frequency sweep test, the samples displayed a solid-like (elastic) behavior, as evidenced by their higher G' values compared to G" values. As the frequency increased, both G' and G" values shifted to higher levels, a common observation in lipid systems and weak gels.

Moreover, the values of the power law model, as shown in Table 11, provide insights into the relationship between G' and the frequency function. In the power-law model equation (G' =

aω^b), 'a' represents the magnitude of G', and 'b' characterizes the slope of G' against frequency rheograms. A 'b' value close to 1 indicates viscous properties, whereas a value closer to 0 indicates higher elasticity of the tested materials (Naeli *et al.,* 2022). In this study, the 'b' values ranged from 0.10 to 0.17, indicating the similar elastic properties and frequency-independent behaviors of the tested fats.

5.1.5.5.3. Temperature sweep analysis

An oscillatory temperature sweep was conducted to determine the point at which the oils exhibit more liquid-like behavior. During each individual test interval, both amplitude and frequency were kept constant. As a result, the temperature-dependent functions of G' and G'' were analyzed in this test (Naeli *et al.,* 2022). The temperature sweep analysis graphs are depicted in Figure 10c. As the oils were heated, both G' and G'' values decreased, indicating a transition of fat properties from solid-like to liquid-like over a specific temperature range. This transition is characterized by a reduction in elastic behavior and an increase in viscous behavior.

For instance, in the case of palm olein hydrolysate, the G' values were slightly higher than the G'' values throughout the entire experiment. On the other hand, for pumpkin seed oil hydrolysate, G'' values exceeded G' values at temperatures above 30°C. Similarly, for deacidified palm olein and rice bran oil hydrolysates, G'' values were higher than G' values at temperatures above 40°C. Notably, rice bran oil displayed solid-like behavior during the temperature sweep.

5.1.5.5.4. Texture

Finally, the hardness of the treated fats was examined and correlated with their rheological behaviors. The hardness of fats is influenced by various factors, including their levels of SFAs, acylglycerol composition, storage conditions, and structural characteristics. The hardness

values of the hydrolysates are provided in Table 11. Consistent with the rheological properties, lower saturation levels resulted in lower hardness of the samples. Among the tested products, the palm olein hydrolysate exhibited the highest hardness values, followed by the rice bran oil hydrolysate and the deacidified palm olein sample. The pumpkin seed oil hydrolysate, as well as the deacidified pumpkin seed and deacidified rice bran oil, were too weak to measure hardness, similar to the inability to measure their rheological values. This experimental analysis further corroborated the findings obtained from the acylglycerol composition, differential scanning calorimetry (DSC) measurements, and rheological measurements (Naeli *et al.,* 2022).

5.2. Section 2 (application of enzymatic reaction to synthesis alkyl rosmarinates after assessment of their antioxidant activity)

The purpose of this section was to study the antioxidant activity of alkyl rosmarinates (methyl and ethyl rosmarinates) compared to rosmarinic acid through radical scavenging activity (*in vitro*) and in various food systems, including bulk oil, structured oil with MAGs, oleogel, O/W emulsion, and gelled O/W emulsion under accelerated oxidation conditions at 35°C in one month. Additionally, the article examines the "Polar Paradox Hypothesis" and "cut-off Effect" by testing the antioxidant effects of the modified antioxidants in both oil systems and emulsions. The research also includes the enzymatic synthesis of alkyl rosmarinates using ethyl rosmarinate as a model and aims to optimize the reaction conditions to achieve the highest yield of ethyl rosmarinate.

5.2.1. Antioxidant activity measurements

5.2.1.1. *In vitro* antioxidant activity of rosmarinic acid and alkyl rosmarinates

In vitro antioxidant activity of rosmarinic acid, methyl rosmarinate, and ethyl rosmarinate are shown in Figure 11. All *in vitro* antioxidant activity measurement results were reasonably comparable with each other. For DPPH assay, rosmarinic acid illustrated high DPPH radical scavenging activities with the IC_{50} value of 10.09±0.92 μmol/L, followed by methyl rosmarinates with IC_{50} value of 12.5±1.43 μmol/L, and ethyl rosmarinate with IC_{50} value of 16.5±1.60 μmol/L (Figure 11.a). As for FRAP, rosmarinic acid also demonstrated much higher ferric ion-reducing activities with the value of 643.87±1.58 (μmol Fe2+/100 g) in comparison to 245.38±2.47 (μmol Fe^{2+}/100 g) for methyl rosmarinate, and 194.45±2.60 (μmol Fe2+/100 g) for ethyl rosmarinate (Figure. 11.b). Likewise, for ABTS assay, rosmarinic acid presented the highest ABTS radical scavenging activities with the IC50 value of 8.15±0.17 followed by methyl rosmarinates with IC50 value of 9.52±0.23 μmol/L, and ethyl rosmarinate with IC50 value of 15.56±0.25 μmol/L (Figure 11.c).

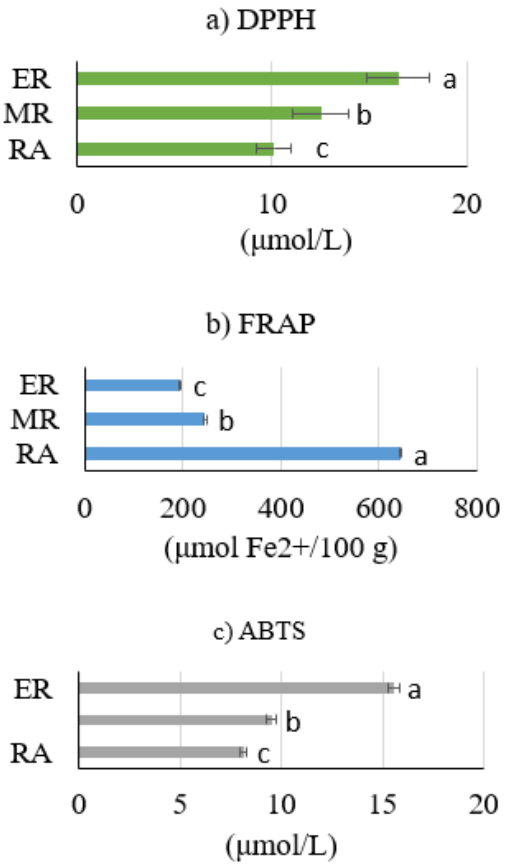

Figure 11. *In vitro* antioxidant activity measurements.

Ethyl rosmarinate (ER), methyl rosmarinate (MR), and rosmarinic acid (RA).

