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Isomerase Activity of Candida rugosa Lipase in Conversion of Racemic Ibuprofen to (S)-Ibuprofen

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ISOMERASE ACTIVITY OF *CANDIDA RUGOSA* LIPASE IN CONVERSION OF RACEMIC IBUPROFEN TO (*S*)-IBUPROFEN

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Dedication

This dissertation is dedicated
to THE ONE and THE ONLY ONE whom we are all from him and will return to him,
to my parents Nahid Rahmani and Hosein Mortazavi,
to my sister and brother, Forough and Hasan;
to my husband, Jalal Rastegary;
to my children, Hosna and Amir Mohammad;
and to my research advisor, Dr. James M. Salvador.

ISOMERASE ACTIVITY OF *CANDIDA RUGOSA* LIPASE IN CONVERSION OF RACEMIC IBUPROFEN TO (*S*)-IBUPROFEN

By

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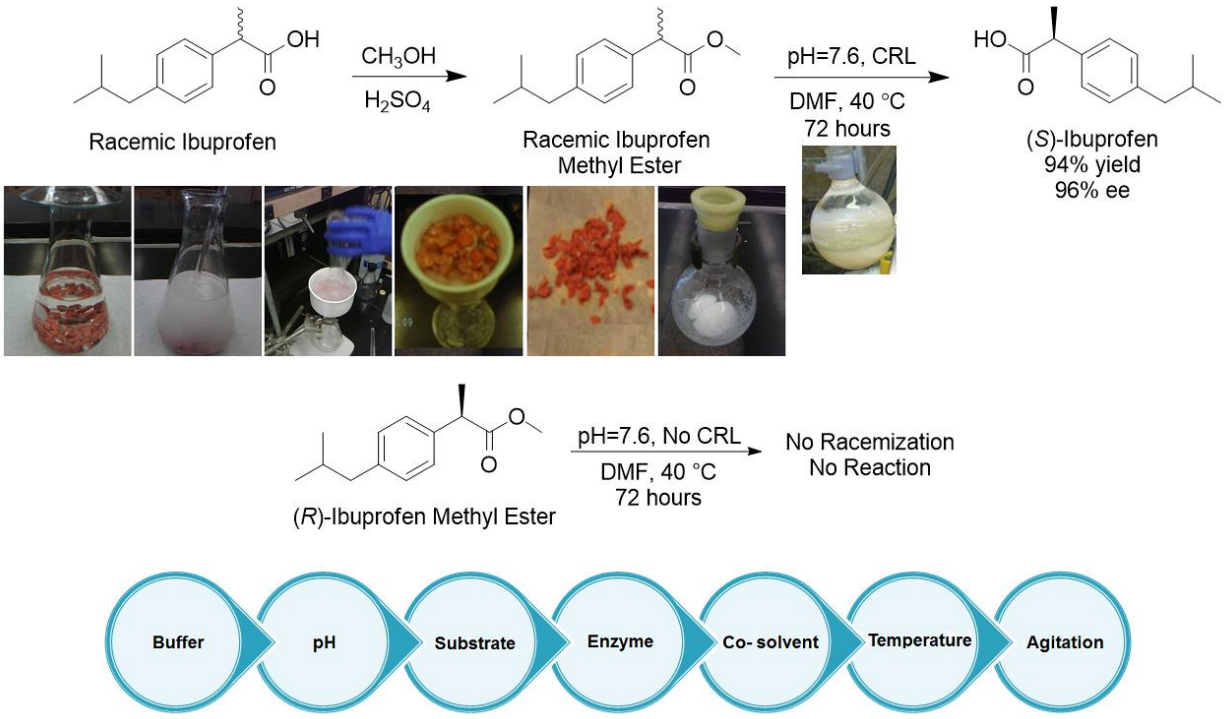
Abstract

The *Candida rugosa* lipase-catalyzed Dynamic Kinetic Resolution of racemic ibuprofen methyl ester was optimal at pH 7.6 to produce (*S*)-ibuprofen in 72 hours. The concentration of various buffers for these reactions ranged from 0.2 to 0.5 M. The commercial lipase was found to be acidic altering the final pH of the reaction mixtures. Dimethylformamide co-solvent maintained the reaction pH better than dimethylsulfoxide, with evidence of the latter functioning as an oxidizing agent. Lower concentrations of ibuprofen methyl ester and higher stirring rates led to faster conversions. The minimal amount of lipase needed was 20 mg/mL buffer. Reaction of (*R*)-ibuprofen methyl ester under the optimized conditions excluding the lipase led to no racemization, demonstrating that the conversion of (*R*)-ibuprofen methyl ester to (*S*)-ibuprofen is catalyzed by the enzyme.



Ibuprofen Extraction, Esterification, and Conversion of Racemic Ibuprofen Methyl Ester to (*S*)-Ibuprofen

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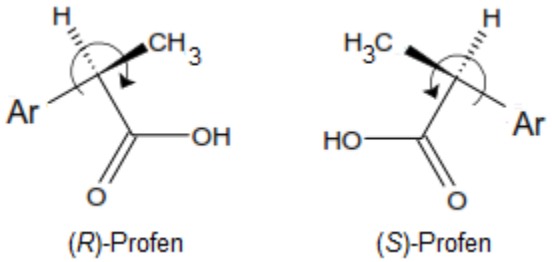
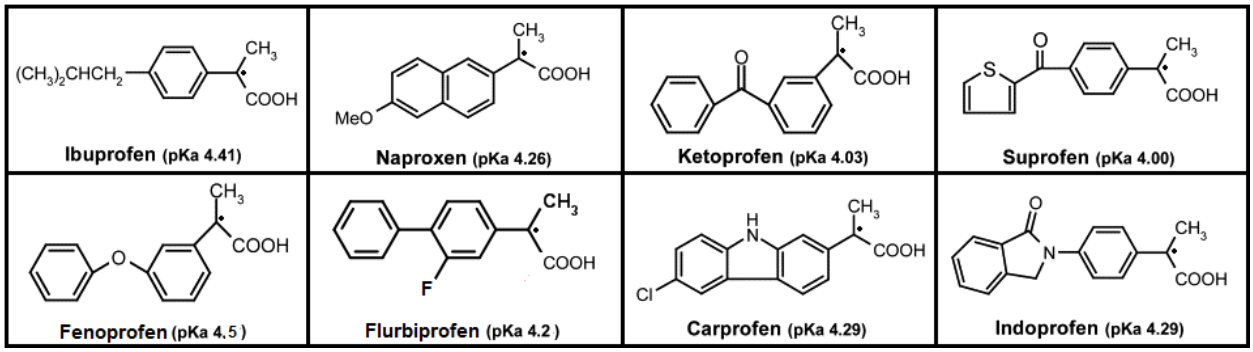
Chapter 1 : Introduction

Biological activity and absolute chemical configuration of a drug are tightly related to each other, where one enantiomer of a racemic drug might show the desired effect, while the other enantiomer might cause side effects or even be inactive [1]. For example, (*S*)-naproxen and (*S*)-ibuprofen are used as anti-inflammatory, antipyretic analgesics to treat arthritis and headache while (*R*)-naproxen and (*R*)-ibuprofen cause side effects such as gastrointestinal disorders [2]. Profens or 2-arylpropionic acids (Schemes 1.1 and 1.2) are a subclass of non-steroidal anti-inflammatory drugs (NSAID), and NSAID in general may induce gastric injury [3]. Profens have the desired anti-inflammatory effect associated with the (*S*)-form. The (*S*)-enantiomer of naproxen and ibuprofen are 28 and 100 times, respectively, more active than the undesired (*R*)-enantiomer. Currently, naproxen and flunoxaprofen are the only profens marketed as pure enantiomers [4].



Scheme 1.1 Generic Racemic Profen Structure



Scheme 1.2 Different Profen Structures [5]

Racemic mixtures can be resolved chemically through diastereomeric salt crystallization processes [6], enzymatically [7] or by using preparatory chiral chromatography columns; however, separation of racemic drugs is a multi-step process and is time consuming [1]. The demand for single enantiomer pharmaceuticals is increasing every year (Figures 1.1 and 1.2). Currently, more than 50% of organic pharmaceuticals are chiral and are produced by chemical syntheses that may be environmentally unfriendly [8] especially considering that half of a racemic mixture may be discarded. Although the principal use of enzymes is to generate optically active drugs, developing environmentally friendly methods to obtain effective single enantiomer drugs merits further attention.

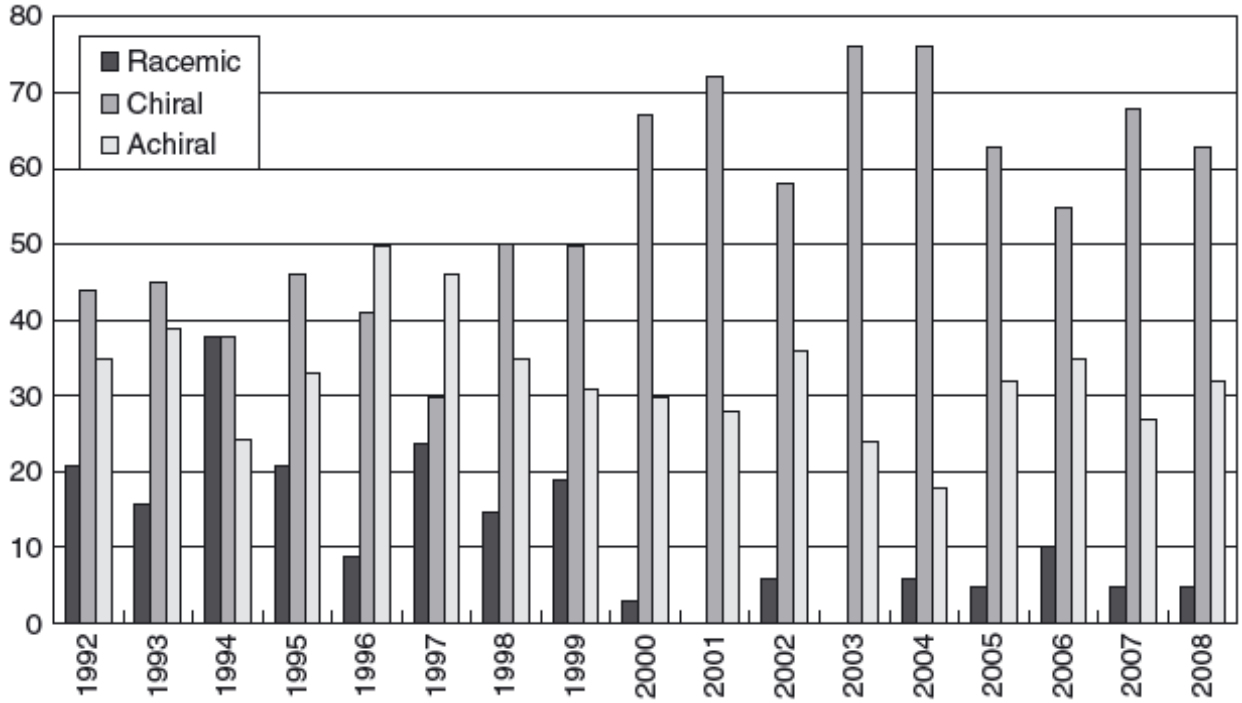


Figure 1.1 Annual Distribution of FDA-approved Drugs [9]

Rank	Product	Active Ingredient	Form of Ingredient
1	Lipitor	Atorvastatin	Single enantiomer
2	Nexium	Esomeprazole	Single enantiomer
3	Plavix	Clopidogrel	Single enantiomer
4	Advair Diskus	Fluticasone salmeterol	Single enantiomer, racemate
5	Seroquel	Quetiapine	Achiral
6	Abilify	Aripiperazol	Achiral
7	Singular	Montelukast	Single enantiomer
8	OxyContin	Oxycodone	Single enantiomer
9	Actos	Pioglitazone	Racemate
10	Prevacid	Lansoprazole	Racemate

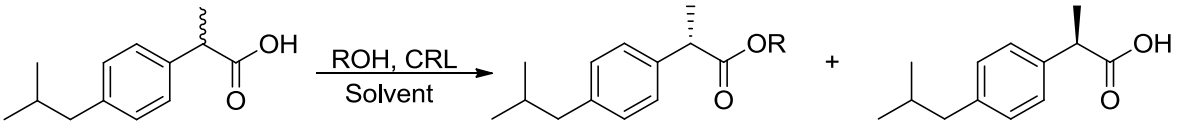
Figure 1.2 Figure Top 10 Best Selling Drugs in the U.S.A [9]

Thomason *et al.* [10] reported that the fungus *Verticillium lecanii* is capable of inverting the chirality of 2-arylpropionic (ibuprofen, ketoprofen, indoprofen, suprofen, flurbiprofen, and fenoprofen) from (*R*) to (*S*)-forms with the exception of ketoprofen. This phenomenon also is observed in mammalian systems including humans.

Isolated enzymes can be used to separate enantiomers of racemic mixtures [7] or to convert racemic mixtures to a single enantiomer [11] where usually one of the enantiomers has a higher preference for an enzyme than the other enantiomer. For instance, an enzyme can selectively esterify one enantiomer of a racemic carboxylic acid mixture or enantio-selectively hydrolyze one enantiomer of a racemic ester mixture to allow the reacted and unreacted enantiomers to be separated. Both these processes are called Kinetic Resolutions (KR). In KR, two enantiomers react at different rates with a chiral catalyst resulting in an enantiomerically enriched product and a leftover reactant.

1.1 Enantio-selective Esterification of Profens Using *Candida* Lipase

Mustranta *et al.* [12] reported the esterification of racemic ibuprofen in different organic solvents using *Candida rugosa* lipase (CRL) and a high enantio-specificity with primary alcohols was observed while yields with secondary alcohols were much lower than primary alcohols and no esterification with tertiary alcohols was observed (Scheme 1.3). CRL was active only in very hydrophobic solvents, and the activity of the lipase was reduced significantly by adding water. Kim *et al.* [13] described the esterification of racemic ibuprofen using CRL in cyclohexane and a 16-fold higher reaction rate and yield was achieved by employing 5% (w/v) silica gel as solid supports, and 3% (v/v) additional water. Hongwei *et al.* [14] reported CRL-catalyzed esterification of ibuprofen with 1-propanol in seven ionic liquids and the results were compared with a control reaction in isooctane, whereas in six ionic liquids the enzyme enantio-selectivity was poor, and only [BMIM]PF_6 was suggested to be used.

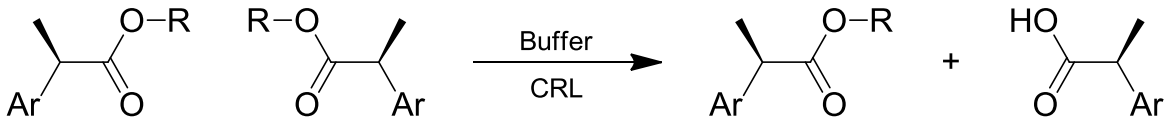


Scheme 1.3 Esterification of Racemic Ibuprofen and Unreacted (*R*)-Ibuprofen

Ducret *et al*. [15] reported the esterification of ibuprofen catalyzed by *Candida antarctica* lipase (type B) with hydrophobic solvents that allowed higher enzyme activity than hydrophilic solvents. However, better enantio-selectivity was obtained in hydrophilic solvents. CRL-catalyzed esterification of flurbiprofen with *n*-butanol is also reported by Bhandarkar [16].

1.2 Enantio-selective Hydrolysis of Profen Esters Using *Candida* Lipase

Lee *et al.* [17] tested the addition of a small percentage of 15 polar organic co-solvents to the hydrolysis reaction of racemic ibuprofen methyl ester (IME) (Scheme 1.4). The co-solvent that best enhanced the CRL enantio-selectivity and reactivity was dimethylformamide (DMF).



Scheme 1.4 KR of Racemic Profen Esters to (*S*)-Profens and Unreacted (*R*)-Profen Esters

Madhav *et al.* [18] examined KR of racemic IME in isooctane using CRL in phosphate buffer as a biphasic system. They reported obtaining a low conversion due to a low interfacial surface area. They tested different co-solvents to increase the reaction rate, and the best one was found to be isooctane along with 37 ℃, a pH of 7.0, and 250 revolutions per minute (rpm) as optimized reaction conditions. (*R*)-IME was inert during the hydrolysis while racemic IME inhibited the reaction. They also found that by increasing the methanol concentration, the rate of enzymatic hydrolysis decreased.

Long *et al*. [19] reported KR of racemic ibuprofen esters in a biphasic enzymatic hollow fiber membrane that operated as a continuous stirred tank reactor. CRL in phosphate buffer was immobilized on a spongy layer and isooctane containing the substrate flowed in the shell side of the membrane (Figure 1.3). The immiscible organic aqueous interface formed in the spongy region of the membrane, and this interfacial area with the immobilized lipase provided a catalytic surface layer for the enzymatic hydrolysis reaction at 40 ℃ to give an enantiomeric ratio [20] (*E*, the ratio of the rate constant for each enantiomer) of 13, and enantiomeric excesses (ee) of 31 and 85% for the substrate and product, respectively. However, the diffusion of substrate and product to and from the enzyme membrane interface where the reaction occurred was rate-limiting.

