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Enantiomer separation through biocatalysis using NADES

Dissertação para obtenção do Grau de Mestre em Biotecnologia

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Setembro 2015

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Agradecimentos

Primeiramente queria agradecer aos meus orientadores, a Professora Susana Barreiros e o Doutor Alexandre, por me terem aceite para realizar este trabalho e por todo o apoio que me foi dado ao longo do mesmo, com eles foi tudo muito mais fácil. Agradeço em especial ao Alexandre, o meu Darth Vader (eheh), que teve que lidar com todos os meus dramas laboratoriais quando nada dava bem e que “festejava” comigo quando finalmente apareciam resultados!

Queria também agradecer ao Ângelo, do Instituto Superior Técnico que contribuiu para que este trabalho pudesse ter bons resultados e à Professora Paula Branco que se disponibilizou para me auxiliar no processo de síntese.

Aos meus coleguinhas de laboratório, Bruno Pedras, João Nogueira, Sandra Abrantes, Francisca Mano, Rita Craveiro, Silvia Rebocho, José Pinto e todos os que por cá passaram e que de forma mais ou menos cientifica contribuíram para uma boa realização deste trabalho.

Fica também um agradecimento aos amiguitos das pausas! Tiago Mestre, Lúcia Pinto, Telma Simões e Luísa Campelo pelos bons momentos passados nas horas livres!

Deixo também um agradecimento aos meus amigos e namorado, que sempre me apoiaram nos bons e maus momentos ao longo deste ano, nem sempre foi fácil, mas eles estiveram sempre por perto para me apoiar e para me ajudarem no que fosse preciso.

E por fim, mas não menos importante, à minha família, que desde o inicio sempre foi uma grande força e inspiração para alcançar este objetivo e ter força para continuar perante as dificuldades, a eles dedico todo este trabalho pois sem o apoio deles nada disto seria possível.

Abstract

Currently, the separation process of racemic secondary alcohols has a great environmental impact because it involves the use of large amounts of organic solvents and energy. Based on the principles of green chemistry, the main purpose of this work is the development of a sustainable process, without the use of organic solvents and using reusable catalysts (enzymes) in order to make the most efficient separation process in terms of energy and reduce the associated environmental impact.

In this work we developed a process for the separating of the enantiomers of (±)-menthol, via a transesterification reaction with vinyl decanoate. At optimum conditions, this reaction will yield the pure enantiomer (-)-menthyl decanoate, while (+)-menthol will remain unreacted. The use of reusable biological catalysts is possible due to the enantioselectivity of the enzyme, allowing the resolution of secondary alcohols to obtain pure enantiomers of high added value.

Candida Rugosa lipase is a selective enzyme capable of selectively converting the (-)-menthol into a different chemical compound and with different characteristics the (+)-menthol.

At the end of the reaction, the menthol enantiomers will be present in the reaction medium in the form of (+)-menthol and (-)-menthyl decanoate. As such it is necessary to use a method based on the properties of alternative solvents to physically separate the compounds.

The main objective of this study is to evaluate deep eutectic solvents as solvents for the transesterification reaction, but also to experiment if whether they themselves are able to act as substrates. Another approach is to test whether they can also act as separating agents of (+)-menthol and menthyl decanoate.

In the first phase it is intended to develop a DES using combinations of choline chloride, urea, and glycerol. The second objective is to synthesize DES based on menthol, containing one or both of the reaction substrates in the composition. The transesterification reaction is conducted in these solvents in order to assess whether the reaction is feasible in the presence of a biocatalyst. After the reaction, the enantiomers can be separated, since they are different entities. This separation is based on the interaction of the compounds with the DES, which allows the physical separation of the two compounds ((+)-menthol and (-)-menthyl decanoate).

Through this work, we got the required proof of concept for all experiments performed in DES.

Using hexane as reaction medium, a (±)-menthol conversion of 26.3 % was achieved, nevertheless controlling the water content in the reaction medium and in the atmosphere a maximum conversion of 50.9 % was achieved after 24 hours at 40°C. In DES ChCl:glycerol (1:2) and ChCl:urea (1:2) conversions are similar to those obtained in hexane at the same conditions, with values of 10.9 % and 10.2 % respectively. In DES menthol:camphor (4:1) was obtained 23% conversion at 40°C after 50 hours. In DES menthol:lauric acid (2:1) and menthol:phenylpropionic acid (1:1) conversions are higher with a maximum of 79.6 % and 62.1 % respectively. For the separation of compounds, DES choline dodecanediote:glycerol (1:1) was most effective with a separation factor of 16±6.5.

Keywords: Candida rugosa lipase, enantioselectivity, racemic menthol, deep eutectic solvents, green solvents.

Resumo

Atualmente, o processo de separação de álcoois secundários racémicos tem um grande impacto ambiental, uma vez que envolve o uso de grandes quantidades de solventes orgânicos e de energia. Com base nos princípios da química verde, o principal objetivo do presente trabalho consiste no desenvolvimento de um processo sustentável, sem a utilização de solventes orgânicos e utilizando catalisadores reutilizáveis (enzimas), a fim de tornar o processo de separação mais eficiente em termos de energia e reduzir o impacto ambiental associado.

Neste trabalho foi desenvolvido um processo para a separação dos enantiómeros de (±)-mentol, por meio de uma reação de transesterificação com decanoato de vinilo. Em condições ótimas, esta reação dará o enantiómero puro (-)-decanoato de mentilo, enquanto o (+)-mentol não reage. A utilização de catalisadores biológicos reutilizáveis é possível devido à enantiosselectividade da enzima, permitindo a resolução de álcoois secundários para se obter enantiómeros puros de elevado valor acrescentado.

Cândida rugosa lipase é uma enzima seletiva capaz de converter seletivamente o (-)-mentol, num composto químico diferente e com características diferentes do (+)-mentol.

No final da reação, os enantiómeros de mentol estarão presentes no meio reacional sob a forma de (+)-mentol e (-)-decanoato de mentilo. Como tal, é necessário utilizar um método com base nas propriedades de solventes alternativos para separar fisicamente os compostos.

O principal objetivo deste estudo é avaliar solventes eutécticos como solventes para a reação de transesterificação, mas também para experimentar se se eles próprios são capazes de agir como substratos. Outra abordagem é para testar se eles também podem agir como agentes separação do (+)-mentol e do (-)-decanoato de mentilo.

Na primeira fase pretende-se desenvolver um DES usando combinações de cloreto de colina, ureia, e glicerol. O segundo objetivo é sintetizar DES baseados em mentol, contendo um ou ambos os substratos de reação na sua composição. A reação de transesterificação é conduzida nestes solventes, a fim de avaliar se a reação é possível na presença de um biocatalisador. Após a reação, os enantiómeros podem ser separados, uma vez que são entidades diferentes. Esta separação é baseada na interação dos compostos com o DES, que permite a separação física dos dois compostos ((+)-mentol e (-)-mentil decanoato).

Através deste trabalho, temos a prova de conceito para todas as experiências realizadas em DES.

Utilizando hexano como meio reacional, uma conversão de (±)-mentol de 26.3% foi conseguida, no entanto, controlando o teor de água no meio reacional e na atmosfera, um máximo de conversão de 50.9% foi atingida após 24 horas a 40°C. Em DES ChCl:glicerol (1:2) e ChCl:ureia (1:2) os valores de conversão são semelhantes aos obtidos em hexano com as mesmas condições, com os valores de 10.9% e 10.2%, respetivamente. Em DES mentol:cânfora (4:1) obteve-se a conversão de 23% a 40°C depois de 50 horas. Em DES mentol:ácido láurico (2:1) e mentol:ácido fenilpropiónico (1:1) obtiveram-se conversões elevadas com um máximo de 79,6% e 62,1% respetivamente. Para a separação de compostos, o DES dodecanediote de colina:glicerol (1:1) foi o mais eficaz com um fator de separação de 16±6.5.

Palavras-chave: Cândida rugosa lipase, enantiosselectividade, mentol racémico, solventes eutécticos, solventes verdes.

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Acronyms

CRL – Candida rugosa lipase

DCC - N,N'-Dicyclohexylcarbodiimide

DMAP - 4-Dimethylaminopyridine

e.e. – enantiomeric excess

e.e.s – enantiomeric excess of the substrate

GC – Gas Chromatography

IL – Ionic liquid

DES – Deep eutectic solvents

NADES – Natural deep eutectic solvents

scCO2 – Supercritical Carbon Dioxide

VOC- Volatile organic compounds

Chapter I

Introduction

I. Introduction

a. Purpose of the work

This work focused on the separation of the enantiomers of (±)-menthol by using green solvents (deep eutectic solvents) as reaction medium. This separation is only possible if there is any change in the properties of the molecules. To this end, a reaction is carried out with an ester, for example vinyl decanoate, which through an enzyme, *Candida rugosa lipase*, will react with (-)-menthol, which is the molecule of interest, changing it.

In the first phase it is intended to develop a DES in which the reaction is feasible, using combinations of common compounds, such as, choline chloride, urea and glycerol. The second objective is to synthesize DES containing one or both of the reaction substrates in its composition. Experiments will be carried out with these in order to evaluate if the reaction is feasible in the presence of a biocatalyst.

Meanwhile, it is intended to develop a DES for the separation of the reaction products. After the reaction, it is possible to separate the enantiomers, as these will be different entities. This separation is based on the interaction of the compounds with a DES molecules, allowing for the physical separation of the enantiomers.

b. Green Chemistry

Currently chemical industry has a very important role in our daily lives because of the many products obtained therefrom which are fundamental to humanity, particularly in the pharmaceutical area with the development of some tons of drugs that allow for better quality of life, but also in other areas such as fuel that had a capacity of about 500,000 tons per year in 1998.[1,2]

However, chemical production creates numerous problems, such as the formation of toxic by-products due to complex processes, environmental contamination caused by the large volume of toxic waste produced in the processes and the contamination of the humans exposed to these xenobiotics.[1]

In recent years concern with these problems has been increasing the pressure on the chemical industry in order to improve the processes to make them less harmful to the environment. It is necessary to always take into account the rule that chemistry should maintain and improve the quality of life for sustainable development, but it was necessary for the acceptance by industries, governments and people in general, which allowed achieve social, economic and environmental goals.[1]

There is then a need to develop processes that decrease the damage to the environment, which requires a new course to improve such chemical processes to reduce the formation of waste, sewage and toxic gases to the environment. This can be done by using alternative reagents, increased selectivity to maximize the use of the starting materials, the use of catalysts to facilitate final separation of the product mixture and also the recycling of reagents and catalysts used in the processes. These changes can be applied in various fields including the chemical industry as an alternative to traditional chemical catalysis seen that with increasing of the selectivity, it is possible to maximize the reactions and reduce the formation of undesirable by-products during the reaction process.[1]

This path outlined for chemistry can be called green chemistry or sustainable chemistry, which is based precisely on this point: "The creation, development and application of chemical products and processes to reduce or eliminate the use and generation of toxic substances".[1]

Green chemistry or sustainable chemistry is an emerging field that is based on the ultimate goal of developing scientific activities and industrial processes that are environmentally friendly. This movement began in the 1990s, more strongly in the USA, England and Italy, which new concepts and values were introduced in many areas of chemistry.[3]

The Green Chemistry goal is then the viability of the processes to minimize the negative impact caused both to humans and to the environment. This feasibility passes through the increase in the safety of processes to solve global problems such as climate change due to pollution caused by the emission of toxic substances to the environment.[3]

Green chemistry is concerned to advance processes so that they are unable to cause pollution. In addition, the decrease of storage and treatment of waste, lead to a decrease in spending which has, in addition to environmental impact, economic impact. This new concept of sustainable chemicals has also recover the bad image of the chemical industry in the press and in the population, and bringing a social impact. Thus, the green chemistry is essential to achieve social, economic and environmental goals.[1,3]

The need to develop more sustainable processes also creates new challenges in chemistry, both for industries and researchers, which can open doors to new discoveries and is an opportunity to develop more and better. Some of these challenges relate to the discovery and development of new synthetic routes using alternative feedstocks or more selective reactions by adjusting the reaction conditions and using alternative solvents. In short, the challenge is to find the ideal synthesis, which should be simple, have a yield of 100%, using natural materials available, be environmentally acceptable, with no waste and by-products, have only one reaction step, be safe and atomically efficient.[2]

Green chemistry is based on three key categories:[3]

i. Use of renewable raw materials;
ii. Increasing energy efficiency to lower power consumption for the same or higher product formation;
iii. Elimination of persistent, bioaccumulative and toxic.

To develop technologies for sustainability principles that must be followed and which involve:[1]

i. Use safe reagents during the process of synthesis to avoid undesirable losses, increasing production yield;
ii. Change toxic solvents for other alternative solvents;
iii. Enhancement of natural processes such as biosynthesis, biocatalysis;
iv. Development of safe compounds, i.e., low toxicity;
v. Development of reaction conditions to achieve higher performance and generation of by-products;
vi. Minimizing energy consumption.

To be applied, these principles must first be an awareness of students and researchers in the chemical area to train skilled professionals for these new scientific and technological concepts that will be responsible for the sustainability of the planet. The emergence of green chemistry in education and research is supported by organizations and scientific societies, governments and industries.[1]

Concern about the quality of life and the environment with regard to practical chemistry led to the formation of the twelve principles of green chemistry:[1]

i. ***Prevention***
It is better to prevent the formation of by-products than treat them later;
ii. ***Atom economy***
Synthetic methods must be developed to maximize the incorporation of atoms of the reactants to the desired final products;
iii. ***Synthesis of compounds with less toxicity***
Wherever possible should be replaced by high toxicity compounds less toxic compounds in chemical reactions;
iv. ***Development of safe compounds***
The chemicals should be developed to possess the desired function, with the lowest possible toxicity;
v. ***Decrease of auxiliary solvents***
The use of auxiliary substances should be avoided where possible, or used in the process innocuous;

vi. ***Energy efficiency***

Synthetic methods should be conducted whenever possible to ambient temperature and pressure, to reduce the energy spent during a chemical process that is both economic and environmental impact;

vii. ***Use of recycled substances***

Products and by-products of chemical processes should be reused whenever possible;

viii. ***Reduction of derivatives***

Derivatization should be minimized or avoided when possible, because these reaction steps require additional reagents and thus may produce undesirable by-products;

ix. ***Catalysis***

The use of catalysts to increase the rate and yield of the chemical processes;

x. ***Development of Compounds to degradation***

Chemicals should be made to the innocuous degradation of toxic products, not to persist in the environment;

xi. ***Real-time analysis for pollution prevention***

The analytical methodologies need to be developed to allow monitoring of the process in real time to control the formation of toxic compounds;

xii. ***Safe chemical to prevent accidents***

Substances used in chemical process should be chosen to minimize potential accidents such as explosions and fires.

These principles are essential to the practice of green chemistry. Industries that undergo these changes will have a commercial advantage because of the application of low-waste processes allowing a huge cost saving.[2]

The growth of green chemistry is closely related to the increased number of conferences in USA, Europe and Asia, which allow the knowledge of the work both in industry and in academia, promoting the application of green chemistry in the chemical industry and other.[2]

Also in terms of publications to note a large growth of articles published in this area which shows the growing concern in developing these greener processes and discover new alternatives that allow for development of the chemical area.[3]

It is estimated that in 2020 biotechnological processes may be involved in the manufacture of 20% of all chemicals, a multi-billion dollar market.[4]

c. Green Solvents

Some examples of applications that are being explored within the chemical industry is the use of water in the subcritical region and CO_2 in the supercritical region as an alternative reagent to organic solvents. Such solvents reduce problems with residues formed at the end of the

reaction due to physical and chemical changes of these solvents. When CO_2 is in the supercritical region, because of its low permittivity, become very soluble organic compounds. When the water is in the subcritical region decreases its dielectric constant, favouring reactions with acidic and basic solid catalysts.[1] Thus these solvents are most promising alternative to organic solvents since it simplifies the final separation of the product into the solvent and are more environmentally friendly.

