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Valorisation of Vegetable Oil Deodorizer Distillate by Enzymatic Reaction and Membrane Processing

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The White Rabbit put on his spectacles.
"Where shall I begin, please your Majesty ?" he asked.
"Begin at the beginning,", the King said, very gravely,
"and go on till you come to the end: then stop."

Alice’s Adventures in Wonderland, Lewis Carrol

So here’s my story...

Abstract

Large amounts of residues are produced by agro-food industries which, without a proper treatment and disposal, may create serious environmental problems. The recovery of added-value compounds from these industrial residues is very interesting from both environmental and economic point of view, especially if green technologies are used. Deodorizer distillates are by-products rich in bioactive compounds, as sterols and tocopherols, produced in the oil refining process. However, they cannot be used directly as food additive due to their high content in pesticides. The valorisation of deodorizer distillates through the recovery of added-value compounds free of contaminants (such as pesticides), is particularly challenging mainly due to the similar molecular weight, volatility and hydrophobicity of the target bioactive compounds and the contaminants.

The present Thesis proposes the production of an extract rich in steryl esters and free of pesticides, through the using of a nanofiltration process. The strategy followed is based on the increase of the molecular size of the target compounds, making possible their separation from pesticides, while maintaining or even increasing the bioactivity of the extract. Steryl esters are known as being more bioactive and soluble in hydrophobic matrices than their free form (sterols), which facilitates their uptake in human or animal consumption. On the other hand, their higher molecular weight (700-800 g mol^{-1}) relatively to pesticides (220-400 g mol^{-1}) enables their easier separation by methods based on molecular weight difference.

Steryl esters can be produced by enzymatic reaction between free sterols (*S*) and free fatty acids (*FFA*), contained in deodorizer distillates. The lipase from *Candida rugosa* is able to catalyse such reaction with satisfactory yields (>80%) in 24 hours and under optimal conditions. The enzyme concentration, water activity in the reaction medium and molar ratio of *FFA:S* were the parameters optimised to assure that the equilibrium is driven towards the esterification of sterols. A mass-balance model describing the transient-state of the reaction system was developed and assessed for different sources of sterols and free fatty acids.

The use of alternative and cheaper sources of free fatty acids (besides oleic acid) that available to the majority of the edible oil producers, such as refined oil and mixtures of deodorizer distillates with different compositions, was assessed for their potential economical advantage.

Ceramic ultrafiltration membranes and PTFE microfiltration membranes were used for a total recovery of the enzyme, but with partial deactivation of the enzyme was observed in consecutive batches. A number of potential causes were identified and their mitigation was investigated in order to establish a strategy to maintain the yield over consecutive batches.

Oleic acid, hexane and ethanol were tested as solvents to perform diananofiltration of the feed mixture for removal of pesticides from esterified deodorizer distillates. The performance of available commercial solvent resistant nanofiltration membranes was evaluated in terms of solvent compatibility and discrimination between steryl esters and pesticides. Additionally, optimised operating conditions were determined.

The efficiency of the process in terms of removal of pesticides and loss of steryl esters was assessed. A mass-balance model of the diananofiltration process was developed and compared directly to experimental observations with good results.

This Thesis shows that the valorisation of deodorizer distillates using an enzymatic reaction coupled to a membrane process is technically possible. Considerations to increase the efficiency of the process were presented and discussed, specifically by identifying the requisites for the membrane in terms of permeability and discriminatory capacity, and necessary engineering modifications to the process.

Keywords: Enzymatic Esterification; Bioactive Compounds; Steryl Esters; Pesticides Removal; Solvent Resistant Nanofiltration; Deodorizer Distillate

Resumo

A indústria agro-alimentar produz grandes quantidades de resíduos que, sem o devido tratamento e eliminação, poderão criar sérios problemas ambientais. A recuperação de compostos com valor acrescentado a partir desses resíduos industriais é muito interessante do ponto de vista ambiental e económico, principalmente se forem utilizadas tecnologias verdes. Os destilados da desodorização são sub-produtos ricos em compostos bioactivos, tais como os esteróis e os tocoferóis, produzidos no processo de refinação do óleo alimentar. No entanto, estes não podem ser usados directamente como aditivos alimentares devido ao seu elevado teor em pesticidas. A valorização de destilados da desodorização através da recuperação de compostos com valor acrescentado, livre de contaminantes (tais como os pesticidas), é particularmente desafiante devido à semelhança do peso molecular, volatilidade e hidrofobicidade dos compostos bioactivos e dos contaminantes.

A presente Tese propõe a produção de um extracto rico em ésteres de esteróis, livre de pesticidas, a partir de um processo baseado em membranas. A estratégia seguida é consiste no aumento do peso molecular dos compostos-alvo, tornando possível a sua separação dos pesticidas, enquanto mantém-se ou aumenta-se a bioactividade do extrato. Os ésteres de esteróis são conhecidos como sendo mais bioactivos e solúveis em matrizes hidrofóbicas do que a sua forma livre (esteróis), o que facilita a sua absorção quando consumidos. Por outro lado, o seu maior peso molecular (700-800 g mol^{-1}) relativamente aos pesticidas (220-400 g mol^{-1}) facilita a sua separação por métodos baseados na diferença do peso molecular.

Os ésteres de esterol podem ser produzidos por reacção enzimática entre os esteróis livres e s ácidos gordos livres, contidos nos destilados de deodorização. A lipase da *Candida rugosa* consegue catalisar essa reacção com rendimentos satisfatórios (>80%) em 24 horas e em condições óptimas. A concentração do enzima, a actividade da água no meio reaccional e a rácio molar entre os ácidos gordos e os esteróis livres foram optimizados de forma a assegurar que o equilíbrio está deslocado na direcção da esterificação dos esteróis. Um modelo baseado no balanço de massa, descrevendo o estado transiente do sistema reaccional, foi desenvolvido e avaliado quando utilizadas diferentes fontes de esteróis e ácidos gordos livres.

O uso de fontes alternativas e baratas de ácidos gordos livres (além do ácido oléico) que estão disponíveis para a maioria dos produtores de óleo alimentar, tais como o óleo refinado e misturas de destilados de desodorização, foi avaliado devido à potencial vantagem económica.

Membranas cerâmicas de ultrafiltração e membranas microfiltração de PTFE, resistentes a solventes, foram utilizadas para a total recuperação do enzima, tendo sido, no entanto, observada

uma desactivação parcial do enzima em batches consecutivos. Pontenciais causas foram identificadas, tendo sido investigado a sua minimização de forma a definir uma estratégia para manter o rendimento ao longo dos batches consecutivos.

O ácido oléico, o hexano e o etanol foram testados como solventes para realizar a diananofiltração da alimentação e, desta forma remover os pesticidas dos destilados de desodorização esterificados. A performance de membranas comerciais de nanofiltração resistentes a solventes foi avaliada em termos de compatibilidade com o solvente e de discriminação entre os ésteres de esteróis e pesticidas. Adicionalmente, foram determinadas as condições óptimas de operação.

A eficiência do processo em termos de remoção dos pesticidas e perda dos ésteres de esteróis foi avaliada. Um modelo baseado no balanço de massa, descrevendo o processo de diananofiltração, foi desenvolvido e comparado directamente com as observações experimentais, tendo sido obtidos bons resultados.

Esta Tese demonstra que tecnicamente é possível a valorização dos destilados de desodorização através de uma reacção enzimática acoplada a um processo de membranas. Considerações para aumentar a eficiência do processo foram apresentadas e discutidas, identificando, nomeadamente, os requisitos da membrana em termos de permeabilidade e capacidade discriminatória, e as modificações de engenharia ao processo necessárias.

Palavras-chave: Esterificação Enzimática; Compostos Bioactivos; Ésteres de Esterol; Remoção de Pesticidas; Nanofiltração Resistente a Solventes; Destilado de Desodorização

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1 Introduction

Summary *This chapter provides an introduction to the concepts presented and discussed in this thesis. Additionally, a summary of the motivation and the research strategy followed in this work are presented in this section, together with an outline of the contents of each chapter that constitute the Thesis.*

1.0.1 Background

Agro-food industries inevitably produce large amounts of agro-residues, which may represent a major waste disposal problem [1–3]. Legislation regulating the management of waste materials was issued by the European Union and has significantly contributed to the development of waste management procedures. A trend of demanding legislation regarding the agro-industrial wastes is expected, increasing the costs of their management. Under this context, the creation of value from such wastes has been highly encouraged and new technologies applying environmentally clean processes have been playing a central role.

One of the most interesting research areas is the recovery of bioactive substances from agro-food wastes using membrane technology [1, 4]. Indeed, after the appropriate pretreatment of the raw material and recovery procedures (usually solvent extraction), most natural residues can provide value-added natural oils, antioxidants, colorants, fragrances and other bioactive substances of great interest to the pharmaceutical, cosmetic and food industries [5]. However, bioactive compounds are present in low concentrations in such natural sources and are present as conjugates and mixtures in extracts, requiring labor-intensive methods for their recovery, purification and concentration. Membrane technology is an alternative technology, remarkably simpler and less energetically intensive than other recovery processes such as distillation and crystallization. Additionally, it enables the production of value-added extracts, without requiring high process temperatures, which typically damage the valuable compounds [6].

The oilseed processing industry generates approximately 10-25 m^3 of wastewater per metric tonne of oil refined produced [7], which represents a significant environmental burden. Chemical refining is the traditional method which comprises four main operations: degumming, neutralization, bleaching and deodorization (Figure 1.1). In the degumming step, the phosphatides are removed from the crude oil, preventing the oil to darken and develop a precipitate during the storage and transport. Subsequently, the free fatty acid are neutralized with caustic soda. The resultant soapstocks are removed by means of centrifugal separators, being mainly valorised as "acid oil" for animal feed. The neutral oils are afterwards bleached to remove essentially pigments (carotenes and chlorophylls), degraded proteins, carbohydrates, polar oxidation products and pro-oxidant metals. The purpose of bleaching is not only to provide a lighter colored oil but also to purify it. Deodorization is the last operation and aims at removing flavors and odours from crude or semi-refined oil to obtain a bland and odorless oil. It consists in a distillation driven by vapour, performed during short periods of time (2-8 h, depending on the operating mode - batch or semi-continuous) at high temperatures (180-280° C) and under a high vacuum (1-6 mmHg). These extreme operating conditions enables the removal of pesticides, aldehydes, ketones, free fatty acids and others volatile compounds responsible for adverse bad flavors and odours of the oil [8–10]. Simultaneously, the content of sterols and tocopherols in the oil is dramatically reduced in about 40% [11, 12], which makes the resulting distillate (about 30 kg/ton of refined oil produced [13]) a by-product with potential for downstream recovery of added-value compounds.

Deodorizer distillates are a complex mixture rich in acylglycerols, free fatty acids and bioactive compounds, such as sterols, steryl esters and tocopherols (Figure 1.2) [16, 17]. The composition of

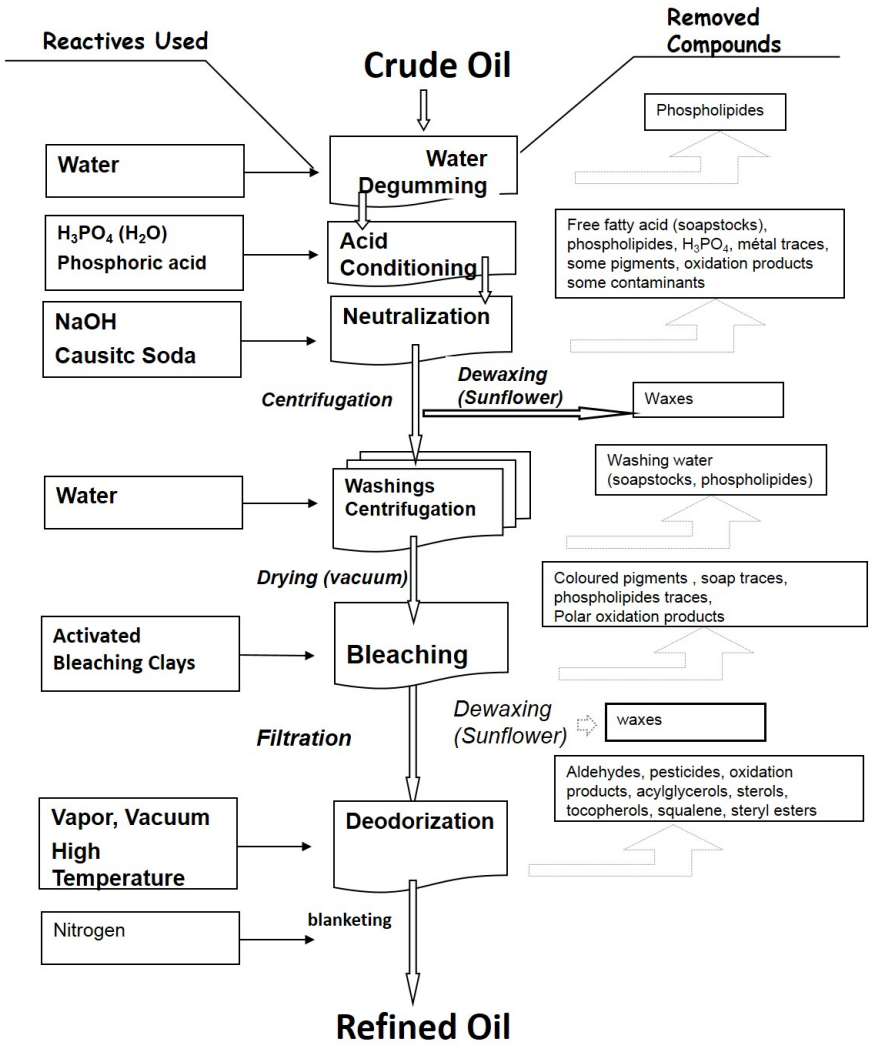


Figure 1.1: Edible oil refining process [14]

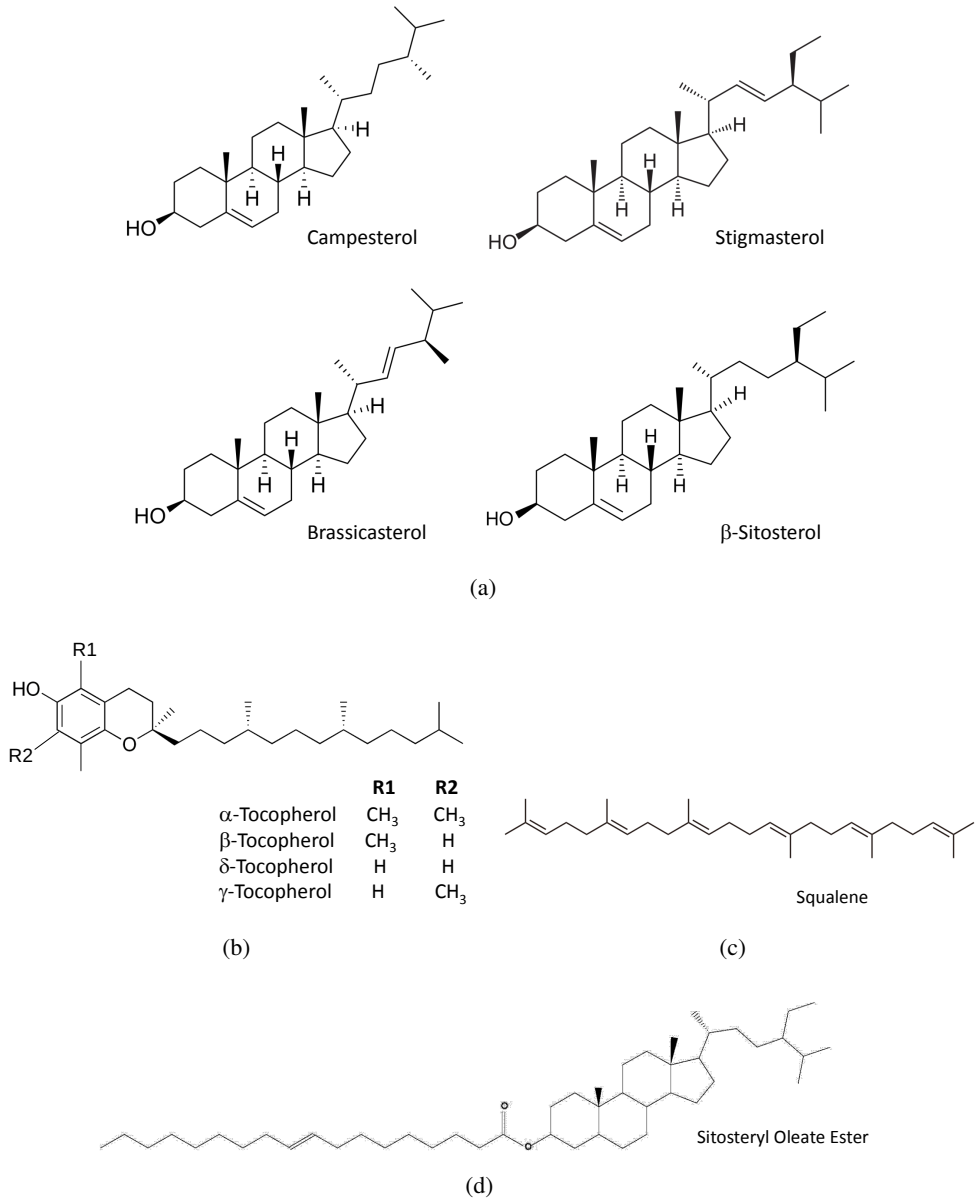


Figure 1.2: Main bioactive compounds present in deodorizer distillates: (a) Sterols, (b) Tocopherols, (c) Squalene and (d) Steryl Esters [15]

deodorizer distillates varies widely, depending on several factors including the source and quality of the seeds used as raw material, the mechanical processes used to extract the oil, and the physical characteristics and operating conditions of the refining plant. The typical average composition of deodorizer distillates is summarized in Table 1.1.

The family of acylglycerols present in deodorizer distillates contains triglycerols, diglycerols, monoglycerols and glycerols. Chemically speaking, tri, di and mono-glycerols are molecules derived from the natural esterification of fatty acid molecules with a glycerol molecule (Figure 1.3). Free fatty acids (FFA) have a general formula: $CH_3(CH_2)nCOOH$ where n is typically an even number between 12 and 22. If a chain contains double bonds, it is called an unsaturated fatty acid; if not, it is called a saturated fatty acid. Oleic acid (C18:1) is a monounsaturated fatty acid

Table 1.1: Typical average composition of deodorizer distillates [18, 19]

Compounds	Composition (% w/w)
Fatty Acids	30-85
Acylglycerols	5-40
Tocopherols	2-10
Free Sterols	2-15
Steryl Esters	0-5
Hydrocarbons	2-5

and it is the most relevant in sunflower and olive oil, being triolein (formed with three molecules of oleic acid) the most prevalent triglyceride [20].

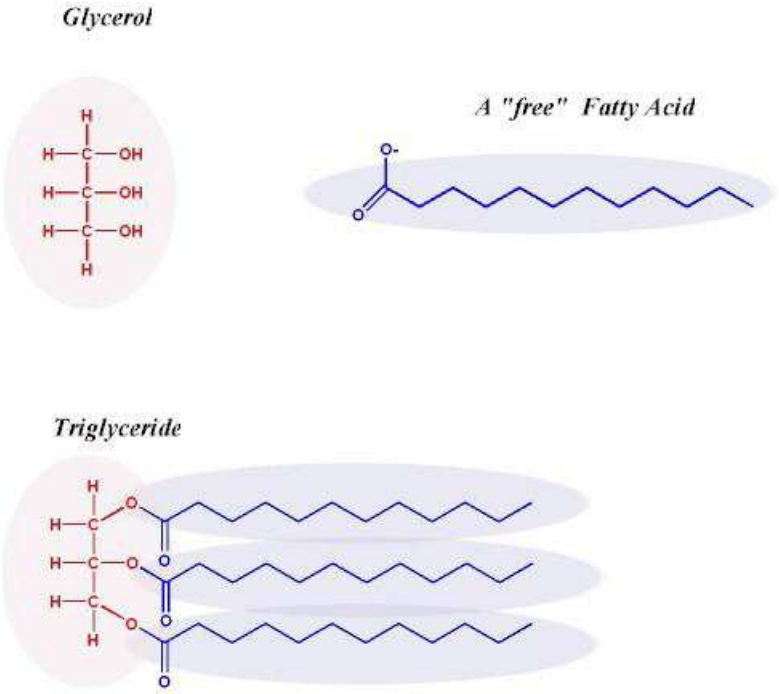


Figure 1.3: Formation of triglycerides from the esterification of glycerols and free fatty acids [20]

Nowadays, and regardless of the interesting composition of deodorizer distillates, they have a very limited commercial value and are sold at a fraction of the oil cost [9, 21]. Their use as animal feedstock or food additive has been discarded mainly due to the high content in pesticides [22, 23]. Maximum residue levels (MRLs) in vegetable oils for human consumption are not specifically set, but according to the article 20 of EU Regulation No. 396/2005, they have to be derived from the MRLs established for seeds and, additionally, the concentration caused by their processing (defined as processing factor) should be taken into account [24]. The concentration of pesticides in seeds should be lower than 0.05 ppm (0.01 ppm is the limit of detection (LOD) for the main lipophilic pesticides). Their concentration in deodorizer distillates can be 500-1000 times higher than the actually allowed values [22, 23]. Therefore, the valorisation of deodistillates as a

food additive rich in bioactive compounds depends on the removal of pesticides, which remains challenging.

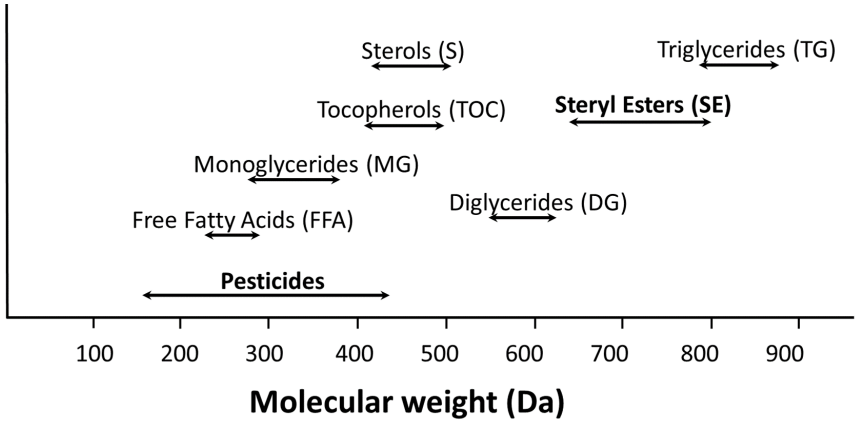


Figure 1.4: Distribution of molecular weight of compounds constituents of deodorizer distillates

Figure 1.4 shows the distribution of molecular weight of compounds constituents of deodorizer distillates. Based only on the molecular weight, it is clear that within the bioactive compounds (comprising sterols, tocopherols and steryl esters) steryl esters (650<MW<800 g/mol) standout as those with the largest potential for separation from pesticides (150<MW<400 g/mol). However, the attempts for valuing deodorizer distillates have been mainly focused on the recovery of sterols and tocopherols due to their higher concentrations comparatively to steryl esters (Table 1.1). The described in the literature include crystallization, supercritical carbon dioxide extraction and molecular distillation [9]. One of the difficulties of recovering sterols and tocopherols from deodorizer distillates by methods based-on their molecular weight and volatility, is the similarity of such properties relatively to pesticides [25, 26]. Additionally, the use of thermal methods (as distillation) induce the degradation of bioactive compounds as well as the conversion of fatty acids into undesirable trans isomeric forms [27]. The use of high amounts of more than one solvent in crystallization procedures, the high operating pressures of supercritical dioxide extraction and the low selectivity of the molecular distillation are other drawbacks of these methods. Membrane technology can be an alternative clean method able to address these limitations. It can be considered advantageous when compared to thermal-based processes, since membrane systems can be operated at room temperature. On the other hand, membrane systems can be operated at relatively low pressures, which constitutes an advantage from a process economy point of view. However, their efficiency rely on the molecular weight difference between target compounds and contaminants meaning that, for this specific application, membranes can be used only for the separation of steryl esters and pesticides. The production of extracts rich in steryl esters is also interesting, since, as sterols, they were approved as ingredients in functional foods by the European Food Safety Authority (EFSA) with regard to lowering cholesterol [28]. In addition, these compounds were identified as anti-cancer [29], anti-atherosclerosis [30], anti-inflammation [31] and anti-oxidation compounds [32]. Moreover, recent studies indicate that sterols in the esterified form are more bioactive than their free form [33] and present a higher solubility in oil phase, making easier their

incorporation in fat-based products.

Typically, the content of steryl esters in deodorizer distillates is very low (0-5% according the Table 1.1) but it can be increased by enzymatic esterification between sterols and free fatty acids, making their recovery more viable. The use of enzymes compared to chemical catalysts is advantageous since enzymes are highly specific, catalyse under mild conditions (decreasing side reactions and saving energy), are more compatible with variations in the composition of the raw material and may be reused. The main drawbacks of using enzymes (depending on the process under consideration) are their low reaction rates, their high price and loss of activity [34].

Lipases (triglycerides hydrolases E.C. 3.1.1.3) are part of the family of hydrolases that act on carboxylic ester bonds. The natural function of lipases is to hydrolyze triglycerides into diglycerides, monoglycerides and glycerol, although, under optimal conditions, they can catalyze other reactions such as esterification, interesterification, acidolysis, alcoholysis, and aminolysis (Figure 1.5). Lipases from *Candida rugosa* and *Mucor miehei* are the most used for esterification applications [35], and more specifically, the lipase from *Candida rugosa* is considered the most appropriate for obtaining yields of production of steryl esters above 80% [36–38]. Since their optimal working temperature is already known (37-40 ° C) [39], the main parameters to be optimized are the enzyme concentration, water activity in the reaction medium (a_W) and reactants molar ratio (free fatty acids:sterols, *FFA:S*). The immobilization of the enzyme in porous carriers is one way of increasing its lifetime and stability. Additionally, handling and recovery of the enzyme are easier if an immobilization process is used. However, limitations to internal mass transport of large molecules through small pores of the carrier to reach the enzyme may be significant, decreasing consequently the efficiency of the enzyme. Furthermore, if the viscosity of the medium is high, as in the use of deodorizer distillates, limitations due to external mass transfer will be necessarily high. In such cases, the use of free enzymes is preferable with the advantage of their higher activity and significant lower price when compared to the immobilized option. The main drawbacks are their reported reduced lifetime and difficulty to handle and reuse [34].

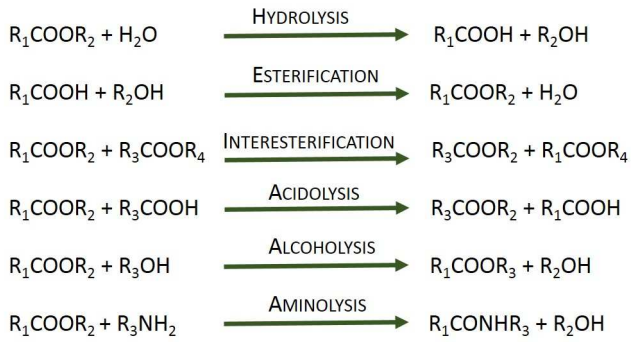


Figure 1.5: Different reactions catalysed by a lipase

The enzymatic enrichment of deodorizer distillates in steryl esters using free and immobilized enzymes is relatively well described in the literature [8, 38, 40–42]. However, it is always coupled to methods as crystallization, supercritical carbon dioxide extraction or molecular distillation, for recovering of the target compounds. The use of nanofiltration membranes was still not considered,

although it may be feasible given the higher molecular weight of steryl esters, 650-800 g mol^{-1}) relatively to pesticides (150-400 g mol^{-1}).

Nanofiltration (NF) is a pressure-driven process, that is typically considered to be between ultrafiltration (UF) and reverse osmosis (RO). Figure 1.6 shows the liquid separation spectrum for the separation of particles and solutes from around 0.01mm to 1Å. Nanofiltration is traditionally used for removing solutes with a size range within 6-13Å, such as sugars, dyes and organic solutes (with a molecular weight (MW) between 200 and 1000 g mol^{-1}), from aqueous feed streams. The key difference between nanofiltration and reverse osmosis is that the latter retains monovalent salts (such as sodium chloride), whereas nanofiltration allows them to partially pass, retaining effectively only divalent and multi-valent salts (Figure 1.7) [43].

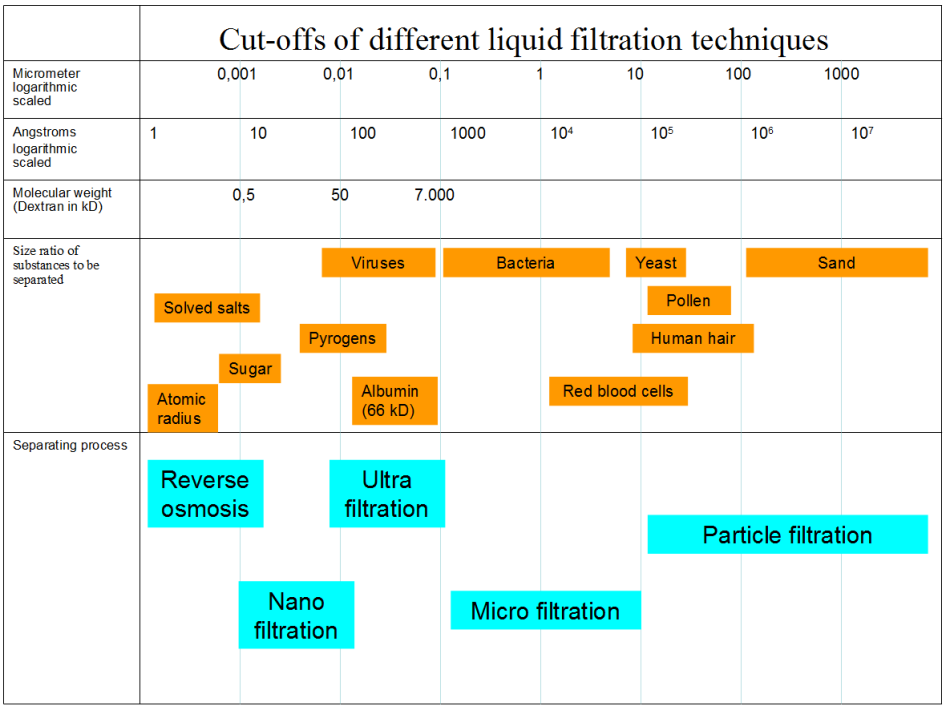


Figure 1.6: Membrane techniques for liquid separation [44]

Nanofiltration was recently applied to molecular-level separations in organic solvent media, with the development of the so-called solvent resistant nanofiltration (SRNF) membranes. Nowadays, SRNF is being used with relative success for pharmaceutical industry applications, such as purification of active pharmaceutical ingredients (API) [46], catalyst recycling [47], removal of genotoxins from APIs [48], continuous solvent exchange [49] and solvent recycling [50]. The use of SRNF in the oil refining industry has been discussed mainly from the point of view of solvent recycling and oil recovery [51–53]. Even so, the only reported application of such technology in large scale is the solvent recovery from lube oil dewaxing (MAX-DEWAX^{TM}) [54].

The development of solvent resistant polymeric membranes is a great challenge for membrane material science, mainly due to their tendency to swell and dissolve when in contact with solvents. This fact may justify the limited number of membrane producers, being the most important

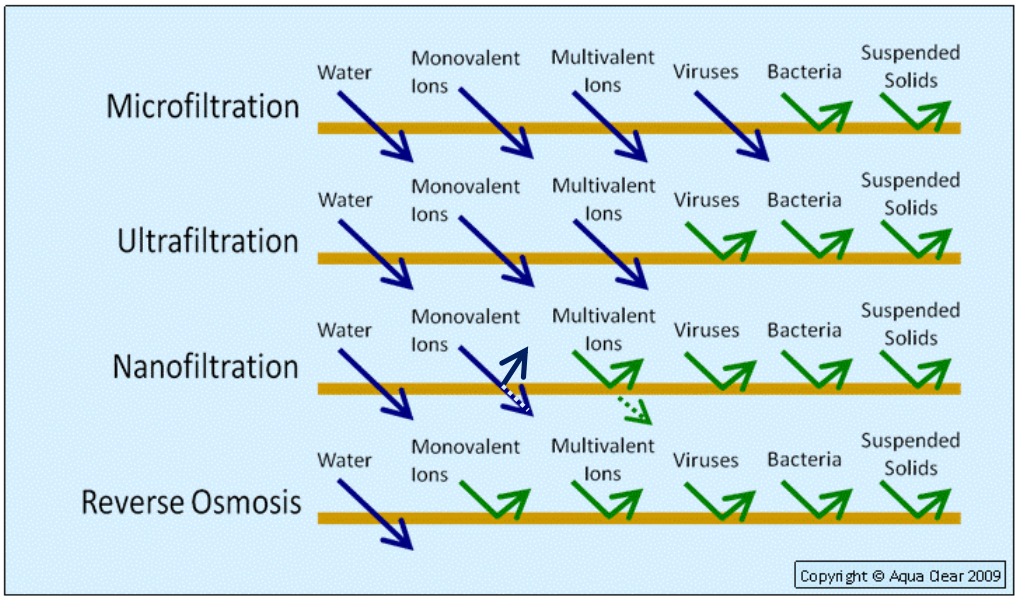


Figure 1.7: Characteristics of membrane separation processes [45]

Evonik [55], GMT [56] and Solsep [57]. Polyimide (PI) crosslinked with PDMS, and polyacrylonitrile (PAN) are examples of materials that were identified to exhibit a satisfactory resistance. Most polymeric membranes used in SRNF are constituted by a porous support with a dense top layer [58]. In the case of non-porous membranes, the transport is hypothesized to be governed by a solution-diffusion mechanism [59]. This model assumes that transport occurs by solute(s) solubilization in the membrane and diffusion through it, being the rejection a function of the diffusivity coefficient of each substance [52]. Other authors [60, 61] present a different approach, in which swollen dense membranes may exhibit an increase in internal microporosity being the rejection of solutes governed predominantly via a size-exclusion mechanism. In this case, the nature of transport of solutes is reported to be mainly convective (Figure 1.8).

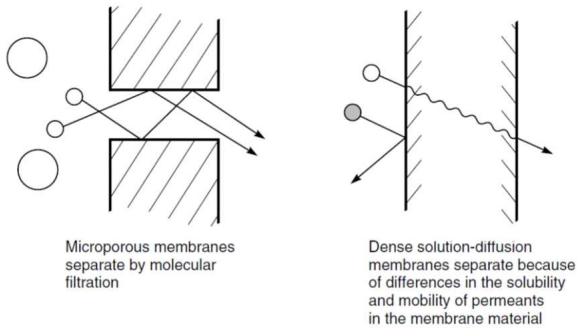


Figure 1.8: Molecular transport through membranes described by the flow through by pores or by solution-diffusion mechanism [43]

Ceramic materials (silicium carbide, zirconium oxide and titanium oxide) are able to endure harsh temperature conditions and show high resistance to solvents being, therefore, an excellent

alternative to polymeric materials. For a long time, the lower end of molecular weight cut-off (MWCO) of these membranes was 1000 g mol^{-1}. However, NF membranes with a pore size of 0.9 nm and a cut-off of 450 g mol^{-1} were developed and commercialized in 2002 by Inopor, a spin-off company of HITK (Germany) [58]. The hydrophilicity of the oxide materials contained in ceramic membranes, lowers the permeability of apolar solvents. Attempts for mixing oxides materials were not successful. The silylation of ceramic membranes has been proposed as a viable solution, however, their commercialization is still restricted, being only manufactured under demand [58].

The fact is that the application of SRNF membranes at large scale constitute a market opportunity, and several efforts have been made in the direction of developing better and more compatible materials in order to increase the efficiency of the separation processes.

1.0.2 Research Strategy

The main objective of this thesis is the development of an integrated process for production of a bioactive extract rich in steryl esters from deodorizer distillates. The valorisation of this by-product depends on the removal of pesticides with a minimal loss of steryl esters.

The solvent resistant membrane-based process developed in this work comprises three steps as illustrated in Figure 1.9.

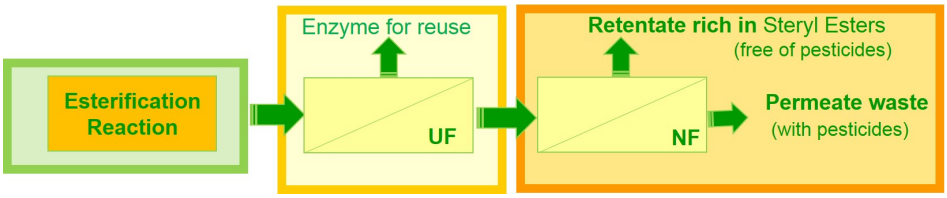


Figure 1.9: Steps of the proposed solvent resistant membrane-based to produce an extract rich in steryl esters from deodorizer distillates

The first step consists in the enrichment of deodorizer distillates in steryl esters. The enzymatic-route was chosen since that it can be carried-out under mild conditions, preventing the thermal degradation of bioactive compounds contained in the deodorizer distillate (which is typically one of the main challenges of the traditional thermal-based separation processes). The lipase from *Candida rugosa* was selected to perform the esterification, since it has been referenced in the literature as the most efficient [36–38]. The enzyme was used in the free form (instead of immobilized), mainly due to the high viscosity of deodorizer distillates, which may induce high limitations to mass transfer. Other advantage is the higher activity of the enzyme in the free form as a direct consequence of the absence of a enzyme-carrier interactions and possible limitations to mass transfer.

The objective of this first step was to obtain at least 80% of yield of production of steryl esters after 24 hours of reaction. The reaction was carried-out in batch mode, since the deodorization can be operated in batch or semi-continuous mode. The reaction time was selected taking into

account that the operation of deodorization can last 2-8 hours [14] and that only 30 kg of distillate is produced per tonne of refined oil [13], being necessary enough time to collect a reasonable quantity of distillate from the deodorization to start a new reaction batch. The temperature of the enzymatic reaction was maintained constant at 40 ° C (optimal temperature, following indications of the suppliers). The concentration of enzyme, the water activity of the reaction medium and the molar ratio of free fatty acids and sterols (*FFA:S*) were the parameters optimized for maximization of the yield of steryl esters production. The importance of the water activity (a_W) in the equilibrium of hydrolysis/esterification reactions, as well as the intrinsic activity of the enzyme has been reported [35]. Therefore, different initial a_W in enzymatic reactions were set (pre-equilibrating the system with different saturated salts solutions, as described by Greenspan [62]) and the impact in the yield of steryl esters production was determined. Finally, the *FFA:S* molar ratio was optimized varying the quantity of free fatty acids in the system by addition of oleic acid (main free fatty acid present in the deodorizer distillates used in this study).