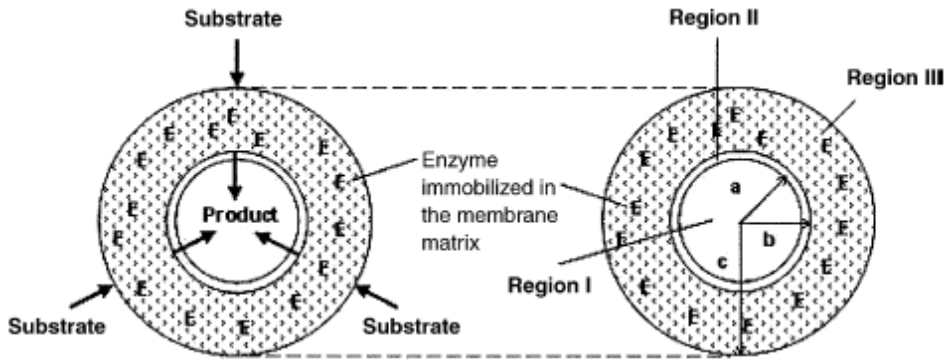
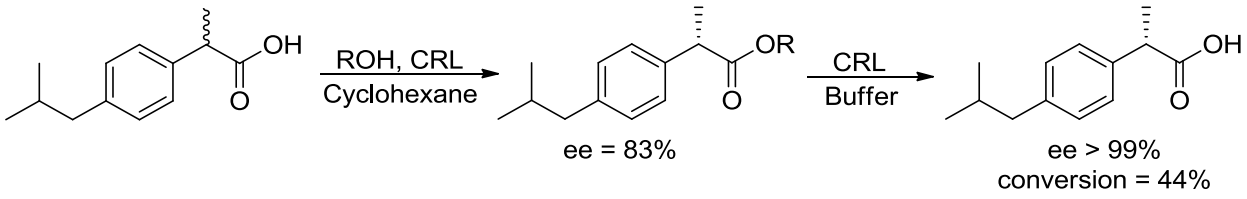


Figure 1.3 KR in a Semipermeable Hollow Fiber Membrane [19]

Zhu *et al*. [21] reported immobilized CRL covalently bound to a pH sensitive support to hydrolyze 2-chloroethyl ester of ketoprofen with 1.5 fold higher activity and 8.7 fold higher enantio-selectivity compared to those of free lipase. Ammazzalorso *et al.* [22] described CRL-catalyzed KR of 3 different racemic 2-substituted aryloxyacetic esters in phosphate buffer containing dimethylsulfoxide (DMSO) and isopropanol co-solvents at 37 ℃ with evidence that for 2-(*p*-chlorophenoxy) acetic acid and 2-*n*-butyl-2-(*p*-chlorophenoxy) acetic acid ethyl esters, DMSO enhanced enzyme enantio-selectivity more than isopropanol. However, both co-solvents moderately improved the lipase enantio-selectivity for 2-phenyl-2-(*p*-chlorophenoxy) acetic acid ethyl ester. More CRL mediated KR of racemates has also been examined and reviewed [23-25].

David Chavez *et al.* [26] in their comprehensive research reported 2 consecutive CRL-catalyzed KR reactions of ibuprofen by first enantio-selectively esterifying racemic ibuprofen with decan-1-ol (in 48 hours) and butan-1-ol (in 96 hours) in cyclohexane with almost 50% conversion to enriched (*S*)-ibuprofen esters and ee of 95 and 69%, respectively. Then upon the enantio-selective hydrolysis of the (*S*)-ibuprofen decyl ester in aqueous phosphate buffer, the reaction was twice as slow as that of the (*S*)-ibuprofen butyl ester because the decyl ester was less soluble. Overall, ibuprofen was isolated in three steps (KR of racemic ibuprofen, separation of unreacted

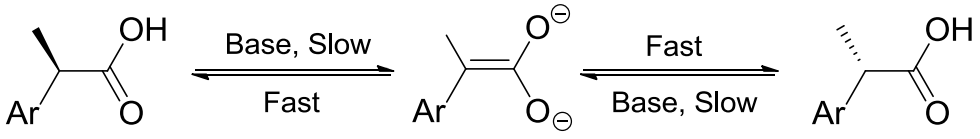
ibuprofen, and enantio-selective hydrolysis of the enriched (*S*)-ibuprofen decyl ester) in 44% yield with an ee greater than 99% (Scheme 1.5).



Scheme 1.5 Esterification of Racemic Ibuprofen to (*S*)-Ibuprofen Ester and Hydrolysis of the Enriched (*S*)-Ibuprofen Ester to (*S*)-Ibuprofen

1.3 Disadvantages of Kinetic Resolution (KR) Reactions

The KR method has the disadvantage of limiting the theoretical yield to a maximum of 50% for the (*S*)-acid enantiomer due to production of 50% (*R*)-acid waste [4, 27-29] (Schemes 1.3 and 1.4). In order to reduce waste, cyclic resolution has been applied to the (*R*)-ibuprofen to racemize this undesired enantiomer (Scheme 1.6) under highly basic conditions and high temperatures. This process is called "Brute Force Racemization" and is followed by resolution of the racemate by enantio-selective esterification of the (*S*)-ibuprofen to its ester to leave behind the unreacted (*R*)-ibuprofen for the next run of racemization [30].



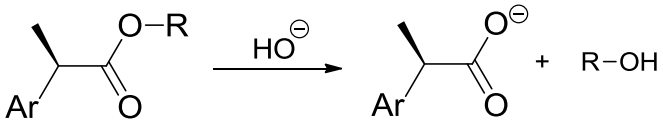
Scheme 1.6 Brute Force Racemization of Profens

1.4 Racemization of Ibuprofen and Ibuprofen Ester Enantiomers

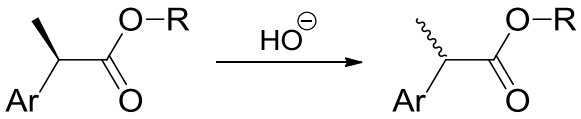
Ibuprofen enantiomers did not racemize at room temperature in 1.25 M NaOH [30], and were also stable in acidic media [4] (up to 3.76 M) [30]. However, the (*R*)-ibuprofen racemization rate (Scheme 1.6) increased at elevated temperatures of 100℃ or above and at higher basic conditions while no racemization occurred in the absence of NaOH [4, 27, 30]. DMF decreased

the racemization rate while DMSO increased it [30]. The higher the DMSO concentration in basic media, the higher the rate of racemization was, but without any base (*R*)-ibuprofen was stable in pure DMSO or DMSO-water mixtures. However, without DMSO under basic conditions only 6% racemization occurred [30].

Xie *et al.* [30] reported that hydrolysis of (*S*)-ibuprofen butyl ester in basic aqueous media (saponification, Scheme 1.7) was slow due to the ester insolubility in water, so the ester was dissolved in a hydrophilic co-solvent, ethanol, to increase the ester solubility in the media. However, (*S*)-ibuprofen could not be obtained using a base-catalyzed hydrolysis because the ester racemized before being saponified (Scheme 1.8). Therefore, the best solvent was determined to be DMSO for complete hydrolysis of (*S*)-ibuprofen butyl ester at 100℃ in 3.76 M H_2SO_4 for 2 hours without any racemization of the ester or product. In a similar work by Liu *et al.* [4], (*S*)-ibuprofen propyl ester was converted to (*S*)-ibuprofen with 1.0 M H_2SO_4 acid-catalyzed hydrolysis in a DMSO-water mixture at 120℃ for 24 hours with no racemization of the ester or product.



Scheme 1.7 Saponification of Profen Esters



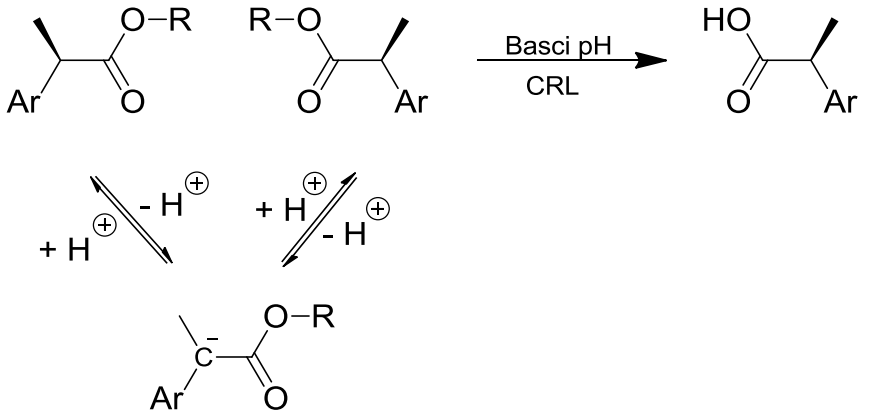
Scheme 1.8 Racemization of Profen Esters

1.5 Dynamic Kinetic Resolution (DKR) Reactions

In-situ isomerization followed by a stereo-selective conversion of one of the isomers leading to a major product is referred to as a Dynamic Kinetic Resolution (DKR) [31, 32]. This process has been applied to large classes of compounds such as secondary [31, 33], allylic [34], and allenyl [35, 36] alcohols [37] ; aldehydes [38]; primary [39, 40] and secondary [41] amines; hydroxy amides [42], amino acid derivatives [43, 44], amino esters [45, 46], azlactones [47], benzyl ketones [48], esters [49], hydroxyl nitriles [50], and hydroxy alkyl sulfones [51]. DKR has also been used to convert racemic profens (2-arylpropanoic acids) to (*S*)-profens, which have the desired anti-inflammatory effect associated with this enantiomer [4, 27, 52], by chemical [53, 54] and enzymatic means [55]. In particular, *Candida rugosa* lipase has been used to produce enriched ibuprofen [28, 29, 56], naproxen [56-59], fenoprofen [60], suprofen [61, 62], and ketoprofen [63] via hydrolysis of various ester derivatives under basic conditions.

1.6 DKR Reactions of Ibuprofen Using *Candida rugosa* lipase (CRL)

Cyclic resolution is a cumbersome and inefficient way to overcome the 50% unused (*R*)-enantiomer. Therefore, a relatively new process called Dynamic Kinetic Resolution (DKR) [31, 32], which attempts to convert the undesired (*R*)-ibuprofen ester to the (*S*)-ibuprofen ester [presumably by *in situ* enolization-racemization and hydrolysis of the (*S*)-ester to (*S*)-ibuprofen], was recently developed (Scheme 1.9).



Scheme 1.9 Proposed DKR Mechanism of Racemic Profen Esters to (*S*)-Profens

The disadvantage of a 50 % maximum theoretical yield led to Fazlena *et al.'s* work [28] which determined the optimum conditions for the DKR of the ibuprofen ethoxyethyl ester, using immobilized CRL at 45 ℃, 20% DMSO in biphasic aqueous isooctane mixture, and a low ester concentration. They examined the impact of base and reported that adding more NaOH (up to 0.5 M) enhanced the hydrolysis, but at certain higher concentrations in isooctane, the hydrolysis slowed down. Also, higher concentrations of the ester generated more (*S*)-ibuprofen product causing enzymatic inhibition and the generated alcohol byproduct reduced the activity by inhibiting the enzyme. They concluded that the (*S*)-ibuprofen synthesis via DKR highly depends on the reaction media, balancing the need for a higher pH for racemization with not too high of a pH for enzyme activity. David Chavez *et al.* [29] in 2010 implemented DKR of racemic ibuprofen methyl ester, using CRL and 20% DMSO in pH 9.8 aqueous $NaHCO_3$ buffer, to obtain (*S*)-ibuprofen in a 94% yield with a 94% ee thus overcoming the 50% maximum yield of KR.

1.7 KR and DKR Reactions of Naproxen Using CRL

Many research groups have worked on CRL-catalyzed KR of naproxen methyl ester (NME). Lee *et al.* [64] reported that acetone treated lipase was more enantio-selective than crude CRL toward the hydrolysis, yet their conversion was only 38%. Xin *et al.* [52] conducted the KR in water saturated isooctane versus a polar hydrophobic water saturated ionic liquid (1-butyl-3-methyl imidazolium hexafluoro phosphate) with an improved conversion from 4 to 31%, respectively. Takac *et al*. [65] reported KR of racemic NME and the enhancement of biocatalytic activity of CRL immobilized on hydrophobic resin Amberlite XAD 7 for hydrolysis in biphasic aqueous isooctane medium at pH 6 and 45 ℃ giving a 49% conversion to (*S*)-naproxen with an *E* of 174.2. They also hydrolyzed NME in an aqueous isooctane biphasic system with maximum 50% conversion using CRL [66]. In a later work [67], under the same reaction conditions with CRL treated at pH 7.5 they only achieved 36% conversion because alcohol treatment of CRL inhibited the lipase for (*S*)-naproxen production. To prevent methanol inhibition in CRL-catalyzed hydrolysis of NME, Xu et al. [68] reported eliminating the problem by introducing methanotrophic bacteria to the hydrolysis reaction which converted methanol into water and CO_2. Both the activity and stability of lipase was improved by methanol removal by the bacteria.

Lin *et al.* [58] developed (*S*)-naproxen production with lipase-catalyzed DKR from racemic naproxen 2,2,2-trifluoroethyl ester with continuous *in situ* racemization of the (*R*)-ester in isooctane and 1,5,7-triazabicyclo [4,4,0]dec-5-ene (*p*-TBD) as an enolizing organic base with a yield of 75% but only a 58% ee of product over 600 hours. In a similar work, Xin *et al.* [59] obtained 60% conversion on DKR of NME in aqueous isooctane biphasic media via CRL hydrolysis and *in situ* racemization by utilizing silicon rubber tubing as a membrane to separate the racemization from the bio catalytic resolution process.

1.8 KR and DKR Reactions of Naproxen Using Other Biocatalysts

Other research groups have examined KR of NME using other biocatalysts such as Carboxyl esterase NP, which possesses more stereo-specificity on (*S*)-NME. For example, Quax *et al.* [69] worked on racemization of (*R*)-NME using diazabicyclo[5.4.0]undec-7-ene (DBU) and Tween 80 to enhance the solubility of the ester substrate. However, Carboxyl esterase NP was inhibited (not toxified) by the acid generated during the hydrolysis [1, 8] due to (*S*)-naproxen reaction with the NH_2 group of the enzyme lysine moiety. To increase the enzyme resistance, site directed mutagenesis was implemented by muting all lysine residues and replacing them with glutamine.

In another cyclic resolution attempt, Steenkamp *et al.* [1] resolved and hydrolyzed racemic NME to (*S*)-naproxen with twice the yield previously obtained using CRL, after 5 hours, by modifying Carboxyl esterase NP with 1% formaldehyde to block the lysine side chain in phosphate buffer and in the presence of 1% Tween 80. The stabilized enzyme gave a 44% yield of (*S*)-naproxen as opposed to a 21% conversion with the untreated enzyme, although a high concentration of the acid product inhibited the enzyme and the generated methanol lowered the reaction rate.

Liu *et al.* [8] implemented a solid-water biphasic system for cyclic resolution of NME using sodium methoxide (DBU was difficult to remove in the work up) by applying mechanical grinding to obtain substrate dispersion and medically pure (*S*)-naproxen with 39% conversion. The enzyme activity decreased at a pH below 7.0 and temperatures above 50 ℃. To solve the enzyme inactivation problem, they auto titrated with aqueous ammonia to maintain a pH of 8.5 and to generate the ammonium salt of (*S*)-naproxen that was harmless to the enzyme.

Finally, Effenberger *et al*. [70] hydrolyzed racemic naproxen amide to enantiomerically pure (*S*)-naproxen in water-saturated organic solvents (butyl acetate with 3% DMSO as co-solvent) using immobilized cells of the bacterial strain *Rhodococcus erythropolis* MP50. After 45 hours they obtained a 48% conversion with 99% ee.