As mentioned before, green technology is a key issue in the field of chemistry because it aims to preserve the environment and reduce the negative influence of human involvement. Green technology makes minimal use of hazardous means, new environmentally acceptable solubilisation techniques, controlling the physical properties of the medium, such as temperature and pressure, and the development of new green solvents. In this context the development of ionic liquids and deep eutectic solvents had received enormous attention from the scientific community in order to replace current aggressive organic solvents.[5]

In recent years, researchers have been continually looking for green solvents, as alternatives to conventional volatile organic compounds (VOCs) in many chemical and industrial processes.[6]

Ionic liquids (IL) have emerged as a new generation of solvents and become interesting for many applications. ILs are composed of discrete cations and anions which form a mixture that are liquid at temperatures equal to or less than 100ºC. They are described as designer solvents since their properties (such as melting point, viscosity, density and hydrophobicity) can be tuned up to meet the needs of a particular process, just changing the structure of the ions. In addition, due to their very low vapour pressures, ILs do not evaporate under normal industrial operating conditions. They have a great ability to dissolve many organic materials, inorganic and organometallic. Moreover, they are immiscible with most organic solvents. However, even this ILs have a great potential for use as "green" solvents for industrial processes, it is still a challenge for its large-scale applications in the industry. This is due to complicated synthetic methods and chemical raw materials expensive to make their synthesis very costly.[7]

To overcome the limitations of RTILs, deep eutectic solvents (DESs) were presented as versatile alternatives.[8]

d. Deep Eutectic Solvents

Deep eutectic solvents (DES) are made by mixing a substituted quaternary ammonium salt and a metal halide or a hydrogen bond donor (HBD). The original precursors generally have high melting points, which form, in a certain composition of a eutectic mixture with a substantially lower melting point; hence, the term deep eutectic solvent.[6] DESs consists of at least one HBD and hydrogen bond acceptor (HBA) counterpart.[8]

DESs have many desirable properties as solvents compared to organic solvents. In fact, they share many characteristics with room temperature ionic liquids (RTILs), such as, liquid wide range, high thermal and chemical stability, non-flammability, and high capacity of solvation. However, DESs are easy to prepare in high purity; thus, they can be synthesised at a considerably lower cost than RTILs. Due to these advantages, the potential for DES viable as solvents are widely explored, with the increasing use in organic synthesis, electrochemistry, and bioorganocatalysis, and materials science.[6]

The first DES was reported by mixing choline chloride with urea. Other similar DESs have also been developed and applied as solvents or catalysts in reactions or bio-transformations, liquid separation and metal electroplating.[8]

During the course of chemical reactions, are often generated different mixtures containing by-products, unreacted components, etc. As a result, it is necessary to develop strategies to purify the desired product to levels within the specifications. This is a challenge for many synthetic reactions, and usually involves time consuming separation procedures, which can even hinder the industrial use of a certain process / reaction. An example could be the catalytic kinetic resolution of racemic alcohols to give optically active compounds. This separation is difficult in many cases, for example, involving time-consuming chromatography steps. The strategy to overcome the downstream limitation may be use of DES, as separating agents. DES are able to dissolve the hydrogen-bond-donor/acceptor molecules (e.g., alcohols), while other molecules non-hydrogen-bond donors/acceptor (e.g., esters, ketones, etc.) remain as a second phase in contact with DES. Based on this property, it is shown here that such mixtures that were difficult to separate alcohols and esters can in fact be separated in an efficient, clean and fast method.[9]

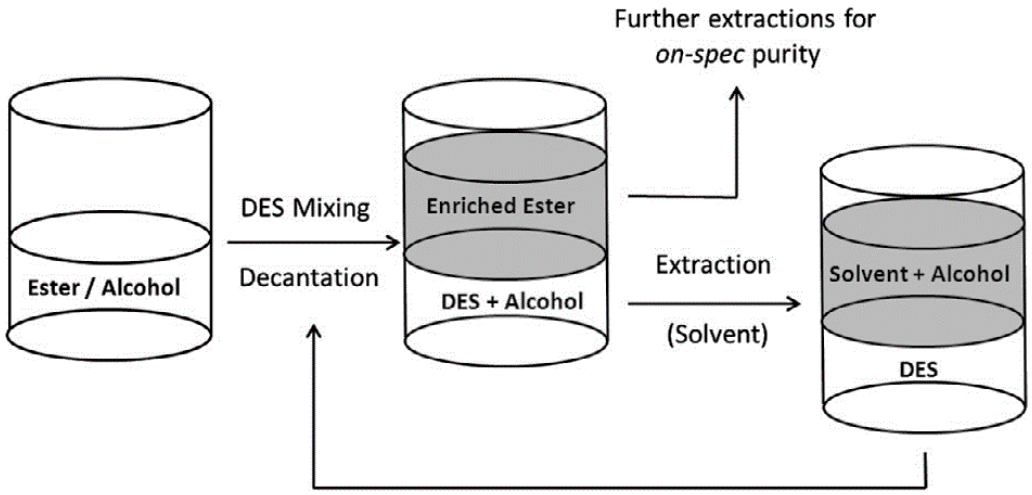

Figure I.1- Scheme separation of alcohols and esters in DES.

It is well established now that many ILs have the strong ability for the dissolution of CO_2. Similar to ILs, DES also have interesting properties solvents for dissolving high amounts of CO_2. Considering that a combination of green CO_2 with DES system has great potential for a variety of chemical processes, studies of the solubility of CO_2 in DES are of paramount importance.[7]

However, to use in the pharmaceutical grade these solvents generate some controversy, due to the compounds used in their synthesis. Overdue to the great development in this sense, today, natural products are indeed a plentiful and ideal source of RTLIs and DES, because of its enormous chemical diversity, biodegradable properties and pharmaceutically acceptable toxicity profile.[5]

Natural deep eutectic solvents are considered today a new generation of solvents, can replace the use of ILs in various application areas, particularly in the pharmaceutical industry.

As noted above, DES is a mixture of two or more organic compounds, which when mixed together in a certain molar ratio form a compound with a lower melting point compared to the individual components, forming liquid mixtures at room temperature. NADES are DES composed by two or more primary natural metabolites, namely, amino acids, organic acids, sugars or choline derivatives. The fact that they are composed by natural metabolites is a great advantage as it makes them totally biocompatible, increasing the possibilities of using these solvents in the pharmaceutical industry.[10]

Because of these attractive benefits and its potential use as friends of the environment alternative solvents, DESs probably present the best answer to the industrial needs for large-scale applications.[7]

e. Biocatalysis

Biocatalysis considers the use of renewable raw materials, energy efficiency and worker safety. Biocatalysis is made from renewable sources, they are biodegradable, non-toxic and their high selectivity simplify the reactions, providing products with higher yields. The biocatalytic processes are also safe because they are typically performed at room temperature, at atmospheric pressure and at neutral pH, preventing the formation of inconvenient by-products.[11]

A promising alternative is the use of enzymes in replacement of chemical catalysts, because enzymes also act as catalysts and thereby decrease the activation energy of the reaction, requiring less energy to the process. The use of microbial activity to catalyse chemical reactions can be successfully applied in various syntheses and is called biocatalysis.[3,4,11]

Over a century, scientists have recognized that live cell components could be applied to useful chemical transformations.

In 1980 and 1990 the first protein engineering technology have extended the range of enzymes to allow the synthesis of intermediates of unusual synthesis. This change expanded biocatalysis to the manufacture of pharmaceutical intermediates and fine chemicals.[11]

In the late 1990s it was developed molecular biology that involves iterative cycles of random changes of amino acids in a protein, followed by selection or screening of libraries with variants resulting from greater stability of the enzyme, substrate specificity and enantioselectivity, this method is called directed evolution. Another method used is site-directed mutagenics that are caused specific changes to required characteristics.[11]

Enzymes are biological catalysts that accelerate the rate of reactions but are not themselves consumed in the reactions; they may be used repeatedly for as long as they remain active. However, in most of the processes, enzymes are mixed in a solution with substrates and cannot be economically recovered after the reaction and are generally wasted.[12]

The main challenge for these applications is the limited stability of the biocatalyst, and these limitations were overcome primarily by enzyme immobilization methods that also facilitated the reuse thereof.[11]

Enzyme immobilization is confinement of enzyme to a phase (matrix/support) different from the substrates and products. It is common to use inert polymers and inorganic materials as carriers matrices. Matrices should be affordable, inertness, physical strength, stability, regenerability, ability to increase enzyme specificity/activity and reduce product inhibition, nonspecific adsorption and microbial contamination.[12]

The immobilization of enzymes have advantages, for example, continuous use of the same enzymes, increased enzyme stability, ability to stop the reaction quickly removing the enzyme from the reaction solution, recovery of product with greater purity, reduction of effluent disposal problems. However, this method can make the process more expensive, it affects the stability and activity of enzymes and may cause difficulties in the diffusion of the substrate access to the enzyme.[12]

There are several techniques for immobilization of enzymes, such as:

- Enzyme adsorption that results from hydrophobic interactions and salt linkages where either the support is bathed in enzyme for physical adsorption or the enzyme is dried on electrode surfaces. Adsorbed enzymes are shielded from aggregation, proteolysis and interaction with hydrophobic interfaces.
- Affinity immobilization exploits specificity of enzyme to its support under different physiological conditions. It is achieved by two ways: either the matrix is precoupled to an affinity ligand for target enzyme or the enzyme is conjugated to an entity that develops

affinity toward the matrix. Affinity adsorbents have also been used for simultaneous purification of enzymes.

- Entrapment is caging of enzymes by covalent or non-covalent bonds within gels or fibers, the enzymes or cells are not directly attached to the support surface, but simply trapped inside the polymer matrix.
- Covalent binding is the most widely used method for immobilizing enzymes. The covalent bond between enzyme and a support matrix forms a stable complex. The functional group present on enzyme, through which a covalent bond with support could be established, should be non essential for enzymatic activity.
- Cross linking is based on the formation of covalent bonds between the enzyme molecules, by means of multifunctional reagents, leading to three dimensional cross linked aggregates.

The variety of existing immobilization techniques for selecting the most suitable for the reaction of interest.[12] Enzymes have several advantages, in particular its regioselectivity, i.e. preference to one of several identical functional groups in the substrate molecule; its enantioselectivity, the preference for one enantiomer of a racemic mixture, and their chemoselectivity, favouring a functional group of the substrate instead of the other.[13]

There are several classes of enzymes:

i. **Class 1: Oxidoreductases.** Catalyse redox reactions, electron transfer, hydride (H^-) or protons (H^+).
ii. **Class 2: Transferases.** Transfer chemical groups between molecules.
iii. **Class 3: Hydrolases.** Use water as the receptor molecules of other functional groups.
iv. **Class 4: Lyases.** Form or destroy double bonds, respectively adding or withdrawing functional groups.
v. **Class 5: Isomerases.** In turn a molecule isomer.
vi. **Class 6: Ligases**. Chemical bonds are formed by condensation reactions, consuming energy in the form of ATP.

The degree of functionalization and complexity present in many natural food difficults the selective chemical modification and normally requires many steps for the protection and deprotection of the compounds of interest, i.e, throughout the reaction, there are steps which add a compound that reacts with the product of interest in order to protect it so that it is not removed in a separation step. After the separation, it is necessary another step to remove this protective compound and obtain the pure product. These additional steps make the reaction more expensive.[13]

A strategy for the efficient synthesis of optically pure molecules that are essential for the production of many pharmaceutical compounds is the use of enzymes. In chemical synthesis the

use of enzymes has several advantages, because the enzymes are not only highly selective, but also operate under mild conditions. This provides the possibility to drastically reduce the number of steps in a process.[14]

The benefits of selective catalysis have resulted in an increase of biocatalytic reactions for synthetic strategies for small molecules and intermediates of pharmaceuticals. There are about 150 biocatalytic processes implemented in industry, most of which are in the pharmaceutical sector.[14]

Recently, new opportunities in biocatalysis offer many possibilities for the manufacture of new chiral compounds with the development of more environmentally friendly and economically competitive processes. New scientific developments in the field of genomics and protein engineering technologies have facilitated the optimization of enzyme properties and increase in the availability of useful enzymes with the specific needs of reaction for a wide range of synthetic and industrial applications.[15]

The discovery of useful enzymes increased the success in areas of biocatalysis. These new opportunities have increased and accelerated the application of biocatalysis in the synthesis of various enantiopure compounds such as alcohols, amines, carboxylic acids, epoxides, among others.[15]

The increased availability of enzymes, created many opportunities for new medical therapies and production of new drugs via biocatalysis. However, some development is still required in biocatalysis, particularly with respect to scale-up, since large scale biocatalysis is currently restricted due to the limited number of commercially available enzymes synthetically useful and its speed of development is limited. Bio-engineering is highly promising because functionalized molecules of interest could be synthesized with high regio and stereo-selectivity.[14]

Moreover, enzymes naturally operate with low substrate concentrations and produce low product concentrations. Such concentrations are not feasible at industrial level, on a large scale, and it is an impediment to apply biocatalysis in the pharmaceutical industry due to the inherent economic implications.[14]

The technical challenges also limits the application of catalysis in industry. The current bio-engineering methods are close to their limits of efficiency, but their costs are still too high for large-scale applications that require thousands of genes.[11]

These are some factors to be developed so that we can increase green chemistry in the chemical and thus be closer to having a more sustainable future.

f. Transesterification

Transesterification is the process of exchanging the organic group R″ of an ester with the organic group R′ of an alcohol. Basically it is the reaction of an alcohol with an ester originating another alcohol and another ester. These reactions are often catalysed by the addition of an acid or base catalyst. The reaction occurs using enzymes as biocatalysts, particularly lipases (E.C.3.1.1.3).

In the transesterification mechanism, the carbonyl carbon of the starting ester ($RCOOR^1$) undergoes nucleophilic attack by the incoming alkoxide (R^2O^-) to give a tetrahedral intermediate, which either reverts to the starting material, or proceeds to the transesterified product ($RCOOR^2$).

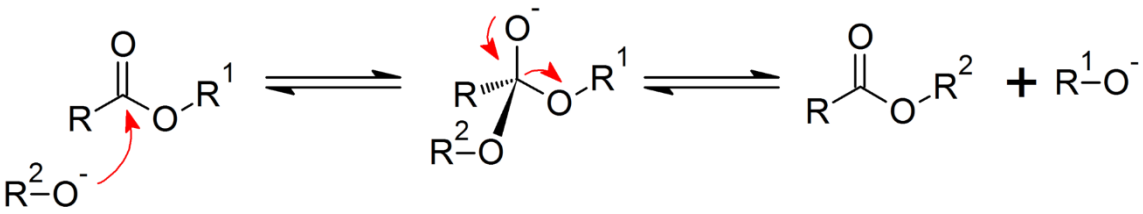

Figure I.2- Scheme for a transesterification reaction.[16]

This reaction is ideally suited to achieve the objective of this job, since we reacting an alcohol ((±)-menthol), with an ester (vinyl decanoate), using an enzyme with specificity for the (-)-menthol, Candida rugosa lipase. Thus originating an ester (menthyl decanoate), which is menthol molecule can be modified interest for their separation. The figure I.3 is a reaction scheme that occurs in this work.