The prediction of the profile of the reactant compounds during the enzymatic reaction is a powerful tool, allowing to describe the transient-state of the system once the kinetic constants were determined. Accordingly, a focus was given to the development of a mass-balance model that could be applied, independently of the source of deodorizer distillates and free fatty acids (necessary to assure that equilibrium is driven towards the esterification of sterols).

The second step of the integrated process is focused on the reuse of the enzyme. The enzyme was recovered using UF/MF membranes and the yield of production of steryl esters was compared between consecutive batches. The effect of water activity, the increasing content of glycerol in the reaction medium (potential inhibitor) due to the the hydrolysis of acylglycerides and the presence of oxidation products were identified as potential causes of the loss of enzyme activity. The understanding of such phenomena was one the objectives, in order to promote the mitigation of enzyme deactivation (if possible) and the reuse of the enzyme, maintaining the yield of steryl esters production higher than 80% over consecutive batches.

The third step consisted on the production of an extract rich in steryl esters and free of pesticides, using SRNF membranes. Diananofiltration is used in this work for the removal of contaminants (such as pesticides) from the process stream. This method consists in continuously feeding fresh solvent at the same rate as permeate is recovered, where steryl esters are retained by the membrane and pesticides are washed-out in the permeate. The selection of solvents to be used in the diananofiltration technique was based on the fact that oleic acid is already present in the original matrix in high concentrations, while hexane is a traditional solvent used in the oil refining industry and, finally, ethanol produced from renewable resources is already considered as an alternative solvent in food applications. Polymeric membranes were chosen since the ceramic membranes still present restricted application to apolar solutions due to their strong hydrophilic character. The performance of available commercial SRNF membranes was evaluated in terms of solvent compatibility and rejection of target compounds. The interaction between the selected solvents and membranes was studied in this work, and associated to the swelling phenomena observed and the viscosity of the solvents. The experimental data of membrane flux and rejection of compounds was used as inputs to a mass-balance model, enabling the prediction of the profile of

the target compounds during diananofiltration processing, as well as the efficiency of the process in terms of loss of steryl esters and removal of pesticides. Such model enabled the comparison of the efficiency of candidate membranes and to select the one that exhibit the better performance for the present application. This model was improved by incorporating a relationship between flux and membrane exposure time to the processing solution (as a result of the interactions between the solvent and the membrane). Finally, the modelling data was compared to the experimentally obtained values and methods for improving the efficiency of the process were proposed and simulated.

1.0.3 Thesis Outline

This Thesis comprises the following chapters:

- Chapter 2 comprises the optimisation of the enzymatic esterification between sterols (*S*) and free fatty acids (*FFA*) contained in deodorizer distillates from different sources. The enzyme concentration, the water activity (a_W) in the reaction medium and the molar ratio between free fatty acids (FFA) and sterols (S) were the parameters optimized, in order to obtain at least 80% of yield of production of steryl esters. A mass-balance model was established and the kinetic constants of all reactions involved were determined. This model allowed to describe the transient state of the system, predicting the profile of concentration of the reactant compounds when using different deodistillates [63]. *DOI: 10.1021/ie1020766*

- Chapter 3 discusses the possibility of using alternative and cheaper sources of free fatty acids (FFA), instead of using oleic acid in the adjustment of the initial excess of FFA (*FFA:S* molar ratio). The use of sunflower refined oil and mixtures of deodorizer distillates with different compositions was evaluated as well as the capacity of the mass-balance model developed in this work for predicting the yield of production of steryl esters. Guidelines to obtain yields of steryl esters higher than 80% are also provided in this chapter.

- Chapter 4 studies the possibility of reusing the enzyme in consecutive reactions. The activity of the enzyme between consecutive batches was analysed and possible causes of loss of activity were identified. In order to maintain the yield of production of steryl esters in consecutive batches, strategies to maintain the yield over batches were assessed and optimised [64]. *DOI: 10.1021/ie202853f*

- Chapter 5 presents the SRNF membranes' screening work to perform the removal of pesticides and recovery of steryl esters. The selection of the solvent to be added to the resulting reaction mixture in order to decrease the viscosity of the reaction medium is also described in this chapter. Furthermore, the optimal operating conditions to obtain the maximum discrimination between steryl esters and pesticides were identified.

- Chapter 6 comprises the development of a mass-balance model able to describe the profile of concentrations of compounds in solution during diananofiltration. The predicted and experimental efficiency of the process in terms of loss of steryl esters associated to the removal

of 99% of pesticides was determined and discussed. As a result, possible improvement solutions are discussed in the basis of the simulations performed.

- Chapter 7 presents a summary of the main findings along with a discussion of relevant questions that have emerged from the present work and that should be addressed in future work.

The scientific work developed in this PhD project is described in Chapters 2 to 6. These chapters are written in the format of scientific papers, being already published the articles related to Chapters 2 and 4.

2 *Production of steryl esters from vegetable oil deodorizer distillates by enzymatic esterification*

Summary

This work is focused on the production of steryl esters by enzymatic esterification of free fatty acids and sterols present in deodorizer distillates. The enzyme selected, a Candida rugosa lipase, shows to be able to synthesize steryl esters with a yield of 80%, after 24 hours, under optimized conditions. These conditions were determined after studying the influence of various reaction parameters such as enzyme concentration, initial water activity in the reaction medium and initial Free Fatty Acid:Sterols molar ratio (FFA:S), at a fixed temperature of 40 °C. The optimal enzyme concentration was found to be 0.5% (w/w) which results from a compromise between the yield of steryl esters formation and the period of time to perform such task, set as 24 hours. The optimal water activity in the reaction medium was found to be in the range between 0.45 and 0.85. The influence of the substrates molar ratio (FFA:S) was also studied and a minimal ratio of 6:1 was found to be the best.

A kinetic mathematical model was established in order to describe the transient state of the reaction system during the enzymatic reaction, as well as to determine the respective kinetic constants, which can be used to predict the evolvement of the composition of the reaction mixture.

The contents of this chapter were adapted from the publication: Teixeira, A. R. S., Santos, J. L. C., Crespo, J. G. (2011). Production of Steryl Esters from Vegetable Oil Deodorizer Distillates by Enzymatic Esterification. Industrial & Engineering Chemistry Research, 50(5), 2865-2875

2.1 INTRODUCTION

Recent efforts from industry to achieve better and improved methods for recovery and purification of phytosterols are related with the growth of the health food market, particularly due to the widespread dissemination of functional food [19]. Under this context, the valorisation of by-product streams rich in bioactive compounds is economically very interesting.

Deodorizer distillate is a by-product stream formed in the last step of the refining process of vegetable oils. It consists on the deodorization of the oil for removal of compounds responsible for bad taste and odor (aldehydes and ketones), pesticides, herbicides and polycyclic aromatic hydrocarbons (*PAHs*). Due to the operating conditions used in this steam-stripping distillation, the resulting distillate also contains sterols (*S*), tocopherols (*TOC*), squalene (*Squal*), free fatty acids (*FFA*), triglycerides (*TG*), diglycerides (*DG*), monoglycerides (*MG*) and steryl esters (*SE*) [9, 17, 65].

Phytosterols have been found to be effective in lowering plasma cholesterol concentrations and prevention of cardiovascular diseases [66, 67], while tocopherols, which are physiologically active as Vitamin E, are natural antioxidants that have an important role in the protection of fats and oils from oxidation [68, 69].

Despite previous efforts [10, 25, 26, 70–76], the valorisation of deodorizer distillates still remains a challenge since the recovery and purification yields of the target compounds are not satisfactory. A high content of pesticides has been found in deodorizer distillates [77], making their valorisation dependent from the removal of pesticides, due to the stringent legislation in the majority of the countries concerning the pesticides content in food additives [17]. It is important to note that none of the works referred indicate the content in pesticides in the product obtained.

The present work studies the enzymatic reaction between *FFAs* and sterols to produce steryl esters for valorisation of deodorizer distillates. Although there is no real consensus, one of the advantages identified by some authors in the literature for producing steryl esters is related with the fact that sterols in the esterified form are known to be more bioactive than in their free form [33]. Also, steryl esters are more soluble in the oil phase than free sterols, making easier their incorporation in fat-based products [67]. Moreover, an increase in the difference of molecular weight between the steryl esters (650-800 Da) and pesticides (150-420 Da) enables a possible recovery of steryl esters by separation processes based on size exclusion such as pressure-driven membrane processes. The molecular weight of the target constituents of the enzymatic reaction suggests that nanofiltration may allow their efficient separation [43], which will be explored in the Chapters 5 and 6.

In the literature, the use of lipases (triglycerides hydrolases E.C. 3.1.1.3) for the synthesis of various steryl esters has been applied in distinct applications. In several published works, *Candida rugosa* lipase is considered one of the most appropriate enzymes for the conversion of sterols to steryl esters [36–38].

Shimada and co-workers authored several studies of enzymatic production of steryl esters followed by distillation [36, 38, 41, 42, 78]. These authors used soybean deodorizer distillates, previously distillated to remove high boiling point substances such as *TG*, *DG* and *SE*, and since the

reaction occurs in a multiphasic system (water content between 20-50%), a subsequent dehydration step (vaccum destillation) is needed. The yield of steryl esters varied between 80-90% after 24 hours of reaction, at 40 °C, with a concentration of *Candida rugosa* lipase (lipase-OY) of 120-250 Units per gram of reaction mixture. The influence of the excess of *FFAs* on the initial reaction mixture was also studied [42], being obtained a positive effect, with a optimal ratio of Deodorizer Distillate/Oleic Acid of 1:2 (w/w), since the reaction is driven towards esterification. The influence of the water content in the steryl esters yield was studied by Shimada and co-workers [42,78] and it was concluded that without adding water to the deodorizer distillate (initial water content = 6500 ppm) the reaction does not occur, being necessary to guarantee an initial water content of 20%.

Torres et al. [40, 79] and Villeneuve et al. [37], also performed the esterification reaction of phytosterols and oleic acid but in a monophasic system since both identified the disadvantage of working with multiphasic systems, namely the need of eliminating solvents from the products. Torres et al. [40, 79] carried out the reaction with a *Candida rugosa* enzyme concentration of 10% (w/w), a molar ratio of substrates (*FFA:S*) of 2, at 40 °C, during 24 hours, obtaining a steryl esters yield production of 90%. Villeneuve et al. [37] carried out the reaction with a *Candida rugosa* enzyme concentration of 5% (w/w), pre-equilibrated to an optimal water activity - a_w - (not especified), a molar ratio of substrates (*FFA:S*) of 3, at 35 °C, during 72 hours, obtaining a steryl esters yield production of 85%.

This work aims at improving the understanding of the enzymatic esterification reaction mechanism in order to maximize the yield of steryl esters. This objective will be accomplished through a mathematical description of the reactions taking place, and an optimization of the different parameters involved.

The conversion of sterols to steryl esters leads to an increase in the molecular weight difference to pesticides that are present in deodorizer distillates. This reaction step thereby facilitates the use of a subsequent membrane-based separation process, such as nanofiltration, for the recovery of steryl esters free from pesticides.

The enzyme concentration is one of the parameters that will be optimized since it has a significant importance in the economic feasibility of the process at a large scale. This work will be focused on the determination of the minimal enzyme concentration necessary to achieve high yields (defined as the moles of steryl esters produced per the initial moles of sterols in the reaction mixture) in a period of 24 hours.

The water activity in non-aqueous media influences the dynamic and catalytic properties of the enzymes, since water acts as a molecular lubricant. Water activity is the parameter that gives a better indication about the hydration state of the enzyme, which plays a crucial role in the catalytic activity, rather than the total water content in solution. Moreover, water has an important role in the equilibrium of the esterification/hydrolysis side-reactions [80–83].

An excess of *FFA* guarantees that the production of steryl esters by esterification of sterols is not limited by the concentration of *FFAs*. Therefore, the effect of the addition of oleic acid (an *FFA* will be evaluated in this work as well as the subsequent and important decrease of the viscosity of the reaction medium, enhancing mass transfer.

2.2 MODELLING OF THE ENZYMATIC REACTION

In order to describe the kinetic behaviour of the enzyme-catalyzed acyl transfer in organic solvents by using esters as acyl donors, a mathematical kinetic model was developed. Since the enzyme has water available to react, the hydrolysis and esterification side-reactions of acylglycerides and sterols will occur simultaneously.

Concerning the mechanism of esterification of the sterols, one mole of sterol (S) reacts with one mole of free fatty acid (FFA), catalyzed by the enzyme (E), releasing one mole of steryl ester (SE) and one mole of water (W) which can be described as:

$$S + FFA + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} SE + W + E \quad \text{(I)}$$

where k_1 and k_{-1} are the kinetic constants that describe the forward and the reverse reaction, respectively.

In the esterification of the acylglycerides, one mole of glycerol (G) is stepwised esterified with three moles of free fatty acid (FFA), by the enzyme (E), to monoglyceride (MG), diglyceride (DG) and triglyceride (TG), releasing three moles of water (W). The following mechanism describe the reactions:

$$G + FFA + E \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} MG + W + E \quad \text{(II)}$$

$$MG + FFA + E \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} DG + W + E \quad \text{(III)}$$

$$DG + FFA + E \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} TG + W + E \quad \text{(IV)}$$

The rate equations for the evolvement of sterols (S) and formation of steryl esters (SE) can be expressed as:

$$\frac{1}{[E]}\frac{\mathrm{d}[S]}{\mathrm{d}t} = k_{-1}[SE][W] - k_1[S][FFA] \tag{2.1}$$

$$\frac{1}{[E]}\frac{\mathrm{d}[SE]}{\mathrm{d}t} = k_1[S][FFA] - k_{-1}[SE][W] \tag{2.2}$$

The rate equations for the formation/consumption of the acylglycerides can be expressed as:

$$\frac{1}{[E]}\frac{\mathrm{d}[G]}{\mathrm{d}t} = k_{-2}[MG][W] - k_2[G][FFA] \tag{2.3}$$

$$\frac{1}{[E]}\frac{\mathrm{d}[MG]}{\mathrm{d}t} = k_2[G][FFA] + k_{-3}[DG][W] - k_{-2}[MG][W] - k_3[MG][FFA] \tag{2.4}$$

$$\frac{1}{[E]}\frac{\mathrm{d}[DG]}{\mathrm{d}t} = k_3[MG][FFA] + k_{-4}[TG][W] - k_{-3}[DG][W] - k_4[DG][FFA] \tag{2.5}$$

$$\frac{1}{[E]}\frac{\mathrm{d}[TG]}{\mathrm{d}t} = k_4[DG][FFA] - k_{-4}[TG][W] \tag{2.6}$$

The free fatty acids (*FFA*) are the acyl donors in all esterification reactions, and their evolvement can be expressed as:

$$\frac{1}{[E]}\frac{\mathrm{d}[FFA]}{\mathrm{d}t} = k_{-1}[SE][W] + k_{-2}[MG][W] + k_{-3}[DG][W] + k_{-4}[TG][W] - k_1[S][FFA] - k_2[G][FFA] - k_3[MG][FFA] - k_4[DG][FFA] \tag{2.7}$$

Water induces the hydrolysis of steryl esters as well as acylglycerides. Its evolvement can be expressed as:

$$\frac{1}{[E]}\frac{\mathrm{d}[W]}{\mathrm{d}t} = k_1[S][FFA] + k_2[G][FFA] + k_3[MG][FFA] + k_4[DG][FFA] - k_{-1}[SE][W] - k_{-2}[MG][W] - k_{-3}[DG][W] - k_{-4}[TG][W] \tag{2.8}$$

In order to guarantee that the water content in the bulk reaction medium is constant during the esterification reaction, it is possible to adjust the water content to a desirable value by equilibration with saturated salt solutions, as described in the Materials and Methods section. In this case, the rate equation for water becomes (assuming a total control of the water activity):

$$\frac{1}{[E]}\frac{\mathrm{d}[W]}{\mathrm{d}t} = 0 \tag{2.9}$$

Concerning the tocopherols and squalene, the enzyme is not able to esterify them, therefore their rate equations were not considered.

If the concentration of all compounds during the enzymatic reaction is known, the kinetic constants can be determined by applying a numerical method to solve the system of differential equations and fitting the model to the experimental values by adjusting the kinetic constants (see the subsection 2.3.4). It should be stressed that different model mechanisms other than the one described here were also evaluated (results not shown). The use of those model mechanisms resulted, in all cases tested, in worse descriptions of the transient species concentration.

2.3 Materials and Methods

2.3.1 Materials

2.3.1.1 Deodorizer Distillate

Sunflower deodorizer distillates were obtained from the producers Lesieur Cristal in Morocoo and Lesieur in France. The composition of the deodorizer distillate was determined in our laboratory. Table 2.1 shows the composition variability of the deodorizer distillates depending from their

Table 2.1: Composition of the Sunflower Deodorizer Distillates used for optimisation of the enzymatic reaction

			Sunflower Deodorizer Distillate		
	Units	Compound	1 [a]	2 [a]	3 [b]
acylglycerides	g/100g	G	N.D. [c]	0.85	N.D.
		MG	3.97	2.52	0.96
		DG	4.92	1.25	2.19
		TG	12.0	18.2	36.0
Tocopherols	g/100g	α-Tocopherol	2.70	2.99	1.74
		δ-Tocopherol	0.34	0.22	0.45
		β/γ-Tocopherol	0.29	0.25	0.08
Squalene	g/100g	Squalene	2.70	2.84	1.72
Sterols	g/100g	Campesterol	1.16	0.91	0.51
		Stigmasterol	0.70	1.59	0.54
		β-Sitosterol	3.30	4.18	2.26
		Others Sterols	1.39	0.41	0.04
Steryl Esters	g/100g	Steryl Esters	5.09	1.17	1.02
Fatty Acids	g as oleic acid/100g	Free Fatty Acids	38.9	20.0	10.9
Water	ppm	Water	509	782	331

[a] From Lesieur Cristal (Morocco)
[b] From Lesieur (France)
[c] N.D.-Not Detected

source and lot. Other compounds that were not quantified in this work may comprise hydrocarbons, aldehydes, ketones, pesticides, herbicides and breakdown products of tocopherols and free phytosterols [10]. All samples were stored in the refrigerator at 4 °C.

2.3.1.2 *Enzyme*

Candida rugosa lipase, Type VII, was obtained from Sigma (Saint Quentin, France). The activity of this lipase as indicated by the supplier is 1535 U/mg of solid, which was experimentally confirmed by using the Sigma Kit for determination of enzymatic activity of lipases using olive oil as substrate. One unit (U) of lipase activity is defined as the amount of enzyme that hydrolyses 1.0 μequivalent of fatty acid from a triglyceride in one hour at pH 7.2 and at 37 °C using olive oil (30 minutes of incubation).

2.3.1.3 *Chemicals*

Analytical-grade chloroform, food-grade oleic acid, N,O-bis (trimethylsilyl) trifluracetamide (BSTFA) containing 1% of trimethylchlorosilane (TCMS) solution (from Fluka) and pyridine were obtained from Sigma Aldrich (Belgium).

All analytical-grade standard substances, squalene (99.3% purity), stigmasterol (97% purity),

β-sitosterol (99%purity), campesterol (99% purity), cholesteryl stearate (96% purity), monoglyceride olein (>99%purity), diglyceride olein (99.7% purity) and triglyceride olein (99.6% purity) were purchased from Sigma (France). A tocopherol kit consisting of α-, β-, γ- and δ-tocopherols was obtained from Merck (>95% purity).

The internal standard heptadecanyl stearate (HDS) was prepared by condensation of heptadecanol and stearoyl chloride, both obtained from Aldrich (Belgium), as described by Verleyen et al. [65].

2.3.2 Analytical Methods

2.3.2.1 Analysis of acylglycerides, Tocopherols, Sterols and Steryl Esters

The following procedure was based on the method developed by Verleyen et al. [65]. In this work, the amount of the reagents used was further optimized.

The deodorizer distillates samples need to be derivatized in order to increase the volatility of the components. For derivatization, samples (≈5 mg) were weighed in a screw-capped test tube and dissolved in 50 μl of pyridine and 100 μl of BSTFA containing 1% of TCMS solution as derivatizating and silylation agent. The test tube was placed in a heating block at 70 °C for 30 minutes to allow the completion of the silylation process. Afterwards, 5 μl of HDS solution (internal standard) with a concentration of 20 mg/ml in chloroform was added to the derivatized sample as well as 840 μl of chloroform in order to obtain a final volume of 1 ml. The samples were transferred to GC vials and injected within 24 hours of preparation.

Separation of the peaks was performed in a Chrompack CP9001 series gas chromatograph with an on-column injection at a set temperature of 60 °C and with a flame ionization detector at 360 °C. A capillary column Zebron-5MS 15 m×0.25 mm, 0.25 μm (Phenomenex, USA) was used. The temperature program of the oven was the following: rinsing at 30 °C/min to 140 °C and continuing the oven heating at a rate speed of 5 °C/min to 235 °C with a 7 min hold and further heating at 15 °C/min to 340 °C with a 30 minutes hold. Helium was used as a carrier gas at a pressure of 100 kPa. The peaks were integrated using the Borwin Software (JMBS Developments, France). Typical retention times for all species identified are depicted in Table 2.2.

2.3.2.2 Analysis of Free Fatty Acids

The free fatty acids (*FFAs*) were determined by titration according to the standard NF.EN.ISO 660-1999. The method was developed and implemented in a titration workstation TitraLab 856 from Radiometer (Denmark). The result is expressed as % of oleic acid.

2.3.2.3 Analysis of Water Content

A Karl Fischer (KF) system of Metrohm AG, Herisau, Switzerland (model 756KF coulometer) was used to analyse the water content. Water content determinations of deodorizer distillates samples were conducted with Hydranal-Coulomat CG, as catholyte reagent free of halogenated hydrocarbons, for a coulometric KF titrator with a diaphragm and with Hydranal-Coulomat Oil as

Table 2.2: Typical retention times (t_R) and relative retention times (RRTs) of identified compounds present in deodorizer distillate

Compound	t_R (min)	RRT [a]
Glycerol	2.8	0.08
Monoglycerides	23.2	0.66
Squalene	24.1	0.69
δ-Tocopherol	25.7	0.73
β-Tocopherol	27.7	0.79
γ-Tocopherol	28.0	0.80
α-Tocopherol	30.3	0.89
Brassicasterol	31.5	0.90
Stigmasterol	31.6	0.92
Campesterol	32.4	0.93
β-Sitosterol	33.1	0.94
HDS (Internal Standard)	34.2	1.00
Diglycerides	37.5	1.10
Steryl Esters	44.5	1.30
Triglycerides	55.5	1.60
Triglycerides	63.7	1.80

[a] Retention time relative to HDS

anolyte reagent for coulometric Karl Fischer titrator, as well as working medium.

Samples are previously weighed (≈1 gram) and injected into the KF titrator. The result is expressed as ppm.

2.3.3 Experimental Procedure Methods

2.3.3.1 Viscosity Measurement

The viscosity was measured using a digital viscometer (Brookfield Engineering Laboratories Inc., USA). The temperature was controlled immersing the samples (17 ml) in a water bath at 40 °C during the viscosity measurement.

2.3.3.2 Water Adsorption Isotherms

Saturated solutions may be used for pre-equilibrating a system to a defined water activity through the vapor phase, since the water activity, at a controlled temperature, will be the same for all phases in contact. Reliable values of water activity for several inorganic salts at different temperatures are tabulated by Greenspan [62]. The salts solutions selected for this study are shown in Table 2.3.

Saturated salt solutions were prepared mixing inorganic salts with distilled water in order to obtain a solution with a high solid fraction. Such solution will allow for a rapid re-equilibration after temperature change. It is important to use inorganic salts with high purity since with large excess of solids, even quite small levels of impurity could depress the vapor pressure of the saturated solution.

Table 2.3: Saturated salt water activities at 40°C [62]

Salt	Water Activity (a_W)
LiCl	0.11
KF	0.23
$MgCl_2$	0.32
NaI	0.33
$MgNO_3$	0.48
$CoCl_2$	0.55
KI	0.66
NaCl	0.75
KCl	0.82
KNO_3	0.89

Oleic acid (*OA*), oleic acid with enzyme and sunflower deodorizer distillates were pre-equilibrated by storage in a closed vessel with the vapor phase in contact with the appropriate saturated salt solution. This solution was placed at the bottom of a jar and the samples to be equilibrated were introduced in an inner recipient with a volume of 5 ml. The jars were sealed in order to prevent evaporation of solvent and to avoid water exchange. Throughout the equilibrium period, the temperature was controlled to prevent cold surfaces from condensing liquid water. The jars were place in an oven at 40 °C, the same temperature at which the enzymatic reaction is carried out.

Since the rate of equilibration depends on the polarity of the samples, the equilibration process required several weeks. This process was monitored by Karl Fisher water analysis of the samples. Brief exposure to the laboratory atmosphere during sampling did not affect the equilibrium except when the total water content was low. Care was needed to ensure re-equilibration after temperature changes. These will normally cause changes in the solubility of the salt used, so that the solution becomes temporarily supersaturated or sub-saturated. A substantial time period (2-3 days) was required before equilibrium was re-established.

2.3.3.3 Water Partitioning Between Solvent and Enzyme

In order to correlate the water present in the reaction system with the hydration degree of the enzyme, the partitioning of water between the solvent and the enzyme was studied.

Solutions of oleic acid with 0.50% and 0.25% (w/w) of enzyme were prepared. Small quantities of water were added, in order to obtain a water content between 0 and 2.0% (w/w). It should be noted that we only considered results from systems were phase separation was not observed. These solutions were mixed during two days at 200 rpm and at 40 °C in an orbital shaker in order to promote the contact and equilibrium between solvent, water and enzyme. After stopping stirring, the total water content of the suspension, as well as in the supernatant after enzyme removal was determined by Karl Fisher titration. The difference in water content between the overall and supernatant solutions corresponds to the water associated with the enzyme. This result was expressed in ppm of water/mg of enzyme. This experiment was carried out using 0.50% and 0.25% (w/w) of

enzyme in order to guarantee that the results are independent from the quantity of enzyme used.

2.3.3.4 Lipase Catalyzed Reactions

Reactions were carried out in a 250 ml hermetically sealed and jacketed vessel in order to maintain the temperature constant during the esterification reaction. Inside this vessel, a smaller recipient of 50 ml was introduced, which may be filled with a defined saturated salt solution if a control of water activity is required during the esterification reaction (Figure 2.1). The smaller vessel is linked to the larger one by fixed glass rods. Hence, mass transfer between both solutions occurs through the headspace of the setup.

The vessel was initially filled with 100 g of the standard mixture (deodorizer distillates with a defined molar ratio of *FFA* to *S*), stirred at 500 rpm and maintained at 40 °C overnight before reaction. If the water activity was controlled during reaction, the inner vessel was filled with a selected salt solution. The *Candida rugosa* lipase was maintained at 40 °C in oleic acid overnight, before adding it to the reaction medium. If a water activity control was used during reaction, the enzyme was also pre-equilibrated at that specific water activity (Table 2.3).

Over the time course of the reaction, samples of $\backsimeq 2$ g were periodically removed from the reaction vessel for *FFA*, Karl Fisher and GC analysis.

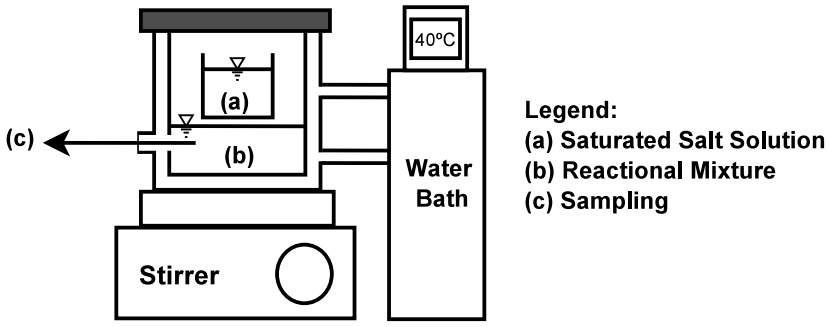


Figure 2.1: Experimental setup of the lipase catalyzed reactions

2.3.4 Numerical Methods

2.3.4.1 Determination of the Kinetic Constants

In order to estimate the kinetic constants of the reactions (I-IV) described in the Modelling of the Enzymatic Reaction section, initial estimates of the kinetic constants and the initial concentration of the compounds were fed to the system of differential equations expressing the mass balance for each one (Eq. 2.1-2.9), at the beginning of the simulation (Figure 2.2). The system of differential equations was implemented on MatLab 7.9 (MathWorks, USA), and solved using the 4th order Runge-Kutta-Fehlberg method [84].

The simulation results were compared with experimental data at each measured point. The deviations between experimental and calculated values were squared and summed up to form an objective function. This objective function was fed into a minimizer routine based on a nonlinear

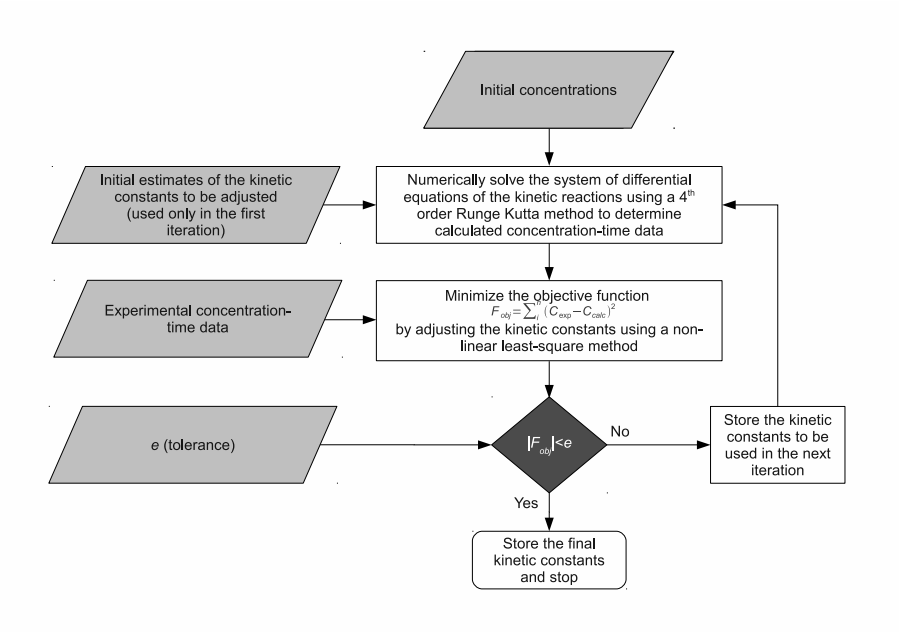


Figure 2.2: Flowchart of the methodology used for the determination of the kinetic constants

least-square method which determines the optimal rate constants.

2.3.4.2 *Methodology for Calibration and Validation of the Model*

The calibration of the model was carried out using as inputs the evolvement of the concentration of compounds during reaction, under optimal conditions as described in the Results and Discussion Section ($\backsimeq$0.50% w/w of enzyme concentration, initial *FFA:S* molar ratio between 6.0 and 6.7 and a_W between 0.45 and 0.85). Information about a_W was also an input to the model as a switch function, that uses Eq. 2.8 if there was no water control, otherwise applying Eq. 2.9. It should be noted that only kinetic experiments with the deodorizer distillate "2" were used for model calibration (see composition in Table 2.1).

The experimental data were split into calibration and validation sets, with respectively 75% and 25% of the data.

In order to verify the robustness of the model, the kinetic constants determined in the calibration step were used to simulate the following conditions, which were selected to be outside the calibration range:

- Lower concentration of enzyme (0.25% and 0.10% w/w);
- Deodorizer distillate from a different source and lot (Deodorizer Distillate "3", see composition in Table 2.1).

2.3.4.3 *Quality of Fit of the Model*

The least square objective function, S_y, may be used to measure the quality of fit of the model. For n compounds, and j data points, the function S_y compares the values predicted by the model

($\hat{\theta}$) and the values actually observed (θ), being expressed as:

$$S_y = \sqrt{\frac{\sum_1^n \sum_1^j \left(\theta - \hat{\theta}\right)^2}{jn-1}} \tag{2.10}$$

Lower values of S_y indicate a good agreement between experimental data and model predictions.

2.4 Results and Discussion

The enzymatic esterification reaction was optimized by studying: i) the effect of the enzyme concentration, which may represent an important cost to the process; ii) the water activity, since water acts as a molecular lubricant and plays a crucial role in the enzyme activity; iii) the reactants (FFA and sterols) molar ratio, because it influences the rate and yield of the esterification reaction.

In the following analysis, all the experiments were carried out at 40 °C in order to avoid the oxidation of fatty acids and preserve temperature sensitive species such as tocopherols and sterols.

2.4.1 Effect of Enzyme Concentration

In this study, different deodorizer distillates from different sources and lots were used, in order to guarantee that the results are valid for a wide range of different deodorizer distillates, independently of their composition.

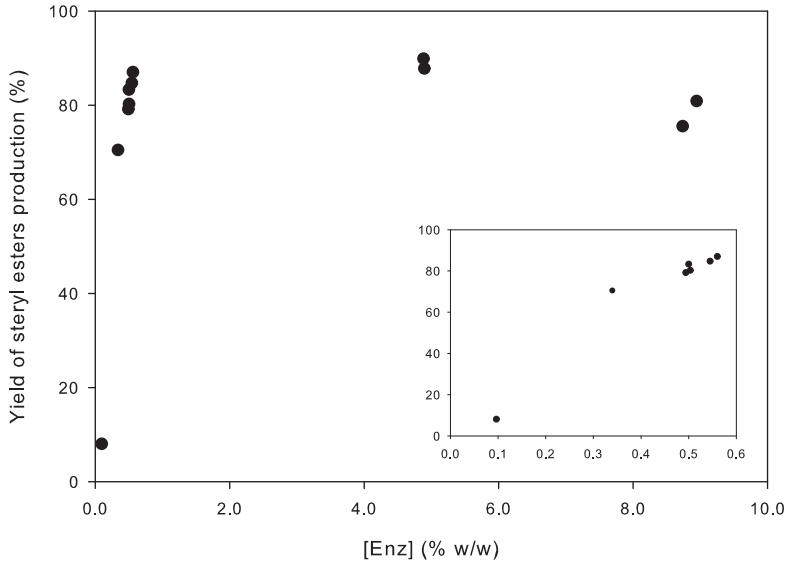


Figure 2.3: Effect of the enzyme concentration ([Enz]) on the yield of the steryl esters production (after 24 hours for reactions carried out with 0.25 and 0.50% w/w of enzyme concentration and after 2 hours for reactions carried out with 5% and 9% w/w of enzyme concentration). See inset figure for enzyme concentrations between 0.0% and 0.6% w/w.

Figure 2.3 shows the yield of steryl esters obtained in kinetic studies performed with different

concentrations of enzyme. The small variation of the initial water activity, a_w, and *FFA:S* molar ratio, between kinetic studies, did not significantly affect the reaction yield since their values are within the optimum range, as it will be shown in the following subsections.

In Figure 2.3 it may be seen that it is possible to use low concentrations of enzyme (0.25 - 0.50% w/w) for obtaining high yields of steryl esters production (70.5-87.7%), similar to those obtained with much higher concentration of enzyme (5.0-9.0% w/w) as reported by Torres et al. [40, 79] and Villeneuve et al. [37]. Below 0.25% (w/w) of enzyme concentration, it is not possible to obtain such high yields within 24 hours. Therefore, for subsequent studies, the enzyme concentration was set at 0.50% (w/w), since we consider this concentration as the best compromise between the efficiency of steryl esters production and the time required to obtain such yield.

2.4.2 Effect of water activity

2.4.2.1 Determination of Water Adsorption Isotherms

Water activity was determined from water adsorption isotherms (water content versus water activity at a fixed temperature) after measuring the water concentration by Karl Fisher titration.

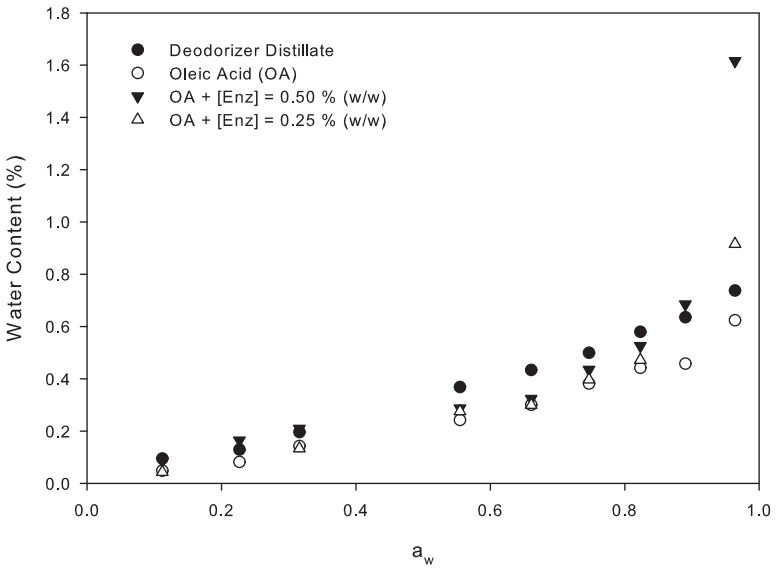


Figure 2.4: Water adsorption isotherms at 40 °C for the (●) - Deodorizer Distillate, (○) - Oleic Acid (OA) , (▼) - Oleic Acid with 0.5% (w/w) of Enzyme (OA+[Enz]=0.5% (w/w)) and (△) - Oleic Acid with 0.25% of Enzyme (OA+[Enz]=0.25% (w/w))

Figure 2.4 shows that the water adsorption isotherms are similar for deodorizer distillate and oleic acid (*OA*), which is consistent with the fact that *FFAs* are one of the main components of deodorizer distillates, representing more than 20% of its composition. On the other hand, it is possible to observe the effect of the presence of the enzyme in the water activity behaviour. The data for oleic acid with enzyme only differ significantly from the data for oleic acid at high values of a_W ($\gtrsim 0.88$). This effect probably occurs because, at lower a_W, water tends to be in solution,

while closer to solvent saturation (at higher a_W) water becomes more available to the enzyme. This hypothesis is discussed in the following subsubsection.

2.4.2.2 Water Partition Between Solvent and Enzyme

In order to correlate the water present in the reaction system with the hydration of the enzyme, the water partitioning between solvent and enzyme was determined by the method described previously (see Material and Methods section).

Figure 2.5 shows how the water partitions between the oleic acid (solvent) and the enzyme at different a_W. At $a_W \leq 0.45$, the water is essentially solvated by the solvent, while between $0.45 \leq a_W \leq 0.85$ the water tends to partition to the enzyme, although its major fraction still remains in solution. At $a_W \geq 0.85$ the solvent is approaching a saturation state, making water more available to the enzyme.

