# Lipase-Catalysed Production of Dietetic Structured Lipids Using Crude Olive Pomace Oils, in Continuous Bioreactors

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## Biotechnology

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## Preface

The work presented in this master thesis was performed at the Instituto Superior de Agronomia - Lisbon University facilities, during the period November 2021 – October 2022.

I declare that this document is an original work of my own authorship and I further declare that I have fully acknowledge of the Code of Conduct and Good Practices of the Lisbon University.

Joana de Souza Gonçalves

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## Abstract

Low-calorie structured lipids, namely MLM-type, are triacylglycerols (TAG) known for their nutrition benefits in our diet. The aim of this work was the lipase-catalysed production of MLM containing caprylic acid (C8:0) or capric acid (C10:0), by acidolysis or interesterification with ethyl esters (C8EE e C10EE), using crude olive pomace oil (OPO) as raw-material. OPO presented high acidity, oxidation products and chlorophylls. Reactions were performed in continuous packed-bed bioreactor (60-122 h), using *sn*-1,3 regioselective commercial immobilized lipases (Lipozyme TLIM or Lipozyme RMIM), in solvent-free media, at 40°C. The results on the production of New TAGs (50-60 % yield) were rather promising, in the range of the assessed acidity. Along with continuous bioreactor operation, both biocatalysts demonstrated high activity and operational stability. First-order model (C8EE + Lipozyme RMIM), linear model (C10:0 + Lipozyme TLIM) and Sadana model (C10EE + Lipozyme TLIM) were fitted to data. Half-lives of 74, 228 and 213 h were estimated, respectively. In the other systems, the biocatalysts did not lose activity along operation. Lipozyme RMIM cost is more than 8-fold the costs of Lipozyme TLIM. Therefore, the use of Lipozyme TLIM and crude OPO will reduce process costs (biocatalyst and oil refining), promoting the sustainability of the process.

*Keywords*: Structured Lipids, Olive pomace oil, Continuous bioreactors, Interesterification, Acidolysis, Lipases.

## Resumo

Os lípidos estruturados de baixas calorias, nomeadamente do tipo MLM, são conhecidos pelos seus benefícios nutricionais na nossa dieta. O objetivo deste trabalho foi a produção de MLM, contendo ácido caprílico (C8:0), ácido cáprico (C10:0), por acidólise ou interesterificação com ésteres etílicos (C8EE e C10EE), utilizando óleo de bagaço de azeitona bruto (OPO) como matéria-prima. O OPO apresentou elevada acidez, produtos de oxidação e clorofilas. As reações foram realizadas em biorreator de leito contínuo (60-122 h), utilizando lipases comerciais imobilizadas e regioseletivas *sn*-1,3 (Lipozyme TLIM e Lipozyme RMIM), em meio sem solventes, a 40ºC.

Os resultados sobre a produção de novos TAG (50-60 % de rendimento) foram muito promissores, no intervalo de acidez avaliado. Ao longo da operação do biorreator contínuo, ambos os biocatalisadores demonstraram alta atividade e estabilidade operacional. O modelo de primeira-ordem (C8EE + Lipozyme RM IM), modelo linear (C10:0 + Lipozyme TL IM) e modelo Sadana (C10EE + Lipozyme TL IM) foram ajustados aos dados. Foram estimados tempos de meia vida de 74, 228 e 213 h, respetivamente. Nos outros sistemas, os biocatalisadores não perderam atividade ao longo da operação.

O custo de Lipozyme RMIM é mais de 8 vezes superiores ao custo de Lipozyme TLIM. Por isso, a utilização de Lipozyme TLIM e de OPO, reduzirá os custos de processo (biocatalisador e refinação do óleo), promovendo a sustentabilidade do processo.

Palavras-chave: Lípidos Estruturados, Óleo de bagaço de azeitona, Biorreatores Contínuos, Interesterificação, Acidólise, Lipases.

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## Abbreviations List

$a_w$ – Water Activity

C8EE – C8 Ethyl Ester

C10EE – C10 Ethyl Ester

CSTR – Continuous Stirred Tank Reactor

DAG – Diacylglycerol

EFA – Essential Fatty Acids

FA – Fatty Acid

FBR – Fluidized-bed Reactor

FFA – Free Fatty Acid

MAG – Monoacylglycerol

MUFA – Monounsaturated Fatty Acid

OPO – Olive Pomace Oil

PA – Palmitic acid

PBR – Packed-bed Reactor

PUFA – Polyunsaturated Fatty Acid

SA – Stearic acid

SFA – Saturated Fatty Acid

SFC – Solid Fat Content

SL – Structured Lipids

TAG - Triacylglycerol

# 1. Introduction and Objectives

In the field of the biotechnology of oils and fats, it is important to consider the lipids as crucial compounds of the population diet providing energy, essential fatty acids, vitamins and antioxidants. Some of them can be considered as functional foods (*e.g.*, virgin olive oil) but others can be modified, being an important subject for different food applications, namely in food industries. However, due to high caloric value and the presence of saturated fatty acids (FA) with negative effects in health, lipids are associated to the major risk sources of cardiac diseases such obesity as well as, in some situations, cancer. Regardless, they play an important role in the acceptability and perception of flavour in a variety of foods. Because some FAs are present in inadequate quantities, these oils and fats must be included into a balanced diet because they are needed for the human body (Mota *et al*., 2020i; Ferreira-Dias *et al*., 2019; Meynier *et al*., 2017; Ferreira-Dias *et al*., 2022).

The production of dietetic triacylglycerols (TAGs) has strongly increased due to their properties according to the nutrition value for population diet. To obtain these new molecules, the use of continuous bioreactors has been increasing, as an innovative method for olive pomace oil using biocatalysts and given their advantages over a batch mode. Thus, the main aim of this master thesis is the production of novel TAG molecules of MLM type, known as low-calorie SLs, using olive pomace oil (with long-chain FAs), before refining process, as raw material, in order to valorise the by-product obtained from olive industry to produce products with improvement in properties for healthy food. The methods used will be (i) acidolysis with FFA (C8:0 and C10:0) and (ii) interesterification with ethyl esters (C8EE and C10EE), in solvent-free medium, catalysed by immobilized *sn*-1,3 regioselective lipases as biocatalysts. Further, operational stability of biocatalysts in continuous bioreactors and, consequently the effect of crude oil quality on biocatalyst stability and activity will be assessed. The reaction kinetics will be assayed by GC column essays and models description of lipase deactivation kinetics will be fitted to the obtained results along bioreactor operation, paying attention on half-life estimation of the catalysts under different reaction conditions.

# 2. Literature Review

## 2.1. Structural diversity and composition of lipids

Lipids, mostly synthesized in the endoplasmic reticulum, are a diverse group of molecules, considered essential structural components of cell membranes, on what their composition of organelles is highly diversified following different functions, which are important in cellular physiology and pathology on distinct live cells. Moreover, lipids are characterized as energy storage components, origin of signalling molecules for cell recognition and immunity, protein recruitment, prevention or treatment of several diseases, providing health benefits and also as protective cover over numerous organisms' surfaces (Zhang *et al*., 2021; Fahy *et al*., 2009; Subramaniam *et al*., 2011).

They are considered soluble molecules in non-polar organic solvents instead of in water, and therefore are classified as biological substances that are hydrophobic. However, they can be also present a hydrophilic part that allow the formation of cellular membranes. Thus, when it is referred to cell membrane lipids, they have an interaction between a hydrophobic and a hydrophilic ends, creating each monolayer, such the case of phospholipids (Fahy *et al*., 2005; Cockcroft, 2021). Lipids of mammalian cells, according to their FA composition, can be distinguished between polar lipids (phospholipids, glycolipids and sphingolipids) and neutral lipids (glycerolipids (TAGs, DAGs, MAGs) and free fatty acids) (Figure 1).

Glycerolipids are defined by having glycerol molecules as their backbone, while sphingolipids present sphingosine (amino alcohol). In latter, sphingoid backbone is characterized by having a hydrophobic tail (Cockcroft, 2021). Phospholipids are considered polar lipids with two FAs, a glycerol unit and a phosphate group and glycolipids are carbohydrate-containing molecules, with hydrophobic FA tail that helps in membrane bilayers arrange (Xie, 2019; Hermanson, 2013).

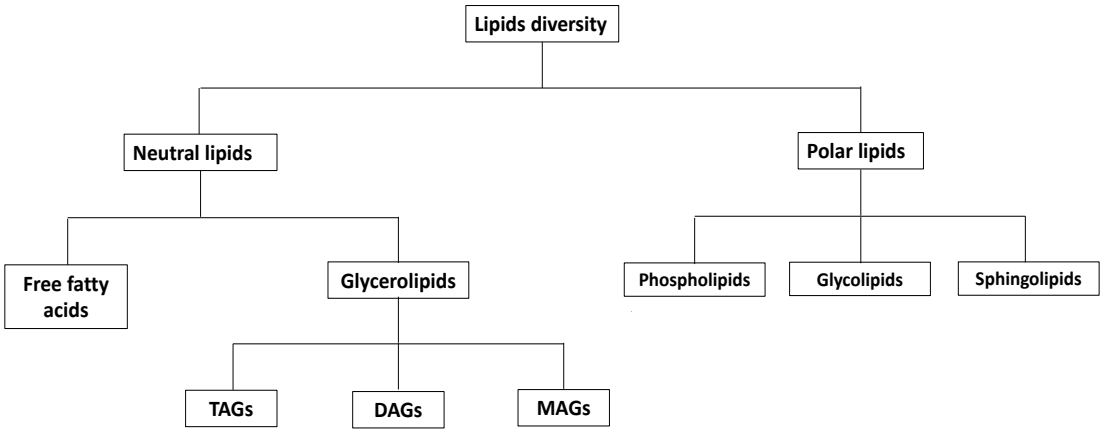


Figure 1. Scheme of structural and molecular diversity of lipids in cells (adapted from Cockcroft, 2021; Meynier et al., 2017).

### 2.1.1. Lipids major compounds

In oils and fats composition are the major components, which are acylglycerols (monoacylglycerols (MAGs), diacylglycerols (DAGs) and triacylglycerols (TAGs)) and free fatty acids (FFA). In the case of minor components, the most important are tocopherols, proteins, sterols, liposoluble vitamins, hydrocarbons and pigments.

TAGs are esters made up of FAs esterified at three distinct positions on a molecule. They are the main component of natural oils and fats (97-99 %), being their composition, structure and stereochemistry, the major determinants on physico-chemical, functional and nutritional characteristics of lipids (Sivakantan *et al*., 2020; Zhang *et al*., 2020).

MAGs and DAGs can be produced through the structure of TAGs by hydrolysis reaction with water, catalysed by lipases. These molecules can be also obtained from incomplete biosynthesis of TAGs. Also exists FFAs, originated from TAGs by cutting ester bonds through lipases action, high temperature and moisture. FFAs when present in high quantity may undergo

oxidation which leads to the increase of a strong taste and flavour in oils and fats, related with rancid (Chew *et al*., 2020; Genot *et al*., 2013). MAGs, DAGs and FFAs are found in much lesser concentrations in oils and fats, although the first two molecules be crucial for emulsification (Ferreira-Dias *et al*., 2022).

Natural oils and fats have their functional and nutritional characteristics associated to FA composition and, in turn, organised in TAG molecules. Thus, TAG nutritional value and physicochemical properties are assessed by the FA composition (chain length and saturation degree) and localization on molecules (Zhang *et al*., 2020; Osório *et al*., 2005). FAs position play an important role in TAGs, since they can determine functional, physical, metabolic and nutritional properties and determine the application and final fate of novel oils and fats (Jala *et al*., 2018; Jadhav *et al*., 2021).

Lipids used for energy storage are the triacylglycerols, where each of the three hydroxyls of the glycerol backbone have FA attached to them. FAs are fundamental components of TAG, as well as phospholipids and sphingolipids, synthesized by chain elongation of an acetyl-CoA unit with condensation of malonyl-CoA groups that contain a cyclic functionality and are substituted with heteroatoms (Fahy *et al*., 2005; Cockcroft, 2021). They present a carboxylic acid with a hydrocarbon acyl chain which can alter in number of carbons in their length ($C_{14}$-$C_{26}$) and degree of saturation, considering the number of double bonds. According to this, FAs can be separated in to short-, medium- and long-chain FAs, according to their chain-length, which determine their solubility and absorption. Furthermore, FAs can be separated in saturated FAs, if the acyl chains do not have double bonds or unsaturated if they have double bonds. In the latter, in turn, they can be divided in to monounsaturated (MUFA) if contain a single double bond or polyunsaturated (PUFA) with multiple double bonds (Cockcroft, 2021; Jala *et al*., 2018). Each FA has a relationship to one of the three positions of the glycerol structure, which are designated by the stereospecific numbering system of *sn* (*sn*-1, *sn*-2 and *sn*-3, respectively), with the percentage of each depending on the lipid supply. FAs have unique rheological and functional characteristics that are crucial for the synthesis of structured lipids (Zhang *et al*., 2020).

## 2.2. Olive oil extraction

The extraction methods of olive oil are divided into three systems: classic, 3-phase and 2-phase (Figure 2). Before all of them, it is important to carry out some previous operations, in order to increase olive oil quality. These operations include harvesting (which should be carried out when most of the fruit is at an optimal state of maturation), transport, cleaning, sorting the fruit and washing. After washing, the extraction systems have different characteristics. In the classical system, the crushing process is followed by malaxation, pressing, and finally decanting. In the 2- and 3-phase continuous systems, crushing is followed by malaxation, decantation and centrifugation.

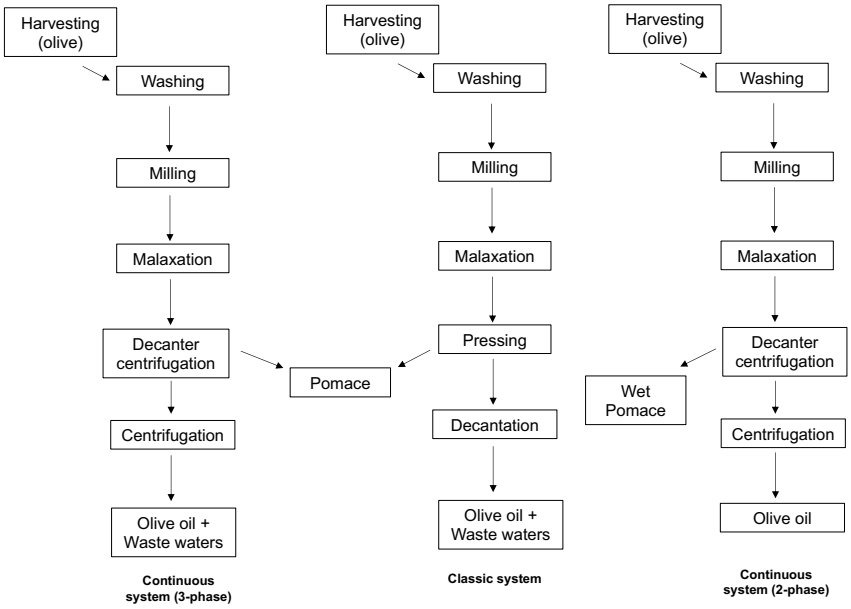


Figure 2. Schemes of olive oil extraction methods: classic, 2- and 3-phase.

The crushing process is characterised as the operation that allows the release of droplets of oils from the vacuole of the fruits. Hammer mills and millstones are used in 2- and 3-phases and classic systems, respectively. This aeration allows the formation of uniform paste, enhancing the effects of crushing. Then, in classic system, before the decantation, it is used pressing with hydraulic press, in order to extract the olive oil and waste waters (liquid phase) and the pomace is retained (Petrakis, 2006).

In two or three-phase continuous systems, olive oil extraction is carried out by centrifugation in horizontal decanters. In three-phase decanters, the pomace (solid fraction) is separated from the vegetation water and the olive oil (liquid fractions). In two-phase extraction systems, a wet pomace is obtained, since it is extracted together with the vegetation water, while the olive oil is extracted separately. Finally, the olive oil is submitted to vertical centrifugation, to remove some pomace particles or traces of water.

The residual oil in pomace, the olive pomace oil (OPO), can be removed by solvent extraction. The OPO presents similar chemical composition when compared with olive oil. The OPO can only be used for edible purposes if it has been refined. Thus, it can be used as raw material to produce structured lipids (SLs), in order to obtain value-added products, being possible to value this oil (Heinzl *et al.,* 2022).

After olive oil extraction, this type of industry presents one of the major environmental and economic problems: production of large amounts of solid waste and by-products. These products, such as olive pomace and olive pomace oil, if they do not undergo a refining process, will not be used for human consumption. Therefore, since they present beneficial compounds and food industry is looking for healthier food products, these residues should be used as attractive low-cost sources for the production of low-calorie SLs or biodiesel (Table 1).

This use and recovery of by-products is related to (i) the requirement to find new sources to increase the availability of new healthier food products and (ii) the concept of a circular economy, in order to recover and value waste, allow reuse and economic growth through waste from industries (Otero *et al*., 2021; Gullón *et al*., 2020; Campos *et al*., 2020). Additionally, the use of crude acidic oils minimizes the costs of raw materials and, consequently, the cost of the olive oil extraction process since oil refinement is not necessary, *e.g.* for TAGs production (MLM, biodiesel and *sn*-2 MAG) (Heinzl *et al*., 2022).