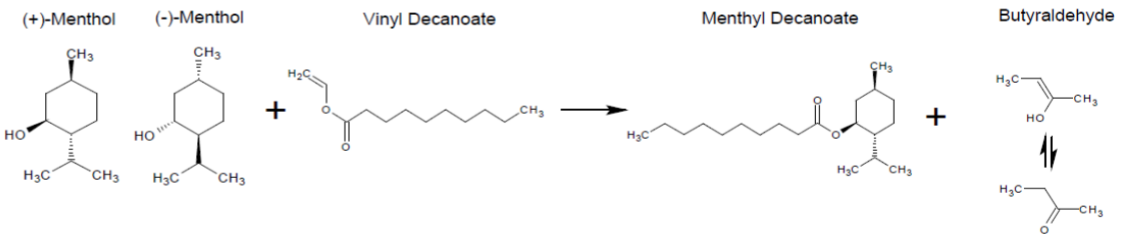

Figure I.3- Scheme transesterification of racemic menthol with vinyl decanoate.

g. Candida rugosa lipase 1

Candida rugosa lipase (CRL), a typical and widely used lipase known as lipase (EC 3.1.1.3). Lipases (EC 3.1.1.3) are commonly used because it catalyses the stereoselective

hydrolysis as well as the reverse reaction (esterification and transesterification). The use of suitable solvents (aqueous or organic) allows to control the direction of reaction. Lipases are used for a variety of biotransformations and in organic synthesis, commonly used in the synthesis of precursors for agrochemicals, pharmaceuticals, or other synthetic targets. [17,18]

The kinetic resolution of racemic secondary alcohols in organic solvents systems is a well studied application of lipases, being advantageous especially in high reaction speed, but also the high solubility of hydrophobic substrates or ease of separation and reuse of the biocatalyst. Also, supercritical fluids have been used as a solvent, thereby facilitating downstream processing.[18]

Lipases are very important in the development of chemical industries, such as pharmaceuticals, food, energy and fine chemicals.[19] For example, one of the methods to improve optical purity of enzymatic reaction products is to select a special acyl donor such as acid anhydrides which make the acyl transfer completely irreversible.[20] However, its high cost and easy inactivation in organic solvents, high temperature and other extreme conditions difficulty the application of lipase in chemical industries. Due to enzymes considerably unstable characteristics, most of the strategies for enzyme engineering are focused on microbiology, genetic engineering, protein engineering, medium engineering, substrate engineering, immobilization on novel supports, process optimization, etc. [17,19]

Candida rugosa lipase (CRL) has been extensively demonstrated to be useful for biotransformation reactions in aqueous and non-aqueous phases due to its high activity and broad specificity.[17]

The yeast Candida rugosa has a family of functional genes codes for several isoenzymes with closely related sequences naming Lip1 to Lip7.[17]

Enzymes are given a classification number, known as "CE" (Enzyme Commission of the IUBMB), which is composed of 4 digits:

1. Class
2. Sub-class within the class
3. Specific chemical groups participating in the reaction
4. The enzyme itself

Candida rugosa lipase is known as EC 3.1.1.3, since it belongs to the class of hydrolases, their sub-class is carboxylic esterase and acts on ester bonds.[21]

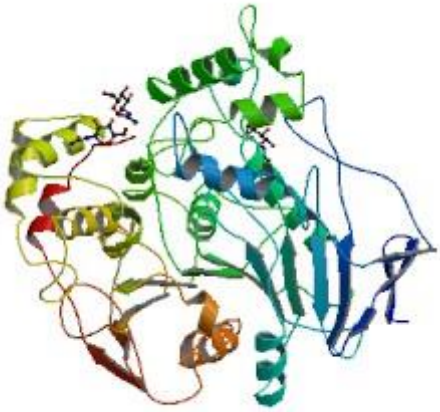

Figure I.4- Structure of Candida rugose lipase 1.[22]

The structure of several lipases have been well studied since 1990 with the structure of Candida rugosa lipase being available since 1993 (Figure I.4). It has 534 amino acids. Candida rugosa lipase displays a broad substrate spectrum with the Candida rugose lipase-catalysed chiral resolution of (±)-menthol representing one of the most thoroughly studied reactions.[18]

h. Menthol

Menthol ($C_{10}H_{20}O$, MW 156.27 g/mol) is a cyclic terpene alcohol, occurs widely in nature and have the chemical properties that make them important fragrance or flavour compound.[23]

Before World War II, Japan and China controlled production of (–)-menthol. During the disruption of trade routes caused by the World War II, Brazil took over as the main producer of Mentha arvensis and became the largest producer and supplier of menthol.[23]

During the 1960s, an oversupply of menthol caused price decrease, and processors reduced production levels.[24]

By 1996, India became the major producer of menthol with a production of 6,000 metric ton of M. arvensis oil. This production was estimated to increase to 20,000 metric tons by 2007. Most of the current production is used for menthol crystallization, however significant amounts of menthol are exported to countries like Brazil, Taiwan and Japan for further purification.[24]

After crystallisation menthol obtain a residual oil which contains 35 % to 45 % menthol and menthones and other typical components of mint. This oil is further modified to have other applications, for example, much of this dementholized oil is rectified by distillation, and sold for

use in toothpaste, mouthwash, shampoo, etc. Moreover, some of these dementholizes oil can be fractionated to isolate menthones, which can be converted by reduction to (-)-menthol.[24]

One of the most important areas in the pharmaceutical and fine-chemical industries is the synthesis of optically pure products. In some cases, the non-target enantiomer has a neutral effect, but it can also impact on a different biological process, and even do so with a deleterious effect.[25]

Optically pure alcohols can be produced in two ways: in aqueous solutions by stereoselective hydrolysis of the corresponding racemic esters or in organic solvents by esterification of the corresponding racemic alcohols. The practical application of the method depends on various factors such as: the desired yield and purity of product, rate of reaction, and enzyme stability.[26]

M. arvensis (cornmint oil) and M. piperita (peppermint oil) are the primary menthol-rich mint species. Menthol is isolated from M. arvensis oil and its application comprises a wide variety of products, including, pharmaceuticals, oral care products, confections, chewing gums, perfumed products, and lotions. Into these products the level of incorporation of menthol ranges from about 0.03% to about 4.0%.[27]

Mint is very popular, not only on its pleasant taste and easy digestibility, but also its increasing association with freshness, cleanliness, and hygiene.[27]

Cornmint oil obtained by steam distillation from the flowering shrub M. arvensis. Contains 70-80% of (-)-menthol that has a slightly herbal minty smell. Pure (-)-menthol can be obtained by recrystallization from low-boiling point solvents.[23]

Peppermint oil made from M. piperita contains up to 50% menthol, but is not used for the production of menthol due to its high price. Peppermint oil is used as a flavouring for toothpastes, other oral hygiene products and chewing-gum.[23]

Peppermint oils are more valuable because it is significantly higher in quality. Their flavour is sweeter and more well-rounded. The tea and herbal aroma components of peppermint oil are more pronounced and produce a fullness of taste. Their impression of freshness, and thus their cooling effect and impact, is more intense than the dementholated cornmint oils.[27]

The largest and most important component of peppermint oil, which makes up 43-50% of the oil, is (-)-menthol.[27]

The characteristic flavour of menthol is dependent on its conformation. Only (-)-menthol have cooling effect.[27]

Menthol has three asymmetric carbon atoms in its cyclohexane ring, and therefore occurs as four pairs of optical isomers; (-)- and (+)-menthol, (-)- and (+)-neomenthol, (-)- and (+)-isomenthol and (-)- and (+)-neoisomenthol, as illustrated in Figure I.5. (-)-Menthol is the isomer that occurs most widely in nature and is the one recognized by the name menthol. It has the characteristic peppermint odour and exerts a cooling sensation when applied to skin and mucosal surfaces. However, the other isomers of menthol have a similar, but not identical, odour and do not have the same cooling action as (-)-menthol. The isomers of menthol each have identical physical properties is (+)- and (-)-menthol. Neomenthol, neoisomenthol, menthol and isomenthol differ slightly in their boiling points with a range of 211.7-218.6ºC. The isomers also differ in their physical characteristics, for example, as at room temperature (+)-neomenthol is a colourless liquid and isomenthol and menthol are white crystals.[23]

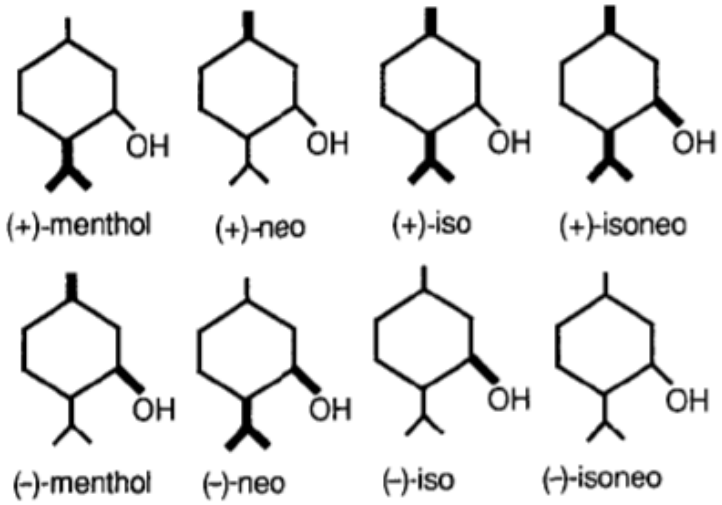

Figure I.5- Isomers of menthol.[24]

M. arvensis oil is normally about 99.0% to 99.6% pure. While, in most cases, the mint oil impurities contribute for a pleasant peppermint aroma, in the other hand, certain impurities can also impart less desirable. This can be adjusted to increase the mint character, if desired, by the addition of a small amount of terpeneless peppermint oils.[24]

Menthol can also be extracted from other essential oils such as cirtonella oil, eucalyptus oil and Indian turpentine oil. Synthesis of menthol from thymol competes with isolation from natural mint oils.[23]

During the 1970s and 1980s, were developed a number of new routes to synthetic (–)-menthol, only two of which led to long-term commercial success, the Haarmann and Reimer (H&R) and the Takasago processes[24].

The procedure developed by Haarmann and Reimer (H&R), represented in Figure I.6, starts from m-cresol and propylene to produce thymol (step 7), yielding the eight isomers of menthol by hydrogenation. A fractional distillation gives racemic (±)-menthol (step 8) which is converted into racemic benzoate (step 9). This mixture is resolved recurring to fractioned cristilization of (–)-menthol via the benzoate ester. By saponification (-)-menthol is obtained whereas the mother liquour gives (+)-menthol. The other seven undesired isomers are recycled in a separate racemization step and reused.[24]

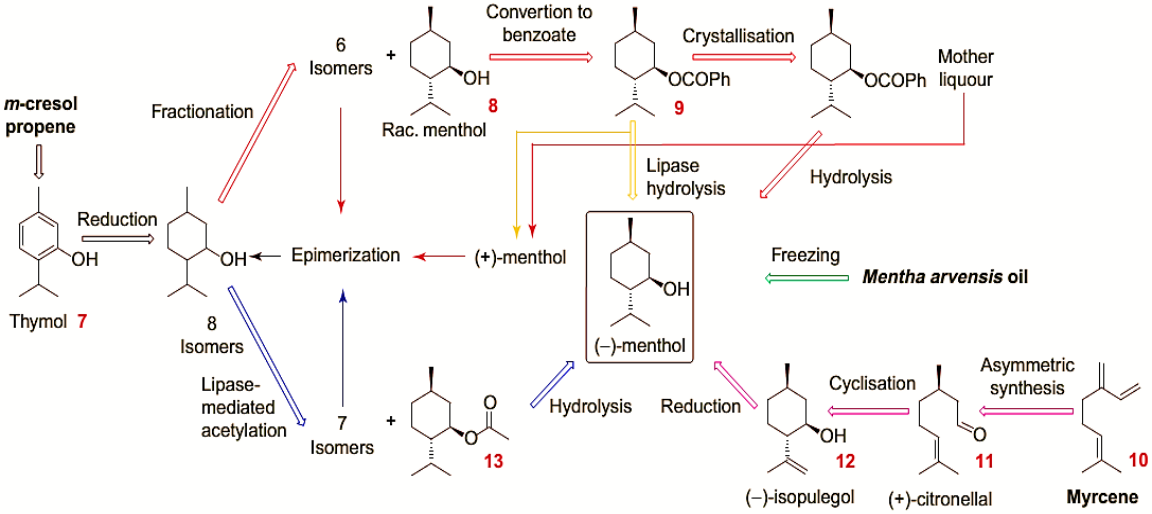

Figure I.6- Industrial production of (-)–menthol. In red we have Haarmann and Reimer process, in green the extractive process and in violet we have Takasago process. Also on the image we have the new biocatalytic process.

In the Takasago process myrcene was udes as the raw material (Figure I.6), which is converted to N,N-diethylgeranylamine (step 10) and then asymmetrically isomerized via the chiral rhodium (S)-BINAP (or SEGPHOS) complex to the optically active enamine of citronellal. Hydrolysis produces (+)-citronellal, which is cyclized to (–)-isopulegol (step 11). By hydrogenation, the isopulegol gives (–)-menthol (step 12) in high optical purity. An alternative starting material is isoprene, which can be dimerized to N,N-diethylnerylamine.[24]

Meanwhile, two other alternatives to the H&R process have been described in the Figure I.6, the first one are based on lipase resolution of racemic menthol benzoate (step 9) using highly enantioselective enzymes like Candida rugosa lipase giving (-)-menthol with a high yield and purity. The other process is based on an enantio- and diastereoselective acylation of the eight isomers of menthol producing a enantiomeric excess over 96%. Then, the ester is separated by destillation from the unreacted isomers and then hydrolysed to give pure (-)-menthol.[24]

As seen above, (-) and (+), menthol have the same physical properties, but different characteristics this makes the separation of enantiomers very complex. Sometimes countless

steps are needed, which generates massive amounts of waste and by-products and low income for the producer.[28]

One way to separate the enantiomers is to transform one into a different molecule, giving it different properties that facilitate the separation and minimize process steps. This way, we can separate efficiently (-) and (+)-menthol using green chemistry and achieving a high value product with extremely purity and low production costs.[28]

A highly selective method for the resolution of (±)-menthol is enzymatic esterification or transesterification. One of the methods to improve optical purity of enzymatic reaction products is to select a special acyl donor such as acid anhydride which makes the acyl transfer completely irreversible; the ester is then easily hydrolysed into free acids and does not produce water as a by-product. Therefore, the biosynthesis of lipase catalysed chemical reactions under mild conditions has been receiving much attention for producing these valuable products. An optimized enzymatic reaction of (-)-menthyl esters synthesis improves the conversion yield and reduces the production costs under most favourable conditions.[28]

Lipases (E.C. 3.1.1.3) have been widely used for the resolution of racemic alcohols through enantiospecific esterification and transesterification. Apart from Candida rugose, lipases from Candida cylindracea and Penicillium simplicissimum were found to be highly selective for the esterification of (-)-menthol.[28]

Chapter II

Materials and Methods

II. Materials and methods

a. Catalysts

- Novozyme 435 (immobilized from iCALB), Sigma Aldrich
  Activity: 5000 U/g, Temp optimum: 30-60 ºC, pH optimum: 5-9.
- Lipozym RM-IM (immobilized from Rhizomucor miehei), Sigma Aldrich
  Activity: 30 U/g, Temp optimum: 30-50 ºC, pH optimum: 6-8.
- Lipozym TL-IM (immobilized from Thermomyces lanuginosus), Sigma Aldrich
  Activity: 100 U/g, Temp optimum: 50-75 ºC, pH optimum: 7-10.
- Lipase from Candida rugosa (CRL), Sigma Aldrich
  Activity: 15-25 U/g, Temp optimum: 35-45 ºC, pH optimum: 6-8.

b. Reactants

- (±)-Menthol, Aldrich, ≥99%
- Vinyl Decanoate, Fluka, 95%
- Tridecane, Sigma, ≥99%
- Camphor, Sigma, ≥99%
- Lauric Acid, Sigma, 99%
- Phenylpropionic Acid, Sigma, 97%
- Choline Chloride, ≥97%
- Glycerol, Sigma, ≥99%
- Urea
- Hexane, Carlo Erba, 95%
- Dichloromethane, Carlo Erba, 99%
- 4-Dimethlaminopyridine (DMAP), Sigma, ≥99%
- Dicyclohexylcarbodiimide (DCC),Sigma, ≥99%

c. Materials

- Karl fischer 831 KF coulometer Metrohn
- Termoquest Trace GC 2000 series
- Auto sampler Thermo Finnigan AS2000
- Asus baberbone PC
- Unimax 1010, Heidolph instruments
- Inkubator 1000, Heidolph instruments
- DU800 Spectrophotometer, Beckman Coulter

d. Procedures

Synthesis of (±)-menthyl decanoate

To a solution of decanoic acid (1.89 g, 11 mmol) in dry dichloromethane (30 mL), DCC (2.26 g, 11 mmol) was added. After 1 h, (±)-menthol (1.37 g, 8.75 mmol) and DMAP (122 mg, 1 mmol) were added. The reaction was completed after 3 h. The mixture was filtered, and the filtrate was washed with H_2O. The organic layer was dried with anhydrous magnesium sulphate, filtered, and concentrated under reduced pressure. Then the mixture was purified by flash chromatography using silica gel and a mixture of hexane and dichloromethane (4:7) as washing solvent. The filtrate was later concentrated by removal of the solvent using a Rotary evaporator (BÜCHI Rotavapor R200) to evaporate it. The product was later evaluated by 1H-NMR and the purity determined by Professor Paula Branco.