Similar results for rosmarinic acid, methyl rosmarinate, and butyl rosmarinate have been reported by Aihaiti *et al*. (2022) isolated from non-volatile compounds of *Hyssopus cuspidatus Bori ss*. In their study, systematic separation and purification of compounds led to the isolation of these three rosmarinic acid derivatives among 34 bioactive compounds with the highest antioxidant activity. In another similar study, Yaermaimaiti *et al.,* (2021) reported that the antioxidant activity against DPPH, ABTS, and FRAP exhibited by these compounds surpassed not only that of positive controls but also demonstrated greater significance compared to other tested phenolic compounds utilizing the same assays (Yaermaimaiti *et al.,* 2021). Lacomte *et al*. (2010) conducted a study where they achieved the hydrophobation of rosmarinic acid using saturated aliphatic primary alcohols with varying chain lengths, ranging from methanol to eicosanol. This modification was accomplished through an acid-catalyzed esterification

process performed in the presence of a highly acidic sulfonic resin. The researchers successfully isolated and characterized the resulting alkyl rosmarinates. To evaluate their antioxidant properties, they determined their global free radical scavenging activity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method under stationary state conditions. Their findings revealed that among the alkyl rosmarinates tested, only the dodecyl ester exhibited stronger antioxidant activity than pure rosmarinic acid (Lecomte *et al.,* 2010). Differences in the reported results of *in vitro* antioxidant activities for alkyl rosmarinates with varying chain lengths can be attributed to several key factors. First, the length of the alkyl chain plays a significant role, with longer chains enhancing lipophilic properties and potentially improving or reducing solubility. Solubility can affect their availability in antioxidant reactions and result in lower measured activity in specific assays. Furthermore, variations in the choice of antioxidant assay, concentration of alkyl rosmarinates, and sample purity can all influence the reported outcomes (Lecomte *et al.,* 2010; Lee *et al.,* 2013; López Giraldo *et al.,* 2007).

5.2.1.2. Antioxidant activity in oil-based model food systems: hydrophobicity of antioxidant versus effect of food matrix

In general, oxidation is triggered when a hydrogen atom attached to an allylic or bis-allylic carbon atom (carbon adjacent to a double bond) is removed, primarily because of its low dissociation energy. Once the hydrogen atom is detached, the resulting alkyl radical gains stability through resonance delocalization. This process may also lead to the formation of trans isomers and conjugated dienes, as seen in polyunsaturated fatty acids. Conjugated dienes are produced when unsaturated fatty acids, comprising two or more double bonds are oxidized, reaching a more stable radical (Mardani *et al.,* 2023). Conjugated diene formation of samples kept at 35 °C on days 0, 10, 20, and 30 are illustrated in Table 12. In addition, the measurement of the p-anisidine value was utilized to assess the presence of secondary oxidation products

(Table 12), demonstrating compounds, such as aldehydes, ketones, alcohols, and hydrocarbons, as a result of the breakdown of conjugated dienes (Mardani *et al.,* 2023).

The oils without and with rosmarinic acid and alkyl rosmarinates showed a comparable lag phase of 20 days. The control oils (with no antioxidant or with BHT) showed a comparable lag phase of 20 days. On day 30, all samples saw a dramatic change in the creation of conjugated dienes and p-anisidine values. However, this change was less visible for oils treated with antioxidants in comparison to the control sample in all food systems.

In the case of bulk oil, although rosmarinic acid caused resistance to oxidation, ethyl rosmarinate, and methyl rosmarinate demonstrated considerably better antioxidant efficiency than rosmarinic acid. In this case, a linear trend was seen in the bulk oil food matrix when assessing both conjugated dienes and p-anisidine value. This finding goes against the polar paradox theory, as it demonstrates that polar antioxidants are typically more effective than nonpolar antioxidants in bulk oil systems. The reason behind this probably lies in the possibility that ethyl and methyl rosmarinates are more accessible where oxidation takes place. Nevertheless, numerous researches have shown that not all antioxidants conform to the phenomenon justified by the polar paradox (Laguerre *et al.,* 2015).

Table 12. Conjugated dienes formation and p-anisidine values of stripped sunflower oil kept at 35 °C.