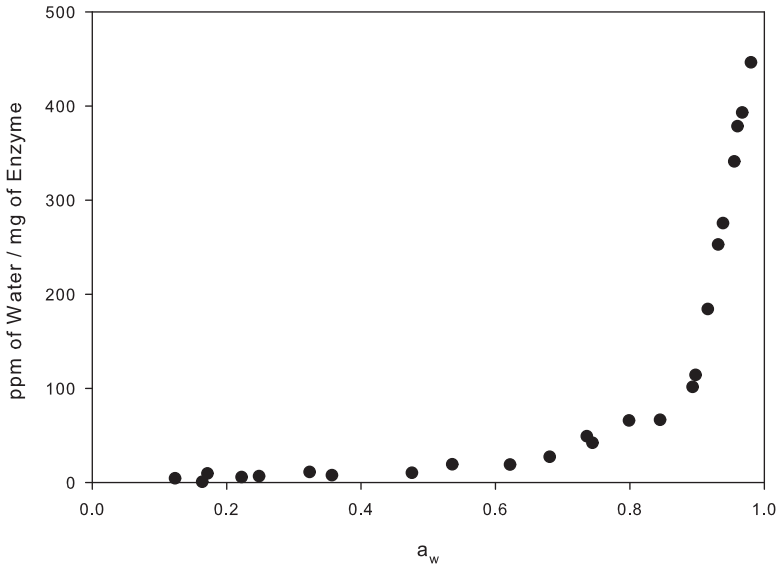


Figure 2.5: Water partitioning between oleic acid and enzyme at 40 °C

Therefore, we concluded that the value of a_W should not be higher than 0.85 since, as it is shown in Figure 2.5, the amount of water associated with the enzyme increases sharply at this value of a_W, driving the reaction equilibrium towards hydrolysis in detriment of esterification, leading to a significant decrease in the yield of steryl esters production (Figure 2.6).

2.4.2.3 Effect of water activity in the yield of the steryl esters production

Kinetic studies were carried out varying the a_W between 0.20 and 1.0 and maintaining the concentration of enzyme and *FFA:S* molar ratio constant at 0.50% (w/w) and 7.0, respectively.

Figure 2.6 shows that the yield of the steryl esters production attains a maximum value at a_W between 0.45 and 0.85. At a_W below 0.45 the yield is lower (<80%) probably due to the lower hydration state of the enzyme, which leads to a more rigid structure with a lower mobility and,

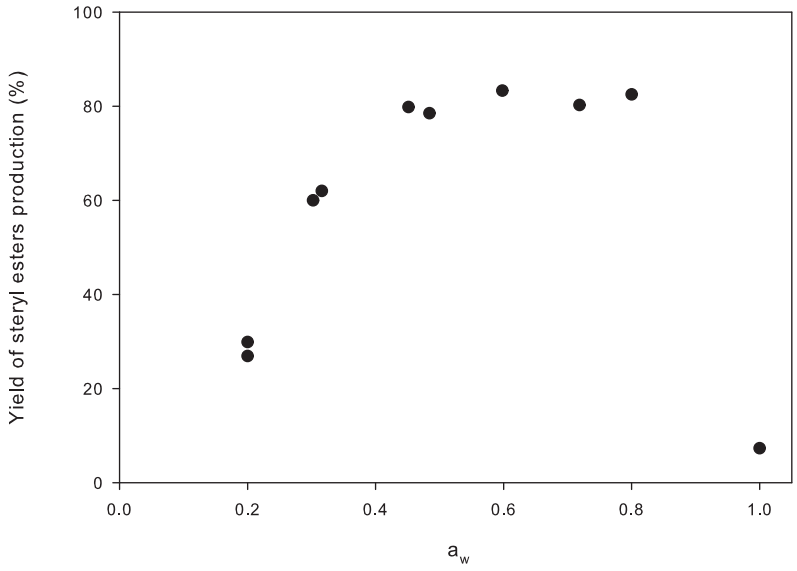


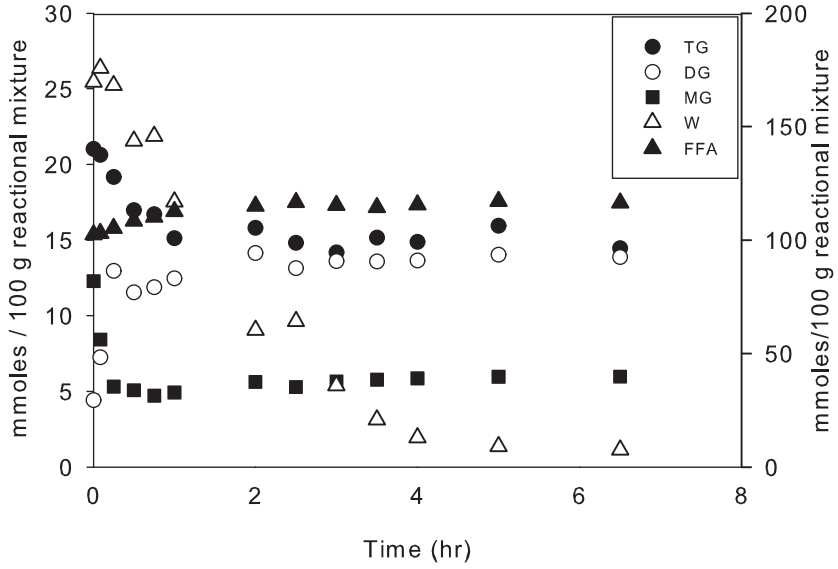
Figure 2.6: Yield of steryl esters production at various water activities (a_W) with [Enz]=0.50% (w/w) and *FFA:S* molar ratio = 7.0 at 40 °C

ultimately, a lower enzyme activity. At a_W higher than 0.85, the solvent approaches saturation with water. Under these conditions, the high flexibility of the enzyme promoted by the high water content seems to cause a decrease of selectivity driving the equilibrium towards hydrolysis, as it is shown in Figure 2.7, where hydrolysis of acylglycerides (Figure 2.7(a)) and the increase of the concentration of sterols (Figure 2.7(b)) are observed.

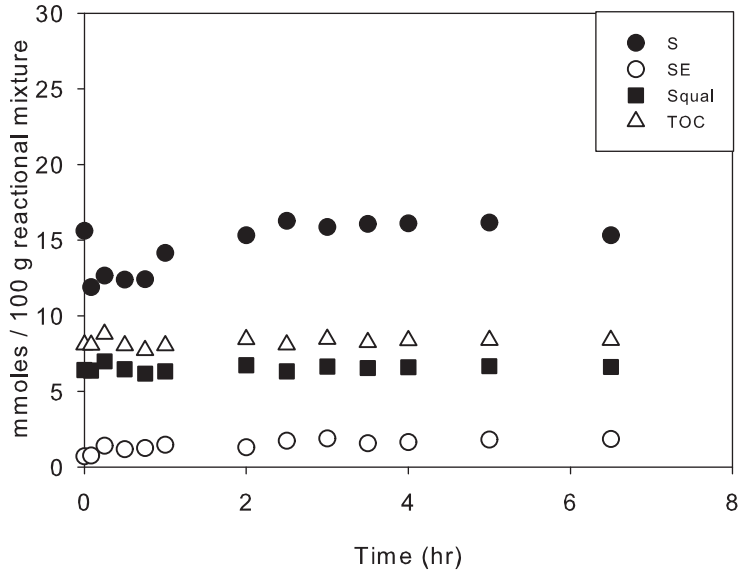
2.4.2.4 Effect of the control of water activity during the esterification reaction

In order to guarantee that the hydration state of the enzyme and the water content in the reaction bulk medium are constant during esterification, it is possible to set the water activity at a desirable value by equilibrating the reaction medium with pre-defined saturated salt solutions.

Figure 2.8 compares the evolvement of the reaction composition during a kinetic study with control of a_W set at 0.66 (Figure 2.8(a) and Fig.2.8(b)) and without control of a_W (Figures 2.8(c) and 2.8(d)). In order to enable an adequate comparison, the initial conditions for the two kinetic studies were similar ([Enz]=0.50% (w/w), a_W=0.66, *FFA:S* molar ratio=6.0). The yield observed was 80.0% for the kinetic study with a_W control, and 78.6% for the kinetic study without a_W control. In the specific kinetic study which was carried out without control of a_W, a small variation of a_W during the reaction occurred (final a_W=0.58) but, even so, the final yield of steryl esters production was not compromised. Apparently, there are no significant advantages in performing the control of a_W in this case, since the value of the water activity during the reaction is within the optimal range (Figure 2.6). These studies indicate that as long as the initial value of a_W in the reaction medium is within the optimal range ($0.45 < a_W < 0.85$), water activity control during the reaction course may not be necessary.



(a)



(b)

Figure 2.7: Evolvement of concentration of target solutes during a kinetic study with a *FFA:S* molar ratio = 6.0, [Enz]=0.50% (w/w) and a_W=1.0 (Figure (a): (•) - triglycerides (TG), (○) - diglycerides (DG),(■) - monoglycerides (MG), (△) - Water (W) represented on secondary axis, (▼) - Free Fatty Acids (FFA) represented on secondary axis; Figure (b): (•) - Sterols, (○) - Steryl Esters (SE), (■) - Squalene (Squal), (△) - Tocopherols (TOC))

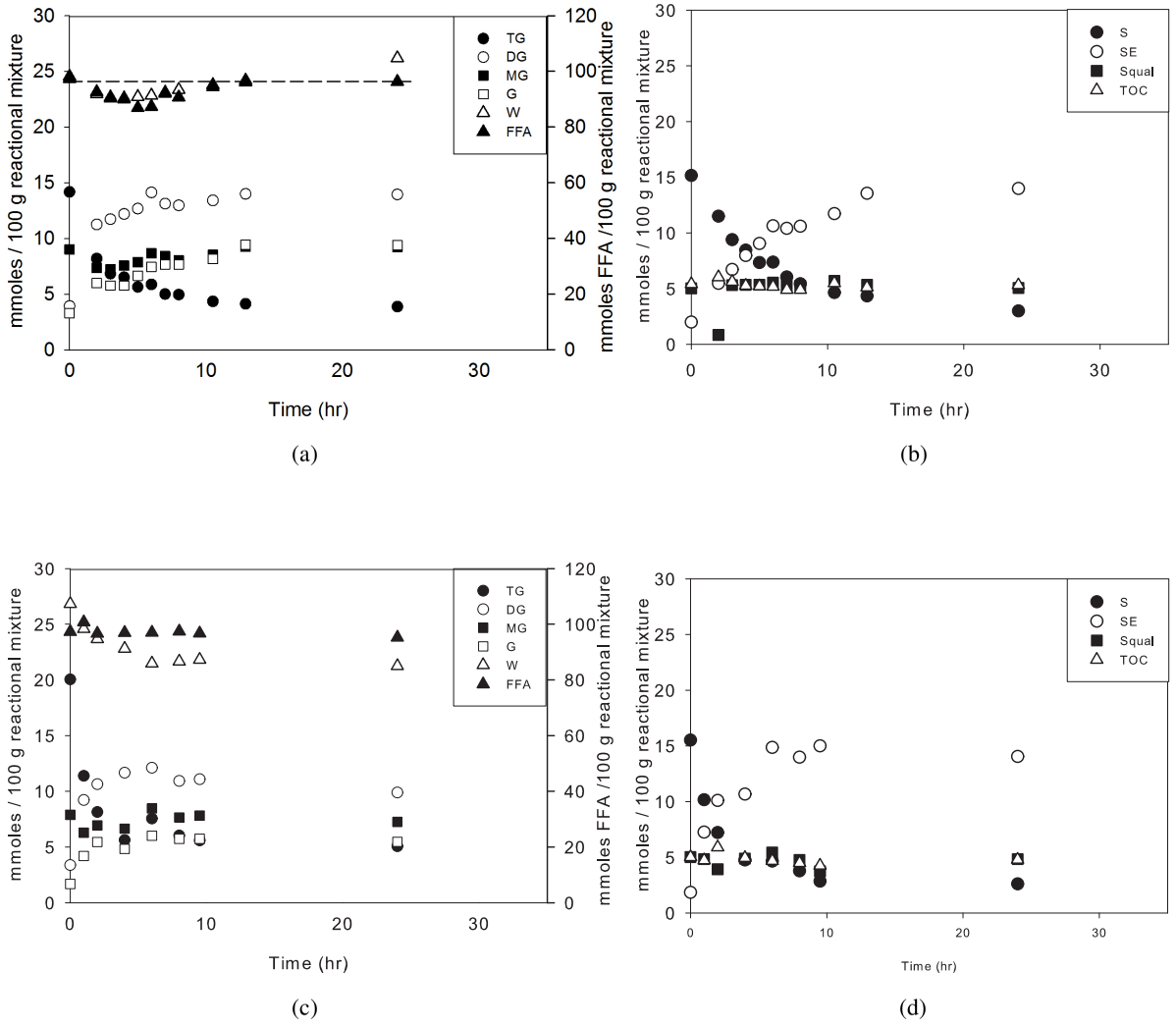


Figure 2.8: Figure (a) and (b): Evolvement of concentration of target solutes during a kinetic study with control of a_W set at 0.66, [Enz] = 0.50 % (w/w) and *FFA:S* molar ratio = 6.0 ; Figure (c) and (d): Evolvement of the compounds concentration during a kinetic study without control of a_W (initial a_W = 0.72), [Enz] = 0.50 % (w/w) and *FFA:S* molar ratio = 6.3 (Figure (a) and (c):(●) - triglycerides (TG), (○) - diglycerides (DG),(■) - monoglycerides (MG), (□) - glycerol (G), (△) - Water (W), (▼) - Free Fatty Acids (FFA) represented on secondary axis; Figure (b) and (d): (●) - Sterols, (○) - Steryl Esters (SE), (■) - Squalene (Squal), (△) - Tocopherols (TOC))

2.4.3 Effect of the *FFA:S* molar ratio

In order to guarantee that the *FFAs* are not the limiting reactant, oleic acid was added to the reaction mixture. Moreover, as it is shown in Table 2.4, the addition of oleic acid leads to an important decrease of the viscosity of the reaction medium, enhancing mass transfer.

Table 2.4: Effect of add oleic acid in the reaction mixture viscosity at 40°C

	FFA:S molar ratio	Viscosity at 40°C (cP)
Deodorizer Distillate "2"	4.5	33.9
Deodorizer Distillate "2" + 5% (w/w) OA	5.7	30.3
Deodorizer Distillate "2" + 10% (w/w) OA	7.2	29.1
Deodorizer Distillate "2" + 15% (w/w) OA	8.8	26.6
Oleic Acid (OA)	–	18.1

In order to determine the influence of the initial *FFA:S* molar ratio in the yield, different amounts of oleic acid were added to the deodorizer distillate "2", whose *FFA:S* molar ratio is originally 4.5. It was observed that such ratio (4.5) is not advantageous for the steryl esters synthesis since the yield obtained was only 7.2% after 10 hours of reaction. Figure 2.9 shows that for *FFA:S* molar ratio values higher than 6, addition of oleic acid does not further improve the yield, reaching a plateau value slightly above 80%. Hence, these results suggest that the optimal *FFA:S* molar ratio is a value at least of 6.

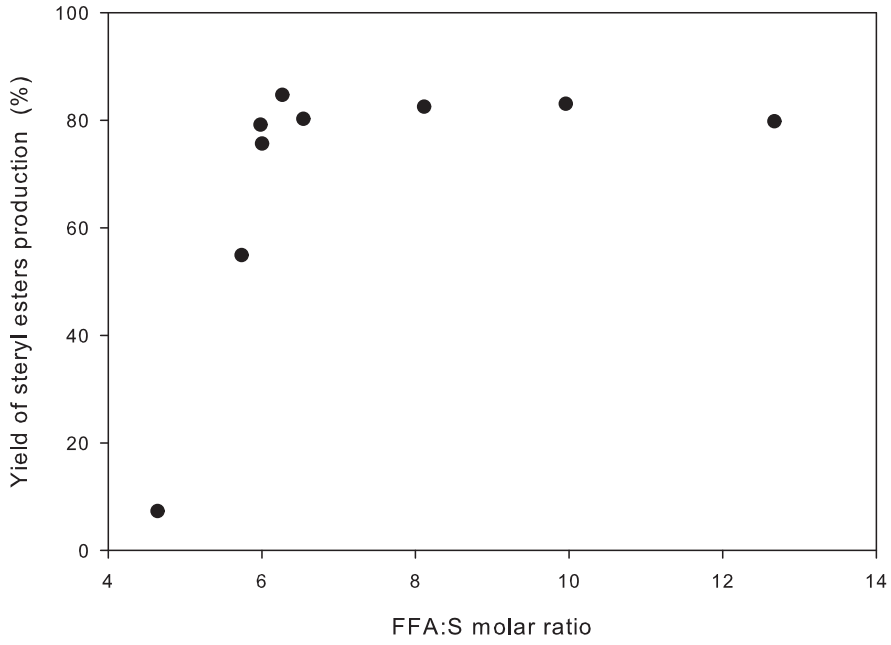


Figure 2.9: Effect of the initial *FFA:S* molar ratio in the yield of steryl esters production after 24 hours of reaction

2.4.4 Modelling of the Enzymatic Reaction

In the present study it was decided to use the established differential equations of compounds mass balance (Eq. 2.1-2.9) to determine the respective kinetic constants. The methodology followed was described in a previous section.

2.4.4.1 Calibration of the Model

The initial conditions of the kinetic studies used for calibration of the model are shown in Table 2.5. From a total of 170 experimental data points, 125 points ($\simeq$ 75%) were used for calibration of the model.

Table 2.5: Initial conditions of the kinetic studies used for calibration of the model

Kinetic Study	[Enz] (% w/w)	a_W	*FFA:S*	Control of a_W
1	0.51	0.64	6.0	No
2	0.49	0.45	6.7	No
3	0.56	0.65	6.5	Yes
4	0.49	0.69	6.0	Yes
5	0.54	0.75	6.3	No
6	0.51	0.63	6.0	Yes

The kinetic constants of Eq. I-IV described previously were determined, as well as the confidence intervals (CI) at 95% for the model predictions, as shown in Table 2.6.

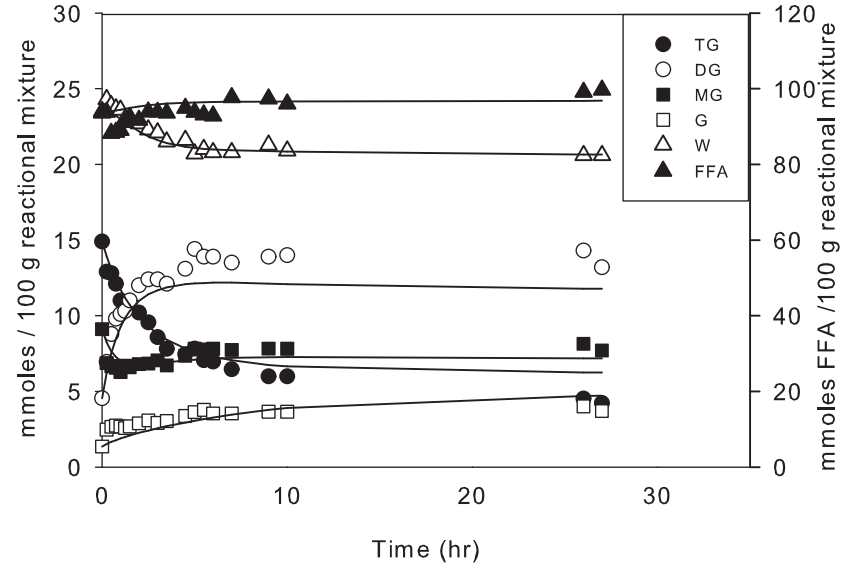
Table 2.6: Kinetic constants of the reactions (I-IV) and the respective error defined as $\frac{CI}{k_x} \times 100$

Kinetic Constant	Value (h^{-1})	Error (%)
k_1	0.152	10.4
k_{-1}	0.267	21.3
k_2	0.143	32.6
k_{-2}	0.445	27.8
k_3	0.691	8.2
k_{-3}	1.980	7.3
k_4	0.170	26.1
k_{-4}	1.508	19.1

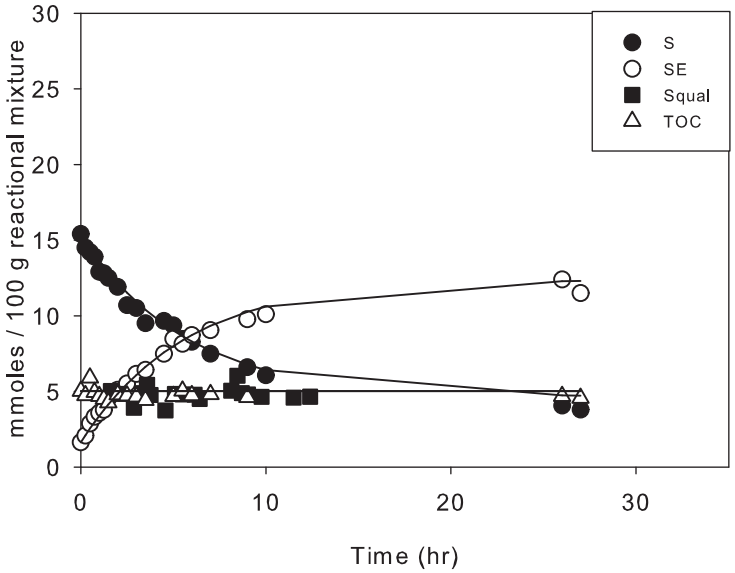
The error associated to the kinetic constants is between 7.3 - 32.6%. The highest error values may be associated to the small variation of the compounds concentration, during the time course of the experiments, such as in the case of k_2 and k_{-2} where the variation of the concentration of glycerol and monoglyceride is not significant (Figure 2.10).

In Figure 2.10 it is shown, as an example, the experimental evolvement of the reaction mixture concentration as well as the concentrations given by the model for the kinetic study "1".

The least square objective function, S_y was used to verify the agreement between the experimental data and model results. Table 2.7 shows the S_y values obtained for each kinetic study used for calibration of the model.



(a)



(b)

Figure 2.10: Evolvement of the reaction mixture concentration for kinetic study "1" (without a_W control) - figure (a) and (b). The points represents the experimental data and the lines the adjustment of the model. (Figure (a) and (c):(●) - triglycerides (TG), (○) - diglycerides (DG),(■) - monoglycerides (MG), (□) - glycerol (G), (△) - Water (W), (▼) - Free Fatty Acids (FFA) represented on secondary axis; Figure (b) and (d): (●) - Sterols, (○) - Steryl Esters (SE), (■) - Squalene (Squal), (△) - Tocopherols (TOC))

Table 2.7: S_y obtained from the estimated compounds concentrations for all kinetic studies used for calibration of the model

Kinetic Study	S_y
1	0.11
2	0.13
3	0.48
4	0.27
5	0.44
6	0.44

A very good agreement obtained for most data points, verified by the relatively low S_y obtained for all kinetic studies, serves as a support for the mathematical modelling approach used.

2.4.4.2 Validation of the model

The initial conditions of the kinetic studies used for validation are shown in Table 2.8. From a total of 170 experimental data points, 45 points were used for validation of the model.

Table 2.8: Initial conditions of the kinetic studies used for validation of the model

Kinetic Study	[Enz] (%w/w)	a_W	*FFA:S*	Observations
7	0.34	0.58	12	Different deodorizer distillate source and high *FFA:S* [a]
8	0.25	0.54	6.2	Low amount of enzyme
9	0.10	0.50	7.4	Very low amount of enzyme

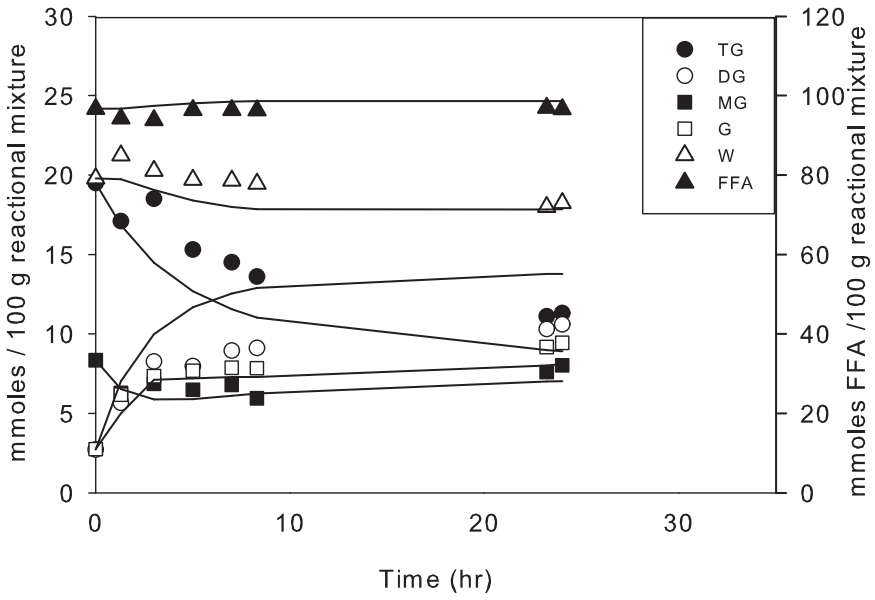
[a] See composition of the deodorizer distillate "3" in Table2.1

Table 2.9 shows the S_y obtained when applied to the estimated solute concentrations (variables) in all kinetic studies used for validation of the model (Table 2.8).

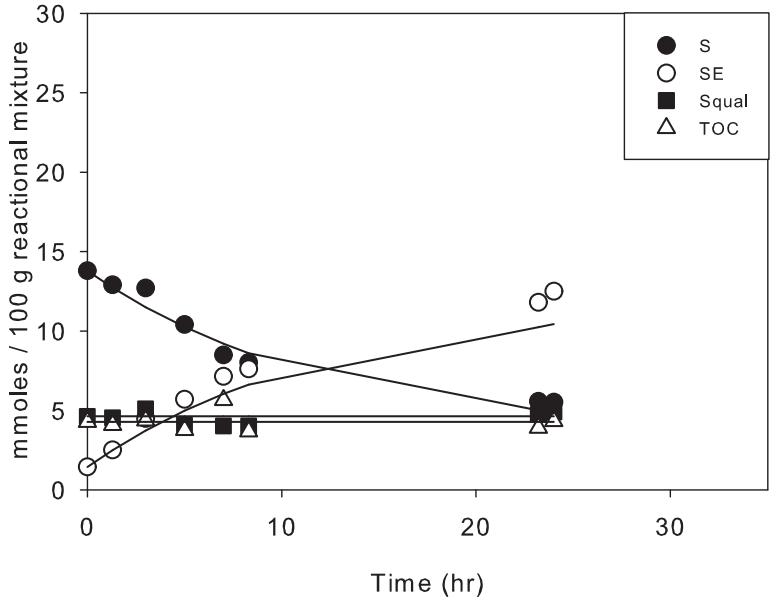
Table 2.9: S_y obtained from the estimated solutes concentrations for all kinetic studies used for validation of the model

Kinetic Study	S_y
7	0.29
8	0.27
9	0.37

Comparing the values of S_y obtained for kinetic studies used for calibration (Table 2.7) and validation (Table 2.9) of the model, it is possible to verify that they are within the same range. Thus, it is possible to conclude that the model developed shows a good extrapolation capacity, enabling the estimation of the concentrations of the compounds in kinetic studies with concentration of enzyme between 0.50 and 0.10 % (w/w) and with high value of the *FFA:S* molar ratio, even when using a deodorizer distillate from a different source and with a composition different from the one used for model calibration.



(a)



(b)

Figure 2.11: Evolvement of concentration of target solutes during the kinetic study 8. The points represents the experimental data and the lines the adjustment of the model. (Figure (a):(●) - triglycerides (TG), (○) - diglycerides (DG),(■) - monoglycerides (MG), (□) - glycerol (G), (△) - Water (W), (▼) - Free Fatty Acids (FFA) represented on secondary axis; Figure (b): (●) - Sterols, (○) - Steryl Esters (SE), (■) - Squalene (Squal), (△) - Tocopherols (TOC))

Figure 2.11 is representative of the good agreement between the model developed and the experimental data for the kinetic study 8, performed with a lower enzyme concentration (0.25 % w/w), which is indicated by its relatively low S_y value (Table 2.9). In this case, the model developed does not describe every component with the same degree of accuracy (for example diglycerides and triglycerides show a worse agreement than other species) but, it is important to notice that sterols and steryl esters kinetics are correctly described.

2.4.5 Optimized Composition of the Deodorizer Distillate after Enzymatic Reaction

Table 2.10 shows the typical optimized composition of the deodorizer distillate after enzymatic reaction.

Table 2.10: Typical optimized composition of the sunflower deodorizer distillates after 24 hours of reaction without control of water activity (deodorizer distillate "2", 0.50% w/w of enzyme, initial a_W=0.54, *FFA:S*=7.0)

	Units	Compound	Before	After
Acylglycerides	g/100g	G	0.25	0.90
		MG	2.98	2.86
		DG	1.68	6.61
		TG	17.3	10.0
Tocopherols	g/100g	α-Tocopherol	0.91	0.96
		δ-Tocopherol	0.51	0.51
		β/γ-Tocopherol	0.39	0.36
Squalene	g/100g	Squalene	1.65	1.75
Sterols	g/100g	Campesterol	1.30	0.49
		Stigmasterol	0.60	0.24
		β-Sitosterol	3.50	1.38
		Others Sterols	0.26	0.16
Steryl Esters	g/100g	Steryl Esters	0.94	8.17
Fatty Acids	g as oleic acid/100g	Free Fatty Acids	26.6	28.8
Water	ppm	Water	3823	3237

The steryl esters are successfully produced while temperature sensitive species such as tocopherols and sterols were preserved which indicate that the temperature of the reaction is adequate. Concerning the content in pesticides, their value in the deodorizer distillate remains constant before and after reaction, which indicates that they are not esterified (data not shown), which is positive aspect if considering a subsequent separation step based on size exclusion.

2.5 CONCLUSIONS

In conclusion, the production of steryl esters by enzymatic catalysis technology proved to be adequate, allowing for obtaining good conversion yields. The production of steryl esters was achieved

with the enzyme *Candida rugosa* under mild reaction conditions (0.25<[Enz]<0.50% (w/w), 0.45 $< a_W <$ 0.85 and *FFA:S* molar ratio higher than 6.0, at T=40 °C) and with a satisfactory esterification degree of phytosterols, typically above 80%.

The kinetic constants determined from the modelling of the reactions that occur in the enzymatic process, allowed for a good prediction of the evolvement of the composition of the reaction mixture in conditions with low concentration of enzyme (0.10% (w/w)), with high *FFA:S* molar ratio, even when using a deodorizer distillate from a different source and with a composition different from the one used for model calibration. In particular, the ability of the model to describe the transient state of the system during the enzymatic reaction when using different deodorizer distillates, is indicative of the good extrapolation capacity of the model.

The production of steryl esters is additionally expected to allow for a subsequent removal of pesticides from the reaction product, using membrane-based separation processes (e.g. nanofiltration), given the difference of molecular weight between the steryl esters (650-800 Da) and pesticides (150-420 Da). This separation process is of utmost importance for the recovery of pesticides-free steryl esters to be used as food additives.

3 Production of Steryl Esters using Alternative Sources of Sterols and Free Fatty Acids - Modelling and Guidelines

Summary *Enzymatic esterification reactions for the production of steryl esters were conducted using different sources of sterols and free fatty from mixtures of vegetable oil deodorizer distillates and refined oil. This work discusses the possibility of using free fatty acids from alternative and least expensive sources, determining their influence in the yield of steryl esters production as well as in the validity of a kinetic model developed in Chapter 2 for defined reaction medium composition. The extrapolation capacity of such model was investigated, identifying the cases where a lack of model fitting was observed and establishing the range of conditions for model applicability. Based on a sensitivity analysis, guidelines to obtain acceptable yields (>80%) were established. This work shows that the use of alternative sources of sterols and free fatty acids for the production of steryl esters is possible, however, there are constraints and guidelines that have to be taken into account.*

The contents of this chapter were adapted from the publication: Teixeira, A. R. S., Santos, J. L. C., Crespo, J. G. (2014). Production of Steryl Esters using Alternative Sources of Sterols and Free Fatty Acids - Modelling and Guidelines. Industrial & Engineering Chemistry Research. Submitted.

3.1 INTRODUCTION

Deodorizer distillates are a residual stream produced by the industry of vegetable oil refining. Their high content in bioactive compounds such as sterols, tocopherols and squalene may vary between 2-20% [17], however, regardless the interesting composition of deodorizer distillates, they have a very limited commercial value due to their high content in pesticides [22, 23].

Steryl esters are relevant ingredients in the preparation of functional foods, being recognized by the European Food Safety Authority (EFSA) as lowering cholesterol agents [28] with higher bioactivity than free sterols [33]. Moreover, the higher molecular weight of steryl esters (650<MW<800 g mol^{-1}) in comparison to their free sterol (400-415 g mol^{-1})) and contaminant pesticides (150<MW<400 g mol^{-1}) opens a potential use of separation processes based on size exclusion such as pressure-driven membrane processes.

In Chapter 2, it was showed that the lipase from *Candida Rugosa* is able to esterify sterols and free fatty acids (FFA) from deodorizer distillates, producing steryl esters under optimised conditions. Accordingly to this study, in order to obtain a satisfactory esterification degree of phytosterols (above 80%) in 24 hours at T=40 °C, the concentration of enzyme and the water activity of the reaction medium (a_W) must be within the range of 0.25<[Enz]<0.50% (w/w) and $0.45<a_W<0.85$, respectively. On the other hand, it is important to assure that the reactants molar ratio (free fatty acids:sterols, *FFA:S*) is higher than 6.0, in order to drive the reaction towards the esterification. The excess of free fatty acids may be adjusted by the addition of oleic acid, as also proposed by other authors [8, 37, 42].

The use of alternative sources of sterols and free fatty acids may be very interesting from an economic and environmental point of view, but increases the complexity of the reaction system. The mathematical model established in Chapter 2 provided the kinetic constants of the relevant involved reactions, enabling, consequently, the prediction of the composition profile during reaction (once characterized the initial composition of the reaction mixture). This model was obtained using different deodorizer distillates as a source of sterols and oleic acid as additional source of free fatty acids. Even though the origin of the deodorizer distillates was different, the initial composition of the reaction mixtures was rather similar. It is expected that this situation may be different if alternative sources of free fatty acids with completely different compositions, are considered to adjust the *FFA:S* molar ratio (in place of oleic acid). This chapter discusses the possibility of using free fatty acids from alternative and less expensive sources, determining their influence in the yield of steryl esters production as well as in the validity of the previous model developed. Additionally, it is aimed to the setting of guidelines for the preparation of the reaction mixture, in order to obtain yields above 80%.

Depending on the operating conditions of the deodorization step, the composition of the respective distillates vary in terms of sterols, tocopherols, steryl esters, free fatty acids and acylglycerols [11]. Since the enzymatic hydrolysis of acylglycerols is a potential source of free fatty acids, a deodorizer distillate, even poor in sterols, may be used to ensure the required excess of fatty acids. Deodorizer distillates from olive oil are typically poor in sterols and rich in free fatty acids, making this by-product an alternative source with an additional high oxidation stability due to their

high content in squalene and tocopherol [9, 85]. Refined oils are products mainly constituted by triglycerides, easily available to the producers of edible oil and less expensive than the oleic acid itself. Therefore, three alternative sources of free fatty acids were considered in our study: a deodorizer distillate and a refined oil from sunflower, rich in triglycerides, and a deodorizer distillate from olive oil.

The applicability of the mathematical model (developed in Chapter 2) to systems with different initial composition was verified by evaluating the quality of the fitting of the model. To accomplish this objective, enzymatic reactions using distinct sources were carried-out under optimal conditions. Deviation of the predicted yield of production of steryl esters to the data acquired experimentally was analysed and discussed, and the limitations to the model extrapolation assessed.

Finally, guidelines for the preparation of the initial reaction mixture were established, in order to obtain yields above 80%.

3.2 Materials and Methods

3.2.1 Materials

3.2.1.1 Sources of Sterols and Free Fatty Acids

Sunflower deodorizer distilates were obtained from Lesieur (France) and Sovena (Portugal). The refined oil with high content in triolein (>90%) and olive oil deodorizer distillate were also a gift from Sovena (Portugal). Food-grade oleic acid with an acid value of 196.0-204 mg KOH/g was purchased from Sigma Aldrich (Belgium). The characterization of deodorizer distillates was made according to the methods described in the subsection 2.3.2 (Chapter 2), being summarized in Table 3.1.

3.2.1.2 Enzyme

Candida rugosa lipase, Type VII, was obtained from Sigma (Saint Quentin, France). The activity of this lipase as indicated by the supplier is 1535 U/mg of solid. One unit (U) of lipase activity is defined as the amount of enzyme that hydrolyses 1.0 μequivalent of fatty acid from a triglyceride in one hour at pH 7.2 and at 37 °C using olive oil (30 minutes of incubation).

3.2.1.3 Chemicals

Analytical-grade chloroform, food-grade oleic acid, N,O-bis (trimethylsilyl) trifluracetamide (BSTFA) containing 1% of trimethylchlorosilane (TCMS) solution (from Fluka) and pyridine were obtained from Sigma Aldrich (Belgium).

All analytical-grade standard substances, squalene (99.3% purity), stigmasterol (97% purity), β-sitosterol (99%purity), campesterol (99% purity), cholesteryl stearate (96% purity), monoglyceride olein (>99%purity), diglyceride olein (99.7% purity) and triglyceride olein (99.6% purity) were purchased from Sigma (France). A tocopherol kit consisting of α-, β-, γ- and δ-tocopherols was obtained from Merck (>95% purity).

Table 3.1: Characterisation of deodorizer distillates

			Deodorizer Distillates		
	Units	Compound	A [a]	B [b]	C [c]
acylglycerides	g/100g	G	0.11	N.D.[d]	N.D.
		MG	0.91	1.83	N.D.
		DG	2.67	1.54	0.44
		TG	52.34	2.20	0.23
Tocopherols	g/100g	α-Tocopherol	1.39	2.07	0.06
		δ-Tocopherol	0.09	N.D.	0.03
		β/γ-Tocopherol	N.D.	N.D.	N.D.
Squalene	g/100g	Squalene	1.96	5.65	8.69
Sterols	g/100g	Campesterol	0.42	0.71	N.D.
		Stigmasterol	0.36	1.29	N.D.
		β-Sitosterol	1.86	5.56	0.27
Steryl Esters	g/100g	Steryl Esters	0.78	0.04	0.05
Fatty Acids	g as oleic acid/100g		10.71	26.98	64.44
Water	ppm		853	786	843

[a] Sunflower deodorizer distillate from Lesieur (France)
[b] Sunflower deodorizer distillate from Sovena (Portugal)
[c] Olive deodorizer distillate from Sovena (Portugal)
[d] N.D.-Not Detected

The internal standard heptadecanyl stearate (HDS) was prepared by condensation of heptadecanol and stearoyl chloride, both obtained from Aldrich (Belgium), as described by Verleyen et al. [65].