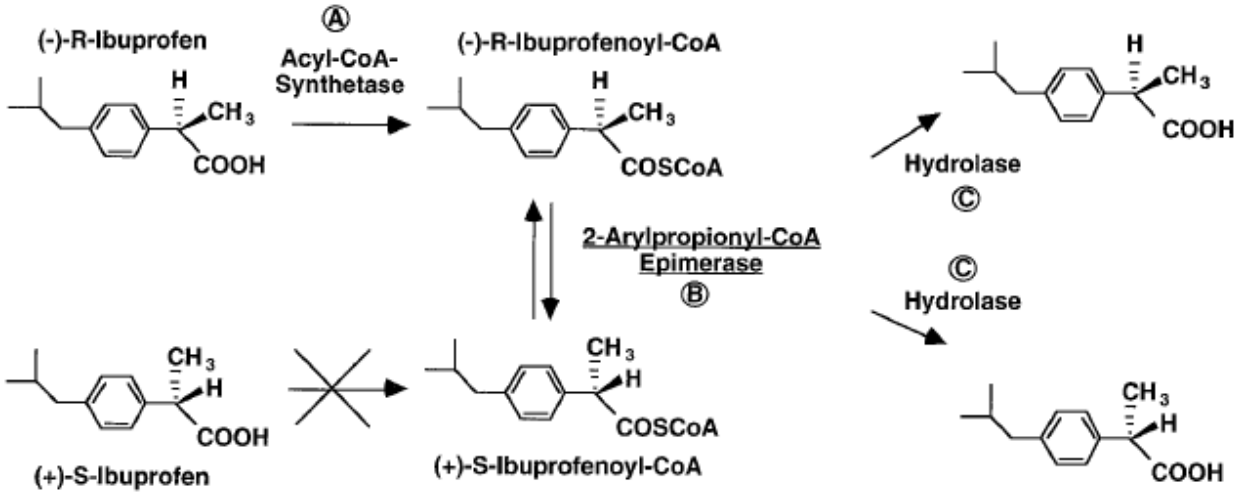
1.9 KR and DKR Reactions of Profen Thio-esters

Racemic profens have been used as substrates in enantio-selective thio-esterifications with lipases. Thio-ester derivatives of profens also have been used in enantio-selective hydrolyses or thio-trans-esterifications employing the same biocatalysts in the presence of organic solvents [56, 57, 60-62]. Racemic thio-esters are employed because they contain an electron withdrawing sulfur that promotes enolate formation at the acidic α carbon and subsequently helps the production of the desired enantiomer. For example, the enhancement of enzyme activity when activated naproxen thio-esters were the substrates is reported by Chang *et al*. [56]. They noted that unlike other lipases, CRL may discern the sulfur and yields lower enzyme activity compared to other corresponding oxygen containing analogues.

A CRL-catalyzed thio-trans-esterification on (*R,S*)-naproxen thio-ester with 4-morpholine ethanol under *in situ* racemization of the (*R*)-thio-ester with trioctylamine in isooctane giving a 65% yield and 95% ee for the desired (*S*)-ester product is reported by Chang *et al*. [71]. They also examined the alcohol inhibition where the initial hydrolysis rate and the yield of the undesired (*S*)-naproxen were suppressed by increasing the alcohol concentration or employing an immobilized lipase containing less water than the free enzyme. In a similar work, Cheng *et al.* [72] reported a CRL-catalyzed thio-trans-esterification on (*R,S*)-naproxen 2,2,2-trifluoroethyl thio-ester with 4-morpholineethanol in isooctane to produce the (*S*)-naproxen ester prodrug with 90% ee by controlling the initial water and alcohol concentration considering the hydrolysis and esterification

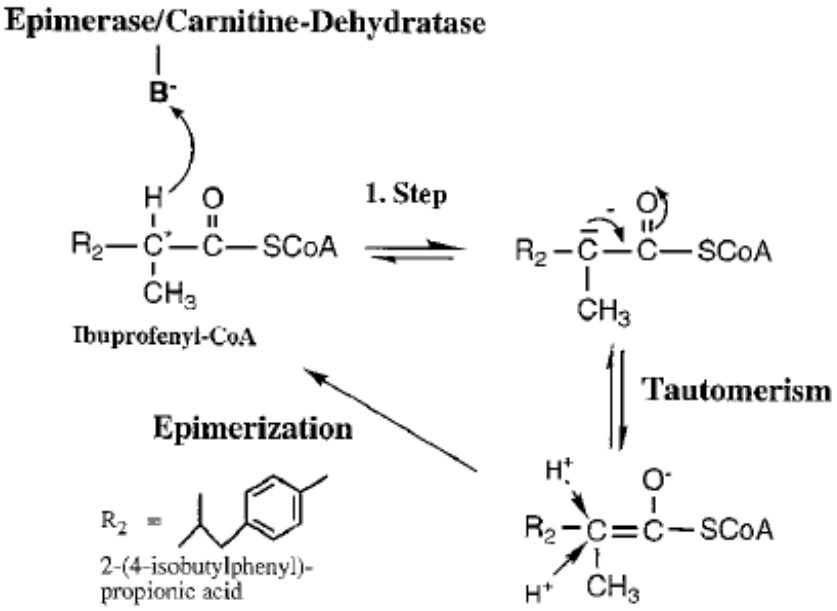
side reactions. Tsai *et al.* [73] also reported the synthesis of chiral 4-morpholino ethyl (*S*)-naproxen ester prodrug from racemic naproxen using biocatalyst lipase MY in cyclohexane as organic solvent.

In a biology oriented work [74], Reichel *et al*. isolated and identified the gene structure of rat 2-arylpropionyl CoA epimerase (a cytosolic and mitochondrial enzyme) and described cloning, sequencing, and expression of the enzyme in Escherichia coli. A pathway for metabolizing 2-arylpropionic acids is chiral inversion of the inactive (*R*)-enantiomers to the (S)-enantiomers catalyzed by 2-arylpropionyl CoA epimerase. The 3-step mechanism encompasses stereo-selective thio-esterification of (*R*)-ibuprofen with acyl CoA synthetase, a microsomal and mitochondrial enzyme, (Scheme 1.10.A) to form an adenylates intermediate of (*R*)-enantiomer. The stereo-selective step of the inversion involves epimerization of (*R*)-ibuprofenoyl CoA thio-ester to the (*S*)-form via 2-arylpropionyl CoA epimerase (Scheme 1.10.B), followed by non-stereo-selective hydrolysis of the thio-esters to (*S*)-ibuprofen (Scheme 1.10.C).



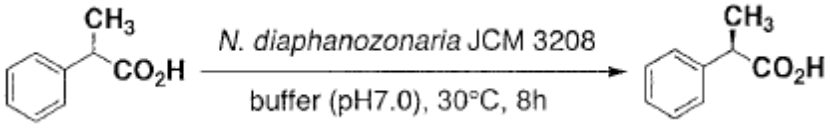
Scheme 1.10 Proposed Mechanism of Chiral Inversion of 2-Arylpropionic Acids [74]

The epimerization mechanism of 2-arylpropionyl CoA epimerase (Scheme 1.11) is a reversible dehydration of substrate which is a common feature of carnitine dehydratase in a similar pathway in which the dehydratase has affinity for CoA linked thio-ester substrates possessing an acidic α carbon. Therefore, they proposed that carnitine dehydratase/epimerase could deprotonate the acidic α carbon of the (*R*)-ibuprofenoyl CoA in the active site of the epimerase that leads to a change of the methyl group configuration, and the resulting carbanion may be reprotonated by solvent.



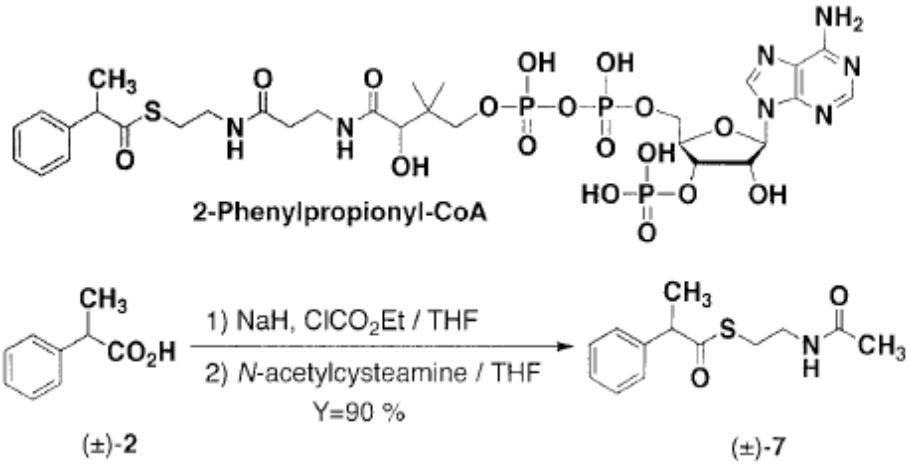
Scheme 1.11 Proposed Mechanism of the Metabolic Inversion of 2-arylpropionic Acids [74]

Mitsukura *et al*. [75] reported synthesis of (*R*)-2-phenylpropanoic acid from racemic acid through isomerization by resting cells of *Nocardia diaphanozonaria* JCM3208 with 92% ee and 92% molar conversion yield (Scheme 1.12).



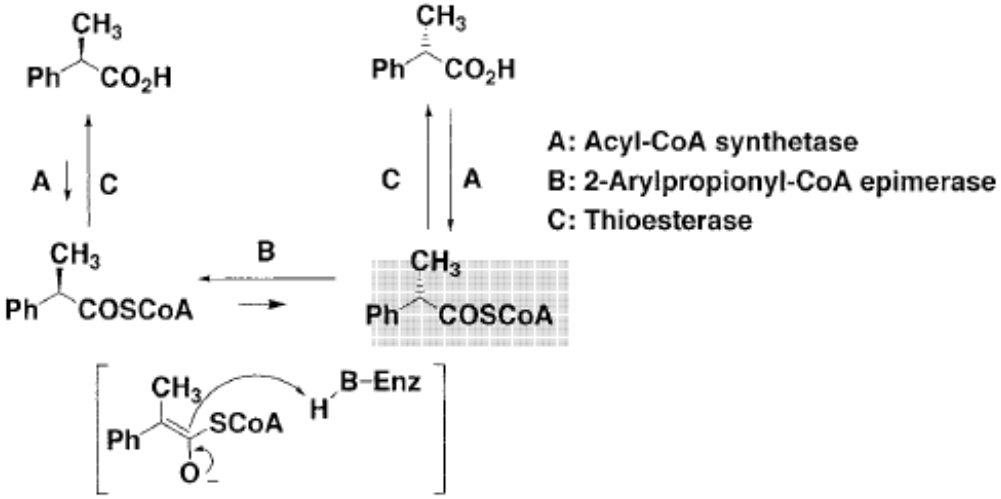
Scheme 1.12 Synthesis of (*R*)-2-Phenylpropanoic acid [75]

To elucidate the isomerization mechanism, they synthesized racemic 2-acetylamino ethyl-2-phenylthio propanoate (7) from racemic 2 as a substitute of a 2-phenylpropionyl CoA (Scheme 1.13).



Scheme 1.13 Synthesis of S-(2-acetylaminoethyl)-2-phenylthiopropanoate [75]

Because the mechanism of racemization of 2-arylpropanoic acids in various species of mammalian liver and microorganisms is almost the same, they proposed similar mechanisms for isomerization in *Nocardia diaphanozonaria*, which are the formation of a CoA thio-ester of 2-arylpropanoic acid by acyl CoA synthetase, the epimerization by 2-arylpropionyl CoA epimerase and the hydrolysis of 2-arylpropionyl CoA by thio-esterase (Scheme 1.14).



Scheme 1.14 Isomerization Reaction Mechanisms of *N. diaphanozonaria* [75]

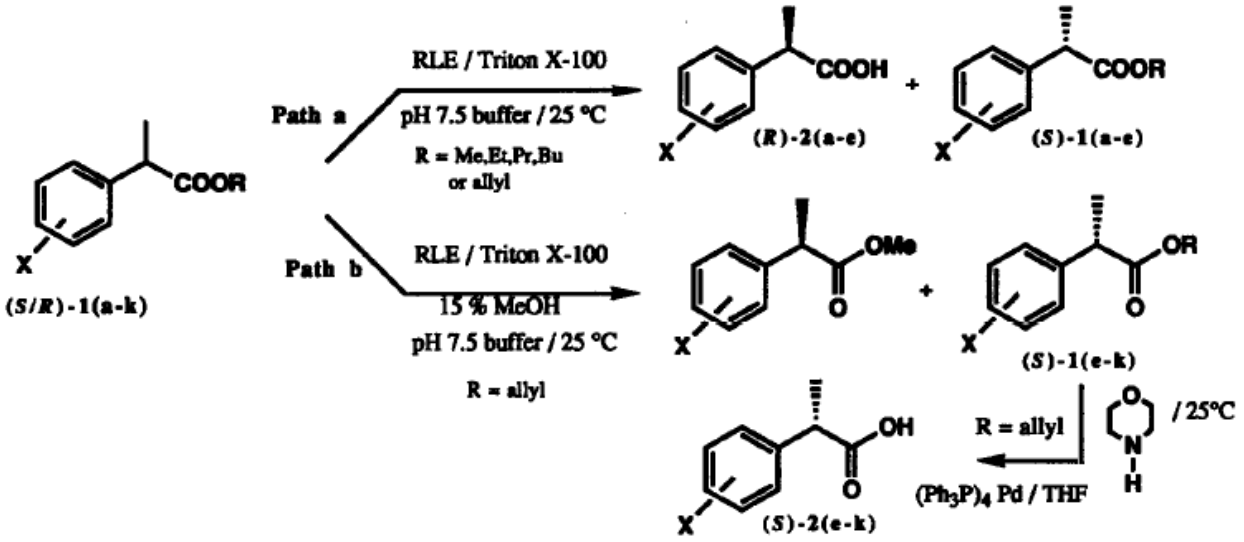
1.10 Biocatalysts and *Candida rugosa* Lipase

Enzymes are powerful, benign, and environmentally friendly biocatalysts in organic synthesis. They can be classified based on their substrate specificity or by sequence alignments which are related to their properties e.g., stereo-selectivity, pH, optimal temperature, and solvent stability [76]. Enzymes can be used in hydrophobic organic solvents at the expense of a lower reaction rate as discovered by Klibanov in the 1980s. However, many lipases, proteases and acylases can maintain their activity even in anhydrous organic solvents [77] although lipases like *Candida Antarctica, Rhizomucor Miehei,* and *Fusarium solani pisi* are insoluble in organic solvents [78]. Hydrolases, namely lipases and proteases, are the most extensively used enzymes in organic synthesis because they are capable of hydrolyzing chiral esters in water, substrate specific, commercially available at low cost, display high stereo-selectivity, and do not require coenzymes [79, 80].

Two major classes of hydrolases are lipases (triacylglycerol hydrolases, e.g., CRL) and esterases (microbial carboxyl ester hydrolases, e.g., Carboxyl esterase NP). Both classes have been discovered, overexpressed, and are capable of catalyzing formation, cleavage, or transfer of the ester functional group, while both are efficient, stable, active in organic solvents, and highly specific for KR of profens. Lipases catalyze reactions of water insoluble substrates (typically triglycerides composed of long-chain fatty acids), and the presence of the water lipid interface is a prerequisite for an efficient catalysis, whereas esterases work on soluble substrates and hydrolyze simple esters (e.g. ethyl acetate) [8, 76].

Esterases are available in animals, plants and microorganisms and show high regio and stereo-specificity which makes them suitable biocatalysts for production of optically pure compounds. They do not require cofactors and are active in organic solvents. The large diversity

of reactions and substrates handled by esterases in nature is still poorly explored. The best studied enzyme among esterases is Carboxyl esterase NP (NP from naproxen), capable of catalyzing naproxen and other 2-arylpropionic acids production with high enantio-selectivity. Carboxyl esterase NP has a molecular mass of 32 kDa, an optimal pH between 8.5 and 10.5 and an operating temperature of 35 to 55 ℃, and is produced as intracellular protein while its structure is unknown [76]. As an example of esterases catalytic power, Senanayake *et al.* [81] resolved (*R*)-2-arylpropanoic acid from (*S*)-2-arylpropanoic acid by enzymatic hydrolysis using rabbit liver esterase (Scheme 1.15). The (*R*)-enantiomer was obtained by enzymatic hydrolysis of the ethyl ester and the (*S*)-enantiomer by trans-esterification of ally propionates to their methyl esters.



Scheme 1.15 Resolution of Racemic 2-arylpropanoic acid by Rabbit Liver Esterase [81]

Lipases are widely distributed in plants, mammals, and microorganisms. Their function is to hydrolyze lipids (triglycerides), and to resolve racemic acids and alcohols. Furthermore, the extracellular microbial lipases catalyze the hydrolysis of natural esters of glycerol and cholesterol as well as the hydrolysis and trans-esterification of unnatural esters as precursors to pharmaceuticals, agrochemicals, and other synthetic targets [82]. Other applications are as industrial catalysts (e.g., detergent industry), in organic syntheses; and in the oils, fats, dairy,

leather, and paper industries [83, 84]. Lipases secreted from different organisms vary in size; the smallest have molecular masses of 20-25 kDa while the largest are 60-65 kDa [85].

Most lipases show a low catalytic activity towards monomeric substrates but become fully active in the presence of a hydrophobic hydrophilic interface which is called interfacial activation. This involves a conformational rearrangement in the lipase where structural elements (lids or flaps) must move to allow a substrate to access the center of the reactions (the active site) of the enzyme [86]. Lipases contain Ser-His-Asp/Glu as catalytic triads [87] which is occluded by the polypeptide flap and is not exposed to solvent (Figure 1.4).