	Conjugated dienes formation				p-Anisidine values			
Sample types	Day 0	Day 10	Day 20	Day 30	Day 0	Day 10	Day 20	Day 30
Bulk oil								
Control	0.31±0.02 [a]	0.53±0.08 [a]	0.88±0.06 [a]	4.87±0.01 [a]	13.11±1.25 [a]	28.42±0.32 [a]	43.23±0.33 [a]	138.32±1.34 [a]
BHT	0.33±0.05 [a]	0.41±0.03 [b]	0.68±0.03 [bc]	3.14±0.04 [b]	12.94±1.48 [a]	13.56±1.38 [bc]	22.48±1.57 [bc]	98.54±2.37 [b]
RA	0.32±0.03 [a]	0.39±0.02 [b]	0.74±0.01 [b]	3.12±0.02 [b]	13.20±2.05 [a]	17.52±1.09 [b]	25.65±0.26 [b]	98.81±0.37 [b]
MR	0.31±0.01 [a]	0.42±0.02 [b]	0.70±0.02 [bc]	2.98±0.03 [c]	12.88±2.51 [a]	14.30±2.90 [bc]	24.33±2.03 [bc]	92.17±1.43 [c]
ER	0.34±0.04 [a]	0.46±0.01 [ab]	0.63±0.04 [c]	2.60±0.05 [d]	12.54±1.35 [a]	12.84±1.10 [c]	21.22±1.83 [c]	87.52±1.50 [d]
MAG-shortening								
Control	0.34±0.02 [a]	0.59±0.02 [a]	0.97±0.05 [a]	5.32±0.03 [a]	14.85±1.10 [a]	29.55±0.26 [a]	45.42±0.74 [a]	142.67±1.95 [a]
BHT	0.34±0.05 [a]	0.46±0.02 [b]	0.78±0.02 [b]	4.07±0.02 [c]	14.53±1.52 [a]	17.27±2.14 [bc]	37.23±1.27 [b]	115.22±2.20 [b]
RA	0.32±0.05 [a]	0.42±0.03 [b]	0.89±0.02 [a]	4.23±0.05 [b]	14.69±1.65 [a]	19.34±1.48 [b]	32.45±2.85 [bc]	113.34±3.54 [b]
MR	0.35±0.08 [a]	0.48±0.01 [b]	0.76±0.05 [b]	3.85±0.06 [d]	14.15±1.54 [a]	14.11±1.19 [c]	28.51±2.15 [c]	101.34±3.04 [c]
ER	0.34±0.03 [a]	0.45±0.05 [b]	0.79±0.02 [b]	4.02±0.09 [c]	14.42±1.13 [a]	16.38±2.30 [bc]	36.48±1.44 [b]	114.65±2.15 [b]
EC Oleogel								
Control	0.35±0.01 [a]	0.57±0.01 [a]	0.91±0.02 [a]	5.11±0.05 [a]	14.22±1.37 [a]	29.67±1.80 [a]	44.34±1.30 [a]	141.23±1.35 [a]
BHT	0.35±0.05 [a]	0.42±0.02 [bc]	0.82±0.04 [ab]	4.20±0.02 [b]	14.35±1.58 [a]	18.83±1.39 [b]	31.24±1.49 [b]	105.12±1.50 [b]
RA	0.31±0.06 [a]	0.43±0.03 [b]	0.65±0.04 [c]	2.75±0.04 [e]	14.11±2.09 [a]	12.23±2.56 [c]	22.14±1.27 [d]	89.75±1.14 [e]
MR	0.34±0.02 [a]	0.38±0.01 [c]	0.78±0.05 [b]	3.27±0.08 [c]	14.61±1.45 [a]	17.68±1.14 [b]	27.03±1.45 [c]	95.68±1.56 [d]
ER	0.32±0.05 [a]	0.40±0.01 [bc]	0.76±0.03 [b]	3.06±0.06 [d]	14.53±1.86 [a]	14.91±1.68 [bc]	25.48±1.38 [cd]	100.84±1.75 [c]
O/W emulsion								
Control	0.33±0.05 [a]	0.55±0.03 [a]	0.87±0.02 [a]	4.86±0.05 [a]	14.94±1.33 [a]	28.93±0.54 [a]	48.43±1.34 [a]	148.21±3.64 [a]
BHT	0.35±0.01 [a]	0.46±0.01 [b]	0.82±0.05 [ab]	4.61±0.03 [b]	15.30±1.04 [a]	24.42±1.25 [b]	46.12±1.34 [ab]	145.54±2.72 [ab]
RA	0.31±0.05 [a]	0.38±0.00 [c]	0.78±0.02 [b]	3.87±0.04 [d]	15.03±1.85 [a]	19.04±0.83 [c]	32.20±0.81 [d]	130.35±1.50 [c]
MR	0.32±0.04 [a]	0.45±0.04 [b]	0.75±0.04 [b]	4.21±0.02 [c]	14.50±1.55 [a]	18.21±1.70 [c]	37.51±0.26 [c]	139.54±1.66 [b]
ER	0.35±0.02 [a]	0.44±0.01 [bc]	0.74±0.01 [b]	4.66±0.02 [b]	15.21±1.70 [a]	22.56±1.20 [b]	45.41±0.45 [b]	141.13±1.92 [b]
O/W emulsion gel								
Control	0.33±0.01 [a]	0.54±0.05 [b]	0.88±0.05 [ab]	4.97±0.05 [a]	14.56±0.65 [a]	28.54±0.52 [a]	46.45±1.32 [a]	140.55±1.65 [a]
BHT	0.33±0.02 [a]	0.53±0.02 [b]	0.83±0.02 [b]	4.03±0.05 [c]	14.50±0.80 [a]	24.15±1.32 [bc]	38.50±1.12 [b]	128.54±1.50 [b]
RA	0.31±0.02 [a]	0.46±0.01 [c]	0.46±0.03 [d]	3.54±0.02 [e]	14.75±2.63 [a]	18.30±0..50 [c]	28.43±1.78 [c]	122.69±1.78 [c]
MR	0.34±0.05 [a]	0.52±0.01 [bc]	0.55±0.01 [c]	3.68±0.01 [d]	14.55±0.60 [a]	21.20±0.20 [bc]	31.21±0.22 [c]	127.53±1.72 [b]
ER	0.34±0.02 [a]	0.72±0.01 [a]	0.92±0.02 [a]	4.87±0.03 [b]	14.93±3.54 [a]	21.50±1.94 [bc]	44.20±1.50 [a]	131.26±1.34 [b]

*Numbers in the same columns for each sample type with different letters are significantly different ($p < 0.05$). RA: rosmarinic acid, MR: methyl rosmarinate, ER: ethyl rosmarinate, EC: Ethyl cellulose, MAG: Monoacylglycerol, BHT: Butylated hydroxytoluene. Controls including samples with no added antioxidant and samples containing BHT at the maximum legally allowed concentration of 200 ppm (0.9076 mM) were employed in the study.

Torres de Pinedo *et al*. (2007) studied the potency of phenolic compounds with different hydrophobic groups as antioxidants in food systems assessed in refined olive oil employing the Rancimat method. They reported a rise in antioxidant efficiency in the bulk oil, and the order of efficiency was found to be dihydrocaffeoyl alcohol > hydroxytyrosol > protocatechuic alcohol, thereby raising questions about the conventional polar theory. They stated that boosting antioxidant capacity is largely influenced by an increase in the number of phenolic hydroxy groups and the presence of a primary alcohol. The polar paradox fails to elucidate the observed antioxidant capacity for these novel phenolic antioxidants (Torres de Pinedo *et al.,* 2007).

In another study, Oh and Shahidi (2022) showcased the application of lipophilized resveratrol derivatives in food systems for the regulation of oxidative processes. They reported that several derivatives exhibited notably superior antioxidant activity compared to resveratrol itself ($p < 0.05$). Their findings also challenged the polar paradox theory, which posits that polar antioxidants are typically more effective than nonpolar antioxidants in bulk oil systems.

On the other hand, in the case of structured fats with MAGs, methyl rosmarinate proved the most effective antioxidant among all samples ($p < 0.05$). This observation is associated with the phenomenon coined as the nonlinear or cutoff effect, which has been reported in various studies involving emulsions and other systems. In a similar study, Laguerre *et al*. (2009) investigated the antioxidant efficiencies of chlorogenic acid and its alkyl esters in emulsion food systems to explore the impact of chain length on their effectiveness. They noted a significant decline in antioxidant efficiency beyond the dodecyl chain. Similarly, Locatelli *et al*. (2008) reported a cutoff effect in relation to the cytotoxic efficiency on L1210 leukemia cells (Locatelli *et al.,* 2008). In their study, a connection between the cytotoxic effect and a limited degree of lipophilicity was seen. They reported that carbon chain lengths of 8 to 12

exhibited higher cytotoxic activity compared to other chain lengths. Moreover, ethyl rosmarinates have been reported to possess the highest inhibitory activity against lung inflammation in comparison to rosmarinic acid or any other rosmarinate esters which strongly endorsed the advancement of this compound as an innovative therapeutic agent for addressing inflammatory lung diseases mediated by macrophages (Thammason *et al.,* 2018).