3.2.2 Analytical Methods

The methods for characterisation of deodorizer distillates are described in the subsection 2.3.2 (Chapter 2)

3.2.3 Experimental Procedures Methods

3.2.3.1 Enzymatic Reactions

Enzymatic reactions were carried out in a 100 ml hermetically sealed and jacketed vessel in order to maintain the temperature constant (40° C). The vessel was initially filled with 50 g of the standard mixture, the water activity was adjusted to a_W=0.8 and 0.5%w/w of lipase from *Candida Rugosa* was added. Eight different mixtures consisting of sunflower deodorizer distillate and an additional source of fatty acids, were prepared accordingly to obtain a molar ratio of free fatty acids (*FFA*) to sterols (*S*) of 7.0, considering the deodorizer distillate as the single source of sterols. The proportion of sunflower deodorizer distillate and source of free fatty acids used for the preparation of the reaction mixtures is summarized in Table 3.2). Over the time course of the reaction,

samples of ≃ 2 g were periodically removed from the reaction vessel for *FFA*, Karl Fischer and GC analysis.

Table 3.2: Proportion of sunflower deodorizer distillate and source of free fatty acids used for the preparation of the reaction mixtures

	Composition	
Reaction	Source of Sterols	Source of FFA
1	70% A	30% B
2	97% A	3 % C
3	98% A	2% D
4	98% A	2% E
5	70% B	30% A
6	81% B	19% C
7	86% B	14% D
8	88% B	12% E

A - Sunflower deodorizer distillate from Lesieur (France)
B - Sunflower deodorizer distillate from Sovena (Portugal)
C - Olive deodorizer distillate
D - Sunflower oil rich in triolein
E - Oleic Acid

3.2.4 Numerical Methods

3.2.4.1 Modeling prediction and Sensitivity Analysis

The dynamic system under study was simulated and analysed using the SimBiology toolbox of MatLab (2011a version).

The software calculated local sensitivities by combining the original ODE system for a model (rate equations defined in the section 2.2, Chapter 2) with the auxiliary differential equations for the sensitivities. This method is sometimes called "forward sensitivity analysis" or "direct sensitivity analysis" [86]. The sensitivity of the species x with respect to the species k was calculated with a full normalization, defined as:

$$\frac{k}{x(t)}\frac{\mathrm{d}x(t)}{\mathrm{d}k} \tag{3.1}$$

This normalization allowed to data be dimensionless and obtain a fair comparison between them.

SimBiology sensitivity analysis uses "complex-step approximation" to calculate derivatives of reaction rates. This technique yields accurate results for the vast majority of typical reaction kinetics.

The sensitivity analysis was conducted to determine the influence of the initial concentration of each compound in the production of steryl esters (after 24 h). The initial composition of the reaction mixture was assumed to be the average of the reaction mixtures used to develop the

model (established in Chapter 2).

3.2.4.2 *Quality of Fit of the Model*

The least square objective function, S_y, was used to measure the quality of fit of the model. For n compounds, and j data points, the function S_y compares the values predicted by the model ($\hat{\theta}$) and the values actually observed (θ), being expressed as:

$$S_y = \sqrt{\frac{\sum_1^n \sum_1^j \left(\theta - \hat{\theta}\right)^2}{jn-1}} \tag{3.2}$$

Lower values of S_y indicate a good agreement between experimental data and model predictions.

3.3 Results and Discussion

The experimental kinetic constants presented in the Table 2.6 (Chapter 2) enable the description of the transient evolvement of the enzymatic reactions given that the initial composition of the reaction medium is known beforehand, independently of the source of the each compounds. Table 3.3 shows the initial composition of the reaction mixtures, as well as the range of concentrations for each compound, where the kinetic constants were validated. In all reactions, there is at least one compound out of the validation range (highlighted in bold). Even so, the kinetic constants were used in a mass-balance model to describe the profile of concentration of the various compounds during reaction and, ultimately, to determine the yield of production of steryl esters.

Table 3.3: Initial concentration of reactant compounds and respective modelling range. Values of concentrations out of the validation range are highlighted in bold.

Compound	Reactional Composition (mmoles/100g) [a]								
	1	2	3	4	5	6	7	8	Model [b]
Glycerol	**4.3**	1.3	1.0	1.0	**4.7**	2.0	1.3	1.0	0.3-3.6
Monoglycerides	2.4	2.4	**1.1**	**1.4**	3.6	3.7	4.1	4.4	2.4-10.2
Diglycerides	**6.8**	4.2	4.0	4.9	3.4	**2.0**	**2.5**	**2.1**	2.7-5.3
Triglycerides	**66.1**	**64.7**	**69.2**	**56.9**	33.4	**6.6**	**44.0**	**8.5**	13.4-37.5
Sterols	11.0	**5.5**	**4.3**	**5.0**	15.7	14.0	15.4	17.0	8.6-17.0
Steryl Esters	**4.6**	1.9	1.5	1.9	3.0	1.4	1.4	**0.9**	1.0-3.2
Free Fatty Acids	**54.6**	**80.6**	**34.5**	103.3	**84.6**	**120.0**	89.5	**122.0**	84.9-111
Water	23.3	21.4	18.7	20.5	23.3	34.4	33.0	35.7	10.3-37.5

[a] See the corresponding reaction mixture in Table 3.2
[b] Validated range of the model, developed in the Chapter 2

The predicted yield of steryl esters production for reactions 3, 6 and 8 shows a high deviation to the corresponding experimental values (Table 3.4). Figure 3.1 compares the profile of the concentration of sterols and steryl esters during the kinetics of reactions 2 and 6, which are examples

Table 3.4: Experimental and predicted yield of steryl esters production and corresponding quality of model fitting measured as S_y (see Eq. 3.2)

	Yield after 24h (%)		
Reaction	Experimental	Predicted	S_Y [a]
1	83.2	76.2	7.8
2	85.9	84.8	3.2
3	83.8	74.2	23.4
4	87.2	86.0	2.7
5	73.3	73.9	6.1
6	51.6	64.9	29.8
7	76.2	79.2	8.8
8	51.6	64.9	19.9

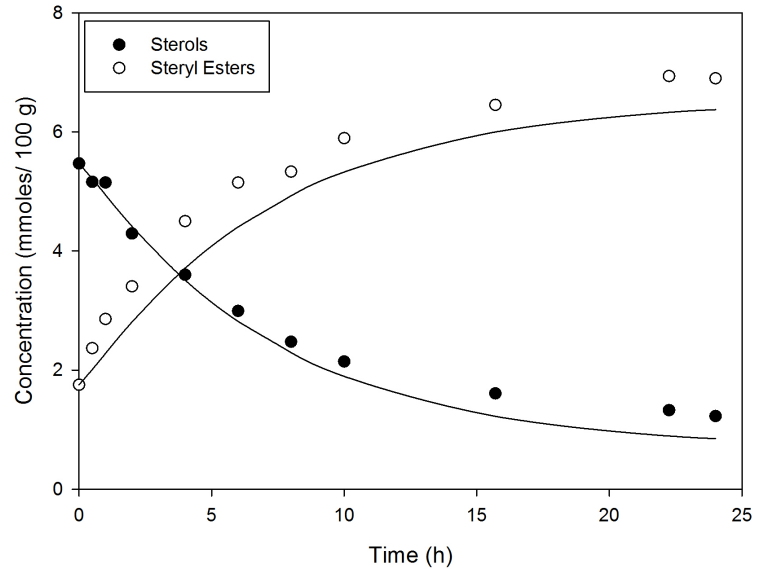
[a] Calculated taking into account the amount of steryl esters produced during 24 hours of reaction

of a good and a bad fitting of the model, respectively. Interestingly, in both cases olive oil (C) was used as a source of free fatty acids, which suggests that the lack of model fitting is not due to the nature of the source. Instead, the reason for the lack of model fitting may be related to the initial concentration of compounds out of the validated range of the model. In fact, reaction 3 shows a concentration of triglycerides, sterols and free fatty acids significantly out of the validated range. In reaction 6, concentrations of diglycerides, triglycerides and free fatty acids were outside the validated range, while in reaction 8, diglycerides, triglycerides, steryl esters and free fatty acids were outside the validated range. The complexity of the system does not allow to conclude directly which compounds may be the responsible for the deviation of the model in predicting the yield of production of steryl esters, since the hydrolysis of acylglycerols releases free fatty acids but it is also a consumer of water from the system, affecting consequently the equilibrium state of the esterification of sterols.

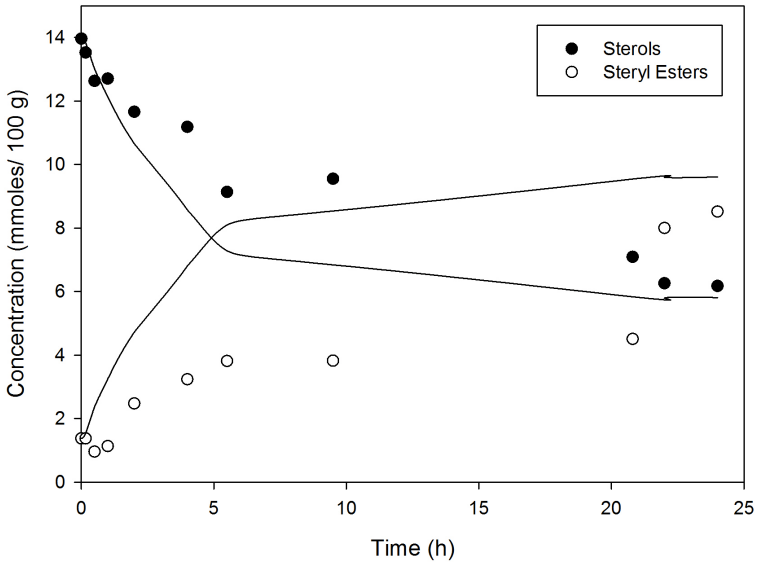
A sensitivity analysis of the model is therefore considered to be critical for identification of the relevant compounds for the production of steryl esters. The ordinary differential equations (ODE) defining the sensitivity of steryl esters production (x) with respect to the initial concentration of each compound present in the reaction mixture (k) were obtained (the detailed procedure and assumptions to solve these ODE system are described in the subsection 3.2.4).

Figure 3.2 shows that the production of steryl esters after 24 hours of reaction is affected mainly by the initial concentration of sterols, followed by the free fatty acids, steryl esters and, finally, triglycerides. The influence of the remaining compounds was found to be not significant. As can be observed, the increase of sterols, free fatty acids and triglycerides has a positive impact in the production of steryl esters, while for an increased initial concentration of steryl esters it is negative. These results suggest that the production of steryl esters is favoured in a reaction mixture rich in sterols, free fatty acids and triglycerides, and with a low initial concentration of steryl esters.

Figure 3.3 shows the normalized response of steryl esters production to a spike in the initial concentration of other individual compounds. A spike in the initial concentration of sterols and



(a)



(b)

Figure 3.1: Examples of a (a) good and a (b) bad model fitting to the profile of concentrations of the sterols and steryl esters during reaction - Reactions 2 and 6, respectively (see table 3.2)

triglycerides has a positive effect in the production of steryl esters (upward trend), while a spike of steryl esters is negative (downward trend). The trend of the curve for a spike in free fatty acids shows an initial increase in production of steryl esters until ≈ 2 hours of reaction time, but then the effect decreases with time (although remaining positive for a 24h time-frame). The influence of the remaining compounds was observed to be not as significant.

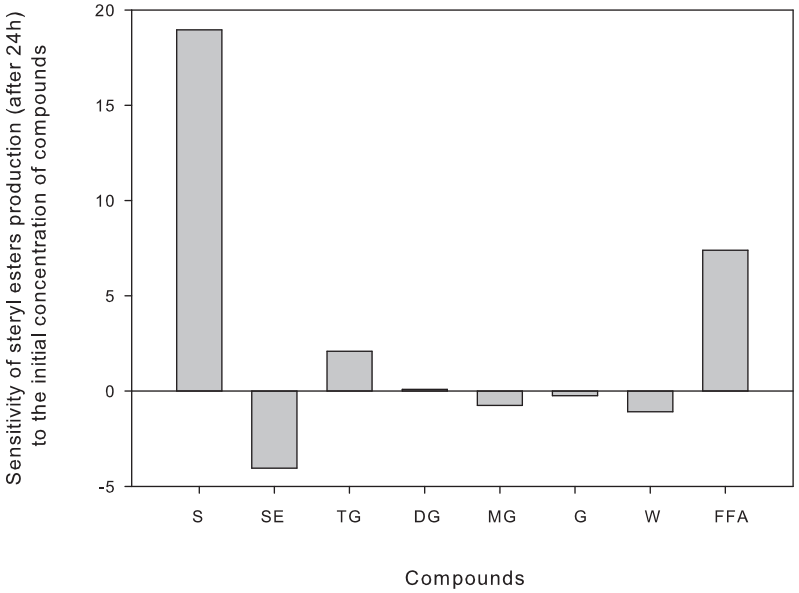


Figure 3.2: Sensitivity of the steryl esters production (after 24 hours) to the variation of the initial concentration of each reactant compound

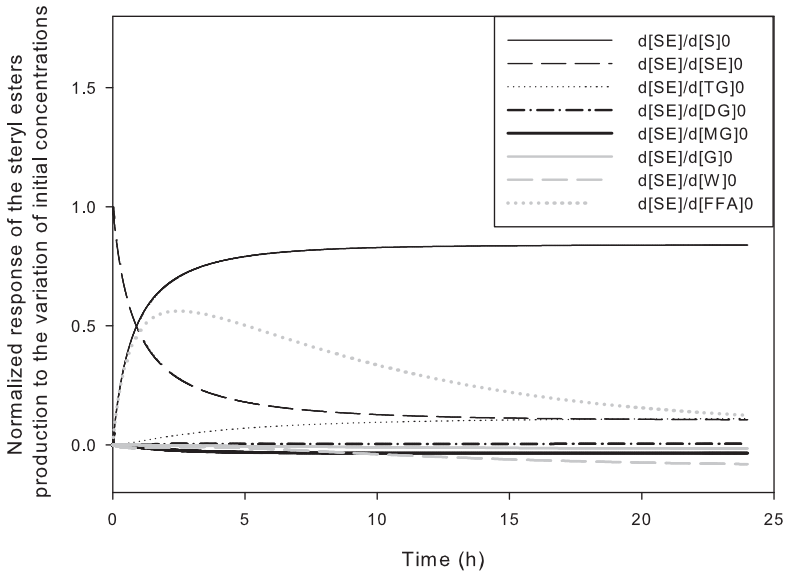


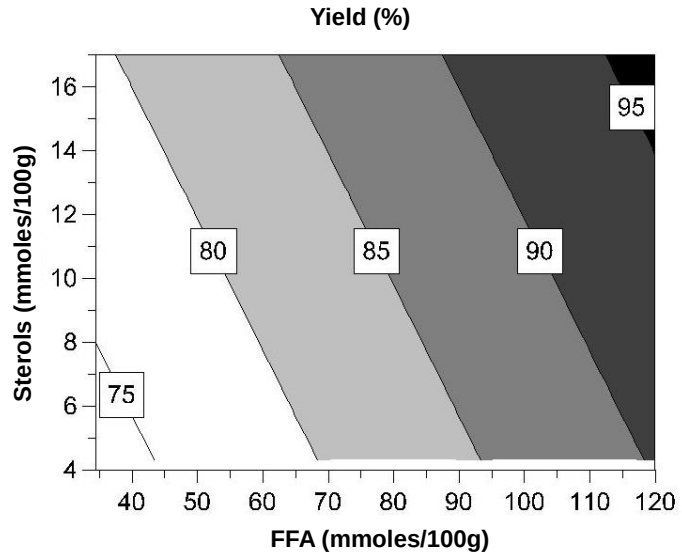
Figure 3.3: Normalized response of steryl esters production, during the reaction, to the variation of initial concentration of each reactant compound

The nature of the impact of the relevant compounds could be anticipated. A high initial concentration of steryl esters drives the equilibrium towards the hydrolysis of steryl esters, having a negative impact in the overall production of steryl esters. Additionally, a high initial concentration of sterols favors the equilibrium towards esterification. On the other hand, the lipase from *Candida*

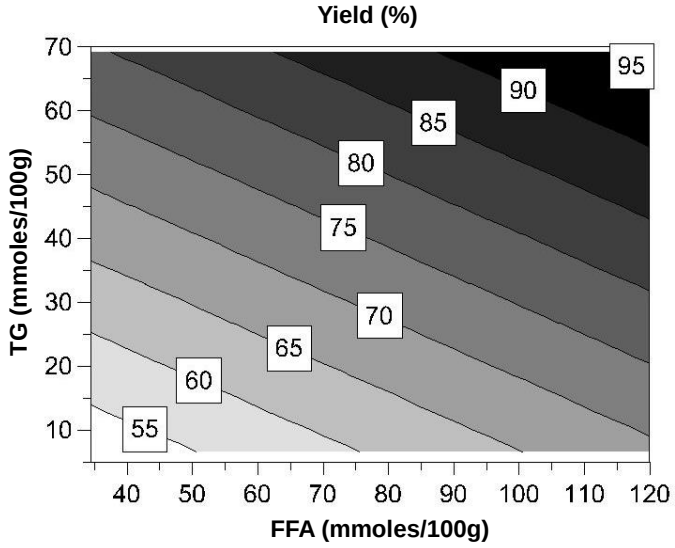
Rugosa is reported to be specific for the hydrolysis of triglycerides, which are their natural substrates. Therefore, once the reaction starts, the enzyme is expected to hydrolyse the triglycerides, releasing molecules of free fatty acids which may be used in the production of steryl esters.

The sensitivity analysis performed in this work suggests that the model is sensible to the variation of the initial concentrations of the compounds identified in reactions 3, 6 and 8 as being out of the validated range. Aligned with this observation, the model presented a lack of fit for those reactions, predicting a higher yield for the reaction 3 e a lower yield for the reactions 6 and 8. An inhibition effect due to a high initial concentration of free fatty acids (>120 mmoles/100g), not captured in the developed model, may be the reason behind the lack of fit. This inhibition could explain a higher prediction of yield in reactions with a low content of free fatty acids (reaction 3) and a lower prediction of yield in reactions with a high content of free fatty acids (reactions 6 and 8).

Based on simulations and experimental observations, it is possible to define guidelines to obtain yields of steryl esters production above 80% in 24 h. These guidelines are hereby presented on the basis of the parameters for which the yield is more sensitive (e.g. steryl esters, sterols, free fatty acids and triglycerides). Therefore, the initial concentration of steryl esters should be as low as possible, to avoid their hydrolysis. This reaction can be mitigated by using an excess of free fatty acids (*FFA*), and consequently, drive the equilibrium towards the esterification of sterols. However, the concentration of *FFA* should not exceed 120 mmoles/100 g, otherwise, an inhibition by excess of subtract may occur. In order to avoid this, it is possible to use a reaction medium rich in triglycerides, since their hydrolysis produces free fatty acids needed for the target esterification. Figure 3.4 shows contour plots of the yield of steryl esters production for a range of concentrations of the three most important components, i.e., triglycerides (*TG*), Sterols (*S*) and free fatty acids (*FFA*). Figure 3.4(a) indicates the relation between S and FFA that should be taken into account to obtain a desirable yield, while the Figure 3.4(b) indicates the relation between TG and FFA (both sources of free fatty acids). For the ranges studied, yield shows a monotonic relation with FFA, S and TG, where these should be as high as possible to improve yield.



(a)



(b)

Figure 3.4: Yield of steryl esters production as a function of the initial concentration of free fatty acids (FFA) and (a) sterols (fixing the concentration of TG in 69 mmol/100g) (b) triglycerides (TG) (fixing the concentration of S in 17mmol/100g)

3.4 CONCLUSION

Enzymatic reactions to produce steryl ester from deodorizer distillates were successfully performed with an acceptable yield (>80%), using least expensive and industrially available sources of free fatty acids and sterols, such as the mixture of deodorizer distillates with different compositions and refined oil rich in triglycerides. The mathematical model developed previously in the Chapter 2, was shown to be able to predict the profile of compounds during the reaction, in conditions of a wide variability of composition of the reaction mixture. The applicability of the

model is only restricted if the initial concentration of FFA is higher than 120 mmoles/100g. In this case, an inhibition by excess of subtract, not captured by the model, may occur.

In order to obtain high yields of steryl esters production, there are other constraints concerning the composition of the initial reaction mixture. A sensitivity analysis performed to the model indicates that the production of steryl esters is affected mainly by the initial concentration of sterols (*S*), followed by the concentration of free fatty acids (*FFA*), steryl esters (*SE*) and, finally, by the triglycerides (*TG*) content. Consequently, guidelines to obtain high yields were only focused on these parameters. It was established that the initial concentration of SE should be as low as possible, and the initial reaction mixture should contain a high content of S, TG and FFA, but the initial concentration of FFA should not be higher than 120 mmoles/100g; otherwise, an inhibition by excess of subtract can occur. Such inhibition can be mitigated using a reaction medium rich in triglycerides as an alternative source of FFA.

4 Lipase-Catalyzed Consecutive Batch Reaction for Production of Steryl Esters from Vegetable Oil Deodorizer Distillates

Summary

A significant decrease of the yield of steryl esters production by esterification of sterols and free fatty acids (FFA), present in vegetable oil deodorizer distillates, was observed when consecutive batch reactions (24 hours each) were carried out using the same enzyme and under optimal operating conditions. This work aims to identify the causes of such phenomena, in order to avoid or minimize it, allowing for reuse of the Candida rugosa lipase catalyst in consecutive batch reactions. The effect of water activity and glycerol produced by hydrolysis of acylglycerides, on the stability of the enzyme was studied. Results show that these effects were not the main reason for the decrease of yield in consecutive batch reactions. The presence of oxidation products proved to play an important role in the enzyme inactivation. Due to the high concentration of antioxidants (phenolic compounds, squalene and tocopherols) naturally present in deodorizer distillates, the inactivation of the enzyme was minimized but, even so, it could not be avoided.

A partial reuse of the enzyme is technically a possible solution, by adding fresh enzyme in the beginning of each batch reaction. Four consecutive batch reactions (24 hours each) were successfully carried out under optimal conditions, keeping a constant yield of steryl esters production higher than 80%.

The contents of this chapter were adapted from the publication: Teixeira, A. R. S., Santos, J. L. C., Crespo, J. G. (2012). Lipase-Catalyzed Consecutive Batch Reaction for Production of Steryl Esters from Vegetable Oil Deodorizer Distillates. Industrial & Engineering Chemistry Research, 51(15), 5443-5455

4.1 INTRODUCTION

Phytosterol esters are commonly used as a functional food ingredient [19] due their known bioactive properties with positive effect in lowering serum cholesterol levels in humans [87, 88].

Deodorizer distillates produced in the final step of the refining oil process (deodorization) are an interesting raw material since they are rich in bioactive compounds such as tocopherols (*TOC*) and sterols (*S*). However, their valorisation relies on the success of the reduction of pesticides content to levels accepted by the stringent legislation concerning pesticides content in food additives [17, 77].

In the Chapter 2, it was proposed a process to produce steryl esters from vegetable oil deodorizer distillates by enzymatic esterification. *Candida rugosa* lipase, in a concentration of 0.5% (w/w), showed to be able to synthetize steryl esters with a yield of 80%, after 24 hours of reaction, under mild operating conditions (at 40 °C). The conversion of sterols (400-415 g mol^{-1}) to steryl esters (650-800 g mol^{-1}) leads to an increase of the molecular weight difference of these compounds when comparing with pesticides (150-420 g mol^{-1}). The esterification reaction step thereby facilitates the use of a subsequent membrane-based separation process, such as nanofiltration [43], for the recovery of steryl esters (in the retentate stream) free from pesticides (which permeate). Moreover, comparatively to free sterols, it is referred in the literature that steryl esters are more bioactive [33] and more soluble in oil phases, making easier their incorporation into fat-based products [88].

A sustainable and economical enzymatic based process requires reuse of the enzyme in a large number of reaction cycles, in order to lower production costs. Taking into account the need for reusing the enzyme, preliminary experiments of consecutive batch reactions were performed. However, the yield of steryl esters produced by esterification of sterols and free fatty acids (FFA) was observed to decrease as much as 30% in the second batch reaction.

Similar observations in similar environments are reported in the literature. Torres et al. [8] observed an inactivation of free *Candida rugosa* lipase in deodorizer distillates modified by a previous removal of high boiling point substances such as steryl esters, diglycerides and triglycerides. Steryl esters were produced in eight consecutive batch reactions, using the same amount of enzyme (10% w/w), with an average yield of 80%. The activity of the lipase after 64 hours (eight batch reactions of 8 hours each), at 40 °C, decreased more than 40%. In another study, Torrelo et al. [89] compared the performance and stability of *Candida rugosa* lipase, both in the free form and immobilized form. Sterols obtained from sunflower oil deodorizer distillates were mixed, in two stages, with a mixture of fatty acids from butter oil, in the presence of 10% (w/w) of free *Candida rugosa* lipase and 5%(w/w) of the same enzyme immobilized in silica. Reactions occurred under vacuum (100 mbar) and mild operating conditions (without solvents, at 40-50 °C), obtaining yields of steryl esters production around 85%. However, after 100 hours at 40 °C, the free enzyme preserved only 35% of its initial activity and the immobilized lipase preserved 57%. These results suggest that inactivation of *Candida rugosa* lipase occurs in this type of media, at similar rates for both free and immobilized enzyme.

While a number of works investigated the inactivation of *Candida rugosa* lipase in consecutive

batch reactions, none of those published studies have so far identified the original cause for inactivation neither an adequate strategy to deal with this problem. There are several agents reported in the literature (applied to other processes and reaction media) that may be potentially responsible for the partial inactivation of *Candida rugosa* lipase, such as excess of water, presence of glycerol and the solvent itself.

In fact, the negative effect of an excess of water on the enzyme activity and stability is known [81,82,90,91]. Most reactions that induce denaturation of the enzyme are hydrolysis and therefore require water. However, a defined amount of water is always necessary to maintain the enzyme activity, since water acts as a molecular lubricant and plays an important role in the equilibrium of the esterification/hydrolysis side reactions. Therefore, water activity in the reaction medium is a parameter that should be optimized.

Concerning the presence of glycerol in the reactional medium, Dossat et al. [92] studied the influence of glycerol produced during the transesterification of high oleic sunflower oil, in the stability of a lipase (immobilized *Candida antarctica* lipase). These authors proposed the formation of a glycerol layer around the enzyme which inhibits the diffusion of the hydrophobic substrates. The removal of glycerol and replacement of enzymatic activity was claimed to be possible by washing the enzyme with a tertiary alcohol (not reactant and only moderately polar) previously equilibrated at the same water activity (a_W) the reaction occurs. Fjerbaek et al. [93] observed that the glycerol effect is more likely due to mass transfer limitations in the carrier support of the immobilized enzyme than an enzyme inhibition effect on the strict sense. However, Virto et al. [94] observed the same negative effect of glycerol with *Candida rugosa* lipase in free and immobilized form.

The inactivation of lipases due to the presence of lipid oxidation products (peroxides and aldeydes) has been reported in the literature [95–98]. Such products are a result of unsaturated fatty acids oxidation when in presence of molecular oxygen and, therefore, they occur naturally in oils. Ohta et al. [95] reported that the inactivation of lipase by polymerization occurred due to the presence of lipid peroxides. Peroxide values higher than 2 mEq O_2/kg were found to be strongly detrimental to the enzyme activity [96, 98]. Additionally, Pirozzi et al. [97] showed that aldeydes formed by secondary oxidation have a stronger effect on the enzyme stability than hydroperoxides from primary oxidation.

The aim of this work is to establish a procedure to reuse the enzyme in consecutive batch reactions of steryl esters production, identifying the inactivating agents of the enzyme and exploring solutions for overcoming the enzyme inactivation observed (and also described in the literature). Firstly, the effect of the control of water activity (during esterification) on the enzyme activity in consecutive batch reactions will be studied. Secondly, since glycerol has been identified as a potential inactivating agent of enzymes, the impact of its removal after reaction will be investigated, by washing the enzyme with a tertiary alcohol [92]. Model reactions without glycerol production will be also conducted, in order to further assess the impact of glycerol in the enzyme deactivation. Finally, the effect of oxidation products present in free fatty acids and acylglycerides (main constituents of the reactional medium) on the stability of the enzyme was evaluated.

A strategy for operation of consecutive batch reactions at constant conversion yield will be

also presented and validated.

4.2 MATERIALS AND METHODS

4.2.1 Materials

4.2.1.1 Deodorizer Distillate

Sunflower deodorizer distillates were obtained from the producers Lesieur Cristal in Morocoo and Lesieur in France. The deodorizer distillate was characterized in our laboratory and the composition is presented in the Table 2.1 (Chapter 2). The peroxide number of this deodorizer distillate is 1.8 mEq O_2/kg and its iodine value is 80.7%. All samples were stored in the refrigerator at 4 °C.

4.2.1.2 Enzyme

Candida rugosa lipase, Type VII, was obtained from Sigma (Saint Quentin, France). The activity of this lipase, as indicated by the supplier, is 1535 U/mg of solid, which was experimentally confirmed (1415±102) by using the Sigma Kit for determination of enzymatic activity of lipases. One unit (U) of lipase activity is defined as the amount of enzyme that hydrolyzes 1.0 μequivalent of fatty acid from a triglyceride in one hour at pH 7.2 and at 37 °C, using olive oil as substrate (30 minutes of incubation).

4.2.1.3 Chemicals

Analytical-grade chloroform, 2-methyl-2 butanol, cyclohexane, octane and acetic acid glacial were obtained from Sigma. Oleic acid food-grade, was obtained from Aldrich (Bornem, Belgium) with an acid value of 196.0-204 mg KOH/g. Triolein technical-grade (>65% purity) was obtained from Sigma (St. Quentin Fallavier, France).

For iodine and peroxide value determination, the Wijs solution 0.1M was obtained from Sigma and analytical-grade potassium iodine from Merck.

The derivatizating and silylation agent, N,O-bis (trimethylsilyl) trifluracetamide (BSTFA) containing 1% of trimethylchlorosilane (TCMS) solution (from Fluka) and pyridine (from Sigma-Aldrich) were both obtained from Sigma (Saint Quentin, France).

All analytical-grade standard substances, squalene (99.3% purity), stigmasterol (97% purity), β-sitosterol (99%purity), campesterol (99% purity), cholesteryl stearate (96% purity), monoglyceride olein (>99%purity), diglyceride olein (99.7% purity) and triglyceride olein (99.6% purity) were purchased from Sigma (Saint Quentin, France). A tocopherol kit consisting of α-, β-, γ- and δ-tocopherols was obtained from Merck (>95% purity).

The internal standard heptadecanyl stearate (HDS) was prepared by condensation of heptadecanol and stearoyl chloride, both obtained from Aldrich (Bornem, Belgium), as described by Verleyen et al. [65].

4.2.2 Analytical Methods

4.2.2.1 Characterization of deodorizer distillates

The methods to characterise the composition of deodorizer distillates are described in detail in the subsection 2.3.2 (Chapter 2).

4.2.2.2 Determination of the Enzyme Content

Nitrogen (N) content is commonly used to quantify protein content in food applications [99]. The nitrogen content was determined by elemental analysis, using a elemental analyzer from Thermo Finnigan-CE Instruments (Italy), Flash EA 1112 CHNS model.

The analyzer operates on the basis of the dynamic combustion of the sample. The sample is weighed in tin capsules and introduced into the combustion reactor.

After combustion, the gases are transported by stream of helium (He) through the reactor, separated by a GC column, and are detected by thermal conductivity (TCD). The results of sample composition on CHNS in expressed as a total percentage of 0.01% and 100% w/w.

4.2.2.3 Determination of peroxide number

The peroxide value (*PV*) is based on the measurement of iodine liberated from the reaction of KI with peroxides in the sample. The PV was determined by titration according to the standard ISO 3960 (2001). The method was developed and implemented in a titration workstation TitraLab 856 from Radiometer (Denmark). The result was expressed as mEq O_2/kg.

4.2.2.4 Determination of Iodine Value

The determination of iodine value was carried out following the ISO 3961 (1996). This method measures the amount of $-C=C-$ (double bonds) present in the product. The result is expressed as g of iodine (I_2) per 100 g of sample. This standard use the reaction of the sample with an excess of Wijs solution followed by determination of excess of Wijs solution using a redox titration with sodium thiosulphate. The method was developed and implemented in a titration workstation TitraLab 856 from Radiometer (Denmark).

4.2.3 Experimental Procedures Methods

4.2.3.1 Water Activity Adjustment in the Reaction Media

Selected saturated salt solutions (with known water activity) may be used for equilibrating a system to a defined water activity through the vapour phase, since the water activity, at a controlled temperature, will be the same for all phases in contact. In this case, oleic acid (*OA*), sunflower deodorizer distillates and 2-methyl-2-butanol were equilibrated in a closed vessel with the vapour phase in contact with different saturated salt solutions (each one corresponds to different characteristic water activities). The jars were place in an oven at 40 °C, the same temperature at which

the enzymatic reaction was carried out. The process of equilibrium was monitored by Karl Fischer titration, and a curve of water content in function of water activity was determined experimentally (data not shown). This experimental curve, allows elimination of the time for equilibrium needed to reach a desired initial water activity (which corresponds to a defined water content) in the beginning of the esterification reaction. A simple measurement of the initial water content of the reaction media allows to determine the quantity of water needed to be added in order to obtain a desired water activity. This procedure was described in detail in the Subsection 2.3.3.2 (Chapter 2).

4.2.3.2 Lipase-Catalyzed Reactions

Reactions were carried out in a hermetically sealed and jacketed vessel in order to maintain the temperature constant during the esterification reaction. The control of water activity during the esterification reaction (if required) was achieved with a defined saturated salt solution placed in a smaller recipient within the reactor vessel, as described above. Mass transfer between both solutions occurs through the headspace (Figure 4.1).

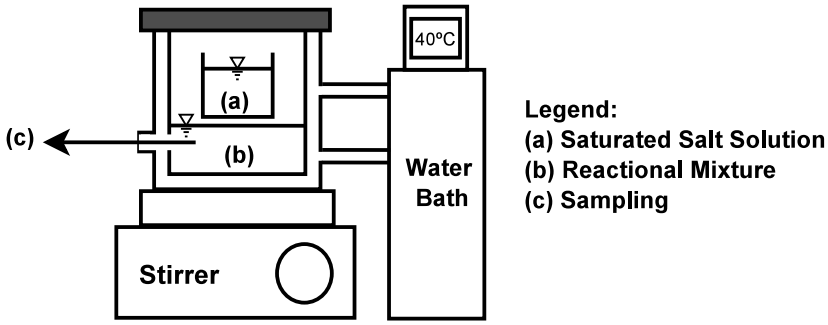


Figure 4.1: Experimental setup of the lipase catalyzed reactions

The vessel was initially filled with 50 g of the standard mixture (deodorizer distillates with a molar ratio of free fatty acids (*FFA*) to sterols (*S*) at least 6.0 or with oleic acid and stigmasterol, when a model reaction was carried out), stirred at 400 rpm and maintained in equilibrium with a selected saturated salt solution, at 40 °C overnight, before reaction. If the water activity was controlled during reaction, the inner vessel remained filled with the salt solution, otherwise it was emptied before starting the reaction. The reaction was initialized by adding 0.5% (w/w) of *Candida rugosa* lipase and finalized after 24 hours by removal of the biocatalyst (procedure described in the following subsection).

Over the time course of the reaction, samples of $\simeq$ 2 g were periodically taken from the reaction vessel for free fatty acids (*FFA*), Karl Fischer and GC analysis.

4.2.3.3 Recovery and Recycling of the Enzyme in Consecutive Batch Reactions

After each batch of lipase-catalyzed reaction, the enzyme was recovered by ultrafiltration of the product mixture (without any addition of solvent) using a METcell dead-end stirred cell (MET,UK). Ultrafiltration was carried out at 3 bar, 40 °C (due to the viscosity of the mixture)

and 400 rpm, using a ceramic membrane Inopor Ultra with a TiO_2 active layer and a nominal pore size of 30 nm. The time necessary for this procedure was short (10 minutes).

The enzyme was recovered and then added to the reaction mixture, initializing, therefore, a new batch reaction. This addition changes slightly the initial concentrations of the compounds in the beginning of each batch. In order to maintain at least the concentration of the catalyst between batch reactions, the mass of original deodorizer distillate was adjusted accordingly.

In the case of the reaction model solution, where the enzyme is suspended in oleic acid (solvent), it is possible to recover the enzyme using microfiltration instead of ultrafiltration, which makes the process faster. Microfiltration was performed using an Amicon stirred cell (Millipore,US) and a PTFE membrane ($0.20\mu m$, Sartorius), at 0.5 bar, 40 °C and 200 rpm.

The enzyme was quantified by measuring the nitrogen content using elemental analysis.

4.2.3.4 Measurement of Enzyme Activity

The enzymatic activity can be measured by the yield of steryl ester production defined as moles of steryl ester produced in 24 hours from 100 moles of sterols. Also, the value of the kinetic constant k_1 was determined, since it reflects the ability of the enzyme to transfer the acyl group from free fatty acids to sterols (see reaction (I) described in the Subsection 2.2, Chapter 2).

4.2.3.5 Identification of Enzyme Inactivation Mechanism

There are several agents that may be potentially responsible for the partial inactivation of *Candida rugosa* lipase, such as excess of water and the presence of glycerol and oxidation products.

Effect of Water Activity

Two series of four consecutive batch reactions (24 hours each) were performed, recovering and recycling the enzyme between batch reactions. Control of water activity was carried out at a defined value in one reaction batch, while a second experiment the water activity was not controlled. The yield of steryl esters production in each batch and the respective kinetic constant k_1 were determined and compared.

Effect of the Presence of Glycerol

- Removal of Glycerol Associated to the Enzyme between Consecutive Batch Reactions

In order to remove glycerol absorbed to the enzyme and replace the enzymatic activity, the procedure described by Dossat et al. [92] was adopted. Such procedure includes enzyme washing using a tertiary alcohols.

Firstly, fresh enzyme was pretreated with 100 grams of 2-methyl-2-butanol with a water activity of 0.54 (water content = 4.5% w/w) and a contact between them was allowed, stirring at 200 rpm at 40 °C, during 15 minutes. The enzyme was recovered either by ultrafiltration or by centrifugation, and subsequently, the enzymatic activity was determined using the Sigma kit.

Secondly, the production of steryl esters was carried out at 40 °C, 400 rpm and under control of water activity (a_W), during 24 hours of reaction. The value of water activity was set at 0.54,

since this value is within the optimal range determined in the Chapter 2. After this first batch, the enzyme was recovered by ultrafiltration. Then, the enzyme recovered was washed with the same tertiary alcohol, for removal of glycerol produced during the reaction. Subsequently, the suspension was ultrafiltrated. The permeate stream was collected and the alcohol was removed by a rotary evaporator (Rotovapor RII from Buchi). The residue was analyzed by GC-FID in order to quantify the compounds adsorbed to the enzyme and recovered by the washing procedure. The enzymatic activity was determined replacing the enzyme a new reactional mixture of deodorizer distillate to produce steryl esters.