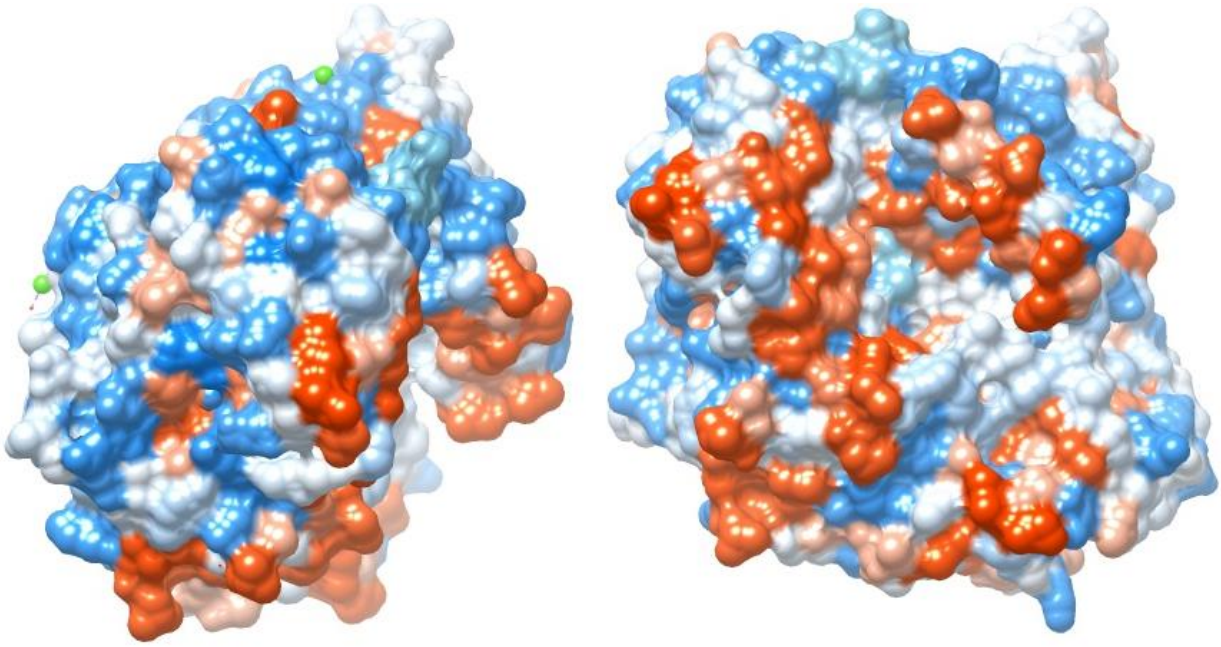
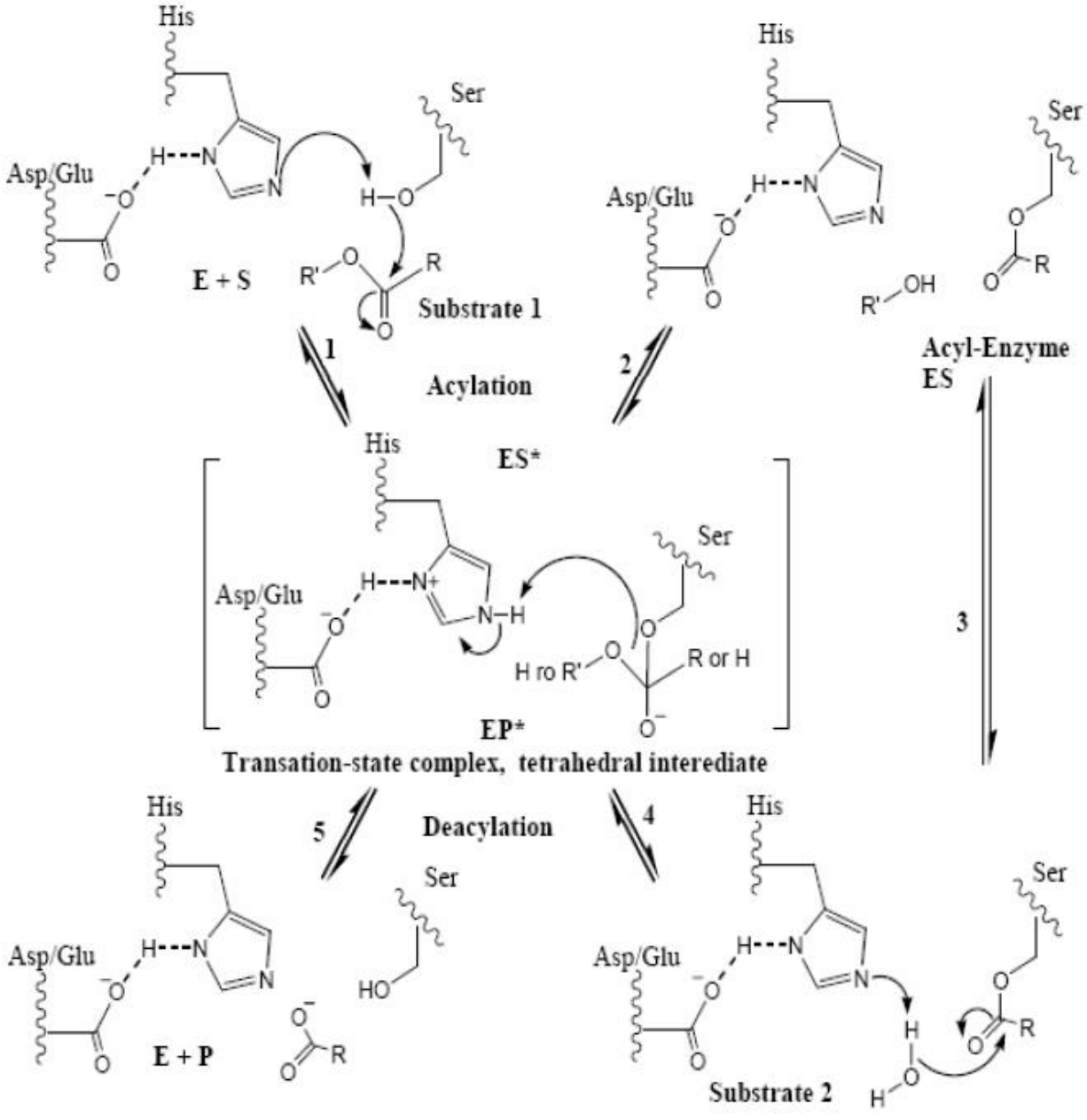


Figure 1.4 Side View of Open Conformation of CRL, Flap on the Right Side (left), CRL Active Site with the Hydrophobic Pocket in the Center (right)

The fungus *Candida rugosa* (formerly *Candida cylindracea*) produces several lipase isoforms, five of which have been cloned and sequenced [88]. One of the commonly used lipases in organic synthesis is CRL used in KR of chiral alcohols and transformations of chiral long chain acyl donors (Scheme 1.16) [7, 89].



Scheme 1.16 Esterification or Hydrolysis Mechanism in the Catalytic Triad of Lipases [7]

The first research group that reported the three-dimensional structures of CRL was Grochulski *et al*. [90]. The active site contains the same catalytic triad as those of Serine proteases. The tetrahedral intermediates of the acylation/deacylation steps of the hydrolysis are the same in all lipases.

Cygler-Grochulski *et al*. [91] determined X-ray crystal structures of CRL covalent complexes with transition state analogs for the hydrolysis of menthyl esters, and proposed that lipases and esterases have similar catalytic triads and alcohol binding sites, and that the catalytic triad of CRL is typical for these enzymes. They concluded that a common feature of the catalytic machinery is that the Serine is embedded in a tight turn at the top of the β-sheet between a β-strand and an α-helix; the triad Histidine is positioned by loops on one side of the Serine and the oxyanion hole is formed on the other side.

Cygler *et al*. [85] in a review described that the active sites of CRL lipases are composed of residues Ser 209, His 449 and Glu 341, which form a catalytic triad similar in arrangement to those of Serine proteases. There is, however, one important difference between the catalytic triads of proteases and lipases, namely that they have opposite handedness.

Lakshmi *et al*. [92] reported (*S*)-ibuprofen docking to CRL in esterification mode using molecular modeling to understand the stereo-specific substrate binding to the active site, and concluded that only the (*S*)-enantiomer is able to bond with the hydroxyl group of the Serine 209 and imidazole base (His 449) whereas the carboxyl group (–COOH) of the (*R*)-enantiomer points away from (His 449) the base of CRL.

Manetti *et al.* [93] reported molecular models of the computer generated complexes between CRL and the Ketoprofen methyl ester and biological assays to conclude that in addition to the residues constituting the catalytic triad and the oxyanion hole, Phe344 and Phe345 are amino

acids involved in the enzyme ligand interactions for stereo chemical preference of CRL to the (*S*)-enantiomer.

Kazlauskas [94] reviewed the X-ray crystal structures of transition state analogs bound to lipases that identified distinct binding regions for the acyl and alcohol portions of esters and suggested molecular level explanations for the enantio-preferences of lipases.

James *et al*. [84] investigated CRL-catalyzed hydrolysis of ibuprofen esters using molecular dynamics simulations under acidic and neutral conditions. They found that CRL preferred IME substrate at an acidic pH, and ibuprofen butyl ester at a neutral pH. For the bulkier substrates with a more hydrophobic side chain at a neutral pH, the flap opened more allowing the ibuprofen butyl ester substrate to gain access to the active site to provide the best enzyme substrate fit. At an acidic pH there was a decrease in the flap opening, accommodating the more compact IME substrate possessing the less hydrophobic side chain since the CRL flap moved towards the closed conformation. Therefore, the choice of substrate depends on pH for CRL, and the motion of the flap is necessary for the substrate entering the active site.

Berglund *et al*. [89] reported molecular modeling for two binding modes of 2-methyl decanoic acid ester enantiomers to the active site of CRL. The fast reacting (*S*)-enantiomer occupied the acyl binding tunnel of the enzyme, whereas the (*R*)-enantiomer left the tunnel empty. The modeling suggested that if both enantiomers were forced to bind to the active site leaving the tunnel empty, the enzyme would reverse its enantio-preference to become (*R*)-enantio-selective. They tested their hypothesis and found that the CRL-catalyzed hydrolysis of the ethyl ester substrate resulted in the conversion of the (*R*)-enantiomer, as predicted by molecular modeling which is kinetic evidence supporting the existence of two binding modes for the substrate in the active site of CRL.

Botta *et al.* [80] suggested that the X-ray crystal structure of CRL might not show the totally open conformation of the lipase. They also proposed that the active site conformational change caused by treating CRL with a polar solvent could be simulated by computational methods.

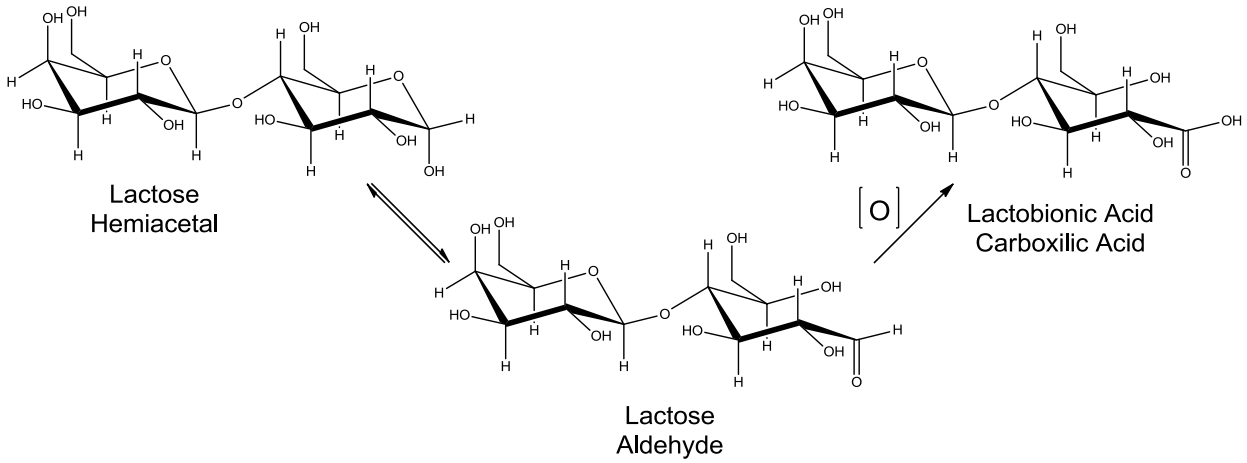
In another publication, Botta *et al*. [82] studied CRL-catalyzed hydrolysis of esters and the relative rates of the hydrolysis where all transformations were highly enantio-specific. The mechanism of enantio recognition was probed by substrate mapping using "Comparative Molecular Field Analysis" (CoMFA) to correlate the extent of substrate hydrolysis and the initial reaction rate with the stereo electronic properties of the substrates.

Longhi *et al.* [95] proposed a structural model for CRL based on the reported X-ray structure of the highly homologous *Geotrichum candidum* lipase conserving the network of interactions around the active site, the salt, and a disulfide bridge. Jayasundar *et al*. [96] investigated the effect of solvent hydrophobicity on CRL activation using molecular dynamics simulations. The closed inactive conformer of CRL was solvated in three alkane aqueous mixtures. Upon analyzing the simulated conformers with the closed conformer of CRL, the flap opened from a closed state by 7.7, 10.2, and 13.1Å for hexane, octane, and decane, respectively.

Salgin *et al.* [97] reported the effects of Li^{+}, Na^{+}, K^{+}, Mg^{+2}, and Zn^{+2} ions on CRL enantio-selectivity in biphasic medium of phosphate buffer and isooctane by performing the hydrolysis of racemic NME. The addition of metal ions increased the hydrolysis rate by 1.31-1.45 times relative to the control, while the ee of product increased slightly.

1.11 Oxidation of Lactose to an Aldonic Acid

In 1909 Mathews [98] reported that sugar solutions including lactose consume oxygen and produce acid when treated with potassium hydroxide solutions. In 1929, Hudson *et al*. [99] made aldonic acids in high yields by oxidizing its corresponding aldoses (lactose, glucose, and xylose) with bromine water. Lactose was oxidized to lactobionic acid (Scheme 1.17) which can be hydrolyzed to galactose and gluconic acid. The reactions were slow since hydro bromic acid was generated, so they used a buffering salt of an organic acid such as barium or calcium benzoate and found that it greatly increased the speed of the reaction.



Scheme 1.17 Oxidation of Lactose to Lactobionic Acid

In 1952, Hardegger *et al*. [100] synthesized aldonic acids with one carbon less than the sugar by oxidative degradation of reducing sugars with oxygen and base. They made 3-[β-D-galactosido]-D-arabonic acid from lactose. Furthermore, they prepared D-arabonic acid, D-lyxonic acid, 3-[β-D-glucosido]-D-arabonic acid, and 3-[α-D-glucosido]-D-arabonic acid from D-glucose, D-galactose, cellobiose, and maltose, respectively.

Because the previously proposed mechanism of DKR reactions is base-catalyzed enolization of ester derivatives before lipase-catalyzed hydrolysis [101] (Figure 1.5 and Scheme 1.9), we investigated the pH at which this enolization occurs for racemic ibuprofen methyl ester (IME) as well as further optimizations of this reaction for the conversion of racemic ibuprofen to its therapeutically effective (*S*)-ibuprofen. The goal of this research is to develop a facile, inexpensive, and environmentally benign methodology for the conversion of commercial racemic profens to their corresponding therapeutically effective enantiomers, (*S*)-profens. To achieve this, the DKR of the racemic IME to (*S*)-ibuprofen was optimized in terms of buffers, pH, substrate concentrations, co-solvents, amounts of enzyme, temperatures, and agitation rates.

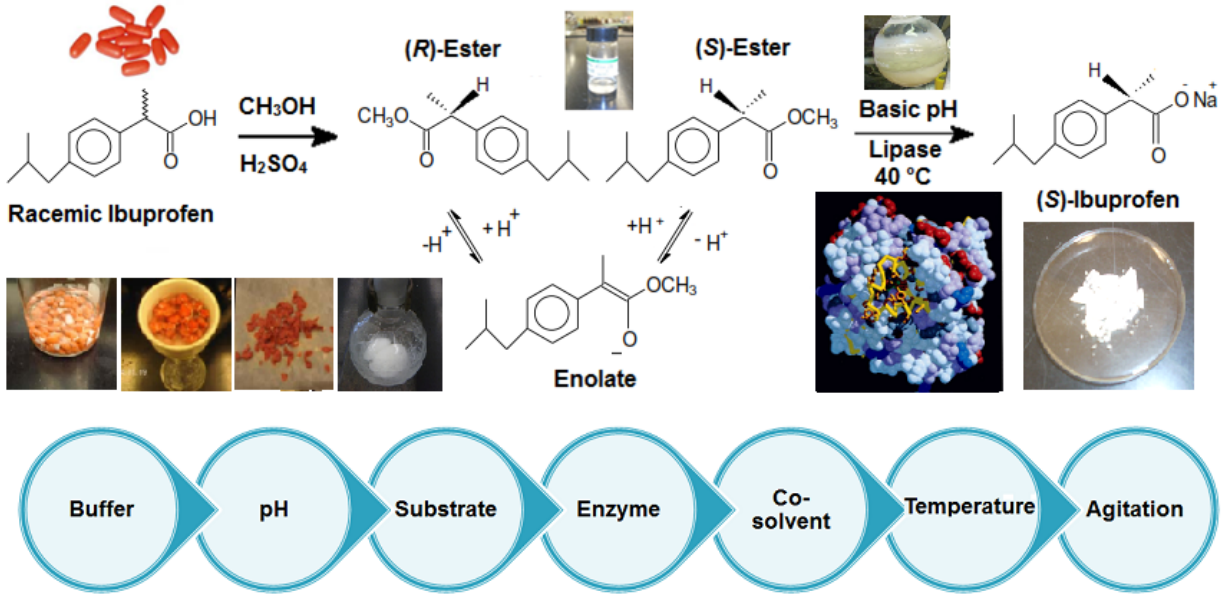


Figure 1.5 Ibuprofen Extraction, Esterification, and Previously Proposed Mechanism of DKR Reactions of Racemic IME to (S)-Ibuprofen [88]

Chapter 2 : Results and Discussion

The Dynamic Kinetic Resolution (DKR) reaction in conversion of racemic ibuprofen methyl ester (IME) to (*S*)-ibuprofen was investigated in full details. First, racemic ibuprofen was isolated from commercial tablets and esterified to make racemic IME. Next, the DKR reaction of racemic IME to (*S*)-ibuprofen was optimized in terms of buffer, pH, substrate concentration, co-solvent, amount of *Candida rugosa* lipase (CRL) enzyme, temperature, and agitation rate by running over 500 small scale reactions. To examine the effect of buffers, several 0.5 or 1.0 M of different buffer solutions were utilized. Based on the achieved optimized parameters, a large scale DKR reaction of racemic IME to (*S*)-ibuprofen was implemented. The Kinetic Resolution (KR) reaction of racemic IME to (*S*)-ibuprofen was performed to isolate (*R*)-ibuprofen methyl ester, (*R*)-IME, from racemic IME. Finally, racemization of (*R*)-IME at different pHs including pH 7.6 was attempted.