Finally, in the case of ethyl cellulose oleogel, emulsion, and emulsion gels, rosmarinic acid displayed the strongest antioxidant efficiency ($p < 0.05$). The observed antioxidant efficiency of rosmarinic acid derivatives in the O/W emulsions (gel and non-gelled) did not align with the polar paradox. Based on the polar paradox theory, nonpolar antioxidants are expected to outperform polar antioxidants in oil-in-water emulsions. This was attributed to the tendency of nonpolar antioxidants to remain near the lipid droplets where oxidation takes place (Laguerre *et al.,* 2015; Laguerre, Sørensen, *et al.,* 2013; Lee *et al.,* 2013). Nevertheless, the results of this study contradict this expectation. In a similar study, Oh and Shahidi (2022) conducted a study where they modified resveratrol to make it lipophilic through esterification and potentially boost its bioactivity. They prepared twelve resveratrol derivatives by employing acyl chlorides of varying chain lengths (C3:0–C22:6) and subsequently evaluated their antioxidant efficiencies. They reported that resveratrol demonstrated the strongest antioxidant efficiency in oil-in-water emulsion in comparison to its derivatives. Costa *et al*. (2013) found that olive oil-in-water nanoemulsion samples containing lauryl gallate demonstrated superior oxidative stability compared to samples containing gallic acid, methyl gallate, and propyl gallate. The researchers reported that the percentage of lauryl gallate in the interfacial region of the olive oil-in-water nanoemulsion was higher than that of gallic acid, methyl gallate, and propyl gallate (Costa *et al.,* 2013).

Furthermore, the emulsifier type and the existence of naturally occurring antioxidants can influence both the overall oxidative stability and the efficiency of added antioxidants, as demonstrated in earlier studies (Sørensen *et al.,* 2017). They evaluated the ability of caffeic acid and various caffeates with different alkyl chain lengths to retard oxidations in various emulsion-based food systems, certain samples were using Tween 80 for stabilization, while others employed CITREM, with and without the existence of tocopherol. They reported that caffeic acid demonstrated the highest antioxidant activity in both emulsions with the existence of tocopherol. However, caffeic acid was shown to be a prooxidant in the Tween-stabilized emulsion system, while the caffeates exhibited high antioxidant properties without the presence of tocopherol. Therefore, multiple factors influence the oxidation reactions in O/W emulsion systems, including oil volume fraction, type of homogenizer, droplet sizes, viscosity, surface charges, pH, interface structure (like thickness and permeability), type of emulsifiers, and the existence or incorporation of different antioxidative agents (Ghelichi *et al.,* 2023).

In addition, Stöckmann *et al*. (2000) delved into the significance of the emulsifier's nature in emulsions. In their study, they examined the behavior of a series of alkyl gallates (ranging from gallic acid to octyl gallate) with regard to antioxidant activity in O/W emulsions prepared from corn oil. Interestingly, substantial variations in antioxidant activity were observed based on the choice of emulsifier, namely lecithin, Brij 58, and SDS. The researchers reported that distinct molecular interfaces occurring among antioxidative agents and emulsifiers were responsible for these discrepancies apparent in various emulsions. Their findings suggested that these connections were probably between the antioxidants and the emulsifier's headgroups, involving potential hydrogen bonds between the hydroxyl group of antioxidants and the emulsifier's charge or alkyl chains of the antioxidants and the lipid chains of the emulsifiers (Stöckmann *et al.,* 2000). Such interactions are believed to influence the diffusions of the antioxidants within the emulsion matrix. Various interpretations have been put forth to account for differences in

the effect of chain length on the nonlinear impact on the efficacy of antioxidants, including partitioning factors, reduced mobility, self-aggregation of antioxidants with long chains, and surface activity characteristics of the antioxidants (González *et al.,* 2015).

5.2.2. Synthesis of ethyl rosmarinate and optimization of the reaction conditions

5.2.2.1.Enzymatic synthesis using ethyl rosmarinate as a model

Figure 12 shows the change in the structure of rosmarinic acid to produce the ethyl rosmarinate by releasing a water molecule which was absorbed by molecular sieves present in the reaction vials to increase the rate of esterification reaction as opposed to hydrolysis reactions.

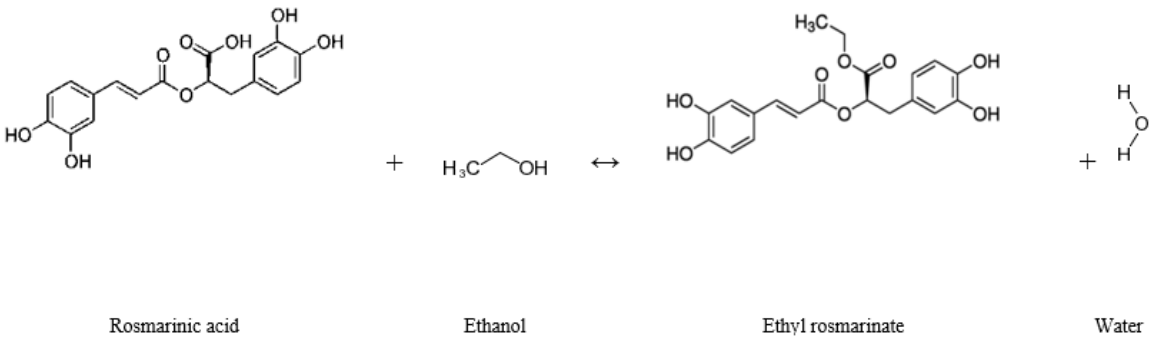

Figure 12. Enzymatic production of ethyl rosmarinates.

The presence of ethyl rosmarinate was confirmed with different detectors before determining ethyl rosmarinate's conversion yield with UV detector determined as the area percentage of product peaks (Figure 13).

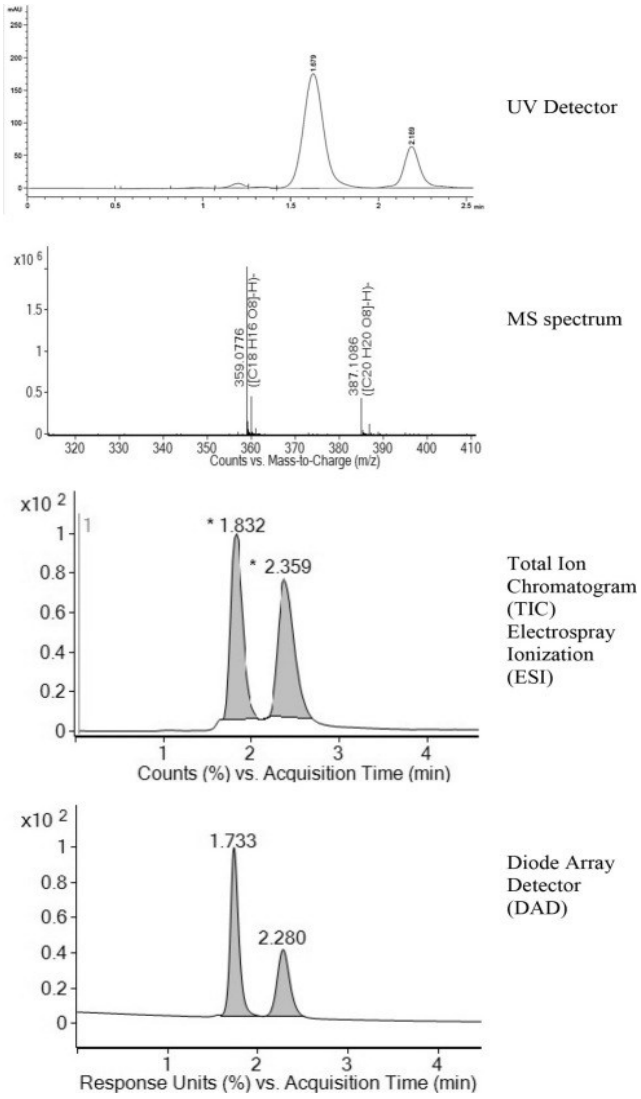

Figure 13. Chromatographic presentation of rosmarinic acid (1st peak) and ethyl rosmarinate (2nd peak).

