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DOTTORATO DI RICERCA IN SCIENZE BIOMEDICHE E BIOTECNOLOGICHE

CICLO XXXIII

COORDINATORE Prof. Pinton Paolo

ENZYMATIC PROCESSES FOR THE PREPARATION OF ESTERS OF POORLY WATER-SOLUBLE CARBOXYLIC ACIDS

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Dottorando

Dott. Zappaterra Federico

Tutore

Prof. Bernardi Francesco

Co-tutore

Dott.ssa Elena Tamburini

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ABSTRACT

The bioavailability of an active ingredient of industrial interest is the parameter that most influences its translation to a marketable bioactive molecule. Bioavailability is largely influenced by the water solubility of the active ingredient. Most of the new chemical entities (NCE) are poorly soluble in water and therefore poorly bioavailable. To overcome these limitations, protocols can be developed to produce pro-drugs with increased solubility in water and, so, bioavailability. The modifications of the active ingredients can take place chemically or, alternatively, enzymatically. This work proposes the development of enzymatic esterification protocols of active ingredients of agri-food and pharmaceutical interest.

Among the active ingredients studied, sorbic acid is the most commonly used preservative in the food industry. The antimicrobial inhibition of sorbic acid could be influenced by its lipophilic nature, which reduces its use in hydrophilic food formulations. Reactions between sorbic acid and glycerol catalyzed by lipases were studied in order to develop a novel sorbic acid derivate with a promising hydrophilic profile. The esterification reaction between sorbic acid and glycerol in a solvent-free system were performed with an immobilized lipase B from *Candida antarctica* (CALB).

Among the active pharmaceutical ingredients studied, ibuprofen is the widely used NSAID limited in bioavailability by its poor water solubility. In this thesis, we proposed an effective optimized enzymatic process for the production of the enhanced-water soluble ibuprofen sorbitol ester prodrug. The direct enzymatic route has been optimized for: biphasic media dynamics, enzyme concentration, water content, temperature, stirring speed, substrates concentrations, and reaction time. ^{1}H, ^{13}C-NMR, and MS confirmed the PPL-catalyzed esterification of ibuprofen with sorbitol. To the best of our knowledge, this is the first time that enzymatic

esterification of ibuprofen with sorbitol (as well as ibuprofen erythritol ester, and xylitol ester), has been proposed. This process initiates the synthesis with the biocatalytic approach of numerous bioactive molecules with increased bioavailability of pharmaceutical-industrial and agri-food interest.

Moreover, we have developed an enzymatic esterification protocol emancipated from the presence of water. This monophasic protocol, for which a patent has been deposited, sees the use of a single organic solvent for the solubilization of all substrates in reaction, avoiding many of the limitations imposed by the canonical O/W biphasic systems.

Finally, the enzymatically synthesized prodrugs were tested for activity. The glycerol sorbate product has been tested against *Streptomices griseus* bacterium and *Saccharomyces cerevisiae* yeast. Results indicate that the esterification of sorbic acid with glycerol does improve its antimicrobial properties against *Saccharomyces cerevisie*. The reported results demonstrate that esterification can be used as a strategy to improve the antimicrobial activity of sorbic acid.

Ibuprofen derivatives were tested *in vitro* via the IB3-1 cell line from fibrocystic patients. The derivatives have been shown to maintain the anti-inflammatory activity of ibuprofen and, in some cases, appear to improve it.

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ABBREVIATIONS

2-MAG - 2-monoacylglycerol
4MCA - 4-methoxy cinnamic acid
A2 - thromboxane
ACS - american chemical society
APCI - atmospheric-pressure chemical ionization
ATP- adenosine triphosphate
AUC - area under the curve
CA - cholic acid
CALB - *Candida anctartica* lipase type B
CDCA - chenodeoxycholic acid
cDNA - complementary DNA

CF - cystic fibrosis

CFTR - cystic fibrosis transmembrane conductance regulator

CFU - colony forming units

Cmax - maximum concentration

CMC - critical micellar concentration

COX - cyclooxygenase

COX-1 - isoform 1 of cyclooxygenase enzyme

COX-2 - isoform 2 of cyclooxygenase enzyme

Ct - threshold cycle

d6-DMSO - deuterated dimethyl sulfoxide

DCA - deoxycholic acid

DCC – dicyclohexylcarbodiimide

DD - disk dsiffusion

DMAP - 4-dimethylaminopyridine

DMSO - dimethyl sulfoxide

dsDNA - double-strand DNA

E2 - prostaglandin

EDTA - Ethylenediaminetetraacetic acid

ESI - electrospray Ionisation

EtOH - ethanol

FAD - flavin adenine dinucleotide

FBS - fetal bovine serum

FCC - food chemicals codex

g/L - grams per liter

G3P - glyceraldehyde 3-phosphate

GAPDH - glyceraldehyde 3-phosphate dehydrogenase

GI - glycemic index

GKD - glycerol kinase

GYM - glucose-Yeast extract-Malt extract

HepG2 - liver cell line

Hex – hexane

HPLC - high performance liquid chromatography

HPTLC - high performance thin layer chromatography

HPTLC-MS - high performance thin layer chromatography – mass

IB3-1 - cellosaurus cell line

II - insulin index

IL-1 - pyrogen interleukin

IR – Infrared

LCA - lithocolic acid

LHC-8 - bronchial epithelial cells medium

logP - octanol/water partition coefficient

Lt - threshold line

m/z - mass on charge

MALDI - matrix in the matrix-assisted laser desorption/ionization

MIC - minimum Inhibitory Concentration

MIC - minimum inhibitory concentration

MS – mass

NAD (+) - nicotinamide adenine dinucleotide

NCE - new chemical entities

NMR - nuclear magnetic resonance

NP-LC - normal phase chromatography

NSAID - non-steroidal anti-inflammatory drug

O/W – oil in water

OD - optical density

PA0-1 - *Pseudomonas aeruginosa*

PBS - phosphate buffered saline

PCR - polymerase chain reaction

PG - prostaglandins

PGH2 - prostaglandin H2

pKa - acid dissociation constant

PPL - porcine pancreatic lipase

ppm – parts per milion

Rf - retention factor

RNA - RiboNucleic Acid

RPM - revolutions per minute

RT - reverse transcription

RT-qPCR - real time quantitative PCR

SV40 - simian virus 40

T_0 - sample at time zero

TLC - thin layer chromatography

TNFα - tumor necroses factor α

UDCA - ursodeoxycholic acid

uHPLC-MS - ultra high performance liquid chromatography- mass

USP - united states pharmacopeia

UV - ultraviolet

UV/Vis - ultraviolet/visible

v/v - volume per volume

Vitamin C - l-ascorbic acid

w/w - weight to weight

YMB - yeast mannitol broth

NMR

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1. INTRODUCTION

1.INTRODUCTION

1.1 ENZYMES: LIPASES

Lipases are hydrolytic enzymes that belong to the subclass of esterases which act on carboxylic bonds C-N, C-O, C-C as well on other bonds, e.g. phospholipases act on the P-O bonds catalyzing the hydrolyzation of the acyl ester bonds in phosphates [1].

These enzymes are fundamental constituents of animals, plants and microorganism and they are involved in biological processes which range from cell signaling, inflammation and meatabolism of lipids, so increasing the amount of the energy production [2]. In humans they break down fats, introduced with food, allowing faster absorption in the intestine, while in plants they hydrolyze oils, producing the energy necessary for carrying out metabolic functions [3].

In particular, they perform their physiological role in the lipolysis process, by converting triglycerides into diglycerides, monoglycerides, fatty acids and glycerol [2]. Because lipases can accept a broad range of natural substrates, i.e. aliphatic, alicyclic, bicyclic or aromatic esters, thioesters, activated amines, [4] [5] [6], the recognized and modified targets can be very different from glycerides.

In fact, *in vitro* lipases exhibit not only their typical natural activity through hydrolyzation [7][8], but they can catalyze also reactions like esterification [9][10], acidolysis [11][12], interesterification [13], transesterification [14][15], aminolysis [16][17] and other hydrolyzations [18] [19]. The variety of sources available for their commercial uses and the versatility of these enzymes associated with their high regio-, chemo- and enantioselectivity leads them to be a good choice in organic synthesis and in biocatalysis, so improving their potential application in several

fields, such as in the food, detergent, pharmaceutical, cosmetic and paper industries [1][20].

1.1.1 The Structure

Lipases are ubiquitous esterases found in several natural kingdoms, thus some of them are located within cells, while others work in the extracellular space. Generally, these enzymes are monomers constituted by acidic glycoproteins and span a wide range of molecular weights, from 16kDa (*e.g. Chromobacterium viscosum I*) to 90 kDa (e.g. *Candida rugosa*). Their tendency to form aggregates and to be present as multimers in solution, account for the highest value of molecular mass [21][16][22]. The structures of lipases have been extensively studied, and it is reported that the primary structure can include from 270 to 641 amino acids and that there is not a preserved sequence belonging to the analyzed species. However, in most of lipases it has been identified a common secondary structure built of alpha-helices alternating with parallel beta-strands, specific but presenting more variations, the more lipases molecular weights differ [20][23]. Two are the active domains, the N-terminal domain which includes the α/β-hydrolase fold with the active site and the C-terminal domain which consists of a beta-sheet sandwich and takes part in the binding process of cofactors [4].

The active site is a catalytic triad of nucleophilic serine and a histidine interacting with aspartic or glutamic acid through hydrogen bonds [24]. Lipases isoenzymes present variability in the sequence of the "lid" or "flat", the mobile amphipathic alpha-loop which shields the active sites from solvents [25]. The mechanism of lid opening may differ and, depending on the presence of specific factors, it modulates the active or the inactive enzyme conformation [21]. In particular, the closed lid maintains the hydrophobic side directed towards the catalytic site, while the hydrophilic towards the solvent [25]: in this conformation, the catalytic site is not accessible [22]. When the displacement of the lid occurs, the hydrophobic side

becomes exposed and it allows the interactions of the catalytic triad with substrates [26]. Crystallography X-ray analysis suggested that the lid opening occurs during the activation at the oil-water interface, allowing access to the substrates at the active site [27].

1.1.2 Physical and Chemical properties

The optimum pH for most animal lipases is alkaline (pH 8-9), but it may be acid depending on some factors: the type of substrate, the presence of salts and the type of emulsifier [28]. Microbial lipases show the highest activity with pH ranging from 5.6 and 8.5 and the maximum stability at neutral pH [29]. In relation to the temperature, majority of lipases optimum is between 30°C and 40°C, while others are active at higher temperatures, e.g. lipases from *Rhizomucor miehei* at 70°C and from *Candida antarctica* at 60-65°C [20].

On one hand, lipases thermal stability varies considerably according to their origin: lipases derived from animals or plants are usually less thermostable than extracellular microbial lipases [30]. On the other hand, it is influenced by the presence of the substrate: it removes the excess of water close to the enzyme therefore, limiting its conformational mobility [20].

1.1.3 Specificity

Lipase specificity is determined by the hydrophobicity or hydrophilicity of the binding sites for the substrates. Unlike majority of enzymes, lipases tolerate organic solvents and, as afore mentioned, are able to accept different types of substrate, other than triglycerides. Thus, by varying the solvent system, lipases can be exploited both for hydrolysis and for synthesis reactions, such as esterification. In presence of racemic esters or substrate including several hydroxyl groups, they perform reactions with high enantio- and regio-selectivity. Their catalytic activity depends on the structure/geometry of the substrate and is independent of its

concentration [20]. Lipases can be classified into three groups based on their ability to hydrolyze triglycerides. The first group includes 1,3-specific lipases that are not able to hydrolyze ester bonds in secondary positions. The second group includes lipases that hydrolyze both the primary and secondary esters and are therefore non-specific. At the third group belong lipases that hydrolyze in non-specific positions but display selectivity for fatty acids, acting only when the fatty acid is of a particular type [20]. All microbial lipases hydrolyze triglycerides in first and third positions but only a few also in the second position (e.g. lipase A from *Candida antarctica*, lipase B from *Geotrichum candidum* lipase from *Penicillium simplicissumum* and lipase from *Candida rugosa*) [31].

1.1.4 Interfacial activation

Lipase's mechanism of action is called interfacial activation. In particular, it allows the adsorption to the lipophilic surface of triglyceride drops. Activation at the interface between the aqueous phase of the enzyme and the organic phase of the substrate strongly increases the enzymatic activity [32]. The main contributions for this particular mechanism of action are: (i) the hydrophobic pocket hindrance close to the active site of the enzyme and (ii) the structural rearrangements. High instability and poor solubility in aqueous media would be expected from an enzyme with a hydrophobic pocket, but the "lid" plays a contrasting role. In fact, in the closed conformation, it segregates and renders inaccessible the active site of the enzyme that remains inactive [33]. The "lid" interacts with its hydrophobic internal face with the hydrophobic areas of the active site, while interacts with its hydrophilic external face with the reaction medium [25]. Furthermore, the "lid" has the possibility to rearrange into the open conformation moving the hydrophobic pocket from the active site to the medium. Thus, the active site is free from the alpha-helix and can convert the substrate into the product. Only lipases in their active conformation at the interface display catalytic activity because the exposed

hydrophobic region becomes the binding site for the substrate [26][34]. Both the active and inactive states of lipases are in equilibrium, but the active state is affected by the presence of oil droplets or organic solvents. The lid opening enables the interaction of lipases with the oil droplets and the consequent entry of triglycerides [27][35].

1.1.5 Inhibitors and activators

The presence of biliary salts is an important influencing factor for the activity of lipases. They enter in lipidic droplets exploiting their liposoluble side so inducing the reduction of triglycerides cohesion and increasing the accessible area for lipases. Metal cations are also an important influencing factor for the substrate's procession by lipases. They induce positive regulations by changing solubility and negative regulations in terms of competition for the active site between ions and substrates [36]. In particular, calcium ions tendentially increase the activity of lipases, while it can be increased or reduced in the case of sodium ions [37]. Moreover, it has been reported that the hydrolytic activity of the lipase deriving from *Aspergillus niger* is increased by ferrous ions and inhibited by ferric ions [38]. However, these effects are all reversible and can be corrected by using chelating agents [39]. According to recent studies, free fatty acids and alcohol tend to inhibit the hydrolytic activity of lipases. In fact, it has been demonstrated that molecules like fatty acids accumulate at the interface so blocking the access for triglycerides to the enzyme. Alcohols with low molecular weight may disturb the three-dimensional structure of lipases [40][41].

1.1.6 Biotechnological production

Lipases from various sources are already commercially available, but the development of innovative techniques of genetic engineering allows the production of more and more recombinant lipases deriving from bacteria and yeasts.

Biotechnological techniques involve the use of genetically modified organisms to obtain enzymes displaying better specific catalytic properties concerning the non-modified enzymes [32]. For example, some of these targeted catalytic properties are (i) enantioselectivity, (ii) regioselectivity, (iii) the specificity of particular substrates, (iv) stability, and (v) high-temperature resistance [1][42]. Large scaled production of lipases is performed in fermenters through the growth of microorganisms and fermentation in a liquid medium. Recently, there has been increasing interest in enzymatic production solid-state fermentation, which offers several advantages: higher product concentration, greater and simpler product recovery, generation of a minimum liquid waste amount [43].

1.1.7 Industrial application

In recent years lipases started to play an important role in the biocatalyst processes. This rising interest in the industry is due to some of their unique features: they are usually stable and perform their function in aqueous media, organic solvents [44][45], ionic liquids [46][47] and supercritical liquids [48][49]. Moreover, lipases are chosen for their easy mode of use, for their high tolerability to substrates, for their stability in different solvents and at high temperatures, and their ever-increasing commercial availability. In fact, lipases are excellent biocatalysts and are widely used as alternatives to classical practices in the selective molecules' conversion. The use of specific natural catalyzers, such as enzymes, can diminish collateral reactions and post-reaction separation problems. Traditional processes include problems like the wide consumption of energy and the production of toxic subproducts [44]. Lipases improve the taste, softness, and texture of several food preparations, such as fruit juices, beer, wine, baked, and dairy products. Thus, they have become an integral part of the food and flavorings industry, offering them great economic benefits [50]. An excellent result of food fermentation processes is the delaying of products' retro-degradation, which usually occurs during storage,

by the oxygen removal in the final steps (e.g. beer production) [51]. These enzymes are employed in the chemical industry to develop pesticides, cosmetics, perfumes, detergents, biosensors, and pharmaceutical products. Moreover, they find application in biodiesel production too, acting as catalysts in the transesterification of oil feedstock, to produce fatty acid alkyl esters (FAAE) [52]. In particular, lipases are exploited for esterification reactions, which are used to develop emulsifiers, surfactants, wax esters, chiral molecules, biopolymers, and modified fats or oils. In recent years, the attention has been shifted to reactions that use enzymes as biotechnological vectors [14][36][43][53][54]. Other examples of industrial applications of lipases from microbial sources are shown in **Table 1.**

Table 1: Examples of Applications of lipases deriving from different microorganism sources

Microorganism	Applications
Candida antarctica	- Esterification/transesterification - Regio-/enantioselective synthesis - Racemic mixtures separation - Polymers production and degradation
Mucor miehei	- Esterification of vegetable oils
Thermomyces lanuginosus	- Detergent productions
Pseudomonas alcaligenes	- Detergent productions
Chromobacterium	- Detergent productions
Caluerpa cylindracea	- Racemic mixtures separation
Geotrichum candidum	- Pharmaceutical products synthesis
Rhizopus nodosus	- Formulations production to treat garments
Pseudomonas aeruginosa LP602	- Wastewater treatment
Pseudomonas cepacia	- Hydrolysis of fats - Transesterification of soy oils
Pseudomonas putida	- Polycyclic aromatic hydrocarbon degradation

1.1.8 Lipase B from *Candida antarctica* (CALB)

Candida Antarctica Lipase – B (CALB) is one of the two variants expressed from the psychrophilic yeast *Candida antarctica,* the alkaline yeast originally isolated from the Lake Vanda in Antarctica. The second variant is lipase A (CALA), but the two enzymes exhibit completely different physiological properties. CALB is a globular protein build-up by a polypeptide chain of 317 amino acids giving a molecular weight of 33kDa and belongs to the α/β-hydrolase fold family. This lipase is stable at pH ranging from 5 to 9, works at temperatures between 30°C and 65°C, and has an isoelectric point (Ip) of 6.0 [22][55][56].

CALB does not perform the interfacial activation and therefore is not considered a true lipase, but it shares several common futures with the other lipases. In particular, as shown in **Figure 1** [57][58], it has the characteristic catalytic triad Ser-His-Asp in its active site and the "catalytic machinery" is complete by the presence of the oxyanion sequence, which permits the stabilization of the tetrahedral intermediate.

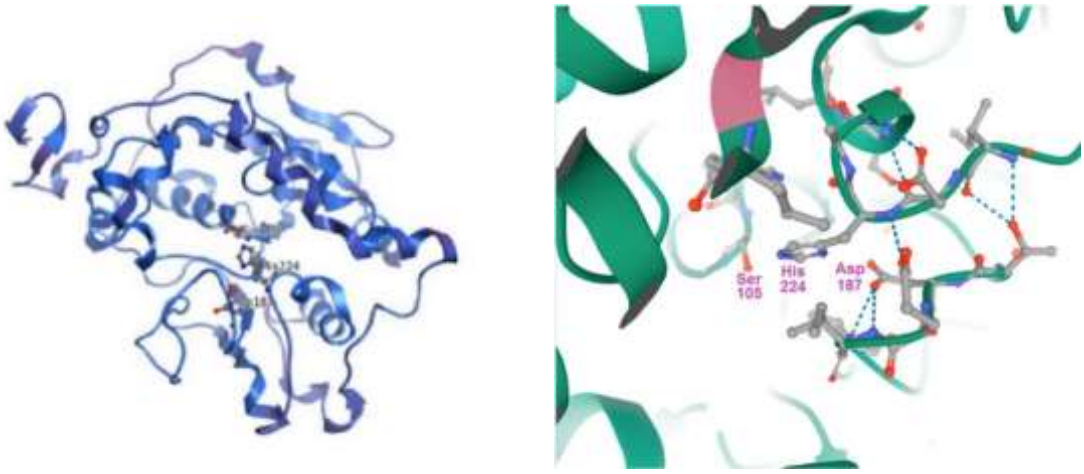


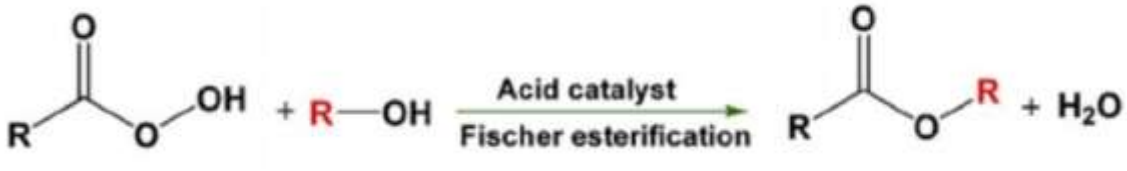
Figure 1: Crystallographic structure of CALB showing the catalytic triad (Ser-His-Asp) in the active site.

The spatial arrangements allow the formation of the oxyanion hole, where the tetrahedral intermediate becomes charged negatively because three hydrogen-bond donors (two from Thr40, one from Gln106) interact with the substrate's carbonyl. The typical lid covering the active site of lipases is absent or very small in CALB. However, there is a small α-loop which partially inhibits the access to the catalytic site. This smaller lid ensures the adsorption of CALB on hydrophobic surfaces, and therefore the interfacial activation. For these reasons, CALB is identified as a lipase, even if its closed conformation does not correspond to those inactive of classical lipases. Moreover, a small pocket with stereospecific requirements gives CALB high enantioselectivity to secondary chiral alcohols. CALB is hence an efficient biocatalyst used for a wide variety of reactions including racemic mixtures resolution, aminolysis, esterifications in solventless media or in polar organic solvents [59][56][60]. This enzyme is the most chosen and stable of the commercial lipases used in large-scale industrial production, however, some enzymatic properties have been improved through genetic engineering techniques. For example, to reach better stability and higher enzymatic yield, CALB has been immobilized on a resin of Novozymes, which is a macro-porous support of acrylic polymers. This matrix (Lewait VP OC 1600) is made up by poly (methyl methacrylate) crosslinked with divinylbenzene. Enzyme immobilization, together with the interfacial activation, induces an ever-active conformation, so avoiding the formation of aggregates, which inhibits its activity [55][61].

1.2 ESTERIFICATION REACTION

The acidic catalyzed condensation between an alcohol and a carboxylic acid followed by the formation of an ester and water is known as Fischer's esterification. The mechanism of this reaction is overall reversible. To drive the reaction to products, it is necessary to remove the formed water or to add an excess of alcohol. The mechanism starts with the protonation of the carbonyl by the acidic catalyst,

which makes the carbonyl a good electrophile. So, it is activated through a nucleophilic attack, performed by the alcohol. A proton is transferred to the oxygen of the original alcohol and, in the end, a molecule of water leaves, while the deprotonation of the carbonyl generates the ester [62] (**Scheme 1**).



Scheme 1: Fischer's esterification.

1.2.1 The role of lipases in the esterification reaction

The research on the production of several types of esters catalyzed by lipases increased during the last decade. Esters are components of fats, oils, natural and synthetic polymers and are useful intermediates or final products for the chemical industry. Esters are obtained by the esterification between carboxylic acid and alcohols or by transesterification processes [3]. Lipases are not only able to perform hydrolysis reaction, but they can also catalyze esterification reactions in the presence of anhydrous medium or less water [63]. The fact that an enzyme catalyzes both hydrolytic and esters-synthesis reactions was not confirmed until he 1960s, even though the enzymatic synthesis of esters was already studied through crude preparations of pancreatic and ricinus lipases. For the first time, Fukumoto et al [64] purified the lipase of *Aspergillus niger* so demonstrating that the triglyceride's synthesis was possible by changing the reaction conditions. Some studies reported the use of lipases from *Aspergillus niger*, *Rhizopus delemar*, *Geotrichum candidum* e *Penicillium cyclopeum* for the synthesis of oleic acid synthesis performed with different primary alcohols [65]. In this field, only the lipase from *G. candidum* allowed the synthesis of esters from secondary alcohols, while tertiary alcohols, phenols, or alcohols from sugars were not synthesized [20]. In thermodynamic

terms, to drive the inverse hydrolysis reaction, it is esterification, is necessary to remove water during the reaction process [63]. So, in the next phases, the research about esterification reactions was driven towards the use of organic solvents and not aqueous [66]. Lipases are highly specific catalysts that typically act in aqueous solvents, although these, like many other enzymes, can maintain their catalytic futures at high concentrations. This, despite being found in organic solvents, where they can catalyze reactions that could not occur in water [20]. Lipases employed in esterification and transesterification reactions in organic solvents showed an increase in enzymatic activity, in stability at high temperatures, in regiospecificity and in stereoselectivity [67]. The knowledge that enzymes can maintain, and in some cases increase, their reaction specificity in almost anhydrous media has improved the perspective to use them in organic chemistry synthesis of pharmaceutic intermediates and chemical products [68]. For this aim, have been performed biphasic system experiments. This, by adding small amounts of organic solvents to aqueous solutions containing enzymes until reaching an ever-greater organic phase. Substrates added in the reaction system pass in the aqueous phase, but, in the end, they convert into products and pass to the organic phase. To facilitate this process is used to form microemulsions by a mechanic or a magnetic agitation, so inducing the enzyme's interfacial activation between two phases [33]. Water is an inappropriate solvent for this type of organic reactions, because organic products are there insoluble, and it can promote unwanted side reactions such as hydrolysis, racemization, polymerization or decomposition, that inhibit the enzymatic synthesis [69]. On the other hand, water is a key element, even when used at low concentrations, in most non-covalent reactions, because it helps to maintain the catalytic enzyme conformation [70]. In biological systems, water can be classified under two categories: the "bulk water", which works as a proper solvent and could be replaced by organic solvents (98% of water) and the "bound water", a minor fraction, which interacts with the enzyme keeping the structure

conformation. The parameter "water activity" (a_w) is used to measure the content of bulk water (i.e., water availability), in reaction moistures. It is an index of the enzyme's hydration grade and gives information about the mass action of water. The "water activity" (a_w) is defined by the following equation:

$$a_w = p_s / p_w$$

i.e., the ratio between the partial water vapor pressure over the substance (p_s) and the partial vapor pressure of pure water (p_w) [Lipases and lipase-catalyzed esterification reaction in nonaqueous media]. Studies demonstrated that in the reaction media containing almost totally anhydrous organic solvents, and a low percentage (10% of the total volume) of water, the detected enzymatic activity was high [71]. In fact, completely anhydrous solvents are not capable to promote enzymatic activity, because some water is always required to keep the native conformation of the enzyme, necessary for catalysis. The exploitation of enzymes in non-aqueous solvents could be a valid alternative to reactions in water, especially for substrates and products poor soluble in aqueous media. Thus, reactions of the enzymatic conversion performed in the presence of organic solvents lead to a lot of advantages [69], below cited:

1. Increased solubility of non-polar substrates and products
2. The possibility to drive the thermodynamic equilibrium, so to favour the ester synthesis than hydrolysis
3. Simplified product extraction from the organic phase with increased performance, and the avoiding of further extractive processes during the product recovery
4. Easier product recovery from solvents displaying low ebullition tension and high pressure
5. Easier enzymes recovery through filtration
6. Often not necessary immobilization given the insolubility of enzymes in organic solvents

7. Inhibition of side reactions water-dependent such as hydrolysis, racemization, or polymerization
8. Reduced enzyme inhibition due to lipophile substrates and products, because their concentration on the enzyme's surface is low
9. Ameliorated thermostability [3]

Nevertheless, the lipase choice must be accurate, because these enzymes display different behaviour depending on the type of solvent. Generally, lipases are more unstable in polar solvents miscible in water, than the ones immiscible. In addition, some lipases exhibit good stability in organic solvents also at high concentrations [66] [72]. Different strategies can be used to shift the thermodynamic equilibrium in lipases catalysis, e.g. using:

1. Organic solvents of different nature
2. Inhibitors of water activity such as polyalcohol or salts
3. Reaction media almost deprived of water, as anhydrous solvents
4. Pure liquid substrates
5. Gas phases

Each solvent can have a different activity on the enzymes or the reaction. For example, non-aqueous solvents can perform specific bindings, compete with the substrates bond, dissociate multimers, shift the equilibrium between two enzymatic conformations, alter the helical structure, react with the enzyme, or affect the catalytic reaction speed [73]. Non-polar solvents shift the equilibrium from the closed to the open conformation of the enzyme, modifying the solubility of the substrates and the products in the reaction medium [44]. A measure of the compatibility of the solvent with the enzyme is defined by the logarithm of the partition coefficient (*P*), abbreviated *log* P. P is defined as the ratio of concentrations of a solvent between water and 1-octanol (biphasic system). Solvents with a *log* P greater than 4 (aromatics and aliphatic) are suitable for biocatalysts, while water-

miscible solvents with a *log* P lower than 2 (short chain alcohol, dimethylformamide, short-chain esters) are not suitable [69]. Reactions catalysed by enzymes can be classified according to whether they occur in a monophasic or biphasic system. Monophasic system includes monophasic solutions of water and solvents miscible in water, or non-aqueous monophasic solutions of organic solvents, immiscible with water. In the first case, the enzymes can be dissolved in water-miscible solvents: the enzyme so as the substrate and product are dissolved in a monophasic solution consisting of water and an organic co-solvent that is miscible in it, such as dimethyl sulfoxide (DMSO), dimethylformamide (DMF), tetrahydrofuran (THF), dioxane, acetone or one short-chain alcohol. Systems of this type are mainly used for the transformation of lipophilic substrates, therefore poorly soluble in an aqueous system. Instead of water, buffer solutions can be used. A study was carried out on the reaction media miscible with water and it was found that as regards lipases, generally, most miscible solvents can be used up to 10% of the total volume. If the proportion of the solvent system exceeds this value, the bound water is removed from the enzyme surface leading to deactivation. It rarely happens that enzymes remain catalytically active in water-miscible organic solvents with extremely low water content. The second case, the organic monophasic system, is considered a non-aqueous system if no more water is added than the absorption capacity of the reaction medium allows. Due to the advantages offered by these systems, there has been a growing interest in recent years on the use of biocatalysts in non-aqueous media. The replacement of all the bulk water with an organic solvent that is non-miscible with water leads to the suspension of the enzyme in a monophasic organic solution. However, it is important that the biocatalyst is hydrated to remain catalytically active. Some systems have proven to be extremely reliable, versatile, simple, and easy to use. The correct selection of solvent will affect the effectiveness of most of the reactions mediated by enzymes in non-aqueous systems. Several attempts have been made to facilitate the selection of solvents by

defying the aforementioned physiochemical parameter *log* P, which gives information on the technical potential of an organic solvent as a reaction medium. As shown in Table 2, hydrophilic solvents miscible with water (DMF, DMSO, acetone and inferior alcohol) are incompatible, while lipophilic solvents not miscible with water (alkanes, esters, or aromatic compounds) prove to be more advantageous than hydrophilic ones, because they have a lower capacity of discarding water molecules needed by the enzyme [20][74]. Some of the solvent's compatibilities based on *log* P are shown in **Table 2**.

Table 2: Compatibility between solvents and enzymatic activity [20].

***log* P**	**Water miscibility**	**Examples**	**Effects on enzymatic activity**
-2,5 – 0	Totally miscible	DMSO	It can be used up to concentration of 50% v/v to solubilize lipophilic substrates without affecting the enzymatic activity
0 – 2	Partially miscible	Dioxane	Limited uses due to the rapid inactivation of the enzyme
2 – 4	Low miscibility	Chloroform, Esan	They cause weak distortions of the enzyme and can be used with caution since changes in activity cannot be predicted
> 4	Immiscible	Heptane	It does not cause enzyme distortion and ensures a good maintenance of activity.

On the other hand, biphasic media are systems containing two different phases, namely an aqueous phase containing the dissolved enzyme and an organic phase. The biocatalyst is in a favorable aqueous environment and not in direct contact with the organic solvent, where most of the substrate is present. The limited concentration of organic material in the aqueous phase is not able to inhibit the enzymes, and the removal of the product from the surface of the enzyme favors the

reaction. In these biphasic systems, the enzymatic reaction occurs exclusively at the interface between the aqueous and the organic phase. A mass transfer of the reagents to the enzyme, and of the products from the enzyme is necessary between the two phases, consequently, fundamental parameters are agitation and mixing. However, care must be taken, because enhanced agitation can improve the transfer, but at the same time can lead to the deactivation of the enzyme [20]. The *log* P, the dielectric constant, the hydrogen bonds, the dipole moment, the polarity of the solvent, the denaturation capacity, the hydrophobicity, the polarity index are, therefore, all parameters that affect the enzymatic activity in a system of organic solvents and determine the stability and catalytic potential of a biocatalyst in an organic medium. Some of the main strategies adopted to improve the activity and stability of these enzymes in water-free environments are the following:

- Protein engineering (site-specific mutagenesis and direct evolution)
- Covalent attack of amphipathic compound (polyethylene glycol PEG, aldehydes and imidoesters): PEG in particular is bound to the surface of the enzyme, altering its activity, stability and selectivity
- Non-covalent interaction with lipids or surfactants
- Trapping in water/oil microemulsion or inverse micelles. With the use of a detergent and small amounts of water or buffer, inverse micelles can be created that contain the enzyme in the aqueous phase while the organic solvent act as a "bulk phase".
- Immobilization on insoluble supports (synthetic polyhydroxy matrices, porous inorganic carriers, polymers, and molecular sieves)
- Use of "solid enzymes" (freeze-dried enzyme powders and cross-linked crystals suspended in organic solvents) [20][69]

Currently the most used strategies in the industrial field concern the use of "solid" and immobilized enzymes [75]. Lipase B from *Candida antarctica* (CALB), used in the experiments showed below, is an example, because it is immobilized on an acrylic resin to minimize the instability.

1.2.2 Esterification as strategy to enhance active molecules properties.

Esterification of lipophilic compounds with water-soluble molecules can increase the polarity and, thereby, improve the aqueous solubility of the resulting ester [76]. The esterification of active ingredients of industrial interest can be carried out in two ways:

• Chemical catalysis; esterification reactions are identified as trans-esterification, in the presence of a base catalyst, and as esterification, in the presence of an acid catalyst [77] (generally sulfuric acid, H_2SO_4, of which antecedents can be found in the literature, for example, in the esterification of ibuprofen [78], flurbiprofen [79], and ketoprofen [80].

• Esterification catalyzed by enzymes; only since the 1990s is the biocatalyst activity of lipases (triacylglycerol hydrolases, EC 3.1.1.3) in esterification reactions calling attention, due to their significant activity and selectivity, properties that are maintained also in organic media, and mild operating conditions, thus favoring their application in the fine chemicals industry, in particular in the presence of thermosensitive products [81]. Lipases of various origins have been used in the esterification of profens, being the lipase B of *Candida antarctica* in its free and immobilized form, is the most used in this synthesis; for example, in the following work on the esterification of ibuprofen [82] [83], naproxen [84] and ketoprofen [85]. Furthermore, several studies have demonstrated antimicrobial activity improvements after esterification of aromatic compounds [86][87].

Furthermore, it is known that esters display good thermodynamic stability *in vivo* [82]. Several publications show enzymatic esterification strategies to improve the water solubility of lipophilic compounds. Jahangiri et al. [76] report a lipase-catalyzed reaction between the water insoluble bixin and sorbitol in order to obtain a novel bixin derivative with a hydrophilic profile.

Biotransformation reactions, specifically the enzymatic esterification lipase-catalyzed, can be used as useful strategies to improve the water solubility of lipophilic molecules. In fact, reduced solubility is synonymous with poor bioavailability [88]. In food and wine products, the poor bioavailability of additives, generally lipophilic, can be a disadvantage for the preservation of beverages or food that contain water, even in small percentage. Bioavailability is a pharmacokinetic property influenced by the solubility of the molecule itself, and it also affects the dosage of the active principle after administration of the medicinal product. It follows that the poor solubility in water and bioavailability of active principles could be a problem in the pharmaceutical-industrial field, in particular for the development and marketing of pharmaceutical formulations [89]. It has been estimated that 40% of the molecules of pharmacological interest are poorly bioavailable [90][91]. The development of protocols for the enzymatic synthesis relating to the enzymatic synthesis of molecules with increased solubility can present a solution for the agri-food sector, e.g., food preservatives, and pharmaceuticals, e.g., anti-inflammatory drugs. The lipase-catalyzed esterification between a lyophilic molecule (food additives or active principles) and one water-soluble, increases the polarity of the ester obtained, and consequently, its solubility in water [88].

The esterification of sorbic acid (lipophilic) and glycerol (hydrophilic) in the presence of CALB, through a solvent-free reaction, in a medium with a water percentage almost null, where the glycerol acts as a reagent and "solvent", allows to obtain glycerol sorbate, that is a hydrophilic ester [92]. Sorbic acid is the most common preservative in the food industry, due to its safety and commercial availability. However, its lipophilic nature is a limit. In fact, the presence of water in food could compromise its effectiveness as a preservative [93]. On the other hand, the characteristic of its glycerol-ester is to be more polar, so more soluble in water.

This increases the antimicrobial and antifungal activity, which makes it a good food and drinks preservative [92].

Ibuprofen is a non-steroidal anti-inflammatory drug (NSAID) used as an analgesic, anti-inflammatory, and antipyretic with low solubility of only 21 mg/L, which severely limits its bioavailability [94]. As demonstrated by Di Guida et al, modifications of drugs such as ibuprofen, aimed at enhancing their solubility, can occur chemically, e.g., by glycosylation [95]. Alternatively, as in the detailed case of this work, through biotransformation reactions with lipase immobilized by CALB. Lipases used as catalysts allow a "green" synthesis between poorly soluble active principles with acid function, and molecules with high polarity, by reducing reaction steps, temperatures [96], and by avoiding the use of chemicals. The result of this type of esterification is a prodrug, that is more soluble than the starting active principle and which potentially has a more effective anti-inflammatory activity.

1.3 SUBSTRATES: ACID

1.3.1 Nonsteroidal anti-inflammatory drugs (NSAIDs)

Natural anti-inflammatory drugs, secreted by the body itself, are derived from corticosteroids, substances of steroidal origin with powerful anti-inflammatory and immunosuppressive action; while, non-steroidal anti-inflammatory drugs (NSAIDs) are a heterogeneous group of drugs with very different chemical structures and not necessarily related to each other, which have anti-inflammatory, analgesic and antipyretic properties, in order to reduce the symptoms of inflammation, pain and fever, respectively. This chemical diversity gives them different pharmacokinetic properties; however, they share some therapeutic activities and relatively frequent adverse effects [97].

NSAIDs represent one of the most prescribed pharmacological groups in the world; They are used in the treatment of inflammation, pain and edema, also in rheumatoid

arthritis, osteoarthritis of various types, myalgia, headache, arthralgia, dysmenorrhea and other minor applications. In addition, epidemiological and experimental studies have documented the beneficial effects of NSAIDs in colon-rectal cancer treatments [98] and Alzheimer's disease [99].

The mechanism of action of NSAIDs is shown in **Figure 2** [100]; In 1971, Vane and Piper demostrated that NSAIDs inhibit the activity of the enzyme cyclooxygenase (COX), from arachidonic acid, resulting in a decrease in the production of prostaglandins (PG) and thromboxanes (A2), which are known cellular mediators in the inflammatory processes [101].

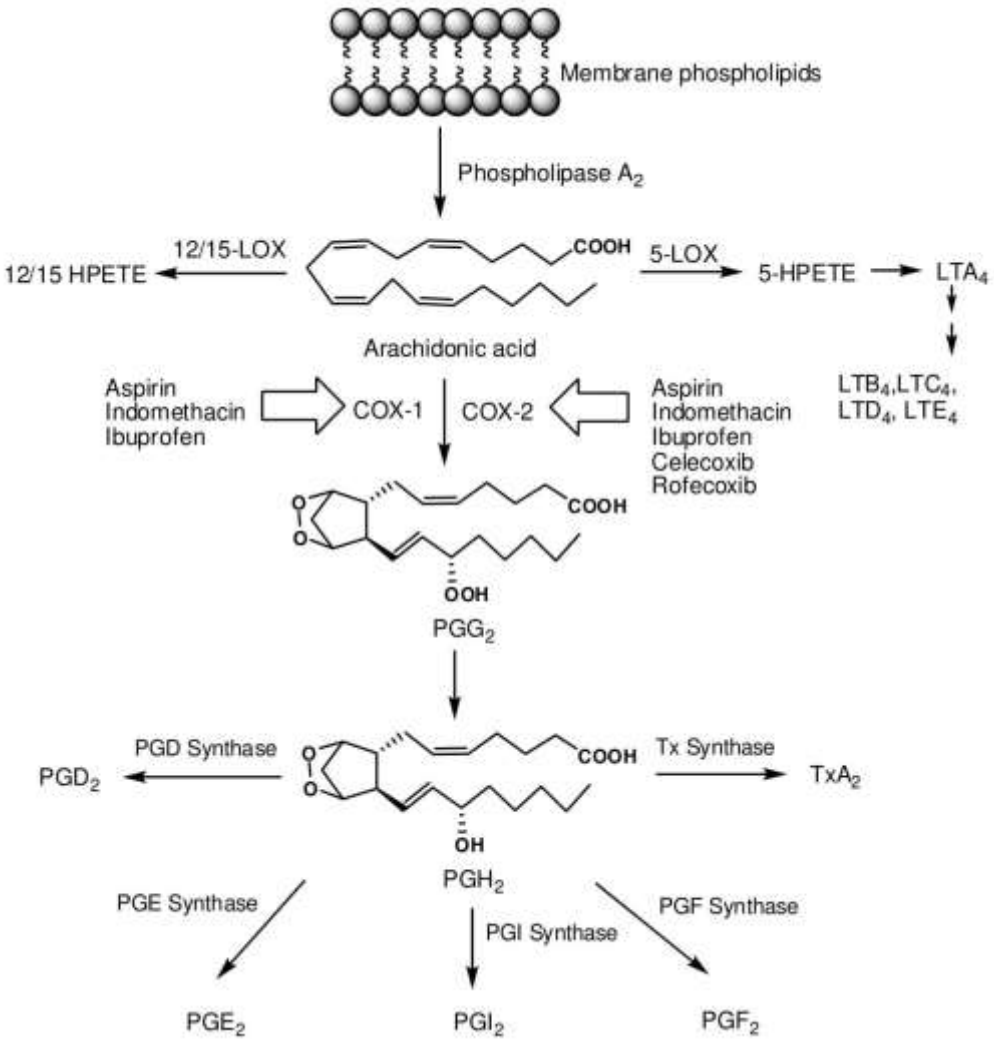


Figure 2: Mechanism of action of NSAIDs.

There are two isoforms of the cyclooxygenase enzyme: COX-1 and COX-2. The first is a constitutive enzyme that is present in the kidney, stomach mucosa, and duodenum; in these tissues, COX-1 carries out the synthesis of prostaglandins that act as cytoprotectors gastric and renal, and in the regulation of platelet aggregation, among other actions. COX-2 is an induced enzyme that is present in the brain, lung, pancreas, placenta, and ovaries; This enzyme is expressed in response to inflammatory processes and stimulates the synthesis of prostaglandins that produces fever, pain or inflammation [102]. Cyclooxygenases, and in particular COX-2, are enzymes that convert arachidonic acid into prostaglandin H2 (PGH2) which, in turn, is converted by other enzymes into prostaglandins, especially responsible for inflammatory effects such as vasodilation or bronchoconstriction. PGH2 can also be converted into thromboxane A2, a molecule involved in the haemostasis process, which stimulates vasoconstriction and platelet aggregation [103].

NSAIDs can be classified according to their mechanism of action as (**Table 3**):

• Traditional NSAIDs that non-selectively inhibit the COX-1 and COX-2 enzymes, to a greater extent COX-1, which results in side effects at the gastrointestinal, renal and coagulation tract levels.

• Selective COX-2 NSAIDs

• Preferential COX-2 inhibitor NSAIDs.

NSAIDs generally have bioavailability after being administered orally, they are absorbed, but their absorption rate varies between NSAIDs, due to differences in their pharmacokinetic properties.

Table 3: Classification of non-steroidal anti-inflammatory drugs.

CLASSIFICATION	GROUP	DRUGS
Traditional NSAIDs	Derivatives of Arylpropionic Acid	Fenoprofen, flurbiprofen, ketoprofen, naxoprofen, ibuprofen, tiaprofenic acid.
	Derived from Acetic Acid	*Arilacetico:* Diclofenac, Aceclofenac, Sulindac, Fentiazac. *Pyrrolacetic:* Tolmentin, Ketorolac. *Indolacetic:* Acemetacin, Indomethacin *Pyranoacetic:* Etodolac
	Derived from N-arylanthranilic Acid	Meclofenamic acid, Mefenamic acid.
	Derivatives of salicylic acid	Diflunisal
Selective COX-2 inhibitors	Coxibs	Celecoxib, Valdecoxib, Lumiracoxib
Preferential COX-2 inhibitors	Non-Acidic Agents-Derivative naphthyl alkanones	Nabumetone
	Oxicans	Meloxicam, Tenoxicam, Piroxicam
	Indolacetic pyran derivative	Etodolac

Among the most important adverse effects of NSAIDs related to chronic use, the following can be mentioned:

a) Gastrointestinal tract: chronic use of NSAIDs administered orally can damage the gastric mucosa, leading to gastric ulcers [104][105]. These effects are associated with the inhibition of COX-1, since prostaglandins have a protective effect on the gastrointestinal mucosa due to the fact that they regulate cell change and repair,

maintain blood flow and stimulate mucus and bicarbonate secretion. The erosive effect is associated with some NSAIDs (weak acids), which become fat soluble at low pH. When administered orally, they cross the lipid membrane and become ionized within the cells of the gastric mucosa, losing fat solubility, therefore, it remains inside the cell, interrupting cell function by affecting multiple cell pathways such as mitochondrial oxidative phosphorylation, synthesis from ATP, and ion exchange (Na^+, K^+ and H^+), among others.

b) Cardiovascular effects: selective COX-2 inhibitor NSAIDs do not inhibit the synthesis of thromboxane A2, therefore, they could increase the risk of thrombosis. In addition, clinical studies indicate that selective COX-2 inhibitors exert significant adverse cardiovascular effects, including an increased risk of myocardial infarction, stroke, high blood pressure, and heart failure [106]. However, most traditional NSAIDs inhibit COX-1 by preventing the formation of thromboxane A2, which induces platelet aggregation [107]. Aspirin is the oldest NSAID, being used predominantly in the treatment of cardiovascular and cerebrovascular diseases, in low doses [108].

c) Renal effect: the inhibition of prostaglandins, generated by the action of COX-1, leads to a decrease in total renal perfusion and redistribution of blood flow in the cortex of the kidneys, a process that culminates in acute renal vasoconstriction, spinal ischemia and, in sometimes in kidney failure [109].

1.3.1.1 Arylpropionic acids

Within the non-steroidal anti-inflammatory drugs, the 2-aryl propionic acids (profen) form a relatively homogeneous group due to their physical and pharmacological characteristics. The first used was ibuprofen, but later a large number of derivatives emerged: naproxen, fenoprofen, ketoprofen, flurbiprofen, suprofen, etc. They are used in rheumatoid arthritis, ankylopoietic spondylitis and

in acute attacks of gout. Some are also used as analgesics (ibuprofen, naproxen), at doses lower than those recommended in rheumatic diseases, and their use in the treatment of dysmenorrhea has recently become popular.

The prophenes are molecules that have a chiral center, therefore they have two enantiomers that exhibit different behavior. According to their spatial orientation, with respect to the chiral center, the following can be distinguished: the S (left hand) enantiomer and the R (right hand) enantiomer. These drugs are marketed as a racemic mixture of both enantiomers.

Ibuprofen

Ibuprofen ((R,S)-2-(p-isobutylphenyl)-propionic acid) is a traditional nonsteroidal anti-inflammatory drug (NSAID) [110] wich belongs to the family of prophenes (2-arylpropionic acid). It was discovered by Dr Stewart Adams (**Figure 3**) in UK in the 1950s, patented in 1961 and first available in 1969 [111]. It was initially licensed to two pharmaceutical companies: Whitehall Laboratories, which sold the product under the Advil and Upjohn brands. After the termination of the patent together with the rights to market, in 1986, ibuprofen tablets were sold under a multitude of trade names, the most common being Advil, Nurofen and Motrin.

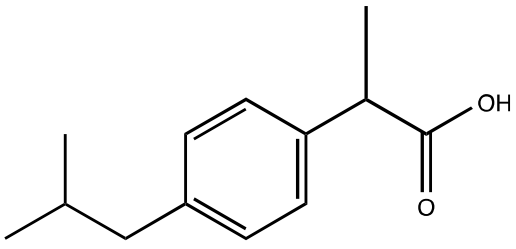


Figure 3: Structure of Ibuprofen. Chemical formula $C_{13}H_{18}O_2$. Log P 3.7; Melting Point 75-77°C

Ibuprofen was developed for the treatment of symptoms caused by arthritis such as swelling, pain and stiffness [112]. It is used in mild-to-moderate pain such as

dysmenorrhea, headaches (including migraine), dental pain, postoperative pain and musculoskeletal/joint disorders including osteoarthritis, rheumatoid arthritis and ankylosing spondylitis [113][114].

The mechanism of action of ibuprofen in different therapeutics purposes is well established. Ibuprofen is a non-selective reversible inhibitor of the cyclo-oxygenase isozymes (COX-1) and COX-2, which are responsible for the conversion of arachidonic acid into prostaglandins including thromboxane and prostacyclin [115].

Because of its chiral center, ibuprofen has two enantiomers [116]. Ibuprofen is a chiral molecule that contains an asymmetric carbon group in the second position, so it is a racemic mixture of two enantiomers R (-) and S (+), **Figure 4**.

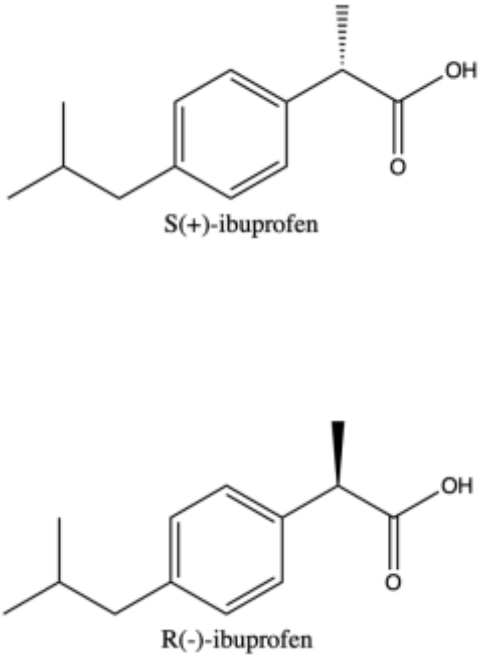


Figure 4: Ibuprofen structure; R (-) and S (+) enantiomers

However, it is well documented that the therapeutic activity of ibuprofen is mainly attributed to the (S) enantiomer, which is 160 times more effective than the (R) enantiomer [117]. R-ibuprofen may have been shown to convert to S-ibuprofen although there are discrepancies on this point [118]. It was reposted how, in vivo,

the unidirectional conversion from R to S occurs: the R isomer can be converted into the S isomer, thanks to a particular isomerase which is α-methylacyl-CoA racemase [116]. Its physicochemical properties are shown in Table 4.

Table 4: Chemical and physical properties of ibuprofen. The table displays the chemical and physical properties values of ibuprofen with the related reference.

Property Name	**Property values**	**Reference**
Molecular formula	$C_{13}H_{17}O_2$	
Molar weight (g mol^{-1})	206.29	
Fusion temperature T_{fus} (°C)	74.0	[119]
Fusion enthalpy ΔH_{fus} (kJ mol^{-1})	25.5	
Thermal capacity ΔCp_{fus} (J mol^{-1})	50.3	
Entropy of fusion ΔS_{fus} (J mol^{-1} K^{-1})	73.0	[120]
Enthalpy of vaporization ΔH_{vap} (kJ mol^{-1})	92.7	
Entropy of vaporization ΔS_{vap} (J mol^{-1} K^{-1})	173.0	
pKa	4.41	[121]
	5.38	[122]

In particular, the analgesic action consists in decreasing the sensitivity of nerve endings to chemical pain mediators such as bradykinin; the effect is rapid and clinically evident earlier than the anti-inflammatory one. The anti-inflammatory

action of the drug, on the other hand, leads to a decrease in vasodilation, edema and pain. Finally, the antipyretic action occurs due to the decrease in the synthesis and release of prostaglandin E2 by the thermoregulatory centers of the hypothalamus, which are stimulated by the pyrogen IL-1 [107].

Ibuprofen is available in numerous pharmaceutical formulations suitable for the different routes of administration. Due to its high patient compliance, cost effectiveness, reduced sterility constraints, flexibility, and and rapid absorption, the most common route of administration for the ibuprofen is the oral [123][124][125] as tablets, caplets or capsules in 200 mg, 400 mg and 800 mg strengths [107][124]. The dose is 200–400 mg (5–10 mg/kg in children), every 4–6 h to a maximum of 1.2 g per day in adults [126].

The area under the curve (AUC), which represents the plasma concentration of ibuprofen as a function of time, is dose-dependent. Ibuprofen binds extensively to plasma albumin (> 98%), in relation to the concentration, but at doses above 600 mg there is an increase in the unbound fraction of the drug, which leads to an increase in renal plasma clearance. The recommended therapeutic dose varies according to the formulation or route of administration, but above all according to age, weight and the symptoms to be treated. The first therapeutic effects are obtained already one hour after administration and plasma Cmax occurs within 3 hours [127][128].

The elimination of ibuprofen occurs via kidney biotransformation, in fact, the conjugated glucuronide metabolites and a percentage of non-metabolized drug are excreted in the urine; excretion varies according to renal function [129]. Liver disease and cystic fibrosis can alter the kinetics of ibuprofen, as well as the interaction with other drugs [107][130][131][132]. With increasing age, blood flow, gastric secretion of bicarbonates and mucus and ability to resist drugs decrease, therefore, although ibuprofen is considered one of the safest NSAIDs and is

generally well tolerated, it is more likely to find clinically evident and severe acute gastric lesions [133].

The acqueous solubility of ibuprofen its 21 mg/L [134]. Ibuprofen is a poorly water-soluble drug, characterized by dissolution-limited oral bioavailability [94]. Because of the presence of a carboxylic group, the rate of dissolution of the currently available solid dosage forms its low, and the consequent poor bioavailability from a high oral dose [135] can cause severe unwanted adverse effects. This drug, to have greater solubility in an aqueous environment, is marketed as ibuprofen lysine or sodium salt, or in the form of derivatives associated or not with other active ingredients [110]. The chronic use by oral long-term administration of ibuprofen drug may causes gastric mucosal damage, more correclty stomach irritation, ulceration, bleading and perfonation [136][104].