Table 1. Applications of crude *vs.* refined oils, in order to valorise the olive pomace oil by-product.

| Type of oil | Application | Reaction Conditions | Bioreactor mode | Reference |
|---|---|---|---|---|
| **Crude** | TAGs of MLM type, Biodiesel (FAME) and *sn*-2 MAG | Acidolysis and Interesterification with ethers, acids or methanol. Lipozyme TLIM and Lipozyme RMIM as biocatalysts | Batch | Heinzl *et al*., 2022 |
| | Low-calorie SLs | Acidolysis and Interesterification with capric acid or ethyl caprate, respectively. ROL in nanoparticles (MNP-ROL) | Batch | Mota *et al*., 2020i |
| | Biodiesel | (Trans)esterification with methanol and sulfuric acid | Batch | Banani *et al*., 2014 |
| **Refined** | Cocoa butter-like fat | Acidolysis with PA and SA acids. Immobilized sn-1,3 specific lipase (from *Mucor miehei*) | Batch | Çiftci *et al*., 2009 |
| | Biodiesel | (Trans)esterification with methanol and sulfuric acid | Batch | Banani *et al*., 2014 |

## 2.3. Structured lipids (SLs)

### 2.3.1. Novel lipids obtained by biocatalysis

Foods enriched in fat substances are possibly the most consumed, being the fat composition and its properties the important factor to attract more consumers, which makes eating a pleasurable action. The diversity of modified lipids, can be increased with changes in food habits

and an eventual introduction of new dietary sources and different ingredients (Meynier *et al*., 2017).

The food industry has been searching to answer and act to reduce the fat consumption. Fat substitutes made of carbohydrate and protein cannot be subject to high temperatures. Thus, they have been trying to produce reduced-calorie fats and improve nutrition properties for a better healthy diet, mimicking all the properties of natural fat (Abed *et al*., 2016). Since fats are characterised by the native TAG structure that affects digestion and absorption and their ingestion can cause many health complications, it is important to take in consideration the substitution of unhealthy fats for more healthy ones, rich in unsaturated or essential FAs (Jala *et al*., 2018; Cozentino *et al*., 2020; Paez *et al*., 2002). One of the solutions is modifying lipids for health, making them functional foods, known as "structured lipids" (SLs) or "tailor-made lipids" for different applications. These SLs are characterised as TAG molecules that contain both long- and medium- or short-chain FA, with each group in its own position (Xu *et al*., 2007). They can be obtained by modifications in the structure (composition and positional distribution of FA) of the native state of TAG, in order to improve physicochemical properties, for instance melting properties, solid fat content (SFC), oxidative stability, digestion, absorption and metabolism (Kim *et al*., 2015; Abed *et al*., 2018; Mensink *et al*., 2016; Institute of Medicine, 2002). They have physiologically-active compounds to provide a health benefit for humans, enhancing their well-being (Mota *et al*., 2020i; Ferreira-Dias *et al*., 2019). Thus, modified fats represent an important growing sector, especially in food industry, in order to provide natural and healthier fats as a response to the current consumer's demand (Costa *et al*., 2017).

Since TAG molecules are the biggest source of dietary FAs (up to 97 % of the energy), it is important to take into account their native arrangement, in order to modify them to give better characteristics to the fat products and reduce calories. Their absorption and nutritional quality will be determined by their distribution among the three positions of molecules (Kim *et al*., 2015; Cockcroft, 2021; Sellapan *et al*., 2001; Osborn *et al*., 2002).

Low-calorie TAGs are one of the examples of SLs with enhanced benefits when compared with ones that exist in nature (Mota *et al*., 2020i). They present medium-chain fatty acids (MCFA) at position *sn*-1,3 and long-chain fatty acids (LCFA) at position *sn*-2, being known as MLM lipids (present lower caloric value (5 to 7 kcal/g) than conventional fats and oils (9 kcal/g)) (Costa *et al*., 2017; Mota *et al*., 2020i). Other examples of SLs that mimics the natural fats are human milk fat (HMF) and cocoa butter (CB) substitutes.

Moreover, MCFAs (C6:0 to C10:0) present high solubility. When released from the TAG molecules, by the action of the *sn*-1,3 regiospecific lipase, they are easily absorbed and digested, due to small structure and low molecular weight, quickly providing energy through oxidation processes (Lee *et al*., 2012). Therefore, they provide rapidly energy to the body and are slowly stored as fat in the adipose tissue, since they are promptly re-esterified, leading to the formation of new TAG molecules as production of dietetic triacylglycerols. However, since they do not contribute with essential FAs, this is considered a disadvantage (Mota *et al*., 2020ii; Nunes *et al*., 2011). In addition, MCFAs have the ability to increase blood cholesterol levels. Thus, they appear

to be best used in a SL that combines their intrinsic mobility, solubility and ease of metabolism. The LCFAs (C12:0 to C18:0) can be saturated or unsaturated (mono- or poli-) and they have a considerable high caloric value (9 kcal/g) and tend to be accumulated in human adipose tissue (Jadhav *et al*., 2021; Mota *et al*., 2020i; Akoh, 1998). However, can have benefits for human body, for instance the role of polyunsaturated FA (PUFA) in the prevention of different diseases, being the most prevalent type of edible oil and some of them recognized as essential FA (linoleic and linolenic acids) (Utama *et al*., 2019). Furthermore, the benefits of both medium- and long-chain FA can be present in MLM structured lipids: the long-chain portions are often contributed by the common vegetable oil (for instance, olive oil), while medium-chain is contributed either by medium-chain TAGs or FA, such as caprylic acid, respectively (Lee *et al*., 2012; Cozentino *et al*., 2020). Thus, and since medium-chain FAs cannot be used alone as source of low-calorie fat, more advantages appear if MCFAs and LCFAs get together on the same molecule, to produce this type of structured lipids, MLM. Reduced-calorie SLs are created by combining the chemistry and functionality of natural lipids with the reduced absorption of LCFAs or the low caloric value of SCFAs and MCFAs. Thus, if the FFA released from lipids after absorption are MCFA or SCFA, they are metabolised readily. LCFAs are easily absorbed. As a result, EFA are most efficiently used in TAG molecules from the *sn*-2 position (Abed *et al*., 2016; Xu, 2000).

These structured TAGs originated through the combination of MCFA with LCFA are designed to produce FA with nutritional health profit that could be important for person with specific diseases conditions (Jala *et al*., 2018; Nunes *et al*., 2012). TAG structure can be modified through different combinations of FAs (modifying their original position) in the glycerol skeleton. The TAG structure has an impact on fat digestion and absorption. Better absorption results from the pancreatic lipases hydrolyses of ester bonds at *sn*-1,3 positions, displaying more activity toward MCFAs than LCFAs (Paez *et al.,* 2022). Thus, novel lipids, namely MLM lipids, can act as functional ones and their benefits focus on the decrease the chances of fat storage in our body due to the presence of medium- and short-chain FAs at *sn*-1,3 location and long-chain FAs at *sn*-2, which are crucial to provide proper functioning to the organism and reduce obesity (Jadhav *et al*., 2021). Therefore, the production of this type of lipids can be considered a “green route”, using immobilized biocatalysts.

### 2.3.2. Immobilized lipases as biocatalysts

Enzymes present a big specificity for the substrate and for the reaction, with the capacity to increase the rate of the chemical reaction by decreasing your activation energy. The reaction rate consists of the substrate consumption (or product synthesis) per unit of time. In addition, enzymes are prone to substrate and product inhibition (Bornscheuer, 2018). In this sense, the use of enzymes, namely lipases became very attractive due to its characteristics, although limitations regarding the high costs, have restrained the industrial implementation of oils and fats interesterification. The low-cost oil valorisation, such olive pomace oil, may be an option to lower process costs (Cabral *et al*., 2003). Especially in the food industry, they have been focused on

the change of inorganic catalysts by the enzymatic ones as an alternative method for lipid modification. Therefore, SLs are able to be synthetized by lipases.

All enzymatic reactions and their implementation in specific bioreactors are planned according to the increase of volumetric productivity factor. For this aim, the enzyme activity should also be expanded, enhancing their concentration in the bioreactor. Thus, the implementation of immobilization process may be able to help the improvement of the quantity of biocatalyst and also its reuse in batch reactors or use in continuous bioreactors (Cooney, 1983). Enzyme immobilization allows the confinement of the enzyme to a restricted region, as bioreactor, what ensures the retention of high catalytic activity and stability, regeneration and reuse possibility in batch or use in continuous mode, in presence of strict conditions, as well as selectivity, specificity and even the reduction of enzyme inhibition by the product (Mota *et al*., 2020ii; Bernal *et al*., 2018; Thangaraj *et al*., 2019).

Immobilization of enzyme is characterized as the confinement to a phase, that can be a matrix or support material. The enzymes can be used non-immobilized, in immobilized state in solid supports (through the attachment to a solid carrier covalently, hydrophobic binding, ion exchange or cross-linking or by holding in a barrier such polymeric matrices) or immobilized by precipitation in organic solvents. The choice of immobilization process should follow different steps such as, the chemistry for enzyme attachment, better conditions for reaction optimization and assessment of biocatalyst behaviour (Stergiou *et al*., 2013; Bernal *et al*., 2018; Wahab *et al*., 2020).

The immobilization in solid supports is able to reduce the costs of biocatalysts for industrial processes, allowing easier product recovery and reuses in bioreactor during the reaction with better operation control. Beyond these advantages, it is easier to separate the biocatalysts from the product using immobilized enzymes, minimizing the product degradation risks, resulting in greater thermostability behaviour. In addition, controlling of bioconversion time and biocatalyst microenvironment, will allow to manipulate the catalytic specificity and activity (Yahya *et al*., 1998). Nevertheless, the use of an immobilization support has some disadvantages. Among them, the loss of catalytic activity during the immobilization process, since the enzymes can be exposed to extreme conditions, such as pH, temperature, organic solvents or situations of shear stress. In addition, during the bioconversion step, loss of biocatalyst or accumulation of inhibitors in the matrix may occur. Furthermore, the physicochemical characteristics of enzymes environment may be altered (Cabral *et al*., 2003; Illanes, 1999; Wahab *et al*., 2020).

Commercial immobilized lipases from microbial sources, such Lipozyme RM IM or Lipozyme TL IM (*Thermomyces lanuginosus* lipase immobilized on silica gel, *sn*-1,3-specific) can have some narrow conditions on their use, such afford high costs, limiting their viability for the production of low-cost products, such as commodity fats, like interesterification fat blends for the margarine industry. However, immobilized enzymes in solid supports must present high catalytic activity as well as high operational stability, which makes easier the use of different continuous and batch bioreactors for oils and fats biotransformation reactions.

Novel supports have been studied such the case of magnetic nanoparticles or hybrid supports from silica and biochar for the enzyme immobilization, in order to promote high catalytic activity and operational stability, ensuring the effective link between enzyme and substrates, for obtained structured TAGs (Mota *et al*., 2020i).

### 2.3.3. Structured lipids production methods

The knowledge about TAG structure to produce MLM lipids through the FA composition is an important topic to obtain the health lipids with improved physical properties of fats and with low calories, when compared with typical edible oils (Meynier *et al*., 2017; Nunes *et al*., 2012; Ferreira-Dias *et al*., 2020; Heinzl *et al*., 2022). By altering the content and position of FAs in the glycerol backbone of acylglycerols, SLs can be produced chemically or enzymatically.

The options for the reactions that are involved in the synthesis of MLM structured lipids, can be: (i) interesterification, (ii) transesterification and (iii) acidolysis (Ferreira-Dias *et al*., 2019).

Interesterification describe the redistribution of different FAs in the TAGs molecules. This process can occur through the random way (chemical) or by enzymes specific action (enzymatic): one TAG molecule reacts with methyl or ethyl esters of medium-chain FA or medium-chain TAG. At the end of the process, it is presented a mixture with a novel TAG profile, where there is an alteration of the chemical and physical composition of oils and fats (Mota *et al*., 2020i; Mensink *et al*., 2016).

Acidolysis is a method with a reaction between an ester molecule (TAG, oil/fat or phospholipid) and a mixture/single FFA, using *sn*-1,3-specific lipase as catalyst (Ferreira-Dias *et al*., 2019).

Another method is transesterification (or alcoholysis) that although less common, is a reaction of exchange of two acyl groups between ester molecules and an alcohol (Ferreira-Dias *et al*., 2019; Zhang *et al*., 2014).

All the reactions can be performed both in solvent or in solvent-free media, in non-aqueous media.

Over the years, recommendations to decrease the amount of saturated FA in food to promote a healthy eating boosted more attention in the use of full or partial hydrogenation, since it is possible a transformation of unsaturated FA rich oils into semi solid fat and improve oxidative stability of unsaturated oils. However, along this process, some of *cis* FA are converted to *trans* FA. Moreover, the hydrogenation will convert PUFA and MUFA into SFA. Given the toxicity risk of SFA and *trans* FA and consequently, negative impact in human health as cardiovascular diseases, the use of interesterification method, although some health implications of long-term consumption less well understood in different studies, was develop to cover these modifications of natural oils and fats in food industry, focused on product and ingredient functionality while also keeping health effects in mind (Sivakanthan *et al*., 2020; Mensink *et al*., 2016).

Although this study is focused on low-calorie TAG molecules production, the application of interesterification on low calorie fats production by altering the structure of TAG molecules for a specific one, this method is able to find in other productions and applications such as cocoa

butter, human milk fats, margarines and even in clinical nutrition to help fat malabsorption disorders (Sivakanthan *et al*., 2020).

The TAG molecules are essential to contribute for the production of MLM lipids using interesterification method. In this reaction, ethyl octanoate (C8EE) or ethyl decanoate (C10EE) are used as reaction substrates together with TAG. Because of the toxicity of the methanol generated during the reactions, ethyl esters are chosen over methyl esters as acyl donors. Furthermore, because ethyl esters are more volatile than FFA they may be collected from the reaction medium more easily by distillation, lowering downstream processing costs (Ferreira-Dias *et al*., 2022).

All the reactions of interesterification, acidolysis and alcoholysis (lipase-catalysed reactions) are divided in two steps: initial hydrolysis and esterification process, modifying molecule's architecture through the redistribution of the two FAs with a TAG or the exchange of the FAs between TAGs, with the help of chemical catalysts, under high temperatures or by *sn*-1,3 regioselective lipases as biocatalysts (Meynier *et al*., 2017; Zhang *et al*., 2020). These reactions involve hydrolysis of ester bonds in TAGs followed by re-esterification, being considered a reversible reaction (Ferreira-Dias *et al*., 2019). In turn, to achieve high conversion product rates, water must be continually removed from the reaction medium to enhance esterification process while reducing hydrolysis. To optimize the first step of the lipase-catalysed reactions, a balance between esterification and hydrolysis should be achieved (Abed *et al*., 2016).

#### 2.3.3.1. Interesterification

*Chemical Interesterification*

The chemical method is extensively used to produce structured lipids by chemical catalysts in the industry, changing composition and physical properties of triacylglycerol molecules. The chemical processes can be hampered by the characteristics of chemical catalysts and the method itself, since is cost-effective and easy to handle when work is developed during upscaling or pilot experiments.

Chemical catalysts (*e.g.*, sodium methoxide) are classified as non-selective, since they catalyse competing reactions, characterized as a random action towards the acyl-positions in TAG molecules, which lead to a lack of specificity. In addition, a lot of undesired by-products are developed, as well as product purification is necessary to remove them. In this sense, this method, although less expensive and the reaction being rapid, compared to the enzymatic interesterification, has low yields and the required products must be purified to remove side-products from side-reactions. Moreover, there is difficulty to inactivate chemical catalysts, high probability of generating pollutant effluents due to hazardous chemicals. Thus, the need for higher quality products with those specific modifications leads to give up the use of chemical pathway and proposes the use of enzymes, such lipases. However, this type of interesterification, for instance, continues to be used in the margarine industry (Ferreira-Dias *et al*., 2019; Mota *et al*., 2020i; Jala *et al*., 2018; Mensink *et al*., 2016).

*Enzymatic Interesterification*

Regarding the enzymatic interesterification method and among the other roles of enzymes, lipases belong to the hydrolases class (EC 3.1.1.3, triacylglycerol acyl-hydrolases) and are able to hydrolyse TAGs to MAGs, DAGs, free FAs and glycerol and when in non-aqueous media they catalyse different reactions mentioned above, such as acidolysis and interesterification. They are considered the most popular group of biocatalysts, promoting different reactions in industrial biochemical processes, such the case of food, pharmaceutical, leather, cosmetic, paper, among others. Within their regioselective classification, based on specificity, they can be classified as non-specific or 1,3-regioselective lipases: the non-regioselective ones do not show distinct specificity regarding the position of acyl group on the glycerol backbone, designing different of configurations, while specific ones have marked preference for the acyl ester bonds at the first and third positions of TAGs (*sn*-1 and *sn*-3) so it will give the desired structure for MLM lipids (Figure 3) (Kim *et al*., 2015; Lee *et al*., 2012). This strategy allows for the control of the FA that is intended to be introduced into the TAG molecule and it has the potential to significantly modify the FA composition of the product's TAG (Rodrigues *et al*., 2010).

This type of enzymes can have different origins such as plants, animals, insects or microbial organisms. One example of fungal source is *Rhizomucor meihei*, with application in food and chemical industry, cosmetics, detergents, agriculture or waste treatment (Sarmah *et al*., 2017; Yahya, *et al*., 1998). Further, catalytic activity of the specific lipases not only depends on the influence of its source, but also on the substrate type and concentration and specificity towards certain FAs as well as the TAGs, DAGs and MAGs (Utama *et al*., 2019).