The DKR reaction of naproxen methyl ester (NME) to (*S*)-naproxen was investigated. First, (*S*)-naproxen was isolated from naproxen sodium salt tablets. An alternative but shorter procedure was performed by direct Fischer esterification of naproxen sodium salt to make (*S*)-NME. Next, the DKR reaction of NME was attempted by using 0.5 or 1.0 M buffer solutions of HEPES or MOPS and utilizing lauric acid as surfactant. Finally, racemization of (*S*)-NME at pHs close to 7.6 was studied.

2.1 Isolation of Racemic Ibuprofen from Tablets

Racemic ibuprofen was isolated by acetone extraction of inexpensive commercial tablets (200 mg/tablet) followed by vacuum filtration of the insolubles, removal of the solvent from the filtrate under reduced pressure, and bulb to bulb distillation of the remaining solid with a Kuelgrohr apparatus to give high yields of racemic ibuprofen (Table 2.1) as white crystals with a melting

point of 75 °C (lit. 75 °C) [102, 103]. Chiral column High Performance Liquid Chromatography (HPLC) spectra with two variations of condensed and baseline resolved peaks of ibuprofen enantiomers are shown in Figure 2.1. The retention time depends on many factors including the solvents ratio, column temperature, the volume of solvents in the bottles, degassing and the amount of air in the pump, and moisture. For example, the less polar the mobile phase, the farther apart the peaks would appear. Proton Nuclear Magnetic Resonance (^{1}H NMR) and Carbon-13 (^{13}C) NMR spectra of racemic ibuprofen are shown in Figures A.1 and A.2 of Appendix A and match the literature [104]. Based on the obtained spectra and melting points, the product was pure racemic ibuprofen with no traces of residual impurities in all the experimental runs. The major factor in recovering a quantitative amount of ibuprofen was sufficient washing of the tablet binder and coating with acetone in the process of vacuum filtration. By implementing this technique, a substantial monetary saving over ibuprofen purchased from chemical suppliers was achieved.

Table 2.1 Yield of Racemic Ibuprofen Isolated from Tablets

Exp #	# of Tablets	Theoretical (g)	Actual (g)	%Yield
1	20.0	4.0	4.0	100.0
2	75.0	15.0	6.1	41.0
3	82.0	16.4	15.5	95.0
4	81.0	16.2	13.7	84.4
5	88.5	17.7	16.6	94.0
6	100.0	20.0	19.9	99.7
7	20.0	4.0	4.0	100.0

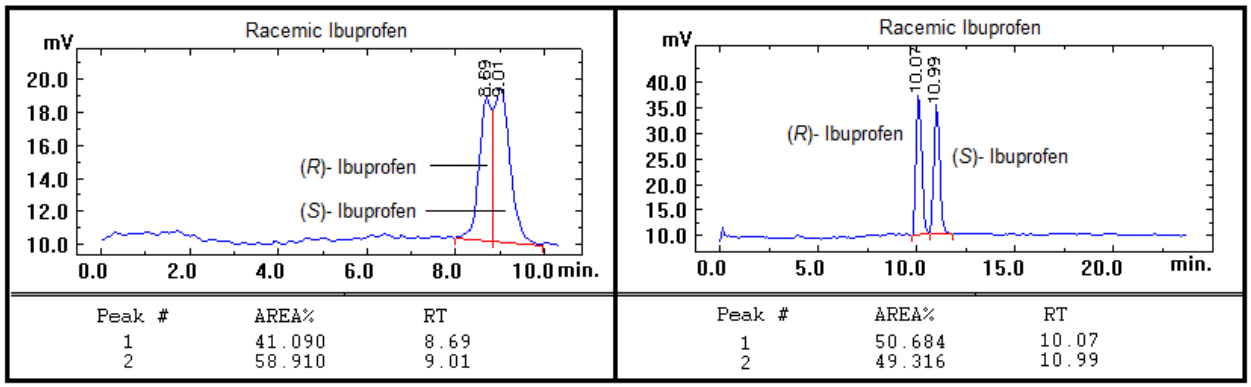
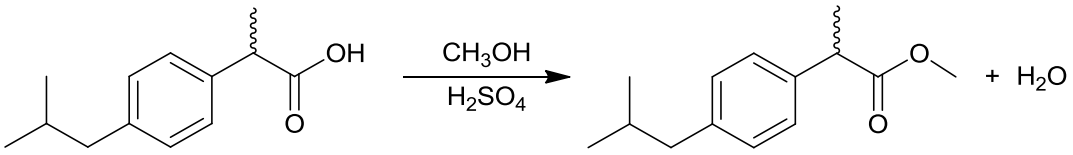


Figure 2.1 Chiral Column HPLC Chromatographs of Racemic Ibuprofen

2.2 Conversion of Racemic Ibuprofen to Racemic Ibuprofen Methyl Ester (IME)

Racemic IME was synthesized overnight, usually in about 12 hours, by Fischer esterification of racemic ibuprofen with excess methanol and acid catalysis (Scheme 2.1). In each run, the ester was extracted with hexanes several times and the solvent was removed under reduced pressure. Starting from run 11 (Table 2.2), each batch of the ester was further distilled bulb to bulb by horizontal distillation. The boiling point range for racemic IME using the Kuelgrohr apparatus was from 90 (0.3 Torr) to 105 °C (0.8 Torr). Better yields were obtained by increased hexanes extractions. Chiral column HPLC chromatographs with two variations of condensed and baseline resolved peaks are depicted in Figure 2.2. Because esters are less polar than carboxylic acids, the retention times of IME enantiomers were less than those of racemic ibuprofen enantiomers. ^{1}H NMR and ^{13}C NMR spectra of racemic IME are shown in Figures A.3 and A.4 of Appendix A and match the literature [105]. Based on the obtained spectra, the purity of the product was proved in all the experiments since no residual impurities were observed and the boiling point was in good agreement with the literature [106]. In summary, a higher yield of racemic IME was obtained by increasing the number of hexanes extractions after Fischer esterification.



Scheme 2.1 Fischer Esterification of Racemic Ibuprofen

Table 2.2 Yield of Racemic IME from Fischer Esterification of Racemic Ibuprofen

Exp #	%Yield after Rotovap	Exp #	%Yield after Rotovap	%Yield after Horizontal Distillation	Exp #	%Yield after Rotovap	%Yield after Horizontal Distillation
1	100.0	11	91.0	59.0	21	93.0	85.0
2	45.0	12	82.0	74.5	22	89.0	80.0
3	73.0	13	90.0	82.0	23	91.0	86.0
4	80.0	14	98.0	79.0	24	98.0	91.0
5	93.0	15	90.0	80.0	25	100.0	97.0
6	95.5	16	90.0	79.0	26	100.0	94.0
7	96.0	17	100.0	88.0	27	100.0	92.0
8	94.0	18	85.0	79.0	28	100.0	92.0
9	94.0	19	93.0	87.0			
10	93.0	20	101.0	88.0			

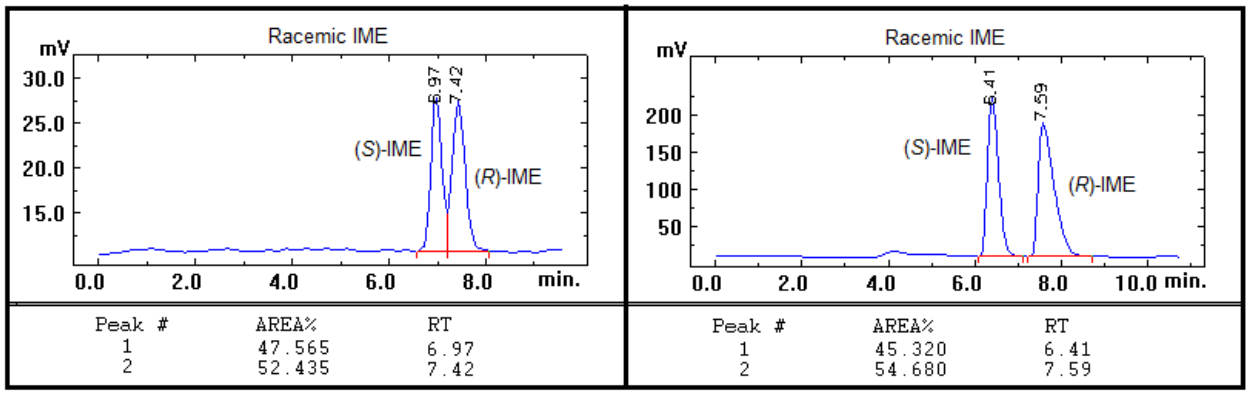
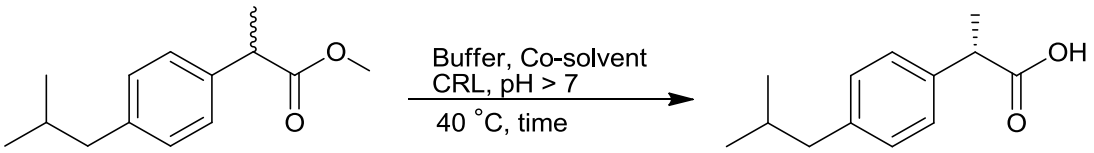


Figure 2.2 Chiral Column HPLC Chromatograph of Racemic IME

2.3 Monitoring DKR Reactions of Racemic IME via Chiral Column HPLC

Hypothesizing that (*R*)-IME was converted to (*S*)-IME via enolization in basic aqueous solutions, racemic IME was subjected to DKR reaction conditions as shown in Scheme 2.2. To monitor DKR reactions of Racemic IME, a 0.2 mL aliquot of the aqueous reaction was acidified to pH 5 and extracted into 0.3 mL hexanes for chiral column HPLC analysis using a Chiralcel-OJ

column. A mobile phase of hexanes/2-propanol (98/2, v/v), a flow rate of 1 mL/min, and helium solvent degassing with 1.0-2.0 μL of analytes for injection into the pump were employed.



Scheme 2.2 DKR Reactions of Racemic IME to (S)-Ibuprofen

Based on this analysis, the order of chromatographic elution was determined to be (*S*)-IME, then (*R*)-IME followed by (*R*)-ibuprofen and (*S*)-ibuprofen. Ester separation was not possible with more than a 2% isopropanol mobile phase concentration. The general pattern of these reactions was the relatively rapid decrease in the amount of the (*S*)-ester with rapid increase of (*S*)-ibuprofen followed by a slower decrease of the (*R*)-ester with a corresponding slower increase of (*S*)-ibuprofen within the course of a few days. Figure 2.3 illustrates chiral column HPLC chromatographs of a typical optimized monitored DKR reaction from the start of the reaction to full completion. Furthermore, chiral column HPLC chromatographs of racemic ibuprofen, (*S*)-ibuprofen, racemic IME, (*R*)-IME, a mixture of both racemic IME and racemic ibuprofen, and a mixture of racemic IME plus enriched (*S*)-ibuprofen are available in Appendix B, Figure B.1.

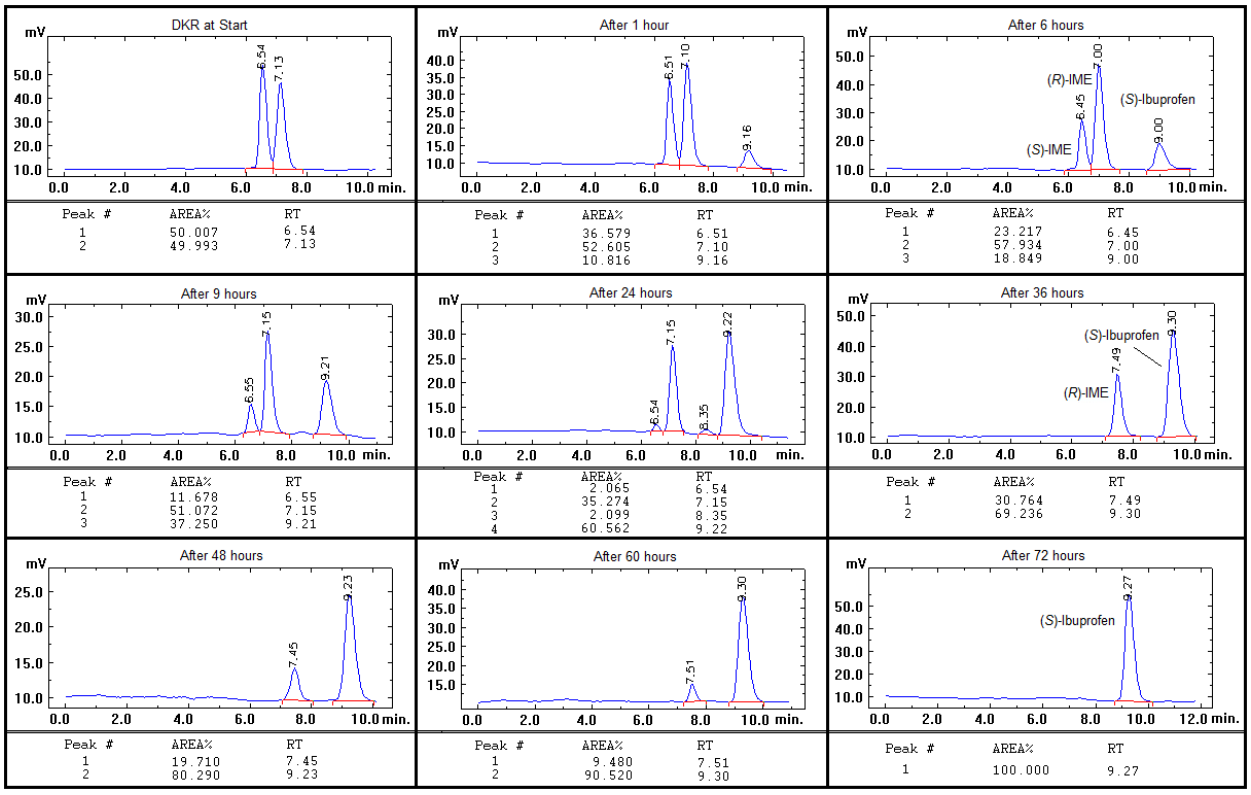


Figure 2.3 General Pattern of DKR Reactions of Racemic IME to (*S*)-Ibuprofen
Panel Order: left to right-top to bottom

2.4 Optimization of DKR Reaction Parameters

To optimize the DKR reaction in conversion of racemic IME to (*S*)-ibuprofen, different buffers, pH, substrate concentrations, co-solvents, amounts of enzyme, temperatures, and agitation rates were examined.

2.4.1 Optimization of DKR Reactions with Respect to Buffers

Due to the enzymatic catalysis and to promote enolization, a right buffer with a right concentration was very important for a successful DKR reaction. Twelve different buffers with reported effective pH ranges from 5.8 to 11 were examined (Table 2.3). Different buffer capacities from 0.1 to 2.0 M concentration, mainly 0.5 and 1.0 M, were tested. After dissolving buffers in Millipore filtered water, the pH of their solutions was measured and adjusted with 1.0 M NaOH or HCl solutions to be within their reported effective pH ranges. Aliquots of each buffer solution

were acidified, extracted with hexanes, and subjected to chiral column HPLC analysis to confirm that none of these buffers contained chromophores that could interfere with the DKR analysis.