A control batch reaction was carried out in similar conditions, although without washing the enzyme between batch reactions. The yield of steryl esters production obtained with and without the washing procedure was compared.

- Model Reaction without Glycerol Production

In order to determine the effect of the absence of glycerol on the enzymatic activity in consecutive batch reactions, a model esterification reaction between stigmasterol (a representative sterol) and oleic acid (a representative free fatty acid) was carried out. Since glycerol is not present nor produced in this model reaction, a possible decrease in enzymatic activity should be a result of an inactivating mechanism not involving glycerol.

The model reaction was carried out under controlled water activity (a_W=0.54, corresponding to 2875 ppm of water content), at 40 °C and 400 rpm. Four consecutive batch reactions were conducted and the respective kinetic constant (k_1) determined. After each batch of lipase-catalyzed reaction, the enzyme was recovered, as described in the Subsection 4.2.3.3.

Effect of the presence of oxidation products

Oleic acid and acylglycerides are the major constituents of deodorizer distillates (representing together 50% w/w) and both contain oxidation products, in similiarity to deodorizer distillates. Therefore, it was opt to perform this study using a simple matrix containing oleic acid/acylglycerides instead of the complex one constituted by deodorizer distillates, eliminating in such way possible interferences from others parameters.

The initial water content of oleic acid was accordingly adjusted in order to obtain a similar initial water activity of the reactional mixture, simulating the initial conditions of the esterification reaction.

Oleic acid was placed in two dark and closed vessels which include a sampling system by septum/syringe. The enzyme was placed in one of them, in contact with oleic acid (in order to guarantee a 0.5% w/w of enzyme and a molar ratio of *FFA* to *S* higher than 6.0), at 40 °C. The other system, without enzyme, consists in our control experiment. In order to promote a good mass transfer, a stirring of 200 rpm was carried out in both vessels.

The evolvement of primary oxidation products in these systems was monitored by measuring the peroxide number. The iodine value was determined in the beginning and end of the reaction. To determine the iodine value, the solutions were previously filtered in order to remove the enzyme.

In order to determine the impact on the enzyme stability, after different periods of contact time,

the mixture (enzyme + oleic acid) was added to the deodorizer distillate (previously equilibrated at the same water activity). The respective kinetic constant (k_1) and yield of steryl esters production were determined.

A similar procedure was performed using the same proportion enzyme/mixture of acylglycerides (86% of triglycerides (65% of Triolein), 13% of diglycerides, 1.9% of monoglycerides and 0.04% of glycerol - determined experimentally by GC-FID, as described previously). It should be noticed that the water activity was set at 0.11 during the contact of the enzyme with the mixture of acylglycerides, in order to suppress the hydrolysis reaction.

After a defined period of time, the enzyme was removed and the acylglycerides were analyzed by GC-FID in order to confirm the suppression of hydrolysis reaction. Subsequently, the enzyme was added to the deodorizer distillate which was previously equilibrated at a water activity of 0.66. The respective kinetic constant (k_1) as well as the yield of the process were determined.

4.2.3.6 Addition of Fresh Enzyme in Consecutive Batch Reactions

After the first batch of production of steryl esters, the enzyme was recovered (as described previously) and added to the reaction mixture, in order to initialize a second batch. Additionally, different quantities of fresh enzyme were added in order to study the impact of such enzyme addition in the final yield of steryl esters production, as well as in the respective kinetic constant, after 24 hours of reaction.

The reactions were carried out under similar conditions (a_W=0.82, initial FFA:Sterol molar ratio = 8.0 and enzyme concentration=0.5% w/w)).

After determining the amount of fresh enzyme needed to maintain the yield in consecutive batch reactions, four consecutive batch reactions were performed (as an illustrative example) and the respective yield and kinetic constant were determined.

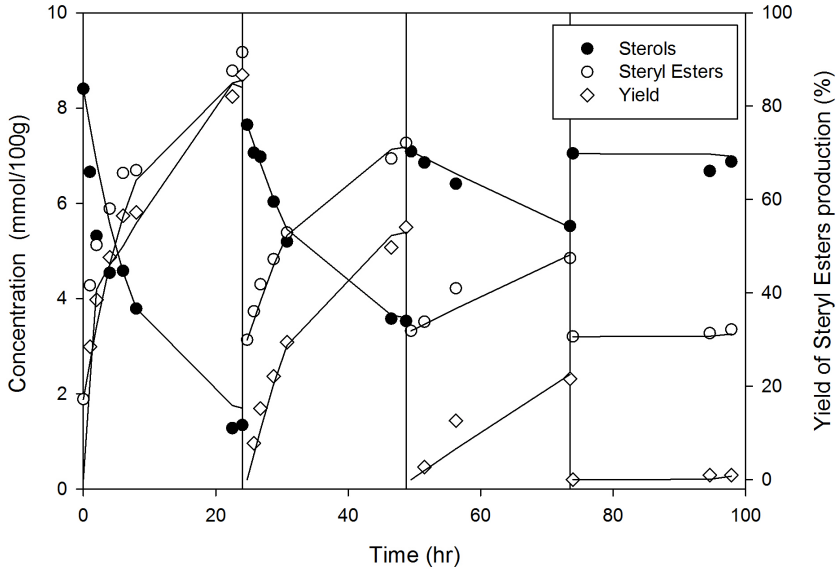
4.3 Results and Discussion

4.3.1 Consecutive Batch Reactions for Steryl Esters Production

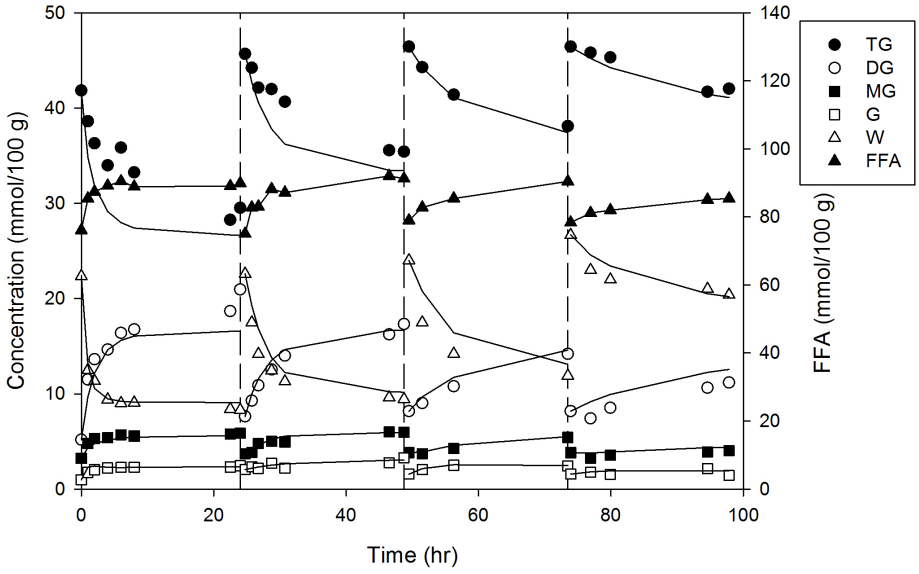
In the Chapter 2 a procedure to produce steryl esters from the esterification of sterols and free fatty acids present in vegetable oil deodorizer distillates was optimized, and high yields of steryl esters production (>80%) were obtained after 24h of reaction. In order to maximize the productivity of this process, reuse of the enzyme is an important requirement. Therefore, four consecutive batch reactions (enzyme concentration at 0.5% w/w) were performed under optimal operating conditions, without water activity control (Figures 4.2(a) and 4.2(b)).

Figure 4.2 shows that the yield of steryl esters production decreases significantly after the first batch. After 98 hours in vegetable oil deodorizer distillate, the *Candida rugosa* lipase is almost completely inactivated. Such inactivation is confirmed by the decreasing value of the kinetic constant k_1 in each batch, as shown in Table 4.1.

In order to validate the procedure of enzyme recovery and confirm that the decrease of the yield of steryl esters production and the respective kinetic constant k_1 observed in consecutive batch



(a)



(b)

Figure 4.2: Evolvement of the yield of steryl esters production and the corresponding concentration of sterols, steryl esters and acylglycerides during four consecutive batch reactions (24 hours each). These batch reactions were carried out using the vegetable oil deodorizer distillate as raw material and 0.5% w/w of enzyme as catalyst, initial molar ratio FFA:Sterol of 10 and an initial water activity of 0.81 (water content=0.41% w/w). Symbols represent the experimental data and the lines the adjustment of the model. ((a):(●) - Sterols, (○) - Steryl Esters (SE), (◇) - Yield of Steryl Esters production, represented on secondary axis;(b):(●) - Triglycerides (TG), (○) - Diglycerides (DG),(■) - Monoglycerides (MG), (□) - Glycerol (G), (△) - Water (W), (▲) - Free Fatty Acids (FFA), represented on secondary axis

Table 4.1: Kinetic constant of the direct reaction I, k_1, in consecutive batch reactions for steryl esters production

Batch	k_1 (h^{-1})	Confidence Intervals at 95%
1	0.157	0.007
2	0.072	0.019
3	0.023	0.002
4	0.003	0.004

reactions are not related with the material loss of enzyme, four consecutive batch reactions were carried out at optimal operating conditions without sampling during esterification. The nitrogen content in the original deodorizer distillate, in the reactional mixture at the end of each batch, and in the permeate of the ultrafiltration step used to recover the enzyme were determined, in order to quantify possible loss of enzyme. The results obtained show that the nitrogen content in the ultrafiltration permeate is lower than in the original deodorizer distillate without enzyme (data not shown), which indicates that the enzyme is fully retained together with other compounds with high molecular weight.

4.3.2 Identification of Potential Mechanisms Responsible for Enzyme Inactivation

Once the procedure of recovery and reuse of the enzyme is validated, the reason behind the decrease in the yield of steryl esters production in consecutive batch reactions remains unknown. Several chemical species are referred in the literature which may be potentially responsible for the partial inactivation of *Candida rugosa* lipase, such as excess of water [81, 82, 90, 91], presence of glycerol [92–94] and the presence of oxidation products [95–98].

The following study allowed assessing the contribution of each potential agent to the inactivation/inhibition of the lipase from *Candida rugosa*, used in this study.

4.3.2.1 Effect of the Water Activity (a_W)

The negative effect of excess of water on the stability of the enzyme, during esterification reactions, is largely referred in the literature [81, 82, 90, 91]. Since vegetable oil deodorizer distillates contain acylglycerides, their hydrolysis occurs even at low water activity, since they are a natural substrate for lipases.

As shown in Figure 4.2, the water content during each consecutive batch reaction decreases; therefore, there is a higher consumption of water by hydrolysis than the water produced by esterification. Consequently, the inactivation of *Candida rugosa* lipase, during the esterification of sterols and free fatty acids in this specific medium, can not be due to an excess of water, as commonly occurs in standard esterification reactions. However, a significant variation of water activity occurs during esterification ($0.82<a_W<0.34$), which may affect the stability of the enzyme. In order

to study the effect of water activity on the stability of the enzyme, when consecutive batch reactions are performed, four consecutive batch reactions were carried out under water activity control (a_W=0.65, water content=0.30% w/w) and within optimal operating conditions (determined in the Chapter 2).

Figure 4.3 suggests that even under control of water activity, it is still possible to observe a significant decrease in the yield of steryl esters production. Therefore, it may be concluded that the decrease of yield and the kinetic constant k_1, obtained in consecutive batch reactions, occurs even under constant water activity conditions.

4.3.2.2 Effect of the Presence of Glycerol

The negative effect of glycerol on the stability of lipases has been reported in the literature [92–94]. The formation of a glycerol layer around the enzyme may inhibit the diffusion of hydrophobic substrates, increasing the internal mass transference resistance. This study allowed evaluate the contribution of glycerol in the inactivation/inhibition process of the enzyme.

Removal of Glycerol Associated to the Enzyme Between Consecutive Batch Reactions

The recovery of enzymatic activity by washing the enzyme with a tertiary alcohol (not reactive and only moderately polar) equilibrated at the same a_W of the reaction was proposed by Dossat et al. [92].

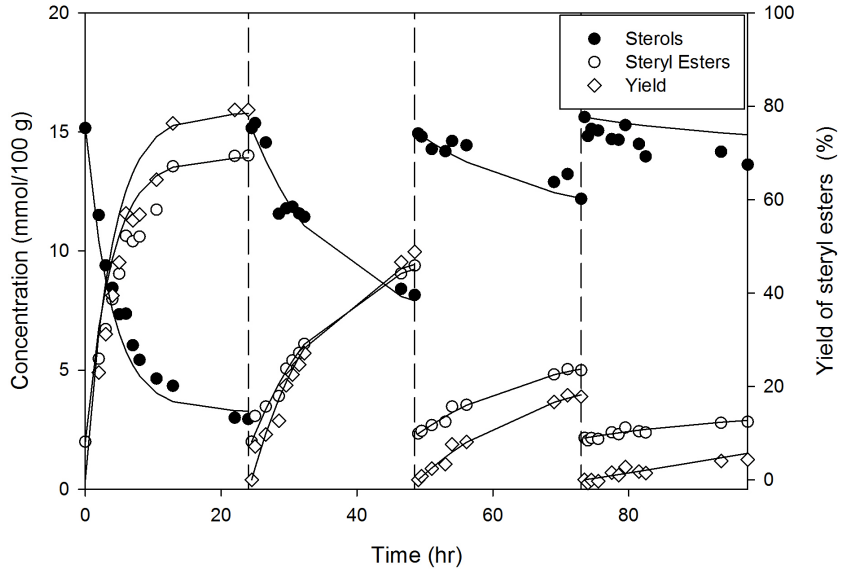
In order to evaluate the effect of the alcohol on the enzymatic activity and simultaneously validate the method used to recover the enzyme, fresh enzyme was washed and, subsequently, recovered either by ultrafiltration or by centrifugation. The enzymatic activity was determined immediately afterwards.

Experimental results show that the washed enzyme recovered by ultrafiltration losses 32% of its initial activity, while the one recovered by centrifugation losses 39%. These similar results demonstrate that the contact of the enzyme with the tertiary alcohol induces a loss of enzymatic activity. This decrease does not depend on the enzyme recovery method used.

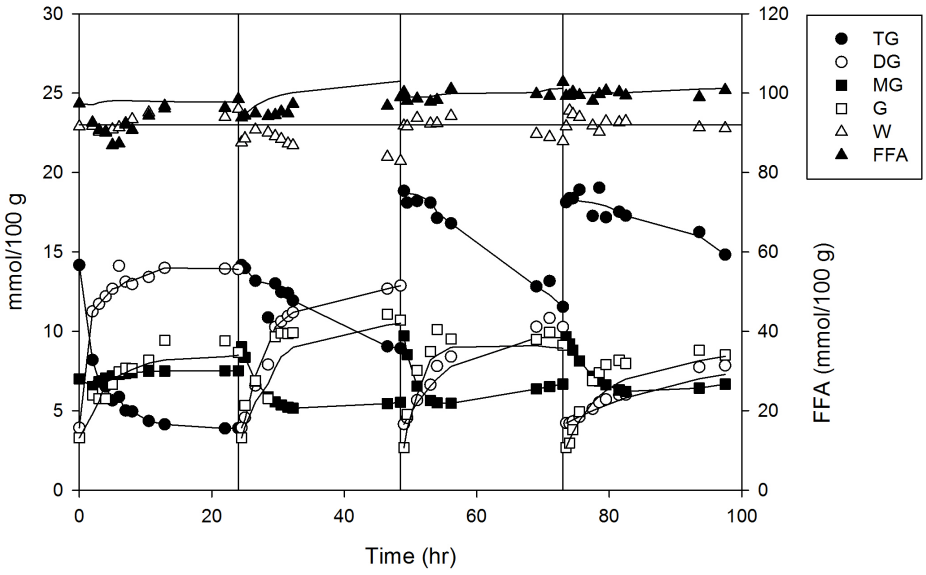
Although, the loss of enzymatic activity induced by this procedure, in order to verify its efficiency in the removal of adsorbed glycerol in the enzyme. A comparison of the yields of steryl esters production obtained in four consecutive batch reactions (24 hours each and similar initial conditions in terms of control of water activity (set at 0.54), initial molar ratio between free fatty acids and sterols (FFA:S set at 6.0) and enzyme concentration (0.5% w/w), with and without subsequent enzyme washing, is shown in Table 4.2.

Table 4.2 shows that the procedure of enzyme washing with an alcohol does not restore the enzyme activity. Therefore, the procedure described by Dossat et al. [92] may be valid for the recovery of the enzymatic activity of immobilized *Candida antarctica* but not for the *Candida rugosa* lipase, in the free form.

In order to verify if the removal of glycerol from the enzyme was efficient, the alcohol used in the washing of the enzyme was evaporated and its residue analyzed. The concentration of glycerol in this residue was found to be three times higher than its concentration in the reactional



(a)



(b)

Figure 4.3: Evolvement of the yield of steryl esters production and the corresponding concentration of sterols, steryl esters (a) and acylglycerides (b) during four consecutive batch reactions (24 hours each). Those batch reactions were carried out with vegetable oil deodorizer distillate, 0.5% w/w of enzyme concentration, initial molar ratio FFA:Sterol of 6.4 and under controlled water activity (set at 0.65). Symbols represent the experimental data and the lines the adjustment of the model. ((a):(●) - Sterols, (○) - Steryl Esters (SE), (◇) - Yield of Steryl Esters production, represented on secondary axis; (b): (●) - Triglycerides (TG), (○) - Diglycerides (DG),(■) - Monoglycerides (MG), (□) - Glycerol (G), (△) - Water (W), (▲) - Free Fatty Acids (FFA), represented on secondary axis

)

Table 4.2: Yield of steryl esters production obtained in four consecutive batch reactions (24 hours each), with and without enzyme washing between batch reactions

	Yield (%)	
Batch	Without enzyme washing	With enzyme washing
1	79.2	79.0
2	55.0	44.9
3	30.1	21.4
4	22.0	19.0

mixture. Therefore, the procedure followed in this work allowed for removing glycerol associated to the enzyme but, apparently, it induced an additional inhibition effect on the enzyme, leading to an even worse result. This experiment reflects a balance between a possible positive effect in the recovery of the enzymatic activity due to the removal of glycerol and a negative effect of the alcohol on the enzymatic activity.

Model Reaction without Glycerol Production

A model esterification reaction between a defined sterol and a free fatty acid was also performed under controlled water activity (a_W=0.54). In this very well controlled condition, where production of glycerol does not occurs, a possible inactivation of the enzyme between consecutive batch reactions means that enzyme inactivation occurs in the absence of glycerol.

The evolvement of the concentration of steryl esters produced by the esterification of stigmasterol and oleic acid and the respective yield is shown in Figure 4.4. After each 24 hours, a spike of stigmasterol was performed in order to maintain its initial concentration (supposing that 80% of stigmasterol was consumed in each batch).

Figure 4.4 shows that the inactivation/inhibition of the enzyme in consecutive batch reactions occurs even in the absence of glycerol and, since the enzyme was not recovered between batches, it demonstrates that the process of inactivation/inhibition of the enzyme does not depend on the recovery method applied.

The inactivation/inhibition is confirmed by the significant decreasing value of the kinetic constant k_1 in each batch reaction (Table 4.3).

Table 4.3: Kinetic constant,k_1, of the model esterification reaction between stigmasterol and oleic acid in consecutive batch reactions

Batch	k_1 (h^{-1})	Confidence Intervals at 95%
1	0.033	0.004
2	0.021	0.006
3	0.012	0.004
4	0.013	0.003

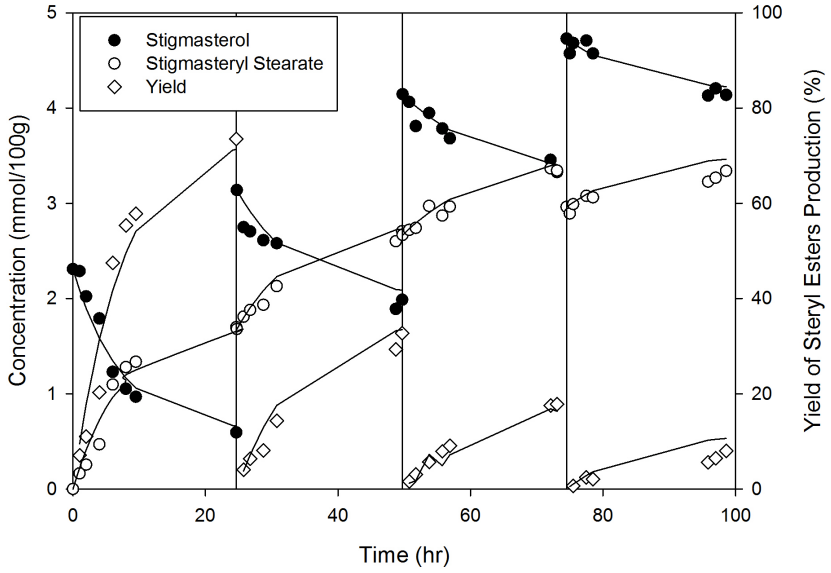


Figure 4.4: Evolvement of the yield of steryl esters production and the corresponding concentration of sterols (stigmasterol) and steryl esters, during four consecutive batch reactions (24 hours each). These batch reactions were carried out under controlled water activity (a_W = 0.54), at 40°C with oleic acid and 0.5% w/w of enzyme concentration. The symbols represent the experimental data and the lines the adjustment of the model. ((•) - Sterols, (○) - Steryl Esters (SE), (◇) - Yield of Steryl Esters production represented on secondary axis))

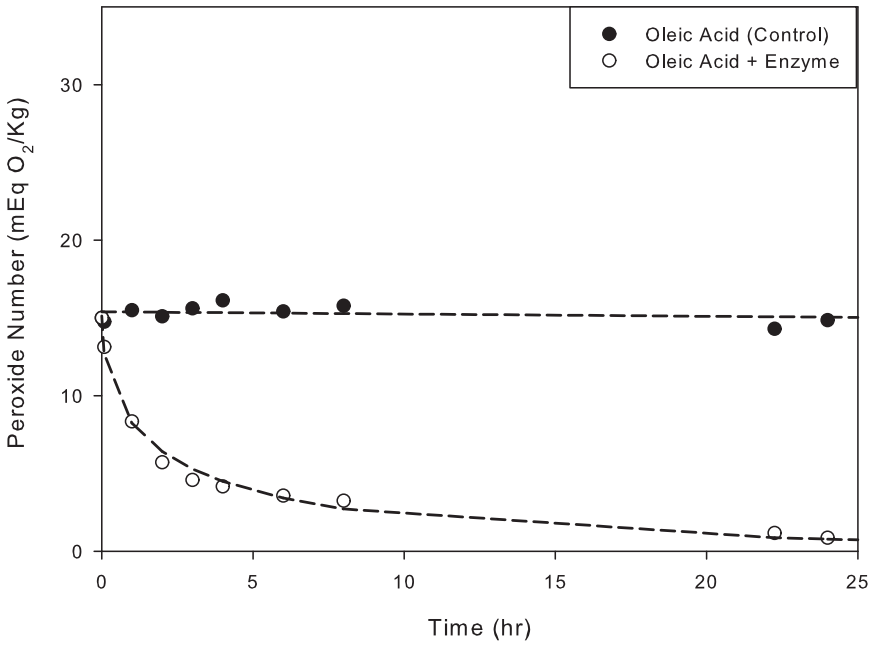
The difference between the absolute values of the kinetic constant, k_1, obtained in this model reaction and in the reaction with deodorizer distillate (Figure 4.2) can be justified since k_1 for the deodorizer distillate reaction is an average of all kinetic constants related to the esterification of all sterol presents in the deodorizer distillates.

4.3.2.3 Effect of the presence of oxidation products

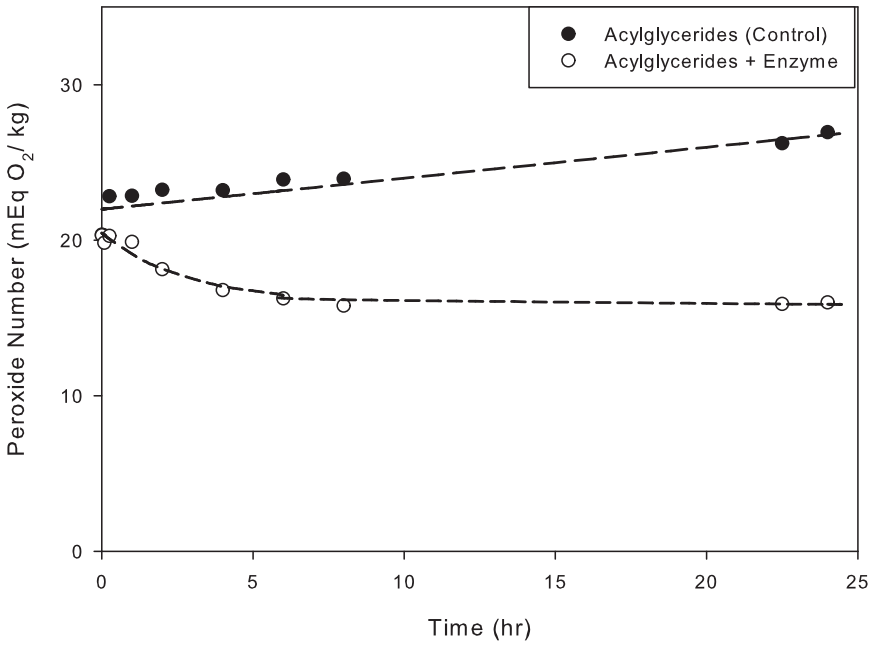
Since the enzyme inactivation/inhibition occurs even in an esterification model reaction with stigmasterol and oleic acid, where glycerol is neither initially present nor produced, this result suggests that oxidation products in oleic acid may be responsible for the decrease in steryl esters production in consecutive batch reactions, in the similarity observed in deodorizer distillates.

A contact between the enzyme and oleic acid was promoted and the evolution of the peroxide number was monitored (Figure 4.5(a)). A similar procedure was performed using a mixture of acylglycerides, the other major constituents of deodorizer distillates (Figure 4.5(b)).

The fact that the peroxide number of the control batch containing only oleic acid (Figure 4.5(a)) remained approximately constant allows to conclude that primary oxidation (production of peroxides by lipid oxidation) and secondary oxidation (decomposition of peroxides to produce aldeydes), during 24 hours at 40°C, are not significant. However, the contact with the enzyme induced a significant decrease of peroxides in solution, which cannot be due to the occurrence of



(a)



(b)

Figure 4.5: Evolvement of the peroxide number in oleic acid (a) and a mixture of acylglycerides (b), during 24 hours and at 40°C. Symbols represent the experimental data. ((a):(•) - Oleic acid (control, without enzyme), (○) - Oleic acid in contact with enzyme ; (b): (•) - Acylglycerides (control, without enzyme), (○) - Acylglycerides in contact with enzyme)

secondary oxidation, taking into account the behaviour observed in the control batch.

The peroxide number of the control batch containing only acylglycerides (Figure 4.5(b)) increased slightly. Since that, no reaction occurred (as proved by GC-FID analysis), this increase was probably due to the higher susceptibility to lipid oxidation.

The fact that there was no decrease of the peroxide number in the control batch, allows to confirm that there was no secondary oxidation (decomposition of peroxides to produce aldeydes), during 24 hours at 40°C. Once again, the contact with the enzyme induces a significant decrease of peroxides in solution.

Ohta et al. [95], proved that the presence of peroxide compounds induces enzyme deactivation by polymerization. Pirozzi et al [97] showed that the enzyme inactivation occurs due to the interaction of oxidation products with SH-groups (cysteine) to form Michael addition products which may further react with the e-amino groups of lysine, leading to protein cross-linking. This phenomena, could explain the decrease of peroxide number due to their reaction with groups of the enzyme. The formation of products from this reaction, could explain the additional C=C in solution, as indicated by the increase of the iodine value observed experimentally (from 58.2 to 60.5% for oleic acid and from 57.0 to 60.0% for acylglycerides).

The decrease of enzymatic activity was confirmed by determining the yield of steryl esters production in a subsequent esterification reaction using deodorizer distillate (Figure 4.6) and by the value of the respective kinetic constant, k_1 (Figure 4.7).

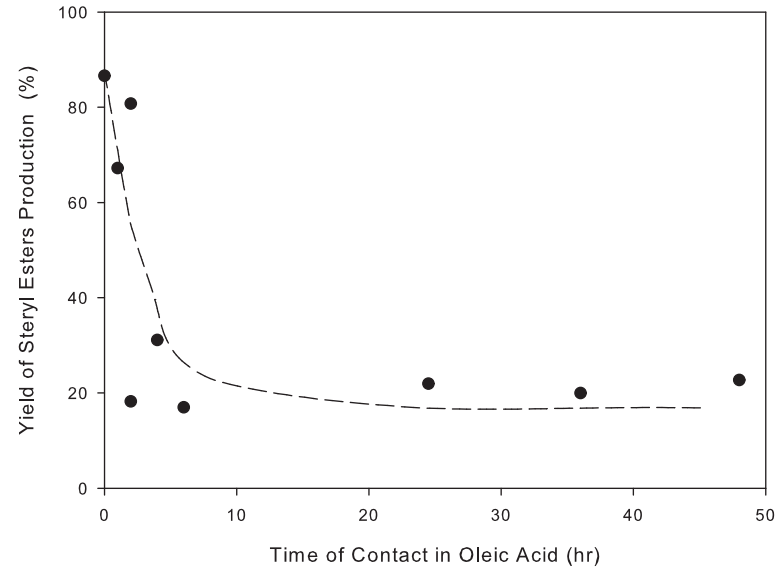
As shown in Figure 4.6, a relatively short time of contact of the enzyme with oleic acid and with acylglycerides induced a significant decrease in the yield of steryl esters production. For longer periods (higher than 10 hours), the conversion yield reaches a plateau. This observation can be additionally confirmed by the decrease of the respective kinetic constant value. The high error associated with determination of the kinetic constant is related with the small variation of the concentrations of the reactant species, due to the inactivation of the enzyme.

In conclusion, the products of oxidation present in the vegetable oil deodorizer distillate have an important role in the inactivation of the enzyme. Unfortunately, this negative effect is not fully avoided by the presence of antioxidants in the deodorizer distillates and, consequently, enzyme inactivation occurs inevitably between consecutive batch reactions.

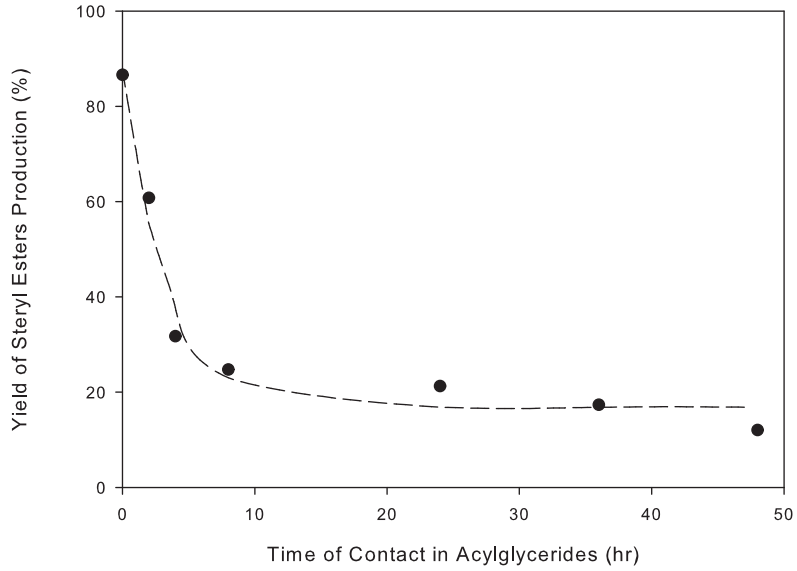
Taking into account the added-value of the deodorizer distillate, the solution to the problem of enzyme inactivation may require the use of methods to selectively remove oxidation products (namely, using absorbents or activating carbon [100]). Alternatively, a precise amount of fresh enzyme may be added at the beginning of each consecutive batch reaction, in order to compensate for the inactivated enzyme. Both options imply additional costs. The first option will be explored in a future work, while the second one is shown and discussed in the following section.

4.3.3 Addition of Fresh Enzyme in Consecutive Batch Reactions

The first step was to determine the amount of fresh enzyme to add in the beginning of a second batch, in order to maintain the yield of steryl esters production. Figure 4.8 shows the yield of steryl esters production obtained in the second batch after 24 hours of reaction, when different amounts



(a)

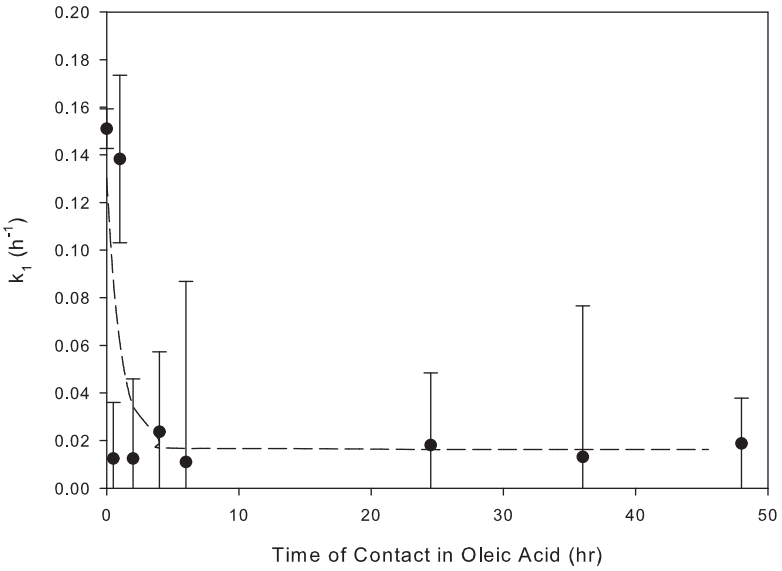


(b)

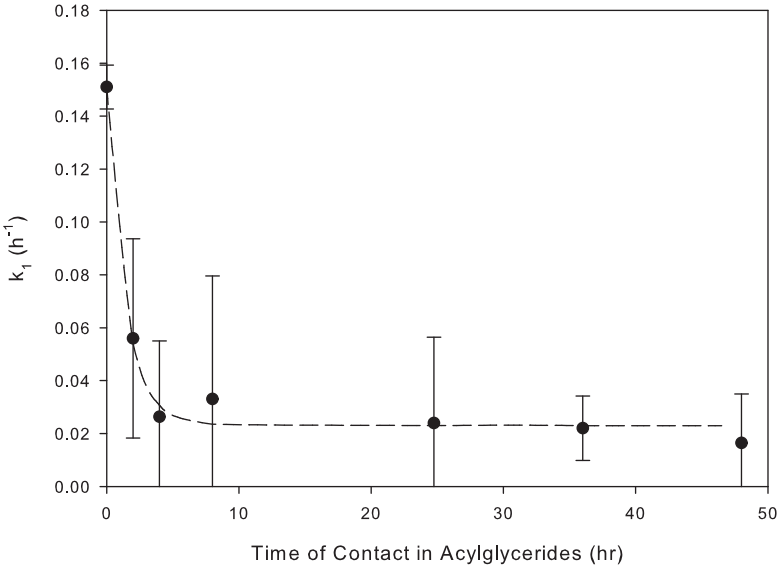
Figure 4.6: Effect of contact between the enzyme and oleic acid (a) and acylglycerides (b) on the yield of steryl esters production during reaction in deodorizer distillate, performed under controlled conditions (constant a_W, set at 0.66), constant temperature (set at 40 °C) and stirred at 200 rpm)

of fresh enzyme were added. The dashed line at a yield of 86% represents the yield of steryl esters production obtained in the first batch.

As it can be observed, an amount of fresh enzyme equivalent to 47% w/w of the enzyme used in the first batch reaction is sufficient to compensate for the decrease observed in the subsequent



(a)



(b)

Figure 4.7: Effect of contact between the enzyme and oleic acid (a) and acylglycerides (b) on the kinetic constant of the esterification reaction (reaction I), k_1, in deodorizer distillate, performed under controlled conditions (constant a_W, set at 0.66), constant temperature (set at 40 °C) and stirred at 200 rpm)

batch, maintaining a constant the yield of steryl esters production.

Concerning the kinetic constant, k_1, the positive effect of an addition of fresh enzyme between batch reactions can also be observed in terms of its absolute value (Figure 4.9).

After knowing that an addition of fresh enzyme of 47% w/w, between batch reactions, allows

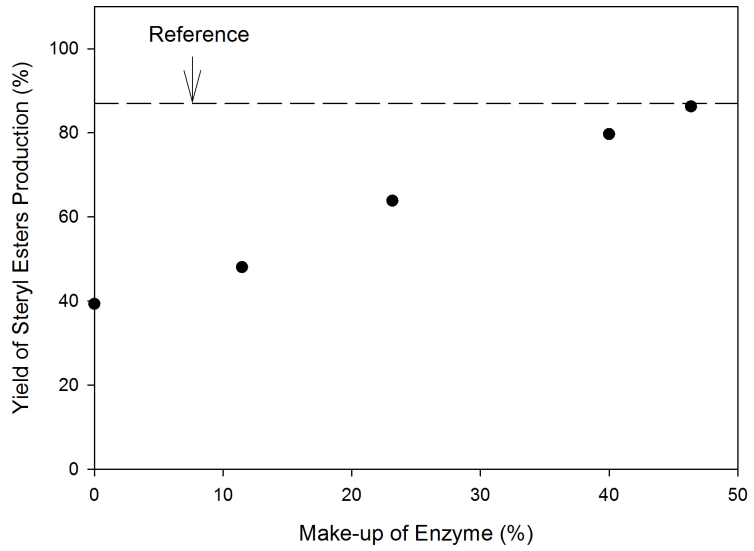


Figure 4.8: Effect of addition of fresh enzyme, between consecutive batch reactions, on the yield of the production of steryl esters. These batch reactions were carried out with a 0.5% w/w of enzyme concentration (first batch), initial molar ratio FFA:Sterol=8.0 and a initial a_W=0.82

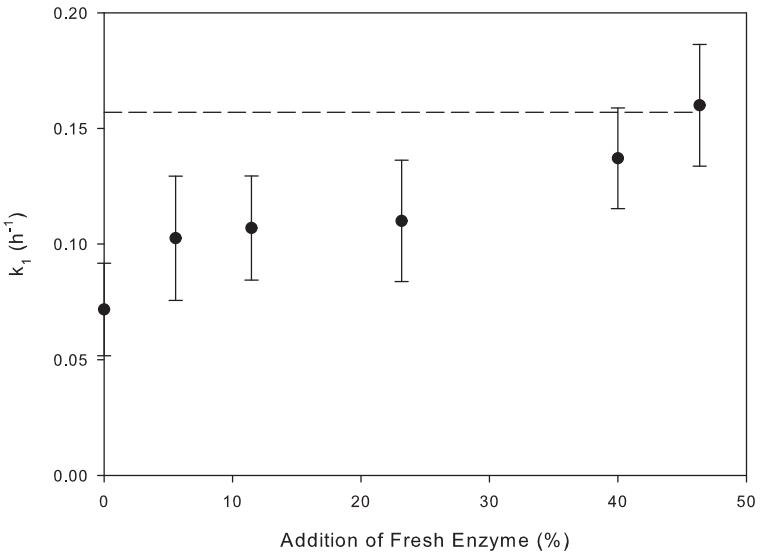


Figure 4.9: Effect of addition of fresh enzyme between consecutive batch reactions, on the kinetic constant,k_1 (reaction I). These batch reactions were carried out with a 0.5% w/w of enzyme concentration (first batch), initial molar ratio FFA:Sterol=8.0 and a initial a_W=0.82

for maintaining the yield of steryl esters production, a series of consecutive batch reactions was performed, adding fresh enzyme between batches. Four consecutive batch reactions were carried out under optimal conditions of water activity (a_W set at 0.82) and an initial FFA:Sterol molar ratio

of 8.0. Figure 4.10 shows that the addition of fresh enzyme between batch reactions allowed to maintain a constant yield of steryl esters production.