Therefore, the use of prodrugs to mask the carboxylic group of 2-aryl-propionic acid has been proposed as a method to improve the bioavailability of the drug in terms of optimizing its absorption, distribution, metabolization and excretion mechanism [137] leading to the reduction of gastrointestinal irritation, due to the fact that the prodrug is released more slowly in the gastrointestinal tract, reducing the inhibition of COX enzymes, an enzyme that catalyzes the binding of oxygen to arachidonic acid, thereby inhibiting synthesis thromboxane A2 and prostacyclins, cellular mediators responsible for the inflammatory process.

So, one way to increase the water solubility of ibuprofen could be an esterification reaction between its carboxylic acid and an alcohol to obtain an enhanced water-soluble prodrug designed to effect better oral availability [138].

As early as 1980, a toxicological and pharmacological study of several derivatives and formulations of ibuprofen showed the prodrug ibuprofen guaiacol ester as suitable form of this drug decreasing toxicity in rat [139]. The design, synthesis and

administration of four brain targeting L-ascorbic acid-prodrugs of ibuprofen exhibit increased level of ibuprofen in brain [140]. The synthesis and test of the N,N-disubstituted aminoalcohol ester of ibuprofen resulted in a significant reduction of ulcerogenicity in the stomach [141]. Furtermore, they have been synthesized, either by esterification reaction prodrugs of profenos, such as: flurbiprofen esters [79][142], naproxen esters [143][84][144], ketoprofen esters [85], and other ibuprofen esters [145][83][136][146]. Corrently, prodrugs have been used in suspensions, emulsions, microemulsions and nanoemulsions [147]. for example, dexibuprofen gel [148], and transdermal derivatives of ketoprofen [80].

1.3.2 Steroids: bile acids

Bile is an electrolyte solution at pH 6.8-8 produced by the liver cells and by the cells of the biliary canaliculi in a daily quantity ranging from 500 to 1500 ml. The bile synthesized by the liver is secreted into the small bile ducts where, passing through the common hepatic duct and the cystic duct, it is stored in the gallbladder (gallbladder). In humans, the gallbladder is located under the anterior border of the right lobe of the liver and its function is to accumulate and concentrate the bile whose composition is shown in **Table 5**.

Table 5: Human bile composition. The table shows the concentrations and percentages of the constituents of the hepatic and gallbladder bile.

	Hepatic bile	**Gallbladder bile**
H_2O	97.5%	82 %
Bile acids	1.1%	3 – 10%
Bilirubin	0.2%	0.6 – 2%
Fatty acids	0.12%	0.3 – 1.2%
Cholesterol	0.1%	0.3 – 0.9%
Lecithin	0.04%	0.1 – 0.4%
Na^+	145 mEq/L	130 mEq/L
K^+	5 mEq/L	9 mEq/L
Ca^{2+}	5 mEq/L	12 mEq/L
Cl^-	100 mEq/L	75 mEq/L
HCO^{3-}	28 mEq/L	10 mEq/L

The most important components of bile are bile acids even if present in small quantities (3-10%).

Bile acids are produced by hepatocytes starting from cholesterol and are subsequently conjugated to two amino acids: taurine and glycine.

At the physiological pH (6.8-8) of bile and intestine, bile acids are found in dissociated form and it is also for this reason that they are also called bile salts. Being the end products of the hepatocytic metabolism of cholesterol, they are classified as steroids. Bile acids are polyhydroxy acids consisting of a basic structure with four condensed saturated rings which takes the name of cyclopentanperhydrophenanthrenic nucleus. In the steroid molecule, the methyls in C-10 and C-13, indicated respectively as C-19 and C-18, are called angular and are perpendicular to the middle plane of the molecule. By convention, in steroids, groups on the same side as angular methyls are referred to as β substituents, while those on the opposite side are referred to as α substituents. The cyclohexane rings A, B and C of steroids have a chair conformation and while the junctions of the rings B, C and D are always trans (in trans the hydrogens in C-8 and C-9 and the methyl in C-13 and l hydrogen in C-14), the junction between rings A and B can be either cis or trans. The hydrogen in C-5, in the case of the trans junction between rings A and B, is oriented downwards: this type of junction is identified with the prefix 5α, **Figure 5**.

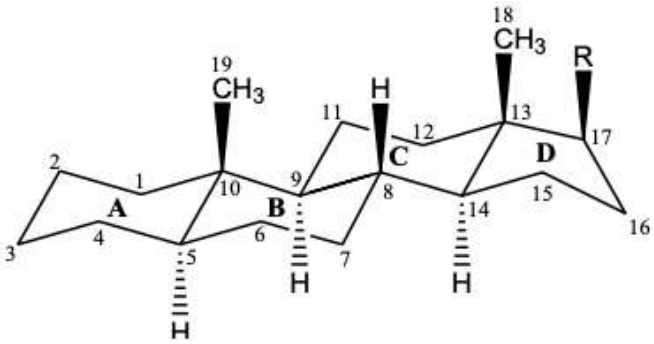


Figure 5: 5α steroids

The 5β structure (**Figure 6**) instead has a cis weld between rings A and B and hydrogen in C-5 is on the same side as the angular methyl.

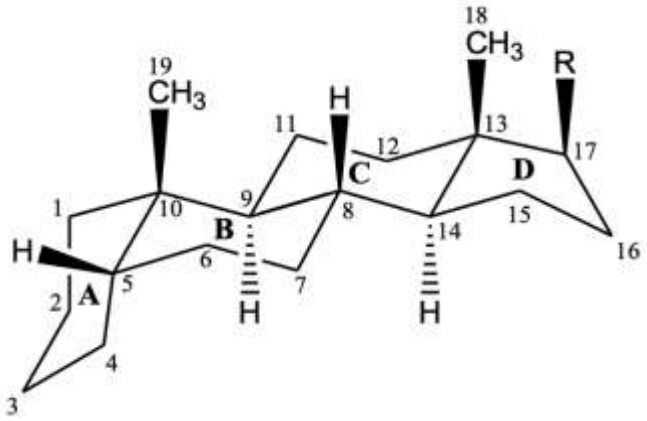


Figure 6: 5β steroids.

Bile acids are 24-carbon steroids of the 5β series.

In human bile there are two types of bile acids called primary:

• Cholic acid (3*α*,7*α*,12*α*-trihydroxycholanic acid, CA), (**Figure 7**);

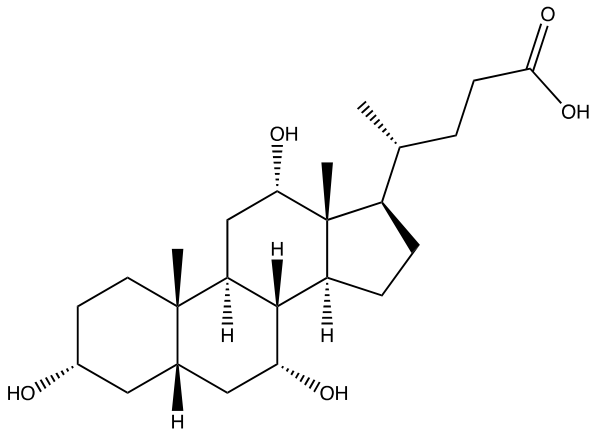


Figure 7: Cholic acid.

• Chenodeoxycholic acid (3*α*,7*α*-dihydroxycholanic acid, CDCA) (**Figure 8**);

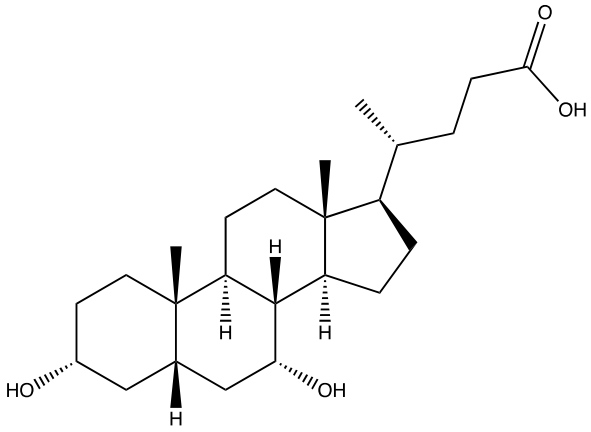


Figure 8: Chenodeoxycholic acid.

These are largely present as conjugates with the amino acids taurine and glycine linked to the carboxyl group by an amide bond.

The pool of bile acids, in addition to CA and CDCA, also includes bile acids called secondary: deoxycholic acid (DCA) and lithocolic acid (LCA) (**Figure 9**), produced respectively by cholic and chenodeoxycholic acids by the flora intestinal bacterial by a dehydroxillation reaction in position C-7.

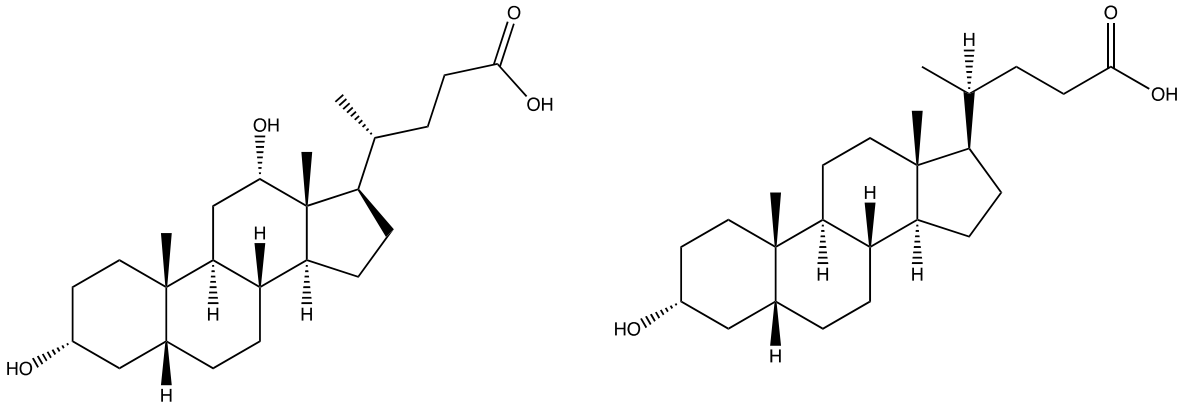
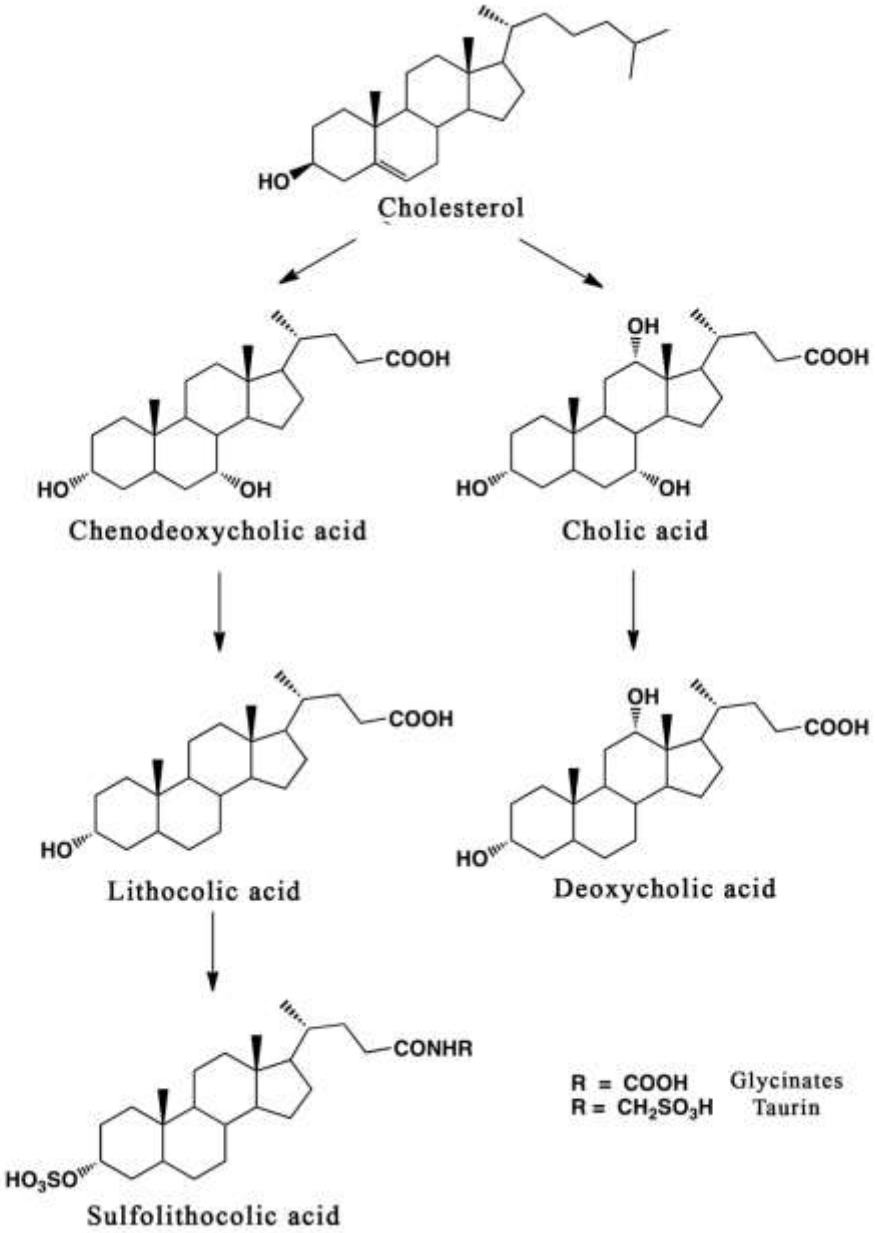


Figure 9: A: deoxycholic acid (DCA); B: lithocolic acid (LCA)

These are absorbed in the proximal jejunum and colon with a passive transport mechanism [149].

After being absorbed from the intestine, the bile acids are returned to the liver where they undergo various transformations that generate tertiary bile acids: for example, lithocolic acid, of which hepatotoxicity is known, is sulphated (thus becoming sulfolithocolic acid) for increase its water solubility and thus decrease its reabsorption. The bile acids thus transformed are conjugated again with taurine and glycine and secreted into the bile (**Scheme 2**).



Scheme 2: Transformations of bile acids.

In the intestine, further reactions catalyzed by the bacterial flora take place such as the hydrolysis of the conjugates and the epimerization in position C-7 of the chenodeoxycholic acid (the substituent from α passes to β) with the formation of ursodeoxycholic acid (UDCA), **Figure 10**.

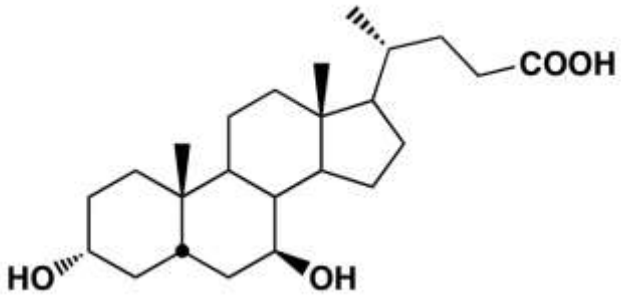


Figure 10: Ursodeoxycholic acid (UDCA).

The various activities carried out by bile acids within the body are linked to the particular structure that these molecules possess; there is a close relationship between physiological function and structure which is defined by the chemical-physical properties of these molecules. In particular, their amphipathic nature guarantees their ability to transport lipid molecules. The amphipathic nature is given by the presence of two regions:

- A polar consisting of hydroxyl groups present in different numbers and positions and α-oriented

- A non-polar compound identifiable with the β-oriented methyl substituents.

The "detergent" and / or lipid transport capacity of bile acids is expressed in the formation of micelles, in which lipophilic compounds find space, obtained by aggregation of many monomers. This phenomenon occurs in a well-defined concentration range, below which only monomers are present, while above the concentration of bile acids aggregated in micelles increases. This parameter is called "critical micellar concentration" (CMC) and can be measured by chemical-physical methods that provide a value of CMC, above which the formation of aggregates

begins. The best known methods are based on measurements of surface tension values, of solubilization of dyes and on activity measurements by means of selective electrodes for bile acids.

The main physiological functions of this type of molecules concern the regulation of bile secretion and transport as well as the ability to emulsify lipids, such as cholesterol, promoting digestion and intestinal absorption.

Due to inflammatory diseases of the gallbladder wall, alterations in fluid dynamics in the biliary tree or bile completely saturated with cholesterol, cholesterol precipitation phenomena can occur in the gallbladder resulting in the need for surgery to remove stones or, in the worst case scenario, the entire gallbladder [150].

In fact, cholesterol precipitates in small crystals which, by agglomerating on a sedimentation core, lead to the production of stones (lithogenic bile).

Until the 1970s, the only therapeutic treatment for gallstones was surgery. Today, however, many patients are treated with chenodeoxycholic acid and ursodeoxycholic acid therapy as some research has shown how gallstones are solubilized using these bile acids as drugs [151].

Ursodeoxycholic acid is the 7β-epimer of chenodeoxycholic acid and represents a small percentage of the total bile acids physiologically present in human bile. In humans, ursodeoxycholic acid is able to increase the ability to solubilize cholesterol in bile, transforming it from lithogenic to non-lithogenic bile.

The mechanisms through which this pharmacological effect manifests itself are more than one:

- decrease in the secretion of cholesterol in the bile, due to the reduction of hepatic synthesis of cholesterol itself and its absorption in the intestine;

- increase in the overall amount of bile acids that promote micellar solubilization of cholesterol;

- formation of a liquid-crystalline mesophase which allows to solubilize the cholesterol more efficiently than the solubilization obtainable in the phase equilibrium;

Ursodeoxycholic acid is considered of greater pharmacological importance than chenodeoxycholic acid in the therapy against gallstones, as it has high efficacy [152]. Given these characteristics, ursodeoxycholic acid represents a product of wide industrial interest worldwide. It is found in fair quantities in the bile of bears (hence its name) but the extraction from the bile of these animals is impractical because it would cause their extinction. This practice has been abandoned by Western countries but continues to be practiced in countries such as China, with somewhat questionable methods. Worldwide, today, the production of UDCA is made starting from the bile acids present in bovine and swine bile. These animals are raised for their meat and the bile is collected, dried and is the raw material for the industrial process.

1.3.2.1 Ursodeoxycholic acid

Ursodeoxycholic acid (UDCA) (3α, 7β-dihydroxy-5β-colanoic acid) **Figure 11**, also known as ursodiol, is a secondary bile acid that derives from the metabolism of cholic acid by the human intestinal microbiota. UDCA is a natural bile acid that is

used to dissolve cholesterol gallstones and to treat cholestatic forms of liver disease including primary biliary cirrhosis.

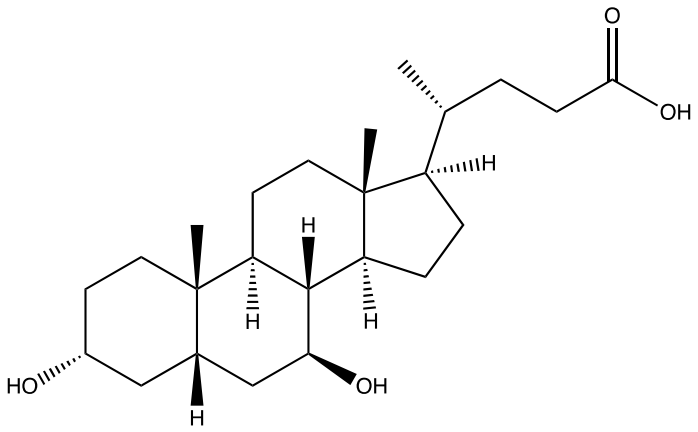


Figure 11: Structure of ursodeoxycholic acid UDCA: chemical formula $C_{24}H_{40}O_4$; PM 392.6; Log P 4.13; water solubility 20 mg/L (at 20 °C).

UDCA appears in solid form in the form of a powder and is considered a secondary bile acid. The other type of bile acid, namely primary bile acids, are produced by the liver and subsequently stored in the gallbladder. When primary bile acids are secreted in the large intestine, they can be broken down into secondary bile acids by the bacteria present in the intestine. Both types of bile acids aid in the metabolism of dietary fats.

Ursodeoxycholic acid regulates cholesterol levels by slowing the rate at which the intestine is able to absorb cholesterol and also acts to break down the micelles, which contain cholesterol [153][154]. Due to this property, ursodeoxycholic acid is used to treat non-surgical gallstones. Ursodeoxycholic acid reduces elevated levels of liver enzymes by facilitating the flow of bile through the liver and protecting liver cells [155]. Although the exact process of action of UDCA is not fully understood, it is thought that the drug is concentrated in the bile and decreases biliary cholesterol by suppressing hepatic synthesis and secretion of cholesterol and inhibiting its intestinal absorption [153]. The reduced saturation of cholesterol allows the gradual solubilization of cholesterol from gallstones, with consequent eventual dissolution of them [156]. UDCA is present in a smaller fraction in the bile acid pool in humans,

but a higher fraction in bears and other hibernating animals [157]. Ursodeoxycholic acid is more soluble in water (hydrophilic) than cholic acid and is less intrinsically toxic to cells [158]. When administered orally, ursodeoxycholic acid becomes an important component of the bile acid pool, the proportion goes from <2% to as much as 65% [159]. In some studies it has been found that by replacing hydrophobic or more toxic bile acids with UDCA, the toxic effects of cholestasis are diminished [160]. Unlike other primary bile acids, UDCA has little agonist activity with FXR, the nuclear bile acid sensing receptor [161]. As a result, UDCA has little effect on the synthesis of cholesterol and lipids and is effective in reducing cholesterol saturation of bile and dissolving cholesterol gallstones. Additionally, UDCA has been shown to decrease serum-increased enzymes in a large number of cholestatic liver diseases including primary biliary cirrhosis, cholestasis of pregnancy [162][163]. In some situations, this improvement in liver enzyme levels has been accompanied by improvements in symptoms, liver histology, and long-term negative outcomes of these diseases (cirrhosis, end-stage liver disease) [164]. UDCA was approved for the dissolution of gallstones in 1987 and as a treatment for primary biliary cirrhosis in 1996. It is also used off-label in other liver diseases. UDCA is available in tablets and capsules of 250 and 300 mg [165][166]. This drug is generally well tolerated; Uncommon side effects can include fatigue, nausea, headache, and weight gain. Less common but potentially serious adverse reactions include hypersensitivity reactions and depression [167]. UDCA has been linked to rare cases of transient and mild elevations of serum aminotransferase during therapy and to rare cases of jaundice and worsening of liver disease in patients with pre-existing cirrhosis

1.3.3 Food Preservatives: Sorbic acid

Sorbic acid, (2*E*,4*E*)-hexa-2,4-dienoic acid, **Figure 12**, is a straight-chain alpha-beta-unsaturated fatty acid first isolated from the oil of unripe rowanberries in 1859 [168]. It appears as a white powder and it's slightly soluble in water. It is tasteless and free of toxicity, but highly irritating to the skin. This molecule occurs naturally in apples, plums and rowan fruits, hence the name [168].

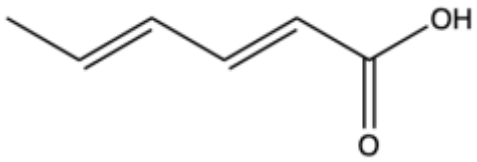


Figure 12: Structure of sorbic acid, chemical formula: $C_6H_8O_2$, molecular weight: 112.13, Log P: 1.26, Solubility: 1.56 g/L (20°C).

Sorbic acid (E200), and its salts (sorbates), are used for their antimicrobial properties as preservatives in foods such as cheeses and dairy products in general, dried fruit, jams, sauces, bread products, drinks, but also food for animals, cosmetics and pharmaceuticals [169][170]. It acts as inhibitor of most molds, yeast, and some bacteria [171]. Sorbic acid, in general, is used both for food preparations or those that come into contact with the human body, and in the polymerization processes of resins, rubbers and plastics. As for the availability at an industrial level, sorbic acid can be extracted from the fruits of the rowan and other rosaceae, however, given the very high demand as an additive, it is also chemically synthesized [172][173].

The antimicrobial action of sorbates occurs at various stages of microbial life cycle (germination, outgrowth, and cell division) [174] and it may result in the alteration of the cell membrane, in the inhibition of transport systems and key enzymes, the creation of a proton flux into the cell, the inhibition of oxidative phosphorylation, or in a synergic effect of two or more of these factors [175][176]. The mode of action

of sorbic acid on bacterial cells and spores has been reviewed by Sofos et al. [175] while York et al. reported the sorbic acid inhibition of the yeast *Saccharomyces cerevisiae* [176].

Sorbates, the more soluble salts of sorbic acid, act as a competitive and reversible inhibitor of amino acid-induced germination [177] of several enzyme systems activity (alcohol dehydrogenase, fumarase, anolase, aspartase, catalase, malate dehydrogenase, alfa-ketoglutarate dehydrogenase, succinic dehydrogenase, and ficin) [178], and of nutrient uptake [179]. The antifungal activity of sorbic acid seems to be related to an interference with the electrochemical membrane potential across the mitochondrial membranes [180].

Potassium sorbate is the most employed salified form of sorbic acid because of its higher solubility in water compared to sorbic acid (only 0.15 g per 100 mL vs. 58.5 g per 100 mL at room temperature) [175][181].

Presence of water, temperature, atmosphere, composition, preparation and storage of the product and pH, individually or in combination, are limiting factors for this preservative and make it vary the minimum antimicrobial concentration required [182].

1.3.4 Aromatic Acids

Phenolic compounds have at least one hydroxyl group linked directly or via a chain to an aromatic ring, phenol being the simplest of them. Starting from this base, there are other compounds that contain more than one hydroxyl group and/or benzene group.

These compounds are present in nature as secondary metabolites in plants, reaching the animal world through ingestion of them. Plants are capable of synthesizing them through two pathways: the shikimic acid pathway or the malonic acid pathway [183]. The classification of phenolic compounds can be carried out according to different criteria, for example, considering the number of carbons, both of the aromatic rings, and those belonging to the chain/s attached to said rings, as shown in **Table 6**.

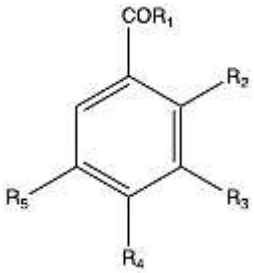
One of the most important groups are the so-called phenolic acids. Phenolic acids comprise two groups: benzoic acids (C6-C1) and cinnamic acids (C6-C3). Derivatives of benzoic acid are found free in nature, mainly as acids (vanillic acid and gallic acid, for example) or aldehydes (such as vanillin and anisaldehyde). They all have a common structure and differ in the number of hydroxyl and methoxy groups, as shown in **Table 7**. Derivatives of cinnamic acid are abundant in plants. Benzoic acids maintain a common structure and their functional groups vary, as shown in **Figure 13**. Phenolic acids, particularly caffeic, ferulic and synapic acids and their esters, are recognized for their antioxidant activity, their capacity as peroxide radical scavengers and as inhibitors of carcinogenesis. Its antioxidant activity strongly depends on its structure and the presence of hydroxyl and methoxy groups in its aromatic structure [184].

At present, phenolic acids have multiple applications, mainly in the pharmaceutical, food and cosmetic industries.

Table 6: Phenols compound classification based on the carbon skeleton.

Carbon skeleton	Groups
C6	Simples phenols
C6 – C1	Benzoic acid and derivatives
C6 – C2	Acetophenone and phenylacetic acid
C6 – C3	Cinnamic acids and derivatives
C6 – C3	Coumarins and derivatives
C6 – C1 – C6	Benzophenone and stilbenes
C6 – C2 – C6	Xanthones
C6 – C3 – C6	Flavonoids and derivatives

Table 7: Benzoic acids classification [185].

Structure	R1	R2	R3	R4	R5	Name
C6 – C1	OH	-	-	-	-	Benzoic acid
	OH	H	OH	OH	OH	Gallic acid
	OH	OH	H	H	H	Salicylic acid
	OH	H	OH	OH	H	Protocatechuic acid
	OH	H	OCH_3	OH	H	Vanillic acid
	OH	H	OCH_3	OH	OCH_3	Syringic acid
	H	H	OCH_3	OH	H	Vanillin
	H	H	H	OCH_3	H	4 - Anisaldehyde

1.3.4.1 Cinnamic acid and derivatives

Cinnamic Acid (*E*)-3-phenylprop-2enoic acid (**Figure 13** and (1) in **Figure 14**) and its derivatives are the major group of phenolic acids with ubiquitous distribution in fruits and vegetables. Recent data support their beneficial effects, including antioxidant [186], anti-inflammatory [187], and anti-cancer activities [188]. Ester derivatives such as ethyl-hexyl methoxycinnamate (octinoxate), isoamyl p-methoxy-cinnamate (amiloxiate), octocrylene and cinoxate are used in cosmetics all over the world as UV filters.

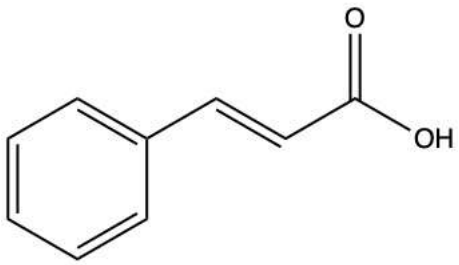


Figure 13: Structure of Cinnamic Acid. Molecular formula $C_9H_8O_2$; Molecular Weight 148.16 g/mol; LogP 2.13; Melting Point 133°C.

Cinnamic acid occurs as white needle-like crystals, insoluble in water, soluble in oils and slightly soluble in alcohol or ether with a bitter taste. This organic compound takes its name from the essential oil in which it is contained, the Cinnamon and exists both as *cis* and *trans* isomer. It is a key chemical found in nature, but it can also be chemically synthesized using different approaches. The oldest on the commercial scale is the Perkin reaction, which is the condensation between an aromatic aldehyde, aliphatic acid anhydride, and the alkali salt of the acid giving as products the cinnamic acid or its derivatives. Other important reactions exploited to obtain it are the Claisen condensation, the Knoevenagel-Doebner condensation, and the Heck reaction [189]. Its physiological biosynthesis occurs, in fact, by deamination of L-phenylalanine mediated by the enzyme *phenylalanine ammonia-lipase* (PAL) [190]. Thanks to its ability to donate electrons

that react with radicals giving stable products, it finds application as central intermediate for several synthesis routes, for example for the production of stilbenes and styrenes [191], taking part in the shikimic and in the phenylpropanoid pathways. Moreover, the presence of the benzene ring and the acrylic acid group leads to the possibility to obtain derivatives of synthesis, in addition to the ones that occur in nature. The type and the position of each substituent determine the biological effect of the derivatives by enhancing or reducing the activity of the original compound [192].

For example, the single substitution in positions 4 of the benzene ring of the cinnamic acid with one hydroxylic group gives the p-coumaric acid ((4), **Figure 14**). It has been reported that this compound displays anti-inflammatory and anti-tyrosinase activities, being a good choice for combined therapies against the hyperpigmentation and erythema [193][194]. In addition, it has the ability to reduce blood glucose and increase insulin, enzymatic and non-enzymatic antioxidants levels [195].

The same double substitution in positions 3 and 4 gives the caffeic acid ((2), Figure 14). This compound has a variety of pharmacological activities, in fact, it acts as anti-inflammatory [196], antioxidant [197], fungicide [198] and anti-cancer [199]. Together with coumaric acid, it acts against possible carcinogens by reducing furan derivatives levels and trapping α-dicarbonyl compounds [200].

Substitutions in 4 with an hydroxylic group and in 3 with a methoxy group generates the ferulic acid ((5), **Figure 14**). This type of reaction occurs in the plant kingdom mediated by the enzyme caffeate O-methyltransferase [201]. Ferulic acid is an organic compound ubiquitous in plants as fundamental constituent of lignocellulose, and it takes the name from *Ferula comunis*. Ferulic acid is a potential anticancer compound because it reduces furan levels, has the ability to arrest the cell cycle, and induces autophagy in cervical carcinoma cells [192][202].

Methoxy and hydroxylic moieties, respectively in positions 3,5 and 4 on the benzene ring, depict the sinapinic acid chemical structure ((3), **Figure 14**). It takes its name from *Sinapis*, mustard, and it is a common constituent of the human diet since it widely occurs in the plant kingdom. Its characteristics to be a protons donator and to adsorb laser radiation leads to its application as a *matrix in the matrix-assisted laser desorption/ionization* (MALDI) mass spectrometry. In addition, it showed antioxidant, anthelmintic [203], anti-inflammatory, and anti-cancer activities [204].

Single methoxy moiety in position 4 on the benzene ring gives the 4-methoxy cinnamic acid (4MCA) ((6), **Figure 14**). This extremely weak basic compound displayed cognition-enhancing activity. It ameliorates the memory deficiency and the impairments of spatial learning mediated by scopolamine [205]. In addition, it can be a potential biomarker for milk (cow) and wild celeries, where it has been detected.

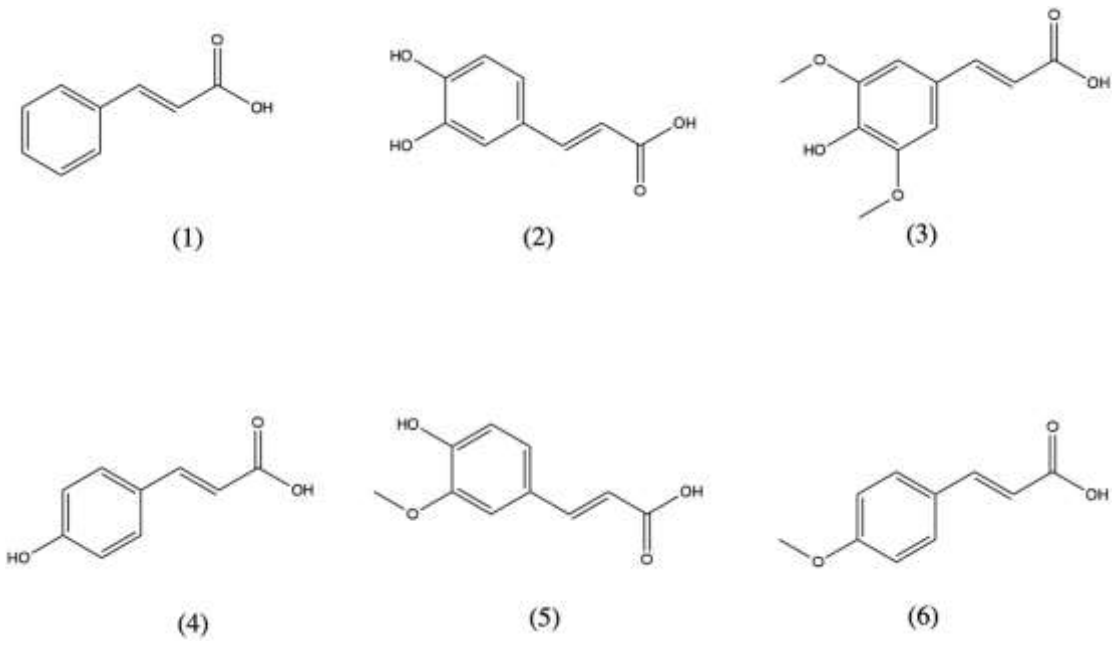


Figure 14: Structures of acids belonging to the cinnamic family are represented: (1) cinnamic, (2) caffeic, (3) sinapinic, (4) coumaric, (5) ferulic, (6) 4 – methoxycinnamic acid.

1.4 SUBSTRATES: HYDROXYL-CARRYING MOLECULES

1.4.1 Glycerol

Glycerol (propane-1,2,3-triol) **Figure 15**, is a propane with three hydroxyl groups in pisition 1, 2, and 3. The presence of three hydroxylic groups confer to the compound the following properties: high hygroscopicity, high boiling point, and low vapor pressure. This nontoxic viscous liquid is widely used in chemical, cosmetic, pharmaceutical, and food industries [82]. It is an intermediate in the metabolism of carbohydrates and lipids, in particular in humans, mice, algae, *Saccharomyces cerevisiae* and *Escherichia coli* [206].

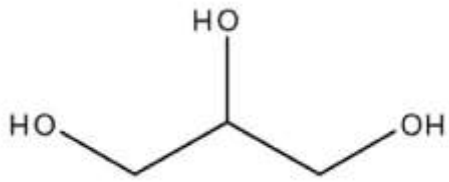


Figure 15: Structure of glycerol; chemical formula $C_3H_8O_3$; PM 92.05; Log P -1.33; miscible in water.

This molecule is non-toxic and appears as a colorless, odorless and sweet tasting liquid; it is characterized by high viscosity, high boiling point and negligible vapor pressure. The presence of the three -OH groups makes glycerol miscible with water in any proportion [207]. This polyol is naturally present in animal and plant cell membranes as part of the phospholipids and triglycerides (fats and oils) that compose them. Thanks to the presence of three hydroxyl groups, glycerol is soluble in water, methanol, ethanol, isomers of propanol, butanol and pentanol, its solubility being lower in dioxane, diethyl ether and acetone, among others, but it is insoluble in nonpolar compounds such as hydrocarbons [208].

Generally, when naming it, the terms "glycerin" and "glycerol" are used interchangeably, but it is important to differentiate between these two terms,

glycerin refers to the product with a high degree of purity, while glycerol is the product that has certain impurities in it.

Glycerol, acting as the backbone for fatty acids, therefore forms a substrate for the synthesis of triacylglycerols and phospholipids in the liver and adipose tissue. In some organisms, the glycerol component, first converted to its glyceraldehyde 3-phosphate intermediate, can be used as a substrate in glycolysis or gluconeogenesis by directly entering the energy and glucose production pathway [209].

The metabolism of glycerol is regulated by the enzymes glycerol kinase, NAD + dependent (cytosolic) G3P dehydrogenase and FAD-bound (mitochondrial) G3P dehydrogenase. Normally, glycerol exhibits rather low acute toxicity so very high oral doses or acute exposures can be tolerated. Chronically high levels of glycerol in the blood are associated, however, with glycerol kinase (GKD) deficiency, which causes a condition of hyperglycerolemia, which is a buildup of glycerol in the blood and urine [210].

The molecule is highly reactive in most typical alcohol reactions. The two terminal primary hydroxyl groups are slightly more reactive than the secondary hydroxyl group. It has hygroscopic properties, at room temperature it quickly absorbs water, it can be oxidized quite easily, the terminal carbon atoms to aldehyde or carboxyl groups and the central carbon atom can be oxidized to carbonyl groups. In addition, it is a stable product under normal storage conditions, compatible with many other chemical materials, non-irritating, with a variety of uses and with no known negative environmental effects. **Table 8** shows the most important physicochemical properties of glycerin.

Table 8: Physico-chemical properties of glycerin [211].

Property	Value
Molecular formula	$C_3H_8O_3$
Molecular weight (g mol^{-1})	92.1
Melting temperature (°C)	18.2
Boiling point (°C)	290
Viscosity (Pa·s)	1.5
Surface tension (nN m^{-1})	64
Density (g cm^{-3})	1.26
Vapor pressure	101

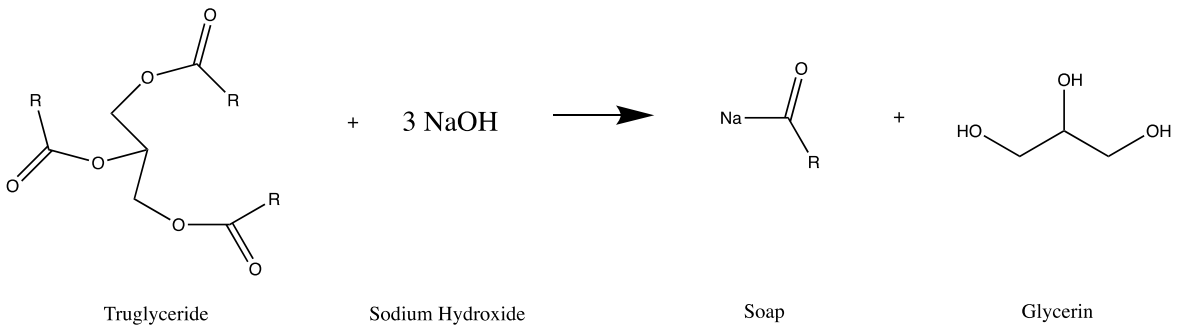
Recently, glycerol availability grew simultaneously with the large increase in biodiesel production according to the international environmental laws (IEL). Glycerol is indeed a major byproduct of the biodiesel manufacturing process, representing approximately 10% w/w of biodiesel. Its consequent higher affordability allowed novel uses, different from the traditional direct ones applied in the food, cosmetic, and pharmaceutical fields. Thus, the use of glycerol as raw material paves the way for the strengthening of sustainable synthesis processes [212]. It is also employed in consumer products such as medicines and cosmetics while in the food industry it can be used as an additive (identified with the initials E422), or as a sweetener or humectant. Furthermore, glycerol is also present in numerous other products such as paints, resins and paper, tobacco or as a lubricant for drain holes in oil and gas fields or as a wetting agent in pesticide formulations [213].

The ability of glycerol and other polyhydric alcohols to confer stability to proteins is a widely recognized phenomenon [214]. Polyols, like glycerol, are frequently used as cosolvent for protein stabilization [215]. Glycerol is known to shift the native protein structure to more compact states [216], prevent the loss of enzymatic activity, increase the thermal unfolding temperature, and inhibit irreversible aggregation of proteins [217]. Moreover, glycerol has been reported as a nontoxic, biodegradable, and recyclable green solvent for high product yields and selectivity in catalysis and enzymatic catalysis [218][207].

1.4.1.1 Obtaining glycerol

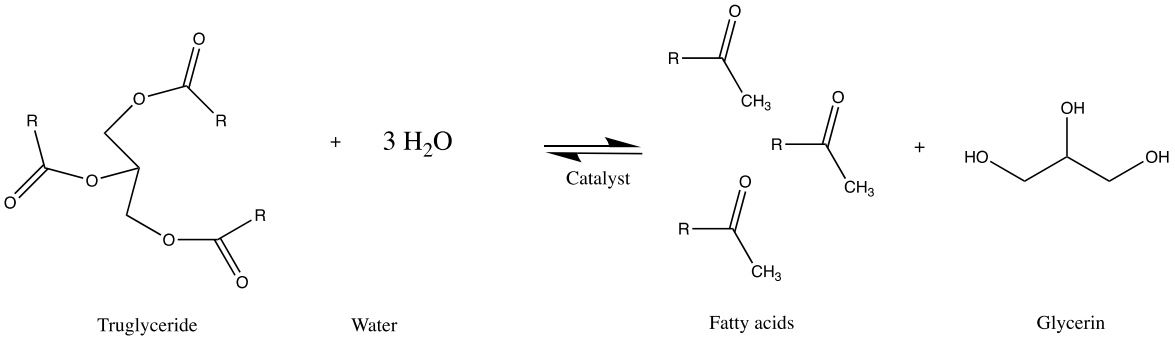
Traditionally, glycerin is obtained as a by-product in saponification reactions, in the production of fatty acids and fatty esters (oleochemistry); in addition, synthetic glycerin can be obtained from propylene [219].

Each of these processes is briefly described below. In the manufacture of soap by saponification of vegetable fats and oils, the reaction of which is shown in **Scheme 3**, the saponification reaction is the hydrolysis of vegetable fats and oils (triglycerides) with soda (sodium hydroxide). The products obtained are soap and a residual bleach where glycerin is found. The yield of the glycerol is approximately 10% of the soap formed [220].



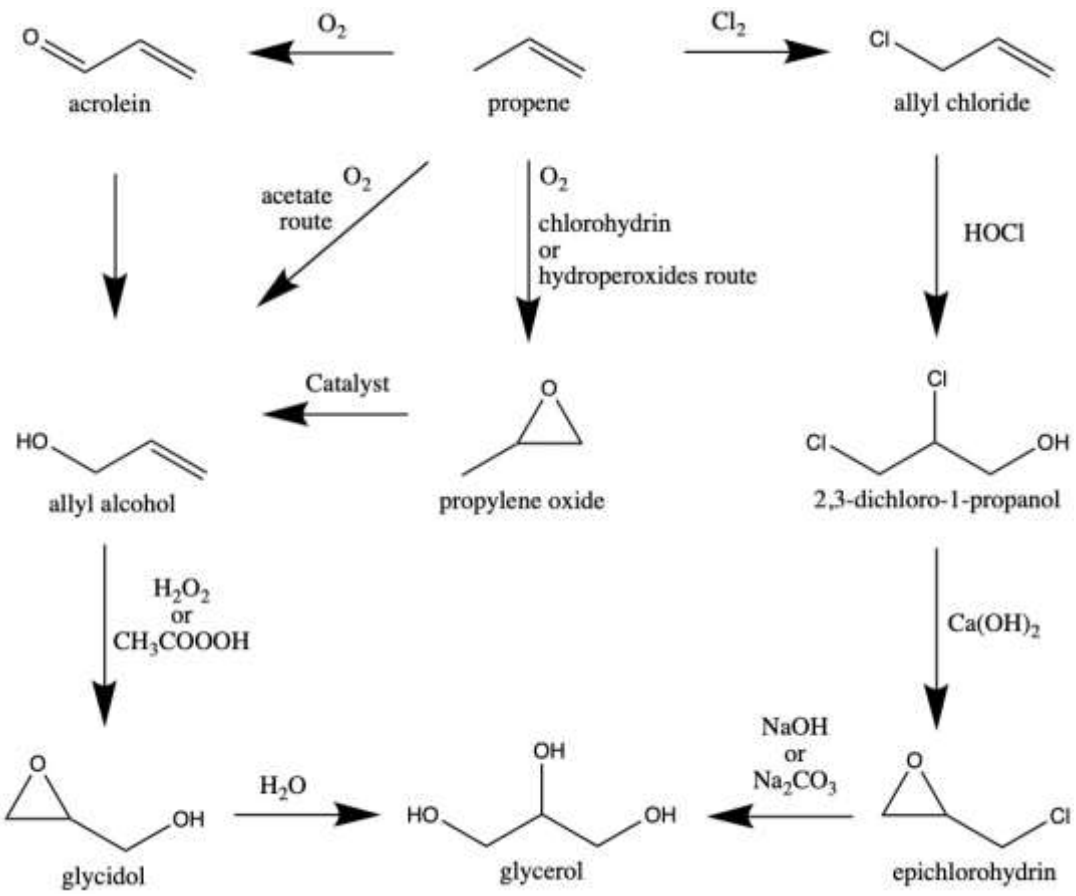
Scheme 3: Saponification of triglycerides to produce soap and glycerin.

Fats and oils (triglycerides) can be hydrolyzed to obtain fatty acids and glycerin. **Scheme 4** shows the hydrolysis of triglycerides, a reversible reaction that consists of the cleavage of the ester bond of the triglyceride molecule to produce the formation of fatty acids and glycerin. Thus, for every 100 g of triglycerides with 61 g of water, 95.7 g of fatty acid and 10.4 g of glycerol are produced [221].



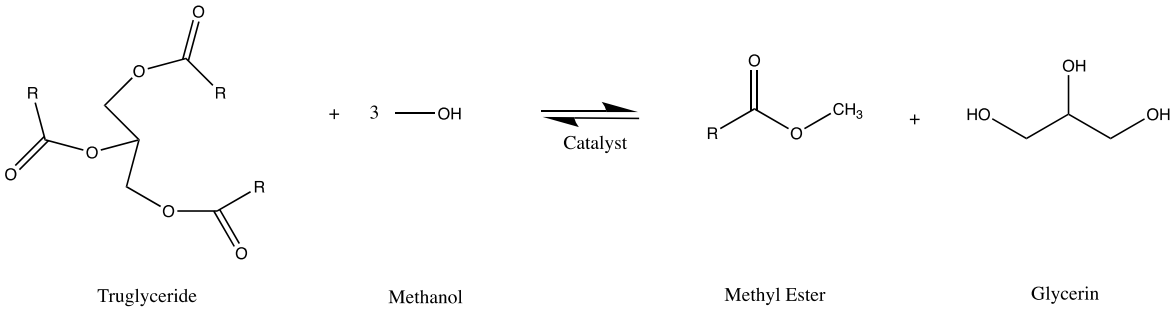
Scheme 4: Hydrolysis of triglycerides to produce fatty acids and glycerin.

In addition, glycerin can be obtained from sources other than fats and oils, from propylene synthetic glycerin is produced. There are two processes: in the first, propylene is treated with chlorine to obtain allyl chloride, which reacts with a hypochlorite solution to give dichlorohydrin, which reacts with a strong kiss to give epichlorohydrin; from it, glycerol is obtained by alkaline hydrolysis. The second process consists of oxidizing propylene to form acrolein, which is reduced to allyl alcohol; This compound can be hydroxylated with hydrogen peroxide in aqueous solution to obtain glycerol directly, or it can be treated with sodium hypochlorite to obtain glycerol-monochlorohydrin which, by alkaline hydrolysis, is converted to glycerin. **Scheme 5** shows the route for obtaining synthetic glycerin through starting from propylene.



Scheme 5: Schematic rouite of glycerin synthesis starting from propene.

In this process, as well as by saponification, hydrolysis and fermentation of sugars, the amount of glycerin obtained is small. However, this situation has changed in recent years, due to the growth in the use of renewable energy sources, biofuels, specifically biodiesel. Glycerin is a by-product of biodiesel manufacturing, where approximately 11 kg of glycerin is produced for every 100 kg of biodiesel. Scheme 6 shows the transesterification reaction (alcoholysis) for the production of biodiesel and glycerin. In it, a triglyceride (vegetable oil, vegetable or animal fat) reacts with an alcohol in the presence of a catalyst to produce glycerol fatty acid alkyl esters. As for alcohol, the most used is methanol (CH_3OH), although for example in Brazil they use ethanol (CH_3CH_2OH), because it comes from the fermentation of sugars in their bioethanol industry.



Scheme 6: Triglyceride transesterification to produce biodiesel and glycerin.

The glycerin obtained from the biodiesel process contains impurities such as: methanol, water, inorganic salts (catalyst), fatty acids, methyl ether and a variety of organic matter, depending on the biodiesel production process [219]. Therefore, it requires a purification process, which consists of the elimination of salts (soap), separation of methanol, pH adjustment by elimination of fatty acids, etc. In this way, the separation of the glycerine waters of the biodiesel process is relatively simple; These waters can be centrifuged, filtered or decanted by gravity once the reaction has finished. Both possibilities are sufficient to achieve a good separation and obtain a glycerol with approximately 80% purity. This crude glycerol is suitable for traditional applications in animal feed or as a carbon source in bioprocesses.

In food, cosmetic, pharmaceutical and chemical synthesis applications, a higher quality of glycerin is required. Therefore, other purification methods are required, the most used being distillation, adsorption and ion exchange, among others. Depending on the degree of purification, different qualities of glycerin can be achieved, there are three degrees of classification of refined glycerin: the technical grade (> 90% purity) used in the chemical synthesis of products, the USP grade (> 99.5% purity) and in food and pharmaceutical products, and the FCC (Food chemicals codex) grade used in food [222]. **Table 9** shows the qualities of the glycerine obtained from the production of biodiesel.

Table 9: Properties of crude, purified and refined glycerin. The table shows the properties range values of crude, pure and refined glycerin [223].

Properties	Crude glycerin	Purified glycerin	Refined glycerin
Glycerol content (% of mass)	60 – 80	99.1 – 99.8	99.2 – 99.8
Water content (% of mass)	1.50 – 6.50	0.11 – 0.8	0.14 – 0.29
Acidity (pH)	0.70 – 1.30	0.10 – 0.16	0.04 – 0.07
Ashes (% of mass)	1.50 – 2.50	0.054	< 0.002
Salts content (soap)	3.00 – 5.00	0.10 – 0.16	0.04 – 0.07

1.4.1.2 Glycerol esterification

The esterification reaction occurs between a carboxylic acid and an alcohol to produce an ester and water. It is a reversible reaction, so the reagents do not completely disappear; Therefore, some authors suggest shifting the equilibrium of the reaction to favor the formation of the ester using different techniques: use of pervaporation, eliminating water using high temperatures or vacuum, causing a change in the state of a product, molecular sieves or hygroscopic salts [224][225] an excess of alcohol has also been considered [207]. With regard to alcohol, short-chain ones such as methanol and ethanol are usually used, since they react faster because they are smaller molecules. In the case of study, glycerin was used as alcohol, due to the considerations established in previous paragraphs. The esterification of glycerin with acids (carboxylic, fatty, phenolic, etc.), due to the presence of the three hydroxyl groups in glycerin, the product of this reaction is usually a mixture of mono, di, and triglycerides, their amount varies depending on the reaction conditions and the catalyst [219]. In general, the applications of glycerin esters are numerous such as emulsifiers, lubricants, foodstuffs, perfume diluents, dispersing agents, solvents for active principles, coatings in food, vehicle for injections, etc.

Furthermore, mono, di and triglycerides (glycerol esters) are widely used in the cosmetic, food, pharmaceutical, plastic and adhesive industries.

The esterification can be carried out by thermal, catalytic and enzymatic routes. Thermal esterification is always desirable, since it occurs in the absence of a catalyst, but it has the disadvantage that it occurs at high temperatures and is usually a slow reaction. In the literature, the esterification of glycerin with cinnamic and methoxycinnamic acid by thermal means at temperatures of 150 and 200 °C has been studied, to obtain monoglycerides and diglycerides used as ultraviolet filters in cosmetics and sun creams [226]. The esterification of glycerin with resin acids has also been studied to obtain triglycerides of resin acids used in the adhesive industry [227].

Catalytic esterification can be in the presence of homogeneous and heterogeneous catalysts [228]. Catalysts can be both basic and acids, the acidic types are generally used since they tend to give up protons to the carboxylic acid. The most used homogeneous acid catalysts are Brönsted acids (HCl, H_2SO_4, PTSA, HBr, H_3PO_4). However, since they are dissolved in the reaction medium, their separation is usually quite expensive. On the other hand, heterogeneous acid catalysts, such as ion exchange resins (Amberlyst, Lewatit, among others), can be separated more easily (by filtration) and are reusable (by acidifying them for a new use). In this sense, the homogeneous catalyst PTSA and the heterogeneous catalyst of mesopororos with sulfonic groups (mesopo-S-phenyl-end-SO3H) have been used in the esterification of glycerin with p-methoxycinnamic acid and methoxycinnamic acid, in obtaining mono and diglycerides of these acids [229].

Enzymatic esterification is increasingly used due to its mild operating conditions of temperature and pressure, enzymes are specific and selective catalysts, as has already been explained. Among the most used enzymes are lipases, especially lipase B from *Candida antarctica* (CALB) or its commercial derivative, the

immobilized Novozym 435, which is produced by the company Novozymes [230]. This enzyme is very selective for the formation of mono, di and triglycerides when the acylating agent is an acid of the aromatic type. The CALB and Novozym 435 enzymes have been used to obtain monoglycerides by esterification of glycerin with benzoic acid [81], in obtaining triglycerides of 4-phenylbutyric acid and phenylalkanoic acids with Novozym 435 [231], and Novozym 435 has also been used in the esterification of glycerin with triacetin [207].

1.4.2 Erythritol

Erythritol ((2R, 3S) -butan-1,2,3,4-tetraol) **Figure 16**, is a polyalcohol naturally present in fruit and fermented foods. It is widely used in both the pharmaceutical and food industries as an additive, sweetener, sweetener and antioxidant [232].