For most buffers (0.8 or 0.95 mL), the pH slightly changed upon adding DMSO (0.2 mL) but did not change upon adding DMF (0.05 mL) as a co-solvent, did not change upon adding racemic IME (50 μL or 222.0 μmol), but decreased considerably after adding CRL (40 mg, Type VII ≥ 700 unit/mg of lactose solid) before commencing DKR reactions (Table 2.3). The most dramatic change was observed with 0.5 M sodium bicarbonate buffer after CRL addition. For example, the pH before adding the lipase to this buffer was 9.23 but after adding CRL it dropped to 7.73. Interestingly, even in 1.0 mL Millipore filtered water, when the lipase (40 mg) was added, the pH decreased from 6.45 to 5.65. Even with higher concentrations of buffer (e.g., 0.8 mL of 2.0 M MOPS, 0.2 mL DMSO, and 50 μL or 222.0 μmol racemic IME), the pH dropped from 7.67 to 7.6 after adding the lipase (40 mg).

Table 2.3 pH of DKR Reactions[a] with Varying Buffers Before and After Adding CRL

Exp #	Buffer[b]	M[c]	pKa at 20 °C[d]	Effective Range[d]	pH after adding water	pH after acid/base adjustment	pH after adding DMSO	pH after adding CRL
1	Imidazole	0.5	7.00	6.2-7.8	10.27		7.68	7.38
2	Phosphate II	0.5	7.20[e]	5.8-8.0	9.29	7.55	7.94	7.43
3	Phosphate II	1.0	7.20	5.8-8.0	9.05	7.36	7.75	7.42
4	MOPS	0.5	7.20[e]	6.5-7.9	3.90	7.87	7.87	7.61
5	MOPS	1.0	7.20	6.5-7.9	3.22	7.66	7.66	7.57
6	TES	0.5	7.50	7.0-8.0	2.90	7.53	7.68	7.37
7	HEPES	0.5	7.55	7.0-8.0	5.05	7.84	7.86	7.65
8	HEPES	1.0	7.55	7.0-8.0	4.76	7.70	7.70	7.62
9	Triethanolamine	0.5	7.80	7.3-8.3	10.70	7.73	7.68	7.64
10	Triethanolamine	1.0	7.80	7.3-8.4	10.08	7.74	7.68	7.67
11	Tricine	0.5	8.15	7.6-8.8	4.54	7.78	7.91	7.62
12	Tricine	1.0	8.15	7.6-8.8	4.46	7.78	7.90	7.80
13	Tris	0.5	8.30	7.0-9.0	10.10	7.77	7.88	7.66
14	Bicine	0.5	8.35	7.8-8.8	5.00	8.09	8.15	7.63
15	Bicine	1.0	8.35	7.8-8.8	4.50	7.82	7.88	7.64
16	Ethanolamine	0.5	9.50	8.5-9.5	10.80	8.83	8.78	6.90
17	Glycine	0.5	9.60	8.6-10.6	6.07	8.55	8.63	7.25
18	Glycine	1.0	9.60	8.6-10.6	5.57	7.95	8.00	6.16
19	Bicarbonate	0.5	10.30	9.6-11	8.67	8.73	9.23	7.73
20	Bicarbonate	1.0	10.30	9.2-10.8	8.50	7.45	8.03	7.30
21	Bicarbonate	2.0	10.30	9.2-10.8	8.12	7.37	7.87	7.27

MOPS: 3-(N-Morpholino)propanesulfonic acid
TES: N-[Tris (hydroxymethyl)methyl]-2-aminoethanesulfonic acid
HEPES: 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
Tricine: N-[Tris(hydroxymethyl)methyl]glycine
Tris: Tris (hydroxy methyl)aminomethane
Bicine: N,N-bis(2-hydroxyethyl)glycine
a 0.8 mL buffer, 0.2 mL DMSO or 0.95 mL buffer, 0.05 mL DMF, 222.0 μmol racemic IME, 40 mg CRL
b Table arranged by increasing pKa of Buffers
c M: Molarity
d Ref. [107]
e Literature value at 25 °C [108]

The acidity of commercial CRL has not been reported before and could be due to oxidation of its lactose filler to aldonic acids [98, 100]. For all DKR reactions, a 0.5 M solution of buffer gave a better result than 1.0 M buffer (see next section). For example, a set of DKR reactions

under the same conditions (0.8 mL MOPS, 0.2 mL DMSO, 50 µL or 222.0 µmol racemic IME, pH 7.6 after adding 40 mg CRL, 144 hours) with 0.5, 1.0, or 2.0 M buffer concentration resulted in a 51, 40, and 40% conversion, respectively. In another example (0.95 mL of MOPS buffer, 0.05 mL DMF, 40 µL or 177.2 µmol racemic IME, pH 7.6 after adding 32 mg CRL, 144 hours) 0.5 versus 1.0 M of the buffer resulted in a 62 and 44% conversion, respectively. More concentrated buffers may prevent DKR reaction progress by disturbing the enzymatic structure.

DKR reactions in conversion of racemic IME to (*S*)-ibuprofen were examined with different buffers. In all these reactions 0.8 mL of 0.5 M of a different buffer solution per experiment, 0.2 mL DMSO, 50 µL (222.0 µmol) racemic IME, and 40 mg CRL were utilized. The pH of the reactions was measured before and after adding the lipase (Table 2.3), and the aqueous mixtures were stirred for 144 hours at 40 ºC followed by HPLC analysis. Chiral column HPLC chromatographs of DKR reactions with different buffers (Figures 2.3 and 2.4) demonstrated that these reactions worked in all the buffers listed in Table 2.3 except for imidazole, ethanolamine, glycine, and sodium bicarbonate while it worked best with MOPS, TES, and HEPES. Therefore, it can be concluded that buffers with a pKa range of 7.20 to 8.35 are suitable for DKR reactions.

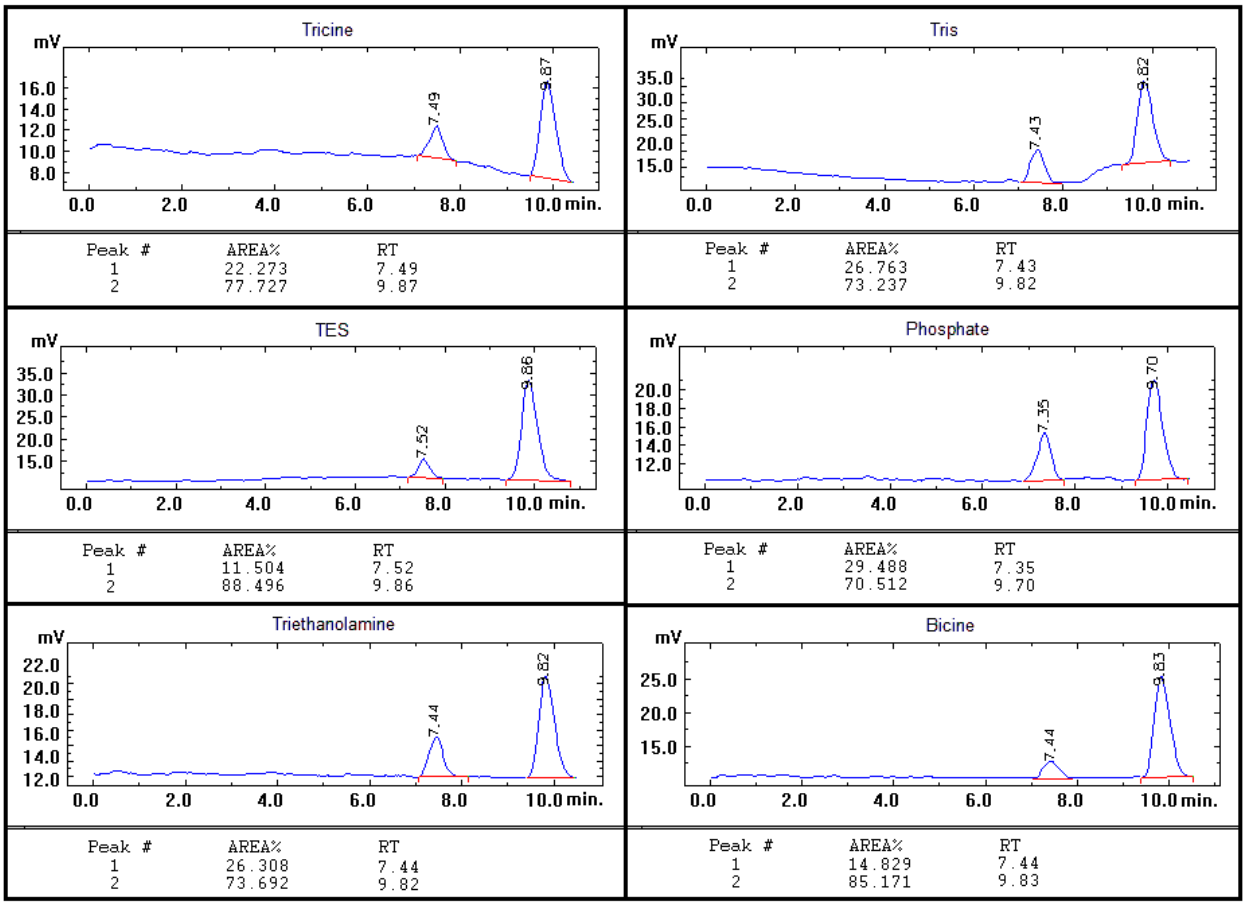


Figure 2.4 DKR Reactions of Racemic IME to (*S*)-Ibuprofen with Different Buffers

2.4.2 Optimization of KR and DKR Reactions with Respect to pH

Several enzymatic hydrolysis in conversion of racemic IME to (*S*)-ibuprofen were examined with different buffers in acidic, neutral, and basic pHs up to 8.7 (after adding CRL) to investigate the pH at which the hydrolysis occurs. In the first set of reactions (Table 2.4, experiments 1-7) at basic pHs, 1 mL of 1.0 M of a different buffer solution per experiment (imidazole, MOPS, TES, HEPES, tris, tricine, or bicine), 50 µL (222.0 µmol) racemic IME, and 50 mg CRL were utilized. The pH of the reactions was measured before and after adding the lipase, and the aqueous mixtures were stirred for 48 hours at 40 ºC followed by chiral column HPLC analysis.

In the second set of reactions (Table 2.4, experiments 8-14) under the same reaction conditions using the same buffers, enzymatic hydrolyses were repeated at a lower pH of 7.6 (after adding CRL) and a shorter reaction duration of 36 hours. In the third set of reactions (Table 2.4, experiments 15-17) under the same reaction conditions using a glycine buffer, the experiment was repeated at acidic, neutral, and basic pHs, 5.77, 7.21, and 8.0 (after adding CRL), respectively, with 24 hours reaction duration. It should be noted that for the third set of reactions with glycine, the pH after adding CRL was 6.16 and adjusted to 8.0 by adding 1.0 M NaOH solution.

Table 2.4 pH Effect on KR of Racemic IME at Basic, Neutral and Acidic Conditions[a]

Exp #	Buffer	pH before CRL	pH after CRL	Duration (hours)	pH	% Conversion
1	Imidazole	7.86	7.93	48	7.87	5
2	MOPS	8.23	7.80	"	7.70	27
3	TES	8.35	8.01	"	8.00	26
4	HEPES	8.30	8.04	"	7.96	32
5	Tris	8.55	8.68	"	8.68	10
6	Tricine	8.56	8.33	"	8.33	6
7	Bicine	8.33	8.05	"	7.90	27
8	Imidazole	7.45	7.50	36	7.60	7
9	MOPS	7.69	7.60	"	7.20	33
10	TES	7.92	7.60	"	7.15	32
11	HEPES	7.69	7.60	"	7.21	51
12	Tris	7.56	7.60	"	7.47	22
13	Tricine	7.81	7.60	"	6.90	36
14	Bicine	7.90	7.60	"	6.70	41
15	Glycine	5.57	5.77	24	5.50	47
16	"	9.50	7.21	"	6.10	45
17	"	8.00	6.16[b]	"	6.70	42

a 1.0 mL 1.0 M buffer, 222.0 µmol IME, 50 mg CRL
b pH adjusted to 8.0

Enzymatic hydrolysis in conversion of racemic IME to (*S*)-ibuprofen took place poorly at a basic pH. A relatively better conversion was achieved (Figure 2.5) with HEPES (32%), MOPS (27%), bicine (27%), and TES (26%) while a poor conversion was obtained with tris (10%), tricine (6%), and imidazole (5%). However, tris and tricine buffers were utilized at basic pHs of 8.68 and

8.33 (after adding CRL). The best buffer in this set of experiments was determined to be HEPES at pH 8.04 (after adding CRL). A higher pH had an adverse effect on Kinetic Resolution (KR) reactions leading to lower conversions due to enzyme inactivation [28]. In fact, at higher pHs there was evidence of base saponification rather than enzymatic hydrolysis by production of racemic ibuprofen. This phenomenon was observed with imidazole, tris, and tricine, at pH 7.93, 8.68, and 8.33 (after adding CRL), respectively. Chiral column HPLC chromatographs of lower conversions above pH 7.6 on KR reactions of racemic IME after 48 hours are available in Appendix B, Figure B.2.

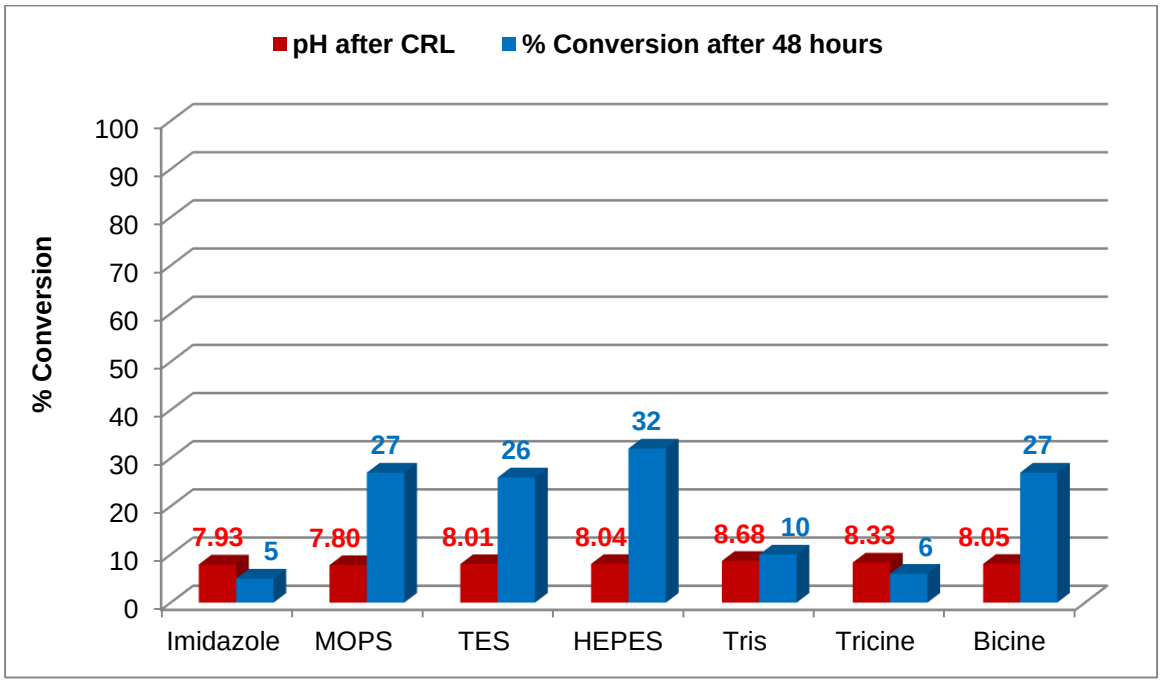


Figure 2.5 Lower Conversions Above pH 7.6 of KR Reactions of Racemic IME

Enzymatic hydrolysis at a slightly basic pH of 7.6 (after adding CRL) in general gave higher conversions. Better conversion was achieved (Figure 2.6) with HEPES (51%), bicine (41%), tricine (36%), MOPS (33%), TES (32%), and tris (22%), while a poor conversion was obtained with imidazole (7%). The best buffer in this set of experiments was determined to be HEPES at pH 7.60 (after adding CRL); in general, the best buffer for performing enzymatic

hydrolysis is HEPES with a pKa of 7.55. Therefore, KR reactions (enzymatic hydrolysis) depend on the pH and the conversion increases by lowering the pH to be slightly basic, around 7.6. Chiral column HPLC chromatographs of increased conversion at pH 7.6 on KR reactions of racemic IME after 36 hours are available in Appendix B, Figure B.3.