Dichloromethane was dried with calcium hydride in a flask at reflux for 2h. This step is important because DCC is hygroscopic and absorbs water from the entire dichloromethane, preventing synthesis.

Calibration curves

After preparing the stock solutions of each compound, various dilutions were made to give a range of concentrations. The concentrations were obtained, in the case of menthol: 5, 10, 15, 20mM; menthyl decanoate: 1.25, 2.5, 5, 10, 12.5, 15, 20, 25 mM; vinyl decanoate: 2.5, 15, 20, 25mM; decanoic acid: 2.5, 5, 10, 15 mM; lauric acid and phenylpropionic acid: 5, 10, 15, 20, 25, 30 mM. The tridecane was present in a concentration of 5 mM in the case of menthol; 6 mM in the case of phenylpropionic acid and lauric acid; 6.25 mM in the case of decanoic acid, vinyl decanoate and menthyl decanoate.

In the construction of calibration curves have taken into account as the measured quantities, for a correction of concentration.

Then the curves were made as presented in appendix 1 for the calculation of each concentration.

Lowry's method

Prepare standard solutions of bovine serum albumin (BSA): 0, 20, 50, 100, 150, 200 mg/mL) was added to 200 µL distilled H_2O.

Prepare Lowry solution: add 250 µL of solution B and C 250 µL of solution and make up to 25 mL with solution A.

Prepare Folin solution: 2 mL Folin solution was added 2 mL distilled H_2O (1:1).

Prepare enzyme solution sample in distilled H_2O: 1 mg/mL may be diluted 1:10 to 1:100.

Experimental Procedure: Add 1 mL of Lowry solution of 200 µL of each sample (enzyme and standard BSA), vortex and wait 10 minutes. Add 200 µL Folin solution to each sample, vortex and wait 30 minutes. Read the absorbance at 750 nm.

For immobilized enzymes prior process is required: add 1 mL 1M NaOH to 10 mg of enzyme. Bath at 100°C for 10 minutes. Vortex and stand on ice for 5 minutes. Vortex and centrifuge at 5400 rpm for 10 minutes. Remove 200 µL of supernatant, representing the enzyme sample.

In this case, BSA standards are made using 1M NaOH solution to bring the volume to 200 µL.

The rest of the procedure is the same.

Enzyme activity

Prepare phosphate buffer pH 7, 0.2 M (32.7 g $Na_2HPO_4.7H_2O$ and 9.4 g $NaH_2PO_4.H_2O$ and 1 L H_2O).

Prepare a solution of p-nitrophenyl butyrate (pNPB) 60 mM in Acetonitrile (10.5 µL pNPB and 989.5 µL Acetonitrile).

Using the same enzyme preparations used in the Lowry method.

Experimental procedure: In a vial add 890 µL of 0.2 M sodium phosphate buffer pH 7 and 10 µL pNPB. Make white in the spectrophotometer. Add 100 µL of enzyme sample. Make reading the absorbance at 400 nm for 120 seconds.

Reactions in hexane with vinyl decanoate

In a 10 mL flask with a 5 mL solution of hexane we added 234 mg of menthol (300 mM), 263.14 µL of vinyl decanoate (300 mM), 200 mg of CRL and 52.16 µL (75 mM) of tridecane, as internal standard to the reaction, that occurs in a pre-heating bath over a hotplate from labnet (model: accuplate). During the reaction 75 µL samples were taken at 0 h, 0.5 h, 1 h, 2 h, 3.5 h, 5 h, 6 h and 24 h added 1050 µL of hexane and filtered using 0.20 µL pore syringe filters. At all time, a reaction media without enzyme was present to serve as blank and samples were taken and analysed with the reaction ones by gas chromatography in a suitable program.

This reaction was performed, in order to absorb the water in the reaction medium, avoiding the formation of Decanoic Acid, in different conditions:

- Addition of molecular sieves, which absorb the water in the reaction medium.

- Controlled atmosphere with lithium salt.

  In a closed flask containing a mixture of lithium salts with distilled water, put into a vail with hexane overnight to saturate the atmosphere. After this vail withdraws and places the vail to the reaction medium. Sampling is made through a needle in order to prevent the opening of the flask.

- Water activity controlled by addition of Na_2HPO_4/$Na_2HPO_4.2H_2O$ salts.

  Add 200 mg of each salt to the reaction medium to have a concentration of 42 g/L.

Synthesis of DES/NADES

DES/NADES were prepared by heating the mixtures of the corresponding reagents with the required molar ratio at the appropriate temperature to form a homogeneous liquid, and then allowed to be cooled to room temperature.

Table II.1- Molar ratio and formation temperature of DES/NADES used for reactions and separation. Note: These molar ratio represented are those used in the experimental work, other molar ratio also allow the formation of DES/NADES.

DES/NADES	Molar Ratio	Temperature
ChCl:urea	1:2	60ºC
ChCl:glycerol	1:2	60ºC
choline acetate:glycerol	1:1	Room temperature
choline dodecanediote:glycerol	1:1	Room temperature
menthol:phenylpropionic acid	2:1	Room temperature

menthol:lauric acid	2:1	Room temperature
menthol:camphor	1:1, 2:1, 3:1, 4:1	Room temperature

Reactions with DES/NADES

Various types of reactions were carried out:

- Use DES only as solvents.
  ChCl:urea, ChCl:glycerol were used as solvents for this reaction.
  In a flask with 4 mL of DES we added 187.2 mg of (±)-menthol (300 mM), 237.6 mg of vinyl decanoate (300 mM) and 160 mg of CRL. We used tridecane as an internal standard 55.2 mg (75 mM). The reactions were performed in a pre-heated bath at 40°C using a magnetic stir plate.
  During the reaction 100 µL samples were taken at 0 h, 1 h, 2 h, 4 h, 6 h and 24 h added 1500 µL of hexane to analyse by GC.

  These DES not dissolve in hexane and therefore the sample had to be done by extraction, so that it was necessary to carry out the “reaction shots”, wherein several replicas of the reaction medium, under the same conditions were used, wherein each replica matches a given reaction time.
  In order to ensure homogeneity of the reaction we made a substrate mixture containing 300 mM of (±)-menthol, 300 mM of vinyl decanoate and 75 mM of tridecane.
  In the case of DES ChCl:urea and ChCl:glycerol added 2 mL of DES in each vail and add 270 µL of a substrate mixture and 50 mg of CRL. The reaction was performed in the shaker at 40°C and 420 rpm.
  The sampling is done by two extractions with 2 mL of hexane each, 4 mL of extracted withdraw 1 mL to which 500 µL of tridecane joined to analyse by GC.

- Use DES as solvent, wherein one of compounds is the reaction substrate.
  For this reaction we use menthol:camphor, wherein only the Menthol is a substrate of the reaction.
  In a flask with 1 mL of DES we added 211.5 mg of vinyl decanoate (1000 mM) and 100 mg of CRL. The reaction was performed at room temperature using a magnetic stir plate.
  In a flask with 1 mL of DES we added 211.5 mg of vinyl decanoate (1000 mM) and 50 mg of CRL. The reaction was performed at room temperature using a magnetic stir plate.
  In a flask with 1 mL of DES we added 211.5 mg of vinyl decanoate (1000 mM) and 200 mg of CRL. The reaction was performed at room temperature and in a pre-heated bath at 40ºC using a magnetic stir plate.

The sampling is done taking a sample 20 µL of the reaction medium and adding 980 µL of hexane and 500 µL of tridecane (internal standard). Subsequently the sample is filtered with a 0.20 µm millipore filter to remove enzyme.

- Use DES as solvent, wherein the two compounds are the reaction substrates.
  For this reaction we use menthol:lauric acid and menthol:phenylpropionic acid, wherein the two compounds are the reaction substrates.
  In a flask with 1 mL of DES we added 62 mg of vinyl decanoate (315 mM) and 40 mg of CRL. We put a reaction in a preheated bath at 40 °C until 76 hours.
  The sampling is done taking a sample 50 µL of the reaction medium and adding 950 µL of hexane and 500 µL of tridecane (internal standard). Subsequently the sample is filtered with a 0.20 µm millipore filter to remove enzyme.
  In a flask with 2 mL of DES we added 396 mg of vinyl decanoate (2000 mM) and 100 mg of CRL. We put a reaction in a preheated bath at 40 °C until 240 hours.
  The sampling is done taking a sample 50 µL of the reaction medium and adding 950 µL of hexane and 500 µL of tridecane (internal standard). Subsequently the sample is filtered with a 0.20 µm millipore filter to remove enzyme.
  In the case of “reaction shots” we add to each vial 120 µL of DES, 12 mg of vinyl decanoate (58 mM) and 6 mg of CRL. We put a reaction in a preheated bath at 40 °C until 96 hours.
  The sampling is done by two extractions with 2 mL of hexane each, 4 mL of extracted withdraw 1 mL to which 500 µL of tridecane joined to analyse by GC.

Separation with DES/NADES

To test separation of the compounds obtained in the transesterification reaction decanoate synthesize menthyl which add menthol in the molar ratio 1:1. This mixture will subsequently be used in all tests separation.

In the case of ChCl:glycerol (1:2) and ChCl:urea (1:2) we used a flask with 1 mL of DES and 100 µL of a mixture containing menthol and menthyl decanoate (1:1). After 24 hours of stirring, withdrawn 50 µL sample of the organic phase and add 950 µL of hexane and 500 µL of tridecane to analyse by GC.

In the case of choline acetate:glycerol (1:1) we used a flask with 168 mg of DES and 100 mg of a mixture containing menthol and menthyl decanoate (1:1). In the case of choline propanoate:glycerol (1:1) we used a flask with 800 mg of DES and 125 mg of a mixture containing menthol and menthyl decanoate (1:1).

In the case of choline dodecanediote:glycerol (1:1) we used a flask with 698 mg of DES and 100 mg of a mixture containing menthol and menthyl decanoate (1:1). After that we made this separation with the following weight ratios (DES:mixture): 1:1, 3:1, 5:1, 7:1.

For each DES, after 24 hours of stirring, we removed the organic phase and withdraw 20 µL of sample and add 780 µL of hexane and 400 µL of tridecane to analyse by GC. After that, 2 extractions were made with hexane, from which we remove 500 µL and added 500 µL of hexane and 500 µL of tridecane to analyse by GC.

Samples analysis

The sample analysis is made by gas chromatography (GC) which is a common type of chromatography used in analytical chemistry for separating and analysing compounds that can be vaporized without decomposition. Some applications include testing the purity of a particular substance, or separating and quantifying the different components of a complex mixture.

The process of separating the compounds in a mixture is carried out between a liquid stationary phase and a gas mobile phase and the column through which the gas phase passes is located in an oven where the temperature of the gas can be controlled.

The GC system (Figure II.1) consists of gas supplies for the mobile phase, flow controls for the gases, a sample injector, an oven for heating the column, a detector, and a data recording device. Carrier gases commonly used are N_2, H_2, or He.

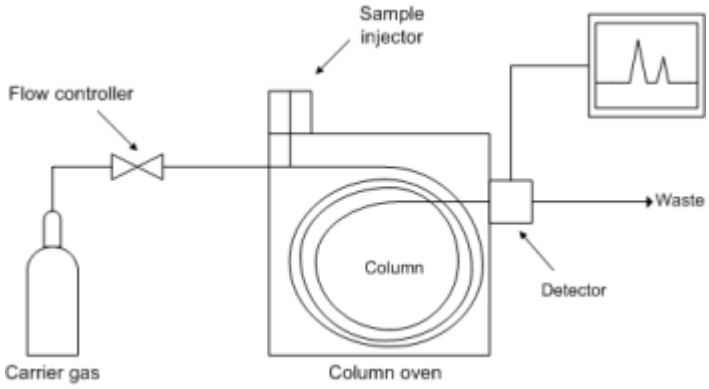

Figure II.1- Scheme of the GC system.[29]

The chromatograph that we used was a TermoQuest Trace GC 2000 Series with an autosampler Thermo Finnigan AS2000. The detector was a flame ionization detector (FID) and the compounds were separated in a Cyclodex B (chiral b-cyclodextrin; J&W Scientific) capillary column (0.25 mm I.D. x 30 m with 0.25 µm film).

This column is specific for the separation of alcohols and esters. With this column it is possible to separate chiral compounds without derivatization.

All the samples were diluted to a concentration present in the calibrations curves presented in Appendix, as internal standard we use tridecane and as solvent we used n-hexane.

The method that we used have a total duration of 90 minutes, beginning with an isothermal of 10 minutes at 90ºC followed by a ramp to 165ºC at a rate of 1ºC/min. The injector and the detector were set at 200ºC and 250ºC respectively. All peaks were identified recurring to Chrom-Card Data System program by Thermo Fisher Scientific and using tridecane as internal standard. This method is showed in the Figure II.2.

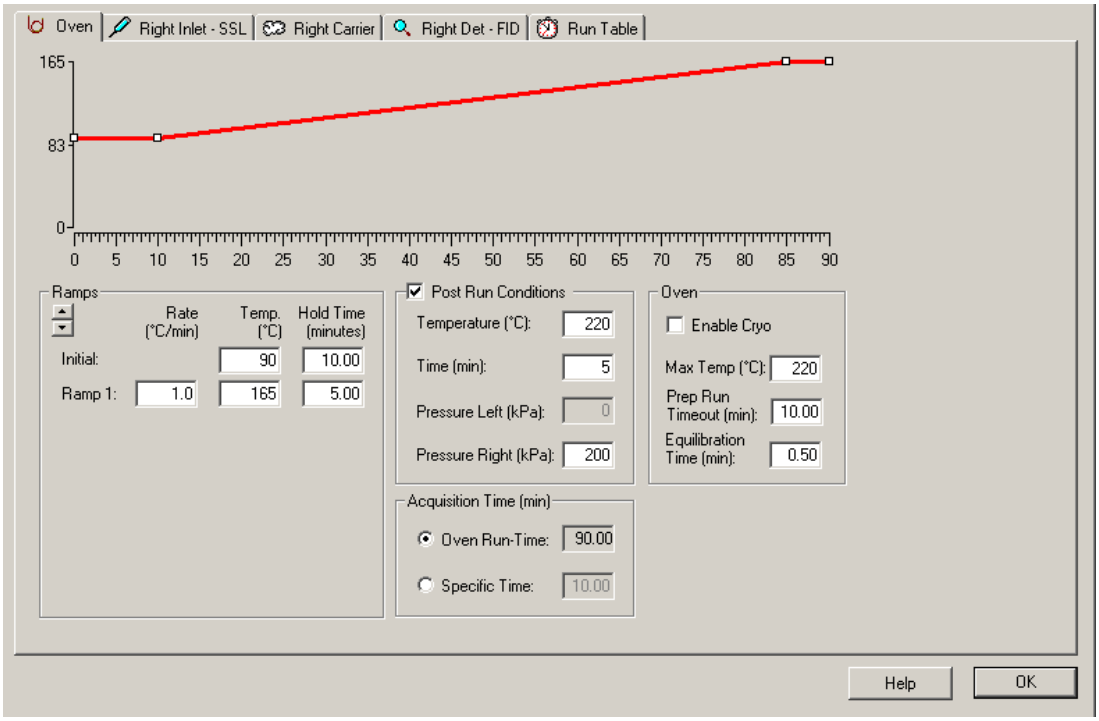

Figure II.2- Trace GC method for transesterification reaction of (±)-menthol and vinyl decanoate.