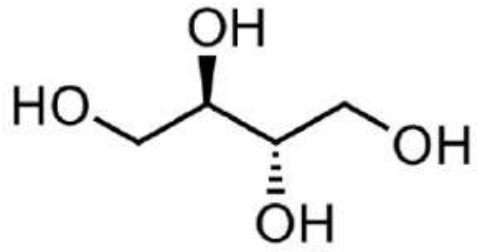


Figure 16: Structure of erythritol, Chemical Formula $C_4H_{10}O_4$; PM 122.12; Log P -2.29; Melting Point 121.5°C

The molecule appears as a white crystalline or granular powder, at room temperature, and has four hydroxyl groups that make it soluble in water and poorly soluble in ethanol.

Erythritol is a four-carbon sugar alcohol (meso-1,2,3,4-butanetetrol) which is 60-80% as sweet as sucrose. It is intended for use as a low-calorie sweetener. Erythritol is manufactured from glucose or sucrose by fermentation with Trichosporonoides megachiliensis or Moniliella pollinis, which are non-pathogenic, non-toxicogenic yeasts. Erythritol also occurs naturally in fruits and mushrooms and is present in

various fermented products, including wine, sake, and soya sauce, generally at a low concentration (700-1300 mg/kg), but in the exceptional case of a single species of mushroom, at 34 000 mg/kg. It is often detected in human and animal tissues and body fluids, including the lens, cerebrospinal fluid, serum, semen, and urine.

The technical characteristics of erythritol, such as its cooling effect and low hygroscopicity, are more like those of xylitol than those of sorbitol, which represents the major market share of sweetening agents. If erythritol were used to replace all xylitol (20% of all polyol use), the projected mean intake would be 1 g/day and the 90th percentile intake would be 4 g/day; if it were used to replace all polyols, the mean intake would be 4-5 g/day and the 90th percentile intake would be 20 g/day on the basis of estimates for diabetic patients [233].

On an industrial level, erythritol is obtained from sugary substrates, such as starch, glucose and sucrose, through microbial fermentation by osmophilic yeasts or by fermentation of Saccharomyches cerevisiae yeast starting from dextrose [234].

In the fermentation broth, in addition to other polyols, large quantities of erythritol are formed. After filtration, ion exchange chromatography and concentration, erythritol is crystallized with a purity greater than 99%.

Erythritol also has an advantageous metabolic profile: thanks to its small size and low molecular weight it can be absorbed up to 90% in the intestine, where it is only partially metabolized, and can be excreted via the urine without undergoing any changes; consequently, the overall caloric value of erythritol varies from 0 to 0.2 kcal/g [235].

Erythritol boasts a glycemic index (GI) and an insulin index (II) close to zero, thus representing a valid substitute ingredient for sucrose in low-calorie and low-calorie diets and suitable for people at risk or suffering from diabetes [236].

Studies have also highlighted the antiradical activity of erythritol to counteract hydroxyl radicals, thanks to its protective properties towards cell membranes; it can therefore act as an antioxidant in vivo, counteracting the effects of free radicals produced by hyperglycemia [237].

Erythritol is also a natural non-cariogenic excipient used in various solid pharmaceutical preparations as a filler or coating for tablets. Erythritol is also used in the food industry as a sweetener for beverages and has been designed for use in dry powder inhalers. Clinical studies have shown side effects similar to those of sucrose [238].

1.4.3 Xyilitol

Xylitol ((2R, 3R, 4S) -1,2,3,4,5-pentanpentanol)) **Figure 17**, is an alditol, a polyalcohol of natural origin derived from the reduction of sugars. Xylitol occurs naturally in fruits and vegetables and is mainly extracted from birch trees to be used as a substitute for traditional sugar (sucrose) [239].

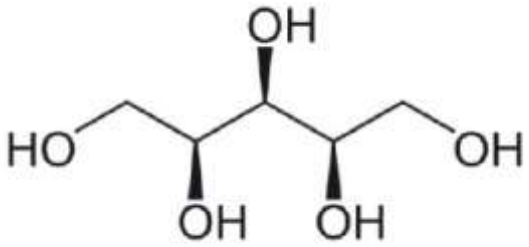


Figure 17: Structure of xylitol. Chemical Formula $C_5H_{12}O_5$; PM 152.15; Log P -3.1; Melting Point 92-96°C.

At room temperature it appears as a white or opalescent crystalline powder in the form of crystals. It is an alcohol with five carbon atoms and the presence in its formulation of five hydroxyl groups makes it particularly soluble in water and poorly soluble in ethanol.

Xylitol is taken through the diet because it is not produced endogenously by humans; is used as a sweetener for diabetics and has practically the same sweetening power as sucrose but with 33% fewer calories [236].

Xylitol is found naturally in many fruits (strawberries, plums, raspberries) and vegetables (for example cauliflower) and, thanks to the consumption of fruits and vegetables, the human body naturally processes 15 g of xylitol per day [240].

On an industrial level, xylitol can be produced starting from raw materials rich in xylan, which is hydrolyzed to obtain xylose; it is extracted from hemicelluloses, almond shells or birch barks (or from wood by-products) [241].

Thanks to its bacteriostatic, sweetening, emollient and stabilizing properties [239], it is widely used in the pharmaceutical industry as a tablet coating and as an excipient in syrups, but also in oral hygiene products such as mouthwashes and toothpastes or sugar-free chewing gum, or in cosmetics (creams, soaps, etc) [242].

1.4.4 Sorbitol

Sorbitol ((2R,3R,4R,5S)-hexan-1,2,3,4,5,6-hexole) **Figure 18**, less commonly known as glucitol, is a polyalcohol with a sweet taste and slowly metabolized by the human body. Most sorbitol is made from potato starch, but it is also found in nature, for example in apples, pears, peaches, and prunes [243].

Sorbitol is a naturally occurring glucose alditol in various fruits and berries and is an isomer of mannitol, in fact they differ in their chemical formula only in the orientation of the hydroxyl on the carbon in position 2.

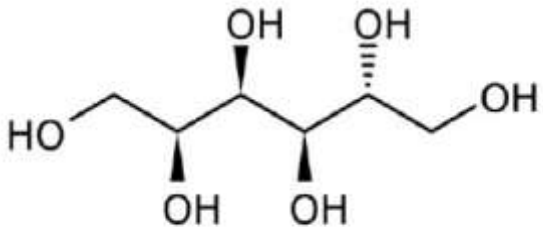


Figure 18: Structure of sorbitol; Chemical Formula $C_6H_{14}O_6$; PM 183.16; Log P -4.67; Melting Point 98-102°C.

The molecule is a white, hygroscopic and crystalline powder with a sweet taste, very soluble in water and scarcely soluble in ethanol.

It can be obtained by reducing glucose, which involves the conversion of the aldehyde group into hydroxyl or it is produced industrially through a hydrogenation process followed by hydrolytic crystallization of the glucose and corn syrup [244].

Sorbitol is often used in modern cosmetics, as a humectant and thickener, and as a cryoprotectant additive in food [245]. It is widely used in the pharmaceutical sector as a coating for tablets, or in the cosmetic sector, or in the food sector mainly as a sweetener [236]. It has a calorific value similar to that of glucose (4 kcal/g).

It is one of the preferred sweetening agents as it has reduced caloric value and low glycemic index (about one half compared to sucrose) and it is free from any possible carcinogenic effects. Another advantage that sorbitol offers is related to its resistance to digestion by oral bacteria and thus has little effect on plaque formation and tooth decay [246].

The human body can transform sorbitol into monosaccharides, especially fructose, without the intervention of insulin: this feature makes it an excellent substitute for sucrose especially in the diets of people at risk or suffering from diabetes [244].

This popular sugar alcohol is also used as an excipient in formulations of various drugs [243] and as plasticizer [247].

1.4.5 Ascorbic acid

Ascorbic Acid (5R)-[(1S)-1,2-dihydroxyethyl]-3,4dihydroxyphuran-2(5H)-one (**Figure 19**) is an organic compound that occurs as a white powder or pale-yellow crystals soluble in water, ethanol, and glycerol, with an acidic and bitter taste. L-Ascorbic Acid (Vitamin C) is an essential water-soluble vitamin naturally present in fruit and vegetables (citrus fruits, kiwis, strawberries, peppers, broccoli) which cannot be synthesized by humans, unlike other mammals, and has to be obtained from diet or food supplements.

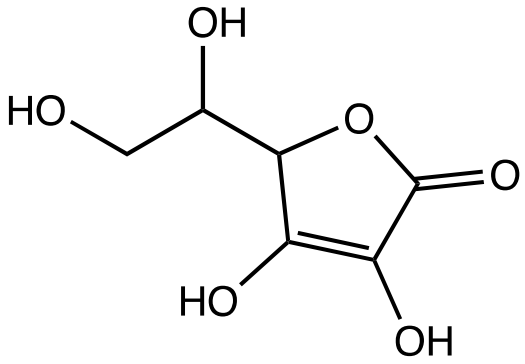


Figure 19: Ascorbic Acid. Chemical Formula $C_6H_8O_6$; Molecular Weight 176.12; LogP -3.36; Melting Point 190-192°C

It is a photo-, humidity- and oxygen-sensitive molecule, which easily degrades with heat. Vitamin C improves the absorption of iron, stimulates the immune system, inhibits the release of histamine, contributes to wound healing, intervenes in the production of bile acids, collagen, and the biosynthesis of some hormones, and has an antioxidant effect with daily intakes of at least 100mg [248] [249] [250].

In industry, ascorbic acid can be chemically synthesized, starting from D-glucose, or by fermentation processes combined with engineered microorganisms [251] [252].

The bactericidal and antioxidant properties are exploited, in particular, in the food industry and for the production of supplements and cosmetics.

Ascorbic Acid, identified as E300, is added to various foods, such as packaged meats, cured meats, fruit juices, frozen vegetables, wine, and beer. It helps both to correct the acidity and aroma of the product and to prevent the browning and the formation of nitrosamines.

Ascorbic acid with its derivatives (salts E301, E302; ester E304) find wide application in industry. For example, the lipophilicity of esters is exploited to prevent rancidity in vegetable fats or oils [248].

It is absorbed starting from the oral mucosa, then gastric mucosa and small intestine, and finally stored in the liver and adrenal gland. In presence of high concentrations, a reduced absorption can be recorded. Thus, the non-stored part is eliminated by urine and toxic effects are rare [249].

2. MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 EQUIPMENT

2.1.1 Equipments for the esterification reaction

The heated magnetic stirrer was model "AREX Digital PRO," by Velp Scientifica SRL (Usmate Velate, Italy).

2.1.2 Analysis Tools

2.1.2.1 HPLC

Reverse phase HPLC analysis of samples obtained throughout each experiment. This technique is based on the separation of the components of the mixture by the greater or lesser affinity that a species has for the stationary phase of a column (hydrophobic in nature) and for the mobile phase (polar eluent in this technique) that is circulating by the system. The separated compounds pass through a detector, their appearance being recorded as peaks in a chromatogram.

The equipment used is a JASCO brand HPLC modular system (LC-2000 series) equipped with reverse phase column (Synergi 4 μm Hydro-RP 80 Å − 250 × 4.6 mm) and shown in **Figure 20**.

Figure 20: Jasco LC-2000 Series Modular Type HPLC Chromatograph.

It consists of the following elements, connected sequentially in the following order:

- Low pressure pump; model Jasco PU – 4180.

Figure 21: Jasco Pump PU – 4180

- Jasco CO - 2065 thermostat to house the column and temper it.

- Refractive index: model RI-4030.

Figure 22: Refractive index: model RI-4030.

• UV/Vis spectrophotometry detector for detection by absorption in the ultraviolet-visible spectrum; model Jasco UV-4070.

Figure 23: UV/Vis spectrophotometry; Jasco UV-4070.

2.1.2.2 uHPLC-MS

The methods for the identification of the compounds generated in the course of the esterification experiments were developed using ultra high performance liquid chromatography (model: Waters Acquity uHPLC) equipped with a ZQ 2000 ESI mass spectrometer (Figure 24; Waters, Milford, MA,USA) and Mass Link software (Waters, Milford, MA,USA).

The ZQ 2000 ESI mass spectrometerr is a universal detector for high performance liquid chromatography (HPLC). The mass spectrometer measures charge / mass ratios of ions, heating a beam of material of the compound to be analyzed until it vaporizes and ionizes the different atoms, the beam of ions produces a specific pattern in the detector that allows the compound to be analyzed.

A 2.6 μm Kinetex 50 × 4.6 mm C18 column was chosen to perform the analysis. This study has been carried out at the Department of Chemistry and Pharmaceutical Sciences, University of Ferrara.

Figure 24: Waters ZQ 2000 ESI mass spectrometer.

2.1.2.3 HPTLC-MS

The HPTLC-MS interface (Camag - Switzerland, **Figure 25**) allows the extraction of compounds from a TLC/HPTLC chromatographic plate and their direct injection into a mass spectrometer via an APCI or ESI interface, for identification purposes. The HPTLC-MS interface has been provided by the Department of Chemistry of the University of Ferrara.

Figure 25: Camag HPTLC-MS interface.

2.1.2.4 NMR

To verify the chemical structure of the products obtained in the esterification reactions, Nuclear magnetic resonance (NMR) spectra of these compounds were recorded with a 400- MHz Varian Gemini spectrometer; Varian, Palo Alto, CA, (USA); **Figure 26**. It's equiped with inverse and direct multinuclear probes, gradients of field. Performs ^{1}H, ^{13}C, and ^{31}P one-dimensional and two-dimensional spectra, homonuclear and heteronuclear at room temperature. The spectrometer has been provided by the Department of Chemistry of the University of Ferrara.

Figure 26: 400- MHz Varian Gemini spectrometer – USA.

2.1.2.5 IR

IR spectra was recorded by Perkin Elmer FTIR Spectrum 100 infrared spectrometer equipped with ATR using a ZnSe Diamond. This study has been carried out at the Department of Chemistry and Pharmaceutical Sciences, University of Ferrara.

Figure 27: Perkin Elmer FTIR Spectrum 100 infrared spectrometer.

2.1.3 Auxiliary Tools

- Analytical Weight Scale; model ENTRIS224-1S; Sartorius s.r.l – Germany.

- Rotatory evaporator; model RE-121; Buchi s.r.l – Cornaredo, Italy.

- TLC viewing Ultra violet UV cabinet; model CN-6, Vilber Lourmat s.r.l – France.

• Lyophilizer; model Lyovapor L-200 di Buchi s.r.l. Italy.



• UV-ray spectrophotometer for RNA quantification; model SmartSpec Plus; BioRad; California – USA.

• UV-Vis spectrophotometer; model UV-30 SCAN; Onda – Italy.

• Coulter Counter; model Z Series; Beckman – USA

• CFX96 Touch Real-Time PCR Detection System, including the Thermal Cycler C1000 Touch as well as the software CFX Manager 184-5000; BioRad; California – USA.

• Thermal Cycler; model GeneAmp PCR System 9700; Applied Biosystem - USA.

2.2. MATERIALS

The solvents, reagents, the antibiotic clotrimazole, the antifungal chloramphenicol and the 60 Å - 63-200 μm silica gel were purchased from the Sigma-Aldrich company. *Saccharomyces cerevisiae* DSMZ 70449 and *Streptomyces griseus* DSMZ 40236 were purchased by the DSMZ company. The cell line used is IB3-1 of immortalized cells with ΔF508 / W1282X mutation, from fibrocystic patients. The ImProm-II Reverse Transcription System cDNA synthesis kit was purchased from the Promega company. The kit for RT-qPCR - iTaq Universal SYBR Green Supermix was purchased by the company BIO-RAD. The TLC Silica gel 60 F254 plates in aluminum 20x20 cm - 0.20 mm thick were purchased by the Merck company. The software used are ChemDraw Professional 16.0, MestReNova, Maestro for CFX Real Time PCR by BIO-RAD, ImageJ.

2.2.1 Enzymes

The development of enzymatic esterification protocols, the aim of this work, involved the use of enzymes in both free and immobilized form. The lipases used derive from the microorganism *Candida anctartica* or from the porcine pancreas.

2.2.1.1 *Candida antarctica* Type B – CALB [253]

CALB is a non-specific lipase from *Candida antarctica B*. This enzyme is stable over a broad pH range, especially in the alkaline pH range. This enzyme exhibits a high degree of substrate specificity, allowing large groups on the carboxylic acid and resulting in highly regio- and enantioselective conversions.

CALB has been used extensively in the resolution of racemic alcohols, amines, and acids, and in the preparation of optically active compounds from meso substrates. The resulting optically pure compounds are highly difficult to obtain by alternative routes and can be of great synthetic value. CALB has also been used as a regio-selective catalyst in selective acylation of different carbohydrates.

The different forms of calb that have been used are given below:

• CALB (lipase B from *C. antarctica* immobilized on acrylic resin immobead 150, recombinant from *Aspergillus oryzae*; ≥1800 U/g) was purchased from Sigma-Aldrich; **Figure 28**.

Figure 28: CALB, recombinant from Aspergillus oryzae – Sigma-Aldrich.

• Novozym 435 and Lipozyme - CALB-L, **Figure 29**, were purchased from Novozymes A / S, Danmark.

(a) (b)

Figure 29: Novozymes Enzymes; (a) Lipozyme CALB-L, (b) Novozym 435

2.2.1.2 Porcine Pancreatic Lipase – PPL - Type II

Lipase from porcine pancreas (**Figure 30**) is widely used industrially for the resolution of chiral compounds and the transesterification production of biodiesel. Funrtermore, is used for the analysis of fatty acid at the 2-position of triglycerides [254], to determine the indigestible fraction of plant source that is resistant to the

Figure 30: Porcine Pancreatic Lipase Type II, Sigma-Aldrich.

action of digestive enzymes [255], as a component of dissolution media to study the dissolution profiles of theophylline [256]. Free PPL (porcine pancreas lipase, type II; lyophilized (cake); CAS: 9001-62-1, EC: 3.1.1.3), was purchesed from Sigma Aldrich, St. Louis, MO – USA.

The biological catalysts used in this thesis are compared in **Table 10**.

Table 10: Specs comparison among the used biocatalysts.

Product name	CALB	Novozym 435	Lipozyme CALB-L	PPL - Type II
Company	Sigma -Aldrich	Novozymes	Novozymes	Sigma - Aldrich
Source	*Candida Antartica B*	*Candida Antartica B*	*Candida Antartica B*	Porcine Pancreas
Activity	1800 LU/g*	10000 PLU/g**	5000 LU/g	100-500 U/mg***
Ph optimum	pH 5-9	pH 5-9	pH 5-9	pH 6-8
Temperature optimum	30-60°C	30-60°C	30-60°C	37°C
Physical state	White colored immobilized granules	Cream colored immobilized granules	Light brown liquid	Lyophilized powder

* One LU (lipase unit) is the amount of enzyme activity when 1 μmol of titratable butyric acid is released per minute
** One PLU (propillaureate unit) is the amount of enzyme activity when 1 μmol of propillaureate is generated per minute
*** One unit will hydrolyze 1.0 microequivalent of fatty acid from triacetin in 1 hr at pH 7.4 at 37°C.

2.2.2 Reagents and solvents

Ibuprofen sodium salt (≥98% pure) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glycerol (≥99%pure), Erythritol, Xylitol, Sorbitol (≥98%pure), N,N-dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), silica gel (60 Å, 70–230 mesh, 63–200 μm), and methanol-d4 degree 99.8% were purchased from

Sigma-Aldrich. All other solvents, other than the methanol for HPLC, were of ACS grade and purchased from Sigma-Aldrich.

2.2.3 Obtaining the acid ibuprofen from sodium salt

In a flask, 5 g of sodium salt of ibuprofen are solubilized in 50 ml of water under stirring (100 rpm) at 50 °C. It is acidified with HCl 37% up to pH 1.

The contents of the flask are then transferred to a separatory funnel in which 50 ml of toluene are added; after having stirred and obtained two phases, the organic phase in which the ibuprofen is contained is separated. A second extraction is carried out by adding another 50 ml of toluene to the aqueous phase; the organic phases are combined and anhydrified with anhydrous Na_2SO_4.

The sodium sulfate is filtered and the solvent is evaporated in a rotavapor. 3 g of ibuprofen are obtained.

2.3 METHODS

2.3.1 Chemical Synthesis of Ibuprofen Sorbitol Ester

For the chemical synthesis of the sorbitol ester of ibuprofen, 0.91g of sorbitol (5 mmol) was dissolved under mild heating in 5ml of dimethylsulfoxide (DMSO) to form a clear colorless solution. To this warm solution 1.03 g (5 mmol) of IBU and 0.06 g (0.5 mmol) of DMAP was added. Subsequently, 1.03g (5mmol) of DCC was added. During addition of DCC white precipitate of dicyclohexyl urea started to form. The mixture was stirred for 20 h, and then cooled in liquid nitrogen to solidify it. This solid mixture was placed to lyophilizator to reduce DMSO amount. After 4 h the content of DMSO was reduced to 3g. Dichloromethane (15ml) was added to the mixture, the mixture was cooled down to 5°C and the solid was filtered off. Filtrate was concentrated in vacuum at 50°C to receive 4.13 g colorless oil.

2.3.2 Stirred Reactor Design

The stirred reactor setup is shown in **Figure 31**. The stirred reactions were conducted using this design.

(a)

(b)

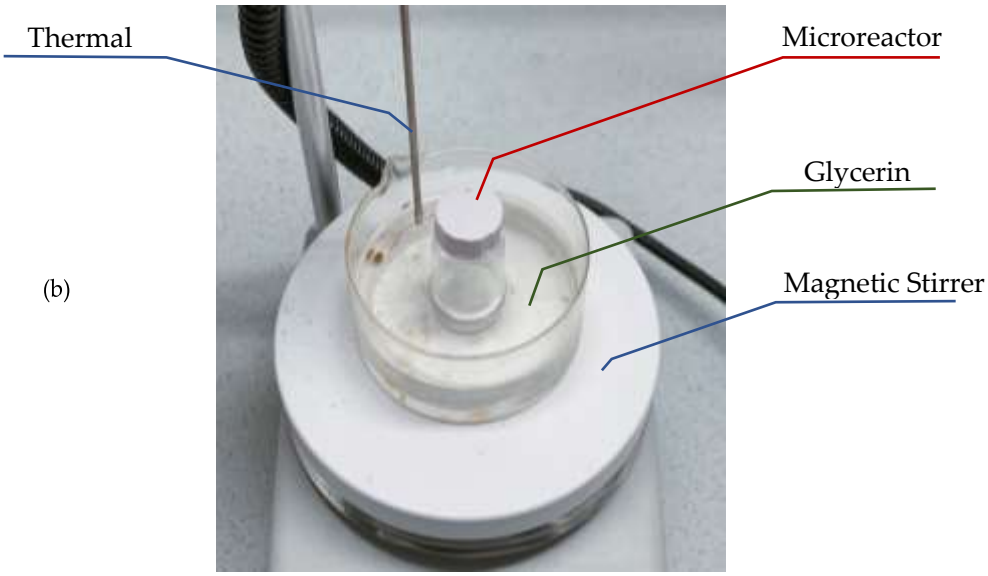


Figure 31: Stirred reactor system for the enzymatic esterification reactions. (a) full view fo the reactor system; (b) Plate view of the reactor with magnetic stirrer, glycerin bath, microreactor and thermal probe.

The stirred reactor design consists of a 20 ml vial. The vial is placed in a glycerine bath atop a heated stir plate. Reactants are added to the vaial in quantities determined by the experimental design. The vial is screw-capped providing a superior barrier for inhibiting the escape of organic solvent vapors from the reaction zone. For the solventless system, the vial is left open. Stirring is accomplished with (1.5 cm in diameter) "double-sided crosshead" magnet (**Figure 32**) driven by a magnetic stirrer within the hot plate.

Figure 32: "double-sided crosshead" magnet associated with the magnetic stirrer.

Heating is provided by an electric resistance heater within the hotplate controlled by feedback from a thermocouple probe. The probe is submerged beneath the liquid level of the glycerine bath to provide an accurate temperature readout of the reactant system. A control loop maintains temperature at the desired setpoint.

2.3.3 Enzymatic esterificatin Protocols: development of an enzymatic esterification

Several enzymatic esterification protocols have been developed. These protocols can be categorized according to how many phases make up the reaction medium (monophasic and biphasic systems) and/or the nature of the medium (solventless or solvent assisted).

2.3.3.1 *Solventless* esterification strategies

The enzymatic syntheses showed in this section exploit a monophasic system where the alcohol which takes part in the esterification reaction represents both the solvent and the reagent. All the enzymatic synthesis experiments were conducted with the setting up of the stirred reactor system described in section "2.3.2 Stirred Reactor Design".

Enzymatic Synthesis Of Monoglyceride Esters

The synthesis experiments of monoglyceride esters were conducted according to the following general method:

1) The active ingredient of interest is weighed on the precision balance. The appropriate amount is weighed to obtain a final concentration of active ingredient of 20-100g L^{-1} in pure glycerol.

2) The reactor with the amount of active ingredient in acid form, and 3-5 mL of pure glycerol, is placed in a glycerine bath, for a preventive solubilization step at a temperature of 50-80 °C. Stirring can help the dissolution of the acid. Once the homogeneous mixture is reached, a first sample (0.25 mL) is taken in the absence of enzyme, considered as a sample at time zero (T_0).

3) The reaction is started by adding the enzyme used in each experiment. The concentration of the catalyst can vary in a range between 10 and 30 g L^{-1}.

4) The samples taken (0.25 mL) during the transformation are frozen, in order to stop the action of the enzyme.

5) The reaction is stopped at 24h.

The specific quantities of reagents used for each monoglycerine ester synthesis reaction are described in the following tables:

[92]	Amount (mg)	Volume (ml)	g L^{-1}	PM
Sorbic Acid	100		1	112.13
Glycerol		10		92.09
CALB	40		4	

[257]	Amount (mg)	Volume (ml)	gL^{-1}	PM
Ibuprofen	50		2.5	206.29
Glycerol		20		92.09
CALB	50		2,5	

	Amount (mg)	Volume (mL)	g L-1	PM
UDCA	150		30	392,16
Glycerol		5		92,09
Lypozime CALB-L		0.1	20	

	Amount (mg)	Volume (mL)	g L-1	PM
UDCA	150		X	392,16
Glycerol		5		92,09
CALB	10		X	

RPM: 720 T: 55°C

Enzymatic Synthesis Of Butyl esters

The monophasic system strategy was also produced with the primary alcohol 1-butanol. The experiments were conducted as in the tables below:

	Amount (mg)	Volume (mL)	g L^{-1}	PM
Sorbic acid	150		50	112,13
1-butanol		3		74,12

Novozym 435	55	18	
Molecular Sieves	98		

55 °C, 130 RPM, 72 h

	Amount (mg)	Volume (mL)	g L^{-1}	PM
Ibuprofen	150		50	206,29
1-butanol		3		74,12
Novozym 435	55		18	
Molecular Sieves	98			

55 °C, 130 RPM, 72 h

2.3.3.2 *Solvent-assisted* esterification strategies

The enzymatic esterification strategies that have seen the intervention of supporting organic solvents can in turn be divided into: monophasic systems and biphasic systems.

Solvent-assisted in monophasic media system

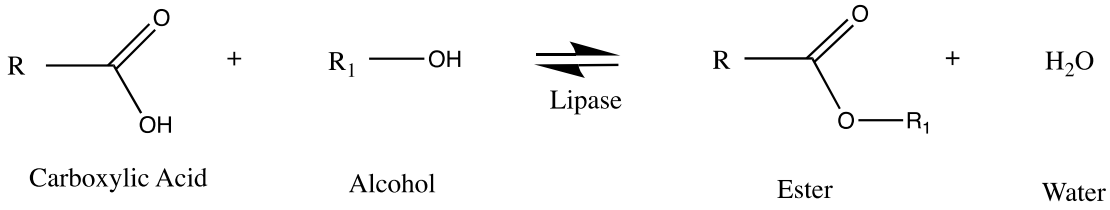
The monophasic systems reported in this section refer to the use of a single organic solvent (tertiary alcohol) as the reaction medium. This is able to solubilize all substrates without however acting as an esterifiable reagent itself.

The method leads to the enzymatic-catalyzed formation of the ester bound between the carboxylic acid of a target molecule and the hydroxyl group of and alcohol, also in solid form. In a dried reactor is placed the predetermined amount of the organic solvent (t-amyl alcohol: from 3 to 5 ml). The solvent acts to suspend both acid (ibuprofen) and alcohol (polyalcohol) in a monophasic system. The form of the reactor is important. In fact, in order to avoid the mechanical disruption of the catalyst, the contact between the walls of the reactor and magnetic stirrer needs to be nearly null. The magnetic stirrer needs to be a "cross" magnet that can minimize the contact with the immobilized catalyst in order to avoid its disruption. In order to solubilize an highly hydrophilic compound (such as: erythritol, xylitol or sorbitol, but also ascorbic acid) in the solvent (t-amyl alcohol) without adding water, a preliminary step is required: the predetermined amount of polyalcohol (or other types of hydroxyl-carried molecules: such as ascorbic acid), is carefully added to the solvent, the reactor is closed in order to avoid solvent evaporation (also if the boiling point of t-amyl alcohol is about 102.4 °C). The reactor is placed on a magnetic stirrer (described in section 2.3.2) and the solvent is heated until the desired temperature. High temperature and vigorous mixing can help the polyalcohol solubilization into t-amyl alcohol.

Temperatures for the different polyalcohol:

- erythritol: up to 55°C to 100°C
- xylitol: up to 60°C to 100°C
- sorbitol: to 65°C to 100°C

Once solubilized, the polyalcohol still in solution also when the temperature of the t-amyl alcohol decreases. At this point, ibuprofen can be easily added for its solubilization (from 100 di 300 mg in 5 ml of t-amyl alcohol). Then, the solvent is allowed to cool in order to avoid the thermal degradation of the enzymatic catalyst. When the solvent temperature is about 80 °C or less, the predetermined amount of enzyme can be loaded (range from 10 to 55 mg in 5ml of t-amyl alcohol). A certain number of "molecular sieves" can be added to the solvent in order to get rid of the water as by-product of the formation of the ester bound (range from 50 to 100 mg in 5 ml of t-amyl alcohol). Less water in the system lead to easier formation of the ester bound as shown in the equilibrium of **Scheme 7.**



Scheme 7: Generic lipase-catalyzed esterification reaction between a carboxylic acid and an alcohol.

The reactor may be preferentially put in glycerin/silicon oil bath placed on the magnetic stirrer in order to maintain the temperature constant. Some magnetic stirrer has a thermometer that, if placed in the glycerin bath, can auto-set the heating force of the stirrer in order to keep the desired temperature constant between the hours. The stirring speed needs to be set from 100 to 200 RMP in order to avoid the catalyst mechanical disruption. The molar ratio acid:alcohol it's 5:1. After 24h, or even less, ester is formed and purifiable from the reaction.

Different ranges were tested:

- reaction volumes: from 3 to 10 mL of t-amyl alcohol

- enzyme concentration: from 5 to 18 g L^{-1}

- molecular sieves concentration: from 10 to 32 g L^{-1}

- agitation speed: from 150 to 220 RPM

The reaction conditions for the esters produced with this strategy are shown in the following tables:

	Amount (mg)	Volume (mL)	g L^{-1}	PM	Ratio
Ibuprofen	126,7	3	42	206,29	5
Erythritol	15	3	5	122,12	
CALB	55	3	18		
Molecular sieve	90	3	30		

	Amount (mg)	Volume (mL)	g L^{-1}	PM	Ratio
Ibuprofen	150	3	50	206,29	5,5
Xylitol	20	3	6,6	152,15	
CALB	55	3	18,3		
Molecular sieve	92	3	30,6		

	Amount (mg)	Volume (mL)	g L^{-1}	PM	Ratio
Ibuprofen	152,9	3	51	206,29	5
Sorbitol	27	3	9	182,17	
CALB	55	3	18,3		
Molecular sieve	93	3	31		

	Amount (mg)	Volume (mL)	g L-1	PM	Ratio

Ibuprofen	304	6	50	206,29	5
Ascorbic Acid	52	6	8	176,12	
CALB	110	6	18		
Molecular sieve	192	6	32		

	Amount (mg)	Volume (mL)	g L^{-1}	PM	Ratio
Mandelic acid	150	3	50	152,15	5
Ascorbic acid	34,7	3	11,5	176,12	
CALB	55	3	18,3		
Molecular sieve	96	3	32		

Solvent-assisted biphasic media system

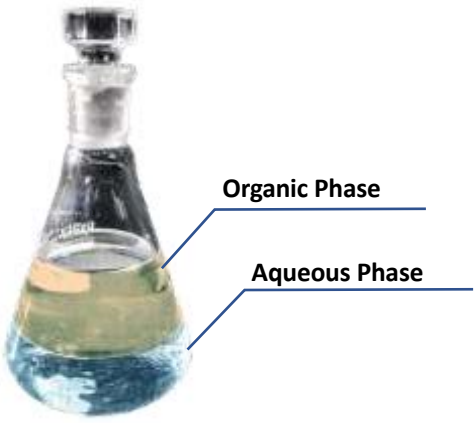


Figure 33: Representative image of a biphasic reaction environment consisting of two solvents that are immiscible to each other.

Ibuprofen (1000 mg), D-sorbitol (30 mg), 50 mg of free PPL, 15 ml of hexane and 1.5 ml (10%) of water were added into a 20 mL capped vial. The reaction mixture was stirred using a magnetic stirrer hot plate at 37 °C and 220 rpm. After 24h of reaction, samples were frozen in order to stop the enzymatic action. A negative control of the reaction was prepared without the use of lipase.

Table 11: Quantities of reagents used for the biphasic esterification of ibuprofen with sorbitol.

[258]	Amount (mg)	Volume (mL)	g L^{-1}	PM	Ratio
Ibuprofen	636		42,40	206.29	5
Sorbitol	450		30	176,12	
PPL	75		5		
Organic Solvent (hexan, toluene, benzene)		12			
Water		3			

2.3.4 Purification Methods

2.3.4.1 Liquid-liquid extraction

Liquid-liquid extraction is an analytical technique that is usually used to separate the components of a solution by means of their division between two immiscible liquid phases. If the solution of a substance in a solvent A is brought into contact with another solvent B that is not miscible with it, the substance migrates from the first phase (solvent A) to the second phase (solvent B) through the contact surface, until it reaches of a distribution equilibrium characterized by a constant K.

In this studies, extraction were used to get rid of the excess of glycerol alfter in the case of solventless monoglyceride syntesis. The extractions were carried out by extracting three times in ethyl acetate.

2.3.4.2 Column chromatography

Column chromatography, also known as liquid-solid or adsorption or normal phase chromatography (NP-LC) is based on the interaction between the active sites of the solid adsorbent with the functional groups present in the molecules of the solute to be separated. This type of interactions is the result of a complex competitive

phenomenon between the molecules of the mobile phase and of the solute for the active sites of the stationary phase.

Example ,purification of glycerol sorbate:

Proceed by preparing the eluent consisting of a 60: 35: 5 ratio of ethyl acetate, hexane and acetic acid respectively, considering a total volume of 200 ml and bring it to condition. We continue with the adsorption on silica of the products:

- The 20 ml resulting from the extraction in ethyl acetate and distilled water are placed inside a balloon;
- 5 g of silica are added;
- The balloon is brought into the rotavapor.

The sample was then dried at a temperature of 60 °C and at a slow rotation (130 rpm) at a pressure of -0.8 bar and a white powder was obtained.

Then the column to be used in chromatography is prepared, the silica is poured so as to reach a height of 8 cm x 2 cm in diameter and proceed with packing, taking care to eliminate the air inside the column, and the sample adsorbed on silica is loaded into the column.

The eluent is taken and added to the column and ends with the elution of the fractions.

65 fractions are collected in 2 ml tubes which have been previously prepared by numbering them.

Once the collection of the fractions is completed, the standards of the reaction (pure glycerol and sorbic acid), the reaction and the various fractions are sown on TLC at intervals of 3 from number 3 to number 65.

2.3.5 Analytical Methods

2.3.5.1 Enzymatic reaction inspection: thin layer chromatography (TLC):

Thin Layer Chromatography (TLC) is a technique that allows to separate the components of a complex mixture and to have qualitative analysis of the reaction in progress.

TLC, being a chromatographic technique, is based on the different affinity of molecules for the stationary and mobile phases. In this case the stationary phase consists of a silica gel, while the mobile phase (eluent) is composed of various solvents. It provides informations on the polar or apolar nature of the molecule through the distances traveled by the bands on the plate during chromatography. The run of a molecule on TLC is influenced by the octanol-water partition coefficient (log P) of the molecule itself, or by its tendency to be more or less soluble in water.

Prepare the chromatographic plate (5 x 10 cm) by tracing the base line about 1 cm from the base and proceed with the sowing of the samples (at equal distance between them) using a glass capillary. Once the plate is dry, this is placed inside a chromatography chamber previously conditioned with the mobile phase (eluent). The solvent will rise by capillarity along the plate and the result will be chromatographic separation **Figure 34**.

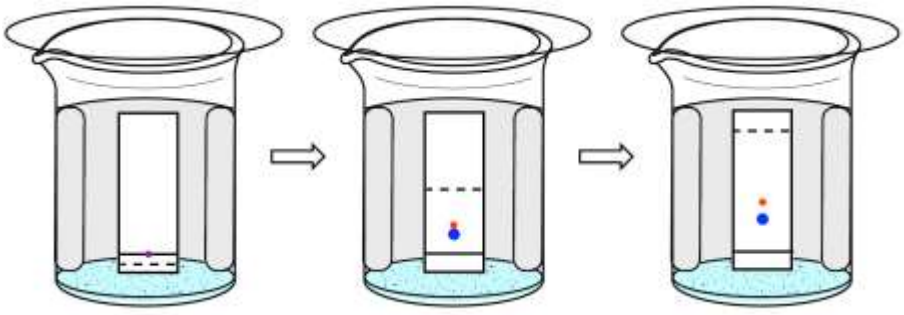


Figure 34: TLC sequence to separate the components of a sample.

The plate is removed from the chromatographic chamber when the solvent has reached the distance of about 1 cm from the upper edge of the plate, stopping the run. A line corresponding to the point of maximum migration of the mobile phase is drawn on the chromatographic plate (solvent front) with a pencil.

Two methods are used to detect bands on TLC plate:

1) Observing it by irradiation with UV rays at a wavelength of 254 nm;

2) by destructive development of TLC with a solution of phosphomolybdic acid;

In the destructive development, the solution is nebulized on the plate until it is completely saturated and is heated up to the development of TLC.

From the bands we obtain information on the greater or lesser affinity to the stationary phase of the separated compounds. Molecules more similar to the stationary phase produce bands closer to the seeding line than less polar ones (and consequently more similar to the apolar mobile phase).

The retention factor (Rf) is calculated for each band. In all likelihood, standards of the same Rf will have the same lipophilic/hydrophilic nature, and presumably a similar chemical nature. The retention factor is calculated:

$$R_f^A = \frac{h_A}{h_S}$$

h_s is the distance traveled by the eluent from the base line to the solvent front;

h_A is the distance traveled by the spot of component A;

There is naturally greater accuracy when the detected Rf values are very different from each other.

2.3.5.2 Analysis of the reaction mixture by HPLC

HPLC chromatography allows to separate complex mixtures and to obtain qualitative and quantitative analyzes.

The chromatograph used, and the related detectors, have been described in section 2.1.2.1. The wavelength of UV/vis reading was set according to the sample of interest. This technique is based on the separation of the components of the mixture due to the greater or lesser affinity that a species has with the stationary phase of a column or the mobile phase (eluent) that is circulating in the system.

Samples derived from *solventless* reactions in glycerol, before being injected into HPLC, are diluted using the following procedure:

0.25 mL samples taken from the micro-reactor are diluted by adding 0.75 mL of methanol (99.9%) to each sample and shaking until its contents are completely dissolved. Methanol allows to decrease the density to proceed to the analysis in HPLC.

To remove from the sample any particles that could clog the HPLC microfluidic system, these are filtered with the aid of a syringe and a microfilter; therefore, filtration helps to eliminate possible impurities that the samples may contain.

The sample is diluted: 0.1 mL of samples in 0.9 mL of methanol.

We proceed with the development and programming of the HPLC separative method. The parameters are as follows:

• Apolar phase: reverse phase column, Synergi 4 μm Hydro-RP 80 Å − 250 × 4.6 mm;

• Mobile phase: Mixture: 90% methanol-10% acidified water (the eluents used are milli-Q water acidified with sulfuric acid at pH 2.2 and high purity methanol).

- Column temperature: 30°C

- Flow: 0.8 mL/min

To quantify the data, the sum of the areas of the starting acid and the ester in question was used as a reference, i.e. the same response is assumed for the two compounds. This value is always kept constant, because the similarity that the acid and the derived ester present in their structure has been considered. In this way the peak area of each compound is the value that is used to calculate its conversion according to the equation (1), considered as the percentage decrease of the reagent or substrate.

$$X = \frac{A_{ester}}{A_{acid} - A_{ester}} \qquad (1)$$

where Aester means area of the ester, and Aacid means area of the acid substrate.

Samples were withdrawn for every concentration and analyzed by JASCO HPLC modular system equipped with reverse phase column at 30 °C, mobile phase 90:10 MeOH/H_2O (pH 2.2), 0.8 mL/min.

In order to determin the conversion yeald, the calibration lines were generated for each substrate with injecting in HPLC the following concentrations: 5, 10, 15, 20, 25 g L^{-1}.

As an example, the calibration lines for hydrocinnamic (**Figure 37**), trans-cinnamic (**Figure 37**) and 3 (4hydroxyphenyl) propionic acids (**Figure 37**) are shown below.

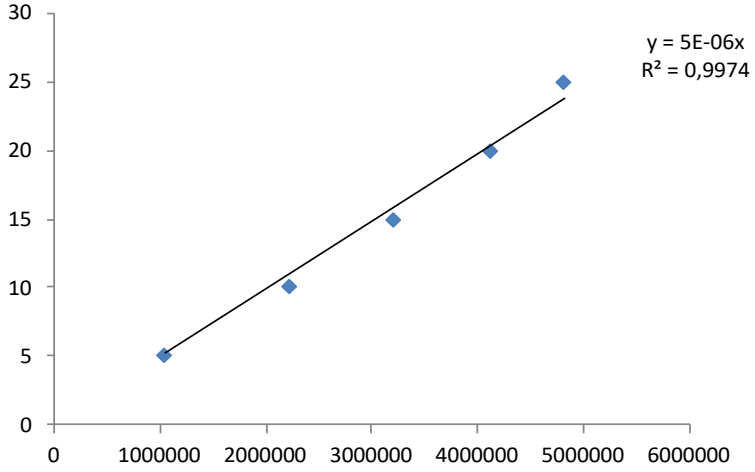


Figure 37: HPLC calibration for Hydrocinnamic acid.

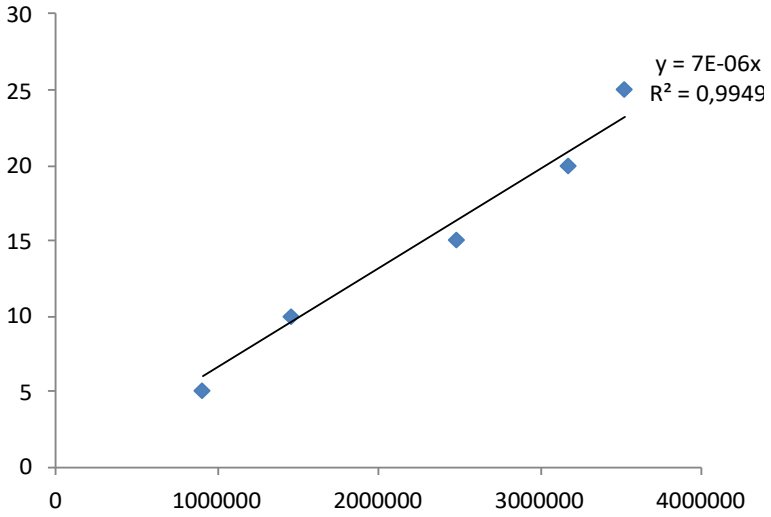


Figure 37: HPLC calibration for Trans-cinnamic acid.

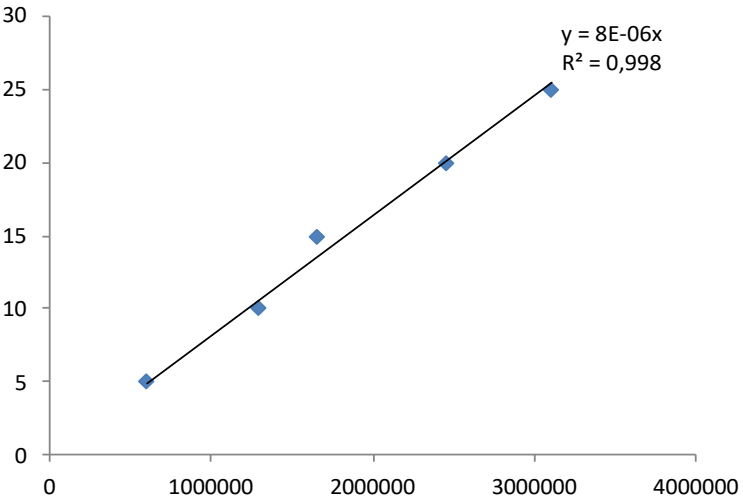


Figure 37: HPLC calibration for 3(4hydroxyphenyl)propionic acid.

2.3.5.3 Chemical Characterization

NMR

Nuclear magnetic resonance, also called NMR (nuclear magnetic resonance) spectroscopy, is a spectroscopic method based on the magnetic properties of the nuclei of certain atoms and isotopes. The purpose of NMR is basically to obtain information on the resonant frequency of active nuclei. The most used are hydrogen, carbon-13 and nitrogen-15. There are two methods to obtain this information: in continuous wave NMR spectroscopy an electromagnet generates the magnetic field necessary to induce the splitting between the energy levels of the atoms active in the sample under examination. At the same time, an electromagnetic wave emitter bombards it at a very precise frequency. The magnetic field of the magnet is then progressively increased: a detector records the absorbance of the sample at each field strength, or how much of the incident radiation is lost as it passes through the medium. As mentioned above, the resonant frequency of an active nucleus is directly proportional to the applied field: as the field increases, the resonant frequencies also increase. When the resonance frequencies become equal to the incident frequencies (which instead is constant), then all the atoms capable of doing so will absorb quanta of energy, and the measured absorbance will be higher. The second method, used by more modern spectrometers, consists in keeping the field constant, instead increasing the frequency of the incident radio wave. From a practical point of view, the result is always a graph with the absorbance on the y axis, and a useful quantity to define the energy supplied on the x axis.

To proceed with the analysis of the sample using this procedure, a range of 1 to 5 mg of the enzymatic reaction product purified with chromatographic column and dried is dissolved in a range of 0.7 to 1 ml of deuterated solvent (CD_3OD, $CDCl_3$, or d6-DMSO) inside a tube for NMR analysis.

NMR Spectra showed the following peaks;

Glycerol Sorbate:

^{1}H NMR (400 MHz, Methanol-d4) δ 7.30 (dd, J = 15.3, 10.1 Hz, 1H), 6.32–6.14 (m, 2H), 5.84 (d, J = 15.3 Hz, 1H), 4.21 (dd, J = 11.5, 4.4 Hz, 1H), 4.12 (dd, J = 11.4, 6.3 Hz, 1H), 3.91–3.80 (m, 1H), 3.57 (dd, J = 5.6, 2.0 Hz, 2H), 1.86 (d, J = 5.9 Hz, 3H).;

^{13}C NMR (101 MHz, CD_3OD) δ 168.93, 147.00, 141.05, 130.97, 119.46, 71.22, 66.52, 64.06, 18.70.

Ibuprofen Monoglyceride:

^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ 7.20 – 7.03 (dd, 4H), 4.10 – 4.02 (dd, 2H), 4.0 – 3.82 (m, 2H), 3.78 – 3.66 (q, 1H), 3,60 (s, 1H), 2.39 (d, 2H), 1.78 (m, 1H), 1.40 – 1.35 (d, 3H), 0.86 (d, , 6H).

^{13}C NMR (101 MHz, $(CD_3)_2SO$) δ 173.95, 137.89, 129.01, 127.06, 69.21, 65.85, 62.52, 44.10, 29.55, 22.15, 18.63.

Ibuprofen Erythritol Ester:

^{1}H NMR (400 MHz, CD_3OD) δ 7.20 (dd, *J* = 8.1, 2.0 Hz, 2H), 7.16 – 7.00 (m, 2H), 4.28 – 4.11 (m, 1H), 4.06 (dd, *J* = 11.6, 6.4 Hz, 1H), 3.84 – 3.60 (m, 3H), 3.64 – 3.46 (m, 2H), 2.43 (d, *J* = 7.2 Hz, 2H), 1.81 (m, 1H), 1.45 (d, *J* = 7.6 Hz, 3H), 0.88 (d, *J* = 6.6 Hz, 6H).

^{13}C NMR (101 MHz, Methanol- d4) δ 175.41, 140.30, 128.94 (4C), 126.88, 74.59, 72.00, 66.07, 63.01, 44.88, 44.60, 30.02, 21.31 (2C), 17.71.

Ibuprofen Xylitol Ester:

^{1}H NMR (400 MHz, CD_3OD) δ 7.21 (d, *J* = 8.2 Hz, 2H), 7.10 (dd, *J* = 8.0, 1.4 Hz, 2H), 4.22 (m, 1H), 4.12 (m, 1H), 3.93 – 3.68 (m, 2H), 3.75 – 3.46 (m, 4H), 2.45 (d, *J* = 7.2 Hz, 2H), 1.84 (m, 1H), 1.46 (d, *J* = 7.2 Hz, 3H), 0.89 (d, *J* = 6.6 Hz, 6H).

^{13}C NMR (101 MHz, CD_3OD) δ 175.10, 139.13 (d, J = 240.3 Hz), 128.95 (4C), 126.86, 72.23, 70.60, 69.92, 65.44, 62.80, 44.86, 44.61, 30.02, 21.29 (2C), 17.63.

Ibuprofen Sorbitol Ester (IBU-sorbitol; [258])

^{1}H NMR (400 MHz, CD_3OD) δ 7.20 (d, J = 8.1 Hz, 2H), 7.08 (d, J = 7.9 Hz, 2H), 4.25 (d, J = 11.4 Hz, 2H), 4.06 (dd, J = 11.4, 6.7 Hz, 1H), 3.95–3.88 (m, 1H), 3.84–3.70 (m, 3H), 3.69–3.53 (m, 2H), 2.46–2.39 (d, *J* = 7.4 Hz, 2H), 1.83 (m, 1H), 1.45 (d, J = 7.2 Hz, 3H), 0.88 (d, J = 6.6 Hz, 6H);

^{13}C NMR (101 MHz, CD_3OD) δ 175.21 (d, J = 29.8 Hz), 140.29, 138.01, 128.92 (4C), 73.61, 72.08, 71.10, 69.49, 65.58, 63.34, 44.87, 44.62, 30.02, 21.32 (2C), 17.64.

Ascorbic acid ester of Ibuprofen [140]:

^{1}H NMR (400 MHz, CD_3OD) δ 7.19 (dd, *J* = 8.2, 2.2 Hz, 2H), 7.08 (dd, *J* = 8.2, 2.7 Hz, 2H), 4.83 (d, *J* = 3.5 Hz, 2H), 3.76 (q, *J* = 7.1 Hz, 1H), 3.65 (q, *J* = 7.1 Hz, 2H), 2.43 (d, *J* = 7.2 Hz, 2H), 1.82 (dq, *J* = 13.4, 6.7 Hz, 1H), 1.41 (d, *J* = 7.1 Hz, 3H), 0.88 (d, *J* = 6.6 Hz, 6H).

Butyl ester of Ibuprofene

^{1}H NMR (400 MHz, CD_3OD) δ 7.23 – 7.16 (m, 2H), 7.12 – 7.05 (m, 2H), 4.06 (t, J = 6.6 Hz, 2H), 3.68 (q, J = 7.3 Hz, 1H), 2.44 (d, J = 7.1 Hz, 2H), 1.83 (dh, J = 13.2, 6.7 Hz, 1H), 1.61 – 1.45 (m, 5H), 1.27 (dq, J = 14.6, 7.4 Hz, 2H), 0.92 – 0.82 (m, 9H).

Butyl ester of Sorbic Acid

^{1}H NMR (400 MHz, CD_3OD) δ 7.30 (m, 1H), 6.80 (d, 1H), 6.35-6.15 (m, 1H), 5.78 (d, 1H), 3.58 (m, 2H), 1.82 (d, 2H), 1.60-1.20 (m, 5H), 0.95 (m, 3H).

^{13}C NMR (101 MHz, CD_3OD) δ 166.34, 145.51, 139.20, 129.67, 118.85, 61.35, 34.47, 18.69, 17.32, 12.88.

UDCA monogliceride (selected peaks):

^{1}H NMR (CD_3OD): δ 0.75 (s, 3H, CH_3-19), 0,92 (d, 3H, *J*=7.2 Hz CH_3-21), 0.97 (s, 3H, CH_3-18), 3,62 (s, 1H, CH-7α), 3,59 (q, 2H, CH_2-27) 3,67 (s, 1H, CH-3β), 4.05 (m, 1H, CH-26) 4.55 (m, 1H, CH-25).

Analysis of the reaction products by uHPLC-MS

Ultra High Performance Liquid Chromatography (uHPLC) is a technologically and instrumentally optimized separation technique in order to generate more performing columns than traditional HPLC. It is characterized by a stationary phase whose particle diameter is more reduced, pumps and mechanical parts able to operate at even higher operating pressures and by a smaller quantity of injected sample volume with consequent saving of eluent necessary for the conduct of separation.

The uHPLC allows to obtain separations of the eluted substances with greater efficiency and in reduced times. During the following tests we will use an uHPLC (model: Waters Acquity uHPLC) associated with a mass spectrometer as a detector (model: ZQ 2000 ESI mass spectrometry).

After a first chromatographic separation with a C18 column with a solvent gradient from 100% H_2O to T_0 to 100% MeCN at T_5 (5 minutes), the eluted components are

analyzed by a mass spectrometer, which will provide information on the atomic mass of the compound in analysis.

Mass spectroscopy is an analytical technique applied both to the identification of unknown substances and to the analysis of substances present in traces. It is commonly used in conjunction with separative techniques such as gas chromatography and liquid chromatography (HPLC). The principle on which mass spectrometry is based is the possibility of separating a mixture of ions according to their mass/charge ratio by exploiting static or oscillating magnetic fields. The molecules are ionized thanks to the passage of a beam of electrons, of known energy, through the sample. The graph showing the abundance of each ion as a function of the mass / charge ratio, known as the mass spectrum, is typical of each compound as it is directly related to its chemical structure and the ionization conditions to which it was subjected. Resolution is the ability of the instrument to distinguish between two adjacent peaks. The resolution necessary to separate A and B is calculated as M/ΔM where:

M is the m/z value of peak A;

ΔM is the difference between the m / z values of two contiguous peaks, peak B and peak A.

The height of the valley between the two adjacent peaks must not be greater than 10% of the height of the peak of lesser intensity for the two peaks to be considered resolved.