As several authors mention, the use of lipases as reaction biocatalysts in these systems has some advantages such as (i) heterogeneity of lipases that operate under milder temperature conditions (between 30ºC-40ºC, most lipases are at their optimally activity), with no thermal degradation, compared to the chemical processes (temperature below 70ºC, under atmospheric pressure), resulting in less temperature-sensitive substrates and products losing their original properties, (ii) present high selectivity (regio-, stereo-, typo-, and substrate) with a decrease in side-reactions and the facility for product recovery and purification, with a few or no formation of byproducts (iii) requirement of a small number of unit operations, (iv) products can be marked as "natural" since they result from a natural process, (v) this type of enzymes do not need co-factors and catalyse a variety of reactions, (vi) they are biodegradable and environment-friendly, since reduces the energy and deleterious reagents, (vii) some lipases may present high stability, according to the reactions, remaining active even under unfavourable conditions and (viii) since lipases can need the interface medium (hydrophobic and hydrophilic) to work well, they should be able to retain the water monolayer to maintain their active structure (Bornscheuer, 2018; Ferreira-Dias *et al*., 2019; Yahya *et al*., 1998; Kim *et al*., 2006). Furthermore, enzymatic interesterification is able to have high efficiency in acyl change, simple and flexible reactions, does not contain hazardous chemicals and formation of large number of by-products and has advantages in terms of saving energy, resulting in a more environment-friendly and safer

hypothesis for different applications. Additionally, product yields can be higher, increasing the amount of final product (Jala *et al*., 2018; Kim *et al*., 2015; Mota *et al*., 2020i; Stergiou *et al*., 2013).

This type of reaction involves the glycerol and FFA molecules as substrates in esterification reaction, with the release of FA from one glycerol molecule and then esterification with other FAs. After, free position on the glycerol molecule will be occupied by different FAs. Thus, the results will show the formation of TAG molecules with different chemical composition, compared with the original ones (Jadhav *et al*., 2021; Kadhum *et al*., 2016; Utama *et al*., 2019). Additionally, the presence of water should be controlled, in order to maintain the enzyme water activity ($a_w$) adequate to promote high reaction rates, but not in excess, which will lead to hydrolysis of TAG (Xu, 2003).

The use of specific enzymes allows the formation of structured lipids with unique physicochemical characteristics (Figure 3), which hydrolyse FAs at the *sn*-1 and *sn*-3 positions, maintaining the FA at *sn*-2 position, preferentially occupied by unsaturated long-chain ones. Thus, due to this lipase's specificity, required acyl group is introduced into a specific position of TAG molecules. In addition, due to these enzyme's properties, MLM lipids can only be synthesized by the enzymatic pathway, in order to maintain their FA characteristics and specific positions of FA. For this reason, in comparison to traditional approaches, enzymes have distinct benefits. Then, progress of the reaction is analysed by the changes in the TAG composition before and after it (Lee *et al*., 2012; Nunes *et al*., 2011).

#### 2.3.3.2. Acidolysis

Acidolysis allows the production of SLs by the reaction between TAG and FFA, where the original composition is modified. An acyl group is transferred between the triacylglycerol molecule and the FFA, as described in the scheme (Figure 3), and in the end of the reaction, new TAGs will be obtained when the hydroxyl group of the MAGs and DAGs reacts with this FFA. This acyl movement depends on several parameters, such as temperature, lipase load, enzyme supports, water content and activity, solvent and reaction system (Nunes *et al*., 2011), which influence the achievement of equilibrium of reversible reaction with a certain yield of the formed product, according to the ratio between FAs and esters used (Xu, 2003).

As in interesterification, acidolysis can have either the action of catalysts (chemical catalyst like acid-base), through a random way or the presence of specific enzyme specificity (Jadhav *et al*., 2021; Kadhum *et al*., 2016). However, the regiospecific activity of enzymes in acidolysis reaction, according to the specific positions of FAs, plays an important role for the synthesis of these SLs (Wang *et al*., 2012). This process, as it happens on interesterification method, to produce MLM lipids, the right type of lipase for the reaction has to be selected, such as the case of the *sn*-1,3 specific Lipozyme RM IM (*Rhizomucor miehei* lipase immobilized on a microporous anion exchange resin, *sn*-1,3-specific), which is commonly used.

For MLM TAGs production, lipase-catalysed acidolysis reaction with medium-chain FAs, such as caprylic (C8:0) or capric (C10:0) acids as acyl donors, and vegetable oils as the source

of glycerol backbone and long-chain FAs in position *sn*-2 is considered one of the most common methods (Nunes *et al*., 2011). Nonetheless, acidolysis demonstrates disadvantages once uses FFA as acyl donor, which may promote enzyme deactivation. In interesterification, the ethyl ester is used as acyl donor as source of FA, instead of FFA, which consequently leads less enzyme inactivation. Moreover, since FFA are less volatile than ethyl ester, FFA recovery is more difficult and more expensive in downstream process (Mota *et al*., 2020i). However, acidolysis is viewed as a very simple method, since both the oil used as raw material and acyl donors are hydrophobic and are present in the same physical phase for various applications, what makes it simpler and easier to perform (Xu, 2003).

### 2.3.4. Solvent-free media vs. organic solvent

The production of low-calorie SLs can occur in organic solvent or in free-solvent media, both in continuous or discontinuous bioreactors.

In three different studies (Ferreira-Dias *et al*., 2019; Nunes *et al*., 2011; Yahya *et al*., 1998), the authors refer that the use of organic solvents has some advantages such as (i) at a room temperature, it is able to solubilise solid hydrophobic substrates and minimize hydrolysis reaction, (ii) easy extraction and final recovery of SLs, (iii) higher reaction rates, since organic solvents are able to maintain a low $a_w$, (iv) promotes the balance of the reaction towards the desired SLs production. However, allowing reactions at a room temperature, the use of organic solvents rise the costs and complexity of the system, making product recovery and its purification more difficult. In addition, it is important the use of biocompatible and food-grade solvent, without problems of toxicity in some applications, mainly for food and pharmaceutical uses. Moreover, the kind of organic solvent used could have a big impact on an enzyme's reaction kinetics and catalytic effectiveness. When selecting a solvent for catalysis reactions, the amount to which the solvent chosen influences the enzyme's activity and stability, as well as the effect of the solvent on the equilibrium position of the reaction should be addressed (Abed *et al*., 2016).

Thus, the preference for the use of solvent-free media is environmentally friendly and more used by the food industry, although sometimes it is required higher temperatures for reactions to melt the fats and, reduce the viscosity of reaction media. It consists in the mixture of substrates in the reaction (oil or fat alcohol or acid) and it is preferable once in the final recovery, purification step it is not necessary the solvent elimination, which makes the separation processes easier and with improvement in the final product quality, as MLM lipids and the problem with acyl migration is also less seen along the reactions (Xu *et al*., 1998; Moreno *et al*., 2004; Kim *et al*., 2006; Lopez-Hernandez *et al*., 2007).

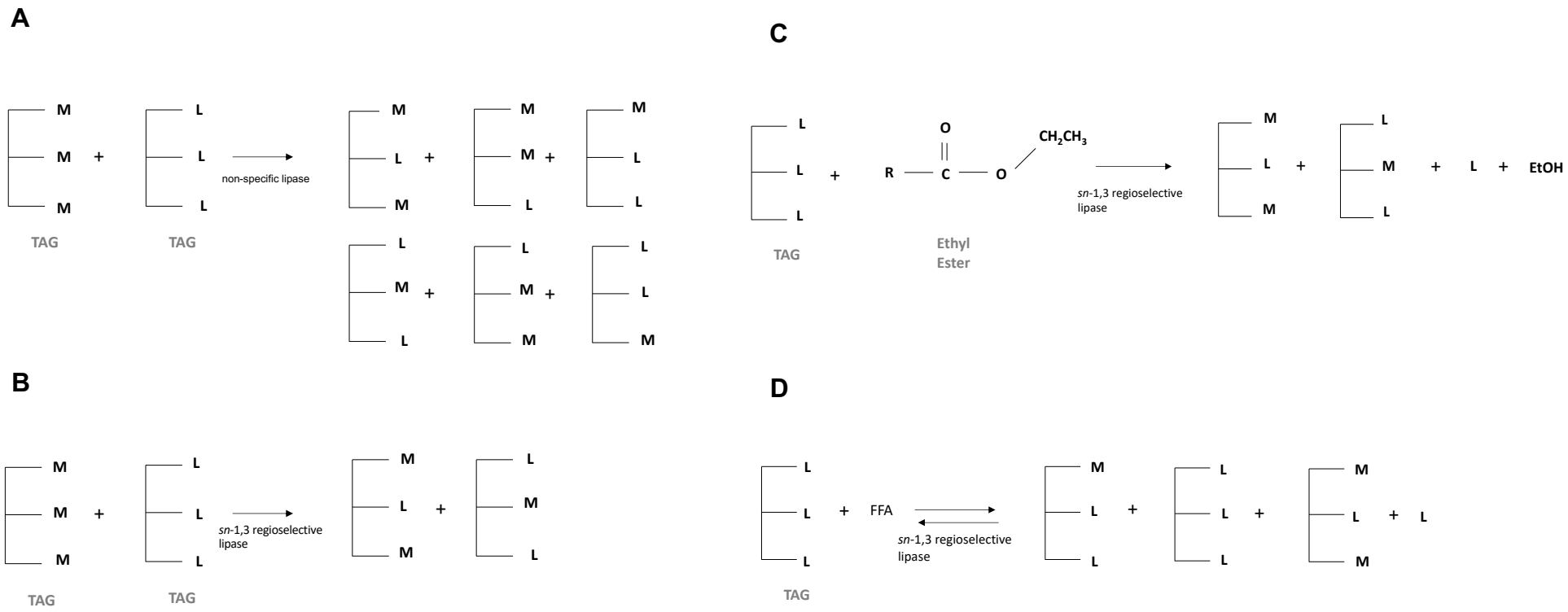


Figure 3. (A) Non-specific enzymes and (B) *sn*-1,3-specific enzymes action and the results originated for structured lipids production, (C) Scheme of enzymatic interesterification reaction between one TAG molecule and an ethyl ester medium-chain FA with *sn*-1,3 specific lipase and (D) Scheme of enzymatic acidolysis reaction between one TAG molecule and free fatty-acid (FFA) with *sn*-1,3 regioselective lipase. It is maintained the long-chain FA (L) at *sn*-2 position by the specificity of enzyme and a FFA release. In all schemes, M and L letters are representations of medium- and long-chain FA, respectively (adapted from Utama *et al*., 2019 and Ferreira-Dias *et al*., 2019).

In the presence of solvent-free systems, downstream processes are more accessible, less components act in the reaction and less waste is produced, decreasing the costs since also high concentrations of product are obtained (Utama *et al*., 2019; Stergiou *et al*., 2013; Zhang *et al*., 2020). Therefore, the presence of systems without organic solvents, can become an advantage for the use of continuous bioreactors, for instance, with high reaction time. On the contrary, the usage of solvent systems can result in toxicity issues, as well as negative impacts on the environment, enzyme function and operational stability (Ferreira-Dias *et al*., 2022).

### 2.3.5. Bioreactors

Bioreactors can be defined as engineered units used for any biotechnological process, in order to improve quality of final products and greatest economical income. They are designed to provide a good and effective environment for enzymes or cells to produce the desired final products in high quantities (Erickson, 2019; Utama *et al*., 2019; Xu, 2003). Many different bioreactors are described. However, for the choice it is important their role regarding the final product produced, including its quality and other several factors, such as (i) flexibility, (ii) efficiency, (iii) stability and reusability of enzymes or cells and (v) the economic costs (Utama *et al*., 2019; Cooney, 1983) as well as other aspects such the biocatalyst and reactional and kinetic characteristics (Cabral *et al*., 2003).

Any bioreactor should be designed in order to reduce some limitations that can occur during the reactions, such as heat and mass transfers and also to control biocatalysts activity and stability, in parallel with the cost reduction of the whole process. Additionally, it is crucial to have in account the impact of conversion and their yield and role in raw material costs. Thus, one of the main objectives of reactor design is to maximize these factors and consequently, improve biocatalyst selectivity, being able to minimize the by-product creation (Cooney, 1983).

For the MLM lipids production, two types of reactors can be developed: batch (or discontinuous) and continuous mode.

The batch or discontinuous mode, beyond the low costs, since less and elementary amount of equipment and the simplest mode of action are required, is considered as the bioreactor more used to collect prior data at laboratory scale or for the production of TAG in small-scale. However, it is difficult to control heat transfer (through internal or external coils or applying a jacketed reactor) and some variations, beyond the accumulation of ineffective time and labour costs, due to constant actions in start and end procedures. In this type of bioreactor, some factors have a considerable weight in yield of MLM synthesis, such as substrate ratio, enzyme load, stirring rate, temperature and reaction time itself (Utama *et al*., 2019; Jadhav *et al*., 2021). Here, the bioreactor feed is done in the start with substrate and immobilized enzyme. The result of this reaction shows the substrate being consumed and the final product increases its concentration, where *X* is described as conversion of the substrate, defined by Eq. 1.

$$X = (S_0 - S)/S_0 \quad \text{(Eq.1)}$$

For this type of production in batch, the most common mode, namely adopted in small industries, is the reactor working with agitation, which allows temperature control, beyond a better mixture of components with the heterogeneous lipases in the middle of reaction media. Even so, batch has some drawbacks such, when immobilized enzymes are in different organic solvents, it may cause some damage due to agitation inside the bioreactor. Also, since the substrate concentration decreases over the reaction time, the reaction rate decreases as well, so a huge degree of conversion is not obtained. Therefore, there are options such as plug-flow or packed bed reactors (PBR), which enable the bioreactor hydrodynamic changes (Jadhav *et al*., 2021).

On the contrary of discontinuous mode, continuous bioreactors have the substrate continually entering in the feed. Hereby, one of the main objectives is to retain the biocatalyst immobilized, allowing to keep constant the quality of products control, as well as the ease of operation. Moreover, with the choice of this type of bioreactor, it is possible to decrease the costs associated with enzymatic processes. It is necessary attention to the residence time ($\tau$) (Eq. 2), related with substrate volumetric flow (Q), working volume inside the reactor (enzyme bed) (V) and the void fraction ($\varepsilon$) (the enzyme volume occupied by the mixture of substrates, where Vs is the volume of the substrates and V is the volume of the enzyme bed) (Eq. 3), developed in previous studies (Xu *et al*., 2002 & 1998), which is an important parameter in the increase of reaction rate and thus, volumetric productivity (Osório, 2008; Utama *et al*., 2019; Utama *et al*., 2020; Osório *et al*., 2006).

$$\tau = (V_{enzyme\ bed} * \varepsilon)/Q \quad \text{(Eq. 2)}$$

$$\varepsilon = Vs/V \quad \text{(Eq. 3)}$$

On one hand, as it occurs in discontinuous mode, in continuous bioreactor also exists the agitation condition, known as continuous stirred tank reactor (CSTR), where the components are homogeneously mixed and the temperature is kept inside the reactor. In addition, the exit flow is identical to the inside bioreactor flow. When the operation starts, the concentration of substrate decreases and of the product increases and after a while, both concentrations remain in steady-state. Its agitated mode allows easy control of temperature and environment, which is closed, namely when performing slower reactions. Furthermore, stirred reactors have greater capacity for multi-phase systems (Paula *et al*., 2015).

On the other hand, it is possible the use of packed-bed reactors (PBR), where the conversion degree alters, with no mixture, with enough residence time provided by reaction mixture in the bed formed, which results in high flow and mass transfers during the reaction, as the substrates cross the reactor tube, which contains the immobilized enzymes. Beyond this, although not very prone for large scale, this second type of continuous bioreactor has some advantages such as the facility of operation, advances in final product, as well as high reaction rate and mass transfer, compared to CSTR. The agitation in CSTR may cause degradation in the

biocatalyst and therefore, possible loss on stability and activity, compared to the fixed-bed option. Moreover, PBR reactors well operate with low amounts of water, since with large quantities the bed is clogged and it is impossible to operate (Zhang *et al*., 2020; Jadhav *et al*., 2020; Xu, 2003).

Within column reactors, it is possible to characterize regarding to the type of flow: fixed (also known as PBR) or fluidized (fluidized-bed reactors (FBR)) flow.

In PBR, the kinetics conditions are more favourable, since it does not have the presence of high tensions created by agitation. The enzymes used in reactions are well positioned and packed in the chamber of the reactor (bed of enzymes), while reaction medium passes through it. Consequently, the medium contacts with the biocatalyst and the reaction occurs and results into a final product. Although having some limitations when talking about substrate and biocatalyst immobilization type, it is considered the best option for enzymatic essays due to ease of operation, high efficacy, application on large-scale, accessible separation of products as well as the reuse of immobilized biocatalysts (Utama *et al*., 2019; Osório, 2008; Cozentino *et al*., 2020; Jahdav *et al*., 2020). In addition, due to the rupture possibility of the supporting materials for immobilized lipases, which allows acyl migration (unfavourable alterations in fatty acid positions) and the creation of many types of medium- and long-chain triacylglycerols (MLCTs) molecules, PBRs are favoured in the enzymatic processes of MLM type (Xu, 2000), since in continuous enzymatic reactors, lipases are immobilised into a column, reducing these events (Mu *et al*., 1998).

In FBR, the biocatalyst is mixed in a fluid phase imposed by fluidized gas or liquid, on the contrary to fixed flow, creating a good fluid vertical movement. In addition, their use tries to correct previous drawbacks of fixed-bed such as mass and heat transfer limitations (Dhyani *et al*., 2019; Guda *et al*., 2015; Rajmohan *et al*., 2020).