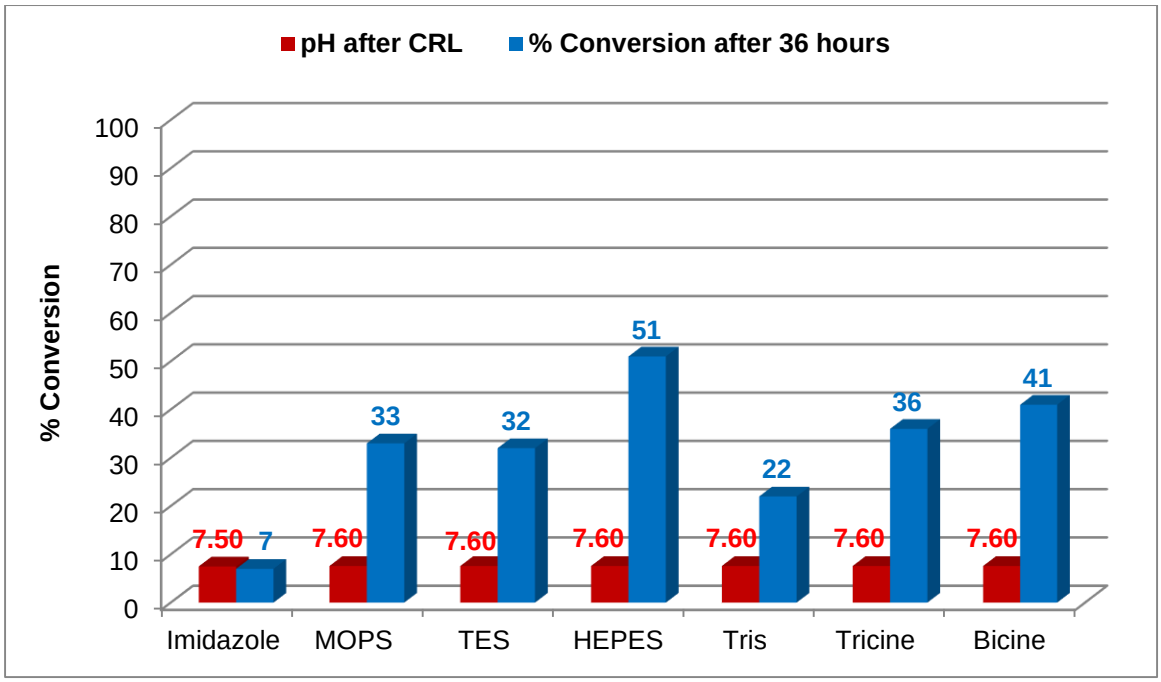


Figure 2.6 Higher Conversions at pH 7.6 of KR Reactions of Racemic IME

Enzymatic hydrolysis under acidic, neutral, and basic pHs using the glycine buffer was successful with a conversion above 40%, since after completion of these reactions the pH was below 7.0 (Table 2.4). The enzymatic hydrolysis with phosphate in conversion of racemic IME to (*S*)-ibuprofen under acidic and neutral condition has been reported by Chavez *et al*. [11, 29]. Furthermore, for running KR reactions (with a maximum 50% conversion) under acidic, neutral or basic conditions no more than 24 hours were needed for the completion of these reactions. Chiral column HPLC chromatographs of the KR reactions with the glycine buffer at neutral and basic conditions are illustrated in Figure 2.7.

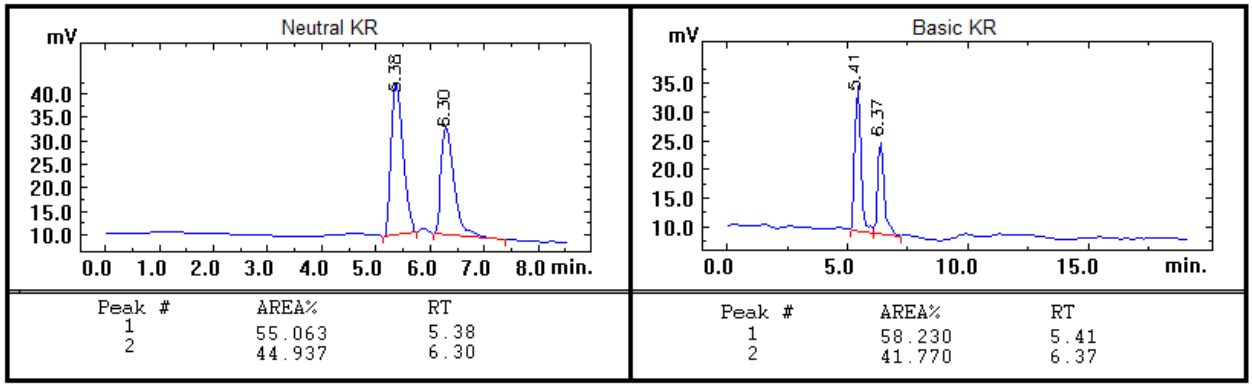


Figure 2.7 KR of Racemic IME with Glycine at Neutral and Basic Conditions

Over 500 small scale DKR reactions with 12 different buffers in conversion of racemic IME to (*S*)-ibuprofen were run (Table 2.3) under acidic, neutral, and basic pHs up to 9.8 and a few above 9.8 (after adding CRL) along with different ratios of reagents to investigate the right pH and optimal conditions for these reactions. However, once it was established that KR reactions do not work well above pH 7.6, DKR reactions from pH 7.30 to 8.0 were examined to find the optimal pH.

For simplicity, 5 sets of DKR reactions are presented. In the first set, 0.8 mL of 0.5 M buffer solution of bicine, 0.2 mL DMSO, 50 µL (222.0 µmol) racemic IME and 40 mg CRL were utilized (Table 2.5, experiments 1-8). In the second set, the same conditions with 50 mg CRL in each reaction were employed (Table 2.5, experiments 9-15). The pH of the reactions was measured before and after adding the lipase. The aqueous mixtures were stirred for 120 hours at 40 ºC followed by chiral column HPLC analysis.

Table 2.5 DKR of Racemic IME at Different pH with Bicine[a]

Exp #	CRL (mg)	pH before CRL	pH after CRL	Duration (hours)	pH	% Conversion
1	40	7.70	7.30	120	6.45	45
2	"	7.85	7.40	"	6.64	47
3	"	7.74	7.50	"	7.13	52
4	"	8.02	7.60	"	7.03	94
5	"	8.10	7.70	"	6.85	52
6	"	8.17	7.80	"	7.00	41
7	"	8..30	7.90	"	7.17	52
8	"	8.41	8.00	"	7.45	45
9	50	7.86	7.28	"	6.67	37
10	"	7.75	7.50	"	6.80	62
11	"	8.12	7.61	"	7.28	90
12	"	8.38	8.05	"	7.46	73
13	"	8.48	8.11	"	7.86	63
14	"	8.60	8.25	"	8.01	55
15	"	8.82	8.44	"	8.28	23

a 0.8 mL 0.5 M bicine, 0.2 mL DMSO, 222.0 µmol IME

In the third set, 0.8 mL of 0.5 M buffer solution of HEPES, 0.2 mL DMSO, 50 µL (222.0 µmol) racemic IME and 40 mg CRL were utilized (Table 2.6, experiments 1-6). In the fourth set, the same conditions with 50 mg CRL in each reaction were employed (Table 2.6, experiments 7-12). The pH of the reactions was measured before and after adding the lipase. The aqueous mixtures were stirred for 120 hours for the third set and 144 hours for the fourth set at 40 ºC followed by chiral column HPLC analysis.

Table 2.6 DKR of Racemic IME at Different pH with HEPES[a]

Exp #	CRL (mg)	pH before CRL	pH after CRL	Duration (hours)	pH	% Conversion
1	40	7.89	7.55	120	7.14	53
2	"	7.82	7.60	"	7.05	47
3	"	8.00	7.65	"	7.14	48
4	"	8.06	7.70	"	7.38	77
5	"	8.15	7.75	"	7.28	65
6	"	8.25	7.80	"	7.30	33
7	50	7.84	7.54	144	7.09	47
8	"	7.66	7.57	"	7.17	53
9	"	7.83	7.63	"	7.22	90
10	"	7.85	7.66	"	7.15	45
11	"	7.96	7.72	"	7.22	25
12	"	7.97	7.73	"	7.31	25

a 0.8 mL 0.5 M HEPES, 0.2 mL DMSO, 222.0 µmol IME

In the fifth set, 1.0 mL of 0.5 M buffer solution of MOPS, 0.25 mL DMSO, 50 µL (222.0 µmol) racemic IME and 40 mg CRL were utilized (Table 2.7). The pH of the reactions was measured before and after adding the lipase. The aqueous mixtures were stirred for 144 hours at 40 ºC followed by chiral column HPLC analysis.

Table 2.7 DKR of Racemic IME at Different pH with MOPS[a]

Exp #	pH before CRL	pH after CRL	Duration (hours)	pH	% Conversion
1	7.78	7.33	144	6.86	47
2	7.86	7.57	"	7.1	49
3	7.91	7.58	"	7.17	59
4	7.69	7.6	"	7.27	75
5	7.9	7.62	"	7.45	66
6	7.95	7.66	"	7.3	61

a 1 mL 0.5 M MOPS, 0.25 mL DMSO, 222.0 µmol IME, 40 mg CRL

Figures 2.8, and 2.9 demonstrate the effect of pH on the conversion of racemic IME to (*S*)-ibuprofen and confirms that DKR reactions worked with a variety of buffers, but only around the optimal pH 7.6 because below a pH of 7.6 enolization decreases and above pH 7.6 the lipase becomes inactive [28]. These results demonstrated that the optimal pH for DKR reactions in

conversion of racemic IME to (*S*)-ibuprofen is 7.6 and that this DKR reaction does not occur at pH 9.8 as stated by Chavez *et al.* [11, 29]. In addition, Figure 2.8 demonstrates 73% conversion with bicine at pH 8.05 due to its higher pKa value of 8.35. Also, a 77% conversion with HEPES (pKa 7.55) at pH 7.70 indicates that bicine and HEPES are capable of working at a slightly higher pH than 7.6.

After determining the optimal pH of 7.6, all further DKR reactions were adjusted by adding buffer or NaOH solutions. The pH was measured at room temperature because at higher temperatures the pH meter indicated lower values [109]. Chiral column HPLC chromatographs of the pH effect on DKR of racemic IME of the first set of experiments (0.5 M bicine buffer at pH range 7.30-8.0) at 40 ºC after 120 hours are available in Appendix B, Figure B.4.

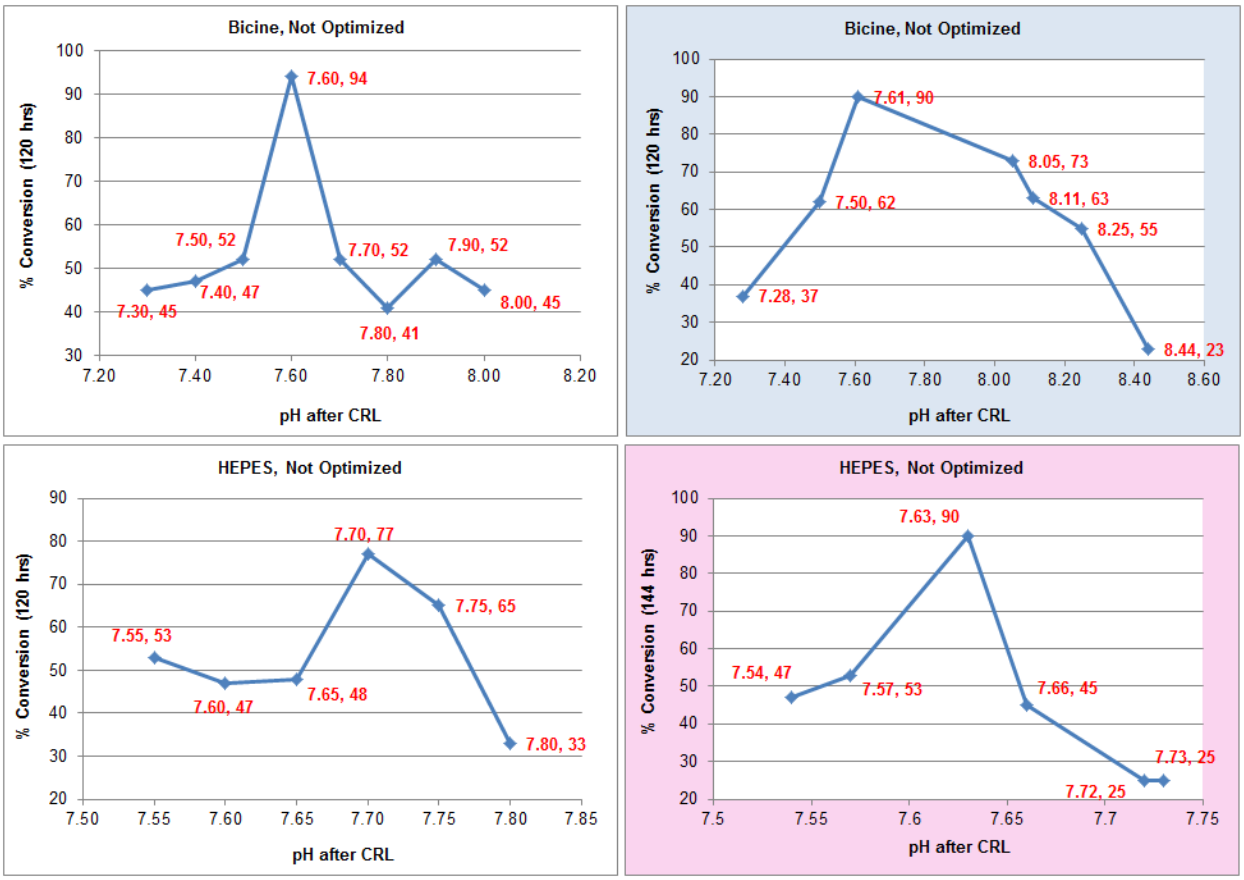


Figure 2.8 DKR of Racemic IME at Different pH with Bicine and HEPES

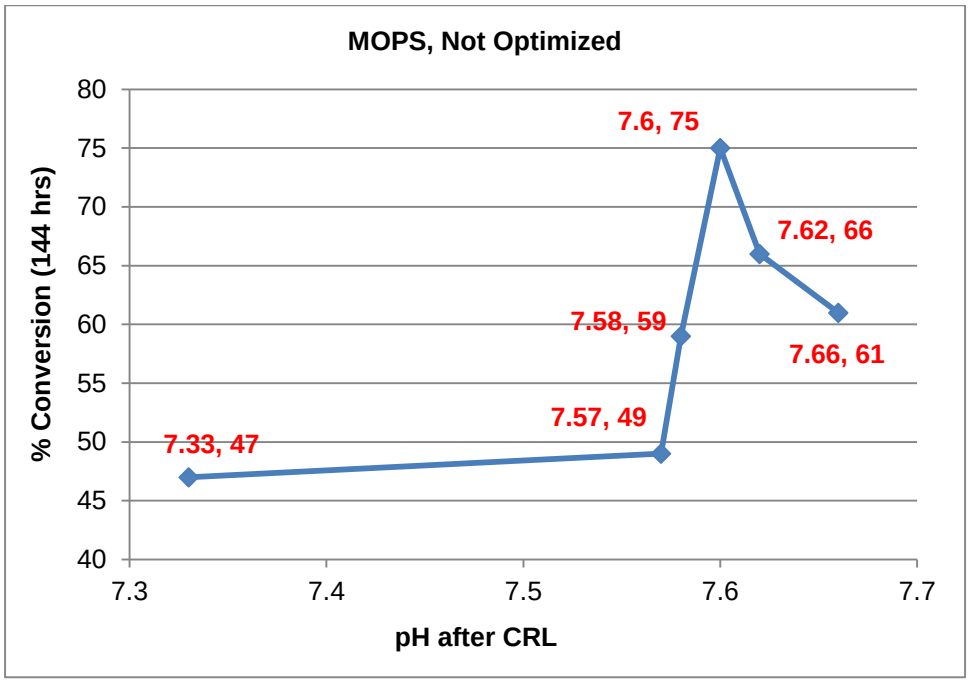


Figure 2.9 DKR of Racemic IME at Different pH with MOPS

2.4.3 Optimization of DKR Reactions with Respect to Substrate Concentration

DKR reactions in conversion of racemic IME to (*S*)-ibuprofen were examined with different amounts of substrate (racemic IME) while all other parameters were held constant and optimized (based on the obtained results up to this point) in order to determine the effect of substrate concentration on these reactions. Therefore 3 sets of DKR reactions are presented. In the first set, 0.8 mL of 0.5 M buffer solutions of MOPS, 0.2 mL DMSO, a different amount of racemic IME per experiment 50 (222.0 µmol), 40 (177.2 µmol), 30 (133.0 µmol), 25 (110.8 µmol), 12 (53.2 µmol), and 10 µL (44.3 µmol), plus 40 mg CRL were utilized. In the second set, under the same reaction conditions 0.95 mL of 0.5 M MOPS, and 0.05 mL DMF as opposed to DMSO were used. In the third set, under the same reaction conditions 0.95 mL of 0.5 M HEPES and 0.05 mL DMF were used. The pH of the reactions was measured before adding the lipase and adjusted to 7.6 after adding the enzyme. The aqueous mixtures were stirred for 144 hours for the first set,

72 hours for the second set, 120 and 72 hours for the third set at 40 ºC followed by chiral column HPLC analysis (Table 2.8).