Chrom-Card Data System program enables us to identify the peaks present in the samples. An image of a typical chromatogram is showed in the Figure II.3.

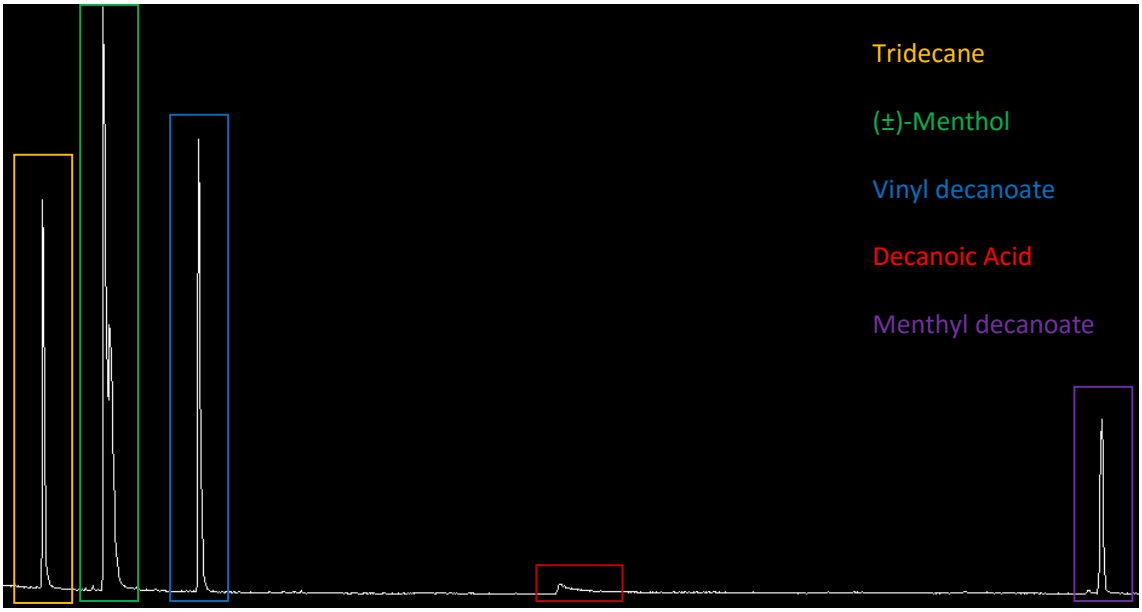

Figure II.3- Chromatogram for the reaction with vinyl decanoate, with identification of each peak.

In the Figure II.3 are presented the peaks obtained in the chromatograms. The first peak corresponds to tridecane, the second corresponds to (±)-menthol ((+)-menthol and (-)-menthol respectively), the third peak corresponds to vinyl decanoate, the fourth peak corresponds to decanoic acid and the last one corresponds to menthyl decanoate.

In the Table II.2 we have the retention time of each peak for the method we use.

Table II.2- Retention times of different compounds using the method for transesterification of (±)-menthol with vinyl decanoate, using tridecane as internal standard. Note: It is also represented the retention time of (±)-phenylethanol.

Compound	Retention time (min)
Tridecane	11.11
(+)-menthol	14.97
(-)-menthol	15.35
Vinyl decanoate	21.98
Decanoic acid	45.78
Menthyl decanoate	84.12
Lauric acid	63.61
Phenylpropionic acid	53.78
(±)-phenylethanol	11.97

Peak separation is good and the integration of all peaks allows to calculate de concentration of each one using calibration standards and recurring to the calibration curve, presented in Appendix.

Chapter III

Results and Discussion

III. Results and Discussion

In this work we intend to develop a simpler method for the separation of racemic menthol resorting to the use of enzymatic reactions. As mentioned above, this separation is possible if we modify one enantiomer selectively, making it different from the initial molecule, including its physical and chemical properties.

In this case, we use selective transesterification / acylation of the (-)-menthol recurring to the vinyl ester, more specifically vinyl deaconate[30] and CRL enzyme that is specific for (-)-menthol and therefore will only modify this enantiomer, making it different from (+)-menthol. Thus we obtain two enantiomers wherein one has been modified and has physical and chemical characteristics different from each other, making it easier and simpler separation process.

An example of this reaction is presented in the Figure III.1:

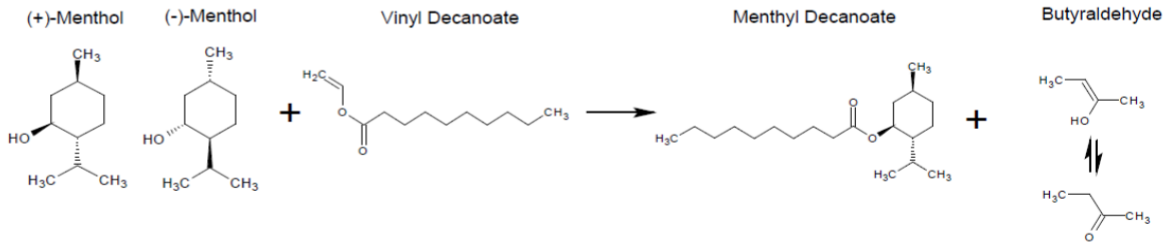

Figure III.1- Scheme of the reaction of (±)-menthol with vinyl decanoate producing menthyl decanoate.

The presence of water in the reaction medium, may promote the hydrolysis of vinyl decanoate, producing decanoic acid as a side reaction (Figure III.2).

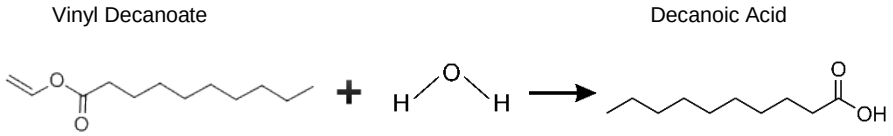

Figure III.2- Scheme of the reaction of vinyl decanoate with water producing decanoic acid.

This reaction was followed by sampling and analysis by GC and menthol conversion determined.

The conversion is obtained by recurring to a calibration curve to acquire the values of concentration of the racemic mixture, and then the conversion is calculated through the following equation:

$$Equation\ 1: Conversion\ (\%) = \frac{initial\ concentration\ (Ci) - concentration\ at\ time\ t\ (C_t)}{initial\ concentration\ (Ci)} \times 100$$

(Used for the calculation of the conversion of racemic menthol through the method described in Appendix)

The main objective of this work is to study the viability of DES for use not only as solvents for biocatalysis but also as substrates.

Therefore experiments were performed, for example, transesterification of menthol with vinyl decanoate in hexane by being a reaction has already been studied and also to be used as a comparison.

ChCl:glycerol (1:2) and ChCl:urea (1:2) were used as solvents for the reaction of menthol with vinyl decanoate.

Finally, to study the viability of DES as substrates we used the DES menthol:camphor (4:1), menthol:lauric acid (2:1) and menthol:phenylpropionic acid (1:1), such as proof of concept.

a. Reactions in Hexane

In order to have a comparison basis of this work it was carried out the reaction of (±)-menthol (300 mM) with vinyl decanoate (300 mM) using tridecane (75 mM) as internal standard and 40 mg/mL of CRL. The reaction was performed at 40ºC until 24 hours.

In the Figure III.3 can be seen the profile of the reaction of (±)-menthol with vinyl decanoate, using CRL.

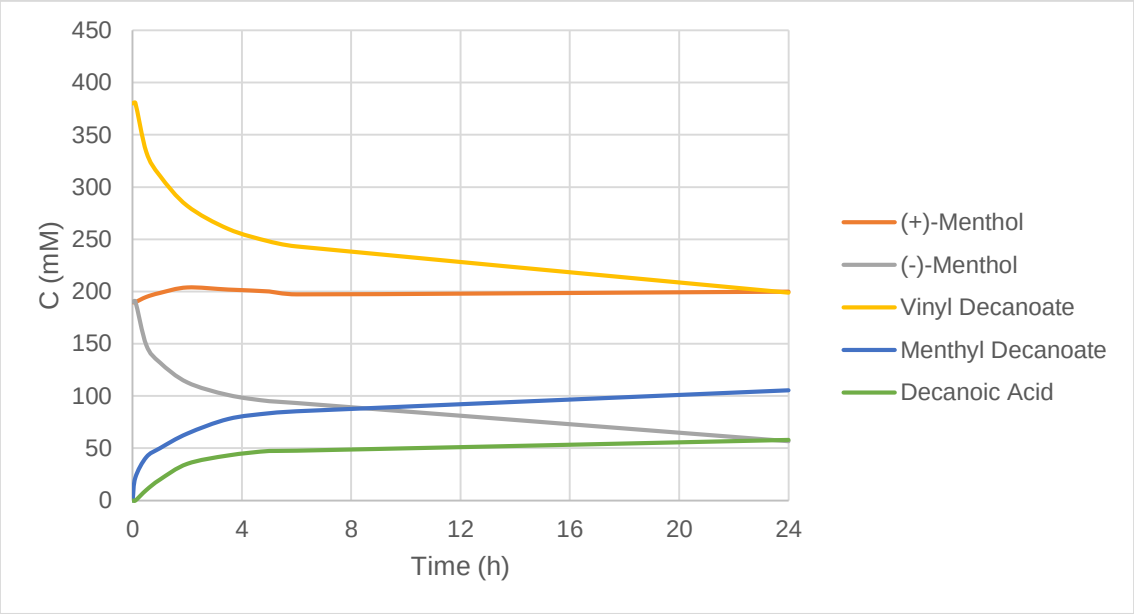

Figure III.3- Concentration values until 24 hours of reaction with 300 mM of (±)-menthol, 300 mM of vinyl decanoate, 75 mM of tridecane and 200 mg of CRL in 5 mL of hexane, at 40ºC.

Analysing the Figure III.3 we can observe the consumption of vinyl decanoate and the (-)-menthol, while the concentration of (+)-menthol remains constant, which shows the enzyme selectivity for the enantiomer R. Initially the concentration of vinyl decanoate is equal to the concentration of (±)-menthol, the latter will be the limiting reagent, and taking into account that only the (-)-menthol is reacted it is expected that vinyl decanoate does not react completely and that its final concentration will be about half the initial concentration.

Nevertheless the formation of decanoic acid resulting from hydrolysis of vinyl decanoate is also observed, lowering the selectivity of the reaction.

The formation of decanoic acid as a byproduct was significant, with a final concentration of 57.8 mM. Decanoic acid is formed by the hydrolysis of the vinyl decanoate. Therefore, the concentration of decanoic acid formed must be consistent with the amount of water in the reaction medium, which may be in the solvent, in the substrate and in the enzyme. Measurements carried out in the Karl Fischer for all components of the reaction medium showed that it contained about 51.5 mM of water and therefore all the water present in the middle, and possibly some coming from the atmosphere, is converted. Thus it is necessary to rigorously control the water content in the reaction media.

Five different methods of water control will be experimented: a normal reaction where no water control is applied; a reaction with addition of molecular sieves, to reduce the water that could be present in the solvent; a reaction in a controlled atmosphere with lithium salts[31]; a reaction with $Na_2HPO_4/Na_2HPO_4.2H_2O$ as internal salts; and a reaction in which the adding of internal salts $Na_2HPO_4/Na_2HPO4.2H_2O$ as conjugated with controlled atmosphere using lithium salts.

These conditions represent a variety of ways to reduce the presence of water in the reaction medium, which will consequently reduce the formation of decanoic acid.

In Figure III.4 we can observe the conversion values of (±)-menthol in transesterification reaction of (±)-menthol (300 mM) with vinyl decanoate (300 mM) using tridecane (75 mM) as internal standard and 40 mg/mL of CRL, at 40ºC until 24 h, using four methods to control water activity. These values were calculated using the equation 1.

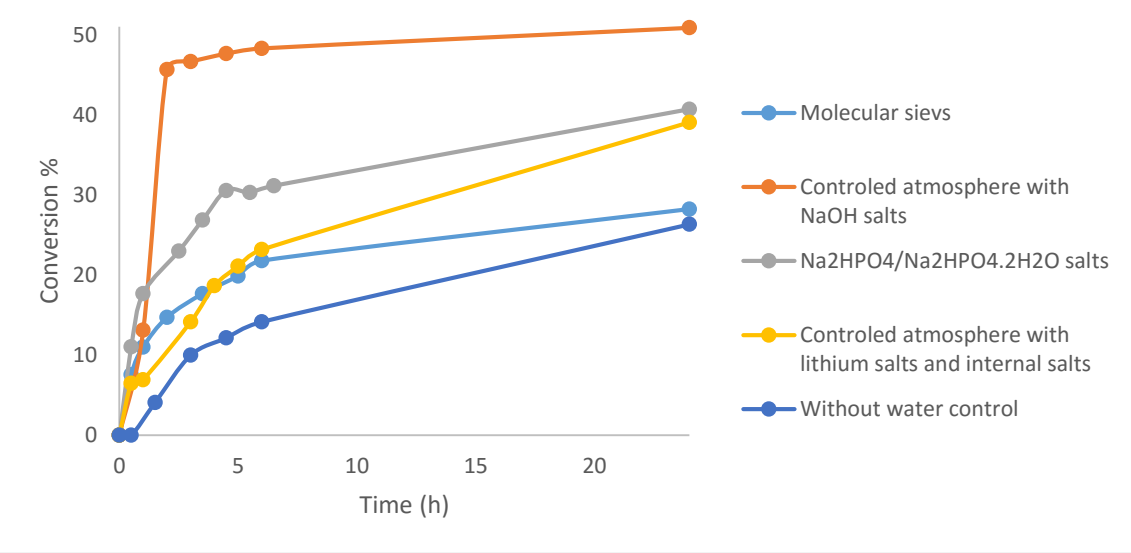

Figure III.4- Comparison of conversion values of (±)-menthol in transesterification reaction of (±)-menthol (300 mM) with vinyl decanoate (300 mM) using tridecane (75 mM) as internal standard and 40 mg/mL of CRL, at 40ºC until 24 h, using four methods to control water activity.

From Figure III.4 it can be seen that the highest conversion values are obtained for the reaction with a controlled atmosphere with lithium salts. We can see that all of the water control conditions applied result in an increase in (±)-menthol conversion, compared to the normal reaction. This is justified by the fact that there is less water available in the reaction medium, which is translated in a lower extent of the hydrolysis of the vinyl decanoate, as we see by the data in Table III.1, and hence the reaction thereof with the (-)-menthol is favoured, reflecting most consumed in a greater extent, favouring the production of menthyl decanoate which is the product of interest.

In Table III.1 are represented conversion values of vinyl decanoate and (±)-menthol, the yield values of menthyl decanoate and decanoic acid, as well the enantiomeric excess.

Table III.1- Conversion values of vinyl decanoate and (±)-menthol, and the yield values of menthyl decanoate and decanoic acid, corresponding to 24 hours of the reaction of (±)-menthol (300 mM) with vinyl decanoate (300 mM) using tridecane (75 mM) as internal standard and 40 mg/mL of CRL.