### 2.3.6. Operational stability

As mentioned in sub-chapter 2.2.2., the utilization of commercial lipases is sometimes restrained by the low operational stability. To surpass this limitation, the immobilization of these enzymes may be a most common solution, being able to achieve some cost benefits on their use. However, there are other possibilities to increase the operational stability such as the addition of stabilizing additives, change of enzyme structure through chemical modification or crystallization (Illanes, 1999).

The use of biocatalysts can be considered often unstable and variable. Thus, in that sense, it was demonstrated that the operational stability of immobilized lipases depends on several parameters such as the characteristics of biocatalyst, the fat water content and the presence of oxidation products, related to the degree of refining of these fats and even the composition of reaction media. In addition, choosing the most suitable bioreactor for the reactions and the microenvironment conditions imposed (temperature, substrate and product concentrations in the microenvironment) will also affect this factor. The use of continuous bioreactors for the production on MLM lipids provides for cost savings on biocatalysts. As a result, the biocatalysts with the highest activity and operational stability should be selected (Ferreira-Dias *et al*., 2022). The stability of a biocatalyst is determined as the ability to retain its activity over

the time along reactor operation. It is one important parameter to control the feasibility of process implementation at industrial scale and one of the main factors to select an enzyme (Osório *et al*., 2009; Illanes, 1999).

The essays to produce new TAG molecules with MLM configuration may be developed using low-cost raw-materials, namely olive pomace oil, which is solvent-extracted from olive pomace after olive oil extraction by mechanical meanings. The composition of olive pomace oil (OPO) composition is similar to that of olive oil. However, since it is extracted by solvent, it must be refined prior to consumption. On olive oil composition, oleic acid (C18:1) is the main component (55-83%), followed by palmitic (C16:0) and linoleic (C18:2) acids (7.5-20 % and 3.5-21 %, respectively). Different studies on oleic acid-rich diets have shown that oleic acid can be promote the reduction of total cholesterol and low-density lipoprotein (LDL) levels and also reduces the ratio of LDL to high-density lipoprotein (HDL) cholesterol. Additionally, oleic acid has shown some positive impact in the prevention of cardiovascular disorders and anticancer effects (Wang *et al*., 2012; Utama *et al*., 2020). Therefore, saturated fats are replaced with oleic acid-rich oils, which provide a variety of health advantages. The use of crude oils as raw materials in structured lipids production, are considered an advantage for the low-cost of enzymatic processes use. Thus, an initial substrate with high quality, instead of crude oils, can decrease the activity and stability of enzymes of MLM production, although their availability and economical costs (Mota *et al*., 2020i; Ferreira-Dias *et al*., 2022).

In addition, it is known that the specificity of position, substrate as well as stereospecificity of lipases is affected by the solvent polarity or partition coefficient (log P) (hydrophobic/hydrophilic coefficient), water activity ($a_w$), immobilization carrier and also reaction environmental conditions. The stability of lipases is considered a crucial topic for different applications given their high economic cost and since it is conditioned by the reaction surroundings, including moisture content, temperature, substrate composition and concentration, lipase and product concentrations, as well as the presence of oxidation products, leading to the loss of 3D-structure of enzymes (denaturation) (Utama *et al*., 2019; Stergiou *et al*., 2013).

Several deactivation models have been applied to biocatalysts during SLs synthesis, in continuous or batch bioreactors, to assess operational stability. The first-order deactivation model and Sadana's series-type deactivation kinetics model (parabolic profile) are the most often used. Then, using these models it is feasible to estimate the half-life time parameter, *i.e*., the enzyme activity it is reduced to 50 % (Ferreira-Dias *et al*., 2022). One of the alternatives for the production of SLs is enzymatic route to study enzyme activity and operational stability in a continuous reaction.

# 3. Materials and Methods

## 3.1. Materials

Three olive pomace oil (OPO) samples from different batches were kindly donated by UCASUL – União de Cooperativas Agrícolas do Sul, from Alvito, Beja, Portugal. The commercial *sn*-1,3 regioselective enzymes Lipozyme RM IM (*Rhizomucor miehei* lipase immobilized on a microporous anion exchange resin) and Lipozyme TL IM (*Thermomyces lanuginosus* lipase immobilized on silica gel) were a gift from Novozymes A/S, Denmark. Current laboratory material was used for all characterization assays of crude oil and MLM lipids production and quantification.

*OPO characterization*

For OPO characterization, the following reagents were used: (i) sodium hydroxide 0.01N, diethyl ether (≥ 99.8%) ethanol and phenolphthalein solution (2% (m/v)) were used in acidity determination, (ii) 2,2,4-Trimethylpentane (iso-octane) 99.8% were used to determine $K_{232}$ and $K_{270}$ and (iii) *n*-hexane (≥ 95%) was used for dilution in chromatic characterization (pigments). For oxidation products and pigments determination, UNICAM UV/Vis spectrophotometer was needed. In fatty acid composition determination, capillary-column gas chromatography was used (Commision Regulation EEC, 1991).

*Interesterification and acidolysis reactions*

Four interesterification and four acidolysis reactions were developed in continuous bioreactor with packed-bed with enzymes (Lipozyme TL IM or Lipozyme RM IM), at the temperature of 40 ºC. Ethylic ester from caprylic acid (C8EE) (> 98 % purity) and ethylic ester from capric acid (C10EE) (> 98 % purity) were used for interesterification and respective acid form (C8:0 and C10:0) (> 98 % purity) for acidolysis reactions, were acquired from TCI Europe, Belgium.

*Gas chromatography and analysis of reaction products*

For the sample preparation, different reagents were used such as *n*-hexane (≥ 95%) p.a. from Fisher Chemical, tetrahydrofuran (≥ 99.9%) p.a. from Sigma Aldrich, monononadecanoine (internal standard in gas chromatography analysis) (> 99%) from Ladoran Fine Chemicals AB, Sweden, piridine p.a. from Fisher Chemical, dichloromethane p.a. from Sigma Aldrich, *n*-heptane (≥ 99.5 %) p.a. from Fisher Chemical and N-methyl-N-trimethyl-silyl-triflouracetamide (MSTFA) (> 90 %) from TCI Europe N.V.

## 3.2. Methods

### 3.2.1. Olive pomace oil characterization

Before starting the characterization, the olive pomace oil was centrifuged (40 ºC, over 30 minute at 10000 rpm) in order to remove the major impurities in the oil.

For the OPO characterization, it was necessary the assessment of three parameters related with oil quality: acidity, oxidation products (regarding the absorbances at 232 nm and 270 nm) and colour, by chlorophyll pigments quantification.

Both oil acidity and oxidation products were determined according to the Commission Regulation Nr 2568/91 (1991), relative to olive and olive pomace oils characteristics and related analysis methods (Commission Regulation EEC, 1991).

Acidity was determined regarding the quantity of free fatty acids on the sample, in this case, respecting to the oleic acid, since it is the major FA of the olive oil. A mass of 1 g of OPO was added to 100 mL of ethanol and diethyl ester solution (1:1 proportion, v:v) as solvent. Then, phenolphthalein (pH indicator) (2/3 drops) was added to the solution of oil and sodium hydroxide solution (0.1 N) was used for FFA titration. At the end, Eq. 4 was used in order to determine the acidity value, in percentage of FFA, where *V*. is the volume spent in titration (mL), *c* represents the titration solution concentration, M the molecular mass of oleic acid (282 g/mol) and *m* the mass of sample weighted. Three repetitions were made.

$$\text{Acidity} = V * c * \frac{M}{1000} * \frac{100}{\text{m}} \quad \text{(Eq.4)}$$

Oxidation products were analysed by absorbances at 232 and 270 nm, which are related with quality of the oil ($K_{232}$: presence of initial products of oxidation; $K_{270}$: presence of final oxidation products). In presence of 2.5 g of oil in 25 mL iso-octane solution (1 % m/v), both absorbances of the sample were read in UV/Vis spectrophotometer with quartz cells (Commission Regulation EEC, 1991).

Colour characterization was assessed by quantification of chlorophyll pigments in the samples. According to Pokorny *et al*., (1995), these compounds are expressed as milligram of pheophytin a in 1 kg of oil, after measurement of absorbances at 630, 670 and 710 nm against air. The pigments content was determined with the use of absorptivity of pheophytins, following Eq. 5, in which *C* represents the content of chlorophyll pigments (pheophytin a; mg/kg oil), *A* the absorbance at 630 ($A_{630}$), 670 ($A_{670}$) and 710 nm ($A_{710}$), respectively, and *L* the thickness of cells used in spectrophotometer.

$$C = \frac{345.3\ x\ (A_{670} - 0{,}5\ x\ A_{630} - 0{,}5\ x\ A_{710})}{\text{L}} \quad \text{(Eq.5)}$$

In addition, FA composition was determined by LET – Laboratório de Estudos Técnicos, ISA, Lisbon using capillary gas chromatography in accordance with Commission Regulation Nr 2568/91 (1991) (Commission Regulation EEC, 1991).

### 3.2.2. Interesterification and acidolysis reactions in continuous reactor

*Reactional media*

To start the interesterification and acidolysis assays, a mixture with OPO (920 g) and ethyl esters or FFA (C10EE (420 g), C10:0 (356 g), C8EE (358 g) or C8:0 (300 g)) was previously prepared, in order to have the substrate mixture for the reaction, in a 1:2 molar ratio of oil/FFA or oil/Ethyl Ester. This mixture was placed in an Erlenmeyer flask, in a water bath at 40 ºC, on a magnetic stirred plate. The water bath was also used for the recirculation system to keep this temperature on column reactor. The amount of 10 g of Lipozyme TL IM or Lipozyme RM IM was added to the reactor column. The height of filled enzyme bed was measured and the respective volume was calculated: an apparent volume of 19.16 mL with TL IM and 31.10 mL with RM IM.

*Flow rate selection*

The bioreactor used consists of a glass column with 2 cm internal diameter and 20 cm height, having a double jacket and a glass sieve (G0) on the bottom (Figure 4). The immobilized enzyme is put in the bioreactor and the continuous flow of reaction medium is upwards to avoid bed clogging and channelling, in a unidirectional way in a steady state.

The calibration of the pump was needed to understand the volumetric flow along the continuous bioreactor equipment, in presence of substrates and a packed-bed in the bottom end. The flow rate will determine the residence time of the substrates in contact with enzyme bed and, therefore, the bioconversion attained. The first sample corresponds to four times the residence time, until reach the steady state (Heinzle, 2009).

To calculate the volumetric flow with the passage of oil through the bioreactor column, different values of the scale of the peristaltic pump were tested. For each setting, several samples were taken to a volume of 5 mL and the time to recover that volume was counted. The flow rate ($Q$) was obtained by the ratio between the volume ($V$, mL) and respective time ($t$, min). Consequently, the residence time ($\tau$) was calculated, according to the Eq. 2. High residence times are required to achieve high quantity of structured lipids.

The flow rates obtained during the interesterification reactions, vary between 0.5-0.6 mL/min, obtaining, theoretically, residence times of 11 and 20 minute for Lipozyme TL IM and Lipozyme RM IM, respectively.

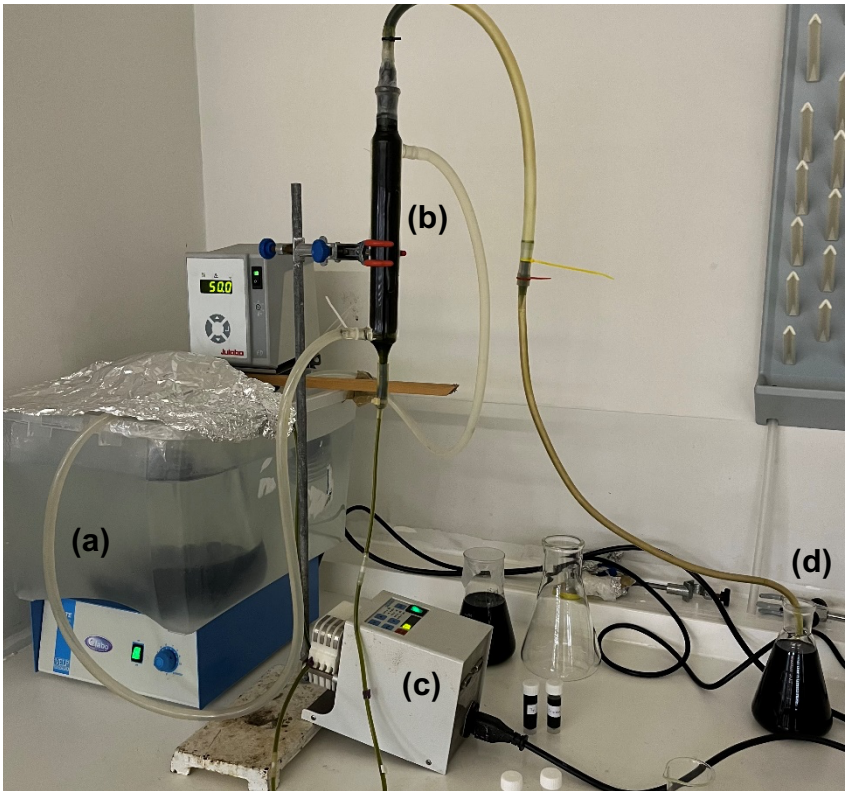


Figure 4. Representation of packed-bed continuous bioreactor for interesterification and acidolysis reactions: (a) 40ºC water bath with substrate reservoir, under agitation, (b) Packed-bed column with water jacket and glass sieve, (c) Peristaltic pump and (d) Product recovery vessel.

*Sampling*

For all reactions, a first sample, without enzymatic modification, was taken before starting the reactions with biocatalysts. The duration of the reactions was 60-122 h, with interruption during the night. For each time, a sample of 5 mL was taken. Over time, the collected samples were frozen at -18ºC for frequent analysis on gas chromatograph equipment (GC).

### 3.2.3. Gas chromatography analysis of reaction products

Before starting the analysis, the samples needed to be derivatized. For each reaction sample, 0.05 g were weighted in a 10 mL volumetric flask and this volume was filled with hexane. Subsequently, 0.5 mL of this mixture was added to a pear-shaped flask, from which the solvent was evaporated using rotavapor equipment (< 120 mbar and temperatures between 30-35 ºC) for 10 minute. To these pear-shaped flasks, with only dry sample, a mixture of 400 μl of internal standard (0.05 g of monononadecanoine in 25 mL of tetrahydrofuran), 200 μl of pyridine and 200 μl of N-methyl-N-trimethyl-silyl-triflouracetamide (MSTFA) was added in the mentioned order, with subsequent waiting period of 15 minute. Then, 4 mL of heptane was added to the flasks, shaken and about 1 mL of this mixture was transferred to the vials used in the GC. The internal standard

(IS) is used to standardize peak areas by dividing each peak are by the area of the IS peak, in each chromatogram.

Before any run of sample sequences on the GC equipment or between runs of large amounts of samples, it was necessary to clean the chromatography column. This cleaning is done in the presence of two solutions: (i) a mixture (1:1) of dichloromethane with methane and (ii) heptane.

Analysis of samples by gas chromatography is initiated by automatic injections on-column of only 1 μL with a syringe. As detector, this equipment uses a flame, created by air and hydrogen. The temperature in the injector was at 53ºC, while in the detector, it is at 380ºC. The gases were applied with a flow rate of 30 mL/min (helium, nitrogen and hydrogen) or 300 mL/min (compressed air).

Each sample is transported through the helium gas (carrier gas) along the chromatography capilar column Agilent J&W 123-5711 (15 m x 0.320 mm x 0.1 μm), where then, the samples were analysed through the separation of its components. This separation was dependent on an increase in detector temperatures from 200 ºC to 380 ºC. Separation step requires the following time/temperature gradient: the temperature increases at a rate of 15 ºC per minute up to 180 ºC, after an initial temperature of 50 ºC during 1 minute. Following this, the temperature rises at a rate of 7ºC per minute until it reaches 230 ºC. At a final rate of 10 ºC/min, the temperature rises to 365 ºC and stays there for 12 minute. In addition, the presence of nitrogen gas helps the transport of the sample to the detector. Each analysis ends after 48 minute (45 of run + 3 of post run), with a subsequent cooling of the oven, before the start of the next injection. Each sample was injected twice. At the end of each injection, the STANDBY method was always applied, so that the runs could occur autonomously and the equipment could access the initial temperatures.

With the analysis of the sample sequences, different chromatograms are generated. They are capable of representing by peaks, the compounds of interest. To identify the compounds, the following standards were used: capric acid (Retention time, RT = 7 minute), caprylic acid (RT = 5 minute), ethyl ester of capric acid (RT = 6 minute), ethyl ester of caprylic acid (RT = 4 minute), oleic acid (RT = 12 minute), monononadecanoine (internal standard) (RT = 18 minute) and triolein (RT = 31 minute). The identification of groups of MAGs, DAGs and TAGs were based on the European Standard (EN 14105, 2011) Therefore, for the detection of new and unknown compounds, the following reference retention times were used: FFA (lower than 17 minute), MAG (between 17.8 and 20 minute), DAG (between 20 and 24 min) and TAG (higher than 24 minute). An example of the chromatograms obtained by OpenLAB CDS program for each interesterification and acidolysis reactions is shown below in Figures 5-8, with the identification of each peak.