IR Spectra

IR spectra was recorded by Perkin Elmer FTIR Spectrum 100 infrared spectrometer equipped with ATR using a ZnSe Diamond.

2.3.6 Activity Tests

2.3.6.1 Evaluation of antibacterial and antifungal activity of Glycerol Sorbate

The methods of Disk Diffusion (DD) [259] and Minimum Inhibitory Concentration (MIC) [260] were used to evaluate the antibacterial and antifungal activity glycerol sorbate.

The culture media used during this tests are:

• GYM (Glucose-Yeast extract-Malt extract) for *Streptomyces griseus*: glucose (4 g L^{-1}), yeast extract (4 g L^{-1}), malt extract (10 g L^{-1}). Before adding the agar, adjust the pH to 7.2;

• YMB (Yeast Mannitol Broth), for *Saccharomyces cerevisiae*: yeast extract (3 g L^{-1}), malt extract (3 g L^{-1}), peptone from soybeans (5 g L^{-1}), glucose (10 g L^{-1});

The disk diffusion technique was used to evaluate the antibacterial and antifungal activity of the sorbic acid and glycerol ester. This technique allows to evaluate the in vitro antibacterial and antifungal activity of compounds and to directly compare the results with the antibiotic and the reference antifungal. The organisms used for this test are *Streptomyces griseus*, a gram-positive aerobic bacterium and *Saccharomyces cerevisiae*, a model yeast.

The disk diffusion technique allows us to evaluate the antibacterial and antifungal activity through the inoculation of the microorganism in a previously sterilized liquid medium (GYM for *Streptomyces griseus* and YMB for *Saccharomyces cerevisiae*).

The culture containing the microorganisms to be tested is left to grow under agitation at a controlled temperature of 28 ° C for 24-36 hours.

After the time necessary for growth, serial dilutions (from 10^1 to 10^8) are performed starting from the mother culture in order to understand, thanks to the cell count,

the order of magnitude of the bacterial and fungal concentration present in the starting flask. Once the concentration of the culture has been established, a series of successive dilutions must be carried out to obtain a concentration of 10^6 colony forming units (CFU/mL).

Later they are sown and dispersed with a sterile loop on a petri dish containing the agar medium, 100 μL of the diluted cultures.

Then three absorbent discs with a diameter of 5 mm are placed on the plate, soaked with 10 μL of the ester to be tested dissolved in an ethanol solution. At the same time, other plates are prepared in the same way in which the disks soaked in the antibiotic and the reference antifungal for the positive control, the ethanol solution for the negative control and the sorbic acid dissolved in the ethanol solution will be added to compare the antibacterial and antifungal activity of the ester. The sorbic acid and the ester placed on the absorbent pads have the same molarity. The antifungal used as a positive control against *Saccharomyces cerevisiae* is clotrimazole at a concentration of 1 mg/mL, while the antibiotic used against *Streptomyces griseus* is chloramphenicol at a concentration of 2 mg/mL. The plates are incubated at 28 °C for 6 days. The final concentrations reached are shown in **Table 12**.

Table 12: Drug concentrations tested against *S. cerevisiae* and *S. griseus*.

Tested Drugs Concentrations (mg/mL^{-1})						
S. cerevisiae	0.05	0.075	0.100	0.125	0.150	0.175
S. griseus	0.15	0.25	0.50	0.75	1.00	1.25

The areas where the growth microorganism was stopped by the antibacterial agents are called inhibition zones. In order to determine the antibacterial activity, the diameters of the inhibition zones for the test organism were measured in millimeters (mm), including disk diameter of 5 mm, and compared to the negative controls. To measure the diameter of the inhibition zone, the software ImageJ has

been employed as image analyzer. Digital images of the Petri dishes were analyzed after calibrating the software. The calibration step correlates pixels of a reference in the image with the real word distance measurement (mm). Inhibition zones were selected, and the diameter of the selection were displayed in the "results" window of the software.

Moreover, minimum inhibitory concentration (MICs) evaluations were performed. MIC were commonly defined as the lowest concentration of compound that completely inhibited (MIC_{100}) or reduced to 50% (MIC_{50}) clearly visible the microbial growth after the whole period of incubation, which was 6 days.

2.3.6.1 Evaluation of antinflamatory activity of Ibuprofen esters

Cell Cultures

To study the anti-inflammatory activity of ibuprofen esters, we started a collaboration with the laboratory of Molecular Biology under the supervision of Professor Ilaria Lampronti. We chose for our experiments *in vit*ro the human *IB3-1* cells, obtained from LGC, Promochem (Teddington, Middlesex, UK). This cell line type has been created in 1991, by the isolation of human bronchial epithelia cells from a patient with cystic fibrosis. The grown primary culture was then subjected to the adeno-viral transformation with the simian virus 40 (SV40), to create an immortalized line, proper for the laboratorial technique. The IB3-1 cells have a genome carrying two mutations: the deletion of phenylalanine in position 508 (ΔF508) on one allele and the W1282X on the other one, representing the most widespread condition causing cystic fibrosis (CF) [261]. They grow in adherence and present a stretched shape – as visible in **Figure 38**.

Figure 38: The pictures show the morphology observable at the optical microscope (20X) of the IB3-1 cells

Subculturing method

The culture method indicated for their growth in healthy conditions includes their passage two times a week, it is when they cover the plate with a confluence of minimum 80%. The subculture process requires the LHC-8 basal medium (Biofluids, Rockville, MD, USA), supplemented with 5% of fetal bovine serum (FBS), in the absence of gentamycin. We kept active their cell cycle letting them divide inside the cell culture flasks T25 with filtered cap (Corning®, Arizona, USA), which were maintained under the following standard conditions: in a 5% CO_2 humidified atmosphere incubator at 37 °C. To avoid contaminations, we performed every single stage of the dilution under sterile hood.

Seeding cells

To perform the experiments, we discarded supernatants above confluent cells and rinsed them with phosphate buffered saline PBS. Then, the removing step required the elimination of PBS and the detachment of cells by adding of a proper volume (1000 μL) of trypsin Trypsin-EDTA (4 minutes at 37°C, 5% CO_2). It followed the

neutralization of trypsin activity with the supplementation of same volume of FBS and the cells resuspension in the final volume (10 mL) with LHC-8 medium. At this step, we took an aliquot (50 μL) of the suspension and we diluted it with 5 mL of physiological solution into the appropriate cup, which is the minimum volume necessary to immerse the capillary of the coulter counter (BECKMAN COULTER® Z Series) used to perform the analysis. The total count (cells/mL) was then calculated multiplying the data displayed by the instrument with the proper related dilution factor (200), specified in the manual. Then, 50.000-100.000 cells/ml were seeded in 12-well plate (1mL/well) and incubated a 37 °C (5% CO_2).

The PA0-1 strain

To evaluate the antimicrobial activity of ibuprofen ad esters, we used the PA0-1 strain, which the most common used for research. The prototype used has been produced by the A. Prince (Columbia University, New York, NY) and it is a non-mucoid strain suited for the laboratorial technique, in fact it gives mild effects on operators. We used both the proper growth mediums, which are the *trypticase soy broth* (TSB) or *agar* (TSA) (Difco). Although PA is an aerobic-anaerobic facultative bacterium, the growth is facilitated in anaerobic conditions, so we chose to seal the tubes during the time of replication. Our aliquots of PA0-1 were stocked in glycerol at -80°C.

Compounds solubilization

Ibuprofen and ibuprofen esters were dissolved in ethanol (EtOH) + 75% H_2O Rnasi free (R.f.), so as all the subsequent dilutions used for the experiments. Lyophilized Tumor necrosis factor alpha (TNFα) powder was reconstituted in sterile PBS at 3% of BSA.

Cells treatment with compounds and stimulation with TNF-α

50.000-100.000 cells/ml of IB3-1 cells were seeded in 12-well culture plates (Sarstedt® Ltd., Leicester, UK) in LHC-8 medium in the presence of 5% FBS. After 48 hours from seeding, we added ibuprofen and esters. Five hours later, we stimulated the cells with TNF-α 100 ng/mL and incubated for further 24 hours. After that, the supernatants were collected, and the total RNA was extracted.

Cell Proliferation Assay

We seeded IB3-1 cells in 12-well plates with LHC-8 medium, supplemented with 5% of FBS. After 48 hours from seeding, we added the volumes of ibuprofen and esters to obtain the indicated concentrations in each well. After 24 and 48 h from treatment cells were rinsed with PBS, detached using Trypsin-EDTA (4 minutes at 37°C, 5% CO_2), neutralized with the same volume of FBS and resuspended in LHC-8 medium. At this step, we took an aliquot (50 μL) of the suspension and we diluted it with 5 mL of physiological solution into the appropriate cup, which is the minimum volume necessary to immerse the capillary of the coulter counter (BECKMAN COULTER® Z Series) used to perform the analysis. The total count (cells/mL) was then calculated multiplying the data displayed by the instrument with the proper related dilution factor (200), specified in the manual and expressed as percentage relative to control cells (untreated). The IC_{50} was calculated after 24 hours and/or 48 hours of culture.

RNA extraction

To evaluate the inflammatory status modulation of cells induced by the TNF-α and contrasted by the ibuprofen and esters, in terms of IL-8 mRNA, we proceeded with the RNA extraction from the cells grown and treated in plates. All the passages required the maintaining of the samples in ice. First, we collected the supernatants of the cells grown in plates into labeled test tubes, then we stocked them at -80°C.

We added 600 μL – 800 μL of Trizol (TRI Reagent®, Sigma-Aldrich, USA) per 1 x 105-107 cells directly to each culture well and incubated for 3 minutes. Then, we fast lysate the cells by vigorous pipetting up and down several times. We transferred the cell homogenate in the corresponding tube test and added 120 μL – 160 μL of chloroform (Sigma-Aldrich, USA), it is the 20% of the volume of Trizol used for the lysis. We mixed the suspensions first through the vortex at maximal velocity for 15 seconds, then incubated for 2 minutes by phase inverting. The suspensions were centrifuged at 12.000 rpm for 15 minutes at +4°C (MicroCL 21R, ThermoScientific, USA). The mixtures separated into three phases: an upper colorless aqueous one, containing the RNA; an intermediate one containing the DNA and a lower one, containing debris, phenol and chloroform. We took the aqueous phase with RNA and transferred into new tubes. Then we added 300 μL or 400 μL of isopropanol (Sigma-Aldrich, USA), the 50% volume of Trizol used, incubated for 10 minutes at room temperature and centrifuged the samples at 12.000 g for 15 minutes at +4°C. We discarded the supernatants and washed the resultant gel-like pellet with 600 μL or 800 μL (equal volume of Trizol), of ethanol 75% (Sigma-Aldrich, USA) and centrifuged for further 5 minutes. Once removed the ethanol, we dried the pellet through the vacuum concentrator SpeedVac (Savant SC110SpeedVac™, Savant instruments inc., USA) for 3-5 minutes. In the end, we suspended the RNAs with 10 μL of ultrapure H2O R.f. and stocked them at -80°C.

RNA spectrophotometric quantification

To quantify the total RNA extracted from the treated cells, we used a single UV ray spectrophotometer SmartSpec™ Plus (BioRad; Hercules, California, USA). First, we calibrated the instrument by adding 50 μL of water in a cuvette and we set the Blank. Then, we prepared samples by diluting 1 μL of RNA (or more if insufficient) with 50 μL of H2O R.f. in a cuvette and analyzed the sample at the optical density of 260 nm (OD_{260}). In order to detect an absorbance between 0,1 and 1, we adjusted

the concentration by diluting with water or by adding extra RNA (maximal 3 μL). We calculated the extracted RNA concentrations of each sample, knowing that 1 OD260 corresponds to a concentration of 40 ng/μL.

Reverse Transcription (RT)

The reaction of reverse transcription was performed using Reverse Transcription System kit (Promega, Madison, WI) and included the previous dilution of 1 μL of Random Primers (Promega, USA) with the previously calculated volume of RNA corresponding to a quantity of 500 - 1000 ng and the volume of water necessary to reach 6,5 μL. The test tubes with these mixes were centrifuged for 5 minutes (Allegra X-12R, BeckmanCoulter, USA) and heated for 5 minutes at 70°C in the Thermal Cycler (GeneAmp® PCR System 9700, Applied Biosystem, USA). Then, we added to each sample, 13,5 μL of the previously prepared Mix RT, consisting of: 4,0 μL of Buffer 5x (RT Buffer 10X, Promega); 2,4 μL of MgCl2 25 mM (Promega, USA); 1,0 μL of dNTPs Mix 50 μg/mL (Promega, USA); 0,25 μL of RNasin® 40 units/μL; 1,0 μL of reverse transcriptase (RT) 160 units/μL (Promega, USA) and 4,85 μL of R.F. water, so to reach a final volume of 20 μL for each sample. The reaction was performed using the following protocol at the Thermal Cycler:

- 5 minutes at 25°C to activate the enzyme
- 60 minutes at 42°C to activate the polymerization
- 15 minutes at 70°C to inactivate the enzyme
- ∞ at 4°C to stock the samples

The cDNAs were finally stocked at -20°C.

Synthetic oligonucleotides

The oligonucleotides used as primers to amplify the IL-8 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the *real time quantitative polymerase chain reaction* (RT-qPCR) genes were produced by the IDT (Integrated DNA Technologies, USA) and by the Sigma (Sigma® Aldrich, USA). The sequences displayed in the table below have been drawn up through the software Primer-BLAST.

Table 13: The table displays the sequences, the length and the melting temperatures (Tm) of the primers used in the RT-qPCR.

Primer	Sequence	Length (#bases)	Tm (°C)
IL-8 R	5′- TTATGAATTCTCAGCCCTCTTCAAAAACT -3′	29	58,6
IL-8 F	5′- GTGCAGTTTTGCCAAGGAGT -3′	20	56,3
GAPDH R	5′- ACTGTGGTCATGAGTCCTTCCA -3′	22	65,9
GAPDH F	5′- AAGGTCGGAGTCAACGGATTT -3′	21	65,7

SYBR GREEN RT-qPCR.

The RT-qPCR is an assay used to quantify nucleic acids through the detection of the fluorescence emitted by a fluorophore. The fluorophore we chose for our experiments was the *SYBR Green*, which is an intercalant that binds the double strand DNA (dsDNA). The technique is similar to PCR, which provides cycles with different temperatures steps, which promote the reactions of denaturation, primers annealing and elongation of the nucleotide sequence.

The SYBR green is inactive in its unbound state, but when it binds to the dsDNA it emits a fluorescent signal, in particular a green light at the wave of length of 488 nm. During the phase of denaturation, the SYBR green is unbound and the fluorescent signal is low. When the primers bind to the template, they provoke the extension phase and the production of the PCR fragments. The SYBR green binds to these fragments and emits as more fluorescence as much template is amplified.

For each PCR cycle, is detected a net increase of the fluorescence signal in than the previous one. The initial quantity of the amplified (cDNA) template is measured through the analysis of the intensifying fluorescence signal.

We performed quantitative PCR reactions using the cDNA obtained from the RNA reverse transcription and specific primers, which bind to the sequences of interest: the IL-8 gene, and the housekeeping GAPDH, used as reference gene. The organization of two independent, but contemporaneous, reactions for each sample was necessary. To obtain a more significant result, we decided to analyze the samples in duplicate, so we had four reactions: two for the IL-8 and two for the GADPH.

Every single reaction mix, having as total volume of 20 μL, consisted of: 1 μL of cDNA or 1 μL of ultrapure H_2O R.f. in the case of the negative control; 5,8 μL of ultrapure H_2O R.f.; 10 μL of *I Taq™ Universal SYBR® Green Supermix 1x* (Abs 520 nm BioRad), containing antibody-mediated hot-start iTaq DNA polymerase, dNTPs, $MgCl_2$, SYBER® Green I dye, enhancers, stabilizers, and a blend of passive reference dyes (including ROX and fluorescein, acting as reference for the normalization of data by the instrument in order to cancel any volume errors made by the operator; 1,6 μL of each primer – forward and reverse (both 50 ng/μL). All reactions were arranged in duplicate on 96-well optical plastic plates (Multiplate™ PCR Plates, BioRad) which we sealed with a plastic cover and centrifuged before the amplification with the CFX96 Touch™ Real-Time PCR Detection System, including the Thermal Cycler C1000 Touch (BioRad) and the CFX Manager™ 184-5000 software (BioRad). The first one phase of DNA denaturation and polymerase activation, requiring 30 seconds at 95°C, allowed the hydrogen-bonds disruption between the nitrogenous base and thus the separation of the two hemihelixes. During the following 40 cycles have been repeated these steps: 5 seconds at 95°C, 30 seconds at 60°C, leading to the annealing and extension of the double helixes of

DNA. The exponential resulting amount of cDNA copy numbers was obtained through the cyclic repetitions and the measuring system is based on the following mathematical relationship: $\Delta Rn = (Rn+) - (Rn-)$, where (Rn+) is the emission of SYBR Green, calculated at each cycle of amplification, and (Rn-) represents the emission before the start of the PCR reaction. Plotting the different values of fluorescence ΔRn detected for each cycle, it is possible to create a spectrogram displaying on the abscissa, the number of cycles and on the ordinate, the computed ΔRn. On the amplification curves of the spectrogram, it is possible to set a threshold line (Lt) above the background signal and at the beginning of the exponential phase. The intersection between the Lt, and each amplification curve corresponds to the value of threshold cycle (Ct), which expresses the detection of the first significant and pure increase of fluorescence intensity. The resultant Ct is inversely proportional to the original relative expression level of the studied gene.

For our studies, we included the use of the reference GAPDH in order to minimize the experimental error and to evaluate the *relative* quantification, which states the expression increment or decrement of the pursued nucleic acid (IL-8) in relation to the reference one (GAPDH), which is stable and basic active in every sample. Our samples were: the not treated cells, as negative control; the cells stimulated with TNFα, as positive control; the cells with the addition of 100 ng/mL of TNFα and the treated with ibuprofen (Sigma – Aldrich) and esters at concentrations ranging from 2 μM to 250 μm. Thus, once obtained the values of *Ct* for the IL-8 and the ones for the GAPDH, we were able to compute the fold of IL-8 as following described. The mathematical equation describing the fold is $2^{-\Delta\Delta CT}$, where the ΔΔCT represents the subtraction of the ΔCT – obtained for each sample – from the ΔCT of the negative control. In particular, ΔCT is the difference between the Ct (averages) acquired by the amplification reference gene and the Ct (averages) acquired by the amplification of the gene of interest.

3. OBJECTIVE AND AIMS OF THE WORK

3. OBJECTIVE AND AIMS OF THE WORK

Poorly water-soluble drugs present ongoing challenges with their translation into viable medicinal products [89]. More than 40% of new chemical entities (NCE) being synthesized by combinatorial screening programs possessing superior pharmacological activities are poorly soluble, which is a great obstacle in formulation development [90][91]. Thus, drugs with poor water solubility require novel formulation approaches to improve their rates of dissolution and oral bioavailability [262]. Often, the solubility of these drugs is limited in the stomach because there is a carboxylic acid in an aqueous acidic media [263]. For this reason, one way to increase the water solubility of poorly water-soluble acid forms of drugs could be an esterification reaction between its carboxylic acid and an alcohol to obtain an enhanced water-soluble drug designed to effect better oral availability [138]. This kind of modification could lead to synthetize a prodrug. A prodrug is a compound that has ability to undergo biotransformation prior to exhibiting its pharmacological properties [264].

Poor solubility of active ingredients does not only concern pharmaceutical products but also food products. Many food preservatives are poorly soluble in water and this results in overdose of the active ingredient used to compensate its poor bioavailability [265].

Enzymatic reactions that involve the union of the active ingredient of interest with highly hydrophilic molecules (glycerol, erythritol, xylitol, sorbitol, ascorbic acid), can be exploited for the production of a molecule potentially more soluble in water than the starting active ingredient.

The purpose of this thesis is the development of enzymatic esterification protocols for the synthesis of esters with increased solubility in water and consequently, bioavailability. The enzymatic esterification targets concern poorly bioavailable

molecules from two different industrial realities: i) ibuprofen, a non-steroidal anti-inflammatory drug from the pharmaceutical industry and ii) sorbic acid, a preservative widely used in the food and wine industry.

The physical state of the hydrophilic portions carrying the hydroxyl groups (polyalcohol and ascorbic acid), which will take part in the esterification reaction as "solubility enhancers", will determine the development of protocols of different nature: *solventless* and *solvent-assisted*. The liquid nature of glycerol allows a *solventless* reaction environment, therefore a mostly monophasic system where the glycerol itself has the role of both solvent and reagent. The esterification reactions with solid polyalcohols of increasing polarity (erythritol, xylitol, sorbitol) and ascorbic acid, instead, will be reached with a *solvent-assisted* system.

For the chemical characterization of the esterification products, the experimental design is based on qualitative analysis techniques such as thin layer chromatography (TLC), nuclear magnetic resonance (NMR), mass spectrometry (uHPLC-MS) and IR. The conversion yield was estimated by HPLC.

To evaluate the effect of esterification in terms of bioavailability, it will be carried out *in vitro*, through the analysis of antimicrobial activity, thanks to MIC (Minimum Inhibitory Concentration) and DD (Disk Diffusion) techniques for the sorbic acid ester, and RT-qPCR analysis for the anti-inflammatory nature of the ibuprofen esters. Microbiological tests involve the use of *Saccharomyces cerevisiae* and *Streptomyces griseus*; while the IB3-1 cell lines anti-inflammatory tests with inflammation induced by Tumor Necrosis Factor (TNF-α).

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4. RESULTS AND DISCUSSION

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Based on the chemical nature of the substrates (solid form or liquid form), the use of *solventless* esterification strategies, in the absence of solvent, and *solvent-assisted*, in the presence of solvent will be evaluated.

4.1.1 ENZYMATIC ESTERIFICATIONS: *SOLVENTLESS* STRATEGIES

We define *solventless* those enzymatic esterification reactions where alcohol is a substrate of reaction but at the same time allows the solubilization of the acid. The scientific literature reports several works where *C. antarctica* lipase B performed efficiently as a biocatalyst of esterification reactions [253] and CALB enzyme is the most widely used for *solventless* esterification reactions because its tiny lid closes only partially the active site of the enzyme [83], making interfacial activation unnecessary. This enzyme has previously proved to be a good choice to work in systems without solvent–water interface [81]. For these reasons, CALB seems to be a good choice for monophasic esterification reactions [266].

In this section, we will report the enzymatic esterification of acid substrates with glycerol and 1-butanol.

4.1.1 Glycerol esters

Until today, enzymatic catalysis in organic media has been widely studied, but this process suffers from several problems regarding the use of volatile and environmentally toxic solvents, and the difficult industrial scale-up due to the large quantities of solvent that must be used and the high energy expenditure in the solvent recovery phases.

Alternatively, ionic liquids (IL) or supercritical fluids were used. In the literature there is the study of the esterification of ibuprofen using various lipases in a system

containing ionic liquids [BMIM] [PF_6] and [BMIM] [PF_4] [145]; on the other hand, the esterification of racemic ibuprofen has been tested using the immobilized enzyme CALB (Novozym 435) in the presence of supercritical CO_2 [267]. But ionic liquids cannot be used in every reaction design. Synthetic chemists should be cautious when designing reactions in ILs depending on reactions they want to do. Indeed, there are reports that show in some cases ILs react with reactants and therefore they cannot be considered as inert solvents [268].

In this context, the liquid nature of glycerol presupposes a *solventless* reaction environment, therefore a system where the glycerol itself plays the role of both solvent and reagent.

Glycerol (propane-1,2,3-triol) is a polyol characterized by the presence of three hydroxylic groups, which confer to the compound the following properties: high hygroscopicity, high boiling point, and low vapor pressure. This nontoxic viscous liquid is widely used in chemical, cosmetic, pharmaceutical, and food industries [82].

The advantages of this type of strategy are several: it does not require a large amount of expensive and toxic solvents, which should be avoided considering the process affordability/cheapness (high product recovery and low energy costs). Novel solutions include hydrofluorocarbons, supercritical fluids, and ionic liquids that may be relevant in the choice of solvents [269], in particular if the final target of the product is the food industry. In this field, dry media reactions offer an economic and environmentally friendly option, avoiding the use of solvents.

Furthermore, glycerol availability grew simultaneously with the large increase in biodiesel production according to the international environmental laws (IEL). Glycerol is indeed a major byproduct of the biodiesel manufacturing process, representing approximately 10% *w*/*w* of biodiesel. Its consequent higher

affordability allowed novel uses, different from the traditional direct ones applied in the food, cosmetic, and pharmaceutical fields. Thus, the use of glycerol as raw material paves the way for the strengthening of sustainable synthesis processes. [212].

The ability of glycerol and other polyhydric alcohols to confer stability to proteins is a widely recognized phenomenon [214]. Polyols, like glycerol, are frequently used as cosolvent for protein stabilization [215].

In recent years, due to the increased production of biodiesel, glycerol is considered not only a by-product but also a waste. For this reason, thanks to the renewable nature of this raw material, and its considerable reactivity at low temperature and pressure, the development of new processes is underway. In particular, thanks to its alcohol groups, it can be used as a reagent for esterification reactions and is also recognized as a "green solvent" in many processes in which catalysts are used [82].

Glycerol is known to shift the native protein structure to more compact states [216], prevent the loss of enzymatic activity, increase the thermal unfolding temperature, and inhibit irreversible aggregation of proteins [217]. Moreover, glycerol has been reported as a nontoxic, biodegradable, and recyclable green solvent for high product yields and selectivity in catalysis and enzymatic catalysis [207][218].

Starting from different types of acids, glycerides syntheses have been reported, leading to its application in several industrial sectors, in particular using chemical and enzymatic catalysts in the pharmaceutical and food industries. An example of this strategy is the solvent-free synthesis of glycerol monobenzoate which has proved feasible thanks to the esterification of benzoic acid with glycerol, using lipase B from *C. antarctica* as a biocatalyst. No inhibitory effect is detected on both substrates and products, and even water seems to have no negative effects on the conversion or reaction rate and, therefore, the production of monoglyceride is

simplified [81]. Ravelo et al. [10] have shown a successful strategy to perform esterification reactions catalyzed by lipase between ibuprofen and glycerol in a solventless system.

4.1.1.1 Synthesis of Glycerol Sorbate

Note: the work explained in this section has been published as "Zappaterra, F.; Summa, D.; Semeraro, B.; Buzzi, R.; Trapella, C.; Ladero, M.; Costa, S.; Tamburini, E. Enzymatic Esterification as Potential Strategy to Enhance the Sorbic Acid Behavior as Food and Beverage Preservative. *Fermentation* 2020." and the title page of the article is visible at page 293.

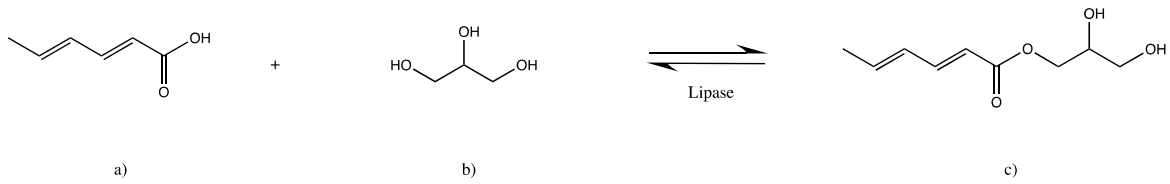
Sorbic acid is the most commonly used preservative in the food industry. Sorbic acid, and especially its more soluble salts, known as sorbates, are used as preservative for food, animal feed, and cosmetic and pharmaceutical products [170].

As is well known, solubility is one of the principal parameters that affects bioavailability [88]. The low solubility of sorbic acid in water can decrease its potential as a food preservative, influencing its bioavailability.

So, we aimed to exploit a solventless media strategy to perform the lipase-catalyzed esterification of sorbic acid with glycerol. The liquid nature of this short sugar alcohol allowed us to remove any organic solvent from our enzymatic synthesis strategy. Indeed, in this reaction, glycerol acts both as reagent and as a solvent. We aimed to employ a monophasic system to reach an effective esterification reaction between sorbic acid and glycerol in order to develop a novel sorbic acid derivate with a promising hydrophilic profile.

In this section, a solvent-free esterification of sorbic acid with glycerol has been carried out using a commercial immobilized enzyme *Candida antarctica* lipase type

B in order to improve water solubility of the resulting ester. The reaction is show in **Scheme 8**:



Scheme 8: Reaction between sorbic acid and glycerol catalyzed by immobilized lipase B from C. antarctica. (a) sorbic acid, (b) glycerol, (c) glycerol ester of sorbic acid.

In particular, glycerol can act as a hydrophilic moiety when combined with sorbic acid, with the concomitant effect of increasing polarity and, consequently, water solubility.

Sorbic Acid (100 mg), pure glycerol (10 mL), and 40 mg of immobilized CALB were placed inside a 20 mL capped vial. The reaction solution was mixed using a magnetic stirrer hot plate at 55 °C and 720 rpm. The use of a crosshead magnetic stirrer was employed to avoid enzyme support disruption. At the end of 24 h of reaction, samples were stocked at −20 °C, aiming to inhibit the enzymatic activity. A negative control of the reaction was prepared without the use of lipase.

Glycerol has proved to be a good candidate for the solubilization of sorbic acid in our experimental conditions. The viscosity of glycerol, together with the choice of a crosshead magnetic stirrer and the right stirring speed, allowed the enzyme to remain highly stable and perfectly separable from the medium. Glycerol viscosity and the selection of the right magnetic stirrer permit minimizing the contact surface between the reactor wall and the stirrer, thus avoiding particle degradation and, in consequence, ensuring the physical integrity of the enzyme support.

Thin layer chromatography was performed to monitor the reaction process; **Figure 39**.

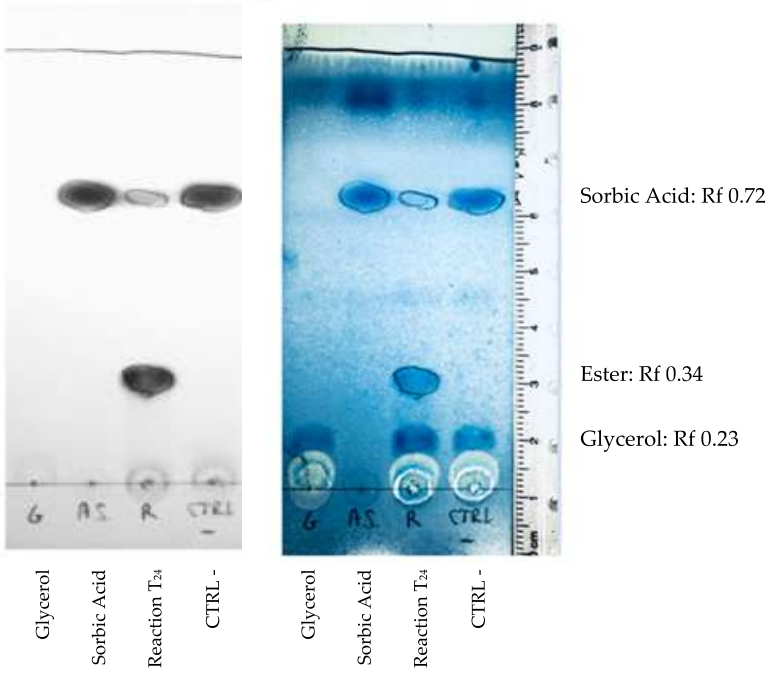


Figure 39: TLC monitoring of the enzymatic esterification reaction of sorbic acid with glycerol. On the left, TLC shown under (UV 254 nm). On the Right, destructive development with phosphomolybdic acid.

The particular viscous nature of glycerol required preliminary solubilization steps of the samples before they could be sown on TLC. 100 μL of the reaction mixtures were diluted in 8 mL of acetone and 1.9 mL of distilled water. This step was performed because of the glycerol high viscosity, which made difficult its sowing on TLC plate. The standard sorbic acid sample has been suspended in methanol (1 mg mL^{-1}). TLC (Silica gel 60, 5 × 10 cm, Merck, Germany) plates were analyzed with the elution system ethyl acetate/hexane/acetic acid 60:35:5 (v/v/v). Starting from the conditioning of the TLC chamber, the mobile phase has been prepared in 10 mL final volume with this proportion: 6.5 mL ethyl acetate, 3.5 mL hexane, and 0.5 mL acetic acid. The compound Rf of sorbic acid was 0.72, while glycerol, more hydrophilic, showed a retention factor (Rf) of 0.23. In these conditions, the Rf of the

glycerol ester of sorbic acid was 0.34. This Rf characterizes a molecule with a higher affinity for the stationary phase of TLC compared to the sorbic acid. Indeed, the presence of two hydroxyl groups in the ester of sorbic acid decreases its logP, and, consequently, raises its hydrophilicity and water solubility. furthermore, the band at Rf 0.34, being visible to UV rays, indicates how the molecule in question possesses double bonds, further suggesting its nature as an ester of sorbic acid with glycerol.

The effect of enzyme concentration on the conversion of sorbic acid has been evaluated through reverse-phase HPLC and allowed us to set up the enzyme concentration to 4 g L^{-1}. As shown in **Figure 40**, the conversion yield increased with the amount of enzyme following a hyperbolic trend typic in the esterification reactions lipase-catalyzed. At higher enzyme concentrations, more active sites are present for substrate binding. Therefore, the reaction rate increases. No improvement is observed after 4 g L^{-1}, so these values were selected as the most adequate enzyme concentration.

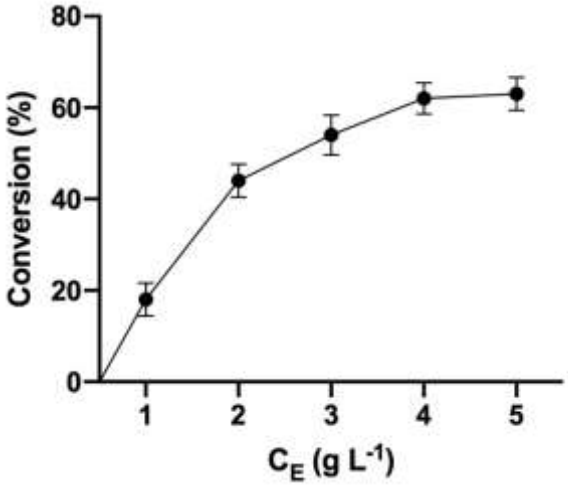


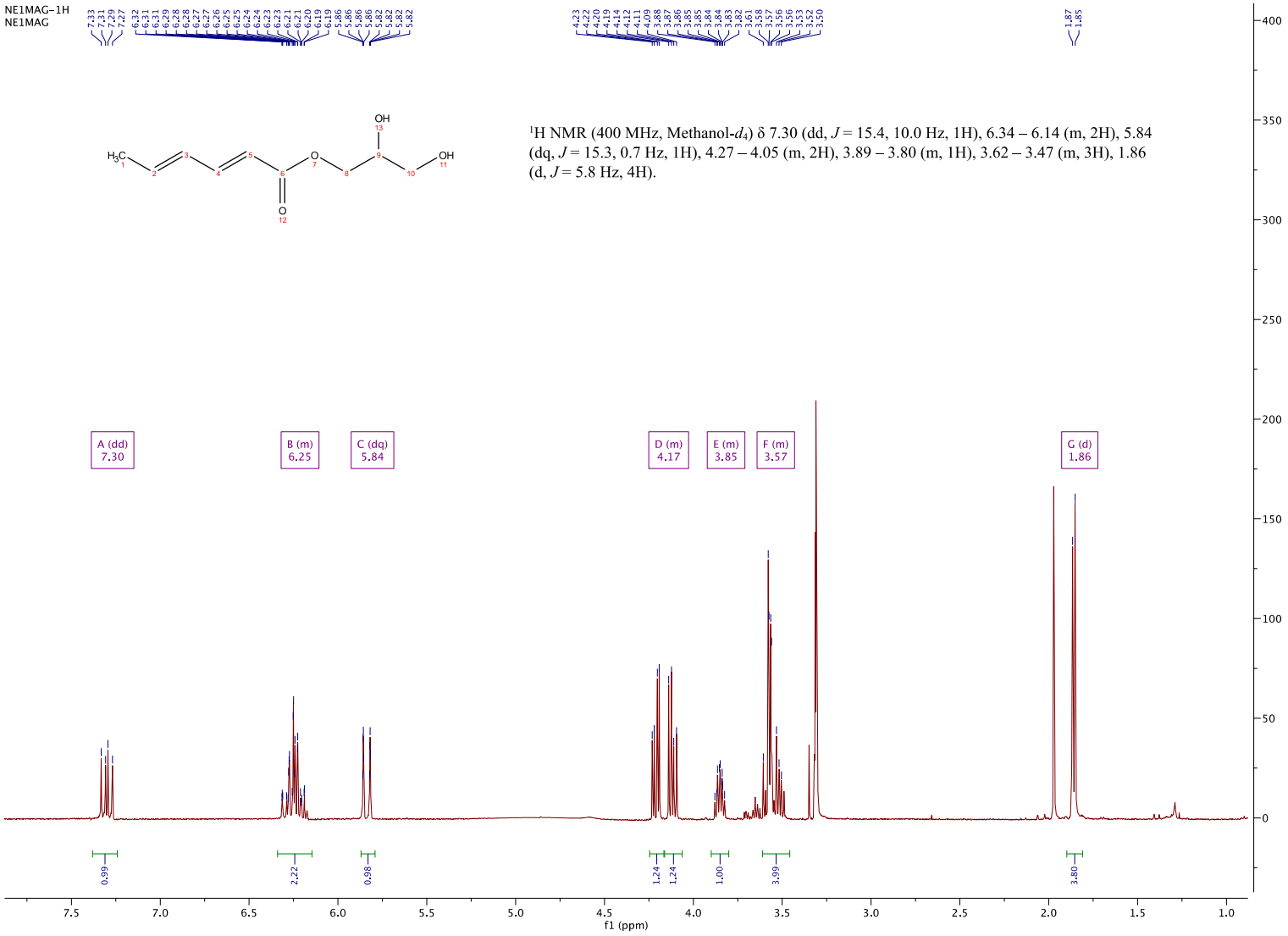
Figure 40: Effect of enzyme concentration at 720 RPM conversion yield of sorbic acid.

It has been previously observed that migration of compounds such as glycerol, 2-hydroxyethyl benzoate, 2-hydroxyethyl sorbate, and sorbic acid occurs from

immobilized forms of CALB, as they are used as stabilizers [270]. In this work, due to the minute concentration in which these compounds are present in CALB and, in some cases (glycerol, sorbic acid), the presence of much higher concentrations of such compounds in the reaction liquid, their effect on the reaction can be considered negligible. In any case, the chromatographic analysis here performed did not report the presence of most of these compounds, despite the fact that they have a very similar LogP to that of the compounds here studied.

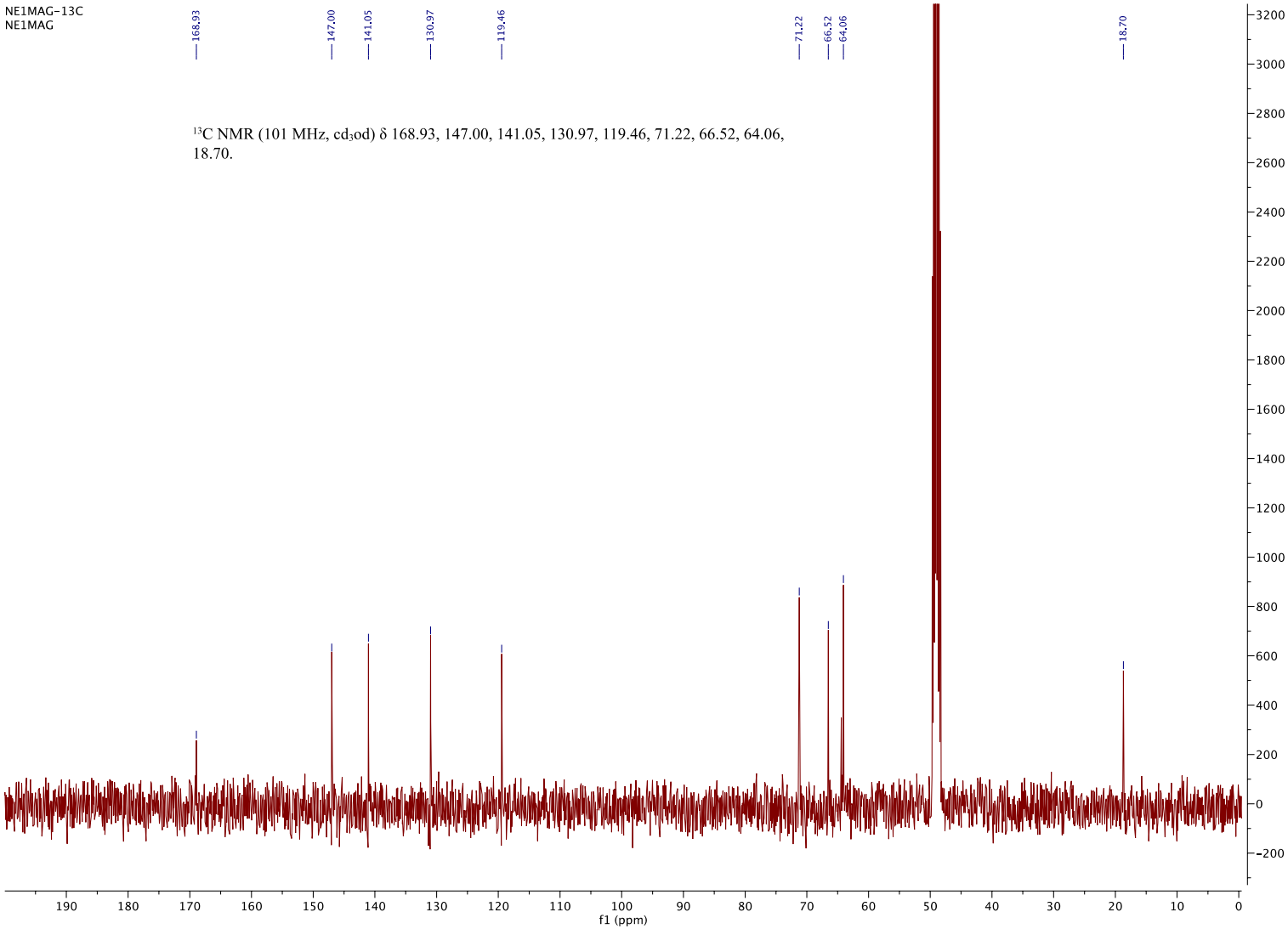
Water activity has been previously reported in literature for this kind of glycerol esterification [271]. In fact, a small amount of water (about 10% *v*/*v*) can be an important strategic advantage. Indeed, water can enhance mass transfer by reducing the viscosity of glycerol [257] and enhance the biocatalyst flexibility, resulting in better esterification activity [217][207].

Before proceeding with the purification, the entire volume of initial sample was washed three times with 10 mL ethyl acetate, in order to remove the excess of unreacted glycerol and extract the ester into the organic solvent. Once the ethyl acetate has been removed with a rotary evaporator, the separation of the glycerol ester was achieved by glass column chromatography. Silica gel column was prepared with the following eluent solution: acetate/hexane/acetic acid 60:35:5 (*v*/*v*/*v*). The eluted fractions were collected, the solvent was removed using a rotatory evaporator, and the rest was analyzed by ^{1}H, ^{13}C NMR (**NMR 1** and **NMR 2**) and IR (**IR 1**)..



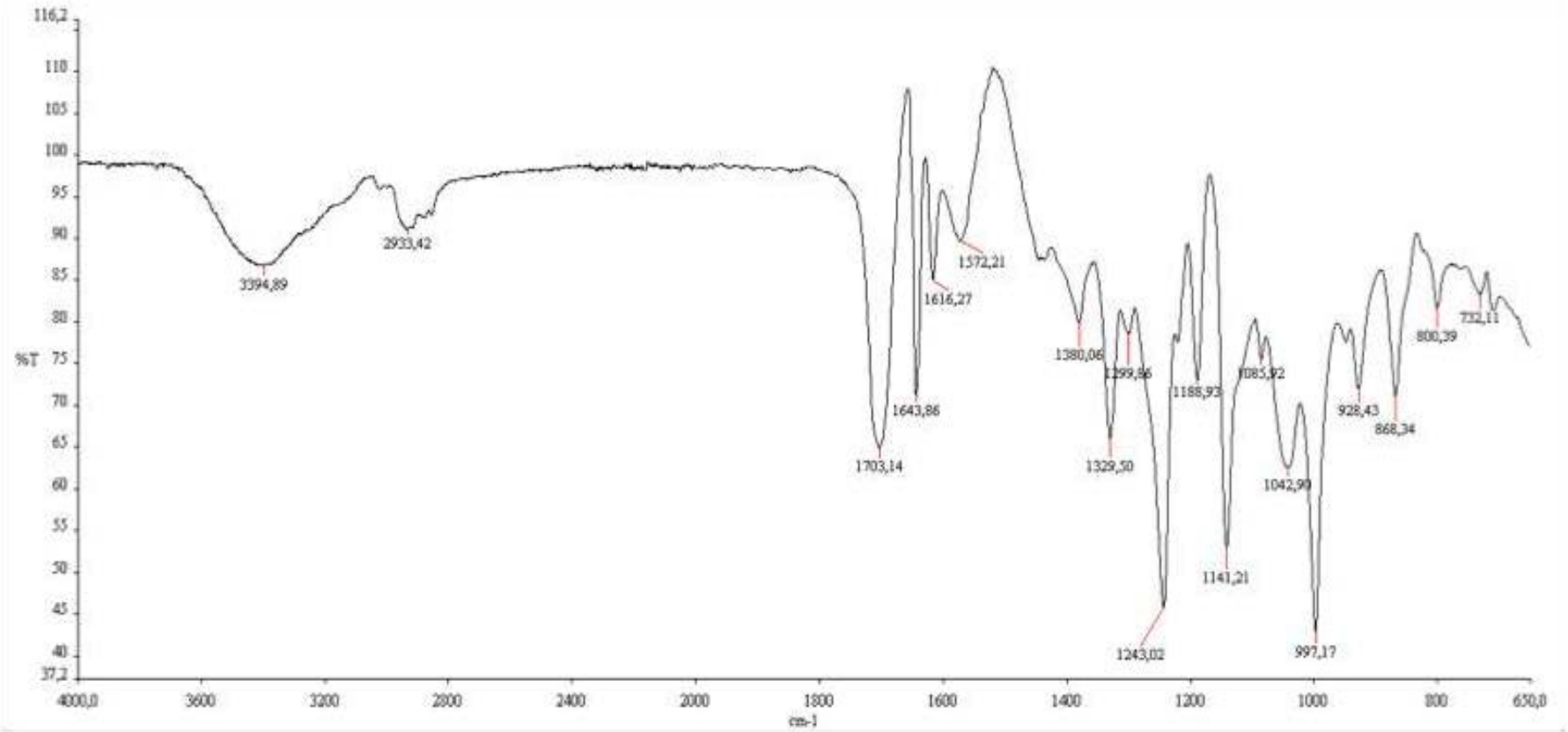
^{1}H NMR (400 MHz, Methanol-d_4) δ 7.30 (dd, J = 15.4, 10.0 Hz, 1H), 6.34 – 6.14 (m, 2H), 5.84 (dq, J = 15.3, 0.7 Hz, 1H), 4.27 – 4.05 (m, 2H), 3.89 – 3.80 (m, 1H), 3.62 – 3.47 (m, 3H), 1.86 (d, J = 5.8 Hz, 4H).

NMR 1: ^{1}H-NMR of Glycerol Sorbate.



NMR 2: ^{13}C-NMR of Glycerol Sorbate.

IR: 3394,89; 2933,42; 1703,14; 1643,86; 1243,02; 1141,21; 997.14 cm^{-1}.



IR 1: Glycerol Sorbate

After IR and NMR, further proof of the effective lipase-catalyzed esterification has been obtained by uHPLC-MS analysis. The result from the reverse phase separation showed that glycerol sorbate eluted at 0.94 min (Figure 41; peak No 1). Because of the reaction conditions (55 °C, 24 h), no 2-monoacylglycerol (2-MAG) regioisomer as by-product seems to have formed. This interesting aspect remains to be investigated and will be the subject of further studies. Curiously, no 1,3-diester was found. Indeed, this compound would be more lipophilic than the sorbic acid monoglyceride and would be held back by the c18 column, producing a peak at retention times higher than the monoglyceride.

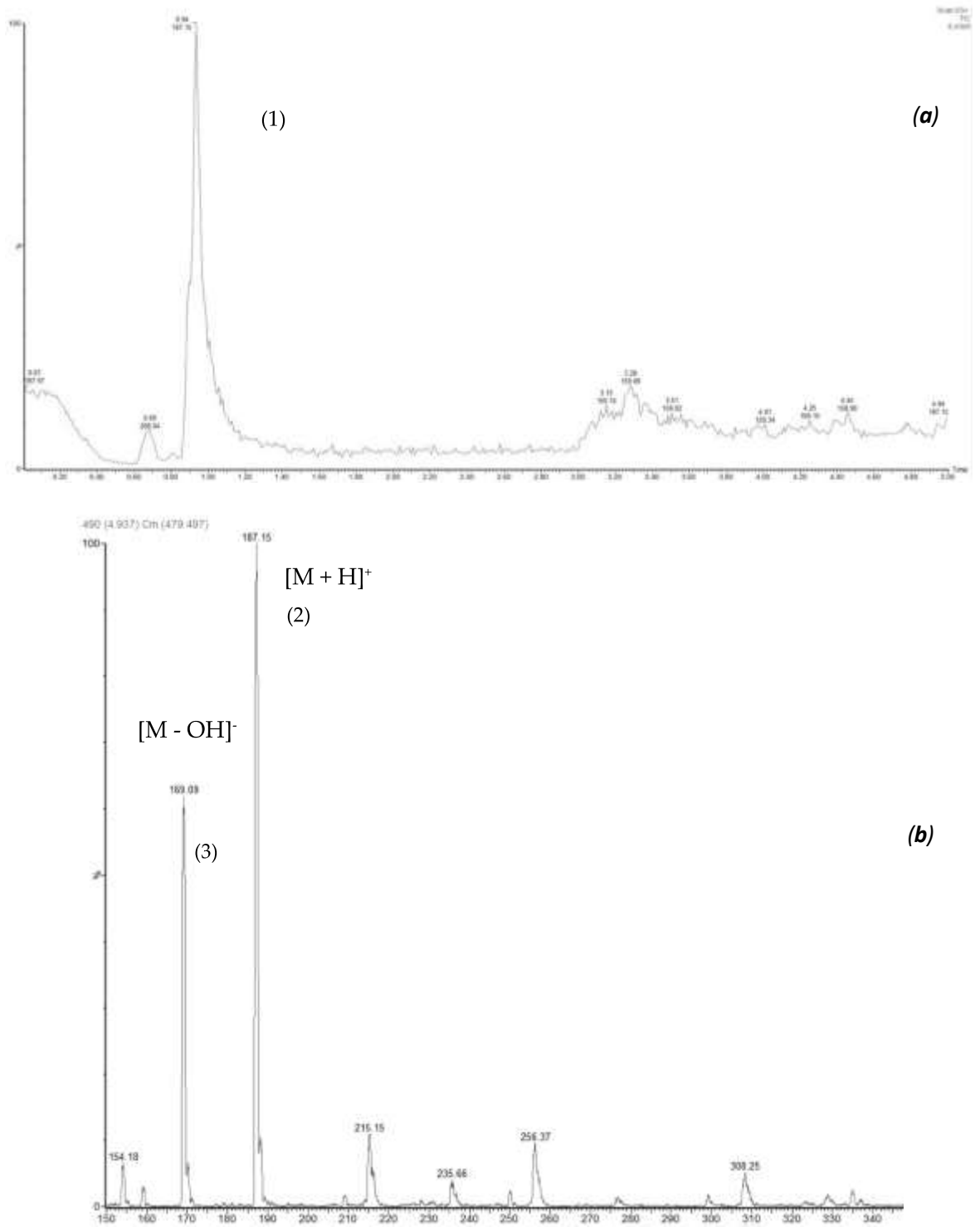


Figure 41: uHPLC/MS analyses of lipase-catalyzed esterification of sorbic acid and glycerol by immobilized CALB in solventless media; (a) uHPLC chromatogram, showing the peak of glycerol sorbate (peak No 1) at 0.94 min; (b) ESI+/MS spectrum of the peak No 1, showing the ester product by electro spray ionization mass spectroscopy.

Electrospray ionization (ESI) was performed on the peaks of the HPLC product separations to obtain mass spectra. The results from the mass spectrometry confirmed that the expected esterification reaction occurs between the carboxylic acid group and oxydril group of glycerol. The newly formed product mass prediction was m/z 186. The fragmentation pattern showed most high peaks at m/z 187 (peak No 2) and 169 (peak No 3), identifying the glycerol sorbate. Peaks appearing at an m/z ratio of 187 and 169 represent the ionized $[M + H]^+$ and $[M - OH]^-$. To the best of our knowledge, this enzymatic esterification of sorbic acid with glycerol is reported for the first time.

Until now, CALB lipase has been proposed as biocatalysts for a similar esterification reaction between benzoic acid and glycerol [81]. This strategy was demonstrated to be an successful way to minimize the effect of water in the esterification/hydrolysis activity equilibrium of the lipase. In fact, a reaction environment completely made of glycerol creates a huge disequilibrium of stoichiometry ratio between alcohol (134 mmoles) and acid (0,89 mmoles) that boosts the esterification reaction, avoids the hydrolysis due to the small amount of water produced (0,89 mmoles at complete sorbic acid conversion into the sorbate), and favors the production of the monoglyceride of the acid, avoiding the formation of di- and triglycerides. Thus, the excess of glycerol is one of the advantages of this esterification approach. This polyol excess shifts the equilibrium of formation of the ester bond towards ester and water products. Moreover, the excess glycerol could easily be recovered and used again as solvent and reagent in batch processing or recycled in a continuous process.

4.1.1.2 Synthesis of UDCA monoglyceride

Bile acids (BAs), are a family of steroids synthesized from cholesterol in the liver. Their primary functions traditionally include regulation of cholesterol homeostasis, its elimination in a soluble form, formation of canalicular and ductular bile, and solubilization of dietary lipids and their intestinal absorption [161].

Among bile acids, Ursodeoxycholic acid (UDCA) is the drug of choice for treating primary biliary cirrhosis and dissolving cholesterol gallstones [165]. UDCA is orally administered and is dissolved in the proximal jejunum in mixed micelles of endogenous bile acids [272]. The administration of UDCA decreases cholesterol saturation of bile [273][274], reduces biliary cholesterol secretion [275], and induces gallstone dissolution [276].

The clinical effectiveness of UDCA includes its choleretic activity, the capability to inhibit hydrophobic bile acid absorption by the intestine under cholestatic conditions, reducing cholangiocyte injury, stimulation of impaired biliary output, and inhibition of hepatocyte apoptosis [277].