The fragmentation of rosmarinic acid molecular ions (358.97) produced two peaks at 179 and 161 (m/z) relating to the dehydrated and deprotonated form of 3-(3,4-dihydroxyphenyl) lactic and caffeic acids. In the case of ethyl rosmarinate, apart from the molecular ion of the ester, fragmentation of molecular ions produced 2 peaks at 179 and 135 (m/z) which can correspond to the deprotonated isomer of caffeic acid and its residues. The dissimilarities in rosmarinic acid and ethyl rosmarinate fragmentations could potentially be attributed to variations in the strength of various ester bonds. Consequently, when the molecular ion of ethyl rosmarinate is fragmented, it theoretically results in the cleavage of the comparatively vulnerable ester bonds

into the deprotonated structure of caffeic acid and 3-(3,4-dihydroxyphenyl) lactates. The findings aligned with the fragmentations suggested by other scientists (Lee *et al.,* 2013; Møller *et al.,* 2007).

5.2.2.2.Optimization of ethyl rosmarinate synthesis

In enzyme-catalyzed reactions, the most critical obstacle in reaching higher production yields within the shortest timeframe is optimizing the reaction circumstances. Numerous factors need to be taken into account, including the nature and quantity of acyl acceptors and donors, the enzymes utilized, the presence or absence of a solvent, reaction time, water activity in the system, temperature, agitation levels, and the use of molecular sieves for water adsorption (Peng *et al.,* 2023). As a result, it is essential to optimize these factors to enhance yield and reduce waste generation. RSM, a commonly employed statistical method in the optimization of enzymatic reactions, facilitates the evaluation of interactions among all reaction parameters, ultimately leading to achieving the uppermost product conversions and the most cost-effective and feasible manufacturing process (Mardani *et al.,* 2022).

Experimental values regarding the optimization of ethyl rosmarinate yield with a UV detector on the conversion of ethyl rosmarinates with Lipozyme 435 are shown in Table 13. A Randomized Box Behnken design suggested 27 runs for optimization of reaction conditions. Conversion yield ranged from around 7.28% to 85.59%. Regression coefficients and significance ($p<0.05$) of involved parameters based on a quadratic model involving all parameters are shown in Table 14. The coefficient of determination (R^2) of the model was 0.96, indicating the model is fitting for representing the connection between the parameters of the reaction condition.

Table 13. Experimental design and results of the predicted and experimental responses

Run	Te (°C)	Eth/RA	En (%)	Ti (days)	Conversion (%)
1	40	1	10	3	7.80±0.01*
2	70	3	16	3	82.75±0.04
3	55	3	4	1	44.2±0.01
4	40	3	4	3	7.28±0.01*
5	55	5	4	3	45.97±0.01*
6	55	5	10	1	63.54±0.01
7	55	5	10	5	80.18±0.00
8	70	5	10	3	12.69±0.02*
9	55	1	10	5	52.74±0.00
10	55	1	4	3	37.44±0.00
11	40	3	10	5	62.08±0.02
12	40	3	10	1	59.94±0.01
13	40	3	16	3	65.41±0.01
14	70	3	4	3	38.57±0.01
15	55	3	10	3	70.22±0.03
16	70	3	10	1	58.66±0.01
17	55	3	10	3	77.09±0.00
18	55	1	10	1	49.58±0.01
19	55	5	16	3	85.59±0.01
20	55	3	16	1	71.13±0.00
21	55	1	16	3	54.75±0.01
22	55	3	16	5	82.79±0.01
23	40	5	10	3	57.65±0.01
24	70	1	10	3	54.66±0.01
25	70	3	10	5	75.07±0.01
26	55	3	4	5	47.71±0.00
27	55	3	10	3	73.28±0.03

Te (temperature), Eth (ethanol), RA (Rosmarinic acid), En (enzyme), Ti (time)
*Rows 1, 4, 5, and 8 were outliers and ignored for this analysis.

The probabilities for the regression of the model were shown to be significant ($p < 0.0001$) with the model F-value of 32.24. There is only a 0.01% chance that an F-value this high can happen because of the noise. Additionally, the predicted R^2 of 0.89 was acceptable similarity to the adjusted R^2 of 0.93. Adeq precision was found to be 19.55. The Lack of Fit F-value of 1.16 implied that the Lack of Fit is not significant relative to the pure error (p-value of 0.55) which showed the model was fit (Table 14).

Table 14. Regression coefficients and significance for the production of ethyl rosmarinate.

Variable	SS	df	MS	F-value	p-value
Temperature	113.63	1	113.63	13.29	0.0065
Ethanol/RA	550.93	1	550.93	64.44	< 0.0001
C-Enzyme	1832.59	1	1832.59	214.36	< 0.0001
Time	238.70	1	238.70	27.92	0.0007
Temperature* Ethanol/RA	0.9925	1	0.9925	0.1161	0.7421
Temperature* Enzyme	66.58	1	66.58	7.79	0.0235
Temperature* Time	50.91	1	50.91	5.95	0.0405
Ethanol/RA* Enzyme	112.37	1	112.37	13.14	0.0067
Ethanol/RA* Time	45.43	1	45.43	5.31	0.0501
Enzyme* Time	16.61	1	16.61	1.94	0.2009
Temperature ²	146.89	1	146.89	17.18	0.0032
Ethanol/RA ²	283.29	1	283.29	33.14	0.0004
Enzyme ²	370.80	1	370.80	43.37	0.0002
Time ²	29.25	1	29.25	3.42	0.1015

SS (sum of squares), MS (mean of squares)

Based on Table 14, the insignificant coefficients were excluded and the model was generated as:

Yield of ethyl rosmarinate (%) = -56.77 + 2.62 Temperature + 11.76 (Ethanol/RA) + 2.82 (Enzyme) – 4.31 (Time) + 0.05 (Temperature*Enzyme) + 0.12 (Temperature*Time) + 0.06 (Ethanol/RA*Enzyme) – 0.03 $(\text{Temperature})^2$ – 2.32 $(\text{Ethanol/RA})^2$ – 0.24 $(\text{Enzyme})^2$

(Equation 11)

This equation can be employed to predict the conversion yield of ethyl rosmarinate for different set levels of parameters determining the reaction conditions. A tendency towards the upper limit of conversion yield at intermediate incubation time was seen, however, the reaction conversion still increased gradually till the last day. Although molecular sieves are added, after some time the reaction would likely have a bit tendency towards hydrolysis. According to the model, the predicted ideal parameters for ethyl rosmarinate conversion fell within the studied experimental range. Additional reactions were done according to the two runs offered by the model, and the predicted values with actual values are shown in Table 15.