Reaction conditions	Vinyl Decanoate conversion (mol %)	(±)-Menthol conversion (mol %)	Menthyl Decanoate yield (mol %)	Decanoic Acid yield (mol %)	$e.e._s$ (%)
Normal reaction	55.6	26.3	25.4	25.9	38.8
Molecular sieves	78.2	28.2	28.4	15.6	57.5
Controlled atmosphere	92.0	50.9	50.9	19.8	100
Internal salts	94.2	40.7	40.7	28.8	78.6
Controlled atmosphere and internal salts	100	39.1	39.1	40.8	100

By analysing Table III.1 we find that the reaction that presents a better yield of menthyl decanoate is the reaction that we applied the condition controlled atmosphere with lithium salts.

We find that the conversion values of vinyl decanoate are always higher than the conversion values (±)-menthol since, in the latter, only the R-enantiomer is reacted with vinyl decanoate and is converted into menthyl decanoate. As vinyl decanoate reacts not only with the (-)-menthol but also with water, it is expected that its conversion is higher, but the greater difference between the conversion of the vinyl decanoate, and (±)-menthol, it means a greater amount of decanoic acid formed. One of the strategies which can be applied to verify in detail the existence of water in the reaction medium would also monitor the reaction with the Carl Fischer and so we would obtain the amount of water in each sample, not just the final sample. The yield of decanoic acid in all cases is less than expected, this may be due to analytical errors as seen by the calibration curve obtained for this compound shown in the Appendix.

The goal of eliminating decanoic acid formation was not successful, despite the strategies used found that continues to occur the reaction of hydrolysis. This is due to the fact that about 96% of water in the medium is from the enzyme.

b. Hexane/hydrophilic DES

As stated above, one of the objective of this study is to evaluate the kinetic resolution of menthol using DES/NADES. DES based in choline chloride (ChCl), in particular ChCl:glycerol and ChCl:urea, are very commonly used in enzymatic transesterification reactions.

In the transesterification reaction of vinyl laurate with three different alcohols (1-butanol, 1-octanol and 1-octadecanol) through iCALB, it was found that both ChCl:urea (1:2) and ChCl:glycerol (1:2) resulted in a conversion and selectivity as high as the reaction in toluene.[32] Thus, to test transesterification reaction of (±)-menthol (300 mM) with vinyl decanoate (300 mM),

using tridecane (75 mM) as internal standard and 160 mg of CRL, we used 4 mL of ChCl:glycerol (1:2) and ChCl:urea (1:2). The reaction was performed at 40ºC until 24 hours.

In Figure III.5 it is shown the conversion values of (±)-menthol obtained over the reaction with vinyl decanoate using DES as solvents. These conversion values were obtained using equation 1.

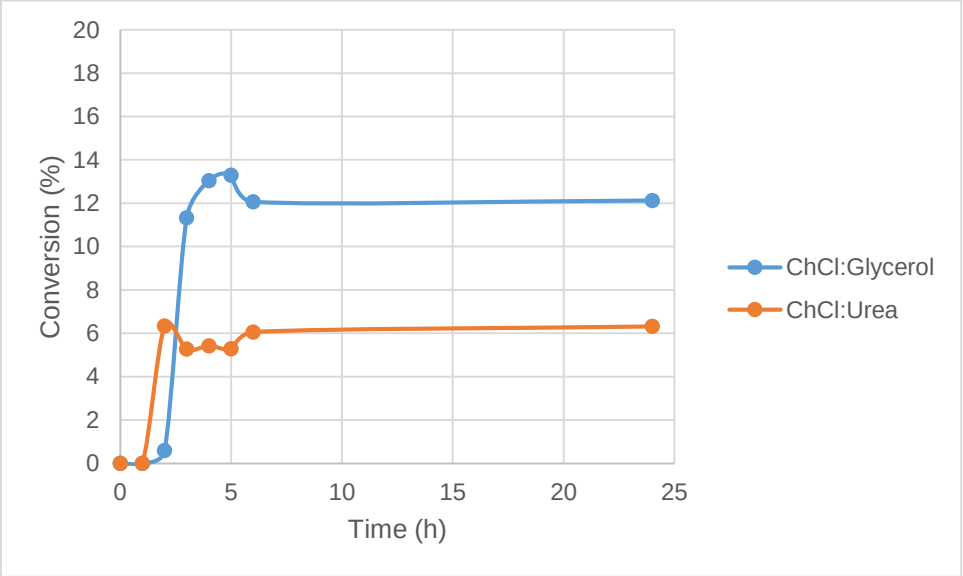

Figure III.5- Conversion values until 24 h of reaction with 300 mM of (±)-menthol , 300 mM of vinyl decanoate, 75 mM of tridecane and 160 mg of CRL in 4 mL of each DES: ChCl:glycerol (1:2) and ChCl:urea (1:2).

Analysing the Figure III.5 for the conversion of (±)-menthol with vinyl decanoate in ChCl:glycerol (1:2) and ChCl:urea (1:2) it is observed that the reaction takes place in both DES. Nevertheless it is also observed that this DES have very low values of conversion and after 3 hours of reaction the conversion of (±)-menthol remains constant over time. These reactions were performed in a 4 mL vial where samples of 100 µL were taken for analysis by gas chromatography. Due to the high viscosity of these DES the sampling could be compromised, which does not guarantee homogeneity of the sample. This viscosity can be measured to better analyze the results and we could measure the water content using the Carl Fischer along the reaction DES, since they are very hygroscopic.

Taking into account this factor, we conducted the experiments in “reaction shots”, in which several replicas of the reaction medium, under the same conditions were used, wherein each replica matches a given reaction time. In each specific reaction time the vial is removed and is

added hexane for the extraction of substrates and products compounds. The extracted portion is then analysed by gas chromatography.

In the Figure III.6 are shown the conversion values of (±)-menthol obtained during the transesterification reaction of (±)-menthol (300 mM) with vinyl decanoate (300 mM), using tridecane (75 mM) as internal standard. This reaction was conducted in “reaction shots” containing 2 mL of DES, 270 µL of a substrates mixture and 50 mg of CRL. The reaction was performed at 40ºC until 24 hours. These conversion values were obtained using equation 1.

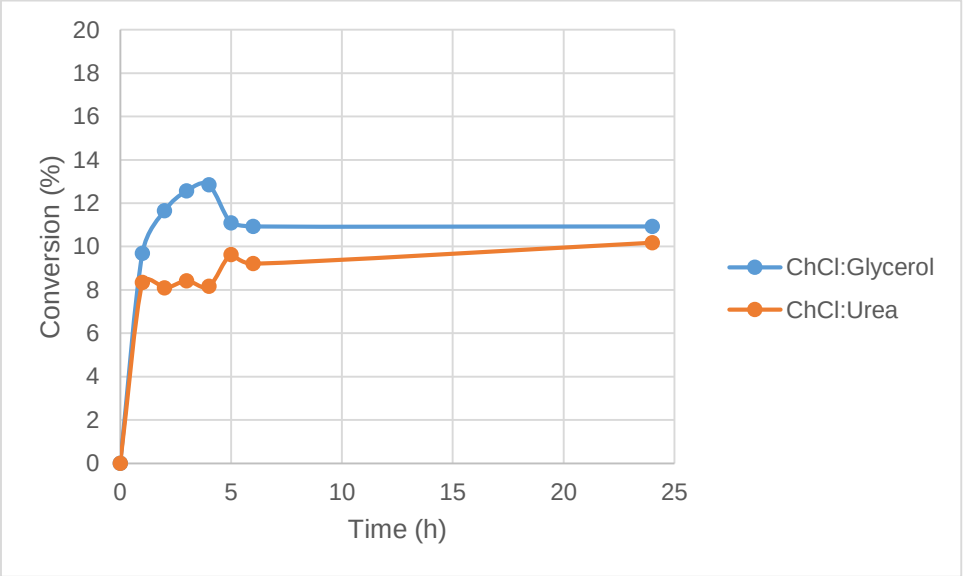

Figure III.6- Conversion values until 24 h of reaction with 270 µL of a Mix (300 mM of (±)-menthol, 300 mM of vinyl decanoate, 75 mM of tridecane) and 50 mg of CRL in 2 mL of each DES: ChCl:glycerol (1:2) and ChCl:urea (1:2).

For the analysis of the Figure III.6 we can see that the conversion values of (±)-menthol are still low and remain constant, in this case, after 2 hours of reaction. The conversion profile in ChCl:glycerol (1:2) is quite similar to the previous experiment, however, the reaction in ChCl:urea (1:2) has a similar profile but with higher conversion values. This may be due to the fact that there were corrections in the sampling, however this difference in values is not very significant.

We found that in both reactions, conversions in ChCl:glycerol (1:2) are always higher than in ChCl:urea (1:2). This fact may be due to the higher viscosity of ChCl:urea (1:2) and therefore higher diffusional limitations.

Comparing with Figure III.4 we can see that there is a similarity in the first hour of reaction, however, while the reaction in hexane the equilibrium is not reached, in the case of DES the reaction stops after one hour. Thus we can see that the solvent may interfere with enzyme activity.

The fact that the values remain constant over time may be due to an inhibition of the enzyme possibly caused by the amount of water present in the reaction medium. As stated above it is important to remove water from the reaction medium to prevent the formation of decanoic acid, but also to maintain the stability of the enzyme in the reaction medium.

The fact that the reaction stops after one hour, means that the enzyme has ceased to catalyse the reaction, i.e., the enzyme is inhibited. As these DES are highly hygroscopic there is the possibility of increasing the amount of water in the reaction over time, altering the acidity of the reaction medium, which may interfere with the conformation of the enzyme, even resulting in its denaturation.

In the literature have already been carried out as successful transesterification reactions in ChCl:glycerol (1:2) and ChCl:urea (1:2).[32]

In order to make a more comprehensive study, we tested the reaction of (±)-menthol with vinyl decanoate with other three enzymes which are used recurrently in transesterification reactions, such as, Novozyme 435, Lipozyme TL IM and Lipozyme RM IM.

To evaluate the catalytic activity of these enzymes in these solvents, we perform all reactions under the same conditions. The reaction was carried out in ”reaction shots”, in which several replicas of the reaction medium, under the same conditions were used, wherein each replica matches a given reaction time. Each reaction vail contained 2 mL of DES, 270 µL of a substrate mixture previously prepared (300 mM of (±)-menthol, 300 mM of vinyl decanoate and 75 mM of tridecane as internal standard) and 100 mg of the respective enzyme. The reaction was performed at 40°C on a shaker.

In the Table III.2 are shown the conversion values of the transesterification reaction of (±)-menthol with vinyl decanoate, in ChCl:glycerol (1:2), ChCl:urea (1:2) and hexane, using CRL, Novozyme 435, Lipozyme TL IM and Lipozyme RM IM.

Table.III 2- Maximum conversion values for (±)-menthol (300 mM) in transesterification reaction with vinyl decanoate (300 mM) , using CRL, Novozyme 435, Lipozyme TL IM and Lipozyme RM IM in ChCl:glycerol, ChCl:urea and hexane.

	ChCl:glycerol	ChCl:urea	Hexane
CRL	10.9	10.2	26.3
Novozyme 435	18.8	19.1	29
Lipozyme TL IM	25.7	22.1	19.5
Lipozyme RM IM	17.9	10.4	10.6

From the data of Table III.2 we can see that in CHCl:glycerol (1:2) and ChCl:urea (1:2), CRL is the enzyme which gives lower conversion values of (±)-menthol. Lipozyme TL IM is the enzyme that has better conversion values, however selectivity in this reaction is lower because this enzyme is not very selective for the (-)-menthol. The fact that the CRL have lower conversion

values may be due to the fact that it is the only enzyme that is not immobilized, and is more likely to suffer inhibition.

To complete this study also tested the transesterification reaction using another alcohol, (±)-phenylethanol. In this case we use the same enzymes and the same conditions, only modify the substrate mixture that contain 300 mM of (±)-phenylethanol, 300 mM of vinyl acetate and 75 mM of tridecane.

In the Table III.3 are shown the conversion values of the transesterification reaction of (±)-phenylethanol with vinyl acetate, in ChCl:glycerol (1:2), ChCl:urea (1:2) and hexane, using CRL, Novozyme 435, Lipozyme TL IM and Lipozyme RM IM.

Table III.3- Maximum conversion values for (±)-phenylethanol (300 mM) in transesterification reaction with vinyl acetate (300 mM) , using CRL, Novozyme 435, Lipozyme TL IM and Lipozyme RM IM in ChCl:glycerol, ChCl:urea and hexane.

	ChCl:glycerol	ChCl:urea	Hexane
CRL	31.8	28.7	17.9
Novozyme 435	27.5	4.9	19.1
Lipozyme TL IM	17.8	34.4	1.6
Lipozyme RM IM	-	20.2	11.2

Analysing the Table III.3 we can see that with the (±)-phenylethanol, CRL gives better conversion values than the obtained in Table III.2. CRL is not immobilized and probably is more susceptible to enzyme-substrate interactions, which may not be favourable to their catalytic capacity.

Analysing the Table III.2 e III.3, we see that there is not a significant difference between the values obtained in the conversion DES and hexane, even increased in some cases.

We intended a proof of concept, which has been successfully obtained, however, these reactions can be further developed and optimized to obtain higher conversion values of (±)-menthol.

c. DES Menthol

As stated above, the objective is to separate the enantiomers of menthol using DES/NADES as solvents. To do so, we first evaluated these solvents as suitable reaction medium. First we used DES based in ChCl, will now test reaction in DES based on menthol. We will synthesize and test reaction in DES containing one of the reaction substrates, in this case Menthol, and DES containing two of the reaction substrates, in this case menthol and a carboxylic acid. The latter approach is very advantageous since it allows us to perform the reaction without

solvent, since the substrates when combined in a given molar ratio are in liquid form and are themselves the reaction solvent. Therefore the reaction becomes more green and sustainable.

In the next section we will deepen the synthesis of DES, as well as the reactions carried out in DES-based menthol with one and two substrates in its composition.

Synthesis of DES composed by Menthol

For the synthesis of DES-based in menthol we had two approaches.

In the first case the DES will be composed by menthol and another compound that forms a DES but does not interfere in the desired reaction. In the second case, we use Menthol and another compound that forms a DES and which can be reacted to give the product of interest, in this case we have used carboxylic acids.

In the Table III.4 are presented the DES which were successfully synthesized as well as the molar ratios and temperatures corresponding to the liquid state.

Table III.4- Composition of DES synthesised and the molar ratios and temperatures corresponding to liquid state.

Compound 1	Compound 2	Molar ratio	Observations
Menthol	Camphor	4:1 – 1:1	Liquid at room temperature
Menthol	Phenylpropionic Acid	8:1 – 1:1	Liquid at room temperature
Menthol	Phenylpropionic Acid	1:2	Liquid above 31ºC
Menthol	Phenylpropionic Acid	1:3	Liquid above 33ºC
Menthol	Phenylpropionic Acid	1:4	Liquid above 40ºC
Menthol	Lauric Acid	8:1 – 2:1	Liquid at room temperature
Menthol	Lauric Acid	1:1	Liquid above 33ºC
Menthol	Lauric Acid	1:2	Liquid above 36ºC
Menthol	Lauric Acid	1:3	Liquid above 39ºC
Menthol	Lauric Acid	1:4	Liquid above 41ºC
Menthol	Miristic Acid	4:1	Liquid above 38ºC
Menthol	Miristic Acid	3:1	Liquid above 39ºC
Menthol	Miristic Acid	2:1	Liquid above 40ºC
Menthol	Miristic Acid	1:1	Liquid above 43ºC
Menthol	Miristic Acid	1:2	Liquid above 46ºC
Menthol	Miristic Acid	1:3	Liquid above 47ºC
Menthol	Miristic Acid	1:4	Liquid above 49ºC

For DES constituted by only one reaction substrate we used menthol:camphor, with molar ratio 4:1, since the menthol to be consumed remains as DES until the molar ratio of 1:1.[33] Other DES mixtures were also experimented, i.e., menthol:ChCl, menthol:urea, menthol:glycerol, menthol:citric acid, menthol:camphor:ChCl, however it was not possible to obtain a DES in any of these cases.