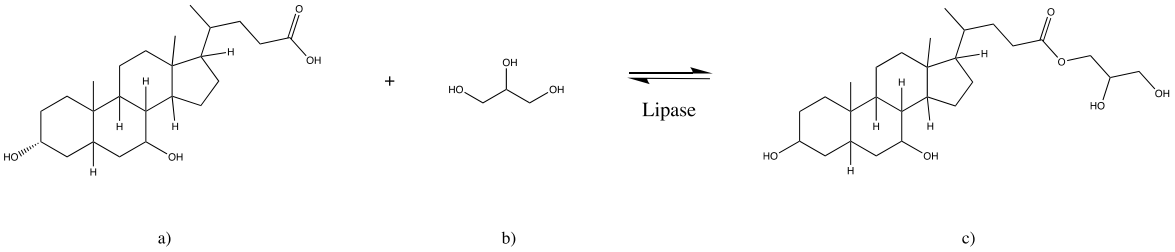
Despite its clinical effectiveness, UDCA is poorly soluble in the gastro-duodeno-jejunal contents and pharmacological doses of UDCA are not readily soluble in the stomach and intestine in healthy man, resulting in incomplete absorption [278]. In patients with cholestatic liver diseases, UDCA hepatic uptake and biliary secretion are impaired [279].

Previous work reported how a galactosylated pro-drug of ursodeoxycholic acid (UDCAgal), with higher solubility than the original UDCA, displayed a higher cell permeation compared to UDCA in liver HepG2 cells [95].

Therefore, we decided to enzymatically synthesize a glyceric ester of UDCA bile acid to produce a more water-soluble molecule. Since the steroid nature of UDCA

is very different from that of sorbic acid, it would have been interesting to understand whether the previously employed esterification strategy would prove to be exploitable in this context.

The enzyme employed for this esterification has been the a immobilized form of CALB and the reaction is shown in **Scheme 9**.



Scheme 9: Reaction between Ursodeoxycholic acid (UDCA) and glycerol catalyzed by immobilized lipase B from C. antarctica; (a) UDCA, (b) glycerol, (c) glycerol ester of UDCA.

UDCA (150 mg) and 5 ml of glycerol are placed in a 20 mL vial. The substrate were solubilyzed at 80 °C. At temperature of 55°C, 10 mg of CALB are added. The reaction is maintained under stirring (720 rpm) at the temperature of 55 ° C for 24 h. At the same time, the negative control of the reaction with the reagents is prepared without using lipase.

At a temperature of 80 °C, glycerol proved to be a reagent capable of solubilizing the acid UDCA. Even allowed to decrease the temperature to 55 ° C and added the biocatalyst, no appreciable problems of acid reprecipitation were encountered.

The high boiling poit of glycerol (over 290°C) allowed us to solubilyze UDCA in this short sugar alcohol without the evaporation problems encountered with canonical organic solvents.

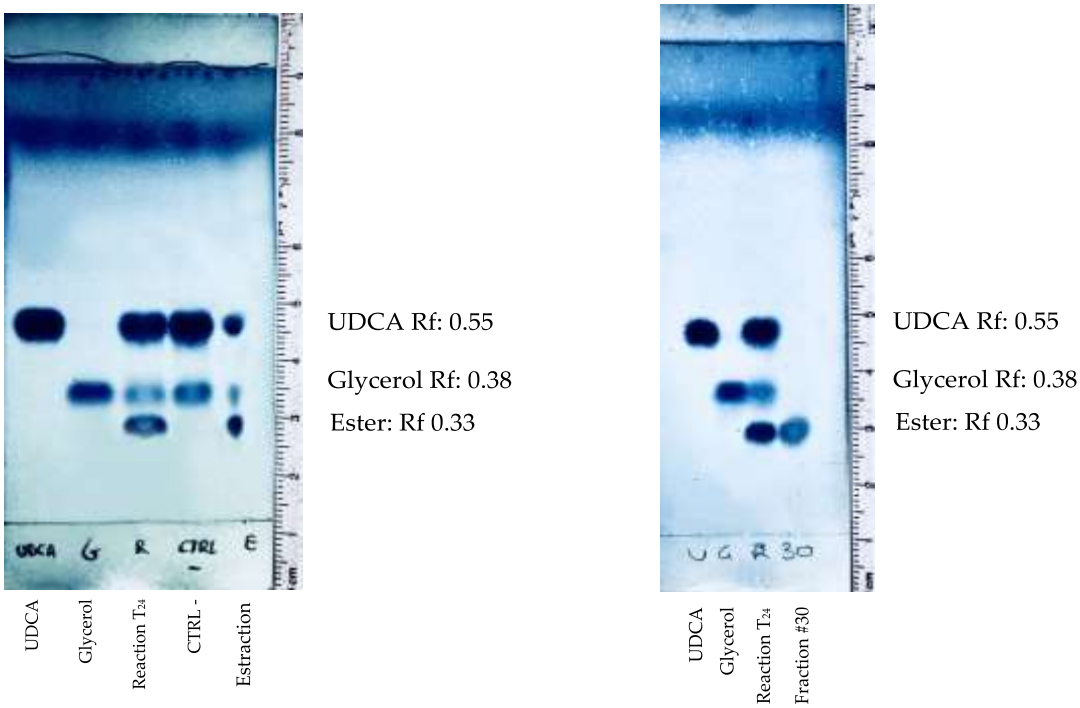
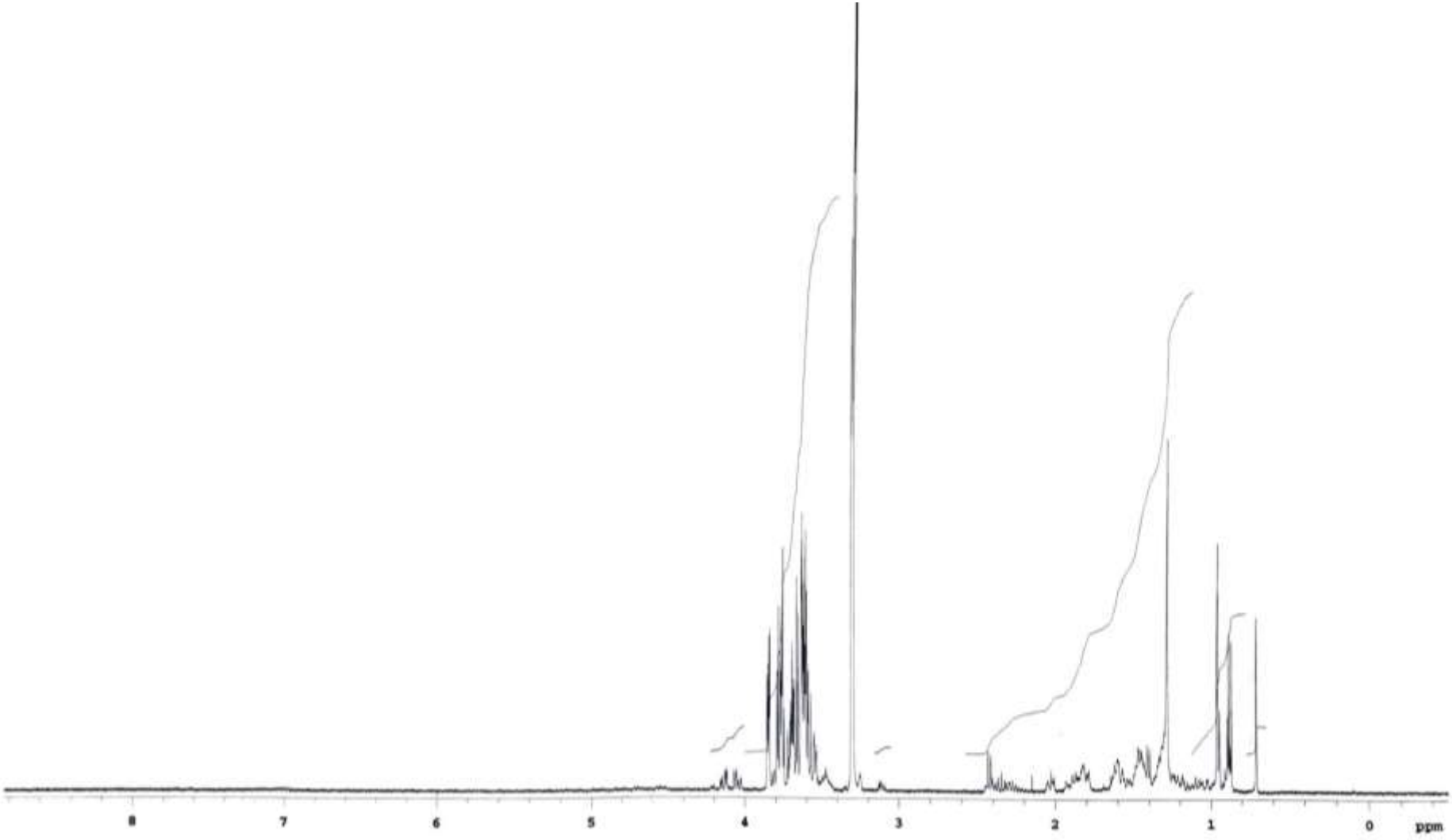


Figure 42: TLC of the enzymatic esterification reaction of UDCA with glycerol. On the left, TLC showing UDCA, Glycerol, Reaction T24, negative control, extract in AcoEt. On the Right, monitoring TLC for the column chromatography purification of UDCA glycerol ester.

After 24h of reaction. The entire volume of reaction glycerol was extracted with an equal volume of ethyl acetate in order to simplify the sample from the excess of glycerol left unreacted. The left part of **Figure 42** shows the monitoring TLC of these steps. Following extraction, the ester was purified by silica gel column chromatography. The TLC on the right, in Figure 42, shows the ester perfectly purified in fraction number 30 of the purification column.

UDCA esterified with glycerol changes its LogP from 4.13 to 3.34. This enhancement in solubility is testified by the Rf of 0.33, instead 0.55.

The chemical characterization of the ester has followed by 1H-NMR and uHPLC-MS where the chromatographic separation was carried out with a C18 column with a solvent gradient from 100% H_2O to T0 to 100% MeCN at T_5 (5 minutes).



NMR 3: ^{1}H-NMR UDCA-Monoglyceride.

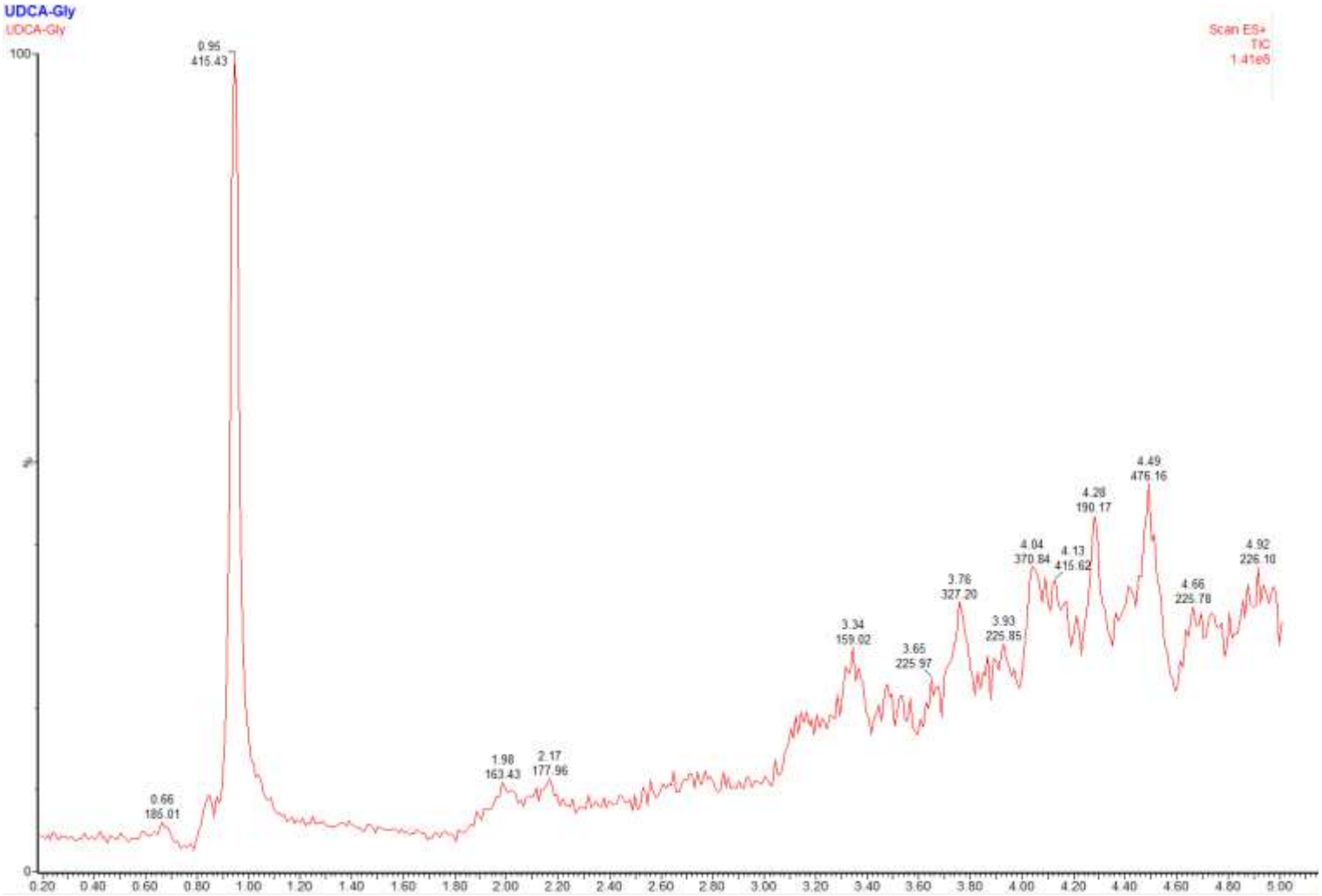


Figure 43: uHPLC chromatographic separation of UDCA monoglyceride.

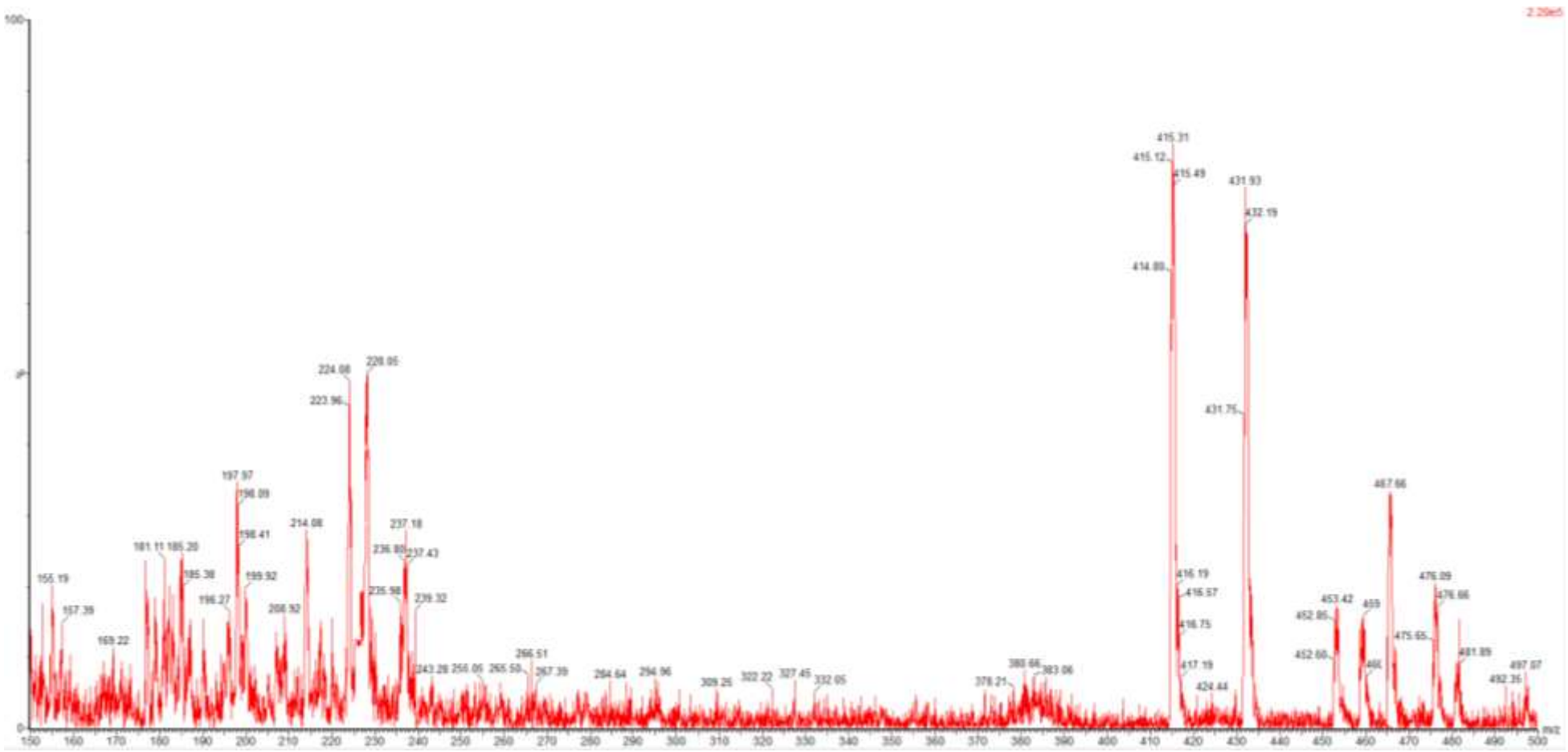


Figure 44: Mass spectrometer detection of monoglyceride UDCA.

UDCA monosgliceride display two more hydroxyl groups than the starting acid. The ^{1}H NMR spectra (NMR 3) confirms the expected structure by showing the characteristic peaks relating to the angular methyl groups in C-18 and C-19 respectively at 0.97 and 0.75 ppm and the glycerol characteristic peakes at 3.6-3.85 and 4.05.

In the uHPLC chromatographic separation, the UDCA ester demonstrates its increased solubility by detecting it at rather short times, 0.95 min (**Figure 43**).The MS analysis (**Figure 44**),related to an expected mass of 466.66 g/mol, confirm the presence of UDCA monoglyceride. This ester showed a strong tendency to undergo the dehydration effect characteristic of the mass spectroscopy technique, forming the radical carbocation. In fact, masses relative to the molecular weight of the ester without 2 and 3 hydroxyl groups (respectively m/z 432 and m/z 415) were observed. Furthermore, the ionized mass value was found (m/z 467).

UDCA monoglyceride was obtained with a yield of 71.4%.

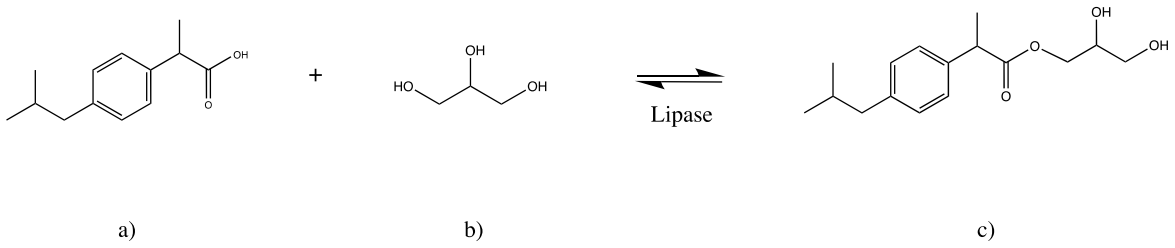
CALB has proved to be the right biocatalyst to perform the enzymatic esterification of the UDCA steroid bile acid with glycerol in order to obtain a molecule polarity enhanced.

4.1.1.3 Synthesis of glycerol esters of Ibuprofen

Ibuprofen is the substrate from which the greatest number of esters were produced during this doctoral work. Of this widespread non-steroidal antinflammatory drug, six esters of different polarity were produced. In this section, we will focus on the production of the glyceric ester of ibuprofen. The binding of ibuprofen to the small three hydroxyl polyalcohol was previously reported by Ravelo et al [82] exploiting a *solventless* strategy involving the use of the lipase form of CALB (Lipozyme CALB-L). To produce this ester we used a solventless protocol similar to the one reported by Ravelo, by using an immobilized form of CALB (Novozym 435) and PPL.

Following purification and chemical characterization with ^{1}H, ^{13}C-NMR, uHPLC-MS and IR, we tested *in vitro* the antinflammatory activity of this and the other ibuprofen esters compared to the starting ibuprofen.

The reaction (**Scheme 10**), was conducted solubilizing 95 mg (19 g L^{-1}) of ibuprofen in 5 mL of glycerol at 80 °C. After solubilization, at 55°C, 10 mg of Novozym 435 are added at the media. The reaction is carried out for 24h at 720RMP.



Scheme 10: Reaction between ibuprofen and glycerol catalyzed by immobilized lipase B from *C. antarctica*. (a) Ibuprofen, (b) glycerol, (c) Ibuprofen Monoglyceride.

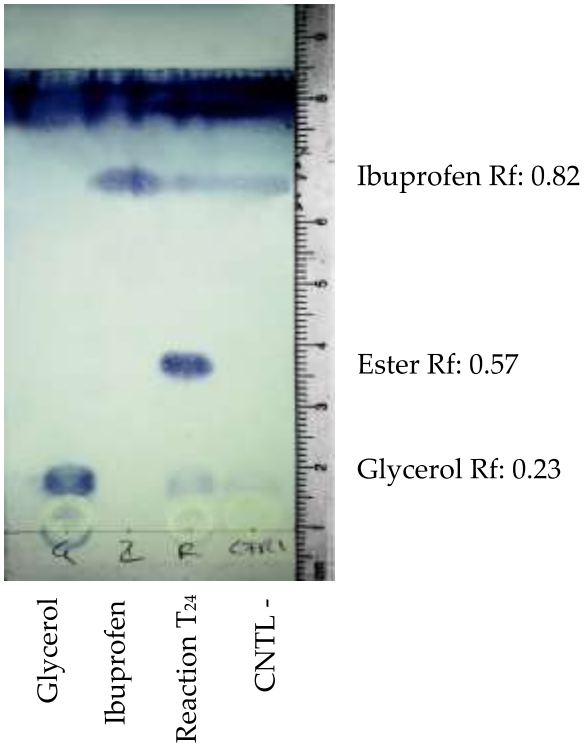
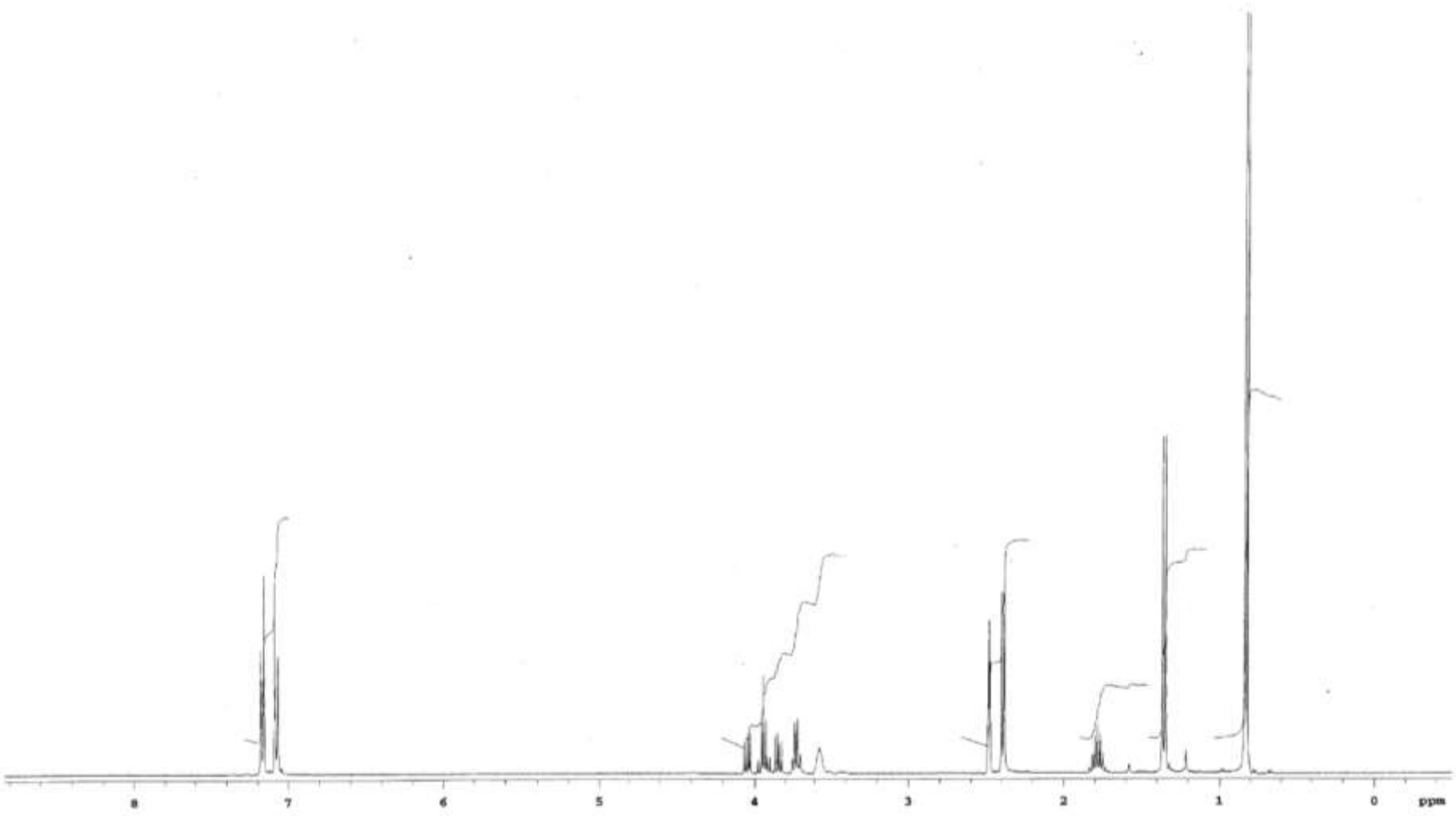


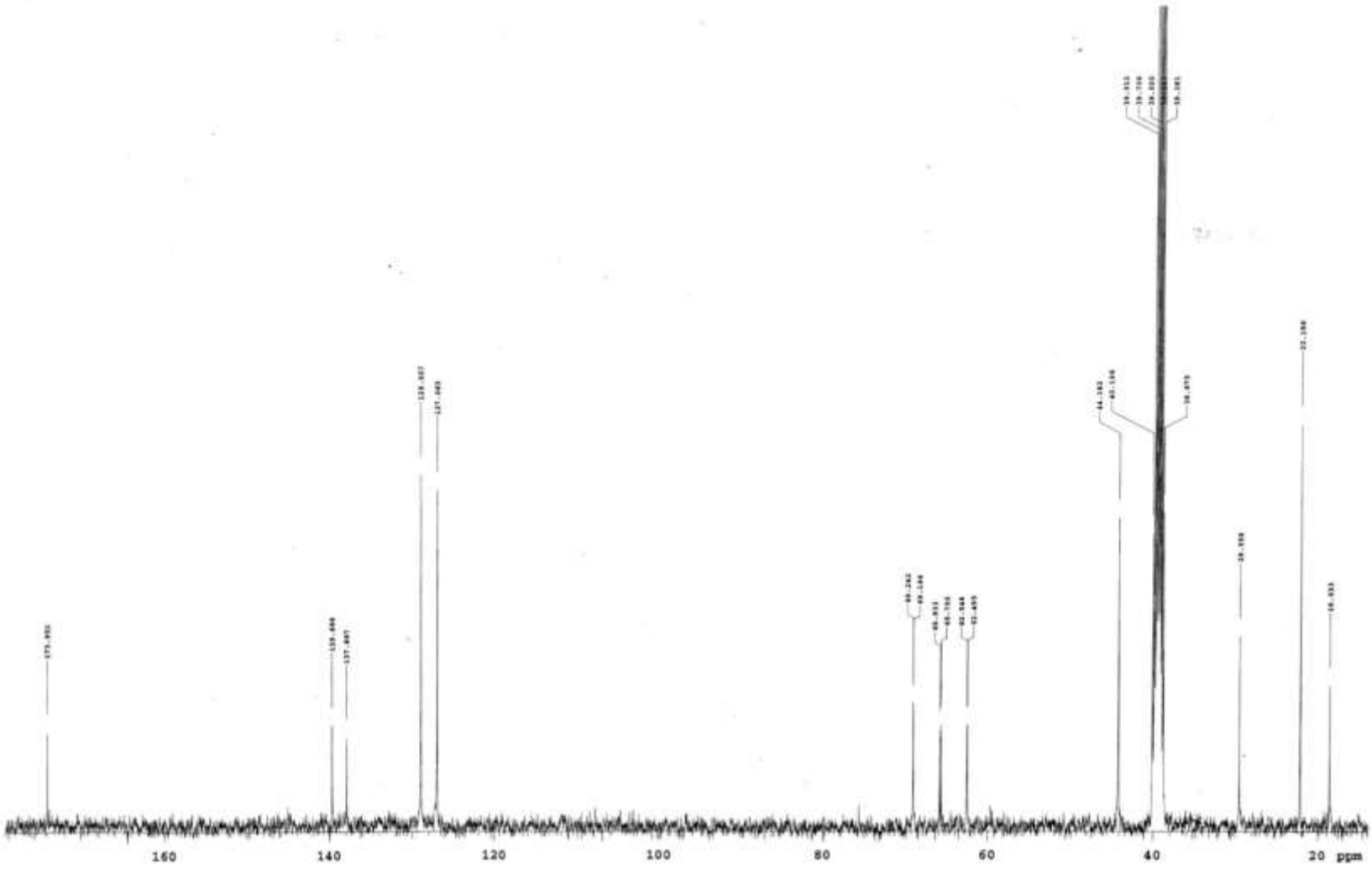
Figure 45: TLC monitoring of the enzymatic esterification reaction of ibuprofen with glycerol.

Similarly to what happened for the sorbic acid ester, also in this case from the monitoring TLC it can be observed that at reaction T_{24} a band is found (specifically with retention time 0.57), which is absent in the negative control. This suggests how, that band, is presumably due to the enzymatic esterification of ibuprofen and glycerol. In support of this, it is observed that the band at 0.57 is characterized by a lower retention factor than that of ibuprofen, demonstrating how the ester actually has an increased polarity and therefore greater affinity for the stationary phase of chromatography.

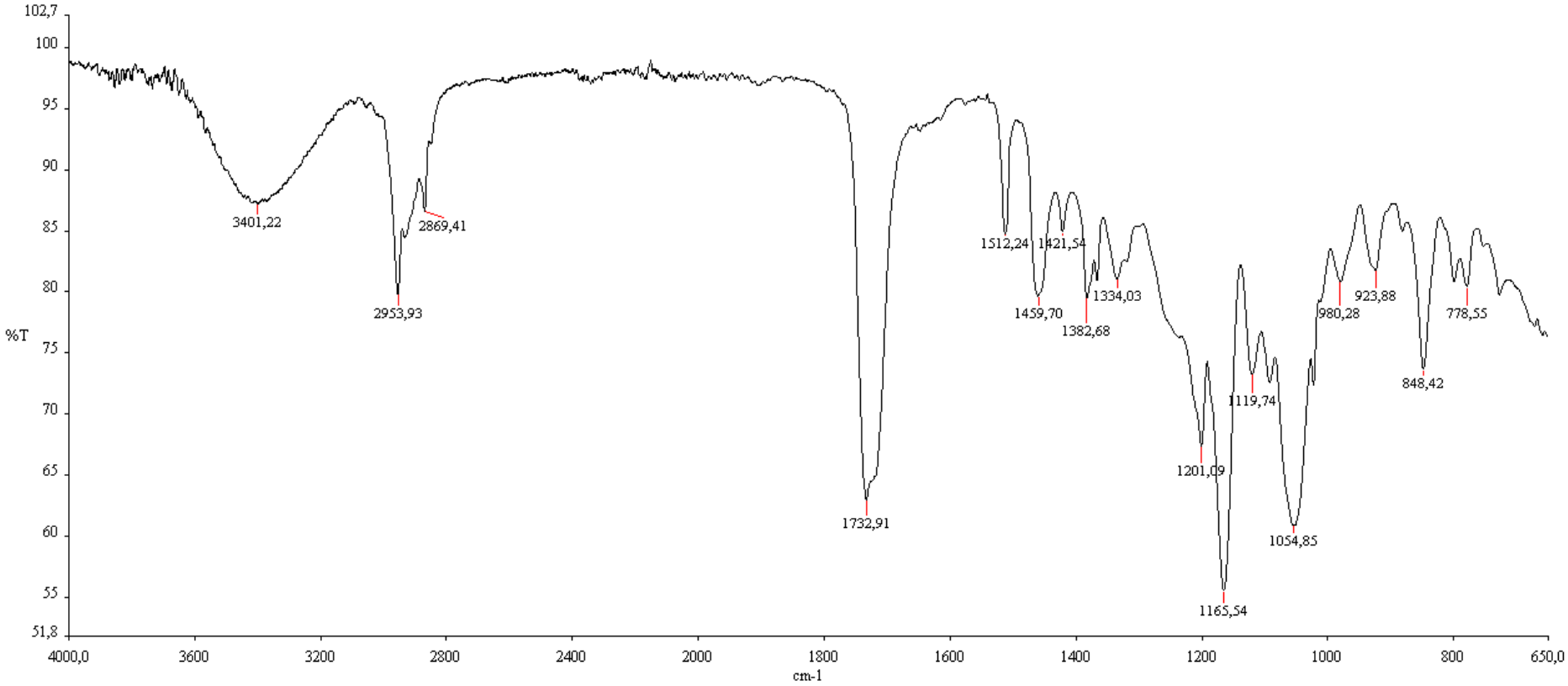
The ester has been purified and chemically characterized by ^{1}H, ^{13}C NMR (**NMR 4** and **NMR 5**), IR (**IR 2**), and uHPLC-MS (**Figure 46** and **Figure 47**),



NMR 4: ^{1}H-NMR of Ibuprofen monoglyceride.



NMR 5: ^{13}C-NMR of Ibuprofen monoglyceride.



IR 2: Ibuprofen Monoglyceride.

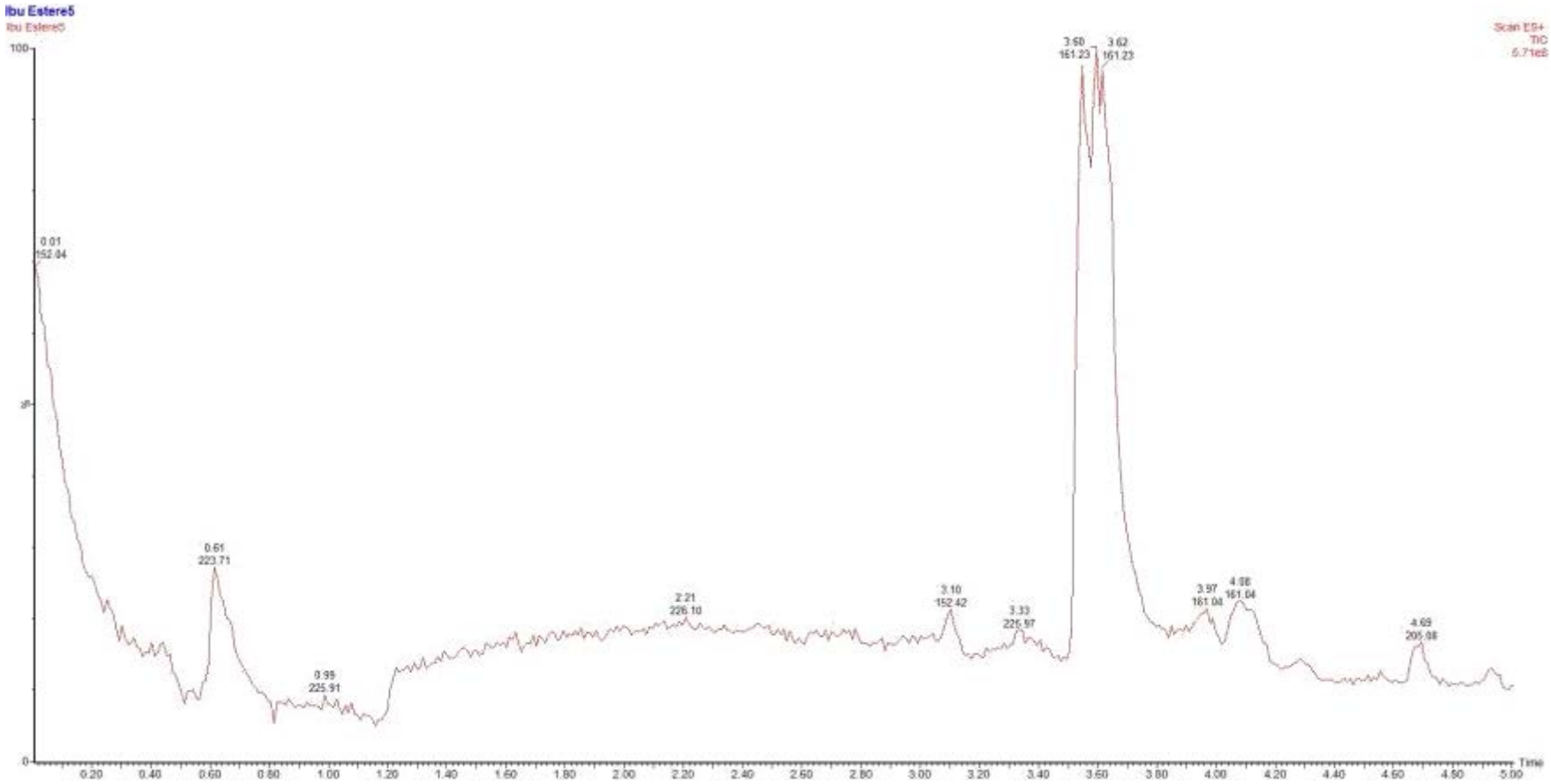


Figure 46: uHPLC chromatographic separation of Ibuprofen Monoglyceride.

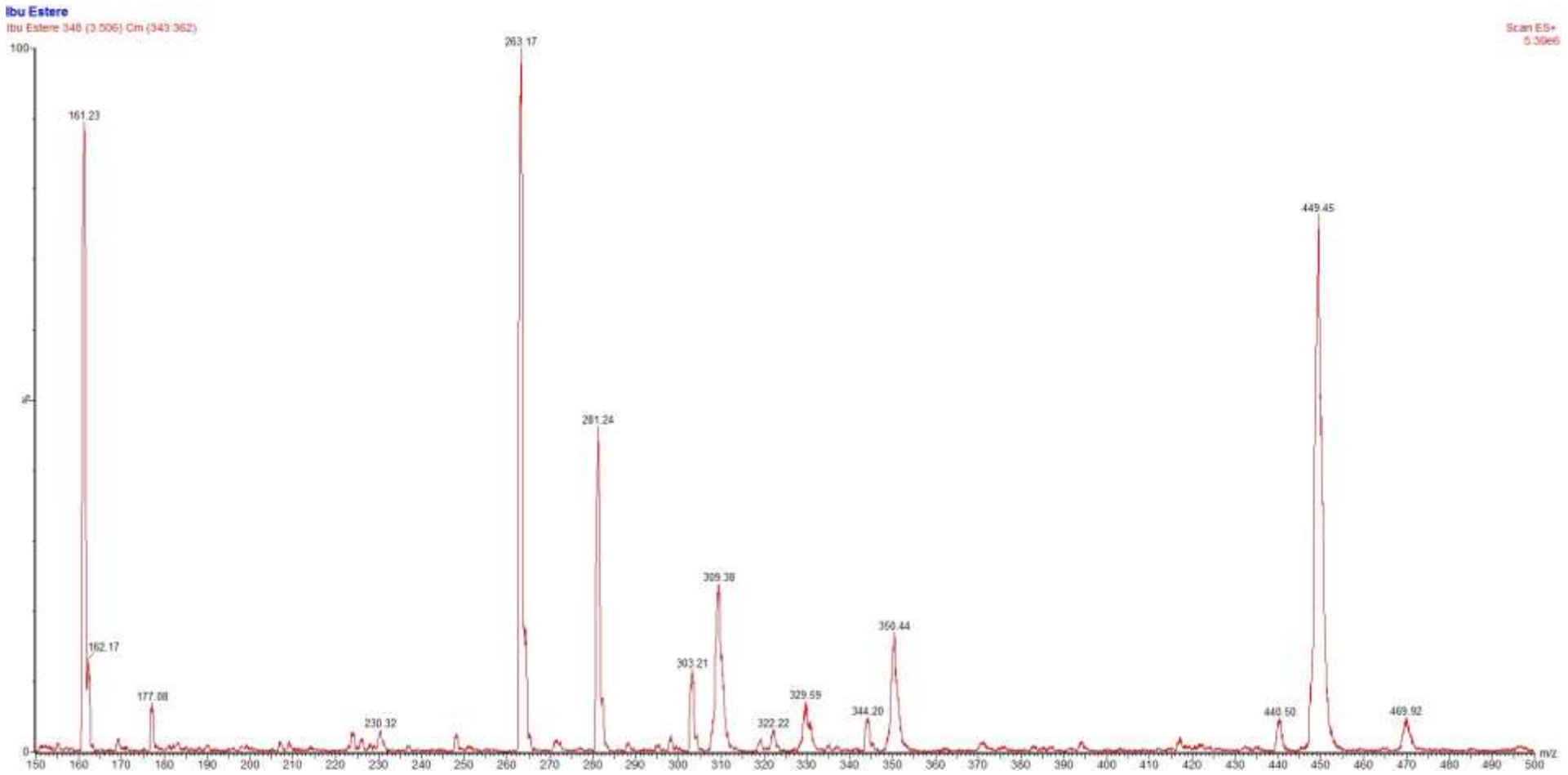


Figure 47: Mass spectrometer detection of monoglyceride UDCA.

NMR spectra, made in d6-DMSO, confirmed the presence of ibuprofen monolycerode showing the characteristic peaks of ibuprofen methyls at 0.87, the aromatic ring at 7.10-7.20 and glycerol peacks at 3.6-4.1.

The chromatography step of the uHPLC-MS analysis display a retention time for the ester of 3.60 min (**Figure 46**). Several form of ibuprofen glycerol-ester are shown at mass spectrophotometry detection step (**Figure 47**). Compared to the expected mass of m/z 280, peaks related to the dehydrated ester (m/z 263), the adduct with hydrogen (m/z 281), and sodium (m/z 303) were found. Interestingly, the masses of diester attributable to two ibuprofen molecules linked to the same glycerol molecule (expected m/z 468) both were found in ESI+ hydrogen adduct m/z 449, and in dehydration effect m/z 469. However, due to the preventive chromatographic separation, we consider the finding of the diester an experimental effect linked to the electro spray ionization of MS. Indeed, a similar molecule would have a different behavior in chromatography, demonstrating retention times other than 3.60 min, being more lipophilic.

The IR analysis (**IR 2**) clearly shows a signal attributable to the hydroxyls of ester at 3401 and to the signals of the carbonyl group at 1732. Methyl groups are also shown at 1459 and 1382.

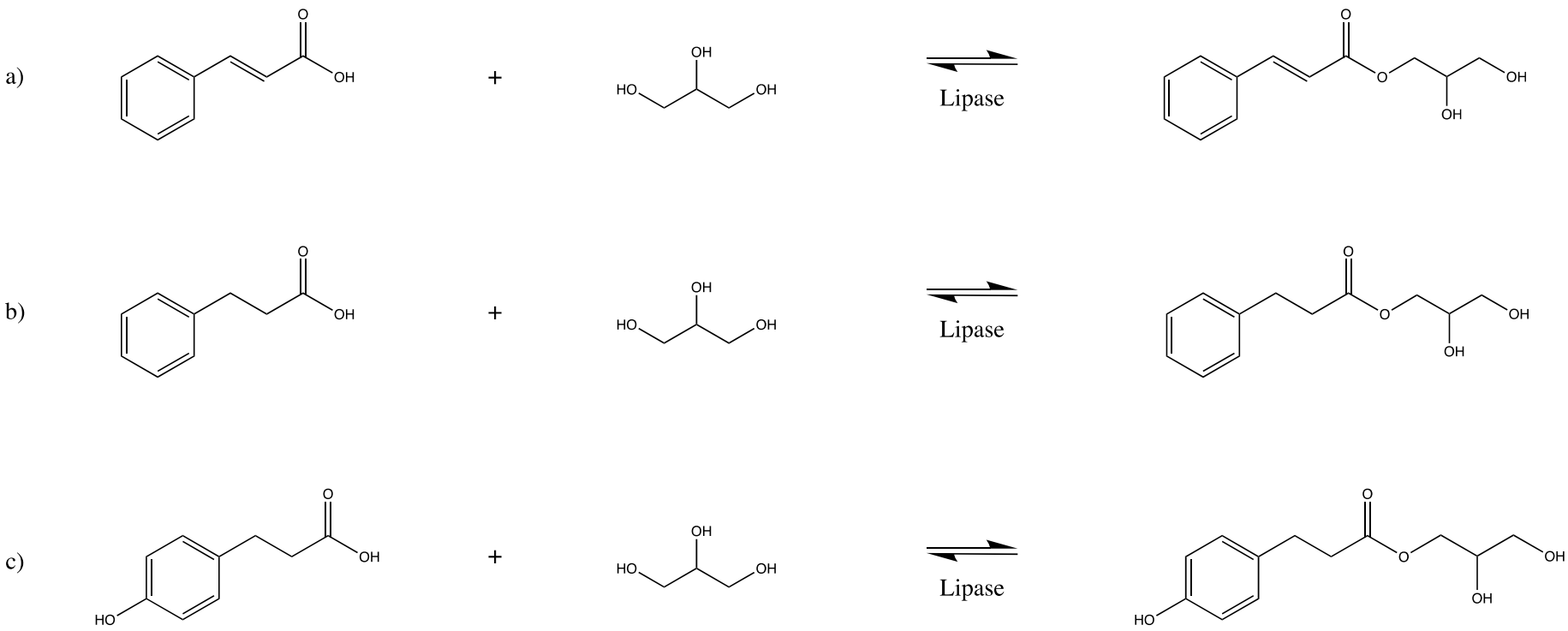
4.1.1.4 Synthesis of glycerol esters of Cinnamic acid

Note: The experiments and analysis of this section were conducted in Chemical and Materials Engineering Department - Complutense University of Madrid, Spain.

Cinnamic acid and its derivatives are the major group of phenolic acids with ubiquitous distribution in fruits and vegetables. Recent data support their beneficial effects, including antioxidant [186], anti-inflammator [187], and anti-cancer activities [188].

Ester derivatives such as ethylhexyl methoxycinnamate (octinoxate), isoamyl p-methoxycinnamte (amiloxiate), octocrylene and cinoxate are used in cosmetics all over the world as UV filters.

The reactions were done using the free form of CALB (CALB-L)



Scheme 11: Esterificazion Reaction between (a) cinnamic acid, (b) hydrocinnamic acid, (c) 3-(4-hydroxyphenyl)propionic acid and glycerol catalyzed by free lipase B from *C. antarctica* (Lipozyme - CALB-L).

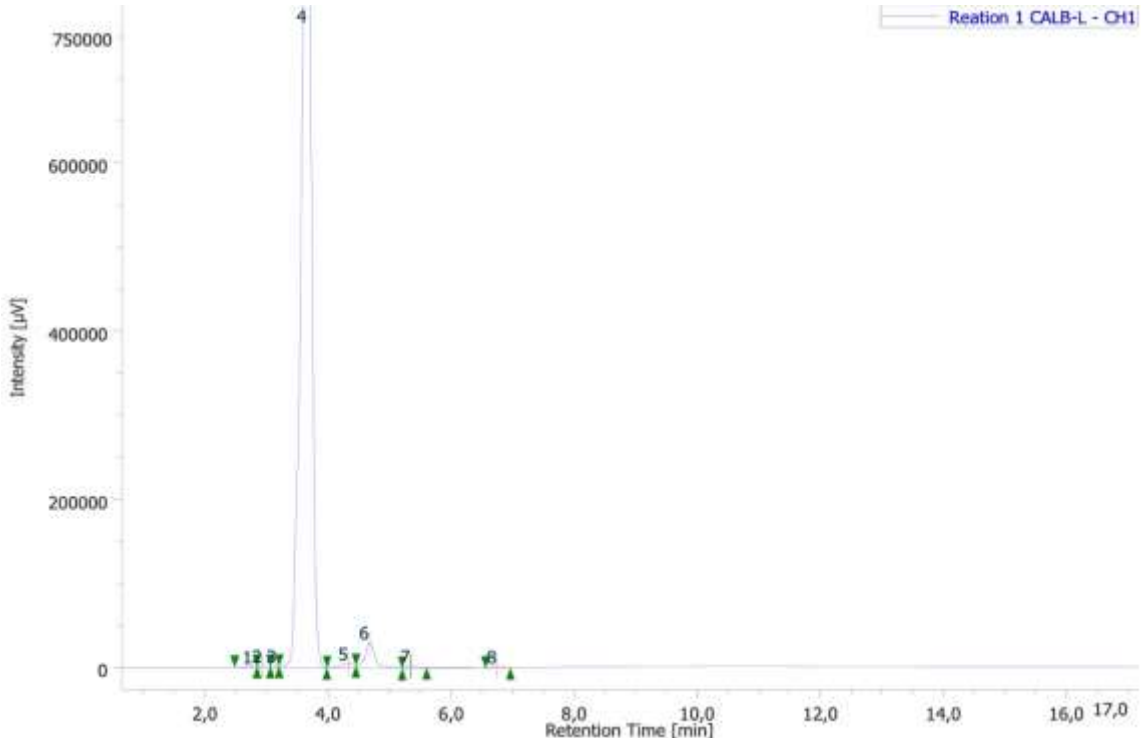


Figure 48: Hydrocinnamic acid monoglyceride HPLC chromatogram, showing the peak of glycerol (peak 4), glycerol ester of hydrocinnamic acid (peak 6) and hydrocinnamic acid (peak 7).

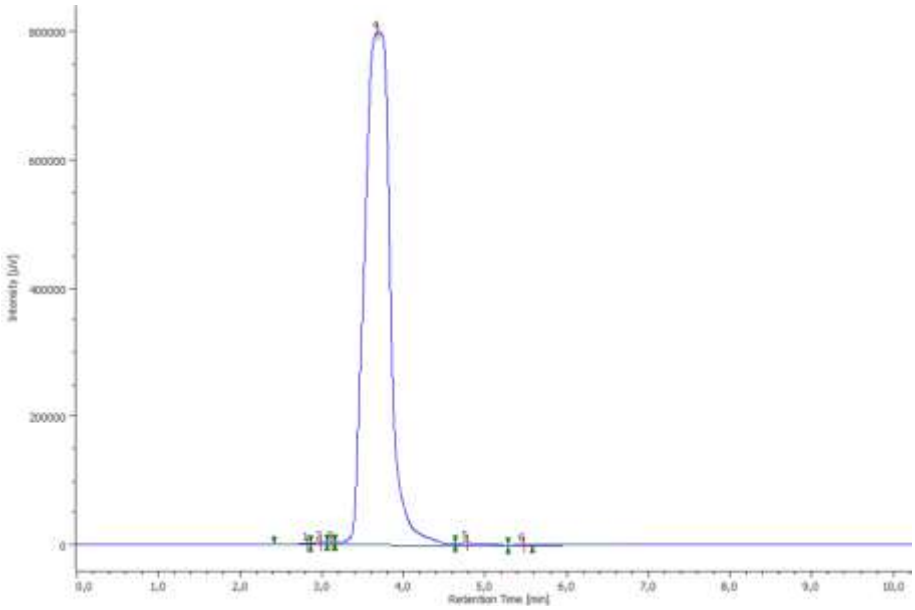


Figure 49: 3(4-hydroxyphenil)propionic acid monoglyceride HPLC choromatogram, showing the peak of glycerol (peak 4), glycerol ester of 3(4-hydroxyphenil)propionic acid (peak 5) and 3(4-hydroxyphenil)propionic acid (peak 6).

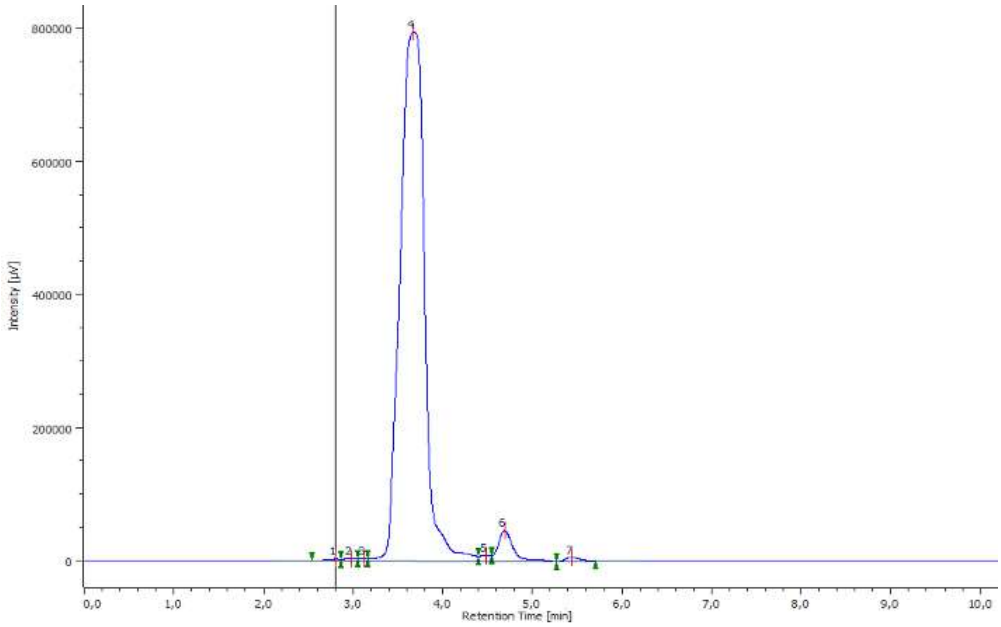


Figure 50: Trans-cinnamic acid monoglyceride HPLC chromatogram, showing the peak of glycerol (peak 4), trans-cinnamic acid glycerol ester (peak 6), and trans-cinnamic acid (peak 7).

The conversion yield for each ester has been: 94% for hydrocinnamic acid glycerol ester, 96% 3(4-hydroxyphenil)propionic acid glycerol ester, and 93% for trans-cinnamic acid glycerol ester.

4.1.2 Lipophilic esters

Although almost all of the esterification protocols presented in this thesis have the aim of producing esters with increased polarity and therefore solubility, we wondered if we could develop a protocol that could produce the opposite result, obtaining molecules with increased lipophilicity.