Table 15. Conditions tested by the model for the conversion of ethyl rosmarinate.

	Run 1	Run 2
Temperature (°C)	55	60
Ethanol/RA	5	3.5
Enzyme (%)	16	10
Time (day)	3	5
Predicted value (%)	85.76	91.01
Experimental value (%)	81.02±0.02	84.04±0.01
Confidence level (95%)	78.90-90.00	81.55-90.01

RA (rosmarinic acid)

The result showed that the experimental values were inside the confidence level of the model (95% confidence level). The desirability was set as the highest amount of conversion yield where all the parameters can be chosen from the range tried by the model. Ultimately, through numerical optimization, it was determined that an ideal approach for synthesizing ethyl rosmarinate could involve different combinations of chosen parameters. Under two specific conditions with the highest desirability, the predicted yield of 85.76 % and 91.01 % was determined. Notably, this closely matched the experimental values of respectively 81.02±0.02 % and 84.02±0.01 %, reinforcing the model's reliability and adequacy by matching the confidence level of the model of 78.90-90.00% and 81.55-90.01%, respectively.

5.2.2.3.Effect of reaction condition on ethyl rosmarinate synthesis

Additionally, the interaction of the parameters on each other and on the conversion rates are shown in contour plots based on the studied model (Figure 14). These plots demonstrate the proportional impact of any two variables while maintaining the other variable(s) at a constant level (central levels based on the model). They are also useful for identifying the optimal values (or minimum levels) of these variables to achieve maximum response.

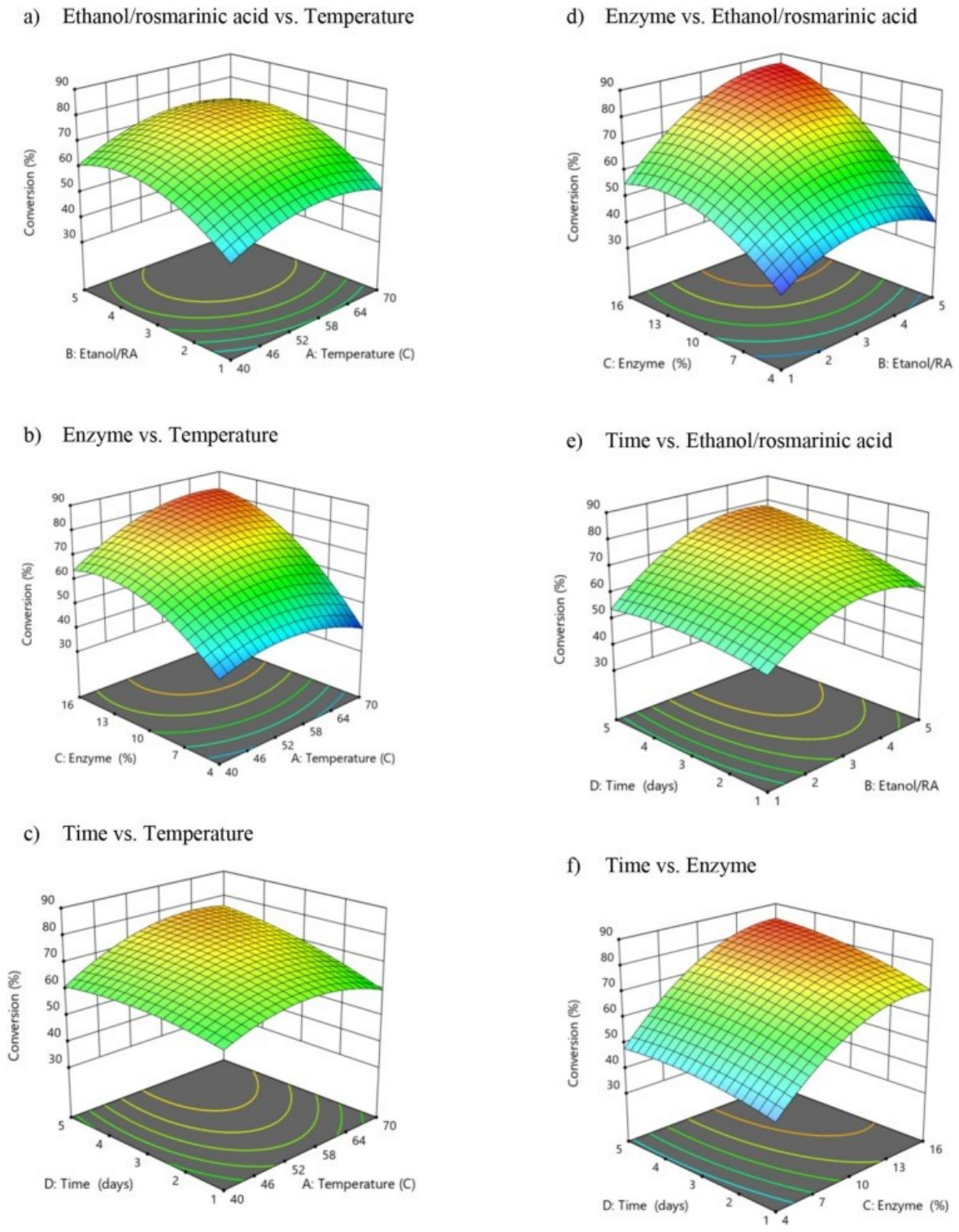

Figure 14. Contour plots of rosmarinic acid conversions to rosmarinate esters.

Fixed factors included time: 3 days, temperature: 50 °C, enzyme-to-substrate ratio: 10 %, and the molar ratio of alcohol to rosmarinic acid: 3.

In this regard, the molar proportions of substrates, which refer to the proportion of acyl donor to acyl acceptor, played a crucial role in determining the conversion yields. Ethanol/RA had

the highest β-coefficients in this study, indicating the highest influence on the efficiency of the enzymatic reaction. Typically, a higher mole ratio (hydrophobic substrates in excess) led to increased acyl incorporation and shorter production times for lipophilized antioxidants. However, it was essential to optimize this ratio to achieve maximum conversion yield while minimizing downstream processing requirements (Yang *et al.,* 2012). In the desirability selection for the model, Ethanol/RA was set as a minimum to reach the highest conversion yield and 3.5 was given as the best proportion. Additionally, when asked to be chosen in the range, the maximum level of 5 was suggested by the model which despite slightly increasing the conversion yield, increases the need for final purification or downstream handling. As shown for the confirmation of the model, increasing the proportion from 3.5 to 5 did not considerably increase the conversion yield. Similar results have been achieved by other scientists on the production of alkyl ferulates (Schär & Nyström, 2015) and esters of dihydrocaffeic acid (Yang *et al.,* 2012).