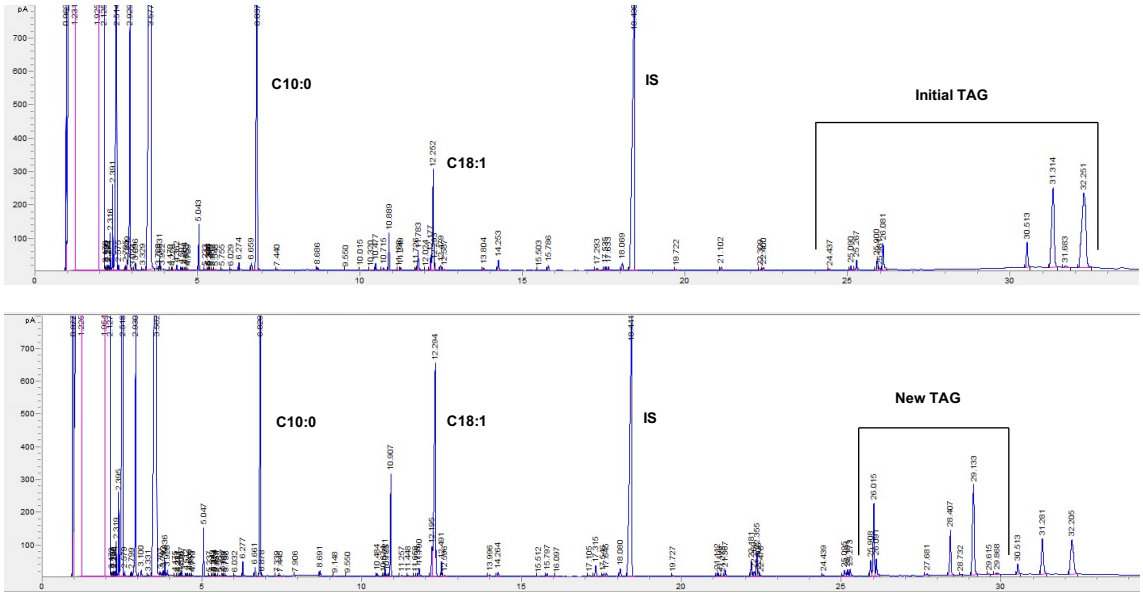


Figure 5. Example of chromatogram of the Lipozyme TL IM-catalyzed acidolysis reaction of OPO and C10:0 at t = 0 h and t = 10.5 h, obtained by OpenLAB CDS program (IS = Internal Standard).

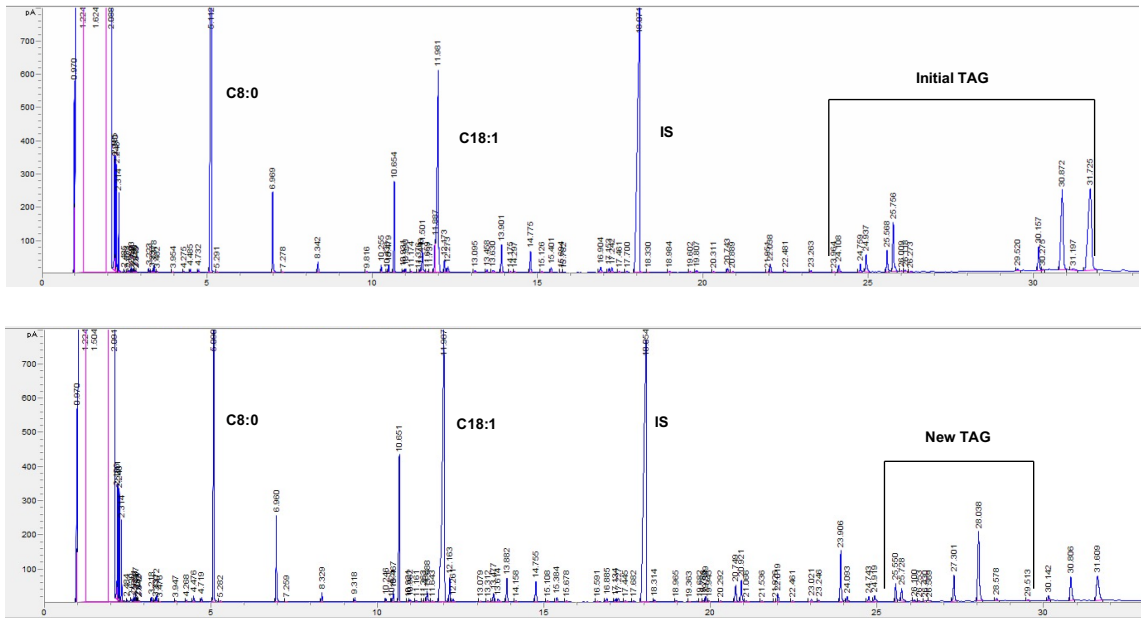


Figure 6. Example of chromatogram of the Lipozyme TL IM-catalyzed acidolysis reaction of OPO and C8:0 at t = 0 h and t = 13.5 h, obtained by OpenLAB CDS program (IS = Internal Standard).

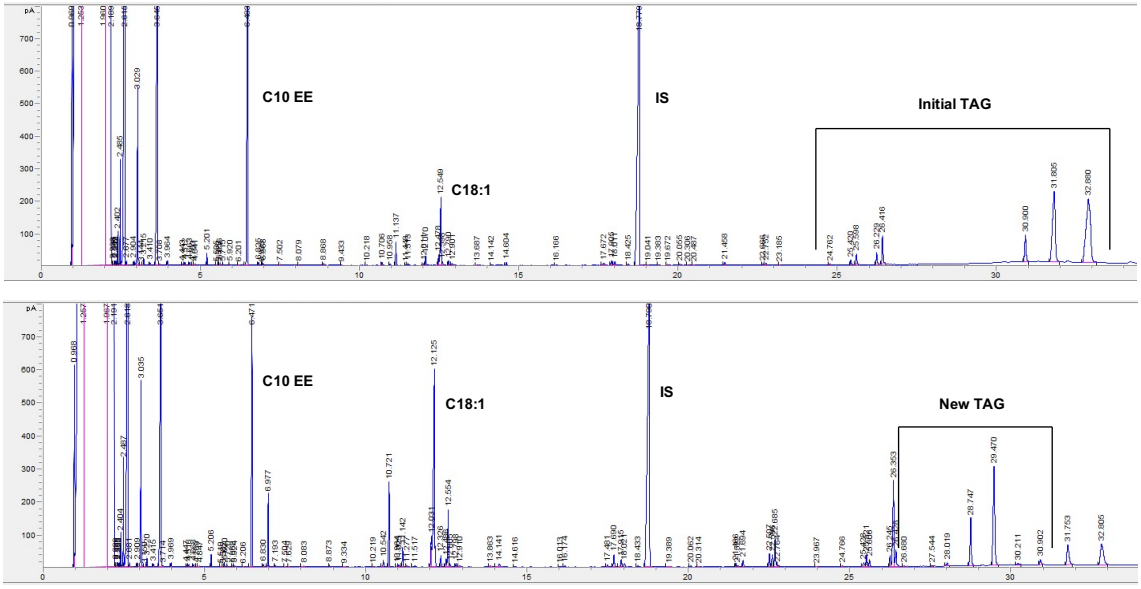


Figure 7. Example of chromatogram of the Lipozyme TL IM-catalyzed interesterification reaction of OPO and C10EE at t = 0 h and t = 11.5 h, obtained by OpenLAB CDS program (IS = Internal Standard).

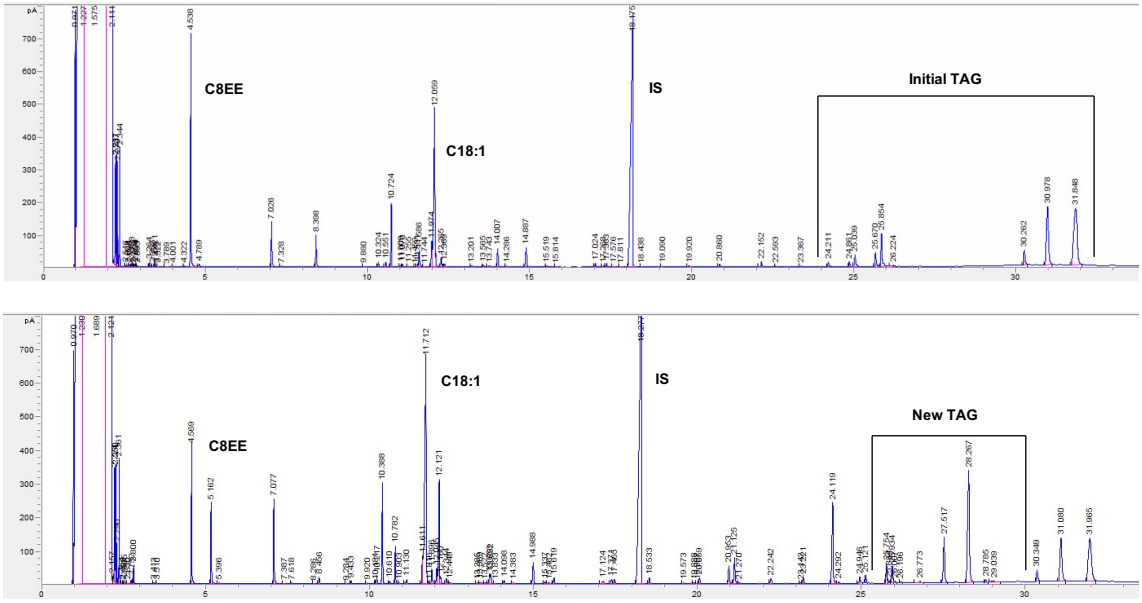


Figure 8. Example of chromatogram of the Lipozyme TL IM-catalyzed interesterification reaction of OPO and C8EE at t = 0 h and t = 10.5 h, obtained by OpenLAB CDS program (IS = Internal Standard).

After obtaining the results, the ratios (R) were calculated, for all peaks, between the area of this peak and the area of the peak of the internal standard (Eq. 6). With this ratio, the new TAG molecules that were formed were quantified through the reaction yield. This yield (Y, %) was calculated through the area of peaks that are identified as new TAGs and the area of peaks that

correspond to the initial TAGs of the sample (in the chromatogram at time 0) (Eq. 7). Furthermore, the degree of conversion (X, %) of TAG and EE/FFA (D) (Eq. 8 and Eq. 9, respectively) was determined.

$$R = \frac{\text{Apeak (a)}}{A\ (IS)}$$

(Eq. 6)

$$Y = \frac{\frac{\Sigma \frac{A_{NEW\ TAG}}{A_{IS}}}{\Sigma \frac{A_{peak}}{A_{IS}}}}{\frac{\Sigma \frac{A_{TAGi}}{A_{IS}}}{\Sigma \frac{A_{peak}}{A_{IS}}}} * 100$$

(Eq. 7)

$$X_{TAG} = \frac{\frac{\Sigma \frac{A_{TAGi}}{A_{IS}}}{\Sigma \frac{A_{peak}}{A_{IS}}} - \frac{\Sigma \frac{A_{TAGf}}{A_{IS}}}{\Sigma \frac{A_{peak}}{A_{IS}}}}{\frac{\Sigma \frac{A_{TAGi}}{A_{IS}}}{\Sigma \frac{A_{peak}}{A_{IS}}}} * 100$$

(Eq. 8)

$$X_D = \frac{\frac{\Sigma \frac{A_{D/Ai}}{A_{IS}}}{\Sigma \frac{A_{peak}}{A_{IS}}} - \frac{\Sigma \frac{A_{D/Af}}{A_{IS}}}{\Sigma \frac{A_{peak}}{A_{IS}}}}{\frac{\Sigma \frac{A_{D/Ai}}{A_{IS}}}{\Sigma \frac{A_{peak}}{A_{IS}}}} * 100$$

(Eq. 9)

### 3.2.4. Operational stability

The activities of the biocatalysts were estimated along the continuous operation, through the results obtained by the GC analysis of the collected samples, as the incorporation of medium-chain FA n the TAGs of olive pomace oil. The operational half-life time (operation time needed to reduce the original activity from 1 to 0.5, or from 100% to 50%) was also estimated through the deactivation profiles of each enzyme.

For each sample, at time *t*, the yield of new TAGs (Eq. 7), conversion of TAGs (Eq. 8) and Ethyl Esters and FFA (Eq. 9) were determined. It is assumed that for time zero (equal to four residence times), the value corresponds to 100 % of the activity. The residual activity during the bioreactor operation was calculated as a function of the initial activity, both for yield and

conversion degrees. The initial activity was considered to be the one that corresponds to the steady state when it is reached, considering the yield obtain for new TAG and conversion degree for TAG and Ethyl Esters/FFA form (Eq. 10 and 11).

$$\mathrm{Y} = \frac{\eta f}{\eta i} * 100 \quad \text{(Eq. 10)}$$

$$X = \frac{Xf}{Xi} * 100 \quad \text{(Eq. 11)}$$

The “Solver” add-in in Excel was used to fit enzyme deactivation models to experimental data. Then, the half-life times were calculated using the fitted models (*e.g.* linear, first-order and Sadana deactivation models).

The first-order deactivation kinetics, in continuous process, is given by Eq. 12:

$$a = A * e^{-k_d t} \quad \text{(Eq. 12)}$$

In this equation, *A* is a constant, *t* corresponds to the bioreactor operating time (h) and $k_d$ is the deactivation rate constant ($h^{-1}$).

In addition, the Sadana model is characterized as the series-type enzyme deactivation kinetics (Eq. 13) (Sadana, 1979) and was also tested.

$$A = 100 - 50 * k_d t^2 \quad \text{(Eq. 13)}$$

By the fitted models, the half-life times, were calculated according to the first-order (Eq. 14) or Sadana models (Eq. 15),

$$t\frac{1}{2} = \frac{\ln 2}{k_d} \quad \text{(Eq. 14)}$$

$$t\frac{1}{2} = {k_d}^{-1/2} \quad \text{(Eq. 15)}$$

### 3.2.5. Cost and specific productivity studies

After monitoring the behaviour of each of the biocatalysts along the reactions, the cost associated to each one was calculated, in order to understand which would be the best choice in

terms of economic viability. This parameter was calculated based on the measured flow rate (Q) of 0.6 mL/min for the interesterification and acidolysis reactions, the mass of enzyme bed used in each continuous operation (10 g), the time (t) in which the biocatalyst maintained its activity and the price for each biocatalyst, given by Novozymes A/S, Denmark (110 €/Kg and 923 €/Kg for Lipozyme TL IM and Lipozyme RMIM, respectively, September 2022). In addition, the specific productivity for the production of New TAGs was estimated, using the average of the absolute New TAG values and also the mass of biocatalyst used.

# 4. Results and Discussion

## 4.1. OPO characterization

The results for the analysis for OPO characterization are described in Table 2. The results are presented by the average of three replicates ± standard deviation for each oil sample (centrifuged, OPO-1, -2 and -3).

Concerning oil quality, the acidity consists of the quantity of FFA resulting from the hydrolysis of TAGs. For OPO, no limit is specified by Regulation 2568/91, while for refined OPO, the acidity must be less than or equal to 0.3. The centrifuged oil samples presented 12.05 % (OPO-1), 15.06 % (OPO-2) and 28.75 % (OPO-3) FFA. This increase in acidity in OPO samples may be related to the storage conditions of olive pomace prior to oil extraction. OPO-1 was obtained in December 2021, while OPO-2 and OPO-3 samples were obtained later from April to June 2022. The oil may undergo certain hydrolysis processes during this stage that are catalysed by lipases and raise its acidity.

The oxidation products are also related with the quality of oil. In the case of crude acidic oils, the absorbances at 232 nm and 270 nm are associated with presence of hydroperoxides and aldehydes, ketones and short chain free fatty acids formation, regarding the first and secondary products of oxidation process, respectively. The values of each absorbance are reported through the specific extinction coefficient (K), and presented in Table 2. Following the Regulation 2568/91, K value for crude OPO is not defined. However, for all samples of OPO, the values are considered high (around K=5) which means that these oils show a high oxidation stage, both for primary products and for secondary products (Absorbance at 232 nm and 270 nm, respectively).

The chlorophyll pigments, which are more readily soluble in the oil and solvent, are result of a solvent extraction of crude oils, responsible for the green intensity of olives. In the olive pomace oil used, their presence is an important parameter for the quality of this oil (Pokorny *et al*., 1995). These pigments have a pro-oxidant activity, which means they will catalyse the oxidation reaction. As for the acidity and oxidation products determination, there is no defined limit for the quantification of chlorophyll pigments. Chlorophyll pigments varied from 367 to 447.5 mg of pheophytin *a* per kg of OPO (Table 2). It is reported that these parameters are associated with the influence of different factors, such as olives cultivars, harvest year, region and olive maturation stage (Ouachab *et al*., 2013; Serafini *et al*., 2019; Yücel, 2012). Furthermore, the observed discrepancies across oil samples might be explained by changes in olive cultivars and phases of fruit development, as chlorophyll pigments reduce through olive ripening (Peres *et al*., 2016).

In terms of fatty acid composition, OPO is quite comparable to olive oil (Commission Regulation (EEC) Nº 2568/91), since oleic acid (C18:1), in the three samples, has the highest content (≅ 69-71 %), followed by palmitic acid (C16:0) (≅ 14 %) and linoleic acid (C18:2) (≅ 10-11 %). In lower amounts, stearic acid (C18:0) accounted for 2.23 % while palmitoleic acid for 2.07 % (Table 2). The harvest year, the cultivar and the region of each sample may be used to explain the small variations between the values of the three oils. These FA composition and levels are consistent with recent research (Heinzl *et al*., 2022) and show that the OPO has good nutritional

quality and is a significant source of essential PUFA, particularly linoleic acid, which is critical in the prevention of cardiovascular diseases. In unsaturated fatty acids, antioxidant properties have also been studied by the food industries, and they can be used as nutraceutical additives in product composition (Durante *et al*., 2017).