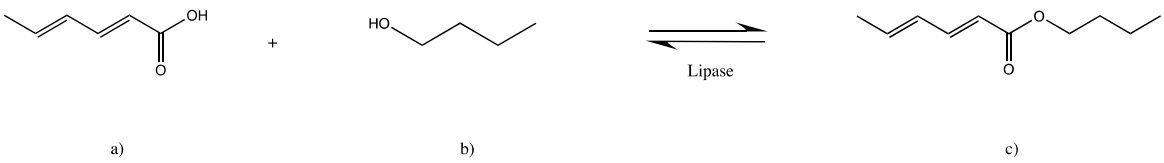
There are several contexts in literature that report how some molecules need modifications that make them more lipophilic rather than hydrophilic. Lipophilic prodrug approaches have been used in the past to take advantage of the intestinal lymphatic transport processes to deliver drugs to the intestinal lymphatics. Lee et at reported a study in which a lipophilic prodrug approach was used to efficiently deliver bexarotene (BEX) and retinoic acid (RA) to the intestinal lymphatic system using lipophilic activated ester prodrugs [280]. Gollnest et al proposed lipophilic prodrugs of nucleoside triphosphates as biochemical probes and potential antivirals [281].

These experiments allowed us to test the catalytic flexibility of type B *Candida anctartica* lipase, which has been shown to be able to produce butyl esters of sorbic acid and ibuprofen.

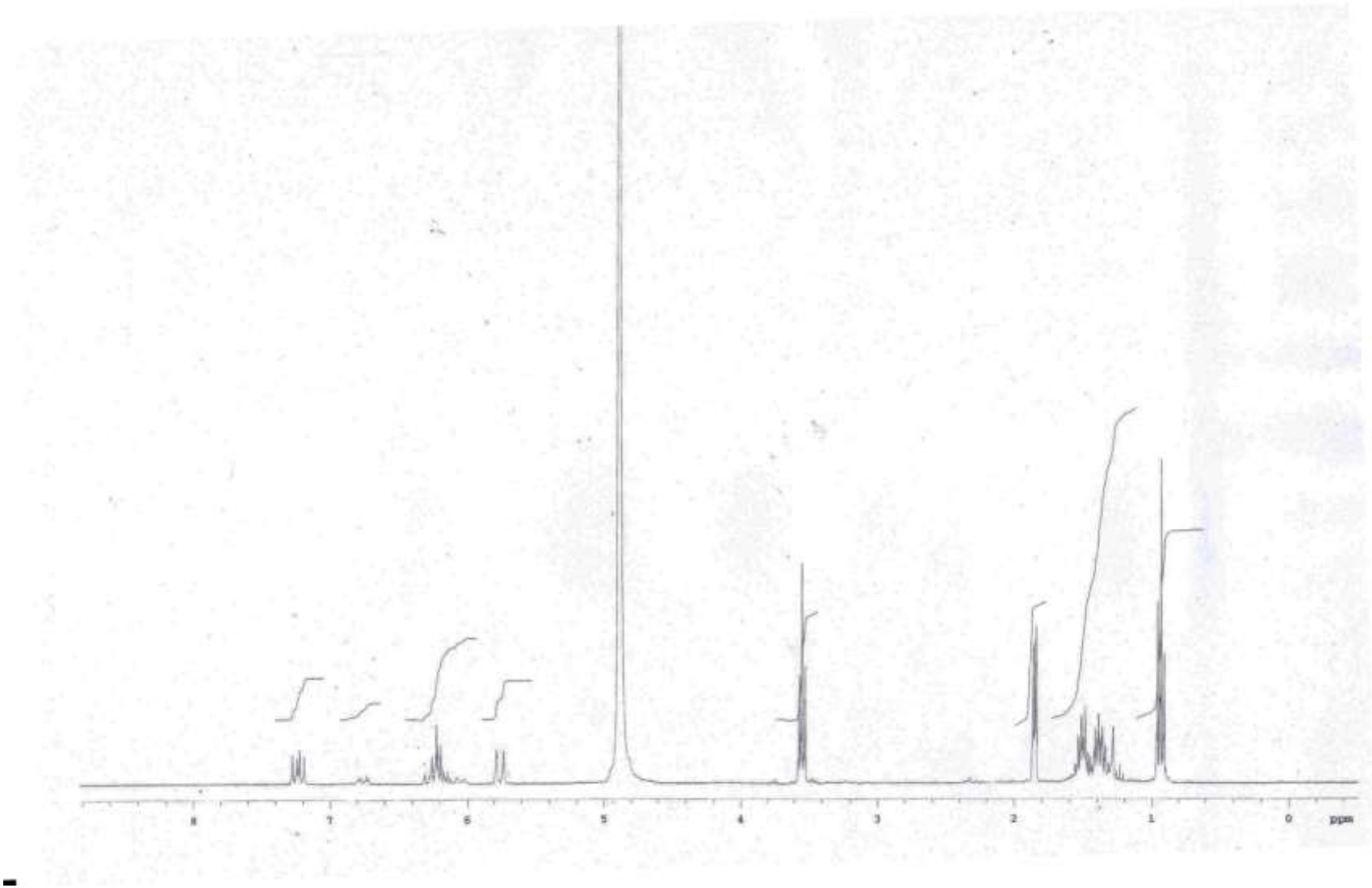
In these first tests, the liquid nature of 1-butanol made it possible to use it both as a reagent and as a solvent.

4.1.2.1 Synthesis of Butanol ester of Sorbic acid

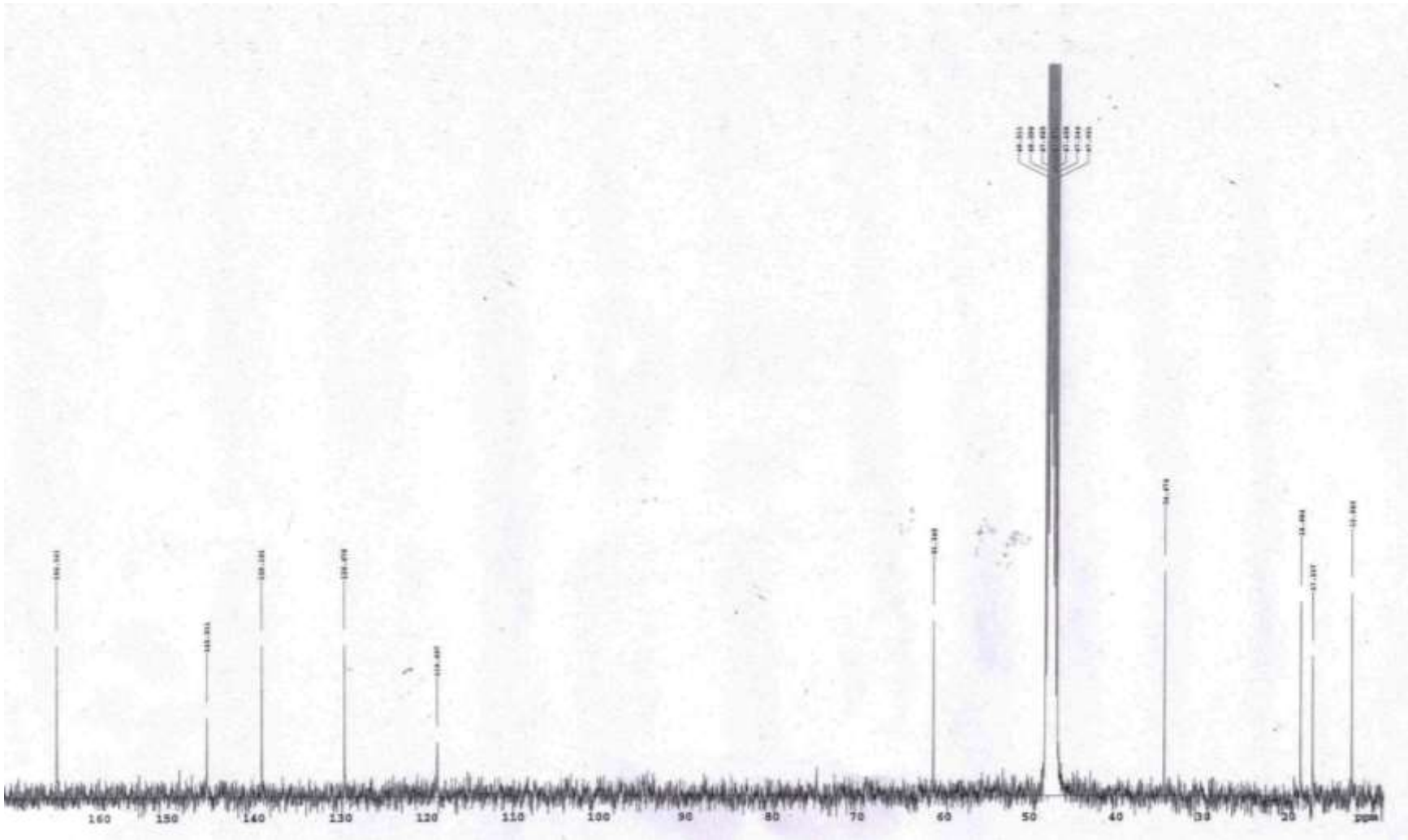
This section report results about the NMR (^{1}H-, **NMR 6** and ^{13}C-, **NMR 7**) characterization for the lipase-catalyzed production of butyl ester of sorbic acid as reported in **Scheme 12**.



Scheme 12: Monophasic esterification reaction between sorbic acid and 1-butanol catalyzed by CALB.



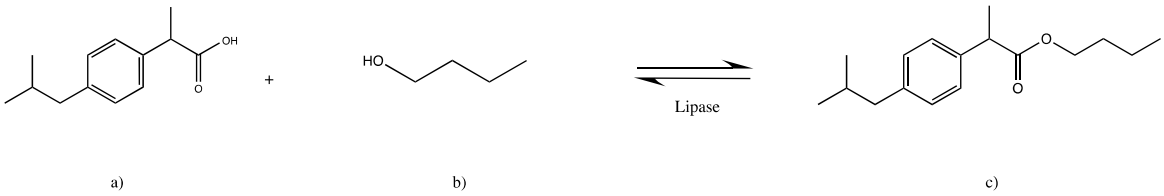
NMR 6: 1H-NMR spectra of sorbic acid butyl ester.



NMR 7: ^{13}C-NMR spectra of sorbic acid butyl ester.

4.1.2.2 Synthesis of Butanol ester of Ibuprofen

The synthesis of butyl ester has also been carried out effectively as regards the anti-inflammatory substrate ibuprofen (**Scheme 13**).



Scheme 13: Monophasic esterification reaction between ibuprofen and 1-butanol catalyzed by CALB.

The ^{1}H-NMR spectrum (**NMR 8**), as evidence of the esterification, is reported on the following page.

NMR 8: 1H-NMR of ibuprofen butyl ester.

4.2 ENZYMATIC ESTERIFICATIONS: *SOLVENT-ASSISTED* STRATEGIES

Although *solventless* esterification strategies, in particular the glycerol strategy, can be an interesting and promising technique for improving the solubility of food and pharmaceutical active ingredients (while also providing an excellent way to recycle glycerol resulting from biodiesel production) it does not it may be the only one.

In the *solventless* strategy, since the entire reaction medium consists of glycerol, there is a substantial imbalance in stoichiometric terms between alcohol and acid by favoring the esterification reaction, compared to hydrolysis, and thus limiting the effects of water are by-product of the reaction.

Anyway, in order to improve the solubility of the esterification products, glycerol is certainly an excellent starting point, but not sufficient. The increase in solubility allowed by the covalent bond of glycerol to the active ingredient of interest remains mostly limited to only the two additional hydroxyl groups that this short polyalcohol is able to provide once the bond has occurred.

The increase, in terms of solubility, allowed by glycerol, can be pushed further by the use of similar polyalcohols but by the greater number of hydroxyls. To operate this type of esterification, however, it is necessary to develop strategies which are no longer *solventless* but which are necessarily assisted by organic solvent.

The polyalcohols chosen as carriers of the hydroxyl group and therefore as moieties capable of increasing the hydrophilicity of the esters were, in increasing order of polarity: erythritol, xylitol, and sorbitol. Also, although it is not a polyalcohol, we found it interesting to produce an ester of ibuprofen with ascorbic acid, due to its intrinsic antioxidant activity.

So, in this section we will focus on lipase catalyzed esterifications that led to the synthesis of erythritol ester of ibuprofen, xylitol ester of ibuprofen, sorbitol ester of ibuprofen and ascorbic acid ester of ibuprofen.

Due to the solid nature of erythritol, xylitol, sorbitol, and ascorbic acid, the esterification reactions with these solid polyalcohols of increasing polarity has been carried out exploiting a *solvent-assisted* system.

In fact, the liquid nature of glycerol allowed the acid substrate to be solubilized directly in it. Instead, due to the solid nature of polyalcohols with a higher number of hydroxyls, it will be necessary to develop new strategies to bring the molecules of interest into solution.

There were developed two *solvent-assisted* strategies: monophasic and biphasic. by monophasic we mean a single organic solvent capable of solubilizing both acid and alcohol. By biphasic we refer to the condition in which two immiscible solvents create an environment in which the enzyme is positioned at the interface between the two solvents which will respectively solubilize one of the two substrates that will take part in the enzymatic reaction.

To produce esters between highly hydrophilic molecules, such as erythritol, xylitol, and sorbitol (logP: -1,86/-3,36) and lipophilic drugs, such as ibuprofen (logP: 3,75), literature reports biphasic organic solvent/water systems.

4.2.1 Synthesis of Ibuprofen esters: Monophasic media *solvent-assisted* strategy

Note: a patent application concerning the protocol discussed in this section was filed on 16/12/2020 with the name "Enzymatic process for the preparation of esters of poorly water-soluble carboxylic acids". The title page of the application is shown at page 289.

Ibuprofen is a poorly-water soluble drug. So, its esterification with hydrophilic compounds can achieve a synthesis molecule enhanced in its water solubility and consequent bioavailability. This strategy can be developed by a biocatalyzed esterification reaction.

In a traditional reaction environment, lipases undergo interfacial activation due to an opposite polarity between the enzyme (hydrophilic) and their substrates (lipophilic). The reaction occurs at the interface between the aqueous and the oil phases where a conformational change associated occurs and increase the enzymatic activity. Hence, interfaces are the key spots for lipase biocatalysis and an appropriate site for modulating lipolysis activity [33]. So, the use of an aqueous-organic solvent biphasic system can produce interfacial activation of the lipases. To enhance the surface of the interface, vigorous mixing of the two phases forms a suspension with a significantly large interfacial area. However, the biphasic environment can often also represent a great disadvantage. In fact, the presence of water inevitably leads to the hydrolysis reaction of the newly ester products due to the stoichiometric equilibrium as can be deduced by the generic lipase catalyzed esterification reaction between a carboxylic acid and an alcohol (**Scheme 7**).

In the light of the above, it is therefore apparent the need to provide a process for the enzymatic esterification of poorly water-soluble carboxylic acids that overcomes the disadvantages of known processes.

Therefore, we aimed to develop a protocol that could limit the disadvantages of enzymatic synthesis in a biphasic system consisting of two solvents that are immiscible to each other. This stategy can lead to an enzymatic-catalyzed formation of the ester bound between the carboxylic acid of a target molecule and the hydroxyl group of a hydroxyl group-carrying molecule such as an alcohol, also in solid form.

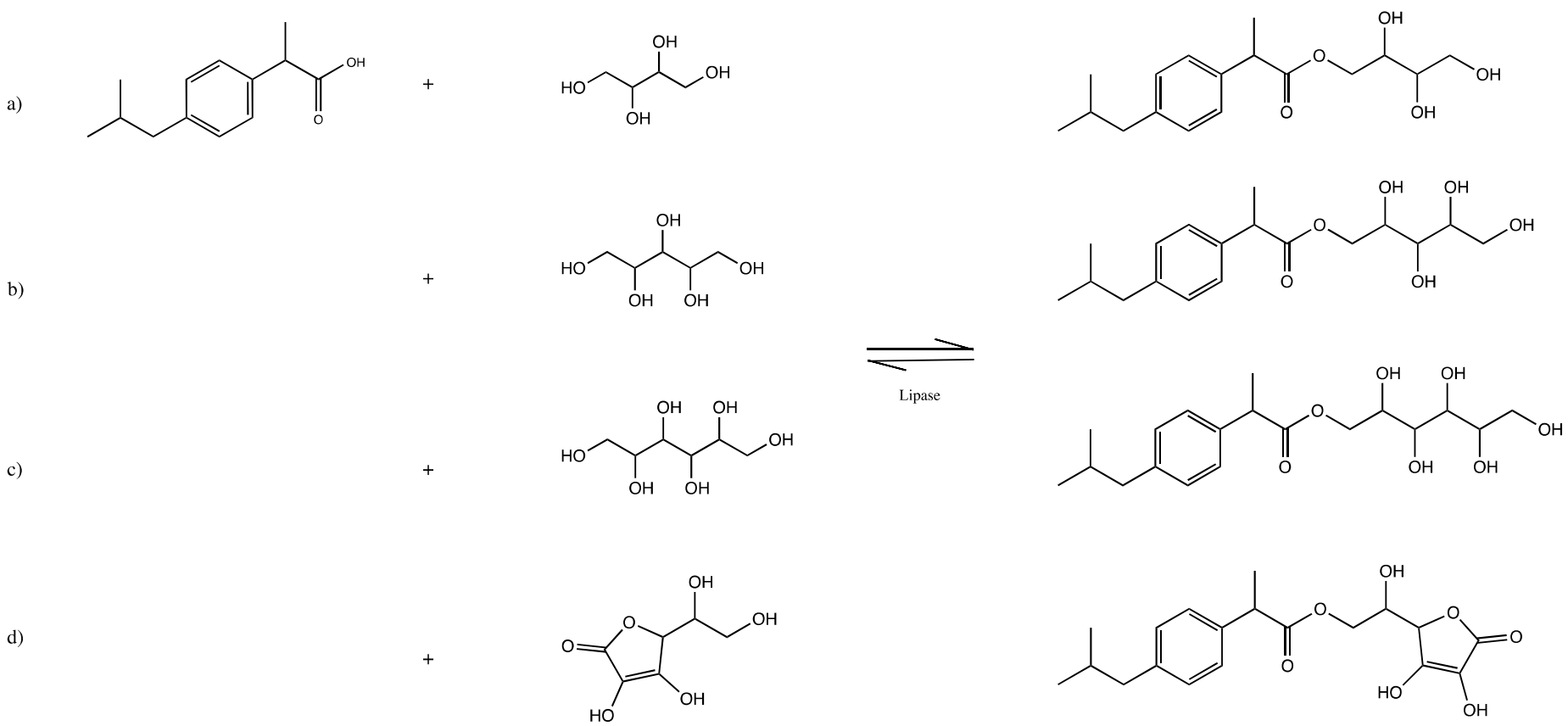
In this section, is reported a process that overcomes the limitations of current biphasic enzymatic esterification strategies. In fact, numerous parameters must be managed in a two-phase system and the presence of water severely limits the possibilities in terms of esterification yield.

In this reaction environment, the solvent (t-amyl alcohol) acts to solubilized both the ibuprofen and the hydroxyl group-carrying molecule in a monophasic system. High temperature and vigorous mixing can help the polyalcohol solubilization into the solvent such as t-amyl alcohol. Ibuprofen can be added for its solubilization (from 100 di 300 mg in 5 ml of t-amyl alcohol). Then, the solvent is allowed to cool in order to avoid the thermal degradation of the enzymatic catalyst. The enzyme can be loaded. Molecular sieves can be added to the solvent in order to get rid of the water as by-product of the formation of the ester bound (range from 50 to 100 mg in 5 ml of t-amyl alcohol). Less water in the system lead to easier formation of the ester bound as shown in the equilibrium reaction reported above. At 180 RPM, after 24h, or even less, ester is formed and is purifiable from the reaction.

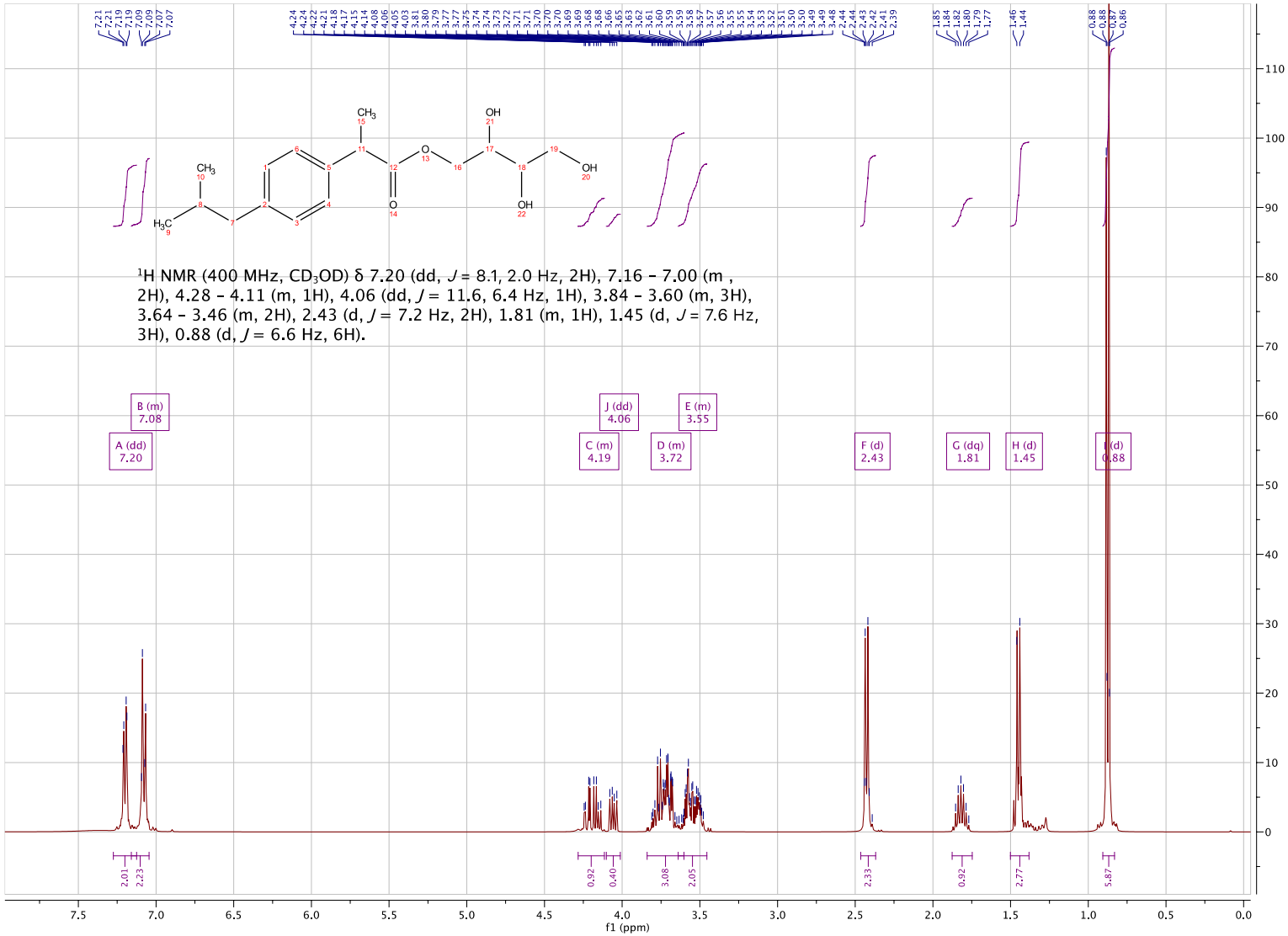
The reactions carried out according to this protocol allowed the enzymatic synthesis of the esters: Ibuprofen erythritol ester, Ibuprofen xylitol ester, Ibuprofen sorbitol ester, Ascorbic acid ester of ibuprofen (**Scheme 14**).

Similarly to what was reported for the previous reactions, the operational workflow regarding reaction monitoring, purifications and characterizations of the esters involved the use of: TLC, column chromatography, NMR, IR, and uHPLC-MS.

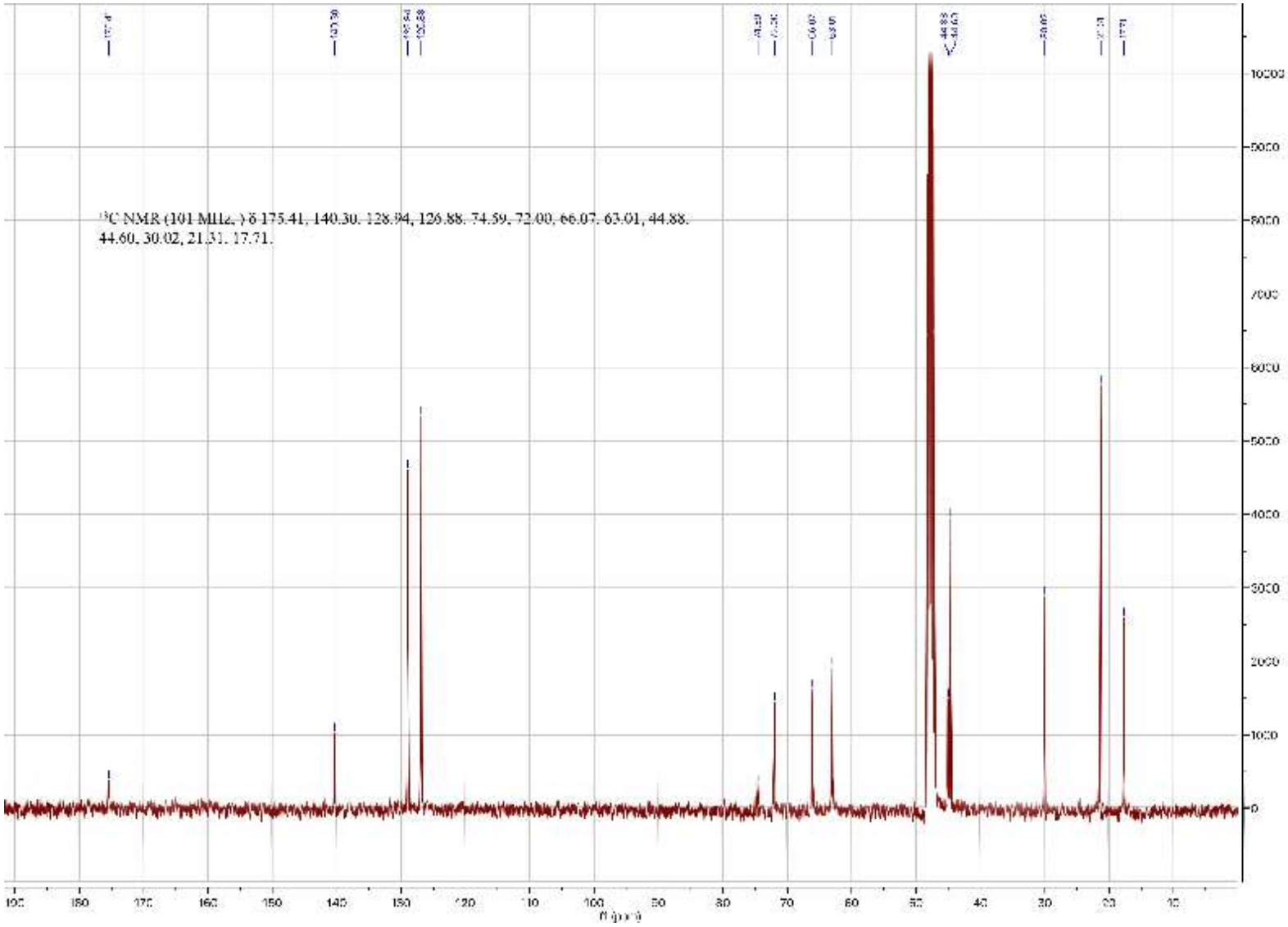
In the following pages the NMR and IR spectra of the ibuprofen esters obtained by this protocol are reported.



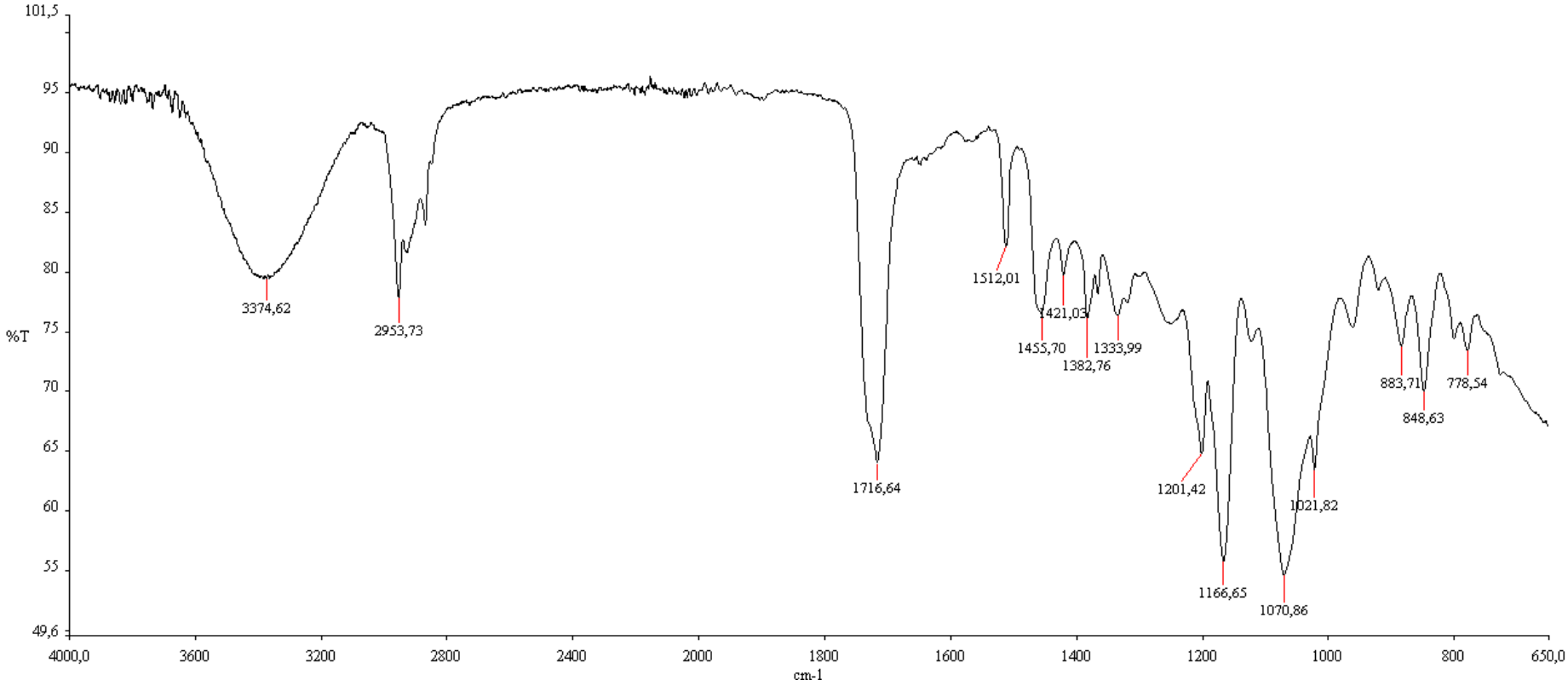
Scheme 14: Esterification reaction of Ibuprofen with: (a) erythritol, (b) xyliyol, (c) sorbitol, and (d) ascorbic acid, catalyzed by Novozym 435.



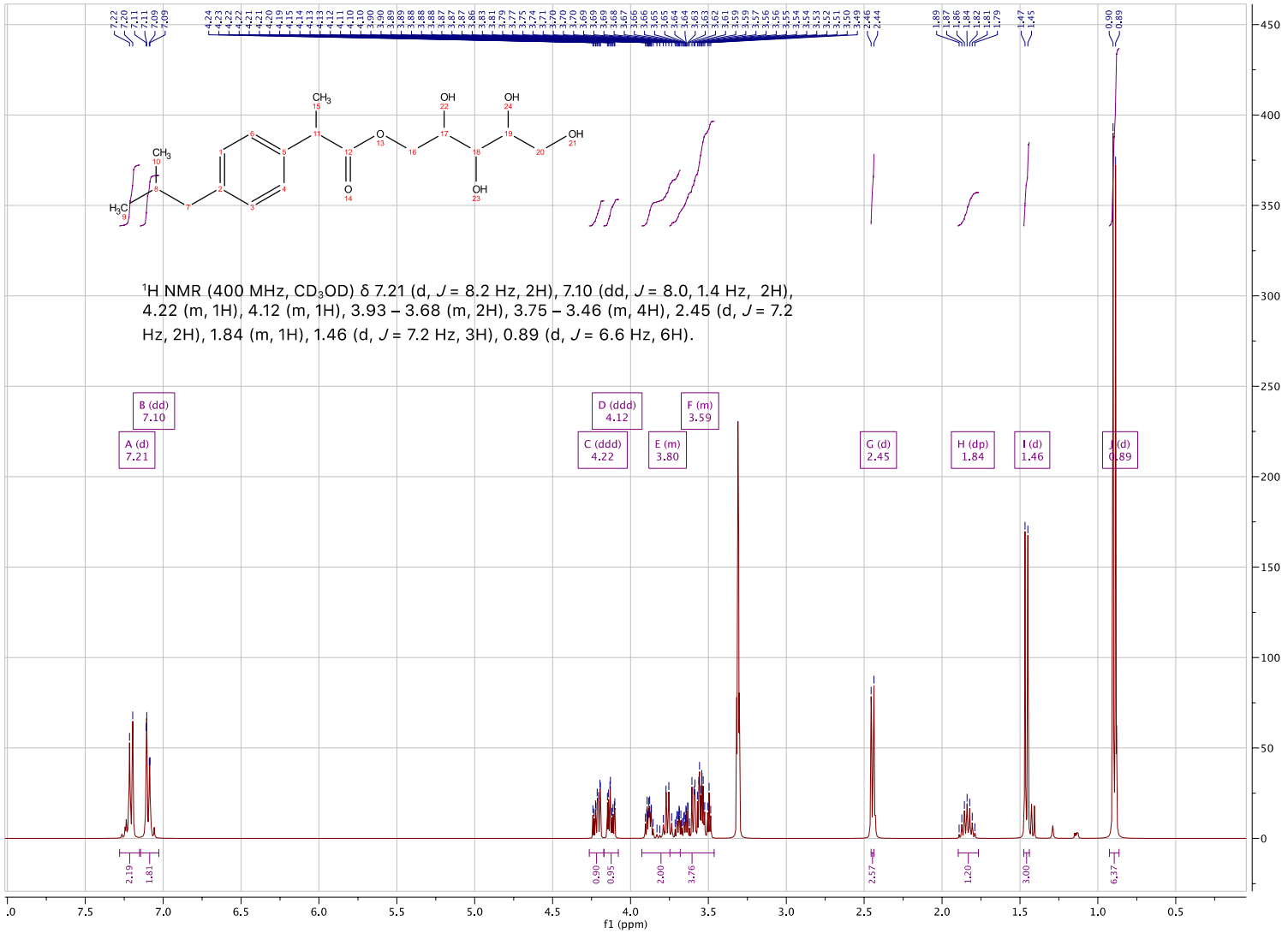
NMR 9: ^{1}H-NMR of Ibuprofen erythritol-ester.



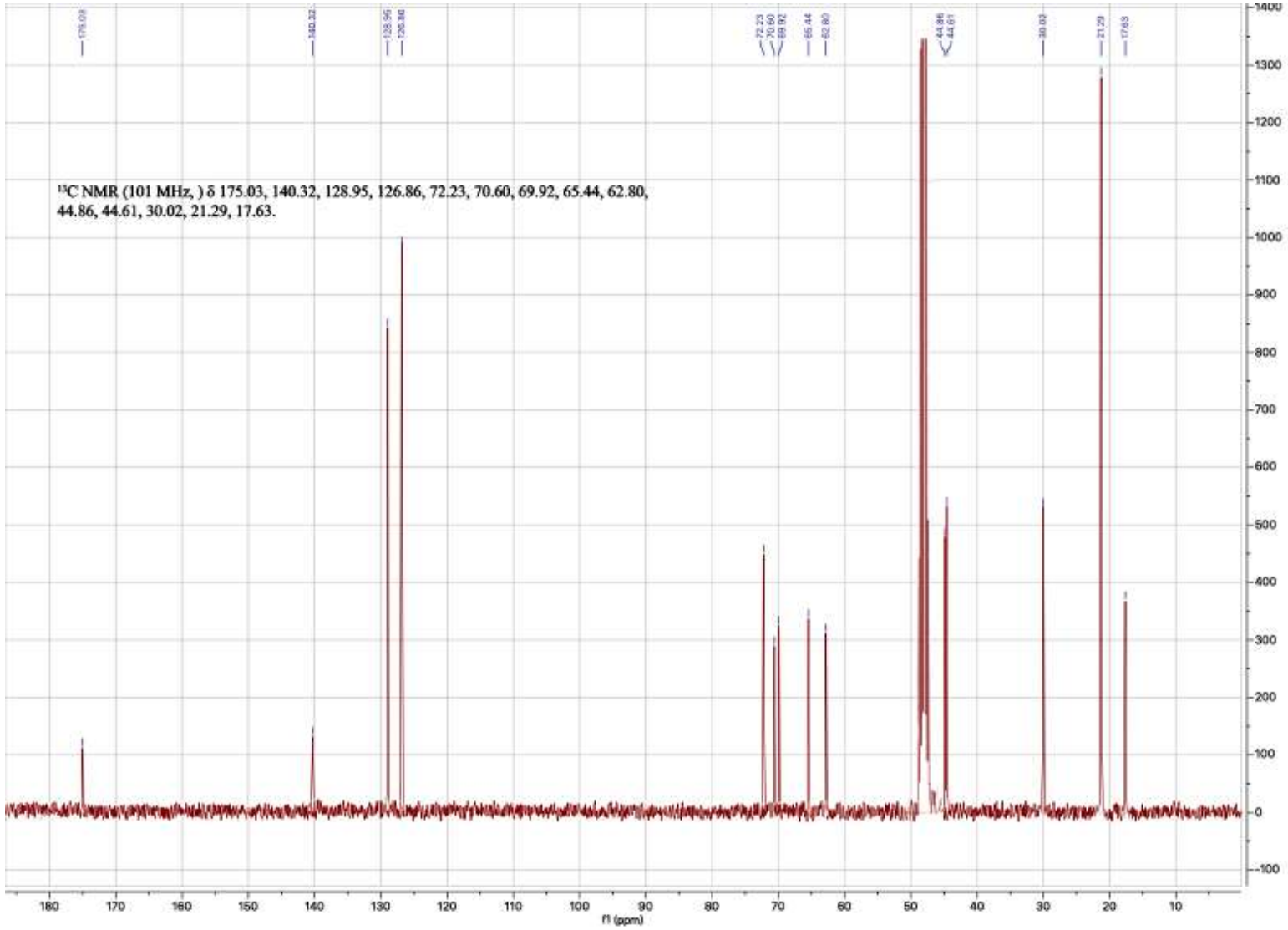
NMR 10: ^{13}C-NMR of Ibuprofen erythritol-ester.



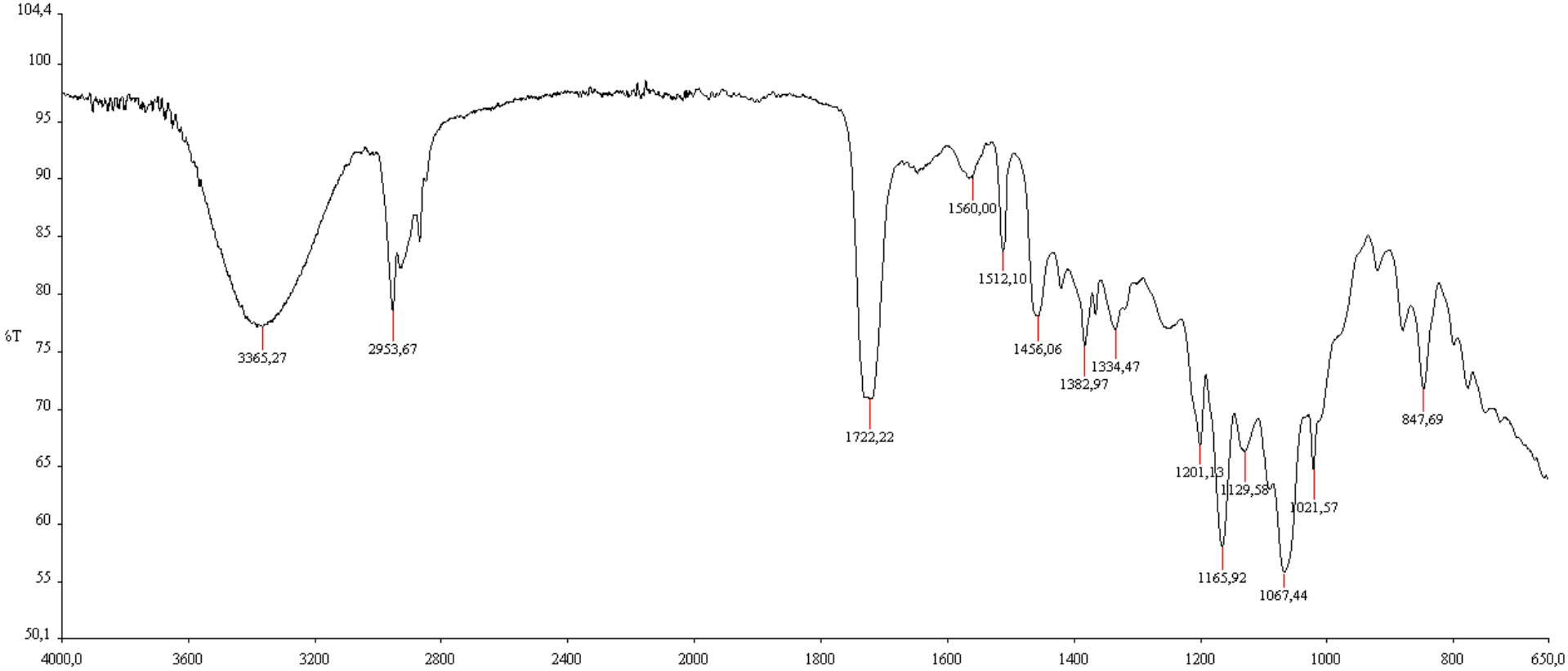
IR 3: Ibuprofen erythritol-ester.



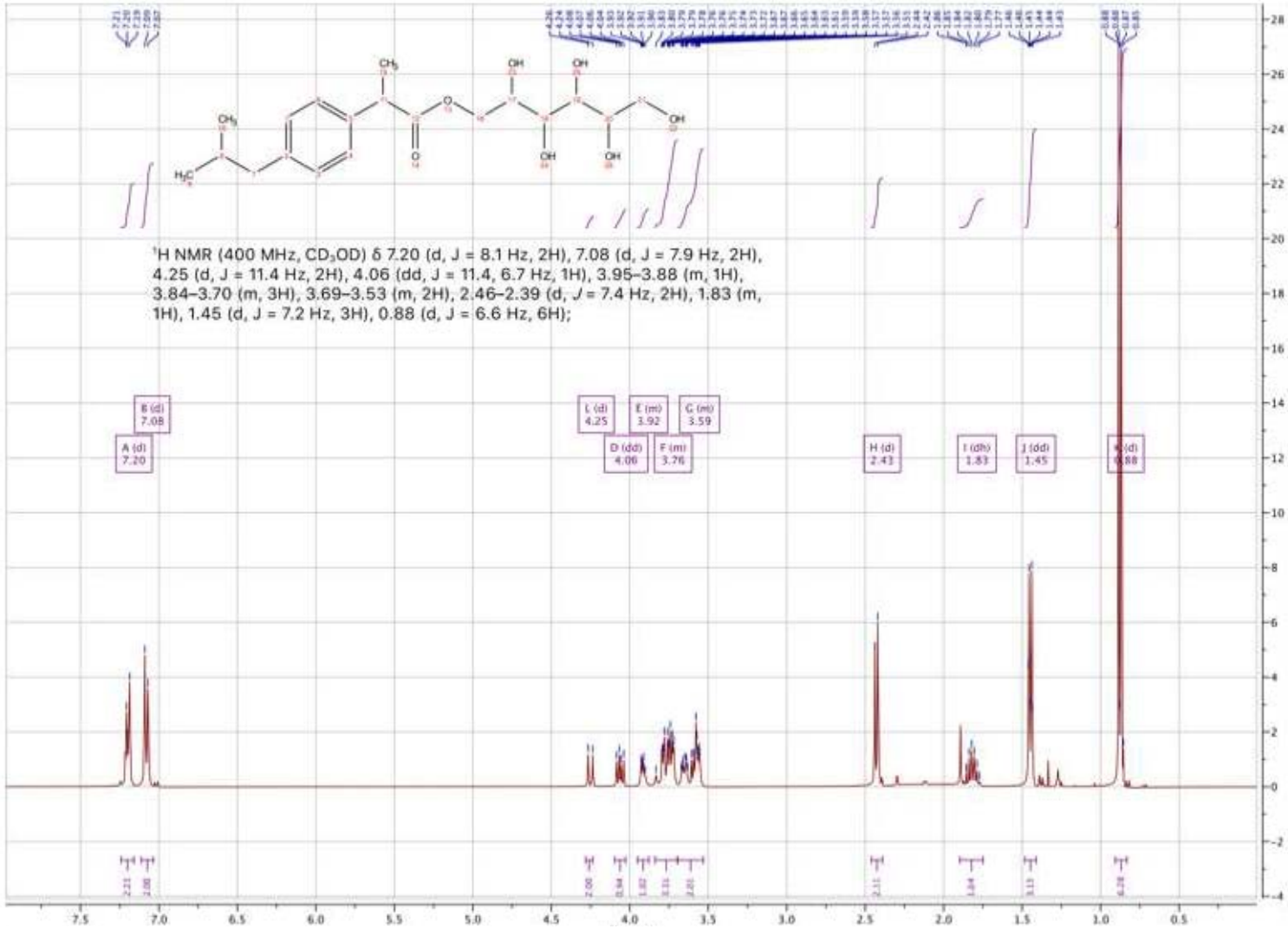
NMR 11: ^{1}H-NMR of Ibuprofen xylitol-ester.



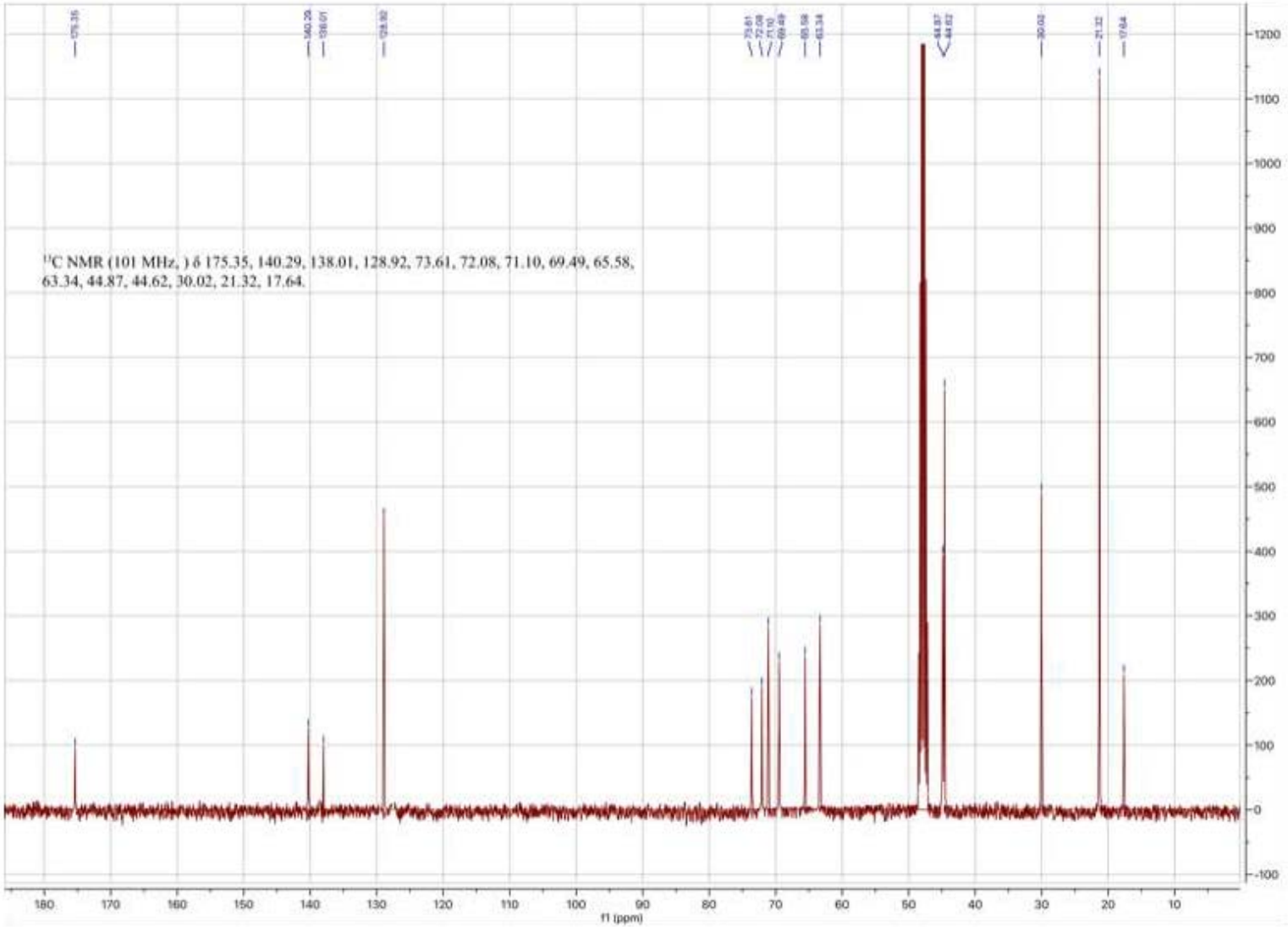
NMR 12: ^{13}C-NMR of Ibuprofen xylitol-ester.



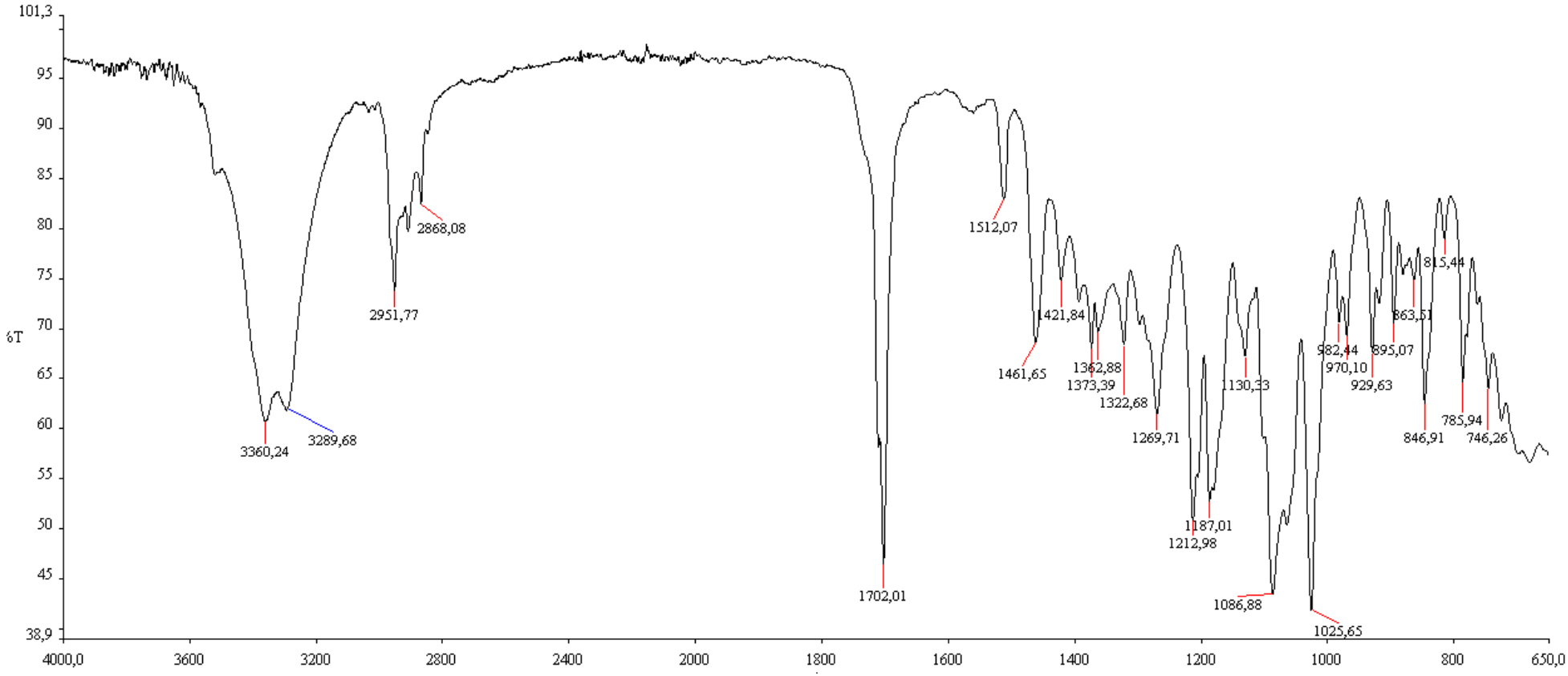
IR 4: Ibuprofen xylitol-ester.



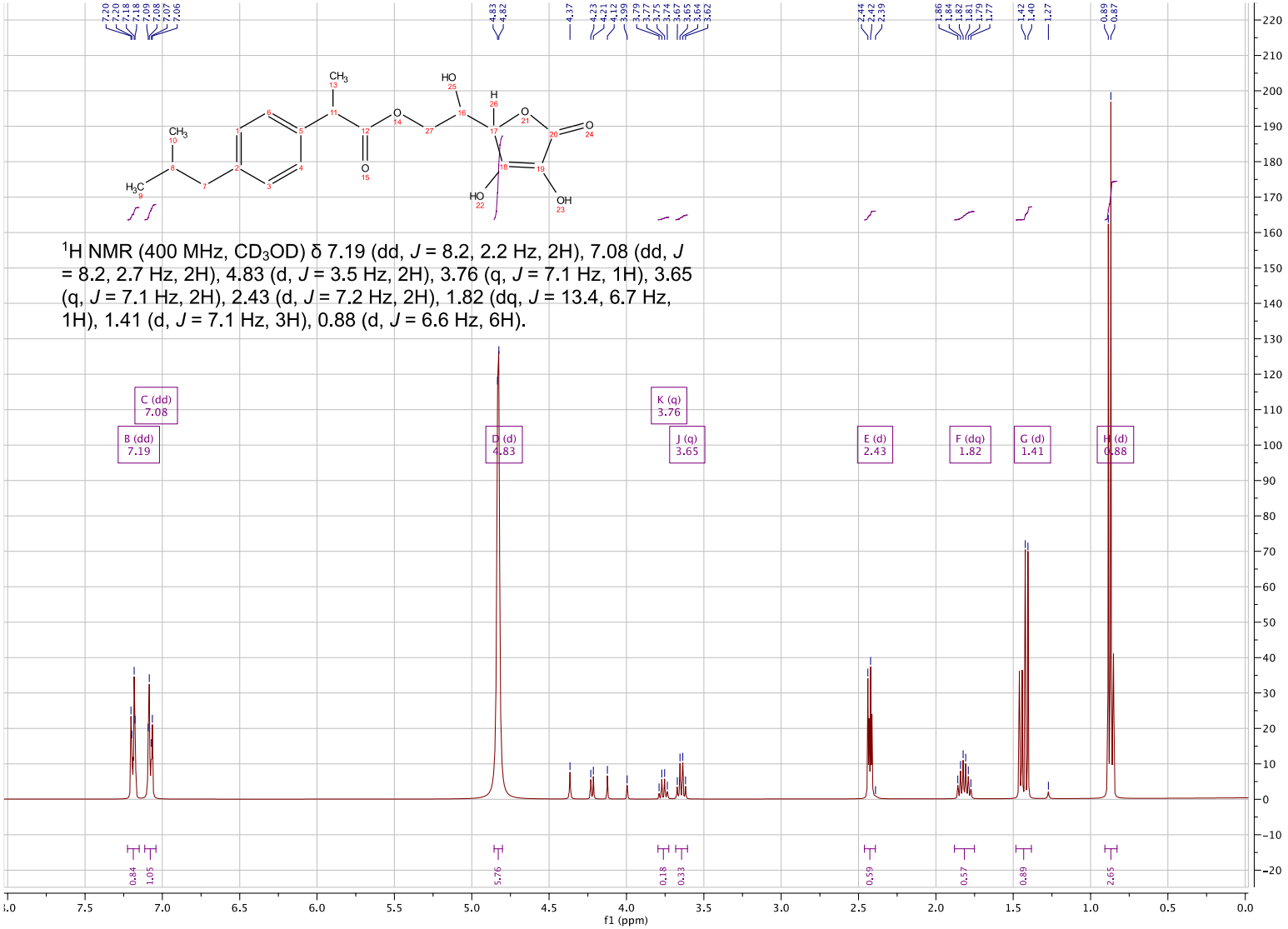
NMR 13: ^{1}H-NMR of Ibuprofen sorbitol-ester.