It is important to notice that similar concentration profiles are obtained in consecutive batches and that they are experimentally in agreement with a kinetic model with only one set of kinetic constants (shown as solid lines in Figure 4.10), which means that the used kinetic model shows a good predicative capacity throughout the consecutive batch reactions.

4.4 CONCLUSION

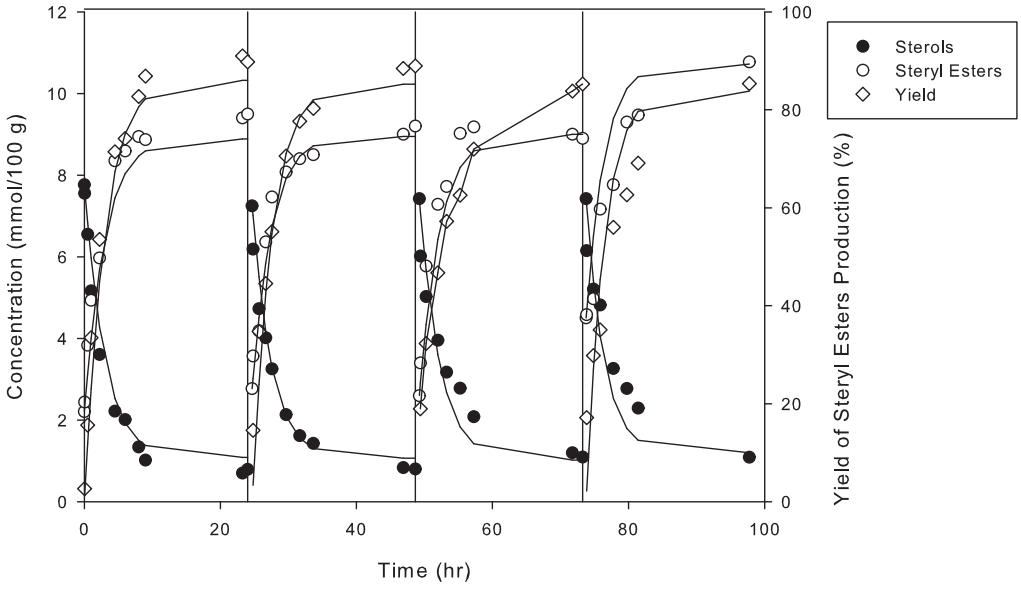
Consecutive batch reactions of steryl esters production from vegetable oil deodorizer distillates were not possible to perform using the same initial quantity of enzyme (0.5% w/w), because after each batch reaction (24 hours) the enzymatic activity in terms of yield of steryl esters production was significantly reduced (Figure 4.2(a)).

Water activity control during esterification reaction, performed to guarantee that the enzyme hydration state was maintained, was not efficient for assuring a constant enzymatic activity.

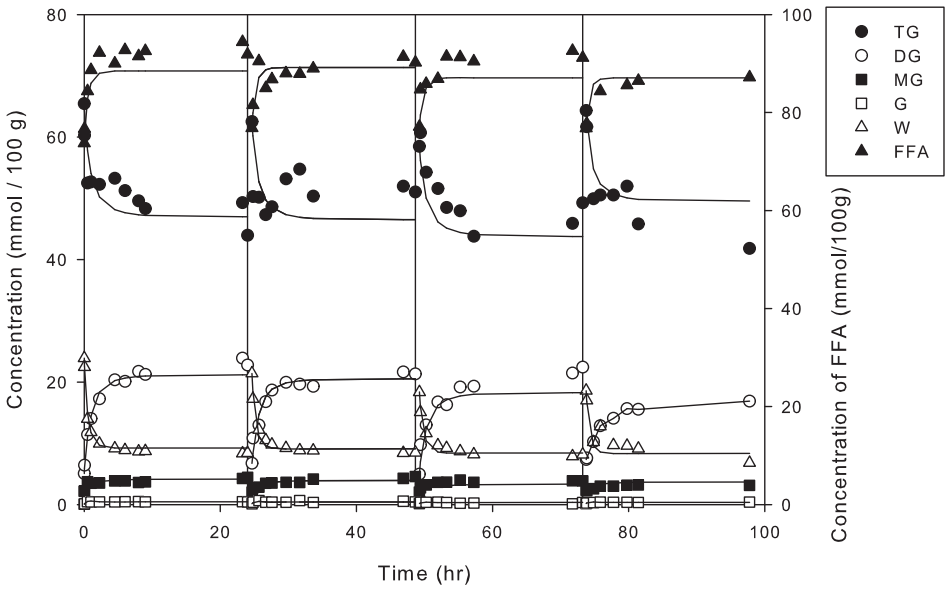
The role of glycerol in the inhibition of the enzyme appears not to be determinant in the enzyme inactivation process, since inactivation of the enzyme occurs in consecutive batch reactions of a esterification model reaction between oleic acid and stigmasterol, where there is no production of glycerol (Figure 4.4).

The products of oxidation present in the vegetable oil deodorizer distillate have an important role in the inactivation of the enzyme. Unfortunately, this negative effect could not be avoided by the presence of antioxidants in the deodorizer distillates and enzyme inactivation occurs inevitably between consecutive batch reactions.

Methods for removing oxidation products should be studied in future work. However, a partial reuse of the enzyme was demonstrated to be technically possible by performing an addition of fresh enzyme (47% w/w) between consecutive batch reactions. This procedure allowed to successfully perform four consecutive batch reactions, maintaining the reaction yield at levels higher than 80%.



(a)



(b)

Figure 4.10: Evolvement of the yield of steryl esters production and the corresponding concentration of sterols, steryl esters (a) and acylglycerides (b) during four consecutive batch reactions (24 hours each), performing an addition of fresh enzyme of 47% w/w between batch reactions. These batch reactions were carried out with a 0.5% w/w of enzyme concentration (First Batch), initial molar ratio FFA:Sterol=8.0 and a initial a_W=0.82. Points represent the experimental data and lines the adjustment of the model. ((•) - Sterols, (○) - Steryl Esters (SE), (◇) - Yield of Steryl Esters production represented on secondary axis)

5 Assessment of solvent resistant nanofiltration membranes for valorisation of deodorizer distillates

Summary

The valorisation of deodorizer distillates from oil refining industry requires a stable membrane suitable to separate steryl esters (bioactive compounds with high molecular weight (MW), 650<MW<800 g/mol) from pesticides (150<MW<400 g/mol), whose content is restricted by the actual legislation. This work aims at identifying a suitable solvent and a solvent resistant nanofiltration (SRNF) membrane to be used in a diananofiltration process for the removal of pesticides.

Hexane, ethanol and oleic acid were investigated as potential solvents. The role of solvent-membrane interactions was found to be important in the permeability of the membrane, supported by a strong relationship with the swelling/solvent viscosity ratio. Selected membranes were compared in a dead-end filtration mode, which allowed for identifying the membrane(s) that showed the best compromise between permeability and discrimination between the target compounds. The membranes with the best performance were GMT-oNF2 from Borsig/GMT, PuraMemS600 from Evonik and 030306F from Solsep. The performance of the process was enhanced while operating in a cross-flow mode, being further improved after optimisation of the concentration of deodorizer distillate and of the processing solutions and the transmembrane pressure.

The membranes identified in this work proved to be suitable for valorization of deodorizer distillates, presenting high rejections of steryl esters (>96%) and significant low rejection of pesticides (<65%), under optimised conditions.

The contents of this chapter were adapted from the publication: Teixeira, A. R. S., Santos, J. L. C., Crespo, J. G. (2014). Assessment of Solvent Resistant Nanofiltration Membranes for Valorisation of Deodorizer Distillates. Journal of Membrane Science. Submitted.

5.1 INTRODUCTION

The production of natural bioactive extracts from agro-industrial by-products for pharmaceutical, food and cosmetic industries has grown significantly in the last years due to the increasing consumer interest for products with a positive impact in health [5]. Deodorizer distillates are a by-product of the refining edible oil industry and have a special interest as source of bioactive compounds (2-20% w/w), such as sterols and tocopherols [17]. However, deodorizer distillates cannot be used directly as additive without a previous purification step mainly due to their high content in pesticides. Maximum residue levels (MRLs) in vegetable oils for human consumption are not specifically set, but it is recommended to be lower than the established for seeds (0.05 ppm for the main lipophilic pesticides) [24]. The concentration in deodorizer distillates can be 800-1000 times higher than the actually allowed values [22, 23]. Therefore, their use as a food additive depends from a quantitative removal of pesticides, which, however, remains challenging.

Methods for the recovery of sterols based-on molecular weight (MW) difference showed to be labor-intensive, time consuming, energetically intensive and inefficient in terms of mass recovery. These methods used include crystallization, supercritical carbon dioxide extraction and molecular distillation [9]. It has been previously shown that steryl esters can be produced by enzymatic esterification of fatty acids and sterols from deodorizer distillates under optimised operating conditions (determined in the Chapter 2). The use of steryl esters as food additive has been intensified mainly after their approval by the European Food Safety Authority (EFSA) as compounds with anti-cholesterol activity [28]. Moreover, there are studies indicating that sterols in the esterified form are more bioactive than their free form [33] and present a higher solubility in oil phase, making easier their incorporation in fat-based products. Therefore, the production of steryl esters is advantageous and, on the other hand, their high molecular weight ((700-800 g mol^{-1}) facilitates their recovery and separation from pesticides (150-400 g mol^{-1}).

Membrane technology is considered as a technology environmentally clean which enables the purification and concentration of natural extracts, without requiring high process temperatures, which typically negatively affect the valuable compounds in natural extracts. Diananofiltration is a membrane-based technique used for the removal of contaminants (such as pesticides) from value-added streams. This method consists in continuously feeding fresh solvent at the same rate as permeate is recovered, where valuable compounds are retained by the membrane, while contaminants are washed-out in the permeate. Therefore, the structural stability of the membrane in the selected solvent is a necessary condition for assuring a successful process. Commercial solvent resistant membranes are available from mid-1990s, however, the number of membrane producers is still limited [58], being Evonik [55], GMT [56] and Solsep [57] the most important. Polyimide (PI) crosslinked with PDMS and polyacrylonitrile (PAN) are examples of materials used to prepare such membranes due their satisfactory resistance to solvents.

SRNF membranes are mostly used in pharmaceutical industry applications, such as purification of active pharmaceutical ingredients (API) [46, 48], catalyst recycling [47], continuous solvent exchange [49], and solvent recycling [50]. Even though, the only reported application of such technology in large scale is the solvent recovery from lube oil dewaxing (MAX-DEWAX^{TM}) [54].

The use of SRNF in oil refining industry has been discussed in the literature, mostly in the scope of solvent recycling and oil recovery [51–53, 101, 102]. This work proposes the investigation of a different perspective, through the use of SRNF in diananofiltration mode for valorisation of by-products, producing extracts that are enriched in bioactive compounds and free of contaminants. Therefore, our work aims at determining the performance of commercial solvent-resistant nanofiltration (SRNF) membranes in the valorisation of deodorizer distillates by the removal of pesticides and simultaneous recovery of steryl esters.

This work addresses the identification of the most adequate solvent to be used for diananofiltration purposes (oleic acid, hexane and ethanol were assessed). The main criteria to select the most adequate solvent and membrane are the membrane permeability in the target solvents and their discrimination capacity between the target compounds, specifically steryl esters and pesticides. Finally, the most adequate membrane and solvent system are assessed in their performance in a cross-flow operation, with the objective of preparing the scale-up of the process.

5.2 Materials and Methods

5.2.1 Material

5.2.1.1 Esterified Deodorizer Distillate

Sunflower deodorizer distillate obtained from Lesieur (France) was characterized in our laboratory (its composition is presented in more detail in the Table 2.1, deodorizer distillate "3") and enriched in steryl esters by an enzymatic esterification between sterols and free fatty acids (FFA). The reaction was carried out under previously established optimised conditions (see Chapter 2).

The esterified deodorizer distillate used in this study is rich in acylglycerides (50%) and free fatty acids (29.5%). It also contains bioactive compounds, such as sterols (0.4%), steryl esters (8.5%), tocopherols (2.3%) and squalene (1.6%). Other compounds comprise hydrocarbons, aldehydes, ketones, pesticides, herbicides and oxidized products from breakdown of tocopherols and free phytosterols. High concentrations of pesticides were detected, namely, 20 ppm of pirimiphos-methyl, 9.7 ppm of chlorpyriphos-methyl and 1.6 ppm of chlorpyriphos.

5.2.1.2 Chemicals

Analytical-grade hexane, ethanol and isopropanol were obtained from VWR (Germany). Technical grade of chloroform (99%) for GC analysis and oleic acid food-grade with an acid value of 196.0-204 mg KOH/g were purchased from Sigma Aldrich (Belgium).

The derivatizating and silylation agent, N,O-bis (trimethylsilyl) trifluracetamide (BSTFA) containing 1% of trimethylchlorosilane (TCMS) solution (from Fluka) and pyridine were both obtained from Sigma (France).

All analytical-grade standard substances, squalene (99.3% purity), stigmasterol (97% purity), β-sitosterol (99%purity), campesterol (99% purity), cholesteryl stearate (96% purity), monoglyceride olein (>99%purity), diglyceride olein (99.7% purity) and triglyceride olein (99.6% purity) were purchased from Sigma (Saint Quentin, France). A tocopherol kit consisting of α-, β-, γ- and

δ-tocopherols was obtained from Merck (>95% purity).

The internal standard heptadecanyl stearate (HDS) was prepared by condensation of heptadecanol and stearoyl chloride, both obtained from Aldrich (Bornem, Belgium), as described by Verleyen et al. [65].

5.2.1.3 Membranes

Five commercial SRNF membranes from different manufactures were selected to be used in this work, namely the 030303, 030306F and 070706 from Solsep (The Netherlands), PuraMem600 from Evonik (U.K.) and GMT-oNF2 from GMT Membrantechnik (Germany). Table 5.1 compiles the most relevant information of each membrane provided by the respective manufacturer.

Table 5.1: Properties of the selected membranes provided by the respective manufacturer

Membrane	Manufacturer	T_{max} (° C)	P_{max}(bar)	Separation	Active Layer
030306	Solsep	150	40	R(99%)~1000 Da (in ethanol)	PDMS [a]
030306F		120	40	R(85%) ~1000 (in ethanol)	PDMS [b]
070706		Not available			
Puramem600	Evonik	50	60	R(90%)=600 Da (in Toluene)	PDMS
GMT-oNF2	GMT	60	35	R(93%)=327 Da (in 2-propanol)	PDMS

[a] Van der Bruggen et al. [103]
[b] 030306 based-membrane

5.2.2 Analytical Methods

5.2.2.1 Analysis of Fatty Acids, Acylglycerides, Tocopherols, Sterols and Steryl Esters

The procedure to analyze fatty acids, acylglycerides, tocopherols, sterols and steryl esters was described in detail in the subsection 2.3.2 (Chapter 2).

5.2.2.2 Analysis of pesticides

Pesticides were quantified by a certified laboratory (Labiagro, Oeiras (Portugal)) according to NP EN 1528-1/2/3/4:2002. The analysis were performed by GC-MS. The results were expressed as mg/kg.

5.2.3 Experimental Procedures Methods

5.2.3.1 Setup

Experiments in a dead-end operating mode were performed in a stainless steel METcell test cell, supplied by Membrane Extraction Technology (MET, UK). The feed reservoir has a total volume of 250 cm^3 and the agitation is promoted by a cross head magnetic bar, providing the adequate fluid dynamic conditions. The pressure applied through the membrane (circular sheet with an

effective area of 51.4 cm^2) was regulated by a pre-assembled gas unit. The permeate was collected in a recipient, during the course of the experiment and the flux was monitored by acquisition of the permeate weight, using an electronic balance with an accuracy of 0.1 g.

Cross-flow experiments were conducted in a Sepa CFII cell (GE, USA), which accommodates a flat sheet membrane with 140 cm^2 of effective area. This cell is reported to mimic closely the flow dynamics of commercial spiral-wound membrane elements, by using a combination of stainless steel shim, feed spacers and permeate carriers. A diamond-type spacer and a steel shim were combined to obtain a channel feed height of 1.55 mm. The feed flow rate was measured using a calibrated rotameter (SK72, Georg Fischer, Switzerland), being imposed by a diaphragm pump (Hydra-cell G-13, Wanner Engineering, USA). The pressure applied to the system was adjusted with a valve assembly and monitored with two transmitters (8864, Trafag, Switzerland) placed in the inlet and outlet of the retentate compartment of the cell. The temperature of the system was indicated by the same transmitters, although, it was controlled by a heat exchanger, using tap water as cooling transfer fluid. The permeate flow rate was monitored by acquisition of its weight, using an electronic balance with an accuracy of 0.1 g, being afterwards, recirculated to a closed recipient. Feed and permeate collectors were hermetically closed, with sampling points and reflux condensers to avoid solvent losses during processing.

5.2.3.2 Solvent Screening

Oleic acid, ethanol and hexane were identified as potential solvent candidates for this separation process. The membrane 030306F (Solsep, The Netherlands) was used as a reference PDMS-based membrane to study the interactions between solvent, solute and membrane. Based on the experimental results, the most adequate solvent was identified. In a dead-end mode, the cell was filled with 100 g of solvent and the flux of different solvents at different pressures was determined. The flux was related with the swelling of the membrane (measurement described in the following subsection) and the viscosity of the solvents, in order to determine the impact of the interaction solvent and membrane in the flux.

Solutions of deodorizer distillate in these solvents (10% (w/w)) were added to the feed reservoir (100 g) of the dead-end cell. The cell was pressurized at 35 bar and the temperature maintained at 20° C. After achieving a reduction of 10% of the initial feed mass, samples of permeate were collected for analysis (±100μL) and the system was slowly depressurised for feed sampling. Samples were analysed by GC-FID and the rejection of compounds in the various solvents systems was determined.

5.2.3.3 Swelling Measurements

The membrane 030306F (Solsep, The Netherlands) was used as a reference PDMS-based membrane to study the solvent-membrane interaction, which may be reflected by the membrane swelling. For the swelling experiments, thick pieces of membrane were used and the procedure described elsewhere [104] was followed. Pre-weighed dry pieces of membrane were immersed in pure hexane and in sunflower oil (5, 10 and 20% w/w) at different time intervals, they were removed from

the solution, the liquid excess wiped and weighed again. After reaching the equilibrium swelling (no further weight increase), the difference between the initial and final weight (M_{dry} and M_{wet}, respectively) was determined. The swelling degree (SD) of the dense PDMS membrane was calculated by:

$$SD(\%) = \frac{M_{wet} - M_{dry}}{M_{dry}} \times 100 \tag{5.1}$$

5.2.3.4 *Membrane Screening*

The membranes under study were compacted in the presence of hexane, at 40 bar and 20±3° C, until reaching a constant flux. Afterwards, the flux in hexane was measured at different pressures to determine the permeability of each membrane. The same procedure was carried out using a hexane-based solution of deodorizer distillate (10% w/w).

In a dead-end mode, the feed reservoir was filled with 100 g of solution and pressurized. For each pressure, and after a redution of 10% of the initial feed mass, samples of permeate were collected for analysis (±100μL). Then, the cell was slowly depressurised, the feed sampled and the remaining permeate returned to the feed reservoir. Samples collected at different pressures were analyzed by GC-FID and the rejection of compounds was determined.

Membranes were compared in terms of permeability and capacity for discrimination between compounds with high and low molecular weight (650<MW<800 g/mol and 150<MW<400 g/mol, respectively).

5.2.3.5 *Determination of the impact of operating in cross-flow mode in the membrane performance*

Operating conditions were optimized using the membrane 030306F from Solsep as reference. This membrane was placed in the cross-flow cell, compacted with hexane, at 40 bar and 20±3° C, until reaching a constant flux, and characterized by determining hexane permeability. Afterwards, the feed tank was filled with 1 L of a solution of deodorizer distillate in hexane (10% w/w). The permeate was continuously recirculated to the feed tank and both the feed and the permeate were sampled, after ≈1 h at constant pressure (varied between 10-40 bar). Permeabilities in hexane and solution, as well as rejection of compounds were compared to those obtained in the dead-end operating mode.

5.2.3.6 *Optimisation of the concentration of the hexane-based solution*

Hexane-based solutions with different concentrations (5, 10 and 20%) were processed at different pressures. In each case, the respective flux was measured and samples from feed and permeate were taken and analysed by GC-FID for determination of the rejection of target compounds.

5.2.3.7 *Effect of transmembrane pressure in the rejection of target compounds*

After optimisation of the concentration, selected membranes were evaluated according to their flux and discrimination between steryl esters and pesticides (target compounds) at different pressures.

5.3 Results and Discussion

5.3.1 Solvent Screening

Solvents are used in diananofiltration to wash out the contaminants (such as pesticides) from valuable streams. In this section it is discussed the selection of the solvent, taking into account the final purpose for which the product is intended (food, cosmetic or pharmaceutical). Oleic acid, ethanol and hexane were chosen as a starting point.

Free fatty acids (FFA) constitute 25-75% of the deodorizer distillate [16], being the oleic acid the most abundant. Its high concentration in the original matrix, makes it a clear candidate as a diananofiltration solvent. On the other hand, hexane is a traditional solvent used in the edible oil refining industry, specially due to its ability to solubilise hydrophobic compounds and its low boiling point which makes its recovery easy. Additionally, edible oil plants are already prepared to process and recovery such solvent under safety conditions. Finally, ethanol has been considered as an alternative solvent in food applications, mainly due to its low toxicity and the possibility of being produced from renewable resources [105, 106].

The viability of the purification process relies on select a solvent compatible with the material of the SRNF membranes. Since the based-material of the membranes used in this study is the same, it is possible to assume that solvent and membrane interactions will be similar (although differences in the proprietary top-layer can introduce some variability). Using the membrane 030306F as a reference, the flux at each solvent at different transmembrane pressures was compared to illustrate the importance of the solvent and membrane interactions in permeability (Figure 5.1).

Oleic acid presented the lowest permeability ($0.0015\,L\,h^{-1}\,m^{-2}\,bar^{-1}$), while hexane presented a permeability higher than ethanol (0.23 and $0.16\,L\,h^{-1}\,m^{-2}\,bar^{-1}$, respectively). Since PDMS is a hydrophobic polymer, a high permeability for hexane could be expected. On the other hand, since oleic acid is a viscous liquid (Table 5.2), transport through a dense membrane was expected to be low.

Table 5.2: Kinematic viscosity (ν) of solvents and their ability to swell the membrane 030306F (PDMS-based membrane), expressed as swelling degree (SD)

Solvent	ν ($\times 10^{-6}\,m^2 s^{-1}$)	SD (%)
Oleic Acid	35.8 [a]	79.4
Ethanol	1.37 [b]	72.2
Hexane	0.49 [b]	49.5

[a] Dynamic viscosity measured in our laboratory using a digital viscometer (Brookfield Engineering Laboratories Inc., USA).
[b] Data from [107]

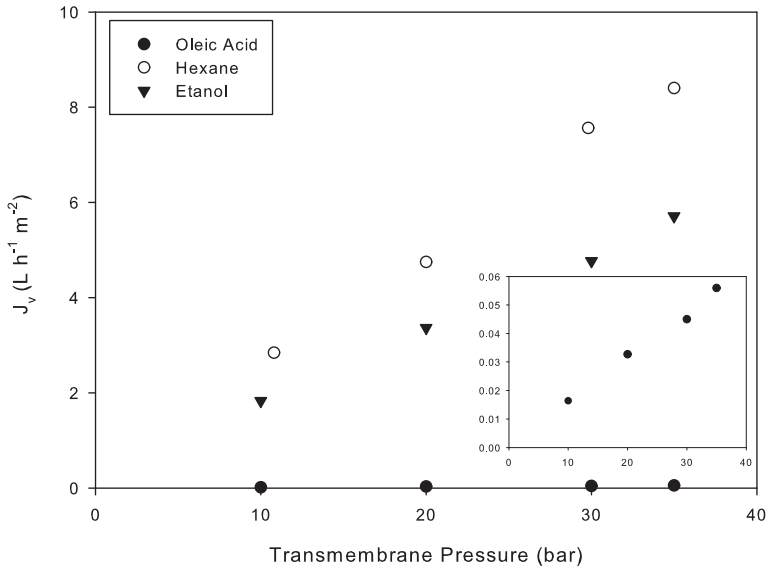
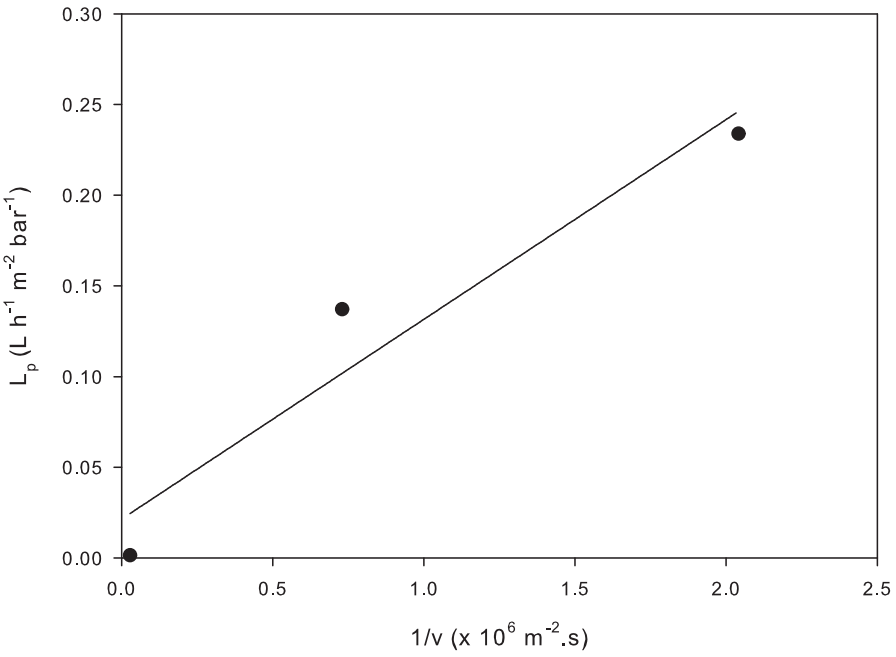


Figure 5.1: Effect of solvent in the membrane flux at different transmembrane pressures and constant temperature (20±3° C). Membrane 030306F from Solsep.((•) - Oleic Acid, (○) - Ethanol, (▼) - Hexane). See inset figure for the curve corresponding to the Oleic Acid.

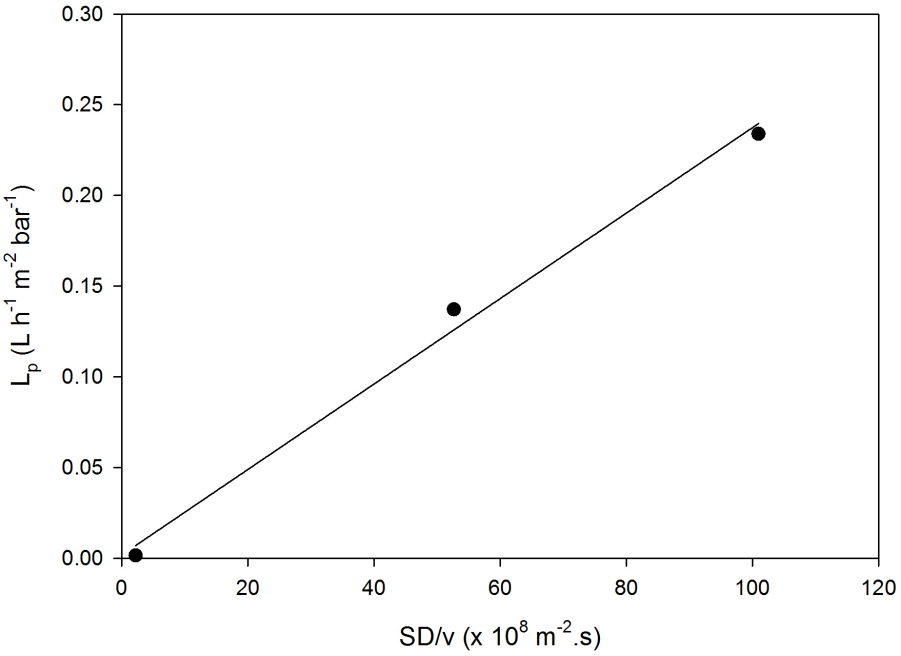
Several authors argued that when swollen, the structure of the dense PDMS layer changes, increasing its free volume, thus, allowing viscous flow [60, 61]. The kinematic viscosity of each solvent as well as its ability to induce swelling in a PDMS-based membrane (expressed as swelling degree, Eq. 6.6) is presented in the Table 5.2.

Contrary to the expected due to its hydrophobic nature, hexane shows the lowest ability to swell the PDMS-based membrane, followed by ethanol and oleic acid. Therefore, the high permeability of the membrane observed in hexane, should be mainly due to its low viscosity, while the extremely low permeability observed in oleic acid may be due to its high viscosity. These results suggest that the pure solvent permeability of the membrane is correlated with its swelling and the solvent viscosity. Figure 5.2(a) shows that the solvent permeability of the membrane (L_p) is inversely proportional to the viscosity of the solvent (R^2=923) - Figure 5.2(b). However, the correlation is improved when the swelling and the viscosity are considered (R^2=0.993). Stafie et al. [104] referred a similar observation in a system of sunflower oil and hexane.

Figure 5.3 shows the curve of rejection of target compounds present in the deodorizer distillate as a function of the molecular weight (MW), when using oleic acid and hexane as solvents. Ethanol was not considered since it was observed a precipitate forming after its addition to the deodorizer distillate. An analysis of this precipitate has shown that it was comprised of 35% (w/w) of acylglicerydes, 31.5% (w/w) of tocopherols, 15.6% (w/w) of sterols, 11.7% (w/w) of steryl esters and 6% (w/w) of squalene. Therefore, ethanol is not a suitable solvent since its addition leads to a partial loss of steryl esters. Oleic acid led to low rejections of pesticides and steryl esters. This observation associated to its extremely low permeability (Figure 5.1), makes oleic acid an unsuitable solvent. Hexane presented a good discrimination between target compounds as well as



(a)



(b)

Figure 5.2: Permeability of solvents (at 20±3° C) as a function of (a) their kinematic viscosity (ν) and (b) their kinematic viscosity (ν) and ability to swell the PDMS-based membrane 030306F (measured as swelling degree, SD)

high permeabilities. Therefore, hexane was selected among the tested solvents.

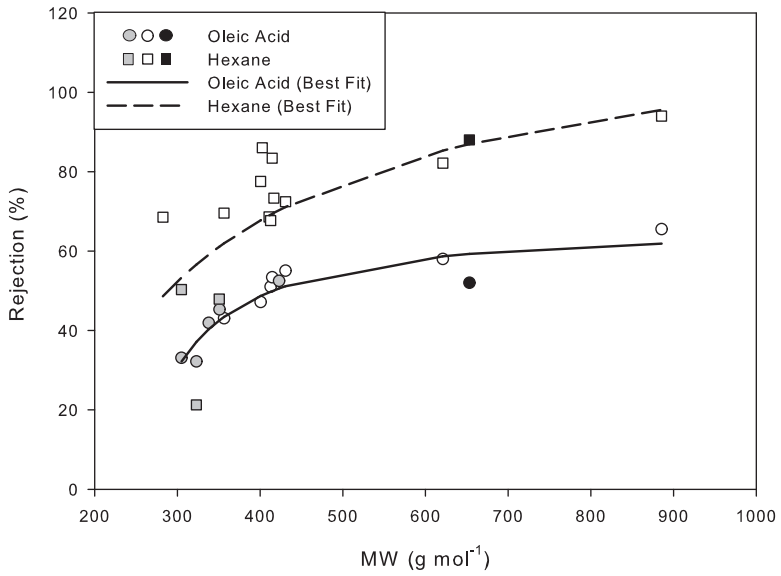


Figure 5.3: Curve of rejection as a function of the molecular weight (MW) of compounds present in a solution with 10% w/w of deodorizer distillate and a corresponding solvent: (circles) - Oleic Acid, (squares) - Hexane. Pesticides are highlighted in gray, while steryl esters are in black. The lines correspond to best fits. Membrane used:030306F from Solsep

5.3.2 Membrane Screening

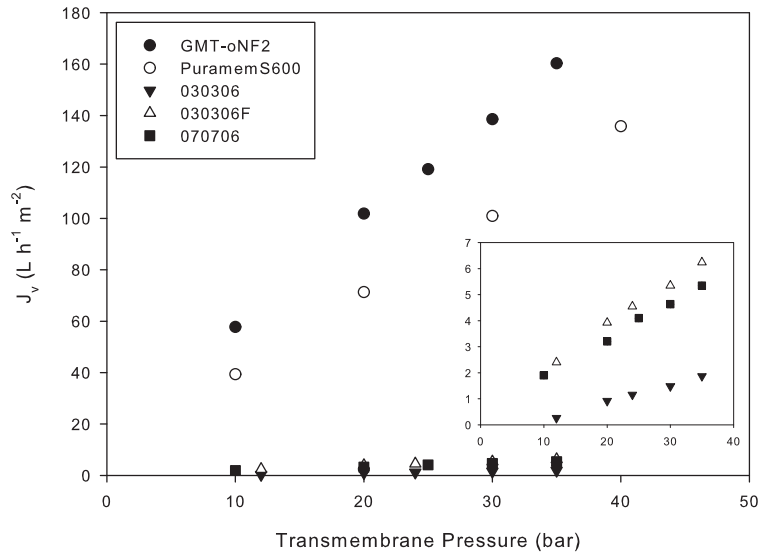
Five commercial PDMS-based membranes were tested in a dead-end operating mode (Table 5.1), in a first selection stage. These membranes are claimed to be hexane resistant, therefore, after compaction, they were tested in terms of flux at different pressures using hexane (Figure 5.4(a)) and, afterwards, using a hexane-based solution of deodorizer distillate (10% w/w) - Figure 5.4(b).

Table 5.3: Permeability of membranes in a dead-end operating mode

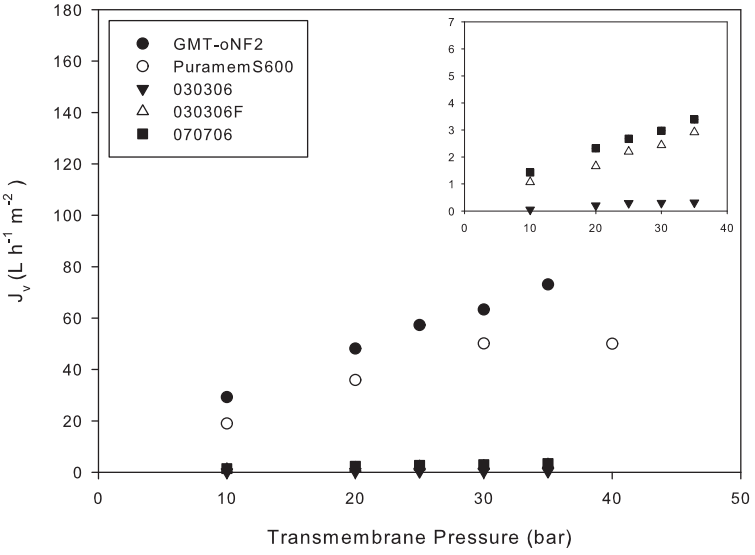
	$L_p{}^{20°C\pm3°C}$ ($L\,h^{-1}m^{-2}bar^{-1}$)	
Membrane	Hexane	Deodorizer Distillate in hexane (10% w/w)
GMT-oNF2	4.0	1.7
PuramemS600	2.8	1.6 [a]
030306	0.049	0.011
030306F	0.23	0.074
070706	0.16	0.077

[a] Determined using the linear range of the function of J_v vs Pressure - Figure 5.4(b)

As expected, the permeability of deodorizer distillate/hexane solutions was lower than the permeability with pure hexane, most likely due to the presence of foulant compounds in the deodorizer distillates (Table 5.3). GMT-oNF2 and PuramemS600 membranes showed the highest



(a)



(b)

Figure 5.4: Effect of pressure in the flux of membranes (a) in hexane and (b) in a solution of deodorizer distillate (10% w/w). See inset for membranes from Solsep

permeabilities, whereas those from Solsep were an order of magnitude lower.

All membranes presented a linear relationship between flux and applied transmembrane pressure, except for the PuramemS600 membrane at higher pressure (Figure 5.4(b)). The non-linearity phenomena was also observed previously by other authors [51, 108–110], where it was related to the effect of the compaction procedure in the effective thickness of the membrane. It was postulated that the increase of pressure makes the top layer penetrating into the supporting porous structure, creating a new sublayer that contributes additionally to the resistance of the membrane.

Figure 5.5 (see pag.92) compares the performance of each membrane in the separation of compounds with high molecular weight (triglycerides,885<MW<975 g/mol and steryl esters, 650<MW<800 g/mol) from compounds with a similar range of molecular weights as pesticides (sterols, 400<MW<415 g/mol and oleic acid, 282 g/mol).