Table 2. Results of characterization essays (acidity, oxidation products, chlorophyll pigments and free fatty acid composition) for three samples of olive pomace oil centrifuged (OPO-1, OPO-2 and OPO-3) (it is represented an average value of 3 replicas ± standard deviation for each sample).

| **Characterization essays** | **OPO-1** | **OPO-2** | **OPO-3** |
|---|---|---|---|
| Acidity (%) | 12.05 ± 0.45 | 15.06 ± 0.93 | 28.75 ± 0.91 |
| $K_{232}$ | 4.92 ± 0.09 | 6.18 ± 0.36 | 6.06 ± 0.71 |
| $K_{270}$ | 1.90 ± 0.08 | 2.36 ± 0.16 | 2.28 ± 0.26 |
| Chlorophyll Pigments (Pheophytin a; mg/kg) | 366.97 ± 31.35 | 447.50 ± 15.84 | 374.35 ± 11.70 |
| FA content (%) | | | |
| C18:1 | 69.05 ± 3.31 | 70.98 ± 3.41 | 69.91 ± 3.36 |
| C16:0 | 14.37 ± 0.69 | 13.50 ± 0.65 | 13.45 ± 0.65 |
| C18:2 | 10.78 ± 1.72 | 9.64 ± 1.54 | 10.60 ± 1.70 |
| C18:0 | 2.11 ± 0.30 | 2.23 ±0.31 | 2.35 ± 0.33 |
| C14:0 | 0.03 ± 0.01 | 0.03 ± 0.01 | 0.04 ± 0.01 |
| C16:1 | 1.46 ± 0.20 | 1.35 ± 0.19 | 1.32 ± 0.18 |
| C17:0 | 0.12 ± 0.02 | 0.12 ± 0.02 | 0.11 ± 0.02 |
| C17:1 | 0.22 ± 0.04 | 0.22 ± 0.04 | 0.20 ± 0.04 |
| C18:3 | 0.84 ± 0.09 | 0.87 ± 0.10 | 0.90 ± 0.10 |
| C20:0 | 0.43 ± 0.05 | 0.44 ± 0.04 | 0.47 ± 0.06 |
| C20:1 | 0.31 ± 0.04 | 0.31 ± 0.04 | 0.31 ± 0.04 |
| C22:0 | 0.18 ± 0.06 | 0.20 ± 0.06 | 0.22 ± 0.07 |
| C22:1 | <0.012 | <0.012 | <0.012 |
| C24:0 | 0.09 ± 0.03 | 0.10 ± 0.04 | 0.11 ± 0.04 |
| *trans* isomer C18:1 | 0.05 ± 0.03 | 0.05 ± 0.03 | 0.07 ± 0.04 |
| *trans* isomer C18:2 and C18:3 | 0.08 ± 0.04 | 0.07 ± 0.04 | 0.12 ± 0.06 |

## 4.2. Flow rate and residence time selection

The flow rate and residence time are related to each other, *i.e.* the calculated value of residence time is directly dependent on the flow rate obtained during the reactions. For all the reactions, the molar ratio between OPO and ethyl ester/acid forms was kept 1:2. The flow rate (Q, mL/min), working volume (V, mL), void fraction ($\varepsilon$) and residence time ($\tau$, min) values of each reaction are presented in Table 3.

In reactions with Lipozyme TLIM, the bed volume filled by the biocatalyst was 19.16 mL. For Lipozyme RLIM, the bed volume filled by the biocatalyst was 30.79 mL. In both cases, the measured flow rate was 0.6 mL/min.

Table 3. Flow rates (Q) and respective residence times (τ) for each enzyme in continuous bioreactor (Lipozyme TL IM and Lipozyme RM IM).

| **Parameters** | **Biocatalysts** | |
|---|---|---|
| | Lipozyme TL IM | Lipozyme RM IM |
| Measured flow rate (Q, mL/min) | 0.6 | |
| Working volume (V, mL) | 19.16 | 30.79 |
| Void fraction (ε) | 0.34 | 0.39 |
| Residence time (τ) (minute) V/Q | 10.86 | 20 |

As previously mentioned, the residence time and the void fraction were calculated by Eq. 2 and Eq. 3. For Lipozyme TL IM, the value for the void fraction was 0.34, and the residence time was 11 minute (Xu *et al*., 1998). For Lipozyme RM IM, void fraction was 0.39 and the calculated residence time corresponded to 20 minute.

The reactor operated with an upward flow, where there was a certain resistance of the enzyme particles, in relation to the fluid of the mixture that passes through the column. The mixture was admitted in the bottom of the column with a flow rate of 0.6 mL/min, through the peristaltic pump. In this way, it allowed longer contact time between the enzyme and the mixture, and therefore, higher residence times were verified for both enzymes.

## 4.3. Low-calorie MLM synthesis

Interesterification and acidolysis reactions of OPO were carried out in the presence of C10EE, C8EE, C10:0 and C8:0, in solvent-free media, with Lipozyme TL IM and Lipozyme RM IM as biocatalysts. Reactions were performed using one of the three batches of OPO (OPO-1, 12.05 % (C10EE with Lipozyme TL IM and Lipozyme RM IM; C10:0 with Lipozyme TL IM), OPO-2, 15.06 % (C10:0 with Lipozyme RM IM) and OPO-3, 28.75 % (C8EE with Lipozyme TL IM and Lipozyme RM IM; C8:0 with Lipozyme TL IM and Lipozyme RM IM). Both interesterification and acidolysis reactions of OPO were carried out in a continuous packed-bed bioreactor at 40ºC, between 60 and 122 h.

In presence of a *sn*-1,3 regioselective lipase, the original FA of the TAG molecule in positions 1 and 3 will be changed, while in position 2 they will be kept. If the replacement of the FA is done in one or two positions, it is possible to obtain DAG or TAG molecules, respectively. In these positions, in turn, the MCFA used as substrate (ethyl ester or acid forms) will be placed (Costa *et al*., 2017).

New TAG yield, TAG conversion and FFA/Ethyl Esters conversion parameters are depicted in Figures 9-14. The formation of new TAGs is observed, together with the consumption

of the original TAG molecules (OPO). The new TAG molecules formed can be of the MLM or MLL type, according to the replacement of FA that was performed with the substrate involved (Figure 3). Since both interesterification and acidolysis reactions generate intermediate molecules, MAGs and DAGs, it is possible to observe that the conversion values of TAGs and esters/acids are, during the reaction operation, higher than those of the yield in new TAGs.

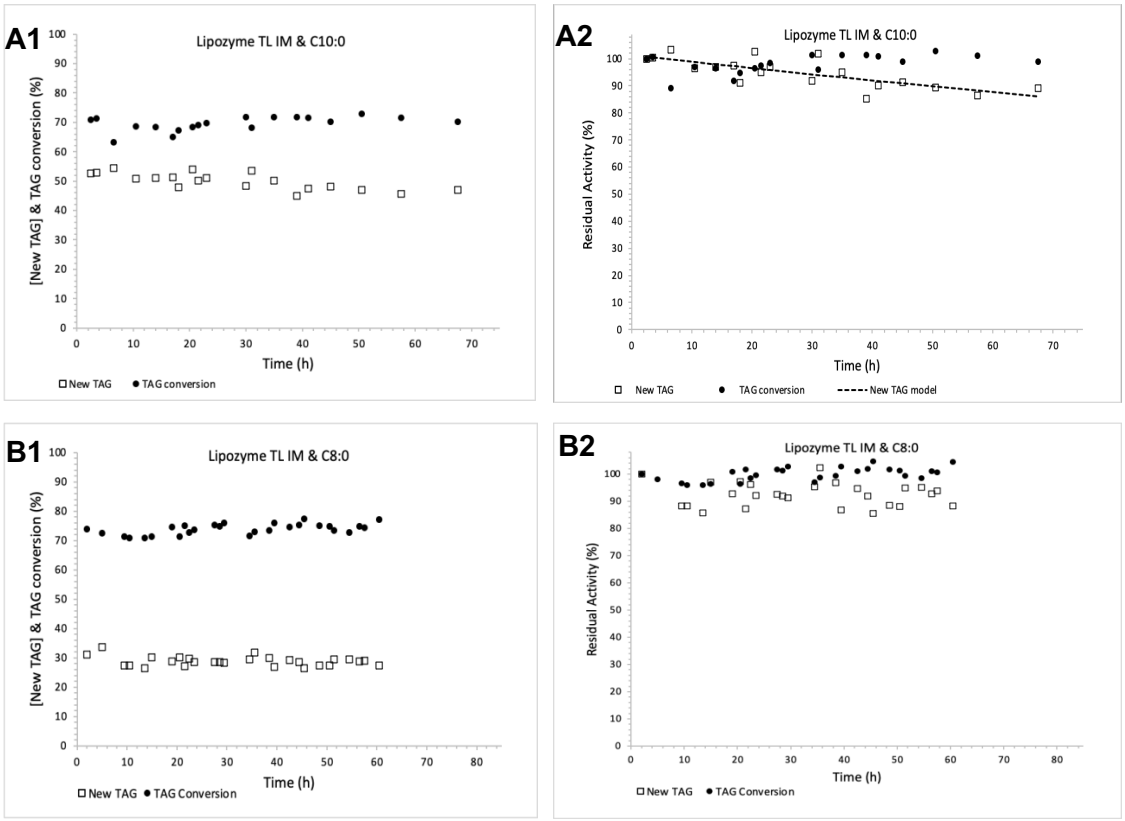


Figure 9. New TAG yield and TAG conversion (%) along acidolysis reaction of OPO with (A1 and A2) C10:0 + Linear deactivation model and (B1 and B2) C8:0, catalysed by Lipozyme TL IM biocatalyst. OPO samples with 12.05 % (A1 and A2) and 28.75 % (B1 and B2) of acidity.

For C8:0 reaction with Lipozyme TL IM, the average of the yield in new TAG is about 29 % and 31 % with Lipozyme RM IM (Figure 9 - B1 and Figure 11 - B1), with 28.75 % of oil acidity. C8EE new TAGs yield values around of 50 % were attained for Lipozyme RM IM and 49 % with Lipozyme TL IM. The conversion of the residual TAGs was above 70 % with both enzymes for the interesterification with C8EE as well as for the acidolysis with the respective FFA (Figure 10 – B1 and Figure 12 – B1), with 28.75 % of oil acidity. These results can be explained by the affinity that the enzymes have for each of the acyl sources. The yields in New TAG being lower for C8:0, indicates a lower affinity of the biocatalysts for medium-chain FA. In fact, caprylic acid is more polar than C10:0 or their respective ethyl esters, and presents a smaller main chain. Between C10:0 and C10EE, no major differences were found (Figures 9-12 - A1), with 12.05 % and 15.06 of oil acidity.

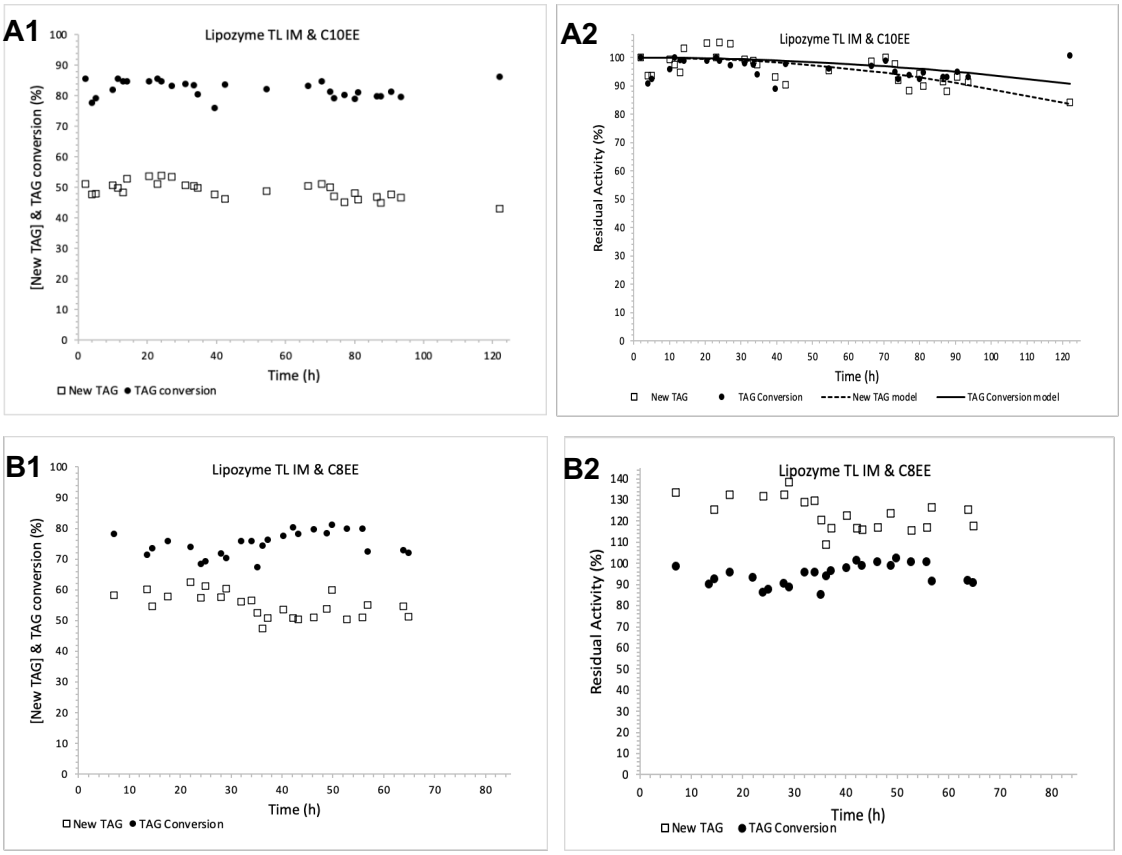


Figure 10. New TAG yield and TAG conversion (%) along interesterification reaction of OPO with (A1 and A2) C10EE + Sadana deactivation model and (B1 and B2) C8EE, catalysed by Lipozyme TL IM biocatalyst. OPO samples with 12.05 % (A1 and A2) and 28.75 % (B1 and B2) of acidity.

For C10EE with Lipozyme RM IM (Figure 14 – B2), with 12.05 % of oil acidity, the ester conversion decreases (from 100 % to 70 %), which could be explained by the formation of TAG molecules with the esterification of only one molecule of medium-chain fatty acid at position *sn*-1 or *sn*-3, i.e., the formation of MLL or LLM molecules. In New TAG profile, higher yield values were obtained in reaction with C10EE as acyl-donor (50-56 %), when compared to results obtained with C8EE (34-54 %). This behaviour demonstrates the preference of the enzyme for the molecule of C10EE, as expected (Figures 10 and 11 - A1 and B1).

With Lipozyme TL IM and Lipozyme RM IM, both reactions with C10:0 and C8:0, presented lower FFA conversions (30-50 %), in relation to the use of ethyl esters (Figures 13 and 14 – A1). This may also be explained by the polarity of the molecules, with the biocatalysts having more affinity for esters than for FFA.

In the characterization of the initial OPO, although the acidity value increased among the samples used (Table 2), the behavior of the biocatalysts along the reactions (Figures 9-14), was not influenced, showing to be independent of the FFA content. The other parameters analyzed (chlorophyll pigments and oxidation products) did not differ much between the OPO samples.

However, on the FFA conversion, there was a slight decrease in the incorporation percentage of the reaction of C8:0 with both biocatalysts with the use of 12.05 % and 28.75 % of acidity (Figures 13 and 14 – A1).

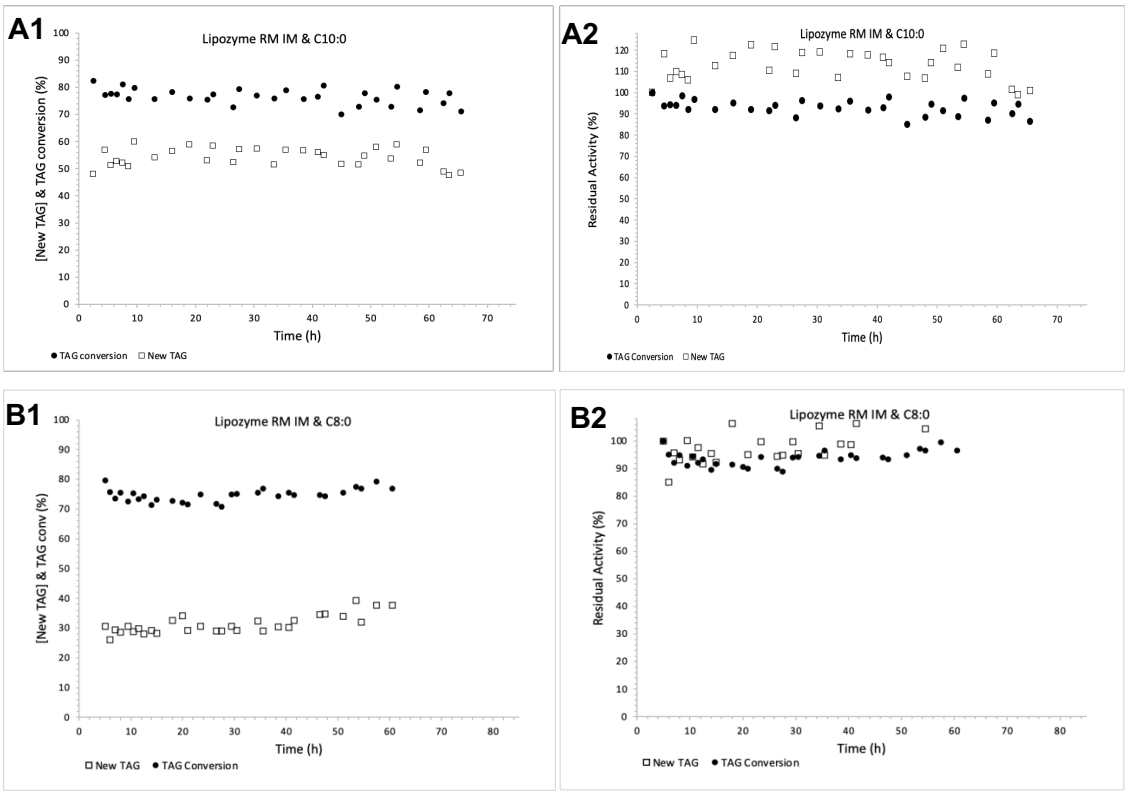


Figure 11. New TAG yield and TAG conversion (%) along acidolysis reaction of OPO with (A1 and A2) C10:0 and (B1 and B2) C8:0, catalysed by Lipozyme RM IM biocatalyst. OPO samples with 15.06 % (A1 and A2) and 28.75 % (B1 and B2) of acidity.