In terms of enzyme content, the increase of enzyme above around 10% did not seem to considerably change the conversion yield in this study. The highest conversion was seen in run 19 with 16% enzyme load, substrate molar ratio of 5, 55 C, and reaction time of 3 days. Nevertheless, at 10% enzyme load conversion yield of more than 80% was observed in a longer time of 5 days. As the concentration of the enzyme increases, the reaction equilibrium shifts towards synthesis until it reaches a constant conversion yield. However, beyond this point, the effect is very low due to steric hindrance caused by an excessive enzyme load. At this stage, there is an abundant amount of substrate, leading to the saturation of almost all enzyme active sites with substrate molecules. This saturation implies that the enzyme molecules are fully occupied with the substrate (Baadhe *et al*., 2014; Bornscheuer *et al*., 2012; Mardani *et al.,* 2015). As a result, any excess substrate molecules remain inactive until the previously bound substrate on the enzymes undergoes a reaction and is subsequently released (Mardani *et al.,*

2022). Consequently, it was crucial to optimize the enzyme addition to ensure economic efficiency and avoid steric hindrance issues.

The effect of time on enzymatic reactions is intricately linked to the enzyme concentration. A higher enzyme concentration can accelerate reaction rates, particularly in the early stages, ultimately demonstrating the significant influence of enzyme amount on the kinetics of enzymatic reactions. The relationship between time and enzyme concentration can be understood through the Michaelis-Menten kinetics equation, which describes the rate of enzymatic reactions. According to this equation, the initial rate of the reaction is directly proportional to the enzyme concentration (Piazza *et al.,* 2007).

Furthermore, to achieve the best results in synthetic reactions, it is vital to conduct them at the optimum temperature. Higher temperatures (above 64 °C) could lead to slight denaturation of the enzyme structure, while lower temperatures (under 52 °C) could decrease the conversion yield, both leading to reduced enzyme activity. This was in agreement with the optimum temperature recommended by the enzyme manufacturer of 30 to 60 °C. Various researchers have reported optimized temperatures for Novozyme 435 ranging from 30 to 70 °C (Mardani *et al.,* 2022).

6. CONCLUSION AND RECOMMENDATIONS

In this thesis, different vegetable oils with varying levels of saturation were subjected to enzymatic reactions after screening various commercial lipases. The aim was to enrich the acylglycerol content, primarily MAGs, and partially DAGs. Four reaction parameters, including water content, enzyme load, temperature, and time, were adjusted to optimize the MAGs and DAGs contents while minimizing FFAs production. The results led to the identification of conditions for maximizing MAGs through a one-step enzymatic hydrolysis reaction. This method presents a significant advantage over glycerolysis reactions, as it allows for the creation of fats with lower saturation levels, particularly useful for cost-effective vegetable oils such as palm oil. However, it was observed that the deacidification reactions led to a high loss of neutral lipids, indicating that alkali refining through saponification may not be a suitable method due to the considerable loss of partial acylglycerols.

Additionally, in this study, the antioxidant activity of alkyl rosmarinates (methyl and ethyl rosmarinates) were compared with rosmarinic acid through radical scavenging activity (*in vitro*) and in different oil-based food systems (under accelerated oxidation condition at 35 °C). The antioxidant activities resulting from *in vitro* analysis were in line with the antioxidant activity of alkyl rosmarinate and rosmarinic acid in ethyl cellulose oleogel, emulsion, and gelled emulsion systems (based on the most effective followed an order of rosmarinic acid > methyl rosmarinate > ethyl rosmarinate). In structured fat with monoacylglycerol, methyl rosmarinate was shown to be the most effective antioxidant (methyl rosmarinate > ethyl rosmarinate > rosmarinic acid). In both oil-in-water emulsion and bulk oil systems, we noticed a discrepancy with the polar paradox. In addition, following the recognition of the significant role played by the food matrix in influencing the antioxidant activity of rosmarinic acid derivatives, the process of lipophilizing rosmarinic acid with ethanol was fine-tuned using Lipozyme 435 (Novozymes) in hexane as a model system. This optimization effort resulted in

an impressive conversion yield reaching as high as 85.59%. In conclusion, further examinations are essential to assess the potential application of each bioactive compound by the relevant industries. In addition to the challenges discussed, microbiological and toxicological analyses are imperative to guarantee the future safe utilization of these modified phenolics.

7. NEW SCIENTIFIC RESULTS

7.1 New scientific findings on enzymatic reactions to solidify liquid vegetable oils

1) Five types of the enzyme were examined for their MAG-producing ability while keeping FFAs as low as possible in palm olein through partial hydrolysis and their ability to produce MAGs, DAGs, and TAGs was compared. Lipase DF Amano 15 was selected for the optimization studies and extending the experiments to other vegetable oils due to production of 9.75 % MAGs in comparison to 0.64-2.35% produced by other enzymes and partial removal of SFAs in the acylglycerol phase.

2) RSM was employed to model and optimize the reaction conditions for Lipase DF. The main effects and interactions between contributing factors that influence the MAG level in hydrolyzed oil, such as enzyme load, temperature, and water content were investigated. The model was validated by experimenting two sets of tests according to the model-predicted conditions. The equation provided in this thesis can be utilized to forecast the production of partial acylglycerols under various predetermined parameters dictating the reaction conditions (Equation 5-10).

3) Using a 2-hour partial hydrolysis, palm olein, rice bran and pumpkin seed oils were converted to plastic fats, which was evidenced by the formation of MAGs, DAGs, and FFAs. All vegetable oils, including palm olein, were liquid at room temperature before partial hydrolysis. After initial hydrolysis, except sunflower oil, all vegetable oils showed a solid like behavior which is attributed to the presence of approximately 10% MAGs and around 30% DAGs. The properties of treated oils were evaluated, revealing that a one-step hydrolysis reaction can yield a high level of MAGs and DAGs (totaling over 40%), with the lowest ratio of FFAs to MAGs achieved at 2 hours. This process enhances the thermal, textural, and

rheological properties of the resulting fats, irrespective of whether FFAs are present or removed.

7.2. New scientific findings on application of enzymatic reactions to synthesis alkyl rosmarinates after assessment of their antioxidant activity in oil-based food systems

4) The impact of the oil-based food matrix including bulk oil, structured fat with monoacylglycerol, ethyl cellulose oleogel, emulsion, and gelled emulsion systems on the antioxidant properties of rosmarinic acid derivatives was investigated for the first time in this study by considering both hydrophobicity of antioxidant and effect of food matrix. In bulk oil, both conjugated dienes and p-AnV values reached a peak in the following order after 30 days: ethyl rosmarinate > methyl rosmarinate > rosmarinic acid = BHT > control. In structured fat with monoacylglycerol, methyl rosmarinate was shown to be more effective than both ethyl rosmarinate and rosmarinic acid. For ethyl cellulose oleogel, emulsion, and gelled emulsion systems, rosmarinic acid was shown to be the more effective.