The membrane 030306 was discarded (Figure 5.5(c)), given the low rejection of all compounds. Membrane GMT-oNF2 presented high rejection for steryl esters (94% at 35 bar), but a poor discrimination between compounds with high and low molecular weight. Membranes 030306F and 070706 had acceptable rejections of steryl esters (88 and 82%, respectively) and a good discrimination between target compounds. The membrane 070706 from Solsep presented negative rejection for compounds with low molecular weight, meaning that these compounds present a higher affinity with the membrane than the solvent itself. Experimental observations suggest that these phenomena is enhanced by the increasing of the transmembrane pressure. However, the low permeabilities obtained for the Solsep membranes (Table 5.3) may compromise their application for the purposes of this work. The membrane that presented the best compromise in terms of permeability and capacity for discrimination of target compounds was the membrane PuramemS600, with a permeability of $1.6\,L\,h^{-1}\,m^{-2}\,bar^{-1}$ and a high rejection of steryl esters of 91% at 35 bar. Taking into account the results obtained in a dead-end operating mode, we selected the membranes with higher rejection for steryl esters, namely, GMT-oNF2 from Borsig, PuramemS600 from Evonik and 030306F from Solsep, to proceed with studies in a cross-flow operating mode.

5.3.3 Impact of operating in a cross-flow mode in the membrane performance

The impact of operating in a cross-flow mode (instead of in a dead-end mode) is well known, being related with a decrease of concentration of polarisation, due to an improvement of fluid dynamic conditions in the feed compartment. The comparison of figures 5.5(d) and 5.6 illustrates this phenomena, when a hexane-based solution of deodorizer distillate (10% w/w) is processed in dead-end and cross-flow mode. Although discrimination between compounds with high and low molecular weight remains, the rejection of steryl esters increased from 91% (in dead-end mode) to 98% (in cross-flow mode) at 35 bar, which represents a significant improvement in the recovery of steryl esters.This study provided further evidence on the improvement of fluid dynamics by operating in a cross-flow mode. However, it should be noted that the variation of the linear velocity in the feed channel showed no significant effect on the rejection of target compounds (data not shown).

5.3.4 Optimisation of the concentration of the hexane-based solution

Hexane-based solutions with different concentrations of deodorizer distillate (5, 10 and 20% w/w) were processed. Using the membrane 030306F from Solsep as a reference, Figure 5.7 (see pag.93) shows that dilution of deodorizer distillate in hexane has a positive effect in the enhancement of membrane flux (see the corresponding permeabilities values in Table 5.4).

Concerning the rejection of target compounds, an improvement of discrimination between

Table 5.4: Permeability of solutions of deodorizer distillate in hexane with different concentrations (operating in a cross-flow mode), using membrane 030306F

Concentration of deodorizer distillates in hexane(% w/w)	$L_p^{20°C \pm 3°C}$ (L $h^{-1}m^{-2}bar^{-1}$)
0	0.25
5	0.064
10	0.033
20	0.016

compounds with high and low molecular weight was observed at low concentrations of deodorizer distillates (Figures 5.6 and 5.8, pag.93-94), which may be related with a decreasing of mass transfer resistance. Therefore, in the following studies a more diluted solution was used (5% w/w), although it should be highlighted that the increase of discrimination between the compounds present will be achieved at expenses of a higher volume to be processed, and therefore, at higher costs.

5.3.5 Effect of transmembrane pressure in the rejection of target compounds

Figure 5.9 (see pag.95) illustrates the flux as a function of the operating pressure for hexane and for a hexane-based solution of deodorizer distillate (5% w/w). As expected, a decrease of flux was observed for the deodorizer distillates solutions, similarly to the results obtained in a dead-end mode.

The permeabilities in hexane measured in dead-end (Table 5.3) and cross-flow (Table 5.5) were within the same order of magnitude, except for the membrane PuramemS600, whose permeability decreased significantly (from 2.8 to 0.33 $Lh^{-1}m^{-2}$, respectively). It should be noted that the membranes tested were from different lots, so that there is a possibility of some variability in the manufacturing procedure of these membranes. An interesting observation was that the typical trend of decreasing permeability when processing pure hexane and solution was reversed in this case.

Table 5.5: Permeability of selected membranes in cross-flow operating mode

	$L_p^{20°C \pm 3°C}$, L $h^{-1}m^{-2}bar^{-1}$	
Membrane	Hexane	Deodorizer Distillate in Hexane (5% w/w)
GMT-oNF2	5.0	4.0
PuramemS600	0.33	0.87 [a]
030306F	0.25	0.064

[a] Determined using the linear range of the function of J_v vs Pressure - Figure 5.9

Figure 5.10 (see pag.96) compares the performance of selected membranes based on their rejection to target compounds. All membranes showed high rejection of steryl esters (>96%) and the discrimination with pesticides was promoted at lower pressures. The membrane GMT-oNF2 had

the lowest discrimination between target compounds, despite being the membrane with the highest permeability. The membrane 030306F presented a very interesting low rejection of pesticides (mostly negative values, which indicates that pesticides permeate trough the membrane faster than hexane), however, the permeability was extremely low. The membrane PuramemS600 was the best balanced membrane in terms of permeability and discrimination between target compounds. An other interesting aspect was the difference between rejections of triglicerydes and free fatty acids at low pressures (>99% and <65%, respectively) observed in the membranes PuramemS600 and 030306F, which make them probably suitable for oil recovery and deacidification.

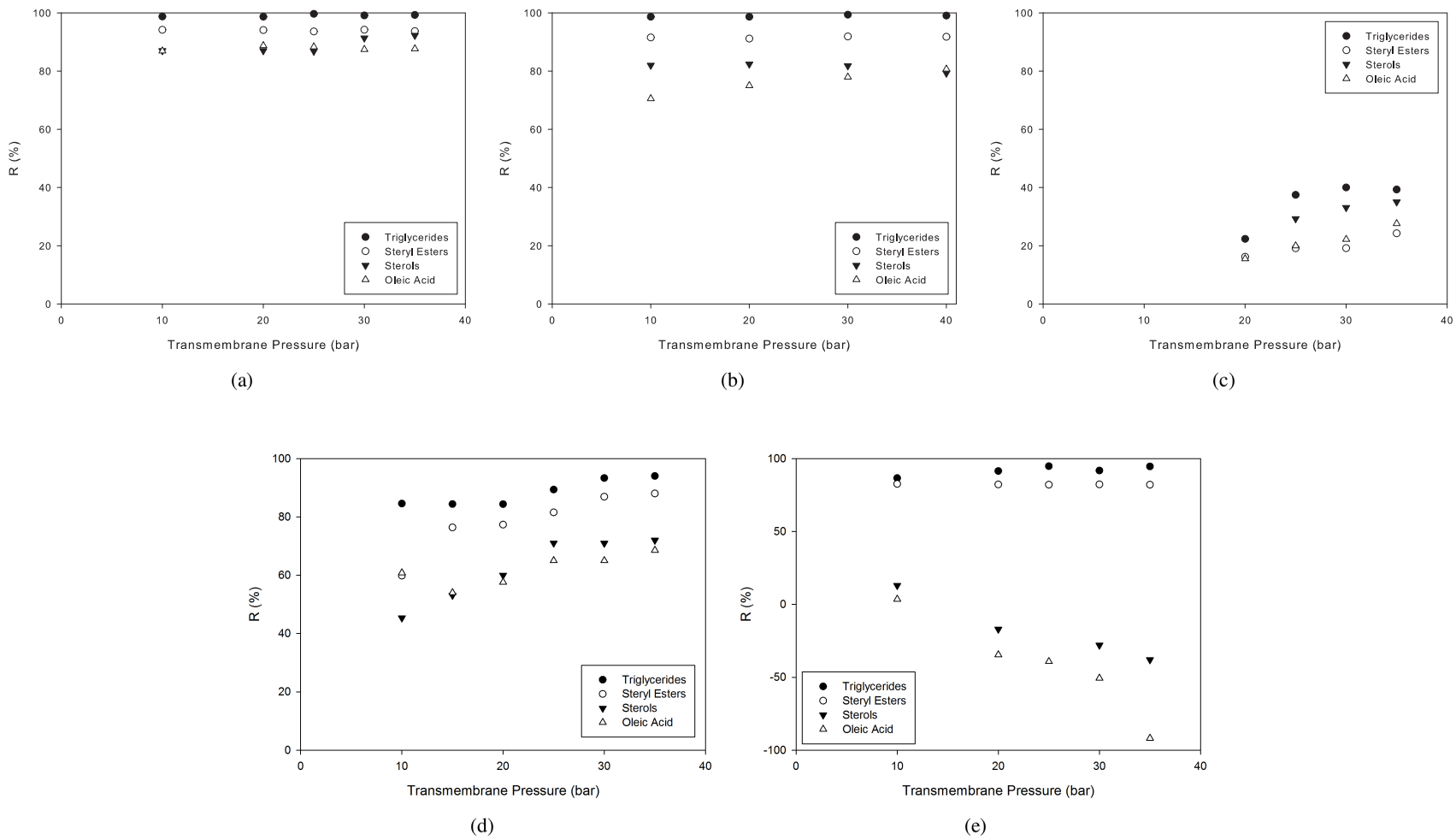


Figure 5.5: Effect of pressure in the rejection of triglycerides (●), steryl esters (○), sterols (△) and oleic acid (▼), using a hexane-based solution of deodorizer distillate (10% w/w) and operating in a dead-end mode. Membranes: (a) GMT-ONF2 (Borsig), (b) PuramemS600 (Evonik), (c) 030306 (Solsep), (d) 030306F (Solsep) and (e) 070706 (Solsep)

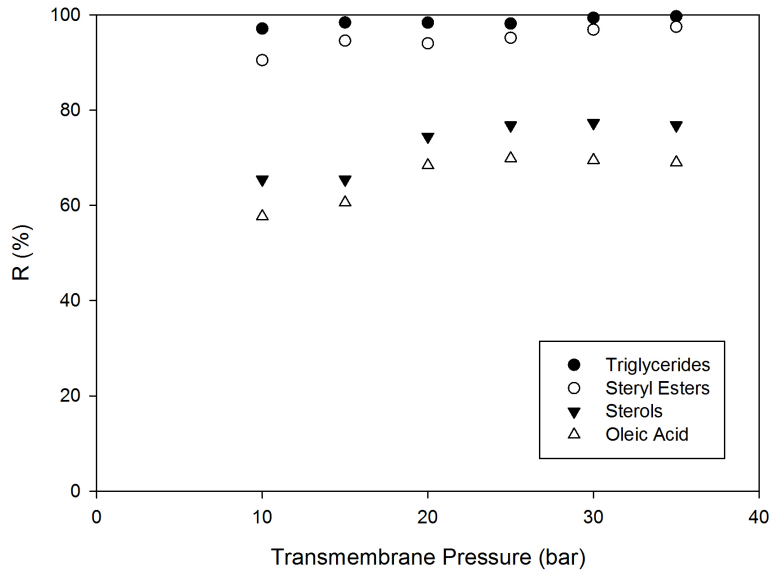


Figure 5.6: Curve of rejection of triglycerides (●), steryl esters (○), sterols (△) and oleic acid (▼) as a function of the pressure, using a hexane-based solution of deodorizer distillate (10% w/w) and operating in a cross-flow mode. Membrane: 030306F from Solsep

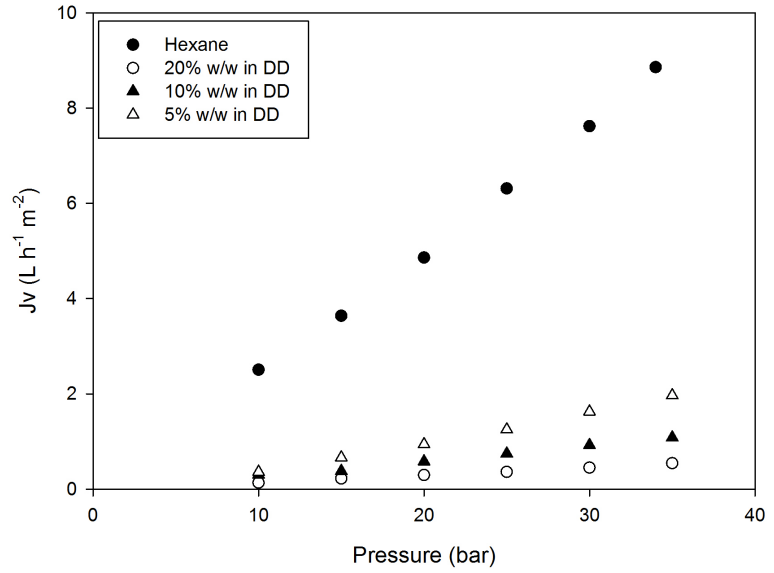
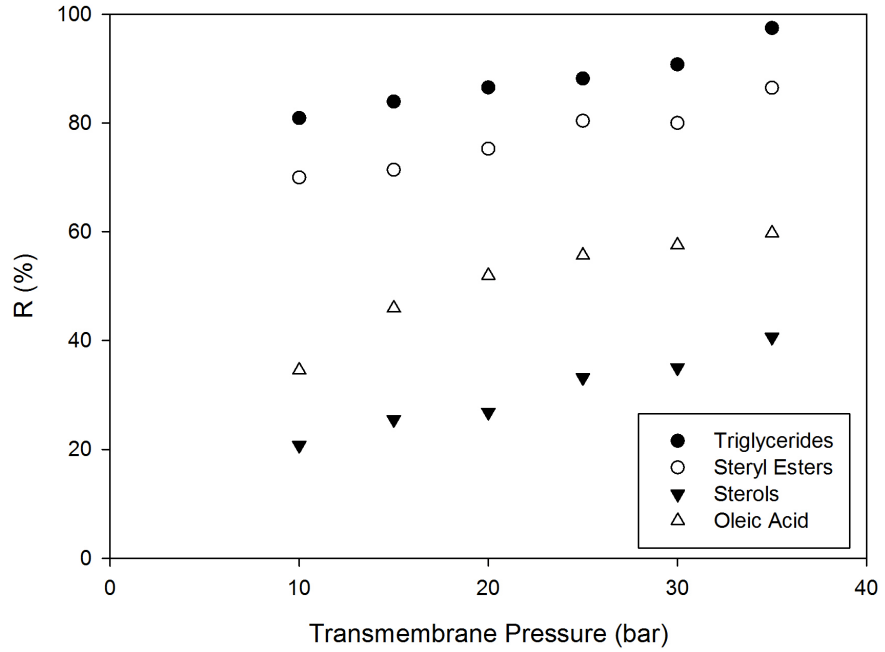
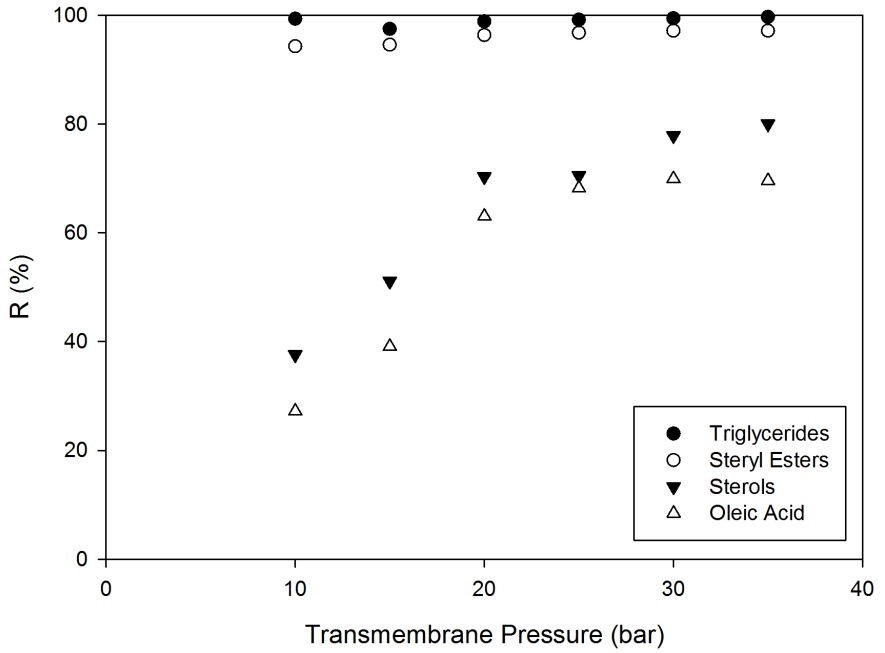


Figure 5.7: Effect of the concentration of deodorizer distillates in hexane on the flux (Jv) of the membrane 030306F from Solsep at different transmembrane pressures

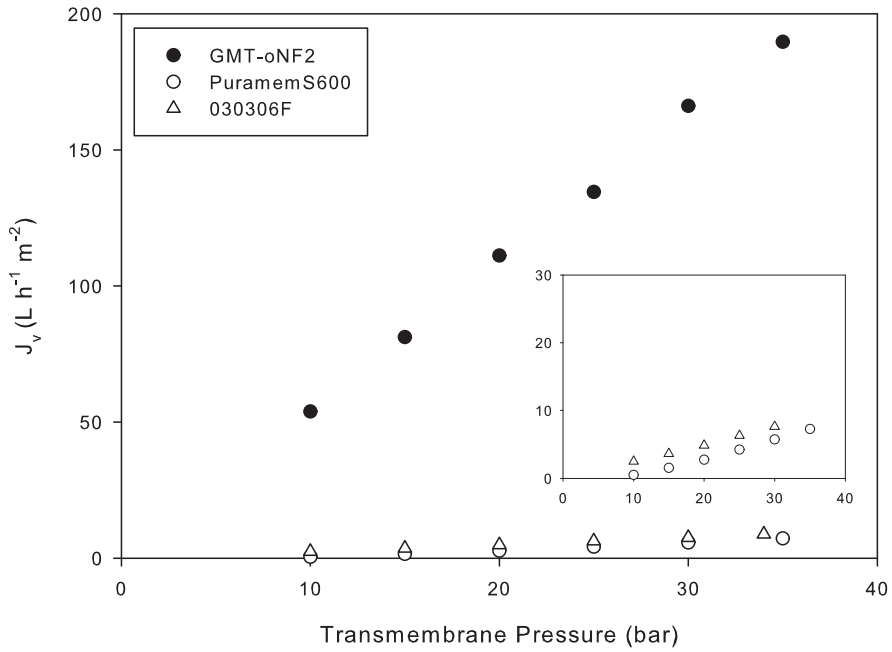


(a)

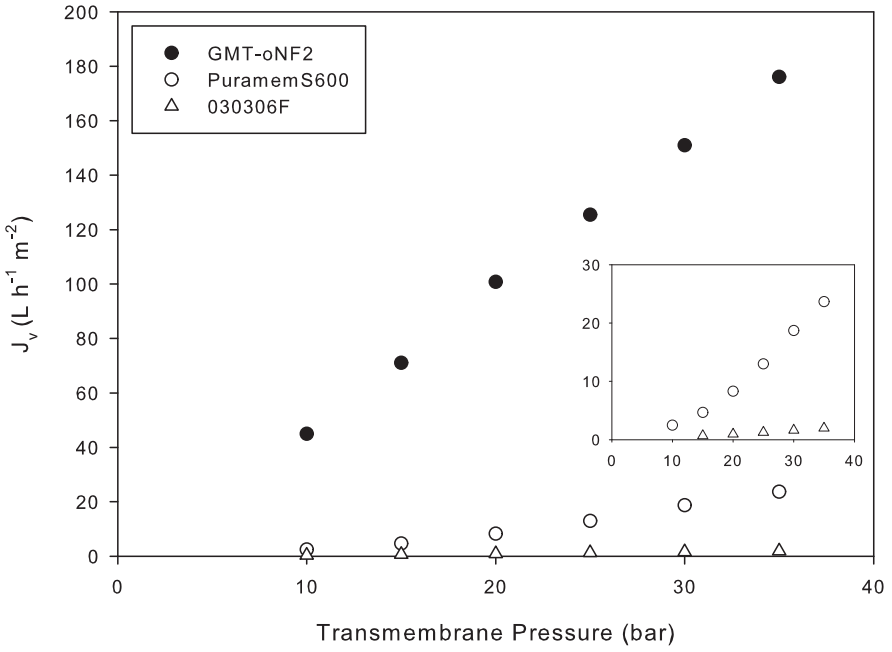


(b)

Figure 5.8: Curve of rejection of triglycerides (●), steryl esters (○), sterols (△) and oleic acid (▼) as a function of transmembrane pressure, using a hexane-based solution of deodorizer distillate with a concentration of: (a) 20% w/w and (b) 5% w/w. Membrane: 030306F from Solsep



(a)



(b)

Figure 5.9: Effect of pressure in the flux of membranes (a) in hexane and (b) in a solution of deodorizer distillate (5% w/w). See insets for membranes PuramemS600 and 030306F

Figure 5.10: Effect of pressure in the rejection of steryl esters (●) and pesticides (pirimiphos-methyl (○), chlorpyriphos-methyl (△) and chlorpyriphos (▼)), using a hexane-based solution of deodorizer distillate (5% w/w) and operating in a cross-flow mode. Membranes: (a) GMT-ONF2 (Borsig), (b) PuramemS600 (Evonik), (c) 030306F (Solsep)

5.4 CONCLUSIONS

This work shows that the use of solvent resistant nanofiltration (SRNF) membranes for the valorisation of deodorizer distillates is technically feasible. The success of this application depends on the stability of the membrane in suitable solvents, the permeability of the membrane for the solvent, and the discrimination of steryl esters (bioactive compounds, 650<MW<800 g/mol) from pesticides (150<MW<400 g/mol).

The role of solvent and membrane interactions was found to be important in the permeability of the membrane, since a strong relationship with the swelling/solvent viscosity ratio was observed. Hexane was selected among the potential solvents tested since it shows a minor impact in terms of swelling, leading to high permeabilities and a good discrimination between target compounds. The membranes GMT-oNF2 from Borsig/GMT, PurameS600 from Evonik and 030306F from Solsep were selected, in dead-end mode, based on the criteria of permeability and discrimination between target compounds. Their performance increased when operating in a cross-flow mode, being further improved after optimisation of the concentration of deodorizer distillate in hexane and the transmembrane pressure. As expected, the cross-flow operating mode showed improved rejection values, most likely due to an improvement of fluid dynamics and mass transfers. Furthermore, processing with a more dilute solution of deodorizer distillate in hexane allowed for a better discrimination of target compounds, although at expenses of a higher volume to be processed (for the same quantity of deodorizer distillate to be treated), and ultimately, at higher costs.

As a general conclusion, the selected membranes proved to be suitable for valorisation of deodorizer distillates, presenting high rejections of steryl esters (>96%) and significant low rejections of pesticides. The selection among these membranes depends on their permeability and discrimination of target compounds during diananofiltration processing, which will be directly reflected in the efficiency of the process, measured by the recovery of steryl esters.

6 Solvent Resistant Diananofiltration for Production of Steryl Esters Enriched Extracts

Summary *Deodorizer distillate is a by-product from edible oil refining rich in bioactive compounds. However, its use as food additive is not allowed due to the presence of pesticides in relatively high concentrations. This chapter discusses the technical feasibility of a solvent resistant membrane-based process for production of steryl esters-enriched extracts, using deodorizer distillates as raw material.*

A mass-balance based model was developed to predict the profile of species concentration during diananofiltration processing of a hexane-based solution containing 5% (w/w) of deodorizer distillate. This tool enabled the comparison of three commercial SRNF membranes in terms of their discrimination between pesticides and steryl esters. PuraMemS600 from Evonik was identified as the best membrane, showing the best compromise between membrane flux and rejection behaviour towards the compounds of interest. This membrane presented a constant rejection of steryl esters (95.5%) and a time-dependent flux, probably associated to swelling effects. Both the rejection and permeability data were used in the simulation of the diananofiltration process, making possible to obtain a good agreement of the model with the experimental data.

The diananofiltration technique investigated in this chapter showed to be suitable for an efficient removal of pesticides, however, at expense of a significant loss of steryl esters of ≈42%. An alternative configuration of two-stage diananofiltration was simulated, suggesting an improvement of the efficiency of the process.

The contents of this chapter were adapted from the publication: Teixeira, A. R. S., Santos, J. L. C., Crespo, J. G. (2014). Solvent Resistant Diananofiltration for Production of Steryl Esters Enriched Extracts. Journal of Membrane Science. Submitted.

6.1 INTRODUCTION

The production of large amounts of wastes by the agro-food industries represents an environmental burden [3]. Demanding legislation concerning waste management has been issued by the European Comission, resulting in a high encouragement to develop and achieve better and improved methods for recovery and purification of value-added compounds from by-products streams. Under this context, the recovery of bioactive compounds has a special interest given the increasing market of functional foods [5]. Deodorizer distillates, a residual stream produced in the vegetable oil refining process, present a high potential for the recovery of added value compounds. Their content in bioactive compounds such sterols, tocopherols and squalene may vary between 2-20% [17] although, their pesticide content is too high [22] to allow their use directly as additive in food, pharmaceutical and cosmetic products [111]. Maximum residue levels (MRLs) in crude and refined oils for human consumption are not specifically set but, according to Article 20 of EU Regulation No. 396/2005, they have to be derived from the MRLs established for seeds (0.05 ppm for main lipophilic pesticides) [24]. The concentration of pesticides in deodorizer distillates can be 800-1000 times higher than the actually allowed values [22, 23]. Therefore, their valorisation as a food additive rich in bioactive compounds depends from the effective removal of pesticides, which remains challenging.

Solvent resistant nanofiltration (SRNF) is a potential alternative technology for recovery of bioactive compounds, being remarkably simpler and less energetically intensive technology than traditional processes such as distillation and/or solvent extraction. Additionally, it enables the purification, fractionation and concentration of natural extracts, without requiring high process temperatures, which typically damage their valuable compounds. SRNF has been applied with relative success in the pharmaceutical industry, such as in the purification of an active pharmaceutical ingredient (API) [46], catalyst recycling [47], removal of genotoxins from APIs [48], continuous solvent exchange [49] and solvent recycling [50]. The use of SRNF in oil refining industry has been discussed, although, mainly from the point of view of solvent recycling and oil recovery [51–53]. The present work discusses a different perspective through the use of SRNF in the valorisation of by-products. Specifically, we propose a solvent resistant membrane-based process to produce an extract rich in steryl esters and free of pesticides, from deodorizer distillates.

The use of steryl esters as food additive has been intensified mainly after its recognition by the European Food Safety Authority (EFSA) as compounds with anti-cholesterol activity. The Scientific Panel decided that the maximum phytosterols intake should not exceed 3 g/day and the content of phytosterols in the ingredients cannot be higher than 30% [28, 112]. However, the regulation express the importance of toxicology analysis to the final product (including pesticides) to guaranty its safety.

Steryl esters can be produced by enzymatic reaction between free sterols and free fatty acids from deodorizer distillates. Their production is advantageous in terms of bioactivity and incorporation in fat-based products, since steryl esters are more bioactive than free sterols [33] and more soluble in the oil phase. Moreover, the molecular weight of steryl esters (650-800 $g\,mol^{-1}$) enables their separation from pesticides (150-400 $g\,mol^{-1}$), if the technology applied is based on

size exclusion. The optimisation of the reaction system as well as the subsequent removal and reuse of the enzyme were the scope of previous studies (Chapters 2, 3 and 4). The present chapter is focused on the purification of the steryl esters rich aiming the removal of pesticides.

SRNF is generally employed in a dianafiltration mode for removal of contaminants with low molecular weight (MW) from valuable products with higher molecular weight. This method consists in continuously feeding fresh solvent at the same rate as the permeate is recovered. In the particular case of this work, steryl esters are to be retained by the membrane while pesticides are to be washed-out in the permeate. One of the main challenges of applying dianafiltration to the present system is the identification of a membrane stable in contact with the processing medium and suitable to perform the required separation. In the previous chapter, the membranes GMT-oNF2 from Borsig/GMT (Germany), PuraMemS600 from Evonik (UK) and 030306F from Solsep (Netherlands) were identified as potential candidates, presenting high rejections for steryl esters (>96%) and relatively low rejections of pesticides in diluted hexane-based solutions (5% w/w). The main objective of this work is to select the most adequate membrane to fulfill the final purpose of removing pesticides from steryl esters-enriched deodorizer distillates and demonstrate that dianafiltration is a suitable technique to achieve that objective. This work should provide the basis for the scaling-up of this process, through demonstration of the concept of producing extracts rich in steryl esters, free of pesticides.

6.2 Modeling of Dianafiltration Process

Dianafiltration in a batch mode of operation at constant pressure and fixed feed volume can be described by a mass-balance for each species i in the system:

$$\frac{\mathrm{d}[V_f C_{f,i}]}{\mathrm{d}t} = -J_v A C_{p,i} \tag{6.1}$$

where V_f (L) is the volume of the feed solution, J_v ($\mathrm{L\,m^{-2}\,h^{-1}}$) is the permeate flux, A ($\mathrm{m^2}$) is the area of the membrane and $C_{f,i}$ and $C_{p,i}$ ($\mathrm{g\,L^{-1}}$) are the bulk concentrations of species i in the feed and permeate, respectively.

The observed rejection (R_{obs}) is defined as:

$$R_{obs} = 1 - \frac{C_{p,i}}{C_{f,i}} \tag{6.2}$$

replacing Eq.(6.2) into Eq.(6.1) yields:

$$\frac{\mathrm{d}[C_{f,i}]}{\mathrm{d}t} = -\frac{J_v A}{V_f} C_{f,i}(1 - R_{obs}) = -\frac{V_D}{t} C_{f,i}(1 - R_{obs}) \tag{6.3}$$

where V_D represents the number of dianafiltration volumes defined as the total volume of permeate collected divided by initial system volume. This dimensionless parameter allows different dianafiltration systems to be compared.

Two parameters were defined for quantifying the efficiency of the system for pesticide (P)

removal (Eq.6.4) and steryl esters (SE) loss (Eq.6.5), defined as:

$$Removal_{P_{(t=i)}} = \frac{M_{f_{P(t=i)}}}{M_{f_{P(t=0)}}} \times 100 \tag{6.4}$$

$$Loss_{SE_{(t=i)}} = \frac{M_{p_{SE(t=i)}}}{M_{f_{SE(t=0)}}} \times 100 \tag{6.5}$$

where M_{pi} and M_{fi} are the mass of species i in the permeate and feed tanks, respectively.

6.3 Materials and Methods

6.3.1 Material

6.3.1.1 Esterified Deodorizer Distillate

The sunflower deodorizer distillate from Lesieur (France) used in this work was characterized in our laboratory and its composition was presented in the Table 2.1 (Deodorizer distillate "3"). Sterols and free fatty acids (FFA) were esterified to produce steryl esters, using a lipase from *Candida Rugosa*. The enzymatic reaction was performed under optimised conditions, that were established in the Chapter 2.

The esterified deodorizer distillate used in this study is rich in acylglycerides (35%) and free fatty acids (29.8%). It also contains valuable compounds, such as sterols (0.27%), steryl esters (6.6%), tocopherols (1.7%) and squalene (1.1%). Other compounds comprise hydrocarbons, aldehydes, ketones, pesticides, herbicides and oxidized products from breakdown of tocopherols and free phytosterols. The pesticides pirimiphos-methyl, chlorpyriphos-methyl and chlorpyriphos were present at high concentration (16.4, 10.5 and 0.27 mg/kg, respectively), as well as oxidized products and aldehydes, given by the peroxide number and the p-anisidine value (15.9 mEq O_2/kg and 1056, respectively).

6.3.1.2 Chemicals

Analytical-grade hexane, ethanol, isopropanol, isooctane, acetic acid glacial and potassium iodine were supplied from VWR (Germany). Technical grade chloroform (99%) for GC analysis and food-grade oleic acid with an acid value of 196.0-204 mg KOH/g were purchased from Sigma Aldrich (Belgium).

The derivatizating and silylation agent, N,O-bis (trimethylsilyl) trifluracetamide (BSTFA) containing 1% of trimethylchlorosilane (TCMS) solution (from Fluka) and pyridine (from Sigma-Aldrich) were both obtained from Sigma (France).

All analytical-grade standard substances, squalene (99.3% purity), stigmasterol (97% purity), β-sitosterol (99%purity), campesterol (99% purity), cholesteryl stearate (96% purity), monoglyceride olein (>99%purity), diglyceride olein (99.7% purity) and triglyceride olein (99.6% purity) were purchased from Sigma (Saint Quentin, France). A tocopherol kit consisting of α-, β-, γ- and δ-tocopherols was obtained from Merck (>95% purity).

The internal standard heptadecanyl stearate (HDS) was prepared by condensation of heptadecanol and stearoyl chloride, both obtained from Aldrich (Belgium), as described by Verleyen et al. [65].

6.3.1.3 Membranes

Three PDMS-based commercial SRNF membranes from different manufactures were studied in this work, namely 030306F from Solsep (Netherlands), PuraMem600 from Evonik (U.K.) and GMT-oNF2 from Borsig/GMT Membrantechnik (Germany). These membranes were characterized according to their permeability and retention of pesticides and steryl esters in the previous chapter (Table 5.5 and Figure 5.10). Table 6.1 compiles the most relevant characterisation data for the membranes used in this work.

Table 6.1: Characterisation of the selected membranes (results from Chapter5)

		Permeability ($L\,h^{-1}\,m^{-2}\,bar^{-1}$)		Pressure	Rejection (%)	
Membrane	Manufacturer	Hexane	Solution [a]	(bar)	Steryl Esters	Pirimiphos-methyl [b]
GMT-oNF2	Borsig/GMT	5.0	4.0	35	96.7	73.3
				20	95.5	66.6
				10	94.0	61.5
PuraMemS600	Evonik	0.33	0.87	35	98.3	74.3
				20	97.1	58.2
				10	96.8	46.6
030306F	Solsep	0.25	0.064	35	97.1	57.9
				20	96.3	19.5
				10	94.2	-5.5

[a] solution of deodorizer distillate (5% w/w) in hexane
[b] main pesticide in the deodorizer distillate used in this study

6.3.2 Analytical Methods

6.3.2.1 Analysis of Fatty Acids, Acylglycerides, Tocopherols, Sterols and Steryl Esters

The procedure to analyze fatty acids, acylglycerides, tocopherols, sterols and steryl esters was described in detail in the subsection 2.3.2 (Chapter 2).

6.3.2.2 Analysis of pesticides

Pesticides were quantified by a certified laboratory (Labiagro, Oeiras (Portugal)) according to NP EN 1528-1/2/3/4:2002. The analysis was performed by a gas-chromatography coupled to a mass spectrophotometer (GC-MS). The results were expressed as mg/kg.

6.3.2.3 Determination of peroxide number

The peroxide value (*PV*) is based on the measurement of iodine liberated from the reaction of KI with peroxides in the sample. The PV was determined by titration according to the standard ISO 3960 (2001). The method was developed and implemented in a titration workstation TitraLab 856 from Radiometer (Denmark). The result was expressed as mEq O_2/kg.

6.3.2.4 Determination of p-anisidine value

The determination of p-anisidine value was carried out following an IUPAC standard method (1987). This method determines the amount of aldehydes (principally 2-alkenals) in oils and fats, which reacting with p-Anisidine during 10 minutes, create a colored complex possible to be measured on a spectrophotometer at 350 nm (Thermo Spectronic, USA). The p-anisidine value is defined as 100 times the absorbance at 350 nm of a solution containing 1 g of the oil in 100 mL of a mixture of solvent (isooctane) and p-anisidine (2.5 g/l in acetic acid).

6.3.3 Experimental Procedures Methods

6.3.3.1 Setup

Diananofiltration experiments were conducted in a cross-flow cell (Sepa CF II (GE, USA)) with a flat sheet membrane with 140 cm^2 of effective area. This cell is claimed to being able to mimic flow dynamics of commercially available spiral membrane elements, by using a combination of stainless steel shim, feed spacers and permeate carriers. A diamond-type spacer and a steel shim were combined to obtain a channel feed height of 1.55 mm. The feed flow was measured using a calibrated rotameter (SK72, Georg Fischer, Switzerland) and set by a diaphragm pump (Hydracell G-13, Wanner Engineering, USA). The pressure applied to the system was adjusted with a valve assembly and monitored with two transmitters (8864, Trafag, Switzerland) placed in the inlet ant outlet of the cell. The temperature of the system was indicated by the same transmitters and controlled by a heat exchanger (using tap water as cooling fluid) placed after the back pressure regulator, in the retentate stream. The permeate flow was periodically monitored by acquisition of its weight, using an electronic balance with an accuracy of 0.1 g and, afterwards, recirculated to the permeate tank. The feed volume was adjusted (to maintain it constant at 1 L), adding continuously fresh hexane by means of a peristaltic pump (Watson-Marlow, 120S-DV). The tanks of feed, permeate and fresh hexane were hermetically closed, with sampling ports and condensers to mitigate solvent evaporation. Figure 6.1 illustrates the implemented process of diananofiltration.

6.3.3.2 Membrane Selection

The membranes listed in Table 6.1 were identified as potential candidates for the separation of steryl esters from pesticides according to the study performed in the previous chapter. Data of permeability in a solution of deodorizer distillate (5% w/w) in hexane and rejections of compounds

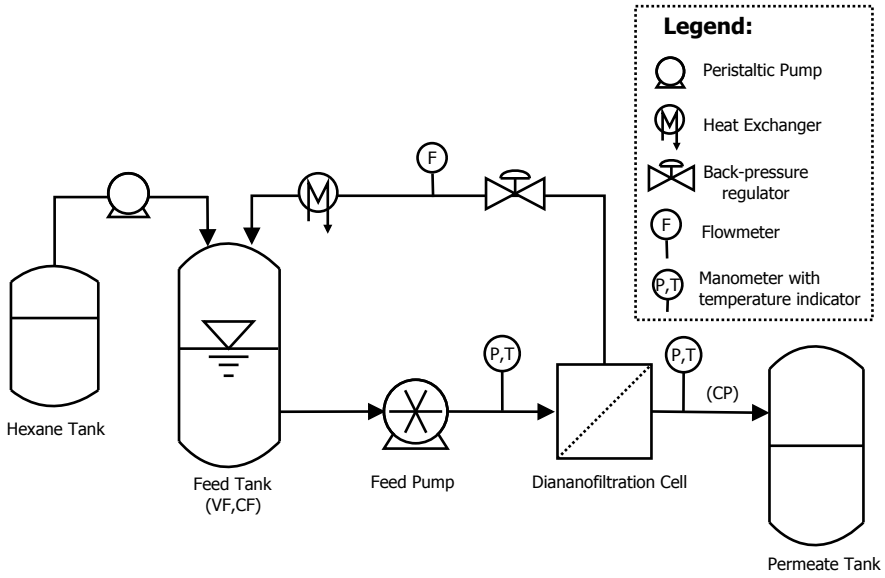


Figure 6.1: Schematic diagram of the process of diananofiltration. The symbols in parenthesis are the variables used in the process modeling (VF - feed volume, CF - feed concentration of the compound *i*, CP - permeate concentration of the compound *i*)

at different pressures were taken into account to predict the processing time and the number of diananofiltration volumes needed to remove 99% of pesticides from deodorizer distillates (assuming a membrane area of 140 m^2) as well as the loss of steryl esters (Eq.6.5) associated to the process. Membranes were compared according to the purification and recovery criteria.