When *sn*-1,3 regioselective lipases are used, the highest value of medium-chain FA incorporation is considered to be 66.70 %, with a yield of new TAG of 100 % (Costa *et al*., 2018; Xu, 2003; Caballero *et al*., 2014).

With the same aim to valorise the use of OPO (Ferreira-Dias *et al*., 2020), Lipozyme RM IM showed a similar behaviour for both types of FAs (C10:0 and C8:0), with yield values of 53-57 %. An identical behaviour of Lipozyme RM IM with C10:0 occurred in this work, with the same oil and biocatalyst, where the yields of New TAG were 50-54 %.

Bassan *et al*. (2019), in batch acidolysis, of grapeseed oil, catalysed by Lipozyme RM IM, C10:0 incorporation values of 34.53 % were obtained. In our study, the incorporation of this FFA into New TAG molecules showed values above 40 %. It will be more favorable that the reaction will occur in a continuous column reactor, without the direct contact with mechanical stirring and allowing the enzyme to remain with its activity and be able to incorporate the FFA into the new acyl positions.

Kim *et al*., (2006) demonstrated that with roasted sesame oil, unrefined and rich in oleic acid (as OPO), the total incorporation of C8:0, using Lipozyme RM IM, reached a value of 42.5 mol %. For OPO, with the same FA composition, a value close to 34.8 mol % was obtained.

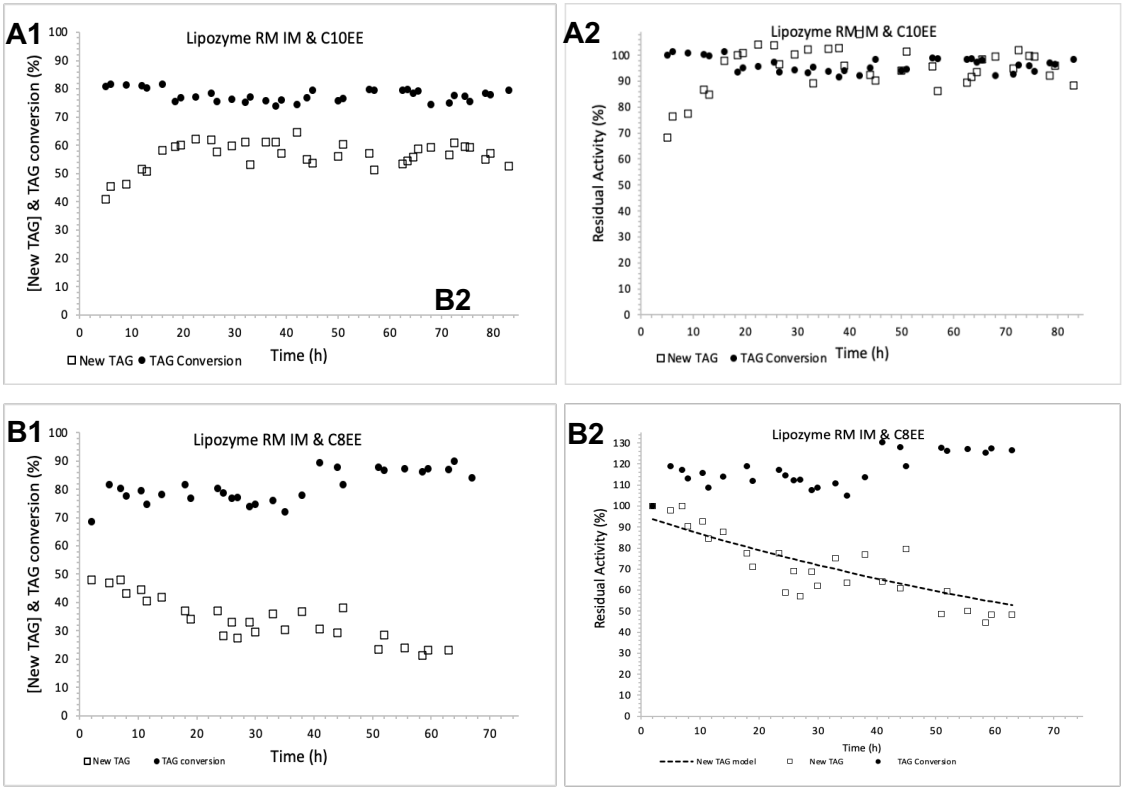


Figure 12. New TAG yield and TAG conversion (%) along interesterification reaction of OPO with (A1 and A2) C10EE and (B1 and B2) C8EE + First-order deactivation model, catalysed by Lipozyme RM IM. OPO samples with 12.05 % (A1 and A2) and 28.75 % (B1 and B2) of acidity.

In batch mode studies, such as Nunes *et al*., (2011), with extra virgin oil as raw material, it was found that in solvent-free media and with molar ratio of 1:2, the incorporation of C10:0 was 19.9 %, while for C8:0 it was 28.8 %, with Lipozyme TL IM as biocatalyst. These values were lower than those obtained in this work. For the same lipase, the values obtained were on average of 36.89 % and 34.11 %, respectively. Therefore, using olive pomace oil with a high acidity for continuous production may bring advantages and better conditions for the formation of MLM lipids.

Similar behaviour was observed by Heinzl *et al*. (2022) for Lipozyme RM IM. They used crude high acidic OPO (3.4–20% acidity) to produce SLs by batch acidolysis with caprylic or capric acids, or interesterification with their ethyl esters, catalysed by Lipozyme RM IM. This lipase showed similar activity in acidolysis and interesterification (yields: 47.8–53.4%, 7 h, 50 $^{o}$C) and was not affected by OPO acidity. Lipozyme TL IM exhibited a clear preference towards interesterification over acidolysis, with new TAGs yields of 17, 20, 42.7 and 51.8 % respectively with C10:0, C8:0, C8EE and C10EE, after 7 h reaction.

In addition, Lipozyme RM IM has been shown to exhibit a higher degree of incorporation than Lipozyme TL IM (Abed *et al*., 2018; Wang *et al*., 2012; Rodrigues *et al*., 2010; Heinzl *et al*., 2022). In our study, Lipozyme TL IM exhibited similar high incorporation values for acyl donors. Furthermore, the high percentage of New TAG yields produced with this enzyme should be mentioned, which is indicative as an advantage, to the use of this biocatalyst in a continuous bioreactor.

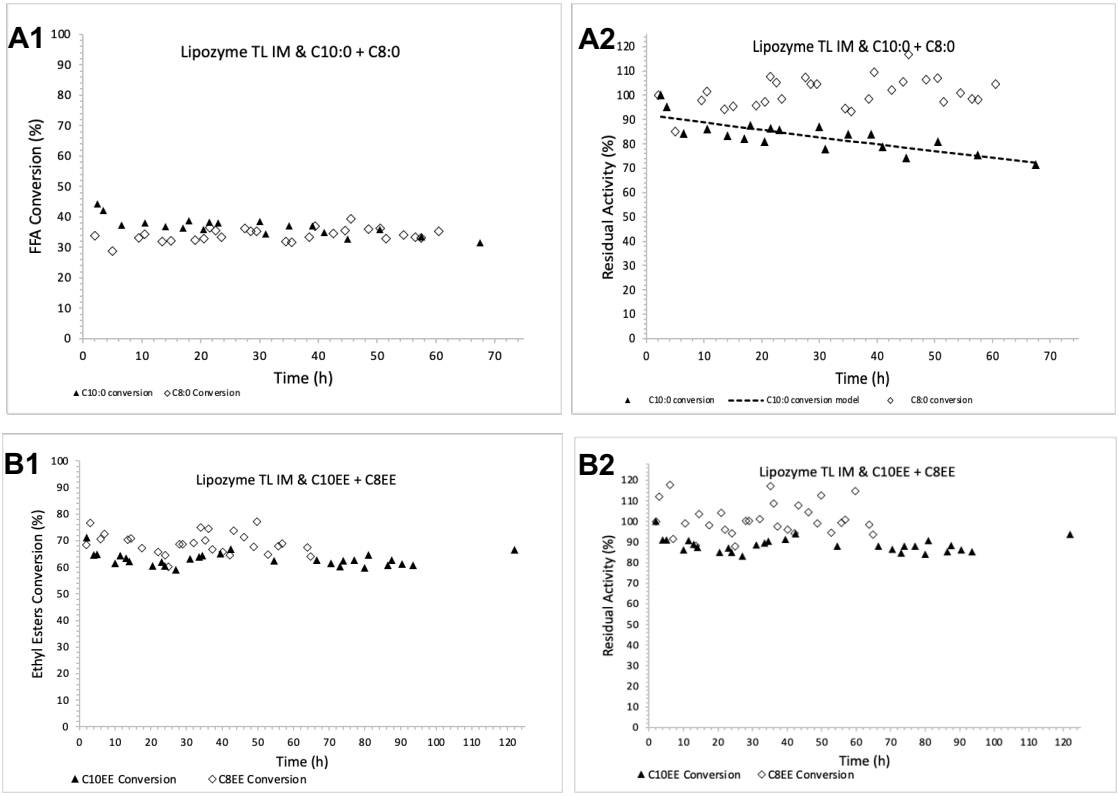


Figure 13. FFA/Ethyl Ester conversion (%) along acidolysis and interesterification reactions of OPO with (A1 and A2) C10:0 + Linear deactivation model and C8:0 and (B1 and B2) C10 EE and C8 EE, catalysed by Lipozyme TL IM. OPO samples with 12.05 % (A1, A2, B1 and B2) and 28.75 % (A1, A2, B1 and B2) of acidity.

Since the interesterification and acidolysis reactions are reversible owing to the activity of lipases, the incorporation of the ethyl esters and acids employed rely on the ratio of the mixture (acyl donors/OPO) used as substrate for the processes. However, the probability that the necessity for acquired product purification will increase with the ratio acyl donors/TAG due to the excess of unconverted FFA or Ethyl Ester in the reaction medium (Xu *et al*., 1998). The reaction eventually reached an equilibrium phase, depending on the ratio of substrate mixture used. Other variables, including the amount of enzyme present or the temperature and reaction system, may also play a role in achieving this steady state (Xu, 2003).

It is important to note that the majority of studies in the literature employed batch reactors to carry out their experiments in the production of MLM lipids. Many factors influence product

output and quality, including reagent molar ratio, substrate composition and bioreactor operation parameters (Cozentino *et al.,* 2020). The approach suggested in this thesis study is a potential system for industrial operations that produces nutritional and functional products for food sector.

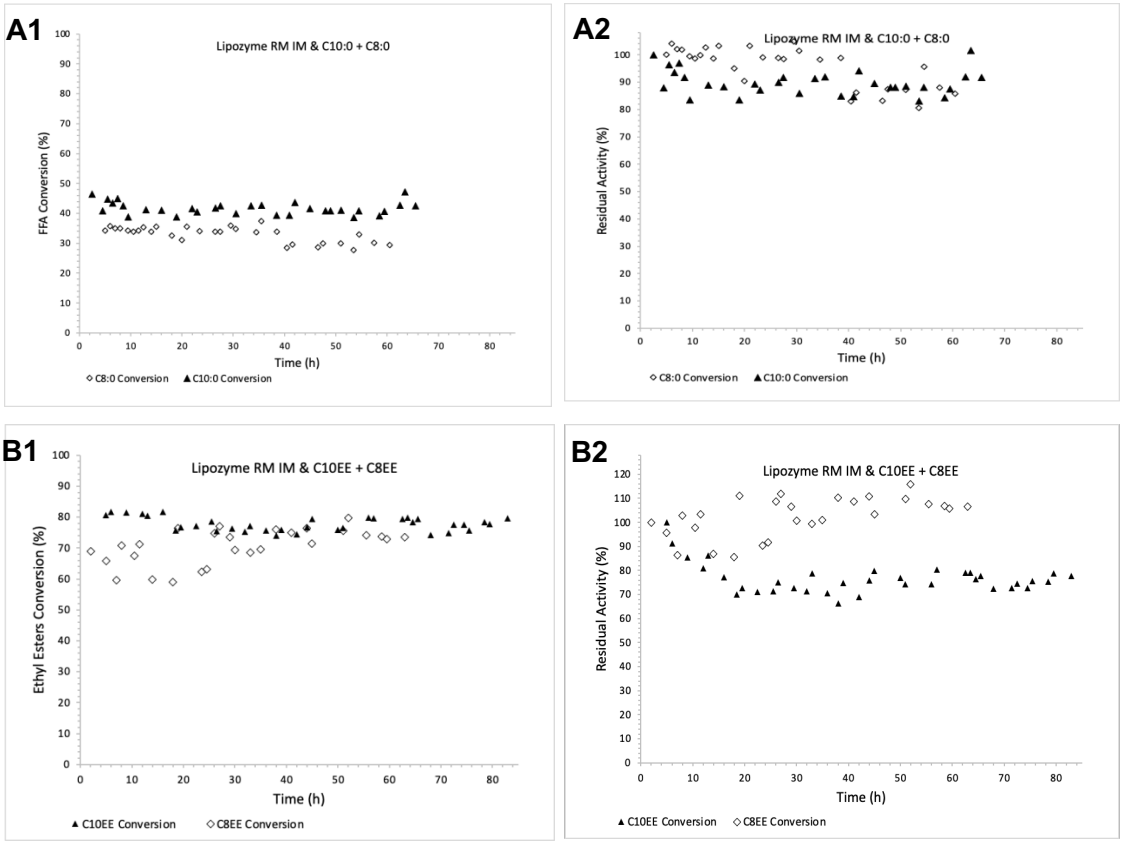


Figure 14. FFA/Ethyl Ester conversion (%) along acidolysis and interesterification reactions of OPO with (A1 and A2) C10:0 and C8:0 and (B1 and B2) C10 EE and C8 EE, catalysed by Lipozyme RM IM. OPO samples with 12.05 % (B1 and B2), 15.06 % (A1 and A2) and 28.75 % (A1, A2, B1 and B2) of acidity.

## 4.4. Operational stability tests

The activities of the biocatalysts were estimated along the reactions.

The data obtained for interesterification reactions were transformed into normalised data, calculating the residual activity of the enzymes along the operation. The ratio between the observed yield for each sample and the initial yield was chosen to measure the residual activity of the biocatalysts. The residual activity during the bioreactor operation was calculated as a function of the initial activity, both for yield and conversion degrees.

The deactivation profiles of Lipozyme TL IM and Lipozyme RM IM and the parameters for each deactivation models fitted (linear, first-order deactivation and Sadana models) to these data points are represented in Figures 9, 10, 12 and 13 and Table 4, respectively.

Table 4. Operational stability parameters of biocatalysts Lipozyme TL IM and Lipozyme RM IM (deactivation model and half-life time, h) for each interesterification and acidolysis reactions, with respective OPO with different acidity and FFA/Ethyl Esters as acyl donor (C10EE, C8EE, C10:0 and C8:0), in continuous bioreactor (n.d.: not determined).

| Type of reaction | FFA/ethyl esters | Biocatalyst | Deactivation model | Half-life (h) | Oil Acidity (%) |
|---|---|---|---|---|---|
| Interesterification | C10EE | Lipozyme TL IM | Sadana | 213.3 | 12.05 |
| | C8EE | | n.d. | - | 28.75 |
| | C10EE | Lipozyme RM IM | n.d. | - | 12.05 |
| | C8EE | | First-order | 74 | 28.75 |
| Acidolysis | C10:0 | Lipozyme TL IM | Linear | 228.3 | 12.05 |
| | C8:0 | | n.d. | - | 28.75 |
| | C10:0 | Lipozyme RM IM | n.d. | - | 15.06 |
| | C8:0 | | n.d. | - | 28.75 |

Both biocatalysts, Lipozyme TL IM and Lipozyme RM IM, presented high operational stability in continuous bioreactor operation.

In acidolysis reaction with C8:0 and interesterification with C8EE, catalysed by Lipozyme TL IM, no deactivation model was fitted (Figures 9 – B2 and 10 – B2), with 28.75 % of oil acidity. The profiles from the reactions catalysed by Lipozyme RM IM, showed that in the acidolysis reactions with C8:0 and C10:0 and interesterification with C10EE (12.05 %, 15.06 and 28.75 % of oil acidity, respectively), the biocatalyst kept its activity along the continuous operation of the bioreactor and therefore have a high operational stability (Figures 11 – A2, B2 and 12 – A2).