For DES consisting of two reaction substrates we used the menthol:phenylpropionic acid in the molar ratio 1:1, menthol:lauric acid in the molar ratio 2:1. menthol:miristic acid was not used for reactions since only reaches the liquid state at elevated temperatures which may compromise the stability of the enzyme.

.**Menthol:Camphor**

Let's look more closely at the reaction in the DES containing just one reaction substrate in its composition.

The fact that the DES menthol:camphor (4:1) has been successfully synthesized, does not mean that the reaction occurred in this solvent. Therefore, since this approach is to be done first, we will focus to see if the reaction is carried out in these DES and subsequently testing some conditions.

Therefore we placed in three vials, 1 mL of DES menthol:camphor (4:1), add vinyl decanoate (1000 mM) and enzyme CRL (50, 100 and 200 mg). This reaction was carried out at room temperature (approximately 20°C) with magnetic stirring. In Figure III.7 are shown the conversion values of (±)-menthol. These conversion values were obtained using equation 1.

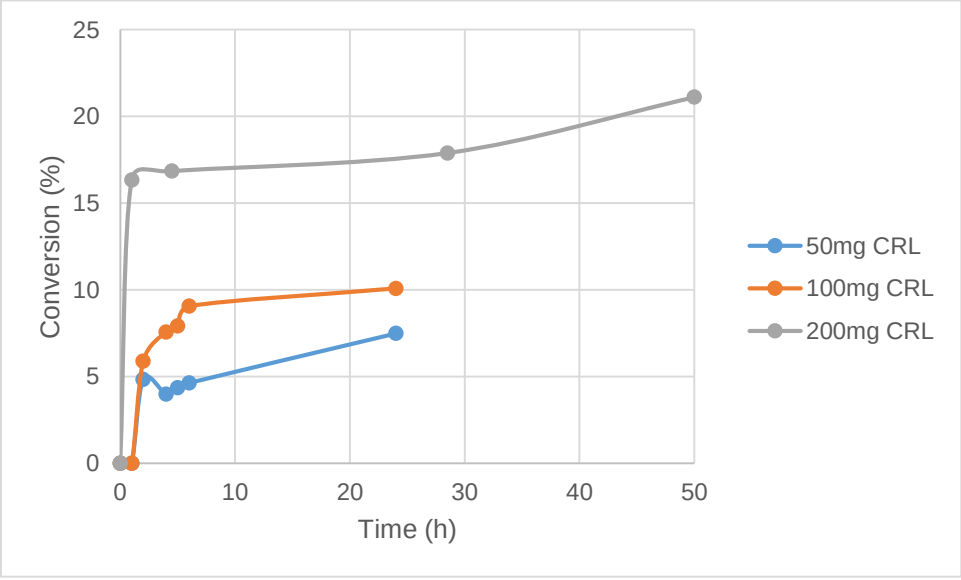

Figure III.7- Conversion of (±)-menthol with vinyl decanoate (1000 mM), using three different quantities of enzyme CRL (50, 100 and 200 mg), until 24h and 50h, at 20 ºC in 1 mL of DES.

From Figure III.7 it can be seen that the reaction occurs in these DES and therefore it is possible to perform the reaction using DES in which one of its compounds is a reaction substrate. We also found that with 200 mg of enzyme (5 times the amount used in Hexane) we obtain a conversion comparable to the reaction conducted in Hexane, and even superior to reactions carried out in ChCl:urea (1:2) and ChCl:glycerol (1:2). This may be due to the fact that these DES are not hydrophilic and thus there is less amount of water present in the reaction medium, hindering the hydrolysis reaction. This reflects what we observe in the reaction analysis in which we found that the amount of decanoic acid formed is much lower than the amount of menthyl decanoate formed, which did not happen in previous reactions.

After we find that with 200 mg enzyme we are able to obtain, at room temperature (approximately 20°C), a conversion similar to that obtained by using hexane as solvent at the same water control conditions, we tested the effect of temperature.

Two reactions were carried out simultaneously, one in which the vial was left with magnetic stirring at room temperature and another in a preheated bath at 40°C.

In the Figure III.8 is shown the conversion values of (±)-menthol over time of the reaction of (±)-menthol with vinyl decanoate (1000 mM), using 200 mg of CRL, until 50 h at 20ºC and 40ºC in 1 mL of DES.

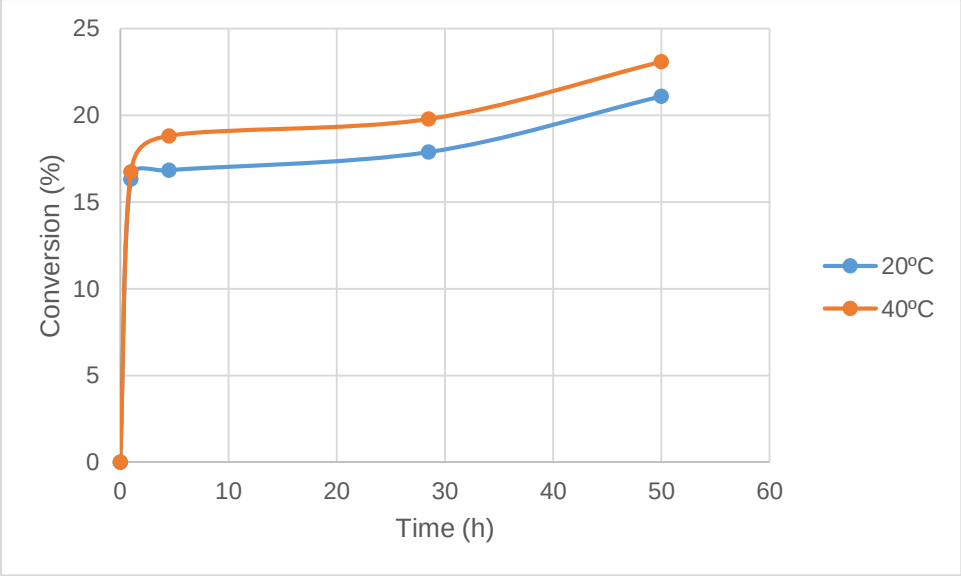

Figure III.8- Conversion in the reaction of (±)-menthol with vinyl decanoate (1000 mM), using 200 mg of CRL, until 50 h at 20ºC and 40ºC in 1 mL of DES.

Analysing the Figure III.8 we can see that increasing temperature is not a significant factor for a higher conversion, with only an increase of 2 % in conversion after 1 hour of reaction.

The main result is the successful proof of concept. In addition to the transesterification reaction be feasible at this DES, we find that the conversion values obtained are similar to those obtained for the reaction in hexane under normal conditions (Figure III.4).

Menthol:Lauric Acid and Menthol:Phenylpropionic Acid

The main objective of this work is to achieve a more simple and effective separation method of (±)-menthol using DES as solvents. Another objective was to test if the reaction occurs in the DES wherein the compounds are the reaction substrates. This concept has never been tested, and so, initially we wanted a proof of concept.

In this case we synthesized successfully two DES using menthol and a carboxylic acid, menthol:lauric acid (2:1) and menthol:phenylpropionic acid (1:1).

Two reactions were performed for each DES. At first, just add the enzyme to the DES to test the reaction of esterification. In the second, added vinyl decanoate and enzyme, in this case we will test the competition between esterification reaction and transesterification.

In the Table III.5 are shown the maximum conversion values for (±)-menthol obtained in reactions in which use the DES menthol:phenylpropionic acid (1:1) as solvent.

Table III.5- Maximum conversion values of (±)-menthol, using the DES menthol:phenylpropionic acid (1:1) as solvent. The conditions of reaction are presented in the table.

Addition of CRL to DES (mol %)	Addition of CRL and Vinyl Decanoate to DES (mol %)	Conditions
56.9	62.1	1 mL of DES, 40 mg of CRL and 315 mM of vinyl decanoate, 40 ºC, 76 h
21.4	17.1	2 mL of DES, 100 mg of CRL and 2000 mM of vinyl decanoate, 40 ºC, 240 h
14.2	-	120 µL of DES, 6 mg of CRL and 58 mM of vinyl decanoate, performed at “reaction shots”, 40 ºC, 96 h

From the data in the Table III.5, we can see that the reaction occurs in this solvent under both conditions. However, results were inconsistent, in that the analytical part is not yet well developed.

The most important thing in this case was succeed proof of concept that we wanted to test and discover that it is possible to carry out the reaction using DES as solvent, in which DES is itself composed of the reaction substrates. Regarding the fact that we use the vinyl decanoate or not, the results were not very conclusive for this DES.

In the case of reactions carried out using the DES menthol:lauric acid (2:1), the results were more conclusive. This carboxylic acid has a different structure from the previous one because it contains a straight chain rather than an aromatic ring. This factor may influence the reaction because, besides being the solvent, this compound is a substrate of the reaction and its structure can difficult the course of the reaction. In the case of lauric acid, the reaction path can be easier due to the similarity of its structure to vinyl decanoate.

In the Table III.6 are shown the maximum conversion values obtained in reactions in which use the DES menthol:lauric acid (2:1) as solvent.

Table III.6- Maximum conversion values of (±)-menthol, lauric acid and vinyl decanoate, using the DES menthol:lauric acid (2:1) as solvent. The conditions of reaction are presented in the table.

Addition of CRL to DES (mol %)		Addition of CRL and Vinyl Decanoate to DES (mol %)			Conditions
Menthol	Lauric Acid	Menthol	Lauric Acid	Vinyl Decanoate	
79.6	100	67.5	61.4	100	1 mL of DES, 40 mg of CRL and 315 mM of vinyl decanoate, 40 ºC, 76 h
45	100	34.1	46.8	71.9	2 mL of DES, 100 mg of CRL and 2000 mM of vinyl decanoate, 40 ºC, 240 h
36.9	94.1	29.6	30.4	86.7	120 µL of DES, 6 mg of CRL and 58 mM of vinyl decanoate, performed at “reaction shots”, 40 ºC, 96 h

Analysing the results in the Table III.6 we can see that the conversions obtained for menthol in this DES are superior to the conversions obtained using the DES menthol:phenylpropionic acid (1:1), in any condition. As noted above, this may be due to the difference in their structures.

Once again it was a successful proof of concept.

In this case we can evaluate the difference between whether or not to add vinyl decanoate. In the case where we only added enzyme, lauric acid is converted in its entirety, whereas in the case where vinyl decanoate is added in the reaction medium the conversion of lauric acid is always smaller than the aforementioned condition and it is also smaller than the conversion of vinyl decanoate.

In the situation we add vinyl decanoate there is competition between the esterification reaction and transesterification reaction and two compounds are formed, menthyl decanoate and menthyl laurate. We can see that the transesterification reaction overlaps the esterification reaction, however, the esterification reaction alone shows very promising results.

The great advantage to perform the reaction without using the vinyl decanoate is that in this case no hydrolysis reaction occurs and therefore no decanoic acid formation nor other by-product.

In the reaction run in these DES, we still find that this method is quite advantageous, since it is not necessary to resort to using solvents and do not give by-products makes this completely green and sustainable reaction.

d. Separation

Through the enzymatic transesterification of (±)-menthol it is possible to convert one of the enantiomers into a different chemical entity. Nevertheless the two compounds, the unreacted alcohol and the ester product, still remain in the reaction medium. A downstream process is necessary for the fractionation and recovery of the enantomerically pure products.

Due to the characteristics of DES it is expected that they will have a higher affinity towards the alcohol than the ester. This affinity is related to the ability to form hydrogen bonds.

The selectivity will be determined by the separation factor, calculated by the equation 2:

$$Equation\ 2:\ \propto = \frac{\left(\frac{Concentration\ of\ menthol\ in\ DES\ phase}{Concentration\ of\ menthol\ in\ organic\ phase}\right)}{\left(\frac{Concentration\ of\ menthyl\ decanoate\ in\ DES\ phase}{Concentration\ of\ menthyl\ decanoate\ in\ organic\ phase}\right)}$$

The first experiments were made with DES ChCl:glycerol and ChCl:urea because the ideal is to have a one-spot reaction.. Although the reaction occurs in these DES, the separation of the enantiomers revealed to be inefficient, since the calculated separation factors were close to 1 (α=1.2 to ChCl:urea and α=1.3 to ChCl:glycerol, in 1:1 DES/mixture mass ratio).

Thus, it was necessary to find other DES that best fit our goal. In partnership with Instituto Superior Técnico, who synthesized three compounds with different characteristics, particularly in the size of the alkyl chain between the functional groups. These compounds, i.e., choline acetate, choline propanoate and choline dodecanediote, were then used for the formation of DES.

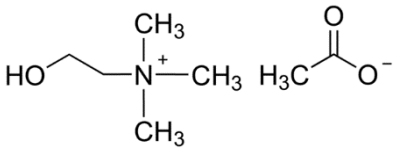

Figure III.9- Choline acetate.

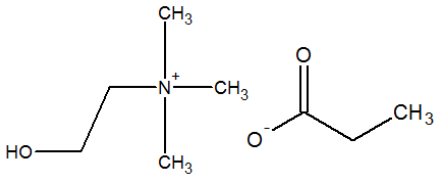

Figure III. 10- Choline propanoate.

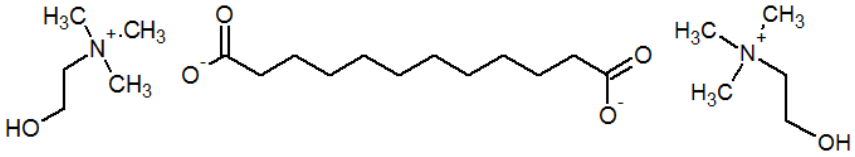

Figure III. 11- Choline dodecanediote.

These compounds were used to form three DES: choline acetate:glycerol (1:1), choline propanoate:glycerol (1:1) and choline dodecanediote:glycerol (1:1).

To test the separation factor, a mixture of (±)-menthol and menthyl decanoate in a 1:1 molar ratio was used.

Table III.7- Separation factor in choline acetate:glycerol (1:1), choline dodecanediote:glycerol (1:1) and choline propanoate:glycerol (1:1).

DES	DES/mixture mass ratio	Separation factor
choline acetate:glycerol (1:1)	1.7	1.1
choline dodecanediote:glycerol (1:1)	7	16.38±6.53
choline propanoate:glycerol (1:1)	6.4	1.5

Analysing the separation factors represented in the Table III.7, we find that DES choline acetate:glycerol (1:1), choline propanoate:glycerol (1:1) and choline dodecanediote:glycerol (1:1) are able to efficiently separate menthol from menthyl decanoate, however in the case of choline acetate:glycerol (1:1) and choline propanoate:glycerol (1:1), this separation is not very significant.

Choline dodecanediote:glycerol (1:1) was the DES that showed better results in the separation of enantiomers.

Solubility studies were also performed for the two compounds in choline dodecanediote:glycerol (1:1) at room temperature. The solubility (±)-menthol and menthyl decanoate, was 0.012 g/mL and 0.006 g/mL, respectively.

To evaluate the importance of the mass ratio of the separation, we conducted a study, shown in Table III.8, which tested the separation of the two compounds on DES choline dodecanediote:glycerol (1:1) using four different mass ratios, 1:1, 3:1, 5:1, 7:1, wherein we use respectively 100, 300, 500 and 700 mg of DES to 100 mg of a mixture containing (±)-menthol and menthyl decanoate (1:1 molar ratio).