5) Additionally, after confirming the importance of the food matrix on the antioxidant activity of rosmarinic acid derivatives, the lipophilization of rosmarinic acid with ethanol was optimized for the first time as a model with Lipozyme 435 in hexane. A conversion yield of as high as 85.59 % for ethyl rosmarinate was achieved, as quantified by HPLC-UV and confirmed by HPLC-DAD-ESI-qTOFMS. The coefficient of determination (R^2) of the model was 0.96, indicating the model is fitting for representing the connection between the parameters of the reaction condition. The offered equation in this study can be employed to predict the conversion yield of ethyl rosmarinate for different set levels of parameters determining the reaction conditions (Equation 11).

8. SUMMARY

Lipase-catalyzed reactions offer promising alternatives to high-temperature chemical processes for producing heat-sensitive MAGs and DAGs enriched with MUFAs and PUFAs. Various enzymatic methodologies have been explored for MAGs and DAGs production in oil mixtures, including glycerolysis and partial hydrolysis. Recent studies have demonstrated the direct structuring of non-solid edible oils using enzymatic glycerolysis, eliminating the need for separate MAGs production. Additionally, enzymatic hydrolysis reactions offer selective options using regioselective lipases, enabling enrichment or removal of specific fatty acids. This approach can lead to improved nutritional properties and healthier alternatives in food formulations.

In the first section, our study aimed to utilize lipases to structure vegetable oils by selectively removing saturated fatty acids and enriching them with MAGs and DAGs, resulting in specialty fats with potential applications in the food industry. Therefore, in the first step, this study proposes an innovative application of enzymatic hydrolysis to maximize MAG production in vegetable oils, while converting liquid oils into structural fats. This was done as a result of the partial transformation of TAGs in liquid vegetable oils into a mixture of FFAs, MAGs, DAGs, and TAGs. Initially, five commercial lipases were tested for their ability to produce MAG. After screening amongst many commercial lipases, the partial hydrolyses of palm olein oil in a solvent-free system were studied to optimize the reaction conditions using Lipase DF (Amano). RSM was used to maximize the proportion of MAG to FFA, by adjusting water content, the enzyme load, reaction temperature, and reaction time in palm olein oil. Subsequently, the model was further studied and extended to four vegetable oils with different degrees of SFAs, including palm olein oil (SFAs of 46.25% and IV of 55.15), rice bran oil (SFAs of 25.98% and IV of 91.68), pumpkin seed oil (SFAs of 18.87% and IV of 100), and

sunflower oil (SFAs of 11.34% and IV of 128.95) while monitoring the acylglycerol composition for 10 hours. Properties of treated oils were assessed and it was shown that one step hydrolysis reaction can produce a high level of MAG and DAG (sum of above 40%), with the lowest proportion of FFAs to MAG at 2 hours, which in turn improves thermal, textural, and rheological properties of the obtained fats, regardless of the presence or removal of FFAs. Finally, FFAs were removed from the treated oils and it was found that deacidification methods can weaken the thermal, textural, and rheological properties of the obtained fats which were proved to be due to the refining loss of neutral lipids and partial removal of saturated fatty acids. This study can open up a new area in the field of structuring oils by simultaneously increasing MAGs and DAGs in the final product and designing fatty acid compositions of the target products for special requirements, as specialty fats, or for mimicking a specific type of behavior or replacing glycerolysis reactions when modification of fatty acid compositions is required.

In the second step, the impact of the oil-based food matrix on the antioxidant properties of rosmarinic acid derivatives with different chain length was studied. This was done in common oil-based food systems under accelerated oxidation conditions at 35 °C, measuring conjugated diene formation and p-Anisidine values. All *in vitro* antioxidant activity measurement results were reasonably comparable with each other. with rosmarinic acid illustrated the highest radical scavenging activities, followed by methyl rosmarinates, and ethyl rosmarinate. In bulk oil, both conjugated dienes and p-AnV values reached a peak in the following order after 30 days: ethyl rosmarinate > methyl rosmarinate > rosmarinic acid = BHT > control. In structured fat with monoacylglycerol, methyl rosmarinate was shown to be more effective than both methyl and ethyl rosmarinates. Finally, for ethyl cellulose oleogel, emulsion, and gelled emulsion systems, rosmarinic acid was shown to be the most effective.

Additionally, after confirming the importance of the food matrix on the antioxidant activity of rosmarinic acid derivatives and as the presence of natural rosmarinate esters in plants is limited and isolating them is challenging, synthesizing alkyl rosmarinates through structural modification of rosmarinic acid is essential. Esterification with aliphatic molecules can enhance solubility and bioavailability, with enzymatic methods being environmentally friendly and requiring less purification. Enzymatic lipophilization offers a practical approach to modify phenolic acids, improving their hydrophobicity while retaining functional characteristics. The lipophilization of rosmarinic acid with ethanol was optimized as a model with Lipozyme 435 (Novozymes) in hexane with conversion yield as high as 85.59% as quantified by HPLC-UV and confirmed by HPLC-DAD-ESI-qTOFMS. The coefficient of determination of the model was 0.96, indicating the model is fitting for representing the connection between the parameters of the reaction condition. The probabilities for the regression of the model were shown to be significant ($p < 0.0001$) with the model F-value of 32.24. There is only a 0.01% chance that an F-value this high can happen because of the noise. Additionally, the predicted R^2 of 0.89 was acceptable similarity to the adjusted R^2 of 0.93. Adeq precision was found to be 19.55. The Lack of Fit F-value of 1.16 implied that the Lack of Fit is not significant relative to the pure error (p-value of 0.55) which showed the model was fit.

9. LIST OF PUBLICATIONS IN THE FIELD OF STUDIES

Mardani, M., Badakné, K., Szedljak, I., Sörös, C. and Farmani, J., 2024. Lipophilized rosmarinic acid: Impact of alkyl type and food matrix on antioxidant activity and optimized enzymatic production. ***Food Chemistry***, p.139518. **Q1 - IF 8.8**

Mardani, M., Badak-Kerti, K., Tormási, J. *et al*. Selective Partial Hydrolysis as a Novel Strategy to Produce Specialty Structured Fats from Vegetable Oils: Optimization of Monoacylglycerol Formation and Assessment of the Final Product 2024. ***Food and Bioprocess Technology.*** https://doi.org/10.1007/s11947-024-03460-7 **Q1 - IF 5.6**

Mardani, M., Badakné, K., Farmani, J. and Shahidi, F., 2022. Enzymatic lipophilization of bioactive compounds with high antioxidant activity: a review. ***Critical Reviews in Food Science and Nutrition*****,** pp.1-18. **Q1 - IF 11.2**

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