In acidolysis with C10:0, Lipozyme TL IM biocatalyst showed a linear deactivation kinetics with a half-life of 228.3 h, either when deactivation was assayed in terms of new TAG or FFA conversion profiles (Figure 9 – A2), with 12.05 % of oil acidity. In interesterification reaction with C10EE, Lipozyme TL IM was slowly deactivated, following a time-series deactivation Sadana model, which was applied in new TAG and TAG conversion profiles with 213.3 h and 283 h of half-life time, respectively (Figure 10 – A2), with 12.05 % of oil acidity. This higher value of half-life for the conversion of TAGs can be explained by the progress of the reaction, which starts with the hydrolysis of ester bonds in TAGs followed by the esterification of FFA in free radicals. Since not all TAGs are converted into new molecules of TAG, intermediate products may be formed, such as MAGs and DAGs. In catalysed-interesterification by Lipozyme RM IM with C8EE, during 67 h of bioreactor operation, a first-order deactivation was observed although the biocatalyst never loses completely its activity (Figure 12 – B2), with 28.75 % of oil acidity.

Lipozyme TL IM showed a high operational stability for both interesterification with C10EE and acidolysis with C10:0 with similar high half-lives and the same oil acidity (Table 4). It means that the lipase has a similar behaviour, independently of the acyl donor.

Reactions that carried out continuously can be classified as steady-state. However, there may be some moments when the steady state does not occur or it takes longer to achieve, such as after some change in the operating conditions of the reaction or during a period of time at the beginning of the reaction. For C8EE with Lipozyme TL IM (28.75 % of oil acidity), a model of enzymatic deactivation is not defined, showing a delay of 13.5 h to reach steady state, probably due to a lack of uniformity in enzyme bed making more difficulty in mass transfer (Figure 10 – B2). In Figure 12 – A2, the same occurs with C10EE reaction catalyzed by Lipozyme RM IM (12.05 % of oil acidity) with a delay of 16 h to reach steady state (Doran, 1995).

The residual activity values of the biocatalysts in acidolysis with C8:0 was lower, compared to C10:0, although the conversion of TAG molecules was similar for both reactions (Figures 9 and 11 – A2, B2).

In C8EE reaction catalysed by Lipozyme RM IM, TAG conversion remains constant over time, with a high residual activity (above the 70 %), indicating that new TAG molecules are being formed with the enzymatic action (Figure 12 – B2).

The conversion parameter of FFA/Ethyl Esters in the reactions is translated into the capacity of these acyl donors to be incorporated into the new TAGs molecules. Both biocatalysts, in C10EE and C8EE reactions (Figure 13 and 14 – B2), did not lose activity and no deactivation model was fitted. Contrary, in the acidolysis with C10:0, Lipozyme TL IM followed a linear deactivation model. However, no loss of activity was observed Lipozyme TL IM in presence of C8:0 (Figure 13 – A2). This suggests a preference of Lipozyme TL IM for ethyl ester molecules.

In spite of differences in bioreactor type, OPO with 3.4-20 % FFA was used by Heinzl *et al*. (2022) for SL synthesis by acidolysis reaction with C8:0 and C10:0, in solvent-free systems, also using Lipozyme RM IM. With this biocatalyst, high operational stability values were observed. This behaviour was similar to this work where enzyme did not lose activity with oil samples with high acidity.

López-Fernández *et al*. (2021) observed that an increase in acidity of OPO, promote a raise in operational stability of the biocatalysts. The results also confirm that lipase activity was not to be affected by the acidity of the OPO used.

With the same type of bioreactor (PBR) and the same operation mode (continuous), using fat mixtures, Lipozyme TL IM showed a first-order deactivation kinetics, with half-lives of 135 h and 77 h, depending of the fat mixture used (Osório *et al*., 2006). Cozentino *et al*., (2020), in acidolysis with C10:0, using Lipozyme RM IM, estimated a half-life value of 209.6 h, which is similar to the value (228.3 h), obtained in this work for the reaction with C10:0 catalysed by Lipozyme TL IM. Lipozyme RM IM showed half-lives of 47 h and 54 h in acidolysis reactions with C8:0 and C10:0 with coffee grounds crude oil, respectively (Mota *et al*., 2020ii). These values represented a much lower operational stability in acidolysis than the results obtained in this study.

In Nunes *et al*., (2011), in batch acidolysis, there was no loss of activity of Lipozyme RM IM, with incorporation of C10:0 in the TAG molecules of olive oil as raw material. However, with the incorporation of C8:0 and in both reactions with Lipozyme TL IM, a deactivation was observed. In addition, in Mota *et al*., (2020ii), a loss of activity of biocatalyst Lipozyme TL IM was observed. This suggests that the operational stability of immobilized enzymes vary if they are used in batch or in continuous bioreactors. In batch mode, there is direct presence of agitation in contact with the enzyme that is placed in the reaction system. In continuous mode, the enzyme is placed in the packed-bed reactor column without agitation, or in a fluidised-bed, helping the integrity of the enzyme.

Lipozyme TL IM used in interesterification reactions of crude olive pomace oil (Mota *et al*., 2020ii) or Lipozyme RM IM in acidolysis of extra virgin oil in Nunes *et al*., (2011), batch reactors, presented the half-life values quite similar to those obtained in the present work.

Heinzl *et al*. (2022), in batch reactions, presented results in which Lipozyme RM IM exhibited better operational stability for the interesterification reactions, with higher half-life values than in the acidolysis reactions. In this work, in the profile of production of new TAG, Lipozyme TL IM presented a similar stability profile in the interesterification with C10EE, to the acidolysis with capric acid. Lipozyme RM IM showed the same situation, but in the interesterification reaction with C8EE, a first-order deactivation model with a half-life of 74 h was fitted.

Immobilised enzymes' operational stability is a crucial parameter to deal with the development of a continuous reaction, especially in industrial processes, since they affect the total cost of the process and influenced by a number of factors, including the linkage of the enzyme immobilisation support, sludge obstruction of the pores, friction support loss and presence of a column in the bioreactor (Paula *et al*., 2015; Poppe *et al*., 2018).

Table 5. Operational stability and residence time parameters, in batch and continuous bioreactors, with Lipozyme TL IM and RM IM as biocatalysts, in solvent-free systems, for SLs production (FBR: Fluidised-bed reactor; PBR: Packed-bed reactor).

| Biocatalysts | Reaction | Bioreactor | Operation mode | Parameters | Reference |
|---|---|---|---|---|---|
| Lipozyme TL IM | Acidolysis | - | Batch | First-order deactivation kinetics<br>Half-life = 50.4 h (C8:0) and 47.2 (C10:0) | Nunes *et al*., 2011 |
| Lipozyme RM IM | Acidolysis | - | Batch | First-order deactivation kinetics with half-life = 299 h (C8:0) and no deactivation (C10:0) | |
| Lipozyme TL IM | Acidolysis | - | Batch | Sadana model with half-lives of 163 h and 220 h | Mota *et al*., 2020ii |
| Lipozyme RM IM | Acidolysis | - | Batch | First-order deactivation kinetics with half-life = 51 h (C8:0) and time-delay + first-order (C10:0) with half-life = 57 h | Heinzl *et al*., 2022 |
| Lipozyme RM IM | Interesterification | - | Batch | Sadana model with half-life = 163 h (C8 ethyl) and first-order with half-life = 189 h (C10 ethyl) | |
| Immobilized lipase/acyltransferase from *C. parapsilosis* | Interesterification | FBR | Continuous | First-order deactivation kinetics<br>Half-life = 9 h | Osório *et al*., 2009 |
| Lipozyme RM IM | Acidolysis | PBR | Continuous | Residence time = 1.5-3.0 h | Nielsen *et al*., 2006 |

| | | | | | |
|---|---|---|---|---|---|
| Lipozyme RM IM | Interesterification | PBR | Continuous | First-order deactivation kinetics<br>Half-life = 7.67 days | Safra *et al*., 2008 |
| Lipozyme TL IM | Interesterification | PBR | Continuous | First-order deactivation kinetics<br>Residence time = 15 min<br>Half-life of fat-blends A and B = 135 h and 77 h, respectively | Osório *et al*., 2006 |
| Lipozyme RM IM | Acidolysis | PBR | Continuous | Residence time = 2 h | Xu *et al.* 1998 |
| Lipase from *Candida antarctica* (Novozym 435) | Interesterification | FBR | Continuous | Sadana kinetics model<br>Half-life = 17 days | Osório *et al*., 2005 |
| Lipozyme TL IM | Interesterification | PBR | Continuous | Residence time = 30-40 min | Xu *et al*., 2002 |
| Lipozyme RM IM | Acidolysis | PBR | Continuous | Half-life = 19.2 days | Kim *et al*., 2006 |
| Lipozyme TL IM | Interesterification | PBR | Continuous | Residence time = 16 min | Zhang *et al*., 2020 |
| Lipozyme TL IM | Interesterification | PBR | Continuous | Residence time = 30-40 min | Utama *et al*., 2020 |
| Lipozyme RM IM | Acidolysis | PBR | Continuous | First-order deactivation kinetics<br>Half-life = 209.64 h | Conzentino *et al.,* 2020 |

## 4.5. Costs and specific productivity studies

Either Lipozyme TL IM or Lipozyme RM IM could be chosen for continuous industrial processes, to produce low calorie lipids. To assess the economic viability of the enzymatic process, the cost associated with each biocatalyst was estimated. For each continuous operation, the time *t* in which the biocatalyst keeps its activity and productivity for the production of New TAGs and respective average yields in New TAGs, are presented in Table 6.

Table 6. New TAG associated costs of each reaction system, catalyzed by both biocatalysts (Lipozyme TL IM and Lipozyme RM IM) used in continuous reaction ($P_s$ = specific productivity, € = costs).

| Biocatalyst | Reaction System | Operation time (h) | New TAG (average Yield, %) | New TAG produced (kg) | Biocatalyst cost (€/ kg P) | $P_s$ (g New TAG/h.g biocatalyst) |
|---|---|---|---|---|---|---|
| Lipozyme TL IM | C8:0 | 60.5 | 28.85 | 0.58 | 0.55 | 0.95 |
| | C10:0 | 67.5 | 49.88 | 1.12 | 0.49 | 1.65 |
| | C8EE | 64.8 | 54.35 | 1.17 | 0.51 | 1.80 |
| | C10EE | 122 | 49.03 | 1.98 | 0.27 | 1.62 |
| Lipozyme RM IM | C8:0 | 60.5 | 31.28 | 0.63 | 4.61 | 1.03 |
| | C10:0 | 65.5 | 54.14 | 1.17 | 4.25 | 1.79 |
| | C8EE | 67 | 34.10 | 0.76 | 4.42 | 1.13 |
| | C10EE | 83 | 56.38 | 1.54 | 3.36 | 1.87 |

The estimated cost of biocatalyst to obtain 1 kg of New TAGs (Kg P) in a continuous reactor was calculated considering the following data: bioreactor flow rate of 0.6 mL/min (*Q*); total operation time (h) (*t*); reaction medium specific gravity of 0.92; biocatalyst load of 10 g, with a cost of 100 €/Kg of Lipozyme TL IM and 923 €/Kg of Lipozyme RM IM (cost available from Novozymes in September 2022).

The total amount of New TAGs (kg) produced after each continuous operation was calculated by the following equation:

$$New\ TAG = (Q \times t \times 60 \times Yield\ \% \times 0.92/100) \quad \text{(Eq.16)}$$

The cost of the biocatalyst used in each continuous operation (10 g) was 1 € of Lipozyme TL IM and 9.23 € of Lipozyme RM IM. The cost of biocatalyst per kg of New TAGs is obtained by dividing the total amount of produced TAGs by the cost of the enzyme bed.

The specific productivity was determined for each reaction in the continuous bioreactor. The data were determined by the ratio of New TAGs produced and total time. Consequently, the

specific productivity was given by the ratio of the productivity by the mass used of biocatalyst (Table 6).

For longer operation times, as is the case of the interesterification reaction of C10EE with Lipozyme TL IM (122 h) or with Lipozyme RM IM (83 h), lower costs are obtained, per Kg P. This relationship between the costs of each biocatalyst and the reaction time indicates the advantage of using these immobilised enzymes over a longer time of operation in the continuous reactor, since they presented high operational stability.

Regarding the specific productivity, the highest values were obtained for the reaction with C10EE and Lipozyme RM IM (Ps= 1.87 g New TAG/h.g biocatalyst), C8EE with Lipozyme TL IM (Ps = 1.80 g New TAG/h.g biocatalyst) and with C10 and Lipozyme RM IM (Ps = 1.79 g New TAG/h.g biocatalyst).

Based on the costs of each biocatalyst provided by Nozoymes A/S, Lipozyme RM IM has a cost over eight times higher than Lipozyme TL IM. However, Lipozyme RM IM presented in some systems better results in productivity than Lipozyme TL IM. Even though, the obtained results suggest that for industrial scale continuous processes, Lipozyme TL IM should be preferred due to its high operational stability in PBR, and to the much lower cost when compared to Lipozyme RM IM. It is worth mentioning that Lipozyme TL IM particles are very susceptible to magnetic or mechanical stirring and, therefore, not adequate for batch reuses or use in CSTR.

# 5. Conclusions and Future Perspectives

The main objective of the experimental work was the production of new MLM-type TAG molecules, through interesterification reactions between the TAGs of crude olive pomace oil and medium-chain fatty acids (or esters form).

Modification of fats and oils is one of the most important sectors in the food industry where new, cost-effective and environmentally friendly methods are required. Thus, natural oils, which are very cheap, can be used as raw materials for the production of structured lipids, which have a significant high added-value. Several structured lipids derived from lipase-catalysed processes are currently available on the market for use in meals or as supplements. It is crucial to look for new low-cost enzymes and encourage the use of low-cost raw materials, as crude olive pomace oil, for the production of lipids with low calories.

After olive pomace oil centrifugation, in order to reduce the solid impurities, three different samples of oil were used in MLM synthesis. All OPO samples had significant levels of oxidation products and chlorophyll pigments, and the acidity ranged between 12-29 %.

The interesterification and acidolysis reactions of olive pomace oil with each respective ethyl ester of FFA form (C10EE/C8EE for interesterification and C10:0/C8:0 for acidolysis) were performed in a continuous column packed-bed reactor, at 40$^{o}$C, along between 60-122 h. All the reactions were developed with molar ratio between OPO and ethyl ester/acid forms of 1:2. Both Lipozyme TL IM and Lipozyme RM IM were tested to study reaction kinetics and respective operational stability.

In acidolysis assays, the reaction with C8:0 as acyl donor, catalysed by Lipozyme TL IM and Lipozyme RM IM, showed a 29 % and 31 % for the yield of production of New TAG. The TAG conversion was above 70 % with both enzymes. In the corresponding interesterification reaction with the C8EE, the yield in New TAG stabilised 50 % values with Lipozyme RM IM and 40 % with Lipozyme TL IM. In the reactions with C10:0 and C10EE, no significant differences were presented (50 % and 49 %, respectively).

With both enzymes, acidolysis reactions showed lower conversion values. The affinity for acyl donors (FFA vs. ethyl esters), may account for these results.

Along the eight continuous runs, the TAG conversion exhibited a higher profile than the production of New TAGs, which demonstrated that throughout the re-esterification of New TAGs and consumption of residual TAGs, MAG or DAG molecules could be being formed.

Lipozyme TL IM was slowly deactivated, presence of C10EE, with Sadana model adjusted in New TAG and TAG conversion parameters, with half-lives of 213 h and 283 h. For Lipozyme RM IM with C10:0 in acidolysis, no deactivation was observed, but with Lipozyme TL

IM, the first-order stability model was applied, with half-life of 228.3 h. In interesterification reaction with C8EE, catalysed by Lipozyme RM IM, a first-order model was also adjusted with half-life of 74 h. Biocatalyst behaviour was not influenced by high acidity, showing to be independent of the oil residue.

These outcomes suggested that in the assays with Lipozyme TL IM, the activity was maintained throughout the reaction, demonstrating high operational stability. For this reason, and given that the immobilized lipase TL IM has a more viable costs results in continuous bioreactor, this biocatalyst may provide economic advantages. Therefore, the system tested in the present study can be submitted for scaling up in the food industry.

This study demonstrated that it is possible to produce MLM SLs in continuous bioreactors with crude acidic oils, decreasing the cost of oil refining and raising the value of olive pomace oil while also enhancing the process sustainability and reducing the environmental effect and promoting circular economy in the olive oil industries.

With the results obtain in present thesis can be used to support future work in the area of structured lipid production, by the use of by-products from the food industry, rich in fat and easily extracted. In this regard, it would be interesting to propose some optimization conditions in the method of producing structured lipids, in continuous reactors, using crude olive pomace oil as raw material. Among them, (i) the use of other immobilized enzymes or supports, in order to study the affinity and the behaviour of the enzymes for the substrates used in the interesterification reactions, evaluating the activity/operational stability relationship, (ii) use of extracted olive pomace as a support for enzyme immobilization, in order to take advantage of this residue, helping the circular economy in industries, (iii) expanding the use of sub-products from food industries for the production of different TAG structures, in continuous mode, (iv) optimization of continuous reaction media, with different conditions, to reduce the extent of degradative reactions of hydrolysis and lipid oxidation, (vi) optimization of the reaction conditions, such as extension of the operation time, in order to decrease the costs of the enzymes used and enhance the productivity, and (vii) use of different reaction substrate ratios, to optimize the yield of MLM structured lipids as a function of the molar ratio.

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