NMR 14: ^{13}C-NMR of Ibuprofen sorbitol-ester.



IR 5: Ibuprofen sorbitol-ester.



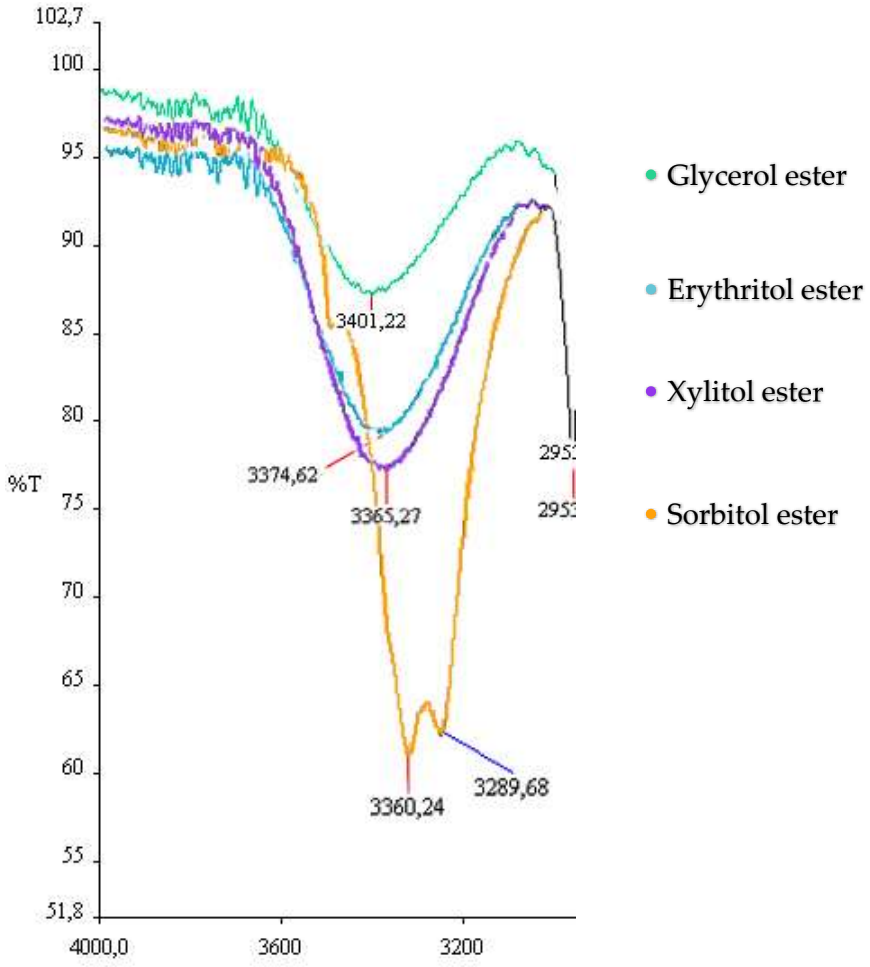
NMR 15: ^{1}H-NMR of ascorbic acid ester of ibuprofen.

All ^{1}H-NMR spectra confirmed the expected structure. The characteristic peaks of ibuprofen, attributable to the aromatic ring (zone 7-7.5 ppm) and to two methyls (0.85 ppm), are always clearly present. Moreover, it can be noted that the signals in the center of the spectrum, in particular in the 3.5-4.5 ppm zone, follow the trend relating to the progressive increase in the length of polyalcohols with increasing polarity: erythritol, xylitol, sorbitol.

The ^{13}C-NMR spectra confirm the expected structure. Interestingly, the two isobutyl methyls are chemically equivalent and therefore both fall to 21.3 ppm. The four unsubstituted carbons of the parasubstituted aromatic ring also fall with the same behavior in zone 128.9 ppm.

To discuss the IR spectra related to the esters of ibuprofen with glycerol, erythritol, xylitol and sorbitol shown on the previous pages, **IR 6** shows a merge of the measured trarmittance percentages and refers to the zone of the spectrum

characteristic of the absorbance produced by the hydroxyl compartment of the molecule under consideration (3000 to 3600 cm $^{-1}$).



IR 6: Merge betweent the IR spectra of ibuprofen esters (hydroxyl zone).

As we can see, with the progressive increase of the hydroxyl groups of the molecule, this results in a higher absorbance and consequently the percentage of transmittance decreases progressively from the glycerol ester towards the sorbitol ester.

Furthermore, the ibuprofen ester with sorbitol shows a signal splitting (we refer to positions 3360.24 and 3289.68 cm^{-1}) which can be attributed to one intramolecular hydrogen bonds between an alkyl hydroxyl and the hydrogen of another hydroxyl. In this case, therefore, the absorption wave doubles because the instrument detects hydroxyls with different behavior. A signal will be determined by alcohol proper,

the second by the alcohol which has producted the hydrogen bond and which therefore generates the splitting at a lower frequency.

The areas of the spectrum around 1700 cm^{-1} and 1380 to 1450 cm^{-1} are characteristic signals attributable respectively to the ester bond carbonyl and the methyl esters of ibuprofen.

We proceeded with the mass spectrophotometric analyzes by uHPLC-MS. The spectra obtained are shown on the following pages.

Ibuprofen erythritol ester: **Figure 51**

Ibuprofen xylitol ester: **Figure 52**

Ibuprofen sorbitol ester: **Figure 53**

Ibuprofen ascorbic acid ester: **Figure 54**

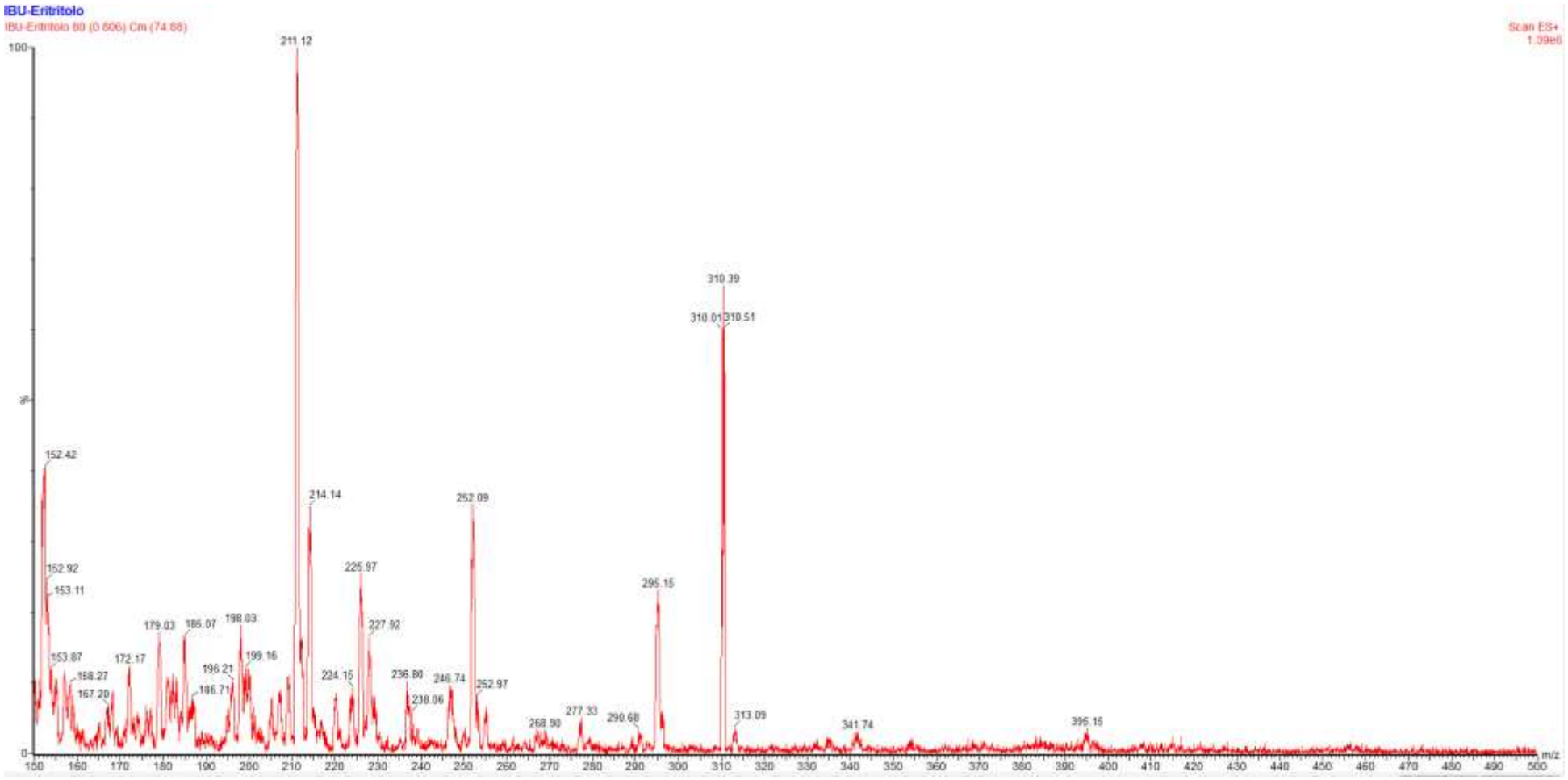


Figure 51: MS spectroscopy detection of Ibuprofen erythritol ester.

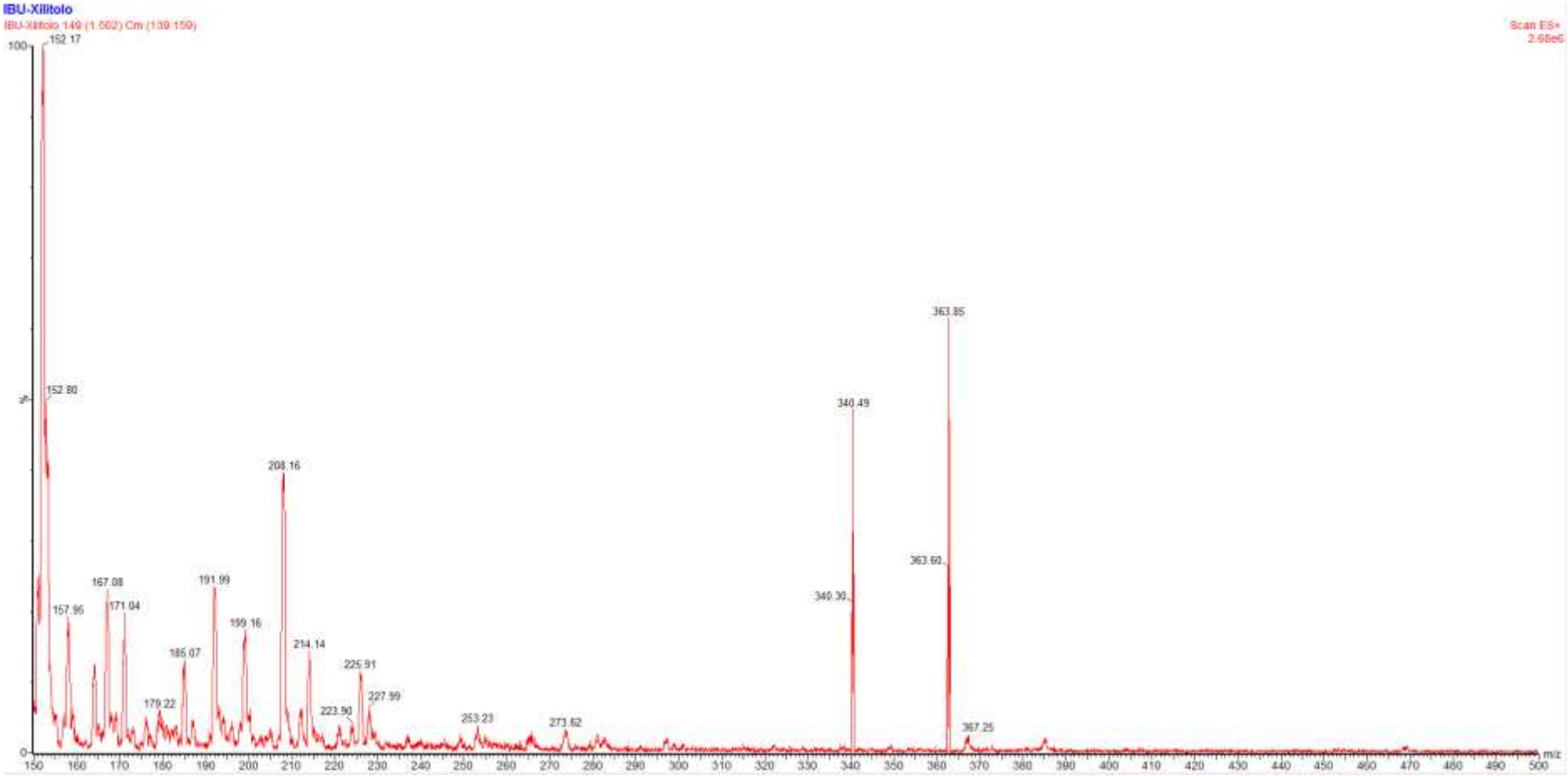


Figure 52: MS spectroscopy detection of Ibuprofen xylitol ester.

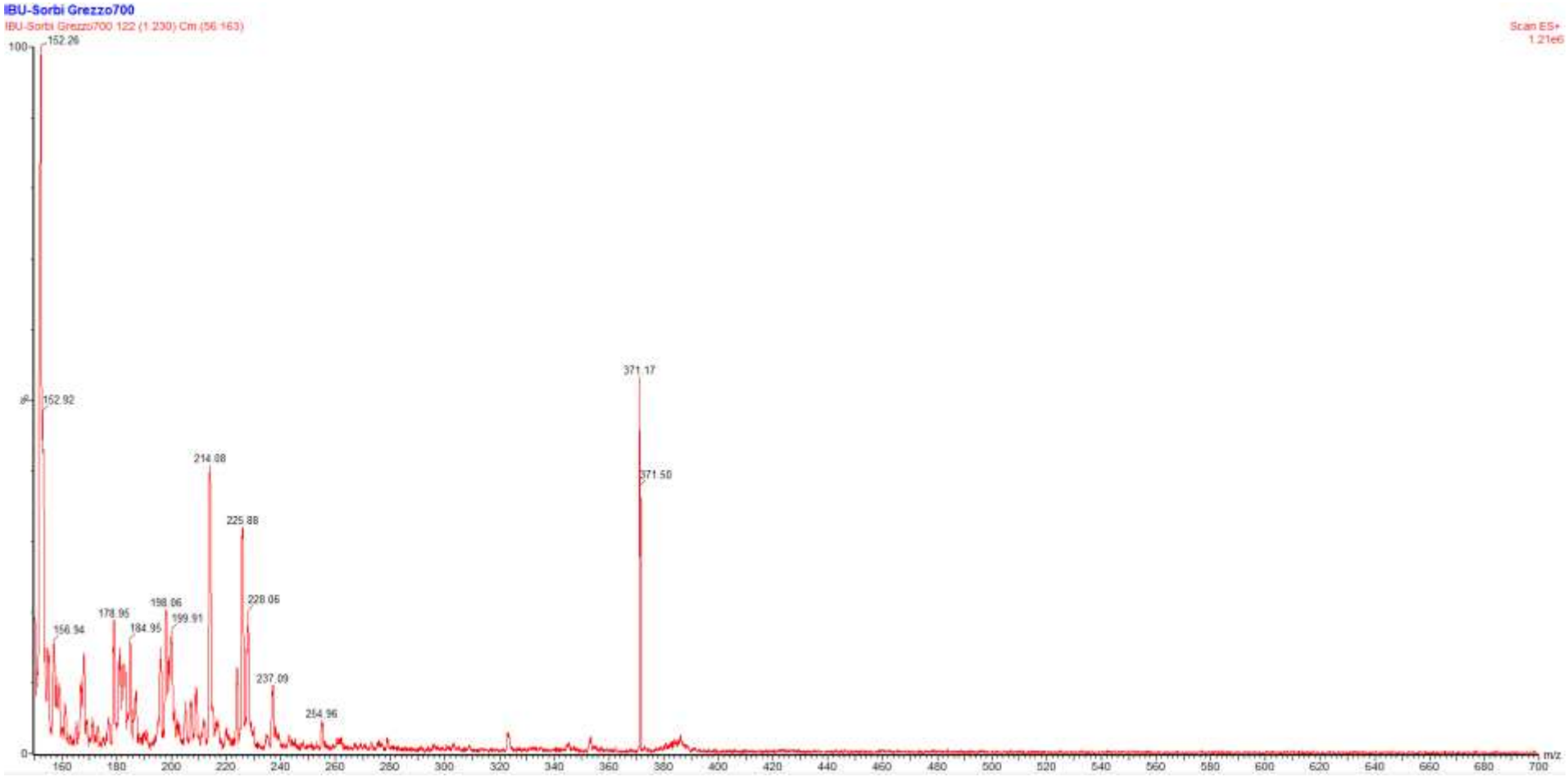


Figure 53: MS spectroscopy detection of Ibuprofen sorbitol ester.

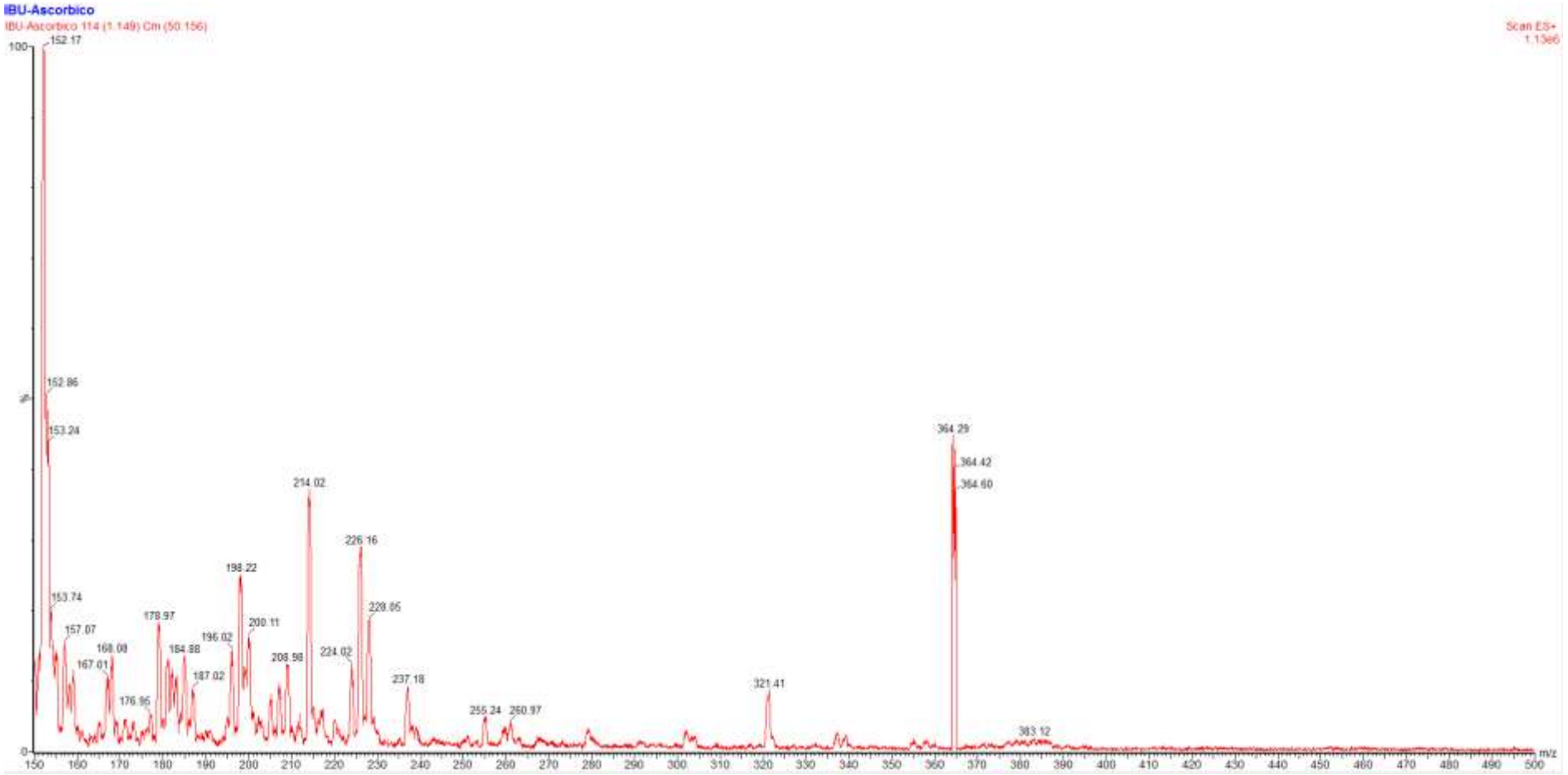


Figure 54: MS spectroscopy detection of Ibuprofen ascorbic acid ester.

The mass spectrometer associated with ultra-high performance liquid chromatography confirmed the expected masses attributable to the esters of ibuprofen with the progressive length polyalcool. The expected and obtained masses, also specifying any sodium and hydrogen adducts, are shown in **Table 14.**

Table 14: Mass analysis of ibuprofen esters with erythritol, xylitol, sorbitol and ascorbic acid.

Ester	Expected mass	Mass obtained	+ Na	+ H
Ibuprofen erythritol-ester	310.39	310.39		
Ibuprofen xylitol-ester	340.42	340.49	363.85	
Ibuprofen sorbitol-ester	370.44			371.17
Ibuprofen ascorbic acid-ester	364.39	364.29		

The mass spectrophotometry detection revealed expected mass of m/z 310.39 of the ibuprofen erythritol-ester. The mass relative to xylitol ester of ibuprofen (m/z 340.42) was also detected for its sodium adduct. Ibuprofen sorbitol-ester was detected as a hydrogen adduct at a mass of 371.17 m/z. Finally, the ibuprofen ester with ascorbic acid resulted in a mass of 36.429 m/z.

The advantages of the process of the present protocol are, for example: better solubility of substrates and product, shifting of thermodynamic equilibria (i.e. synthesis takes place instead of hydrolysis), possible use of the enzyme directly in an enzymatic step of a chemical process, and solubilization of hydrophilic and lipophilic compounds in the same organic phase.

The monophasic system according to the present protocol is greatly easier in terms of reaction-management compared to the biphasic W/O system. Therefore, much less variables have to be considered in comparison to the variables in biphasic

systems. In the process of the present protocol water is not required, and this shifts the hydrolysis/synthesis equilibrium towards the synthetic behavior of the lipase. The use of a commercially available immobilized enzyme lead to highly reproducible organic synthesis with highly stereo- and regio- specificity. Furthermore, the immobilized enzyme is stable and perfectly separable from the medium in order to be reutilized.

A suitable enzyme for the process of the present protocol is CALB. Since CALB lacks in lid covering the active site, it does not need any interfacial activation, therefore, it can be used in a monophasic system like the one of the present protocol. Other suitable lipases are Lipases from *Thermomyces ianuginosa* and *Rhizomucor miehei,* which are considered similar to CALB in terms of catalytic capacity for different substates. In addition, no toxic solvents are used.

Unlike many enzymatic syntheses reported in the literature, which require reaction times of even 1 week, the process according to the present invention allows a conversion yield that ranges from 65 to 100% after only 24 hours. Reaction-volumes are small: the esterification can be achieved also in reactors or microreactors of 2-5 ml. This small-scale process can be easily scaled up. The process of the present protocol advantageously provides a way to solubilize two substrates, i.e. the carboxylic acid and the hydroxyl group-carrying molecule such as a polyalcohol, in a single organic solvent.

It is important to note that not all the organic solvents can lead to this kind of esterification process. Indeed, the right solvent choice is crucial. Although several solvents were used in enzymatic synthesis, no one are suitable for the process of the present invention. **Table 15** shows the organic solvents tested in attempt to produce the ibuprofen stereos in a monophasic system.

Table 15: Tested solvents attempting the monophasic esterification of ibuprofen and polyalcohols.

Tested solvents
Acetone
Acetonitrile
Benzene
Cyclohexane
Diethyl Ether
Dimethyl Carbonate
Dimethylformamide
Hexane
Ethyl Acetate
Isoamyl Alcohol
Methanol
Heptane
Pyridine
Tetrahydrofuran
Toluene

However, it has also been reported that activity might be affected by the solvent without cor- relation to the logP [282][283]. The literature reports how apolar organic solvents (down to logP 2) can strip away the water layer that allows the right enzyme conformation, thus proving less suitable solution for biocatalytic purposes [284]. Much polar solvents can't solubilize the carboxylic acid such as ibuprofen. DMSO deactivates the enzyme. Some organic solvent (such as methanol) are alcohols so small that interacts with the active site of the enzyme, inactivating it. Other long-chain alcohol (for example: octanol) can take part in the reaction as substrate. Furthermore, none of the common organic solvents, which have been tested, can solubilize both the carboxylic acid such as ibuprofen and the polyalcohol

(log P from: +3,75 to -3,36). For this reason, in our opinion, the success of our esterification experiments in t-amyl alcohol is due to the tertiary nature of this alcohol which is able to solubilize both substrates but does not interact with the active site of the enzyme. part of the reaction and not causing inactivation of the biological catalyst.

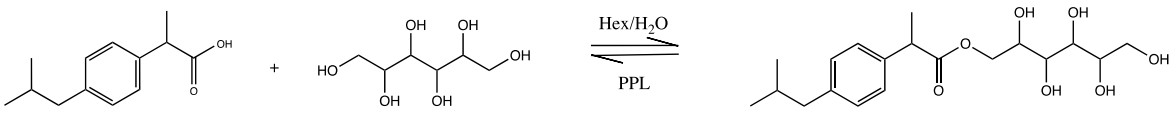
The developed synthesis protocol can be used for economic, and simple scale up synthesis, for the production of highly pure and selective pharmaceutical esters. The three chemicals, synthesized with the protocol in question, can find application as new non-steroidal anti-inflammatory drugs for the treatment of pain (headache, dysmenorrhea, dental pain) up to musculoskeletal disorders such as osteoarthritis, and rheumatoid arthritis. This technique can be exploited by different industries, from pharmaceutical to food industries. This method can easily achieve to solubility enhanced prodrugs, preservatives of or flavor esters.

4.2.2 Synthesis of Ibuprofen Sorbitol ester: Biphasic *solvent-assisted* strategy

Note: this work has been published as "Biocatalytic approach for direct esterification of ibuprofen with sorbitol in biphasic media", 17 Mar 2021, *International Journal of Molecular Sciences*. The title page of the article is visible art page 290.

The poor water-solubility of ibuprofen characterizes its dissolution-limited oral bi-oavailability and this can cause problems of drug tolerability [16]. For the covalent attach of hydrophilic functions to this poorly soluble active ingredient, as a possible strategy for improving its solubility, we focused on lipase-catalyzed esterification of ibuprofen with sorbitol.

The aim of this experiments have been to develop a protocol in order to obtain an effective lipase-catalyzed esterification of ibuprofen with sorbitol using free Porcine Pancreas Lipase (PPL) in biphasic media (**Scheme 15**).



Scheme 15: Lipase catalyzed esterification of ibuprofen and sorbitol with PPL in a biphasic environment; Hex/H_2O: solvents, hexane/water; PPL: porcine pancreas lipase.

Indeed, althrough some lipases (such as *Candida antarctica* lipase B) have shown catalytic activity without interfacial activation [266], PPL needs acquous-organic biphasic media interface to catalyze the reverse reaction of synthesis [285][286]. Most lipases, such as PPL, have an α-helical fragment (termed the "lid") that covers the active site. Conformational change of PPL at the oil–water interface can open the lid producing lipase activation [287]. In addition, water molecules on the enzyme surface has been described as a molecular lubricant of enzymes [63] and this increase its internal flexilility facilitating the movements necessary for catalysis [288]. Furthermore, in a biphasic W/O condition, highly hydrophilic compounds such as sorbitol are solubilized by the water phase, allowing to amplify the spectrum of organic solvents that can be used as a means for enzymatic catalysis (having to solubilize only the ibuprofen and not both reaction substrates).

Althorugh immobilized lipases have been shown to display better catalytic activity compared to the non-immobilized lipases in non-aqueous media, we decided to exploit and test the less expensive free form of porcine pancreatic lipase (PPL).

For this purpose, several factors were considered to establish the operating conditions: the selection of the organic solvent, enzyme concentration, water

content, temperature, stirring speed, molar ratio, and reaction time. The conversion yield of the substrate was studied to evaluate the effect of changes in the parameters of the enzymatic reaction.

The reference standard of the sorbitol ester of ibuprofen was chemically synthesized. Subsequently, enzymatic synthesis of IBU-sorbitol ester prodrug was achieved by operating under mild reaction conditions. The purified product has been analyzed using NMR and HPTLC-MS.

A ratio of 5:1—solvent/water (water: pH 7; finale volume: 15 mL)—was used to synthesize ester with 5 g L−1 of free PPL, along with an acid/alcohol molar ratio of 5:1. Reactions were carried out in screw-capped 20 mL vials immersed in a glycerin bath placed on a magnetic stirrer equipped with a temperature probe capable of self-regulating the temperature of the heating plate to maintain the temperature constant over time (35 ◦C and 400 rpm). After 24 h (unless otherwise indicated) of reaction time, the catalyzer was filtered out and the biphasic media was dried with a first rotatory evaporator step (RE-121; Buchi s.r.l–Cornaredo, Italy) and final lyophilization.

Chemical Synthesis of the IBU-Sorbitol Ester

Since information on the target compound is not commercially available except for the unknown degree of purity, it was synthesized chemically according to a procedure in the literature [69]. The thusly obtained compound was utilized as a reference compound to allow calibration. The mass spectrometry testifying to the esterification of IBU-sorbitol is reported in the **Figure 55**.

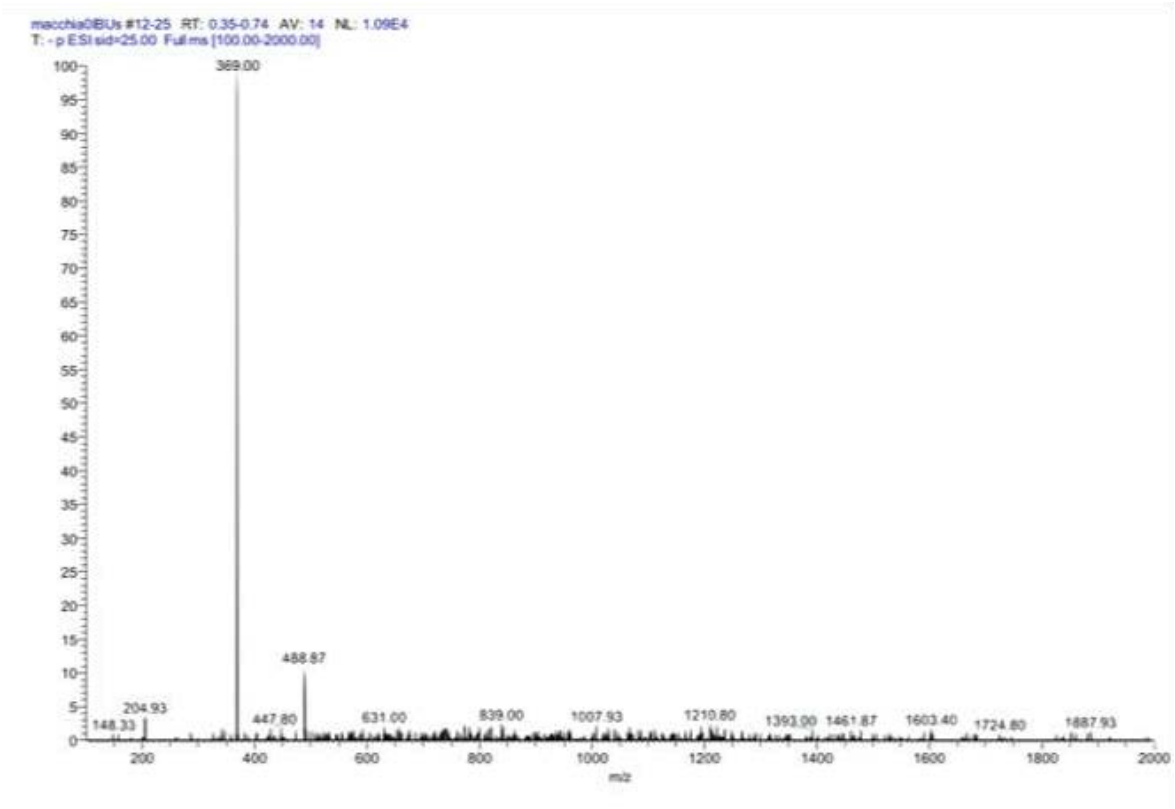


Figure 55: HPTLC-mass spectra in negative ionization mode of the chemically synthesized IBU-sorbitol ester (m/z 369).

Preliminary experiment: Selection of suitable organic solvent

The ability of the free PPL to catalyze the synthesis of IBU-sorbitol ester was investigated starting from the selection of the suitable organic solvent.

Previously, polyalcohol glycerol was chosen as a hydroxyl groups-carrier molecule to increase the polarity of the resulting ester and thus increase its bioavailability [92]. Ibuprofen was esterified with glycerol in a system defined as solventless [82].

In fact, due to its liquid nature, glycerol can act as both a solvent and a reagent. Despite this, the increases in solubility resulting from the covalent attachment of glycerol to poorly soluble water-soluble active ingredients are strongly limited by the only two additional hydroxyls that this polyalcohol can provide following the ester bond. Therefore, to maximize the hydrophilizing effect of adding a polyalcohol to ibuprofen, we decided to choose sorbitol by virtue of its six brought hydroxyls, against the three of glycerol. Although according to this thesis, sorbitol is a better hydrophilic agent than glycerol, its solid nature requires its solubilization in an aqueous medium, thus not being able to exploit solvent-free systems such as those reported for glycerol.

So, this lipase-catalyzed esterification needs a biphasic organic solvent/water media but not only due to the solid nature of sorbitol. Indeed, although some lipases (such as *Candida antarctica* lipase B) have shown catalytic activity without interfacial activation [266], PPL needs an aqueous-organic solvent interface to catalyze the reverse reaction of synthesis [72, 73]. Most lipases, such as PPL, have an α-helical fragment (termed the "lid") that covers the active site. Conformational change of PPL at the oil–water interface can open the lid producing lipase activation [287].

In the biphasic system reported in the present work. The organic solvent will have a solubilizing role towards the lipophilic compound, ibuprofen, and the water will solubilize the sorbitol polyalcohol. However, finding an adequate organic solvent can be a challenge. Indeed, it's generally reported that solvents with logP < 2 are less suitable for a biocatalytic purpose [284] greatly influencing enzyme activity and substrate specificity [289]. The polarity of organic solvents affects the amount of water in the aqueous layer around the catalyst where the enzymatic reaction occurs influencing its conversion yield [290]. So, based on the logP of these 3 solvents we have chosen to test hexane, benzene, and toluene as organic solvents. Ibuprofen, due to its strong lipophilicity, proved to be highly soluble in all three organic

solvents tested. Furthermore, Ravelo et al reported the solubility test of ibuprofen in different solvents [291].

LogP is an extensively used parameter to express the polarity of a solvent (or compound) and its possible effect on the enzyme activity in lipase-catalyzed esterification [292]. To determine the effect of the reaction medium on the esterification, IBU-sorbitol ester was synthesized with PPL in these three solvents monitoring the reaction by TLC and calculating the conversion yield by HPLC (**Table 16**).

Table 16: Effects of various organic solvents IBU-sorbitol ester production by PPL.

Solvent	LogP	Solubility in water	Conversion (%)
Hexane	3.9	9.5 mg mL^{-1}	18 ± 1.3
Toluene	2.43	526 mg mL^{-1}	11 ± 1.1
Benzene	2.13	1790 mg mL^{-1}	-

The reaction conditions 5 g L^{-1} of free lipase, solvent/water, and acid/alcohol ratio 5:1, incubation at 35°C with stirring at 400 rpm; 24h.

In a reactor final volume of 15 mL, 20% of water was included because it is well known that the quantity of water in the reaction medium must be lower than that of the organic solvent to push the lipase towards its synthetic behavior, avoiding the hydrolytic behavior. This amount of water also allowed the complete solubilization of sorbitol.

Interestingly, there appears to be a correlation between the polarity of the organic solvent, its consequent miscibility in water, and the percentual conversion yield. In fact, data of these tests show how the less polar solvent, ie hexane, produced a yield of 18% against the yield of 11% obtained with toluene. Furthermore, with regards to the esterification with benzene medium, this did not give any positive results, in fact, no TLC band attributable to the retention factor associated with IBU-sorbitol ester was found.

This catalytic behavior of the PPL enzyme can be explained by its interfacial activation mechanism. More apolar organic solvents, such as hexane, having limited solubility in water, produce clearer solvent/water interfaces. The better the interface, the better the emulsion produced by, agitation and the greater the space that the lipase has to be able to undergo the phenomenon of interfacial activation and therefore be catalytically active. The dispersion of the organic solvent in water was evaluated. The same organic/water media, (solvents only) were analyzed at the same reaction conditions. The presence of organic solvent in water was evaluated after 24 hours of controlled agitation and temperature. Spectrophotometric readings allowed us to evaluate how, under these experimental conditions, toluene (260 nm) is 70 times more present than hexane (290 nm) in the aqueous phase separated from the organic one. The greater miscibility of toluene in water than hexane could explain the lower conversion efficiency of lipase in this reaction context, which could suffer the effects of a worse interfacial activation.

Furthermore, the differences in substrate conversion yield may be explained by the capacity of the solvent to stabilize the product IBU-sorbitol. For the reaction to take place effectively, the reaction product needs to be in an organic layer that allows its stability. The nature of the organic solvent strongly influences the enzymatic esterification process, in some cases up to cancel the ester formation (as it was for benzene). Given that toluene is a more polar solvent than hexane, it would be expected that this can better solubilize the reaction product. However, the data show that the conversion is better, albeit slightly, in hexane. This suggests that the conversion yield is a measure influenced by several factors, which see the concomitant effect on the interface and solubilization of the ester produced. Except for a few literary sources, this ester has not been extensively studied and its distribution mechanisms between organic solvents of different polarity are not thoroughly known. So, the purified ester was loaded into biphasic hexane/water and toluene/water media under the same conditions (volumes, temperature and

stirring, time) as the enzymatic reaction. The system was stopped, and the partitioning of the ester was evaluated in the organic phase compared to the aqueous one. Only one in ten parts of IBU-sorbitol ester was found in hexane, in contrast, two of ten parts were found in toluene. Thus, due to its higher polarity compared to ibuprofen, IBU-sorbitol ester could split between the organic and aqueous phase in an emulsified biphasic system but preferentially remaining in the aqueous solvent. Moreover, we studied the octanol/water partition coefficient (LogP) of the ester of ibuprofen with sorbitol using the shake flask method [293]. IBU-sorbitol ester was found to have a LogP of 1.37 ± 0.09 compared to 3,9 of ibuprofen. This aspect, together with the influence that the solvent has on the interface, may explain the different percentage conversion yields between the solvent hexane and toluene, although slightly. Similar results related to the increment in ester yield in the organic medium were reported in the literature earlier. Been Salah et al [294], observed that the hydrophobicity of organic solvent greatly affected and improved the esterification activity. They found higher conversion rates in hexane media. Moreover, Hazarika et al [295] reported a good correlation between initial esterification rates of PPL and the increase of the hydrophobicity of the solvent.

For this reason, hexane was chosen as the organic medium for further experiments due to higher ester productivity with this solvent. Also, hexane has been shown as the suitable organic solvent for the PPL-catalyzed esterification of glycerol and oleic acid [296], acetylenic acids with n-butanol [297], and for the enzymatic synthesis of ethyl oleate [295].

Furthermore, we evaluated how IBU-sorbitol ester is freely soluble in ethanol and 50% EtOH/water solvent. This could allow evaluating the anti-inflammatory activity of this ibuprofen derivative on cell lines *in vitro*.

To eliminate the partitioning variable of the product between solvents, these were evaporated before the evaluation of the conversion yield, as reported in the materials and methods section.

OPTIMIZATION OF THE LIPASE-CATALYZED SYNTHESIS OF IBU-SORBITOL

Effect of enzyme concentration

After the selection of the organic medium, the effect of the concentration of enzyme used was studied and is reported in **Figure 56**.

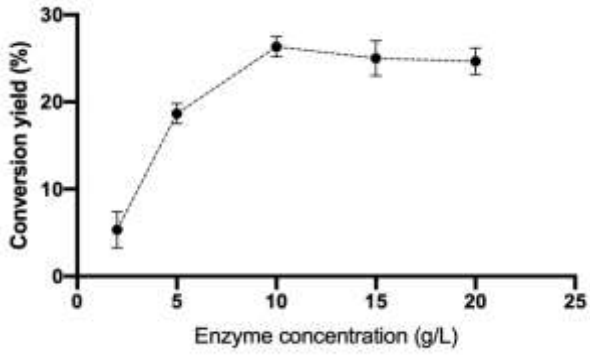


Figure 56: Effect of enzyme concentration on the conversion yield of IBU-sorbitol ester catalyzed by PPL. Reaction time: 15h (equilibrium condition). All measurements were performed in triplicate.

To evaluate the effect of enzyme concentration on conversion yield, five concentrations of free PPL enzyme in a range of 2 to 20 g L^{-1} were tested. As shown in **Figure 56**, the conversion yield increased with the amount of enzyme following a hyperbolic trend typic in the esterification reactions lipase-catalyzed. At higher enzyme concentrations, more active sites are present for substrate binding. So, the reaction rate increases. However, after 10 g L^{-1}, a small decrease in conversion yield is reported. Probably, in a complex system like the biphasic one, since water is present in the system, at high concentrations of enzyme this could hydrolyze the newly formed ester bond.

For this reason, the value of 10 g L^{-1} was selected as the most adequate enzyme concentration for further tests. The enzyme activity of the porcine pancreatic lipase has been carried out. The results showed which enzyme units are 0.035 ± 0.01 units/mg.

Effect of initial water content

Handle a biphasic-media esterification can be challenging. Not only the type of organic solvent chosen is decisive, but it is also essential to be able to create a net interface, control the amount of initial water, and be able to determine sufficient agitation for the system to produce an effective emulsion. The volume of organic solvent and water needs to be finely regulated, as well as substrate concentrations and molar ratio.

On this basis, various authors have reported the effect of organic solvents on the performance of lipases. Generally, the hydrophobicity of solvents and the water content are the most important factors influencing the improvement of lipases [298]. Water molecules on the enzyme surface have been described as a molecular lubricant of enzymes [63] and this increases its internal flexibility facilitating the movements necessary for catalysis [288]. Also, highly hydrophilic compounds such as sorbitol need a water phase to be solubilized. Indeed, the solubility of sorbitol in organic solvents is too low to reach adequate concentration levels for direct lipase-catalyzed esterification [299]. In contrast, ibuprofen shows higher solubility in non-polar solvents [300]. Despite the organic synthesis with lipases possible, must be considered that alcohol and solvents have great effects on the performance of the lipases [301].

As for the water, the effect of initial water content on enzymatic activity was examined through the initial addition of an amount of water ranging from 5 to 30 % (v/v) of the total amount of the reaction mixture (15 mL).

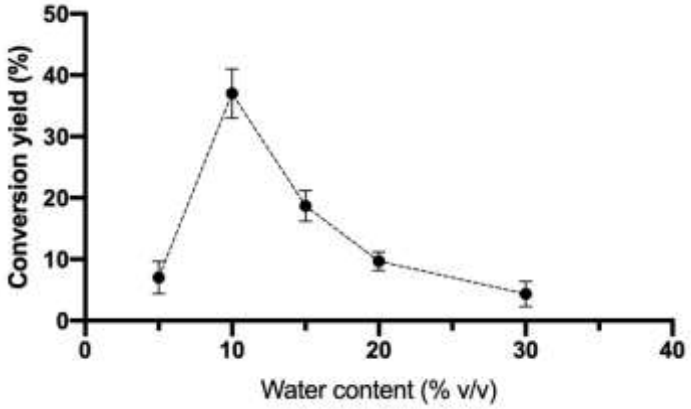


Figure 57: Effect of the amount of water on the ester yield for PPL. Reaction time: 15h (equilibrium condition). All measurements were performed in triplicate.

The results of **Figure 57** show how at low water content (5% v/v) the activity of pancreatic porcine lipase is strongly limited by the scarcity of the aqueous compartment of the system. Probably, the enzyme is not fully hydrated resulting in a decreased three-dimensional conformation of the latter. Even more likely, the amount of water corresponding to 5% v/v is not sufficient to generate and maintain a stable and clear solvent/water interface. This eliminates the interfacial activation effect of the PPL, severely limiting its activity.

At a water content corresponding to 10%, a significant increase in the conversion yield of the substrates is observed. At this amount of water, the lipase is correctly hydrated and manages to meet the ibuprofen and sorbitol substrates respectively solubilized in hexane and water.

As the amount of water in the system increases, there is a sharp decline in the conversion yield. This can be explained by the hydrolytic-synthetic balance characteristic of the lipase **Scheme 7**.

Based on this balance, excess water in the system leads the enzyme to produce hydrolysis of the ester bond (as physiologically occurs for the hydrolysis of triglycerides). A similar pattern has been shown in previous studies that report how

the excess of the aqueous compartment in the system has destabilizing effects for porcine pancreatic lipase [302].

In a biphasic system such as the one reported in the present work, the reaction medium consists largely of hydrophobic organic solvent, which could determine an accumulation of water near the active site of the lipase, in particular near-polar amino acid residues. Therefore, the addition of high quantities of water to the system can increase the size of the interfacial area and facilitate the hydrolysis of the ester. Due to this, the production of the ester can be reduced [92, 78].

Following this experimental evidence, the value of water content was selected at 10% v/v for further experiments.

Effect of temperature

The effect of temperature on the progress of the PPL-catalyzed esterification reactions was tested at 6 different temperatures between 30 and 55 °C, and results were given in **Figure 58**.

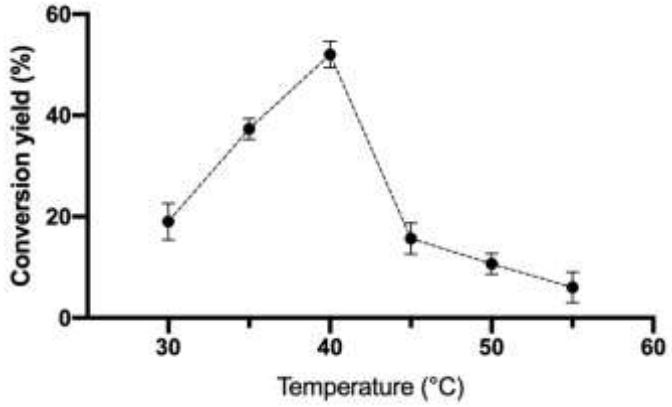


Figure 58: Effect of temperature (°C) on the esterification yield of IBU-sorbitol ester. Reaction time: 15h (equilibrium condition). All measurements were performed in triplicate.

Net changes in the conversion profile of IBU-sorbitol ester were rising the temperature from 30 to 40 °C. The rate of esterification increased enormously with the increase of reaction temperature from 30 to 40 °C, in absence of catalyst inactivation. However, after 40 °C the ester yield decreases tremendously. Being the reported one a biphasic system, the influence of the highly hydrophobic organic solvent is to be considered in the advent of mechanisms related to enzymatic instability. The result shown in **Figure 58** supports this thesis. In fact, since PPL is free, or not-immobilized, it is clear that the increase in temperature causes instability of the said enzyme, considering the difficult biphasic environment. At higher temperatures, enzymatic instability occurs which can be observed through visual inspection. The denatured enzyme remains adhered to the walls of the microreactor.

Besides, temperature massive influences the physical state of substrates (such as solubility, ionization, etc). The chemical reactivity and the reaction equilibria of the substrate are governed by its thermodynamic properties [304]. However, since this enzyme is in free form the effect of changes on mass transfer can be considered negligible. Furthermore, when enzymatic esterification involves the use of polyalcohol, the use of low temperatures pushes the enzyme to covalent attack against the primary hydroxyl groups [92]. Other studies reported similar behavior of the porcine pancreatic lipase [94, 95].

Effect of stirring speed

Given the highly lipophilic and hydrophilic nature of ibuprofen and sorbitol, respectively, the choice of a biphasic esterification system was obligatory. This is a difficult to manage system with many variables involved. One of the key aspects in managing a medium like the biphasic one is finding the right degree of agitation. In fact, the agitation affects the "interfacial quality" of the interface [307]. Since the reaction medium is a water-in-oil dispersion, the conversion rate would be determined by the interfacial area. The interfacial area of a water-in-oil dispersion

determines the conversion rate because it's a function of the speed of agitation and the ratio of the volumes of the aqueous organic phases (in our case the better ratio was 10:1 oil/water). The interfacial area is dependent on the speed of agitation [308]. As this area increases, a greater number of enzyme molecules will become adsorbed onto the interfacial sites. One all the molecules have occupied sites at the interface, however, any increase in the interfacial area due to the increases in agitation speed will not affect.

Conversely, in a system mainly consisting of hydrophobic organic solvent, excessive agitation, added to the administration of heat, can causes enzymatic denaturation [33]. At the same time, if the system is not stirred sufficiently, the emulsion between the organic and aqueous phase is not obtained; this limits the space of the interface where the enzyme can undergo the phenomenon of interfacial activation and thus be catalytically active.

In our case, the choice of the inexpensive PPL enzyme in its free form further complicates matters. In fact, it is reported that the immobilization of enzymes has the effect of increasing their stability against temperature and organic solvents [98, 99], an advantage that in this context has disappeared. However, we decided to use a free form of PPL to propose a system that exploits an enzyme without the need for pretreatments, inexpensive, and thus highly reproducible.

By stirring speed [311], we mean the angular speed with which, through the magnetic stirrer, we can produce motion to the system. The effect of the stirring speed in the esterification of ibuprofen with sorbitol in organic/water media was studied by varying the stirring speed between 100 and 600 RPM. These tests allowed us to observe and determine any mass transfer limitation in the biphasic reaction system. The results are shown in **Figure 59**.

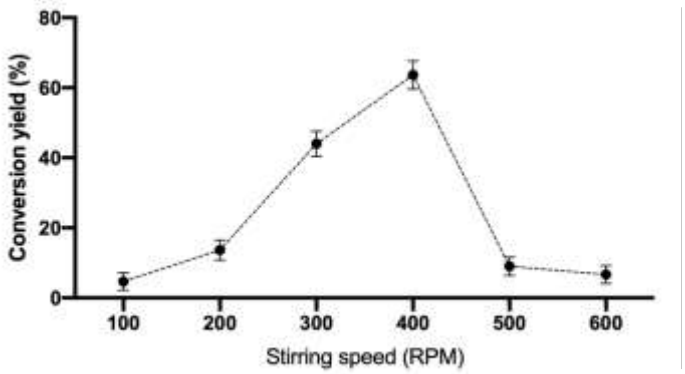


Figure 59: Effect of stirring speed on the enzymatic esterification of ibuprofen with sorbitol. Reaction conditions: PPL = 10 g L^{-1}, T= 40 °C, C_{IBU} = 42 g L^{-1}, $C_{Sorbitol}$ = 30 g L^{-1}, C_w = 10% v/v, volume ratio hexane/water = 10:1. Reaction time: 15h (equilibrium condition). All measurements were performed in triplicate.

As shown in **Figure 59**, enhancing the stirring speed there is an increase in the initial rate of esterification. Being hexane immiscible in the water present in the reaction medium, at low speeds, the contact between the phases is too poor to ensure the meeting of the substrates. The contact between the phases and therefore the meeting between the substrates increases as the agitation increase up to the value of 400 RMP, beyond which, a reasonable decrease in the rate of esterification is observed. The changes in the observation of the esterification rate are attributable, similarly to what has been seen for the increase in temperature, to phenomena of enzymatic instability. This data is answered by the nature of the free enzyme PPL, (in an organic solvent, at 40 degrees) which, at an agitation of 500 RPM and up, is very likely to undergo denaturation phenomena. In this system, the optimum stirring speed is reached at which mass transfer limitations and enzyme denaturation can be neglected. Therefore, the value of 400 RMP was chosen for subsequent experimental runs.

Effect of substrate concentration

Two primary forces determine the acid and alcohol selectivity of lipases: steric hindrance and hydrophobic interactions [101, 102]. Testing the effects of acid and alcohol concentrations on the productivity of PPL in catalyzing the esterification reaction of ibuprofen with sorbitol, we first set the alcohol value at 30 g L^{-1} and varied the acid concentrations in a range between 30 and 80 g L^{-1}. Secondly, having obtained information on what was the best concentration of ibuprofen for enzymatic esterification and holding that fixed, we varied the concentrations of polyalcohol sorbitol in a range between 5 and 40 g L^{-1}.

As seen in **Figure 60 (a)**, when the concentration of ibuprofen increases, there are no inhibitory effects on the enzyme. In any case, given that no further increase in conversion yield is observed beyond the ibuprofen concentration of 60 g L^{-1}, this value was taken as a reference for subsequent tests.

After investigating the effects of acid concentration, the alcohol concentration was varied between 5 and 40 g L^{-1}. As shown in **Figure 60 (b)**, the highest yield was obtained at an alcohol concentration equal to 20 g L^{-1}.