6.3.3.3 Influence of the membrane exposure time to a hexane-based solution of deodorizer distillate

A solution of deodorizer distillate (5%w/w) in hexane was continuously recirculated, with total recirculation of the permeate and the retentate to the feed tank. The pressure was set at 20 bar and the flow rate of the permeate was periodically measured. In order to correlate the variation of the permeate flux with membrane swelling, thick pieces of the membrane were used and the procedure described elsewhere [104] followed. Pre-weighed pieces of dry membrane were immersed in the same solution and, at different time intervals, they were removed, the liquid excess wiped and weighted again. After reaching the equilibrium swelling (no further weight increase), the difference between the initial and final weight (M_{dry} and M_{wet}, respectively) was determined. The swelling degree (*SD*) of the dense PDMS membrane was calculated by:

$$SD(\%) = \frac{M_{wet} - M_{dry}}{M_{dry}} \times 100 \tag{6.6}$$

6.3.3.4 Determination of rejection of compounds in total recirculation mode

A solution of deodorizer distillate in hexane (5%w/w) was processed, with total recirculation of the permeate and the retentate for the feed tank. The pressure was set at 20 bar and samples of feed and permeate were periodically collected during 8 hours to determine the evolving of the rejection of compounds.

6.3.3.5 Dianano filtration Procedure

The membrane selected was compacted with hexane, at 40 bar, until attaining a constant flux. Then, the flux of the membrane was measured at different pressures to determine its permeability in hexane. Afterwards, the feed tank was filled with a hexane-based solution of deodorizer distillate (5% w/w) and the pressure was set at an optimal value (that would be defined in the course of the work). The solution circulated at 350 L/h and the permeate was collected in the corresponding tank. Samples of both permeate and retentate were periodically taken for GC analysis to assess the performance of the membrane.

6.3.4 Numerical Methods

6.3.4.1 Modeling of the Dianano filtration Process

A system of differential ordinary equations is obtained by applying the Eq. 6.3 to each compound present in the deodorizer distillate. This system was implemented and solved on MatLab 7.9 (MathWorks, USA), assuming as constant the volume of the feed (V_f=1 L), the flux of the membrane and the rejection of target compounds (summarised in the Table 6.1). This first approach allowed to compare the predicted performance of each membrane in the removal of pesticides and loss of steryl esters, operating in dianano filtration mode. After selecting the membrane and observing a variation of the flux as a result of the contact of a new membrane with the medium, the same system was solved taking into account the flux as a function of the processing time and assuming the average value of the rejection observed during the processing a solution of deodorizer distillate (5%w/w) in hexane for eight hours, in total recirculation mode. In all simulations was considered a feed volume of 1 L.

6.4 RESULTS AND DISCUSSION

6.4.1 Selection of membrane for dianano filtration processing

Following guidelines of the European Food Safety Authority (EFSA), the concentration of pesticides in edible oil for human consumption should be lower than 0.05 ppm for the main lipophilic pesticides (the same value established for seeds) [24]. Therefore, this is the maximum concentration of pesticides that can be detected in the enriched-food product after adding the extract rich in steryl esters. Furthermore, the intake of phytosterols should not exceed 3 g/day, being 30% (w/w) the maximum phytosterols content in the extract. Since fruits and vegetables daily consumed provide already about 400 mg of phytosterols [113–115] and the oil itself contains phytosterols (see

Table 6.2: Typical content of phytosterols in the main oils, amount of extract to add, dilution factor (DF) of the extract in the final product, and removal of pesticides ($Removal_P$) necessary in order to assure a concentration of pesticides below 0.05 ppm

Oil	Phytosterols (mg/100g) [a]	Extract to add (%) [b]	DF [c]	Removal$_P$ (%) [d]
Rapeseed	735	28.8	4.5	99.2
Corn	733	28.8	4.5	99.2
Corn margarine	442	30.3	4.3	99.2
Canola	649	29.3	4.4	99.2
Sunflower	302	31.0	4.2	99.2
Soybean	239	31.3	4.2	99.2
Soybean margarine	164	31.7	4.2	99.2
Olive	156	31.7	4.2	99.2

[a] Sum of free and esterified phytosterols typically presented in oils, according to Phillips et al. [116]

[b] Amount of extract needed to add (subtracting the phytosterols provided by the daily consumption of fruits and vegetables (400 mg) as well as the typical content of phytosterols in oils to the recommended daily intake (3 g/day) and assuming that the phytosterols content of the extract produced by diananofiltration is 20% w/w and that the daily consumption of the enriched-oils is 40 g (equivalent to four tablespoons))

[c] Dilution Factor defined as the final volume after extract addition divided by the added extract volume

[d] Removal of pesticides (Removal$_P$) needed based on the initial content of pesticides in the deodorizer distillate (27.2 ppm) and knowing that the concentration of pesticides in the phytosterol-containing food products must be lower than 0.05 ppm (value established for seeds)

the typical content in Table 6.2), the addition of extract to edible oil is limited.

Table 6.2 shows the typical content of phytosterols in several oils [116], the amount of extract to add in order to fulfill the recommended daily intake of phytosterols, the dilution factor (DF) of the extract in the enriched-food product, and the removal of pesticides ($Removal_P$) necessary to assure a concentration of pesticides below 0.05 ppm. In order to obtain such values, it was assumed that the phytosterols content in the extract produced by diananofiltration is 20% w/w (below 30% w/w, the maximum allowed) and that the daily consumption of the enriched-oils is in average 40 g (equivalent to four tablespoons). As it can be observed, 99.2% of pesticides present in the steryl esters enriched-extracts (produced by diananofiltration) must be removed in order to assure a concentration of pesticides below 0.05 ppm in the enriched-food product. The final concentration of steryl esters in the enriched-oils, after addition of the extract, would be around 6.5% (w/w).

The use of the mathematical model in Eq. 6.3 along with the specific rejection and permeability data for each membrane in Table 6.2 enabled the determination of the concentration of species during diananofiltration (see procedure description and assumptions in the subsection of Numerical Methods). The modeling strategy was to solve the equation for a 99.2% pesticides removal, the loss of steryl esters, the processing time and the amount of fresh hexane necessary to be added to the feed tank (expressed as a number of volumes of diananofiltration). Table 6.3 shows the simulated results for a removal of 99.2% of pesticides from deodorizer distillates, assuming that a Sepa CF II membrane system with 140 cm^2 membrane area is used.

These simulations show that membrane GMT-oNF2 presents a relatively high loss of steryl esters (between 42 and 50%) for removing 99.2% of the pesticides from deodorizer distillates.

Table 6.3: Simulation results for the selection of the membrane at different operating pressures (see procedure description and assumptions in the subsection of Numerical Methods). Determination of the processing time and the number of diananofiltration volumes (V_D) needed to remove 99% of pesticides, as well the loss of steryl esters ($Loss_{SE}$) associated to the process

Membrane	Pressure (bar)	V_D [a]	$Loss_{SE}$(%) [b]	Time of operation (days) [c]
GMT-oNF2	35	16.6	42.3	0.35
	20	13.4	45.4	0.50
	10	11.5	49.8	0.85
PuraMemS600	35	17.3	25.3	1.7
	20	10.7	26.7	1.8
	10	8.4	23.6	2.9
030306F	35	10.5	26.3	14.0
	20	5.7	19.0	13.3
	10	4.4	22.4	20.5

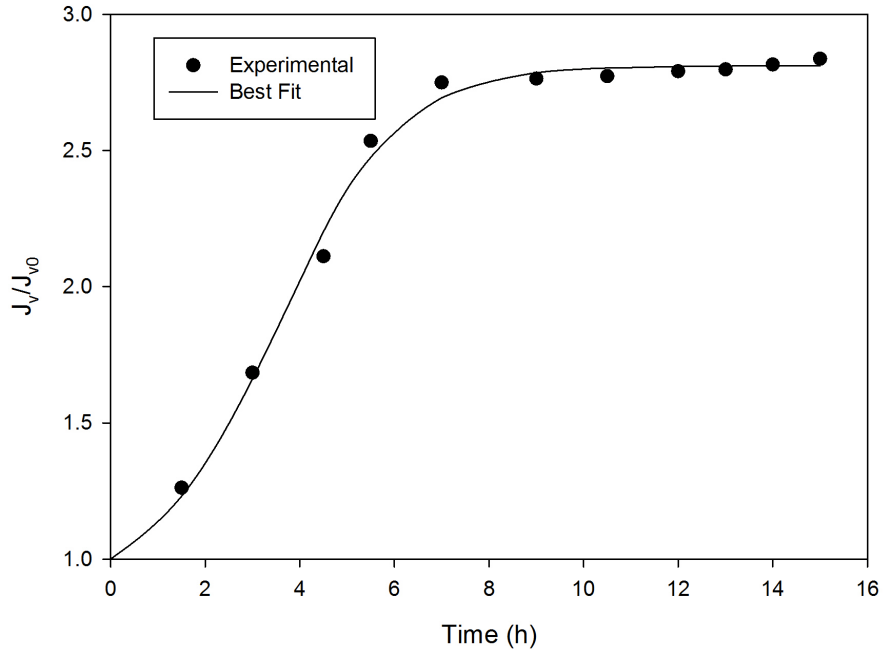
[a] Defined as the total volume of permeate collected divided by initial system volume
[b] Defined by the Eq. 6.5
[c] Assuming a membrane effective area of 140 cm^2

The loss observed in the two other membranes was significantly lower (between 19 and 27%). Comparing these membranes, membrane 030306F is the one that requires the lowest amount of fresh hexane (reflected in a lower number of V_D), presents the lowest loss of the steryl esters, but the processing time is considerably higher than for PuraMemS600, given its very low permeability (0.064L h^{-1} m^{-2} bar^{-1}, Table 6.1). The PuraMemS600 membrane is the one that shows the best compromise in terms of processing time and the number of diananofiltration volumes to fulfill the criterion of pesticide removal. Therefore, the PuraMemS600 membrane was selected to be used in the subsequent diananofiltration experiments. The pressure was set at 20 bar, which shows the best compromise in terms of processing time, hexane consumption and loss of steryl esters (26.7%).

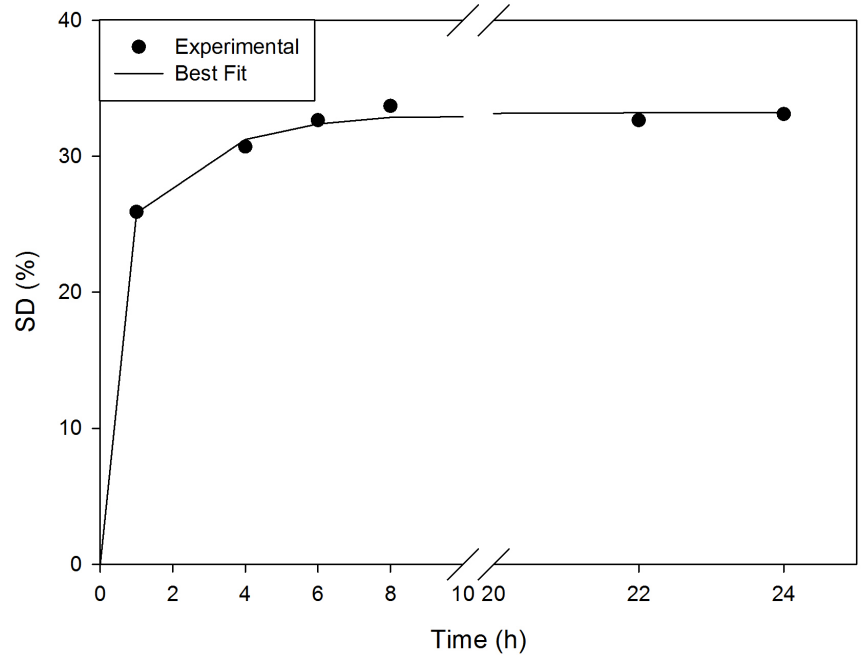
6.4.2 Influence of the membrane exposure time to a hexane-based solution of deodorizer distillate

Figure 6.2(a) shows that during processing of a solution of deodorizer distillate (5% w/w) in hexane at 20 bar, the flux of the PuraMemS600 membrane increases in the form of a sigmoidal curve, reaching a plateau after ≈ 8 hours.

A similar curve was obtained through the representation of the evolvement of the membrane swelling was exposed to the same solution (Figure 6.2(b)). These curves were obtained in independent essays, and the similarity of both curves suggests an underlying relationship of increasing flux with swelling. Furthermore, the effect of swelling was observed to be irreversible after the membrane was contacted with the solution for 16 hours since, after this contact, the permeability of the membrane in pure hexane increased 20% in relation to its initial value (from 0.27 to 0.33 L h^{-1} m^{-2} bar^{-1}). Therefore, in the simulation of the diananofiltration process, Eq. 6.3 must



(a)



(b)

Figure 6.2: (a) Increase of the flux of the membrane PuraMemS600 (measured as J_v/J_{v0}) during the processing of a hexane-based solution of deodorizer distillate (5% w/w) at 20 bar and 20±3° C, with a total recirculation of the permeate and retentate to the feed tank (b) Swelling of the membrane PuraMemS600 (measured as swelling degree, SD, Eq.6.6)

be solved taking into account the curve represented Figure 6.2(a), whereby the flux (J_v) is a function of the exposure time of the membrane to a hexane-based solution of deodorizer distillate (5% w/w), which is given by following equation:

$$J_v(t) = a + \frac{b}{1 + e^{\frac{-(t-c)}{d}}} \tag{6.7}$$

where a=0.916, b=1.90, c=3.55 and d=1.27. It should be noted that the use of the Eq.6.7 assumes the use of a new membrane for t=0.

6.4.3 Determination of rejection of compounds in total recirculation mode

The degree of swelling due to the contact between the membrane and an organic solvent was reported to induce changes in the membrane free volume and, under such conditions, its rejection behaviour is expected to change [60, 61, 117]. The monitoring of rejection of compounds during eight hours, in total recirculation mode, showed that it remains constant, with no significant variations being observed (Table 6.4). These results suggest that, even if swelling of the membrane and change in the free volume take place, these were not sufficient to affect significantly the rejection of the solutes under study. Constant rejection values are, therefore, a reasonable assumption for dianafiltration modeling purposes.

Table 6.4: Average rejection observed (R_{obs}) of compounds, at 20 bar and 20±3° C. Membrane:PuraMemS600 from Evonik

	Compound	R_{obs} (%)
acylglycerides	G	73.0±10.7
	MG	70.7±3.7
	DG	91.5±1.3
	TG	99.4±0.2
Tocopherols	α-Tocopherol	88.0±1.4
	δ-Tocopherol	90.1±1.4
	β/γ-Tocopherol	85.4±4.5
Squalene	Squalene	83.4±2.6
Sterols	Campesterol	78.4±2.4
	Stigmasterol	75.3±3.3
	β-Sitosterol	78.6±2.6
Steryl Esters	Steryl Esters	95.5±1.2
Fatty Acids	Oleic Acid	79.0±2.2
Pesticides	Pirimiphos-methyl	58.2 [a]
	Chlorpyriphos-methyl	31.8[a]
	Chlorpyriphos	45.6[a]

[a] Only the last sample was analysed

6.4.4 Dianafiltration

6.4.4.1 Flux evolvement during processing

Figure 6.3 shows that flux increases during the processing of a hexane-based solution of deodorizer distillate (5% w/w), in a dianafiltration mode. This increase of flux during dianafiltration may be explained by the swelling of the membrane (supported by Figure 6.2), as well as by a decrease of feed viscosity associated to the permeation of compounds present in deodorizer distillate and the permanent addition of hexane (kinematic viscosity of esterified-deodorizer distillate=76.8×10^{-6} m^2 s^{-1} ; kinematic viscosity of hexane= 0.49×10^{-6} m^2 s-1).

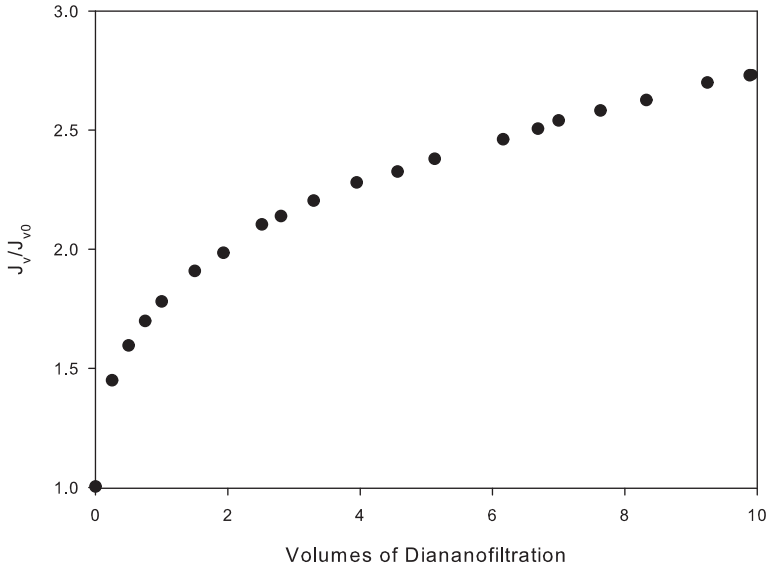


Figure 6.3: Increase of the flux of the membrane PuraMemS600 (measured as J_v/J_{v0}) during dianafiltration of a hexane-based solution of deodorizer distillate (5% w/w), at 20 bar and 20±3° C

The effect of swelling was shown to be an irreversible phenomenon, changing the initial permeation characteristics of the membrane. In fact, after dianafiltration, the permeability of the membrane in pure hexane increased 91% in comparison with its initial value (from 0.33 to 0.63 L h^{-1} m^{-2} bar^{-1}), suggesting that a long exposure of the membrane to a solution of deodorizer distillates affects it irreversibly.

6.4.4.2 Evolvement of mass of compounds present in the deodorizer distillate

In the simulation of dianafiltration was considered the initial concentrations of the different compounds and rejections presented in Tables 6.5 and 6.4, respectively. It was assumed a fixed feed volume of 1 L and a variation of the flux given by Eq. 6.7. Accordingly to the simulation under these conditions, 10.2 volumes of dianafiltration would be needed to remove 99.2% of pesticides, resulting in a loss of steryl esters of 37%.

Figure 6.4 shows that the simulation results of mass of compounds present in the deodorizer

distillates, during diananofiltration, are in good agreement with the experimental data. Therefore, the assumptions considered proved to be adequate to describe the physical system.

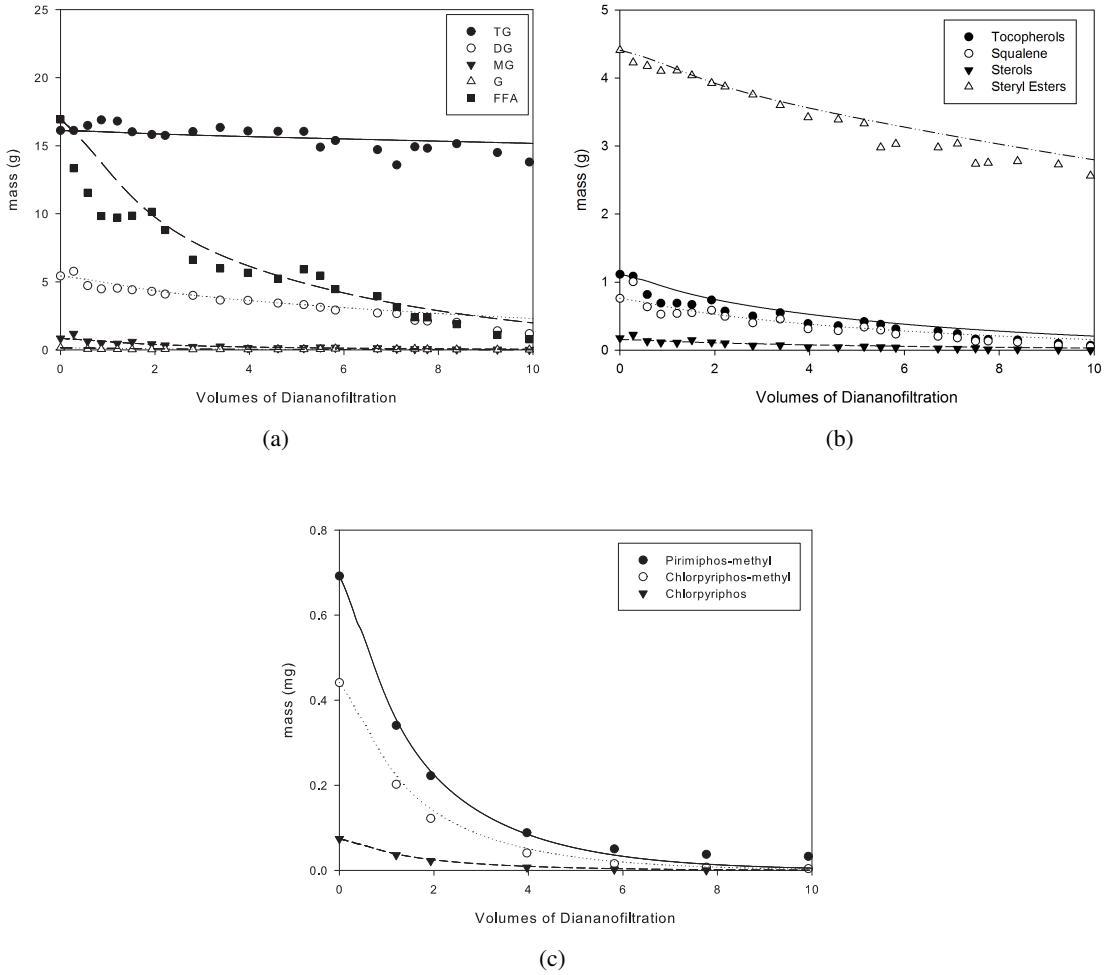


Figure 6.4: Evolvement of mass of (a) acylglycerides (TG- Triglycerides, DG- Diglycerides, MG- Monoglycerides, G- Glycerol and FFA - Free Fatty Acids) (b) bioactive compounds (Tocopherols, Squalene, Sterols and Steryl Esters), (c) pesticides (Pirimiphos-methyl, Chlorpyriphos-methyl and Chlorpyriphos) during diananofiltration processing. The points represents the experimental data and the lines the simulated data

The extent of loss of a compound depends on its rejection and increases with the number of volumes of diananofiltration (if not totally retained by the membrane). The high rejection of the triglycerides by the membrane (99.4%, Table 6.4) enabled to recover 94% of the triglycerides present initially in the deodorizer distillates, while the tocopherols, squalene and sterols (bioactive compounds) presented a significant loss (93.6% in average) as a result of their lower rejection (see Table 6.4). Steryl esters presented the lowest loss within the bioactive compounds (42%) due to their higher molecular weight, also indicated by in their higher rejection. This fact reinforces the need for the strategy used in this work in performing a previous step of esterification of the deodorizer distillate to produce steryl esters.

The composition of the esterified deodorizer distillate before and after diananofiltration is summarised in Table 6.5. The extract produced is rich in steryl esters (21.1%) and, like the edible oil, the triglycerides are the major constituent (53.9%). Contaminants, such as pesticides and aldehydes, were significantly reduced (99.2% and 92%, respectively) as well as promoters of oxidation as peroxides (52%) and free fatty acids (75%).

Table 6.5: Composition of the esterified deodorizer distillate before and after diananofiltration

			Diananofiltration	
	Units	Compound	Initial	Final [a]
acylglycerides	g/100g	G	1.1	0.08
		MG	1.3	0.1
		DG	8.2	9.8
		TG	24.2	53.9
Tocopherols	g/100g	α-Tocopherol	1.3	0.31
		δ-Tocopherol	0.34	0.26
		β/γ-Tocopherol	0.07	N.D. [b]
Squalene	g/100g	Squalene	1.1	0.4
Sterols	g/100g	Campesterol	0.02	N.D.
		Stigmasterol	0.01	N.D.
		β-Sitosterol	0.2	N.D.
Steryl Esters	g/100g	Steryl Esters	6.6	21.1
Fatty Acids	g as oleic acid/100g		23.8	6.0
Pesticides	mg/kg	Pirimiphos-methyl	16.4	0.4
		Chlorpyriphos-methyl	10.5	0.02
		Chlorpyriphos	0.2	< 0.01 [c]
Aldehydes	p-anisidine Value		1056.0	93.1
Peroxides	mEq O_2/kg		15.9	7.6

[a] After 10 volumes of diananofiltration
[b] N.D. - Not Detected
[c] Limit of Detection (LOD)

The value of pesticides removal was set considering that the extract produced in diananofiltration contained 20% (w/w) in steryl esters. The content obtained is very similar to the value assumed (21.1%) as well as the removal of pesticides needed (99.2%) to assure a concentration of pesticides in the final enriched-food product (after addition of the produced extract) below than 0.05 ppm.

6.4.4.3 *Efficiency of the process*

The efficiency of the process was assessed in this work using two parameters: the removal of pesticides (Removal_P, Eq. 6.4) and the loss of steryl esters (Loss_{SE}, Eq. 6.5). Figure 6.5 shows the impact of pesticides removal in the loss of steryl esters. As can be observed, a removal of

99.2% of pesticides was achieved at expense of a significant loss of steryl esters ≈ 42%.

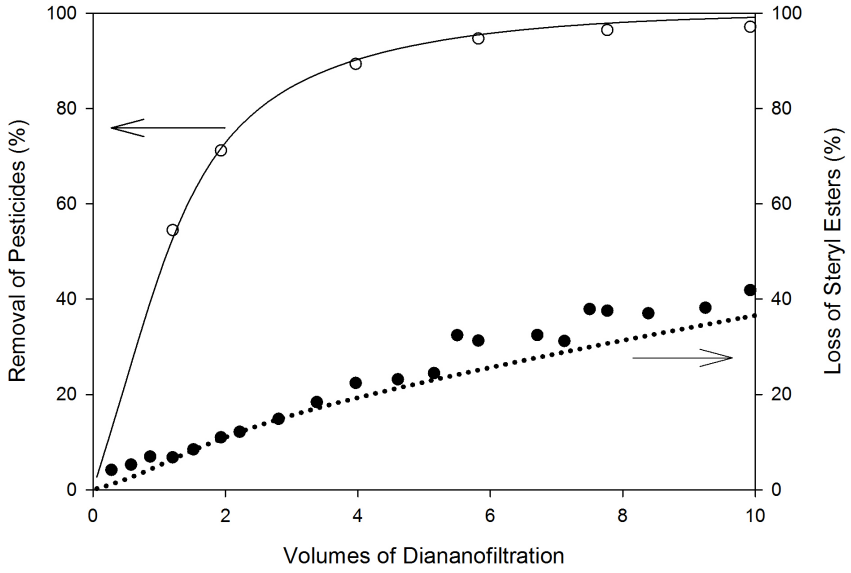


Figure 6.5: Removal of pesticides and loss of steryl esters as a function of the number of diananofiltration volumes

The main reason for such high loss of steryl esters is related to their relatively low rejection by the membrane. To the authors' best knowledge currently there are no other membranes available with a higher rejection for steryl esters that could be used in this process. A different alternative may be to use a two-stage diananofiltration process instead of a single-stage, as described recently Kim et al. [118].

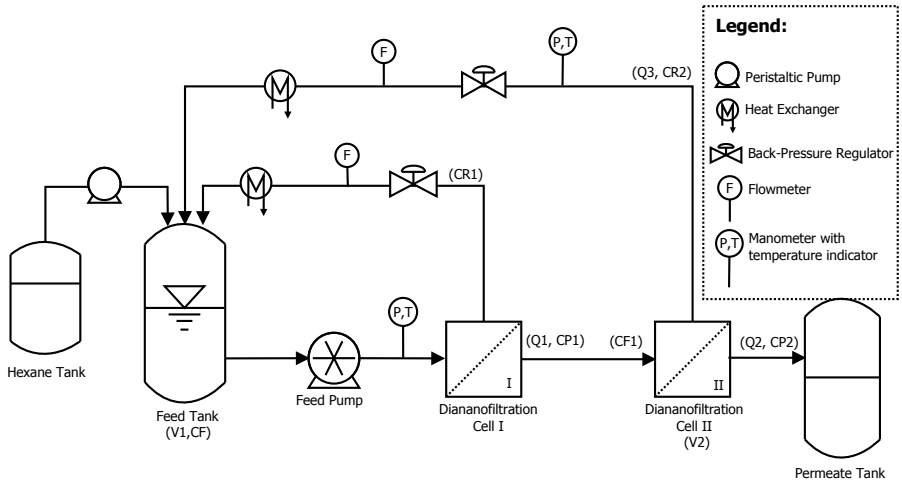


Figure 6.6: Schematic diagram of a two-stage of diananofiltration. The symbols in parenthesis are the variables used in the process modeling

Figure 6.6 illustrates a schematic diagram of a two-stages configuration, where the permeate

from the first stage is directly connected to the feed of the second stage. The flow rate through the first and second membranes (Q1 and Q2, respectively) is controlled by a back-pressure valve opening at the retentate side of the second stage. An exercise was conducted in this work through the simulation of a two-stage diananofiltration process, using the initial conditions and membrane data used previously in the single stage simulation. The feed pressure in the first stage was maintained at 20 bar, whereas for the second stage a pressure of 10 bar was assumed. Additionally, it was assumed that the volume of the feed in the second stage corresponds to the dead-volume of the system (V2=0.4 L).

Figure 6.7 shows that operating with a two-stage diananofiltration can lead to a significant improvement in terms of steryl ester depletion from the feed solution. Indeed, a 99.2% removal of pesticides leads in this case to ≈11% loss of the initial amount of steryl esters (instead of a ≈42% loss obtained with a single stage). This improvement is, however, accomplished with a use of a larger number of diananofiltration volumes (16.3 instead of 10.3 volumes) and an expected higher operating and capital investment costs associated to a more complex process design.

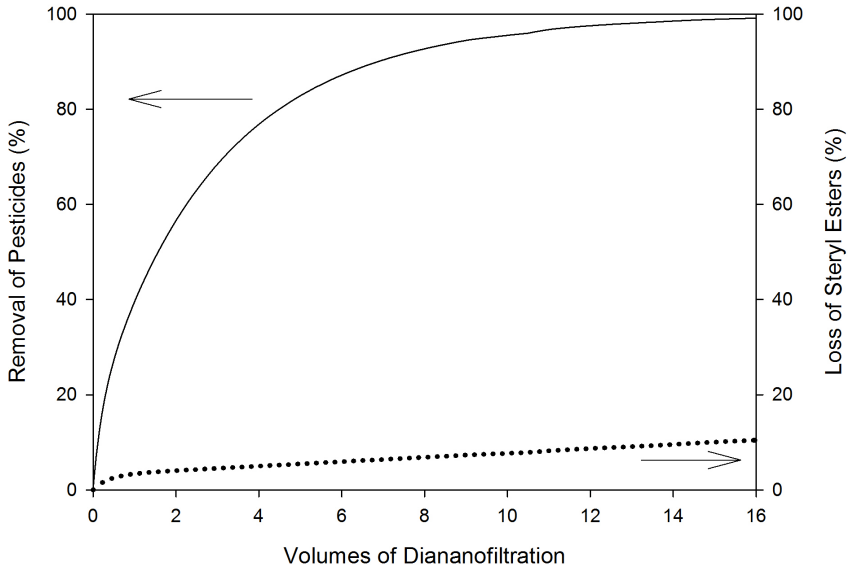


Figure 6.7: Removal of pesticides and loss of steryl esters, as a function of the number of diananofiltration volumes, for a two-stage diananofiltration

6.5 CONCLUSIONS

This work shows that the production of extracts rich in steryl esters and free of pesticides, from deodorizer distillates, could be achieved through the use of solvent resistant nanofiltration (SRNF) membranes.

Among the three commercial membranes studied, PuraMemS600 from Evonik was selected as the most adequate to produce an extract that could meet the specifications required. This membrane enabled the removal of 99.2% of the pesticides from esterified-deodorizer distillate, below

0.05 ppm in the enriched-food (which is a necessary condition). The diananofiltration technique showed to be efficient to fulfill such objective, however, at expense of a significant loss of the initial amount of steryl esters (≈42%).

The mass-balance model developed in this work described with a good accuracy the diananofiltration process, showing a good agreement with the experimental data. This model assumed that the rejection of compounds and the feed volume remained constant during processing. Additionally, it took into account the influence of the membrane exposure time to a hexane-based solution of deodorizer distillate (the flux was observed to increase during processing, most probably due to swelling).

Strategies for future work should focus on the development of membranes that can better reject steryl esters, and on engineering modifications of the process. It was shown by simulation that the use of a two-stage diananofiltration could decrease significantly the depletion of steryl esters, which could pave the way for a short term adoption of these processes based on SRNF membranes.

From an environmental point of view the diananofiltration process investigated in this work was shown to be highly solvent intensive. Therefore, for application purposes, this process should be ideally integrated with a solvent recovery operation, using distillation and/or organic solvent reverse osmosis, depending on the solvent boiling point and the respective energy requirements.

7 Conclusions and Future Work

Summary *This chapter presents some of the main overall conclusions that resulted from this thesis. In addition, some aspects that could be addressed in the future are discussed.*

7.1 CONCLUSIONS AND FUTURE WORK

The concept of a solvent resistant membrane-based process to produce an extract rich in steryl esters was demonstrated in this Thesis.

Similarly to the main steps of this process, this Chapter is divided into three sections:

- Enrichment of Deodorizer Distillates in Steryl Esters
- Reuse of the Enzyme
- Production of an Extract Rich in Steryl Esters

7.2 ENRICHMENT OF DEODORIZER DISTILLATES IN STERYL ESTERS

The enzymatic esterification of sterols and free fatty acids was shown to be adequate for the production of steryl esters. A lipase from *Candida rugosa* in its free form showed to the able to catalyse such reaction with satisfactory yields (>80%), in 24 hours, under optimal conditions. These conditions were identified as being the following: temperature set at 40 °C, enzyme concentration and initial water activity (a_W) within the range of 0.25-0.50% (w/w) and 0.45-0.85, respectively, and initial molar ratio of free fatty acids (FFA) to sterols (S) higher than 6.0. Furthermore, it was found that the control of the water activity (a_w) during the reaction is not required as long as the initial value is within the optimal range.

The mass-balance model developed was able to describe the transient state of the reaction system during the enzymatic reaction when using different deodorizer distillates and sources of free fatty acids, which is indicative of the good predictive capacity of the model. The use of alternative and cheaper sources of free fatty acids (other than oleic acid), available to the majority of the oil producers, such as refined oil and mixtures of deodorizer distillates with different compositions, was considered to be feasible which may be very interesting from a process economy point of view. However, the proportion of each stream should be adjusted in order maximize the concentration of sterols, triglycerides and free fatty acids (*FFA*), without exceeding the initial concentration of *FFA* in 120 mmoles/100g; otherwise, an inhibition by excess of substrate may occurs. The use of sources rich in triglycerides is an alternative to avoid inhibition, since the hydrolysis of triglycerides constitutes a source of free fatty acids, assuring the equilibrium is driven towards the esterification of sterols.

Complementary to this study, the use of soapstocks as a potential source of free fatty acids could be considered. Soapstocks are a by-product produced during the neutralisation step of the oil refining, whereby free fatty acids are removed. Nowadays, this residual stream is valorised as "acid oil" for animal feed due to its high content in fatty acids, varying between 30-60% (depending on the respective source) [9].

7.3 REUSE OF THE ENZYME

Consecutive batch reactions of steryl esters production from vegetable oil deodorizer distillates were not possible to be conducted without a significant decrease of enzymatic activity, observed

in terms of the yield of steryl esters production.

The products of oxidation present in the vegetable oil deodorizer distillate have an important role in the inactivation of the enzyme. Unfortunately, this negative effect could not be avoided by the presence of antioxidants in the deodorizer distillates and enzyme inactivation occurs inevitably between consecutive batch reactions.

Four consecutive bacth reactions with yield at levels higher than 80% were successfully carried out, however, it was necessary to add fresh enzyme (47% w/w of the initial amount) between batches.

Methods for prior-removal of oxidation products, such as adsorption, should be studied in a future work. Bhattacharya et al. [119] showed the efficiency of adsorbents, such as magnesol, activated carbon, silica gel and aluminum hydroxide, in the reduction of oxidation products contained in thermal degraded of frying oils. The characteristics of such oils are similar to deodorizer distillates, since the later was obtained by a distillation at high temperature (180-280° C). If the efficiency of this procedure is high for in the removal of oxidation products, the possibility of reusing the enzyme in consecutive batches should be verified. Furthermore, the additional cost of adsorbents should be compared with the cost of performing a make-up of enzyme.

On the other hand, the lifetime of the enzyme in the free and immobilized forms could be compared, however, the additional cost of immobilization must be taken into account.

7.4 Production of an Extract Rich in Steryl Esters

This work showed that the use of polymeric solvent resistant nanofiltration (SRNF) membranes for the production of an extract rich in steryl esters, free of pesticides, is technically feasible. The success of this application depends on the stability of the membrane in suitable solvents, and the discrimination of pesticides (contaminants, 150<MW<400 g/mol) from steryl esters (bioactive compounds, 650<MW<800 g/mol).

Puramem S600 from Evonik showed a good compatibility with hexane and a satisfactory discrimination between steryl esters (R=96%) and pesticides (R=32-58%). The diananofiltration technique showed to be efficient, removing 99.2% of the pesticides (necessary condition to obtain a concentration of pesticides in the extracts to target values for their use as food additive), but with a loss of 42% of the initial amount of steryl esters. A mass-balance model to describe the process of diananofiltration was developed with independent measurements and showed a good agreement with the experimental data.

The insufficient rejection of steryl esters by the membrane leads to a significant loss of these compounds, decreasing the efficiency of the process. The use of two-stage diananofiltration could address such drawback. Simulation results predicted that it is possible to remove 99.2% of the pesticides, losing only 10.6% of the initial amount of steryl esters (instead of 42% obtained with a single stage), although, at expense of a higher number of volumes of diananofiltration (16.3 instead of 10.3 volumes). In the future, if membranes with higher performance are developed, the processing in a single-stage should be reconsidered. For now, it is recommended the implementation of a two-stage diananofiltration process and validation of the simulation results.

The use of ceramic membranes with solvents is remarkably simpler given their compatibility and widely acceptation by industry, however, their application in nanofiltration separations with apolar solvents is still limited. Further investments in designing ceramic materials able to perform such separations are needed.

From an environmental point of view, diafiltration may be considered as a solvent intensive technique. Therefore, the process discussed should be ideally integrated with a solvent recovery step, using distillation and/or organic solvent reverse osmosis, depending on the solvent boiling point and the respective energy requirements. Solvent-resistant reverse osmosis membranes are still under development, so this possibility depends from new advances on adequate membrane materials.

The concept of producing extracts rich in steryl esters with efficient downstream recovery was successfully assessed in this Thesis. The next step should involve a scaling-up study. An important point to address by the industry in the future is the manufacturing of reliable SRNF spiral-wound elements. Indeed, the materials of construction of these elements need to be compatible with the aggressive processing solutions, which is an important challenge, in addition to the compatibility of the membrane itself. The availability of such robust membrane elements is mandatory for providing structural integrity of the element in terms of mechanical and chemical resistance.

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