Table III.8- Separation factor for different mass ratio of DES:mixture using the DES choline dodecanediote:glycerol (1:1).

Mass Ratio DES:Mix	1:1	3:1	5:1	7:1
Separation factor	0.75±0.12	1.66±0.002	2.65±0.45	16.38±6.53

We observed that increasing the weight ratio is favourable for an increase in separation factor.

Possibly, when we have a larger mass ratio, more hydrogen bonds available to dissolve the (+)-menthol, contrary to ester which does not have as much ability to form hydrogen bonds.

Chapter IV

Conclusion

IV. Conclusion

The objective of this work was the study of the transesterification reaction of (±)-menthol with vinyl decanoate using DES. Moreover, we wanted to test DES, not only as solvents, but as separating agents to achieve the second goal of this work which is the physical separation of the reaction products.

First we performed the transesterification reaction in hexane for comparison between the reaction using DES as solvents and using organic solvents. In this case, using hexane as reaction medium, a (±)-menthol conversion of 26.3 % was achieved. Hereupon, this reaction was tested by varying the requirements as to control the water content in the reaction medium and in the atmosphere, where a maximum conversion of 50.9 % was achieved after 24 hours at 40°C. Despite the strategies used, we found that the decanoic acid formation had only a slight decrease and the strategies used were not successful. After analysis of the amount of water in the solvent, substrates and enzyme, we found that about 96 % of water existing in the medium is from the enzyme. In the future it is necessary to develop an effective strategy to remove the water present in the enzyme.

Using DES only as solvents, ChCl:glycerol (1:2) and ChCl:urea (1:2), we achieved conversion values of 10.9 % and 10.2 % respectively. These values are not as high as expected, and the reaction reach the equilibrium (stopped) before the 2h reaction time. Nevertheless it was found that the conversion values are similar to the ones obtained from reactions carried out in hexane, at the same conditions. In the future, should be conducted more tests regarding catalytic stability of the enzymes, especially CRL, in these DES.

Using DES containing (±)-menthol, namely, menthol:camphor (4:1), a conversion of (±)-menthol with vinyl decanoate of 23 % was obtained at 40°C after 50 hours. In the future, should be applied other conditions to the reaction, namely, initial concentration of substrate, reaction time, temperatures, etc.

Using DES as both substrates of the reaction, i.e., menthol:lauric acid (2:1) and menthol:phenylpropionic acid (1:1), the (±)-menthol conversion value obtained are higher with a maximum of 79.6 % and 62.1 % respectively. In the esterification reaction, there is no formation of decanoic acid. Using this strategy there is no need for the use of additional solvents,turning this process into a more sustainable and “green” alternative. In the future, the analytical part must be optimized.

For the separation of (±)-menthol and menthyl decanoate using DES, we tested ChCl:glycerol (1:2), ChCl:urea (1:2), choline acetate:glycerol (1:1), choline propanoate:glycerol (1:1) and choline dodecanediote:glycerol (1:1). It was found that the latter was the most effective with a separation factor of 16±6.5.

Chapter V

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V. Bibliography

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Chapter VI

Appendix

VI. Appendix

Calibration curve for (+)-menthol, (-)-menthol and (±)-menthol

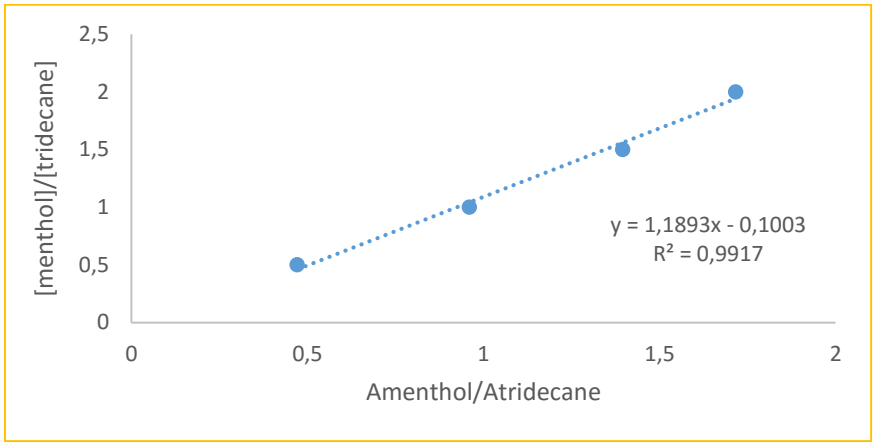

Figure VI.1- Graphic for the calibration curve of (+)–menthol.

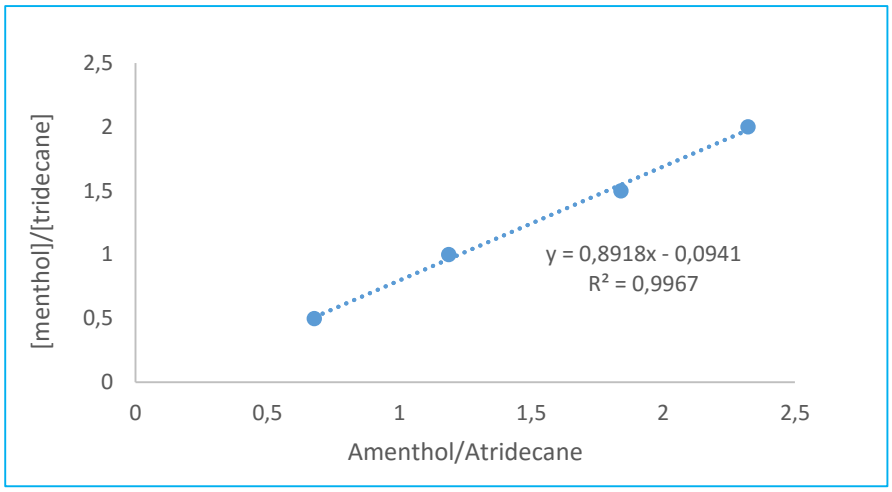

Figure VI.3- Graphic for the calibration curve of (-)-menthol.

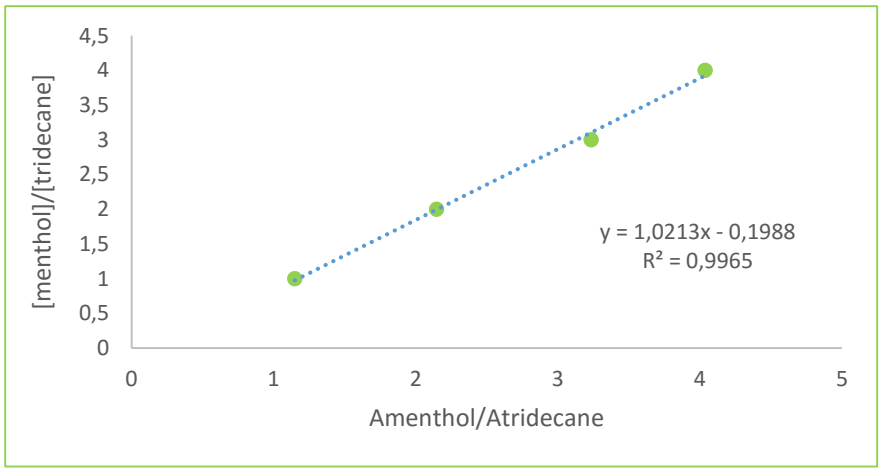

Figure VI.2- Graphic for the calibration curve of (±)-menthol.

Calibration curve for vinyl decanoate

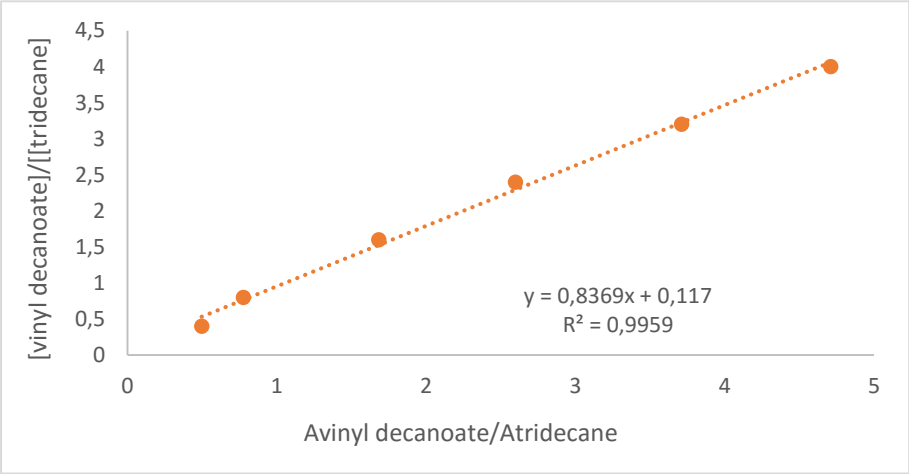

Figure VI.4- Graphic for the calibration curve of vinyl decanoate.

Calibration curve for decanoic acid

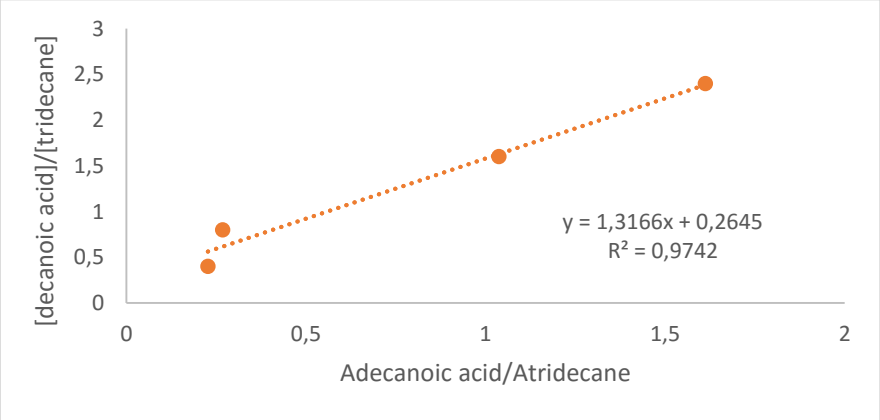

Figure VI.5- Graphic for the calibration curve of decanoic acid.

Calibration curve for menthyl decanoate

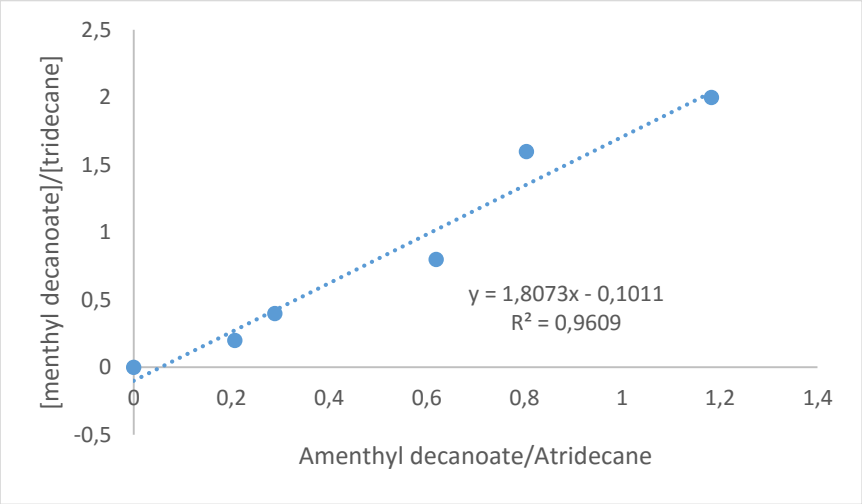

Figure VI.6- Graphic for the calibration curve of menthyl decanoate.

Calibration curve for phenylpropionic acid

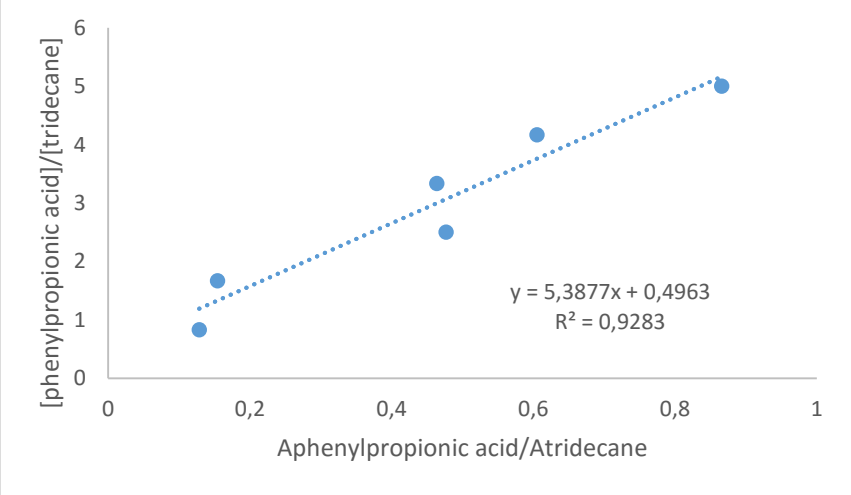

Figure VI.7- Graphic for the calibration curve of phenylpropionic acid.

Calibration curve for lauric acid

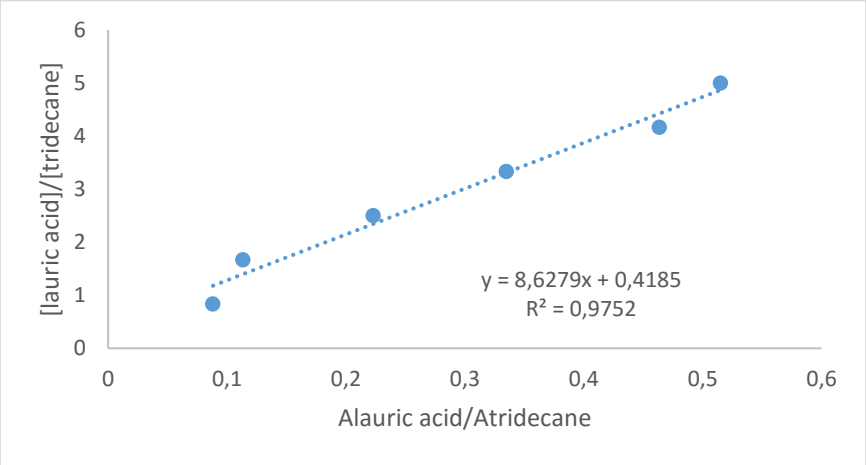

Figure VI.8- Graphic for the calibration curve of lauric acid.

Calibration curve for (±)-phenylethanol

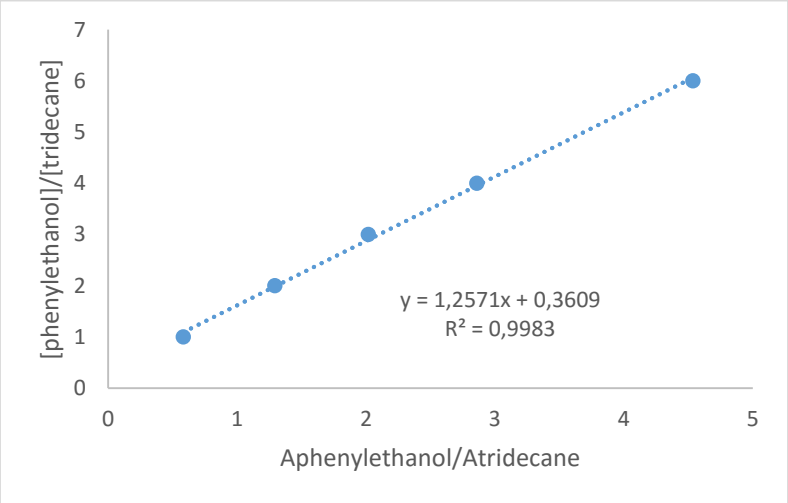

Figure VI.9- Graphic for the calibration curve of (±)-phenylethanol.