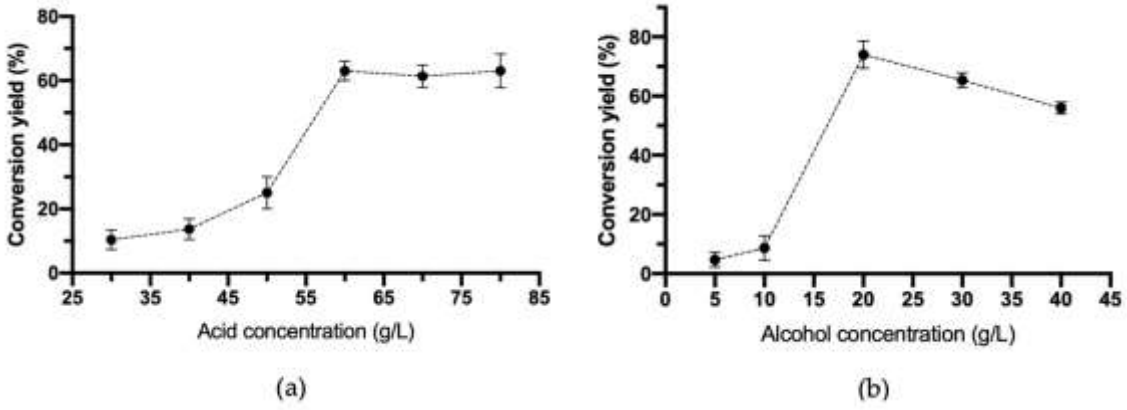


Figure 60: Effect of substrates concentrations on ester yield for PPL; (a) 30 g L^{-1} constant sorbitol concentration; (b) 60 g L^{-1} constant ibuprofen concentration; Reaction conditions: PPL 10 g L^{-1}, Cw= 10% v/v, 40 °C, 400 RPM, 24 h. All measurements were performed in triplicate.

In this PPL-catalyzed esterification strategy, estimation of how much alcohol can be added to the reaction medium is essential. Indeed, Brockerhoff et al reported that hydroxyl groups reduce the activity of PPL [101][286]. Moreover, it has been reported that substrates comprising alcoholic functions are a limiting factor in the esterification yield due to the tendency to localize themselves at the interface between the organic solvent and water, stealing space for the enzyme which therefore cannot undergo interfacial activation and reach the catalytically active form [314]. The literature reports how three main characteristics of the alcohol in question influence the conversion rate of the enzyme: molecule size, hydrophobicity, and solubility. The binding energy, influenced by the size of the molecule carrying the hydroxyl groups, is the force that allows the conversion of the enzyme into its activated form, when the substrate binds to the active site. When the binding energy is low, the lipase is not in its optimal three-dimensional conformation, and the reaction proceeds slowly. Moreover, the more the hydroxyl carrier molecule is soluble in water, the more the enzyme is exposed to alcoholic functions, the more it undergoes denaturation and therefore inactivation. The great limitations related to the choice of sorbitol as a hydrophilic agent carrying hydroxyl groups have strongly influenced the entirety of the experimental design of the lipase-catalyzed esterification of ibuprofen with sorbitol. First of all, a biphasic system was chosen where there was a good quantity of water capable of solubilizing the sorbitol substrate, secondly, the ratio between acid and alcohol has always provided an excess of acid compared to the amount of sorbitol due to its effects on the conversion yield that prevented from being able to do the opposite.

In our tests, beyond the concentration of 20 g L^{-1}, a decrease in the concentration rate of IBU-sorbitol ester is observed. Probably, the high concentrations of polyalcohol sorbitol can be considered as facilitators of protein denaturation by solvating the hydrophobic amino acid residues within the active site and stabilizing the denatured rather than the native conformation [308].

Effect of reaction time

IBU-sorbitol ester synthesis was attained at its optimal conditions at several reaction times between 5 and 40h and results were given in **Figure 61**.

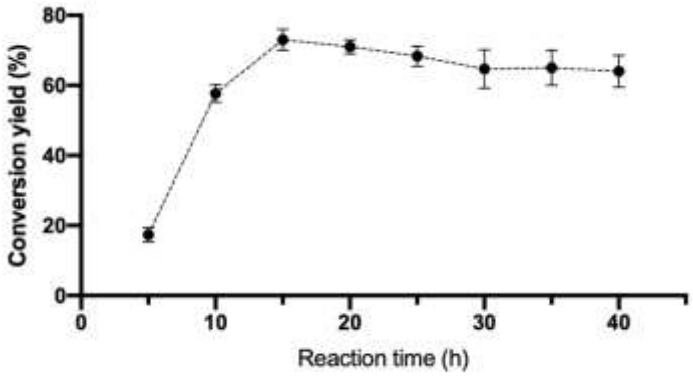


Figure 61: Ester productivity at different reaction times between 5 to 40h. All measurements were performed in triplicate.

Ester production grows with time up to 15h of reaction time and then moderately decreases. The increment of the amount of water produces as a by-product of the catalysis of the ester bond may explain the decrease in conversion yield. Probably, increased water may lead to hydrolysis of the ester previously produced [230]. Furthermore, this decrease may be described also by instability phenomena of PPL in hexane after hours of agitation and heating. Similar results were obtained by Ozyilmaz et al [315] using PPL for the production of aroma ester.

IBU-sorbitol ester: MS spectroscopy characterization

Positive electrospray ionization (ESI+) was carried out on the band with Rf of 0.14 (IBU-sorbitol ester) of the TLC reaction separations of the enzymatic reaction product to achieve mass spectra. The mass spectrometry results confirmed that the esterification reaction took place between the carboxylic acid group of ibuprofen and a hydroxyl group of sorbitol. The esterification product formed has a mass prediction of m/z 370.

The fragmentation pattern showed in **Figure 62** describes the m/z of IBU-sorbitol ester as the ionized m/z 371 $[M + H]^+$ adduct (visible both in (a) and (b)) and m/z 393 [M + Na]+ adduct, confirming the presence of the enzymatically synthesized prodrug.

To the best of our knowledge, although Douša et al [316] have chemically produced the ibuprofen ester with sorbitol, this is the first protocol that proposes effective enzymatic esterification of ibuprofen with a highly polar polyalcohol such as sorbitol, besides with a free enzyme. This strategy was demonstrated to be a successful way to produce a prodrug of ibuprofen by direct enzymatic esterification in a biphasic W/O system.

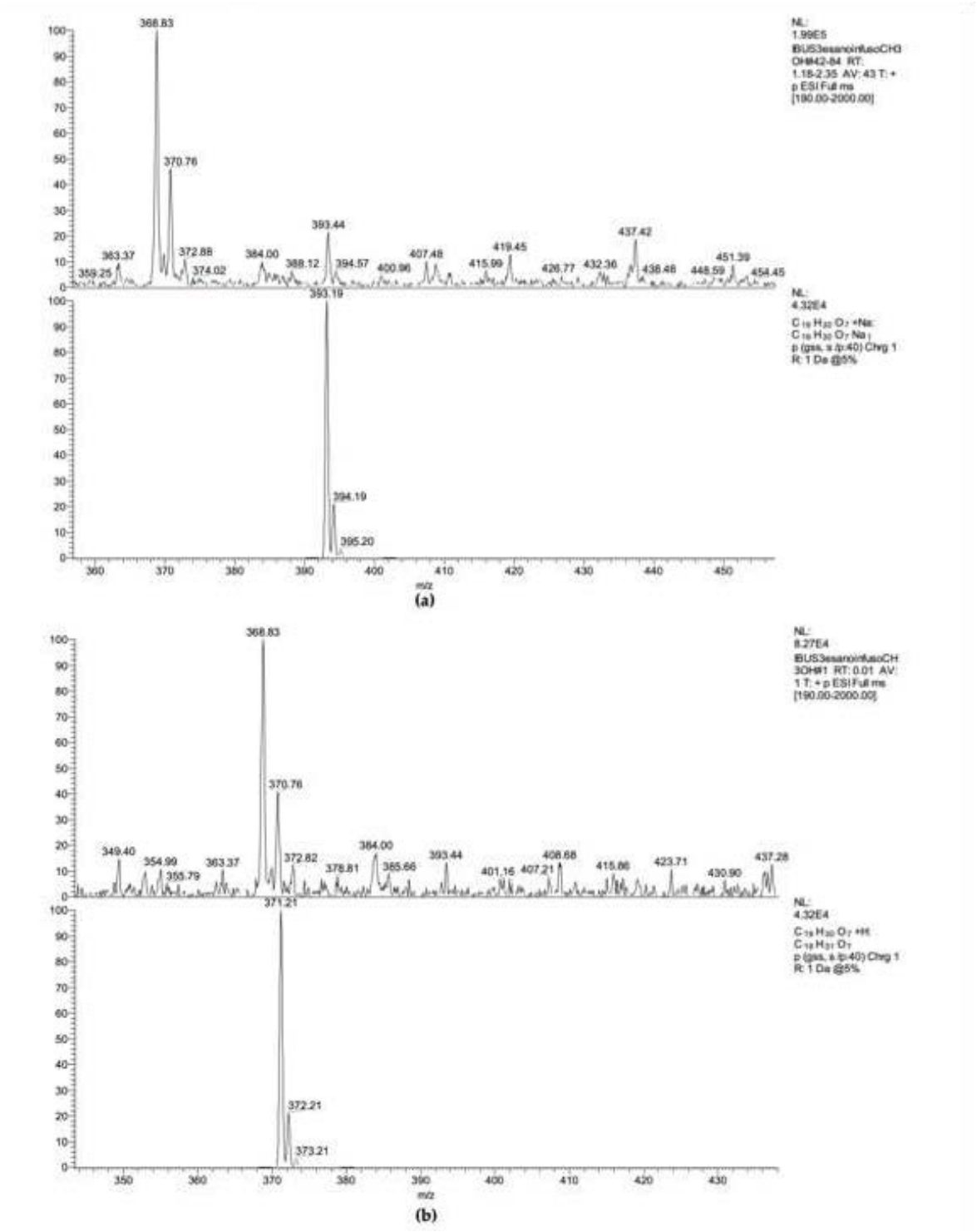


Figure 62: ESI+, HPTLC-MS analysis of lipase-catalyzed esterification of ibuprofen with sorbitol by free PPL in biphasic media. (a) Na^+ adduct of IBU-sorbitol ester. (b) H^+ adduct of IBU-sorbitol ester.

4.3 Correlation Between Polarity And Phase Affinity: Chromatographyc Analysis (TLC, HPLC)

The purpose of this thesis was to produce enzymatic esterification protocols of poorly soluble active ingredients to, mainly, increase their solubility in water as a result of increasing the polarity of the molecule.

The active ingredient ibuprofen was then esterified with polyalcohols from the increasing number of hydroxyls and therefore from the increasing polarity.

The increase in the polarity of the esters resulting from the combination of ibuprofen with polyalcohol such as glycerol, erythritol, xylitol and sorbitol, compared to ibuprofen in acid form, can therefore be studied using crematographic techniques.

In fact, chromatographies allow us to evaluate the different polarity of different molecules depending on the affinity for a stationary phase or a mobile phase.

This study then proceeded with the separation of the esters of ibuprofen, and acid ibuprofen, by means of thin layer chromatography TLC and high performance liquid chromatography HPLC.

Figure 63 shows the result of the chromatographic separation of the ibuprofen esters.

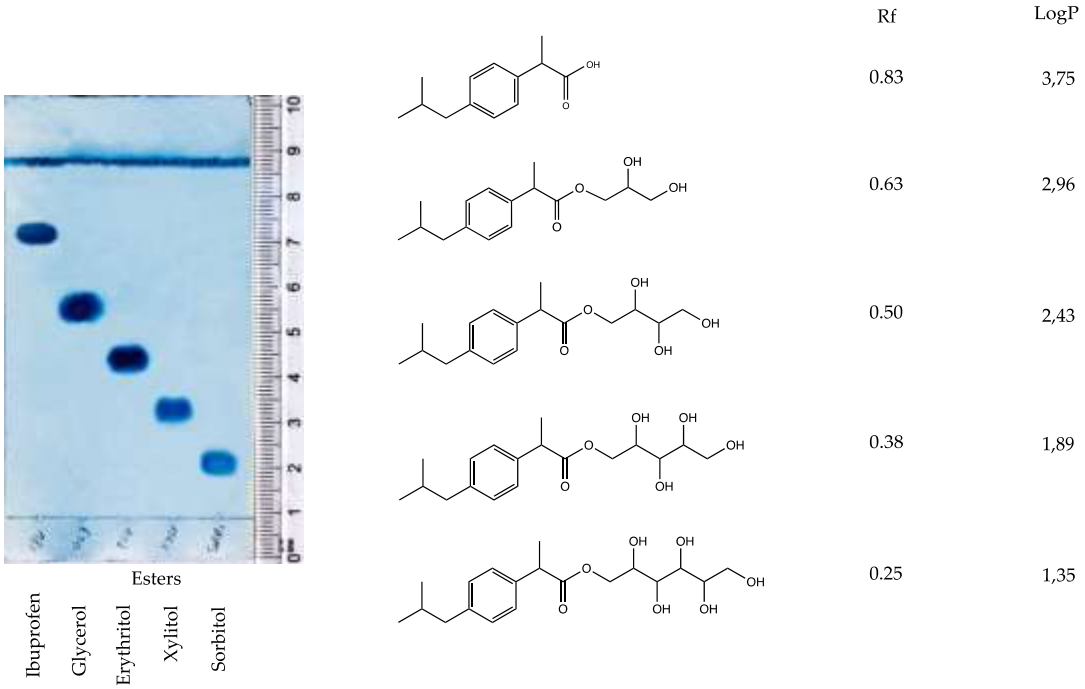


Figure 63: Correlation between length of sugar alcohols and logP of esters.

Through thin layer chromatography it was possible to demonstrate the presence of an inverse correlation between the number of hydroxyls introduced into the ester and the partition coefficient.

Figure 63 shows, in fact, how the affinity of the ester towards the mobile (apolar) phase decreases as the number of hydroxyls of the molecule linked to ibuprofen by ester bond increases. As you can see, the RF of the products progressively decreases as well as the logP of the molecules, while the polarity increases with the increase in the number of hydroxyls that are added to the ibuprofen.

Further evidence of the gradual increase in polarity of the ester with the increase in alcoholic functions, compared to the starting ibuprofen, was provided by chromatographic separation by HPLC.

Liquid chromatography (**Figure 64**) shows the retention times of the ibuprofen

esters on the C18 column. Since the C18 column is apolar, more polar molecules such as ibuprofen sorbitol-ester will have a lower tendency to remain retained by the stationary phase of the column and will be eluted at shorter retention times. Therefore, as expected, the ester with sorbitol is the first to be eluted, followed by: ibuprofen xylitol-ester, ibuprofen erythritol-ester, ibuprofen glycerol-ester and only last will be eluted l'ibuprofene.

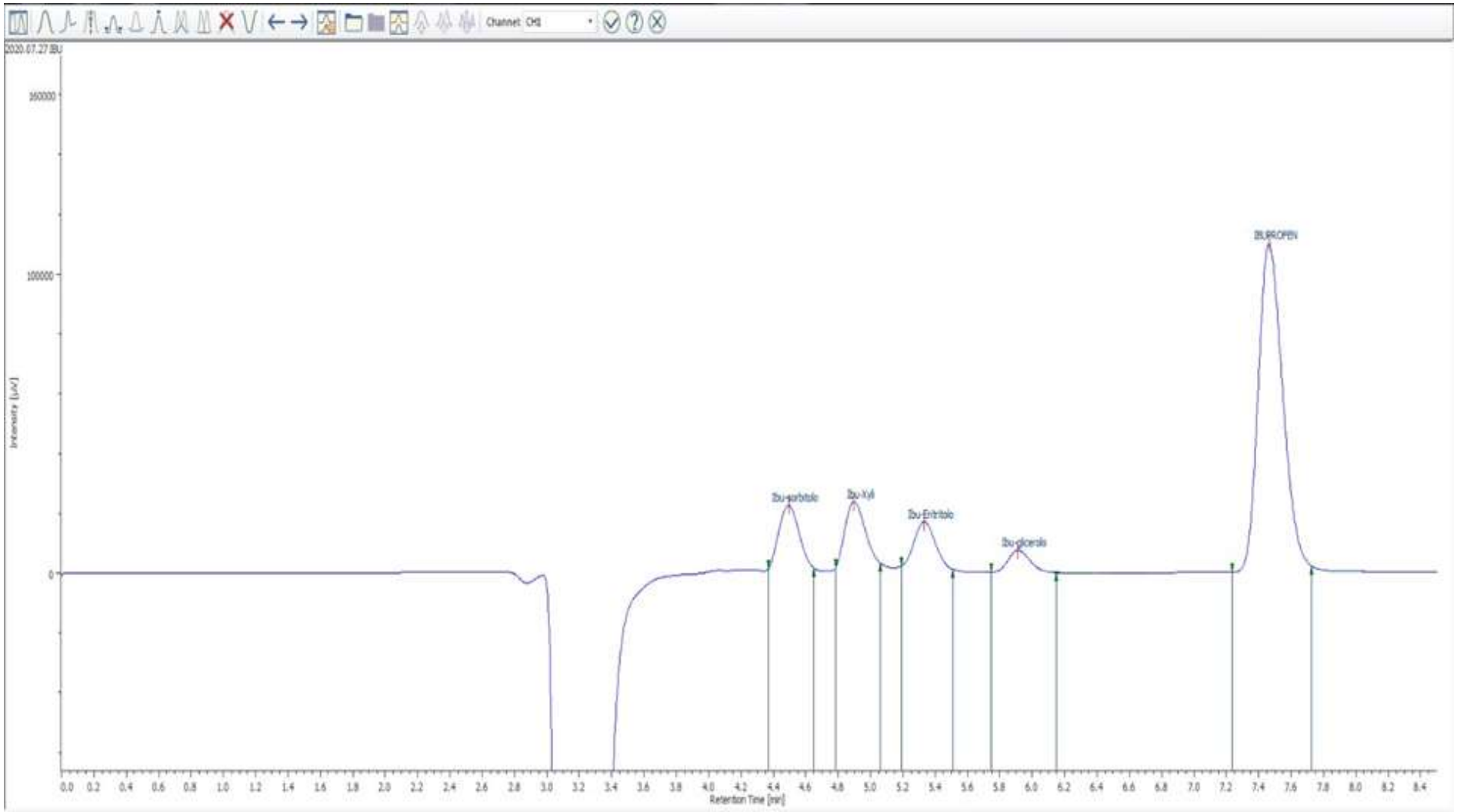


Figure 64: HPLC reverse-phase C18 column separation of Ibuprofen esters.

Table 17 shows the ibuprofen esters and their retention times on the C18 column.

Table 17: Retention times of ibuprofen esters on column C18; mobile phase: 83% mehtanol, 17% H2O (pH 2.2).

Molecule	Retention time (min)
Ibuprofen sorbitol-ester	4.5
Ibuprofen xylitol-ester	4.9
Ibuprofen erythritol-ester	5.35
Ibuprofen glycerol-ester	5.94
Ibuprofen	7.50

4.4 ACTIVITY TESTS

With the enzymatic esterification protocols discussed in the previous section, we synthesized esters with increased polarity that could potentially result in food preservatives or pharmaceutical products with increased solubility and consequently bioavailability.

The glyceric ester of sorbic acid was then evaluated for antimicrobial activity with disk diffusion DD and minimum inhibitory concentration tests MIC.

Ibuprofen esters were evaluated for anti-inflammatory activity by qRT-PCR.

4.4.1 Evaluation of antibacterial and antifungal activity of Glycerol Sorbate

A comparison in terms of antimicrobial activity of pure sorbic acid and its glycerol-ester against the yeast *Saccharomyces cerevisiae* and the bacteria *Streptomyces griseus* is also provided in this section. This yeast was previously reported as model in microbial stability of ready-to-drink beverages studies [176].

Leterature report esterification as strategy to enhance antimicrobial activity. Effective esterification of eugenol in solventless systems has been reported, with augmented antimicrobial activity against several bacteria compared to pure eugenol [317]. Encouraging results were also obtained considering the antifungal activity of the eugenol isobutyryl and acetyl esters that inhibit dermatophytes *M. gypseum* and *T. mentagrophytes* growth [318].

So, we esterified sorbic acid with glycerol to obtain a chemical compound with potentially increased antimicrobial activity as result of better solubility and consequent better bioavailability. Furthermore, potassium sorbate, a very popular food and drink preservative, shows a much higher solubility than sorbic acid, but has some negative aspects. In fact, due to its pKa, when the pH is up to 5, potassium sorbate is charged and relatively impermeable to the membrane. Thus, to achieve the desired antimicrobial activity, the concentration of potassium sorbate often needs to be increased above the maximum regulatory levels allowed in foods and drinks. For this reason, an ester between sorbic acid and glycerol could be a valuable solution to the membrane permeability issue of potassium sorbate.

Two models were chosen to test the antimicrobial activity of the ester; the bacterium Streptomyces griseus, and the yeast Saccharomyces cerevisiae. Disc diffusion (DD) and minimal inhibitory concentration assays (MIC) were performed. Cultures of a bacterium (*S. griseus*) and a yeast (*S. cerevisiae*), with a cell density of 7×10^6 CFU/mL, were exposed to different concentrations of the preservative and its ester (**Table 12**).

The disc diameters of the inhibition zone are shown in **Table 18.** The antimicrobial activity of the ester was compared to sorbic acid, the currently used antimicrobial agents often used as preservatives in food object of this research. The antimicrobial activity of potassium sorbate, the potassium salt of sorbic acid, was also assessed because of its enhanced solubility compared to sorbic acid. This parameter is the

one that identifies potassium sorbate as the current choice of microbial control in foods.

Table 18: Antimicrobial activity of sorbic acid, glycerol and the two positive controls chloramphenicol and clotrimazole.

Streptomyces griseus	**DD**
Chloramphenicol	18 mm ± 0.3
Sorbic acid	/
Glycerol sorbate	/
Potassium sorbate	/
Saccharomyces cerevisiae	
Clotrimazole	18 mm ± 0.2
Sorbic acid	10 mm ± 0.4
Glycerol sorbate	11.5 mm ± 0.1
Potassium sorbate	12.6 ± 0.2

The antifungal effect of sorbic acid, as well as glycerol sorbate, against *S. cerevisiae* was clearly confirmed by the disk diffusion experiments. The diameters of the inhibition zone of the newly synthesized glycerol ester of sorbic acid were 11.5 mm for *S. cerevisiae*, while sorbic acid caused an inhibition zone of 10 mm. YMB was pH 6.5. Due to its pKa (4.76), potassium sorbate was partially charged. However, its antimicrobial activity was better than the sorbic acid ester. For this reason, no tests at different pH ranges have been conducted. The yeast growth inhibition of the ester was higher than the not esterified sorbic acid. Therefore, esterification could be an interesting strategy to increase antimicrobial activity of food preservatives. This strategy will be further investigated by designing other esters with increased water solubility and, consequently, antimicrobial activity. According to the results presented in **Table 18**, it can be noted that glycerol sorbate as well as sorbic acid showed a good inhibitory effect against the yeast specie tested but not for the bacterium. However, we found that glycerol sorbate, as well as sorbic acid, does not inhibit growth of the Gram-positive bacterium *S. griseus*. This is probably due to the

more complex cell wall structure of Gram-positive bacteria, which has additional lipopolysaccharides on the outer surface that generally reduces the ability of most antibiotics and extracts to penetrate the bacterial cells [319]. Furthermore, it has been reported how some microorganisms, for example *Acetobacter* species, are able to degrade sorbic acid using it as carbon source [171].

The MIC values of glycerol sorbate on *S. cerevisiae* were on the range of 0.14 mg/mL (**Table 19**). Natural products presenting MICs of ≤1.00 mg mL^{-1} are normally considerate noteworthy in terms of antimicrobial activity [320]. With this assumption, although potassium sorbate is the compound with the lowest MIC, the antimicrobial activity of the sorbic acid ester in this work results to be good for the control of the yeast *S. cerevisiae*. Moreover, the growth inhibition of the glycerol ester of sorbic acid resulted to be better compared to the not-esterified food preservative sorbic acid.

Table 19: Susceptibility of *S. cerevisiae* to sorbic acid and glycerol sorbate.

	S. cerevisiae	
	MIC_{50} [1]	MIC_{100}
Sorbic acid	0.097 ± 0.0016	0.150 ± 0.001
Glycerol sorbate	0.090 ± 0.001	0.140 ± 0.0008
Potassium Sorbate	0.080 ± 0.001	0.125 ± 0.003

[1]MIC: Minimum Inhibitory Concentration (mg/mL^{-1}), described as the lowest concentration of the compound that totally inhibited (MIC_{100}) or reduced growth to 50% (MIC_{50}).

Nevertheless, additional safety information regarding this ester is required to obtain approval as food, beverage, or cosmetic preservative. As widely discussed in the literature, health information will need to investigate the effects of these additives and the consequences of their long-term dietary consumption. Indeed, though the actuals salts benzoate and sorbate are widely used compounds for food and beverage preservation, there continues to be a concern about their complete

safety. Cell culture studies and model organisms have shown some issues. Benzoate and sorbate are reported to results in chromosome aberrations in cultured human lymphocytes. Thus, exposure to these agents should be evaluated in terms of downsizing [321]. About this, the positive aspects reported in the literature regarding the use of monoglycerides as preservatives in food and drinks can be of great interest. Monoacylglycerols, like monocaprin and monolaurin, used as fruit juice preservatives, suppressed or prevented the growth of filamentous fungi in vitro [322]. Monoglycerides also inhibited the germination of bacteria like *Bacillus cereus* and *Clostridium botulinum* [323]. Model membrane-based biophysical measurement techniques showed that fatty acid and monoglycerides as antimicrobial destabilize phospholipid membranes, causing several direct and indirect inhibitory effects [324]. Therefore, the importance of developing monoglycerides-based preservatives, as well as deeply testing their effects on human safety, turns out to be very essential.

4.4.2 Evaluation of antinflammatory activity of Ibuprofen Esters

Enzymatically synthesized ibuprofen esters were investigated in terms of anti-inflammatory activity.

The covalent attack of hydrophilic portions to the starting active ingredient could increase the anti-inflammatory capacity of the prodrug. In fact, it is reported in the literature that the development of prodrugs with increased solubility can represent an effective strategy to improve the bioavailability of the active ingredient under consideration [140].

As ibuprofen is one of the most widely used anti-inflammatory drugs for the treatment of cystic fibrosis (CF), the cell line IB3-1 was chosen for analysis [325]. This cell line, with the ΔF508/W1282X mutation in the CFTR gene, derives from the bronchial epithelium of patients with cystic fibrosis [326]. Since cystic fibrosis is a

disease characterized by an important inflammatory state at the bronchial and pulmonary level, the choice of this cell line, prone towards inflammatory states, can be considered an interesting model for the evaluation of the anti-inflammatory activity of the ibuprofen prodrugs synthesized via enzymatic route [327]. The need for the development of new ibuprofen derivatives as anti-inflammatories for the treatment of CF is driven by the adverse effects of chronic and high-dose use of ibuprofen [328].

In CF, neutrophils, which in CF cause lung damage, are recruited to airways by overabundant interleukin 8 (IL-8) [329]. CF respiratory epithelium secretes exaggerated amounts of IL-8, either spontaneously [330] or in response to pro-inflammatory cytokines [331]. Down-regulation of IL-8 production in CF respiratory epithelium could attenuate neutrophil-dominated inflammation, so we tested IB3-1 cell line as model for our tests.

To induce inflammation in a controlled and measurable way, the cells were treated with Tumor Necrosis Factor-α (TNF-α). The expression of the interleukin 8 gene was evaluated by qRT-PCR and the expression folds were calculated by setting the untreated positive control at 100% inflammation. From this it can be deduced that the lower the expression of the chemokine IL-8, the greater the anti-inflammatory effect of the drug administered.

The anti-inflammatory activity of the esters of ibuprofen with erythritol, xylitol, and sorbitol was evaluated *in vitro* and compared with that of ibuprofen in its native form. The results are shown in **Figure 65**.

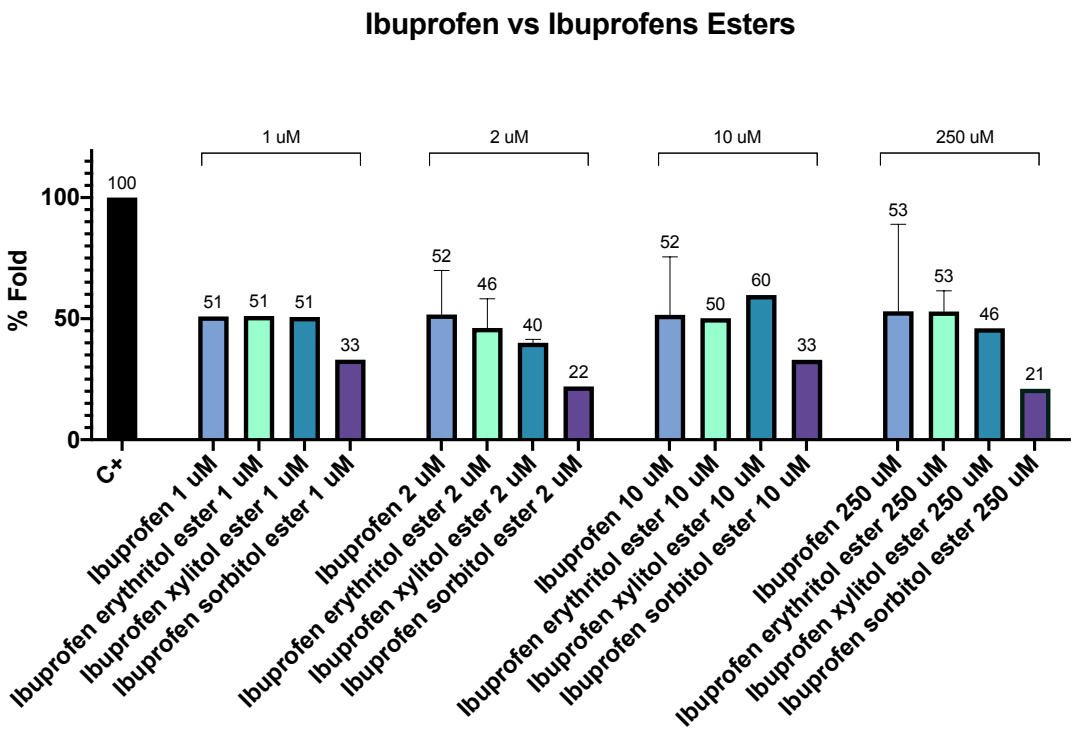


Figure 65: Fold (%) of the anti-inflammatory activity of ibuprofen and derivatives grouped by concentrations of administration.

Ibuprofen and the three esters were tested at concentrations of 1 μM, 2 μM, 10 μM, and 250 μM, in order to assess whether a dose-response to increased concentrations of the drug. At the concentrations studied so far, this does not appear to have occurred. Probably, the concentrations of ibuprofen and derivatives can be further increased. In fact, the evaluation of the antiproliferative activity on IB3-1, initially conducted concerning ibuprofen xylitol ester and ibuprofen sorbitol ester, reports that the IC_{50} for these derivatives is, respectively, of 492.9 μM and 742.5 μM. The increased solubility of the ibuprofen sorbitol ester could result in the greater anti-inflammatory effect it appears to have, as well as its decreased toxicity, which is why it appears to be the most promising ester for future experiments. In fact, although the statistical validity of the presented data is an important aspect yet to be evaluated, the trend seems to show ibuprofen sorbitol ester as the favored derivative for the control of the inflammatory response in the IB3-1 cell line, by

decreasing the expression of the interleukin 8 gene more than ibuprofen and the other derivatives tested. Anyway, these preliminary results provide us with information on how the covalent attack of hydrophilic molecules on ibuprofen, thus generating a pro-drug, did not negatively affect the anti-inflammatory capacity of the NSAID. In fact, even the prodrug with a lower number of hydroxyl groups produced results comparable to those of ibuprofen but with a more soluble derivative of the starting active ingredient. The aspect of the increased solubility of derivatives on the anti-inflammatory activity, the core of pro-drug design, has not yet been evaluated. Indeed, it was decided to standardize the solubilization vehicle of ibuprofen and derivatives to 75% EtOH/H_2O, when, the esters, need less ethanol to be solubilized. This could result in lower concentrations of pro-drug administration with the same effect or in increased anti-inflammatory abilities. At the current state of research, knowing that the addition of polyalcohols to ibuprofen does not result in inactivation is relevant information for the design of upcoming esterification strategies and for the set up of pharmacokinetic tests.

The data shown photograph the state of an experimental work still in progress. The next experiments in evaluating the anti-inflammatory activity of ibuprofen esters will also include the use of ibuprofen glycerol ester and ibuprofen ascorbic acid ester. The latter will be evaluated last by virtue of the complex molecular mechanisms that the ascorbic acid hydrophilizing portion can generate as a bioactive molecule in its own right.

Furthermore, new concentrations of use of ibuprofen and its esters will be evaluated since, often, in CF ibuprofen is administered at high doses, due to which adverse effects are often manifested.

Moreover, the aspects and effects that the different polarity of the esters can influence the timing of the prodrug action will be evaluated. In fact, for lipophilic

molecules, a change in polarity could influence the timing of drug efficacy due to the nature of its passage through cell membranes.

5. CONCLUSIONS

The solubility in water of active ingredients of industrial interest is the main parameter that influences their bioavailability. The purpose of this thesis was the development of enzymatic esterification protocols for the production of esters with increased solubility and therefore bioavailability.

The protocols developed were different, including monophasic or biphasic solventless or solvent-assisted strategies. These esterification protocols have proved flexible and lead to the enzymatic synthesis of several derivatives starting from substrates of agro- and pharmaco-industrial origin (e.g. sorbic acid and ibuprofen). These methods can easily achieve solubility-enhanced prodrugs, preservatives of or flavor esters.

As biocatalysts, we have chosen lipases from two organisms: pancreatic porcine lipase (PPL, type II), in free form, and lipase from *Candida anctartica*, in free (Lipozyme CALB-L) and immobilized form (Novozyme 435).

Each of these enzymes led to the development of tailor made esterification protocols. In particular, Type B *Candida anctartica*, a lipase known for its ability to process solubilized substrates even in monophasic reaction environments, has proven to be an efficient enzyme in the production of highly polar esters under the reported experimental conditions.

In particular, the monophasic solvent-assisted system proposed in this invention is greatly easier in terms of reaction management. Compared to the biphasic system, it will be considered much less variable. Water is not required, and this shifts the hydrolysis/synthesis equilibrium towards the synthetic behavior of the lipase. The use of a commercially available immobilized enzyme leads to highly reproducible organic synthesis.

The developed synthesis protocol can be used for "green", economic, and simple scale-up synthesis, for the production of highly pure and selective pharmaceutical esters. Unlike the chemical route, no preliminary step of protection of functional groups is needed.

Although many enzymatic syntheses reported in the literature require reaction times of even 1 week, the reported protocols can lead after only 24h the substrate conversion yield that ranges from 65 to 100%. Reaction volumes are small: the esterification can be achieved also in reactors or microreactors of 2-5 ml. This small-scale process can be easily scaled up. Exploiting a way to solubilize two substrates in a single organic solvent, it's not required for the one substrate to be liquid. This system works at low temperatures, at atmospheric pressure and the reactor is not filled with nitrogen or other gases.

Qualitative separative and spectrophotometric analyzes allowed us to investigate the nature of the enzyme synthesis product. ^{1}H-NMR, ^{13}C-NMR, and uHPLC-MS are techniques that have confirmed the effective organic synthesis of the ester derivatives.

The *in vitro* activity tests of the new derivatives have shown how these esterification strategies may be potential candidates for the synthesis of esters with increased solubility that maintain, or improve, the antimicrobial or anti-inflammatory properties of the poorly bioavailable active ingredient.

The antimicrobial activity of glycerol sorbate against *S. griseus* and *S. cerevisiae* has been evaluated, giving encouraging results for the control of the fungal model. The project will be extended to a greater number of microorganisms considered food contaminants, to have a broader and more detailed view of its potential.

The ibuprofen esters, synthesized with the protocol in question, could find application as new non-steroidal anti-inflammatory drugs for the treatment of pain

headache, dysmenorrhea, dental pain) up to musculoskeletal disorders such as osteoarthritis, and rheumatoid arthritis.

6. REFERENCES

6. REFERENCES

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7. LIST OF PATENT/PUBLICATIONS

This section contains the list of publications and patents drawn up during the three years of the doctorate in Biomedical and Biotechnological Sciences, cycle XXXIII.

Patents:

• "Enzymatic process for the preparation of esters of poorly water-soluble carboxylic acids." December 2020. Number: 102020000031025.

Tamburini E, **Zappaterra F,** Costa S.

Articles:

• "Biocatalytic Approach for Direct Esterification of Ibuprofen with Sorbitol in Biphasic Media". Mar 2021, *International Journal of Molecular Sciences*;

Zappaterra F, Rodriguez Maldonado M.E., Summa D, Semeraro B, Costa S, Tamburini E.

• "Biotransformation of Cortisone with Rhodococcus rhodnii: Synthesis of New Steroids". Mar 2021, *Molecules*;

Zappaterra F, Costa S, Summa D, Bertolasi V, Semerato B, Pedrini P, Buzzi R, Vertuani S.

• "Fermentation as a Strategy for Bio-Transforming Waste into Resources : Lactic Acid Production from Agri-Food Residues". December 2020, *Fermentation*;

Costa S, Summa D, Semeraro B, **Zappaterra F**, Rugiero I, Tamburini E.

• "Enzymatic Esterification as Potential Strategy to Enhance the Sorbic Acid Behavior as Food and Beverage Preservative" October 2020, *Fermentation*;

Zappaterra F, Summa D, Semerato B, Buzzi R, Trapella C, Ladero M, Costa S, Tamburini E.

• "Δ1-Dehydrogenation and C20 Reduction of Cortisone and Hydrocortisone Catalyzed by Rhodococcus Strains" May 2020, *Molecules*;

Costa S, **Zappaterra F**, Summa D, Semeraro B, Fantin G.

8. APPENDIX

Ministero dello Sviluppo Economico

Ricevuta di presentazione

per

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International Journal of *Molecular Sciences*

MDPI

Article

Biocatalytic Approach for Direct Esterification of Ibuprofen with Sorbitol in Biphasic Media

Federico Zappaterra [1], Maria Elena Maldonado Rodriguez [2], Daniela Summa [1], Bruno Semeraro [3], Stefania Costa [1,*] and Elena Tamburini [1]

[1] Department of Life Sciences and Biotechnology, University of Ferrara, Via L. Borsari, 46, 44121 Ferrara, Italy; zppfrc@unife.it (F.Z.); smmdnl@unife.it (D.S.); tme@unife.it (E.T.)
[2] Department of Biotechnology Engineering of the RRNN, Salesian Polytechnic University, Av. 12 de Octubre y Wilson, Quito 170109, Ecuador; mmaldonado@ups.edu.ec
[3] GATE SRL, Via L. Borsari, 46, 44121 Ferrara, Italy; bsemeraro@gategreen.it
* Correspondence: cstsfn1@unife.it

Abstract: Ibuprofen is a nonsteroidal anti-inflammatory drug (NSAID) introduced in the 1960s and widely used as an analgesic, anti-inflammatory, and antipyretic. In its acid form, the solubility of 21 mg/L greatly limits its bioavailability. Since the bioavailability of a drug product plays a critical role in the design of oral administration dosage, this study investigated the enzymatic esterification of ibuprofen as a strategy for hydrophilization. This work proposes an enzymatic strategy for the covalent attack of highly hydrophilic molecules using acidic functions of commercially available bioactive compounds. The poorly water-soluble drug ibuprofen was esterified in a hexane/water biphasic system by direct esterification with sorbitol using the cheap biocatalyst porcine pancreas lipase (PPL), which demonstrated itself to be a suitable enzyme for the effective production of the IBU-sorbitol ester. This work reports the optimization of the esterification reaction.

Keywords: ibuprofen; sorbitol; esterification; porcine pancreas lipase; prodrug

Citation: Zappaterra, F.; Rodriguez, M.E.M.; Summa, D.; Semeraro, B.; Costa, S.; Tamburini, E. Biocatalytic Approach for Direct Esterification of Ibuprofen with Sorbitol in Biphasic Media. *Int. J. Mol. Sci.* **2021**, *22*, 3066. https://doi.org/10.3390/ijms22063066

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1. Introduction

Ibuprofen ((R,S)-2-(p-isobutylphenyl)-propionic acid) is a traditional nonsteroidal anti-inflammatory drug (NSAID) [1] that was developed in the late 1960s [2] for the treatment of symptoms caused by arthritis such as swelling, pain, and stiffness [3]. Ibuprofen, like other nonsteroidal drugs such as ketoprofen and flurbiprofen, is widely used for its analgesic, anti-inflammatory, and antipyretic properties [4]. It is used for mild-to-moderate pain such as dysmenorrhea, headaches (including migraine), dental pain, postoperative pain, and pain caused by musculoskeletal/joint disorders, including osteoarthritis, rheumatoid arthritis, and ankylosing spondylitis [5,6]. The mechanism of action of ibuprofen for different therapeutic purposes is well established. Ibuprofen is a non-selective reversible inhibitor of cyclo-oxygenase isozyme (COX)-1 and COX-2, which are responsible for the conversion of arachidonic acid into prostaglandins, including thromboxane and prostacyclin [7]. Due to its chiral center, ibuprofen has two enantiomers [8]. However, it is well documented that the therapeutic activity of ibuprofen is mainly attributable to the (S) enantiomer, which is 160 times more effective than the (R) enantiomer [9]. It has been reported that, in the human body, (R) ibuprofen can undergo "metabolic inversion" to produce (S) ibuprofen [10]. Due to its high patient compliance, cost-effectiveness, reduced sterility constraints, and flexibility, the most common route of administration for ibuprofen is the oral [11] via tablets, caplets, or capsules at 200, 400, or 800 mg strengths [12,13]. The dose is 200–400 mg (5–10 mg/kg in children) every 4–6 h for a maximum of 1.2 g per day in adults [14]. Due to its aqueous solubility of 21 mg/L [15], ibuprofen is a poorly water-soluble drug, characterized by dissolution-limited oral bioavailability [16]. The low rate of dissolution from the currently available solid dosage forms, and the consequent poor

molecules

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Article

Biotransformation of Cortisone with *Rhodococcus rhodnii*: Synthesis of New Steroids

Federico Zappaterra [1], Stefania Costa [1,*], Daniela Summa [1], Valerio Bertolasi [2], Bruno Semeraro [3], Paola Pedrini [1], Raissa Buzzi [1] and Silvia Vertuani [4]

[1] Department of Life Sciences and Biotechnology, University of Ferrara, Via L. Borsari 46, 44121 Ferrara, Italy; federico.zappaterra@unife.it (F.Z.), daniela.summa@unife.it (D.S.), pdp@unife.it (P.P.), raissa.buzzi@unife.it (R.B.)
[2] Department of Chemical and Pharmaceutical Sciences, University of Ferrara, Via L. Borsari 46, 44121 Ferrara, Italy; valerio.bertolasi@unife.it
[3] GATE SRL, Via L. Borsari 46, 44121 Ferrara, Italy; bsemeraro@gategreen.it
[4] Department of Life Sciences and Biotechnology, Master Course in Cosmetic Science and Technology (COSMAST), University of Ferrara, Via L. Borsari 46, 44121 Ferrara, Italy; silvia.vertuani@unife.it
* Correspondence: stefania.costa@unife.it; Tel.: +39-0532-455-708

Abstract: Cortisone is a steroid widely used as an anti-inflammatory drug able to suppress the immune system, thus reducing inflammation and attendant pain and swelling at the site of an injury. Due to its numerous side effects, especially in prolonged and high-dose therapies, the development of the pharmaceutical industry is currently aimed at finding new compounds with similar activities but with minor or no side effects. Biotransformations are an important methodology towards more sustainable industrial processes, according to the principles of "green chemistry". In this work, the biotransformation of cortisone with *Rhodococcus rhodnii* DSM 43960 to give two new steroids, i.e., 1,9β,17,21-tetrahydoxy-4-methyl-19-nor-9β-pregna-1,3,5(10)-trien-11,20-dione and 1,9β,17,20β,21-pentahydoxy-4-methyl-19-nor-9β-pregna-1,3,5(10)-trien-11-one, is reported. These new steroids have been fully characterized.

Keywords: biotransformation; cortisone; *Rhodococcus rhodnii*; steroids

Citation: Zappaterra, F.; Costa, S.; Summa, D.; Bertolasi, V.; Semeraro, B.; Pedrini, P.; Buzzi, R.; Vertuani, S. Biotransformation of Cortisone with *Rhodococcus rhodnii*: Synthesis of New Steroids. *Molecules* **2021**, *26*, 1352. https://doi.org/10.3390/molecules26051352
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1. Introduction

Steroid drugs are used for various therapeutic applications and represent the most marketed drug category after antibiotics, with an annual production of over one million tons. To date, there are about 300 steroid-based drugs on the market, and this is constantly growing [1]. In this field, corticosteroids are a group of hormones produced by the adrenal gland cortex. This type of molecule is used for its anti-inflammatory, immunosuppressive properties and for its effects on metabolism. In particular, steroids belonging to the glucocorticoid class can control the metabolism of carbohydrates, lipids, and proteins, while mineralocorticoids control the concentration of electrolytes and, consequently, the amount of water present in the blood [2].

Glucocorticoids play an important role in anti-inflammatory and immunosuppressive therapy [3] and are widely used in the treatment of allergic reactions and inflammatory and autoimmune diseases, as well as in the prevention of rejection in transplants and the treatment of hematological neoplasia [4].

Unfortunately, the use of these drugs (also known as corticosteroids) has a long series of side effects. On the other hand, it is known that even small structural differences can significantly modify the power and duration of an action.

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Fermentation as a Strategy for Bio-Transforming Waste into Resources: Lactic Acid Production from Agri-Food Residues

Stefania Costa [1,*], Daniela Summa [1], Bruno Semeraro [2], Federico Zappaterra [1], Irene Rugiero [1] and Elena Tamburini [1]

[1] Department of Life Sciences and Biotechnology, University of Ferrara, Via L. Borsari, 46, 44121 Ferrara, Italy; daniela.summa@unife.it (D.S.); zppfrc@unife.it (F.Z.); irene.rugiero@unife.it (I.R.); tme@unife.it (E.T.)
[2] GATE SRL, Via L. Borsari, 46, 44121 Ferrara, Italy; bsemeraro@gategreen.it
* Correspondence: stefania.costa@unife.it; Tel.: +39-0532-455-708

Abstract: Lactic acid (LA) obtained by fermentation of carbohydrates is well-known and widely used in the food sector. This process is as an alternative to the chemical synthesis and ensures several advantages especially in terms of environmental sustainability. In particularly, the opportunity to use agro-food residues as fermentable raw materials could improve the overall process sustainability, without considering the indisputable advantages in terms of waste reduction and residual biomass valorization, in a bio- and circular economy perspective. This research deals with the study and development of the fermentation processes of various waste biomasses from the agro-food industries, including milk whey (MW), ricotta cheese whey (RCW), pear processing residues (PPR), potato pomace (PP), tomato pomace (PT), in order to obtain an experimental protocol applicable to the production of LA. *Lactobacillus casei* DSM 20011 (ATCC 393), a homofermentative L(+)-LA producing bacterium has been used, starting from small-scale tests to verify of the microorganism to grow in complex medium with different carbon sources and the possible presence of potentially toxic substances for microbial growth. Yields from 27.0 ± 0.3% to 46.0 ± 0.7% have been obtained. Then, a scaling-up was performed in a 1 L batch fermenter, using a mixed medium of RCW and PPR in different ratio. The best LA yield was 78.3% with a volumetric productivity of 1.12 g/L·h in less than 60 h.

Keywords: lactic acid; PLA; *Lactobacillus casei*; agri-food waste; circular economy

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1. Introduction

Agri-food residues refer to organic waste that results from the processing and packaging of animal and/or plant products by the agri-food industry [1]. The Food and Agriculture Organization (FAO) estimates that about one-third of food worldwide produced for human nutrition is yearly lost or wasted [2]. Considering the entire supply chain, including agricultural production, for the EU-27 food waste can be quantified in about 129 million tonnes per year, with about 25% accounted for the sole postharvest and manufacturing stages [3]. Such a large quantity poses the question of high costs of management for collection and transportation, as well as the need of adequate treatment facilities. In fact, the Directive (EU) 2018/850 requires to reduce to 10% waste disposal by landfilling and prohibits landfilling for waste that can be recovered in line with the waste hierarchy [4]. In particular, the separate collection of bio-waste and the enhancement of its valorization rates are still a key issue in EU [5]. This ensure that economically valuable waste materials will be reinjected into the EU economy and prevent detrimental consequences for human health and the environment [6].

However, more studies are needed to foster the switch to more sustainable agri-food system and to promote the adoption of circular economy concepts [7]. As it is well known,

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Enzymatic Esterification as Potential Strategy to Enhance the Sorbic Acid Behavior as Food and Beverage Preservative

Federico Zappaterra [1], Daniela Summa [2], Bruno Semeraro [2], Raissa Buzzi [1], Claudio Trapella [3], Miguel Ladero [4], Stefania Costa [1,*] and Elena Tamburini [1]

[1] Department of Life Sciences and Biotechnology, University of Ferrara, Via L. Borsari, 46, 44121 Ferrara, Italy; zppfrc@unife.it (F.Z.); raissa.buzzi@unife.it (R.B.); tme@unife.it (E.T.)
[2] GATE SRL, Via L. Borsari, 46, 44121 Ferrara, Italy; smmdnl@unife.it (D.S.); bsemeraro@gategreen.it (B.S.)
[3] Department of Chemistry and Pharmaceutical Sciences, University of Ferrara, Via Fossato di Mortara, 17, 44121 Ferrara, Italy; trpcld@unife.it
[4] Department of Materials and Chemical Engineering, Chemical Sciences School, Complutense University of Madrid, 28040 Madrid, Spain; mladerog@ucm.es
* Correspondence: cstsfn1@unife.it

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Abstract: Sorbic acid is the most commonly used preservative in the food industry. The antimicrobial inhibition of sorbic acid could be influenced by its lipophilic nature, which reduces its use in hydrophilic food formulations. Reactions between sorbic acid and glycerol catalyzed by lipases were studied in order to develop a novel sorbic acid derivate with a promising hydrophilic profile. The esterification reaction between sorbic acid and glycerol in a solvent-free system were performed with an immobilized lipase B from *Candida antarctica* (CALB). The glycerol sorbate product has been tested against *S. griseus* bacterium and *Saccharomyces cerevisiae* yeast. Results indicate that the esterification of sorbic acid with glycerol does improve its antimicrobial properties against *Saccharomyces cerevisie*. The reported results demonstrate that esterification can be used as a strategy to improve the antimicrobial activity of sorbic acid.

Keywords: sorbic acid; glycerol; esterification; lipase; CALB; antimicrobial

1. Introduction

Sorbic acid is a straight-chain alpha-beta-unsaturated fatty acid first isolated from the oil of unripe rowanberries in 1859 [1]. It acts as inhibitor of most molds, yeast, and some bacteria [2]. Sorbic acid, and especially its more soluble salts, known as sorbates, are used as preservative for food, animal feed, and cosmetic and pharmaceutical products [3]. The antimicrobial action of sorbates occurs at various stages of microbial life cycle (germination, outgrowth, and cell division) [4] and it may result in the alteration of the cell membrane, in the inhibition of transport systems and key enzymes, the creation of a proton flux into the cell, the inhibition of oxidative phosphorylation, or in a synergic effect of two or more of these factors [5,6]. The mode of action of sorbic acid on bacterial cells and spores has been reviewed by Sofos et al. [5], while York et al. reported the sorbic acid inhibition of the yeast *Saccharomyces cerevisiae* [6]. Sorbate acts as a competitive and reversible inhibitor of amino acid-induced germination [7], of several enzyme systems' activity (alcohol dehydrogenase, fumarase, anolase, aspartase, catalase, malate dehydrogenase, alfa-ketoglutarate dehydrogenase, succinic dehydrogenase, and ficin) [8], and of nutrient uptake [9]. The antifungal activity of sorbic acid seems to be related to an interference with the electrochemical membrane potential across the mitochondrial membranes [10]. Potassium sorbate is the most employed salified form of sorbic acid

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Δ1-Dehydrogenation and C20 Reduction of Cortisone and Hydrocortisone Catalyzed by *Rhodococcus* Strains

Stefania Costa [1,*], Federico Zappaterra [1], Daniela Summa [2], Bruno Semeraro [2] and Giancarlo Fantin [3]

[1] Department of Life Sciences and Biotechnology, University of Ferrara, Via L. Borsari, 46, 44121 Ferrara, Italy; federico.zappaterra@unife.it

[2] GATE SRL, Via L. Borsari, 46, 44121 Ferrara, Italy; daniela.summa@unife.it (D.S.); bsemeraro@gategreen.it (B.S.)

[3] Department of Chemical and Pharmaceutical Sciences, University of Ferrara, Via L. Borsari, 46, 44121 Ferrara, Italy; giancarlo.fantin@unife.it

* Correspondence: stefania.costa@unife.it; Tel.: +39-0532-455-708

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Abstract: Prednisone and prednisolone are steroids widely used as anti-inflammatory drugs. Development of the pharmaceutical industry is currently aimed at introducing biotechnological processes and replacing multiple-stage chemical syntheses. In this work we evaluated the ability of bacteria belonging to the *Rhodococcus* genus to biotransform substrates, such as cortisone and hydrocortisone, to obtain prednisone and prednisolone, respectively. These products are of great interest from a pharmaceutical point of view as they have higher anti-inflammatory activity than the starting substrates. After an initial lab-scale screening of 13 *Rhodococcus* strains, to select the highest producers of prednisone and prednisolone, we reported the 200 ml-batch scale-up to test the process efficiency and productivity of the most promising *Rhodococcus* strains. *R. ruber*, *R. globerulus* and *R. coprophilus* gave the Δ1-dehydrogenation products of cortisone and hydrocortisone (prednisone and prednisolone) in variable amounts. In these biotransformations, the formation of products with the reduced carbonyl group in position C_{20} of the lateral chain of the steroid nucleus was also observed (i.e., 20β-hydroxy-prednisone and 20β-hydroxy-prednisolone). The yields, the absence of collateral products, and in some cases the absence of starting products allow us to say that cortisone and hydrocortisone are partly degraded.

Keywords: cortisone; hydrocortisone; biotransformations; prednisone; prednisolone; 20β-hydroxy-prednisone; 20β-hydroxy-prednisolone; *Rhodococcus* spp.

1. Introduction

Steroids are lipids belonging to the terpenes class, and from a chemical-structural point of view they contain a tetracyclic system of carbon atoms (cyclopentanoperhydrophenanthrene). This type of compound is widespread in nature: thousands of steroids have been identified in living systems. Over 250 sterols and related compounds have been reported to occur in plants (e.g., phytosterols, diosgenin, and brassinosteroids), insects (e.g., ecdysteroids), vertebrates (e.g., cholesterol; corticosteroids: glucocorticoids, mineralocorticoids; sex hormones: androgens, estrogens; bile acids, vitamin D; and neurosteroids), and lower eukaryotes: yeasts and fungi (e.g., ergosterol and ergosteroids) [1–3].