Table 2.8 Effect of Substrate Amount on DKR of Racemic IME at Optimal pH[a]

Exp #	Conditions	pH before CRL	pH after CRL	Duration (hours)	pH	IME (µL)	IME (µmol)	% Conversion
1	0.8 mL MOPS 0.5 M 0.2 mL DMSO	7.90	7.60	144	7.41	50	222.0	51
2	"	7.90	7.60	144	7.25	40	177.2	54
3	"	7.90	7.60	144	7.24	30	133.0	61
4	"	7.90	7.60	144	7.26	25	110.8	64
5	"	7.90	7.60	144	7.31	12	53.2	85
6	"	7.90	7.60	144	7.47	10	44.3	91
7	0.95 mL MOPS 0.5 M 0.05 mL DMF	7.90	7.60	72	7.30	50	222.0	44
8	"	7.90	7.60	72	7.27	30	133.0	57
9	"	7.90	7.60	72	7.47	25	110.8	62
10	"	7.90	7.60	72	7.49	12	53.2	82
11	"	7.90	7.60	72	7.56	10	44.3	91
12	0.95 mL HEPES 0.5 M 0.05 mL DMF	7.93	7.63	120	7.45	50	222.0	71
13	"	7.93	7.63	120	7.35	40	177.2	46
14	"	7.93	7.63	72	7.61	10	44.3	91

a 40 mg CRL

In this set of experiments with 50 (222.0 µmol), 40 (177.2 µmol), 30 (133.0 µmol), 25 (110.8 µmol), 12 (53.2 µmol), and 10 µL (44.3 µmol) of the substrate, it was verified that the lower the racemic IME concentration, the higher the conversion of the reaction (Figure 2.10), with some reactions only taking 72 hours to convert most of the racemic IME to (*S*)-ibuprofen. This is reported to be due to higher amounts of (*S*)-ibuprofen and methanol products inhibiting or deactivating the enzyme [1, 8, 28, 59, 66, 69]. In particular 90% of 10 µL (44.3 µmol) of racemic IME could be converted to (*S*)-ibuprofen in about half the time than previously reported, e.g., 120 [28] and 144 hours [29] with 85 and 94% conversions, respectively. In another example with 0.95 mL of 1.0 M HEPES buffer, 0.05 mL DMF, pH 7.62 after adding 40 mg CRL, 50 µL (222.0 µmol)

or 40 µL (177.2 µmol) racemic IME resulted in a 41 and 68% conversion, respectively after 120 hours.

As depicted in Figure 2.10, a conversion of 71% with HEPES indicates the flexibility of this buffer compared to MOPS. Chiral column HPLC chromatographs of the DKR reactions (for the first set) of various amounts of racemic IME versus % conversion to (S)-ibuprofen after 144 hours are depicted in Figure 2.11.

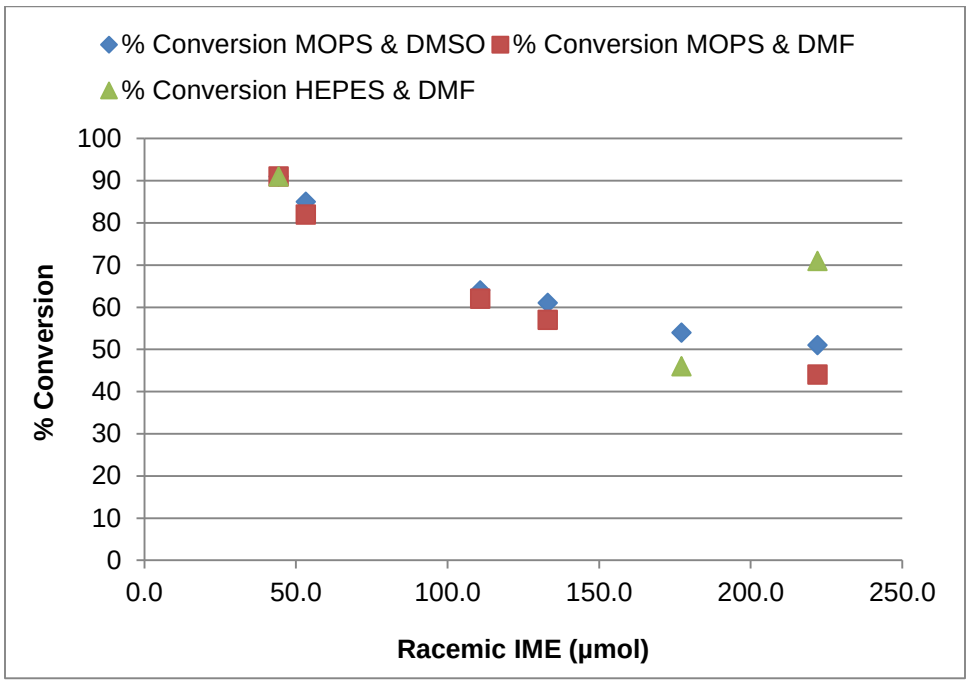


Figure 2.10 Effect of µmol of Substrate on DKR of Racemic IME at Optimal pH

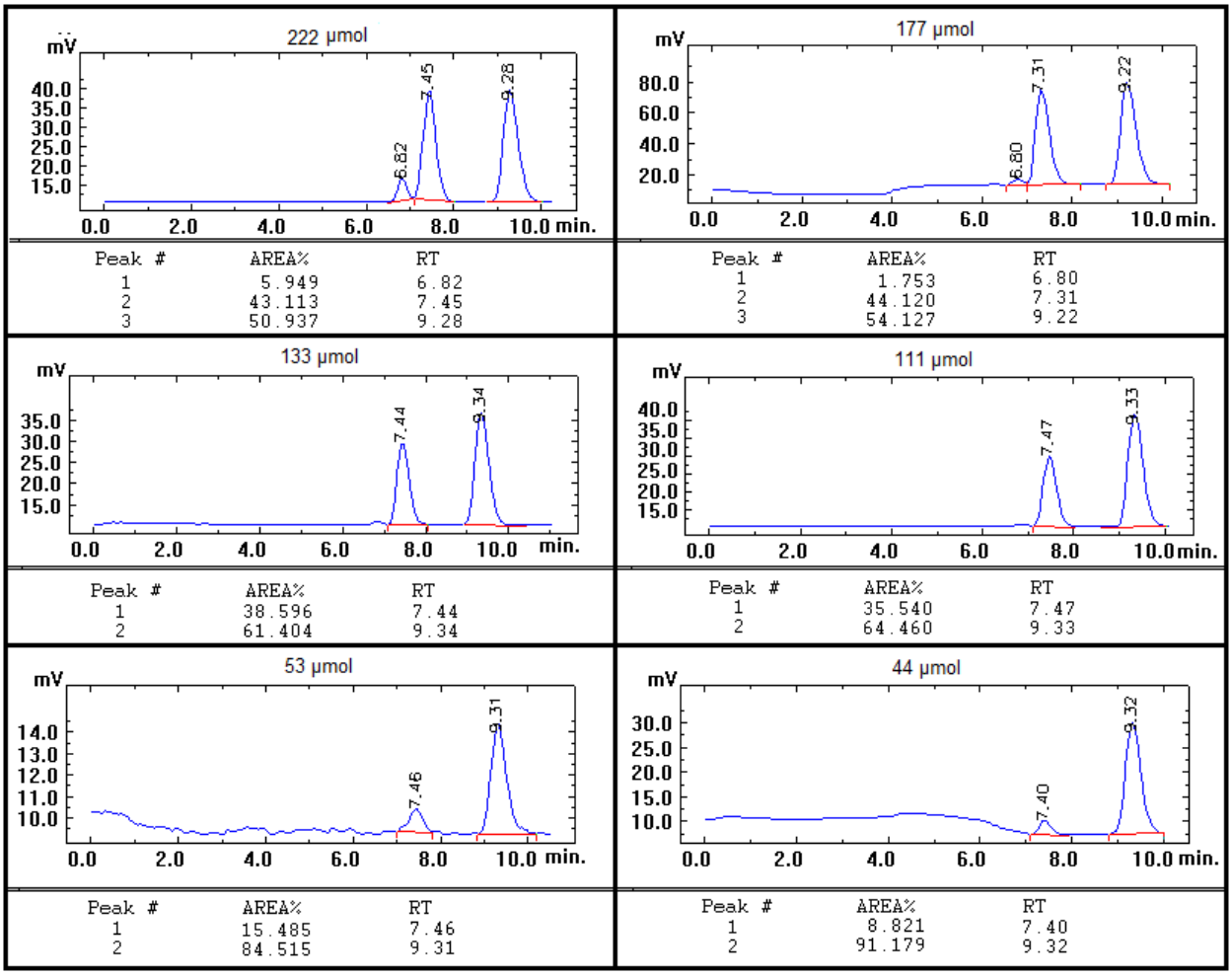


Figure 2.11 Effect of µmol of Substrate on DKR of Racemic IME at Optimal pH

2.4.4 Optimization of DKR Reactions with Respect to Co-solvent

Once it was verified that a lower substrate concentration would lead to a more fruitful conversion, the next step was examining different co-solvents. Since the literature states that DMF is more enantio-selective in KR reactions than DMSO [17], DKR reactions in conversion of racemic IME to (*S*)-ibuprofen were examined with the two co-solvents while all other parameters were held constant and optimized based on the results obtained up to this point.

Two sets of experiments were conducted with different buffers with all other parameters constant and optimized. In the first set of experiments, 0.8 mL of 0.5 M of a different buffer solution per experiment (bicine, HEPES, MOPS, phosphate, tricine, TES, tris, or triethanolamine),

0.2 mL DMSO, 10 µL (44.3 µmol) racemic IME, and 40 mg CRL were utilized while in the second set of experiments the same reaction conditions were applied with 0.95 mL of 0.5 M of the same buffer solutions, and 0.05 mL DMF. The pH of the reactions was measured before adding the lipase and adjusted to 7.6 after adding the enzyme. The aqueous mixtures were stirred for 72 hours at 40 ºC followed by chiral column HPLC analysis. Generally, after adding DMF to all the buffers, little or no change of pH was observed.

Although racemic IME is insoluble in water, it was visually observed that both the addition of DMSO [28, 29] and DMF [17], as well as increasing the pH above 9.0, increased the solubility of the substrate in the buffer solutions. In addition, DMSO and DMF were used as co-solvents in DKR reactions to enhance the solubility [29, 110] of racemic IME in the aqueous buffer and the enantio-selectivity of CRL at concentrations previously used to perform DKR and KR, namely 20% for DMSO and 5% for DMF [17, 28, 29]. These solvents were also theorized to increase the rate of enolization of racemic IME at higher pH [28, 29, 110]. In addition to observing a greater rate of reaction for DMF over DMSO, the pH of the reactions with the former co-solvent were more stable whereas the pH with the latter co-solvent always decreased over time. This may indicate that DMSO is helping to convert lactose aldehydes to organic acids [111]. In hindsight, DMSO should be avoided for DKR reactions, whereas DMF was found to be the best co-solvent for these reactions as it was reported for KR [17]. An example of chiral column HPLC chromatographs of the DKR reactions is depicted in Figure 2.12 which demonstrates the enhanced performance of the DMF versus DMSO reactions with all other variables constant (0.8 or 0.95 mL of 0.5 M MOPS buffer, 0.2 or 0.05 mL of DMSO or DMF, respectively, 10 µL or 44.3 µmol racemic IME, and 40 mg CRL). In this example, after 72 hours the pH decreased by 0.6 for DMSO (the pH after 3 days was 7.0), while it did not change for DMF (the pH after 3 days was 7.60).

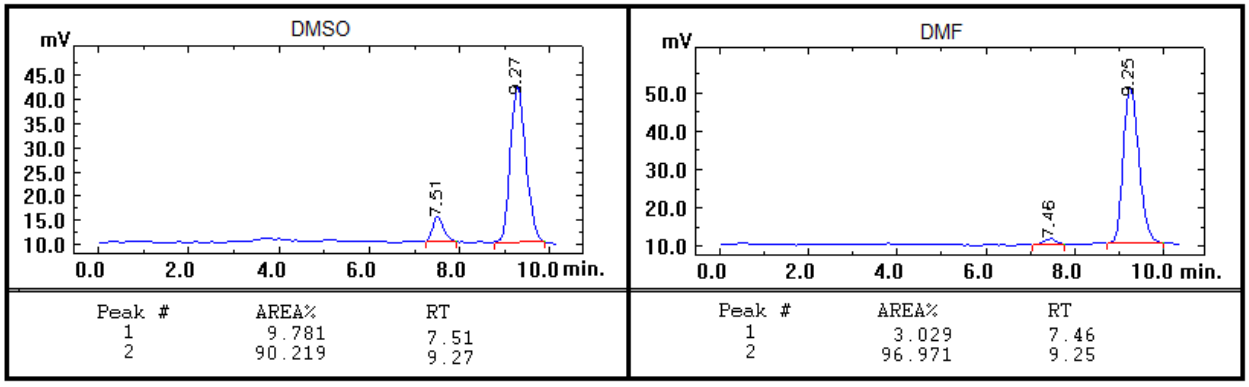


Figure 2.12 DMSO versus DMF in DKR of Racemic IME

2.4.5 Optimization of DKR Reactions with Respect to Mass of CRL Enzyme

DKR reactions in conversion of racemic IME to (*S*)-ibuprofen were examined with a set of reactions by varying the amount of CRL to observe the effect of this parameter while all other parameters were optimized and held constant. Therefore, 0.95 mL of 0.5 M buffer solutions of HEPES or MOPS, 0.05 mL DMF, 10 µL (44.3 µmol) racemic IME, and different amounts of CRL (50, 40, 30, 20, 15, and 0 mg) were utilized. The pH of the reactions was measured before adding the lipase and adjusted to 7.6 after adding the lipase (Table 2.9). The aqueous mixtures were stirred for 72 hours at 40 ºC followed by chiral column HPLC analysis. Within these 72 hours, the reactions were exposed to air 4 times.

Table 2.9 Various Amounts of CRL in DKR Reactions of Racemic IME[a]

Exp #	Buffer	pH after CRL	Duration (hours)	pH	mg of CRL per mL of Buffer	% Conversion
1	HEPES	NA	72	7.60	0	0
2	"	7.63	"	7.52	15	60
3	"	7.63	"	7.52	20	89
4	"	7.63	"	7.57	30	89
5	"	7.63	"	7.61	40	91
6	"	7.63	"	7.60	50	89
7	MOPS	NA	"	7.60	0	0
8	"	7.60	"	7.58	15	55
9	"	7.60	"	7.55	20	82
10	"	7.60	"	7.57	30	81
11	"	7.60	"	7.55	40	80
12	"	7.60	"	7.59	50	81

a 0.95 mL 0.5 M buffer, 0.05 mL DMF, 44.3 µmol IME

Since the amount of enzyme required to convert racemic IME to (*S*)-ibuprofen was varied, it was confirmed that the lowest and optimal amount of lipase for a 72 hour-reaction was 20 mg/mL of buffer using 44.3 µmol of substrate, Figure 2.13. That additional lipase was not required is important to reduce costs and simplify purification. The latter is a concern because the lipase-lactose mixture creates emulsions that are difficult to workup. Because these reactions were exposed to air 4 times, a lower conversion was achieved. Chiral column HPLC chromatographs of the DKR reactions in conversion of racemic IME to (S)-ibuprofen at optimal conditions with various amounts of CRL after 72 hours are depicted in Figure 2.14.

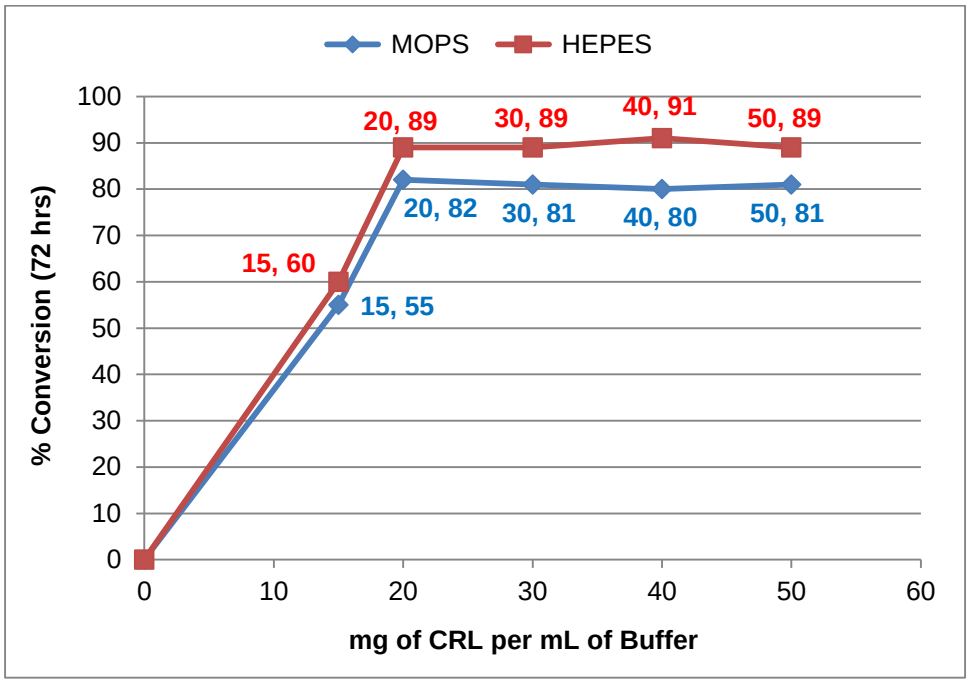


Figure 2.13 mg of CRL versus % Conversion of Racemic IME to (*S*)-Ibuprofen

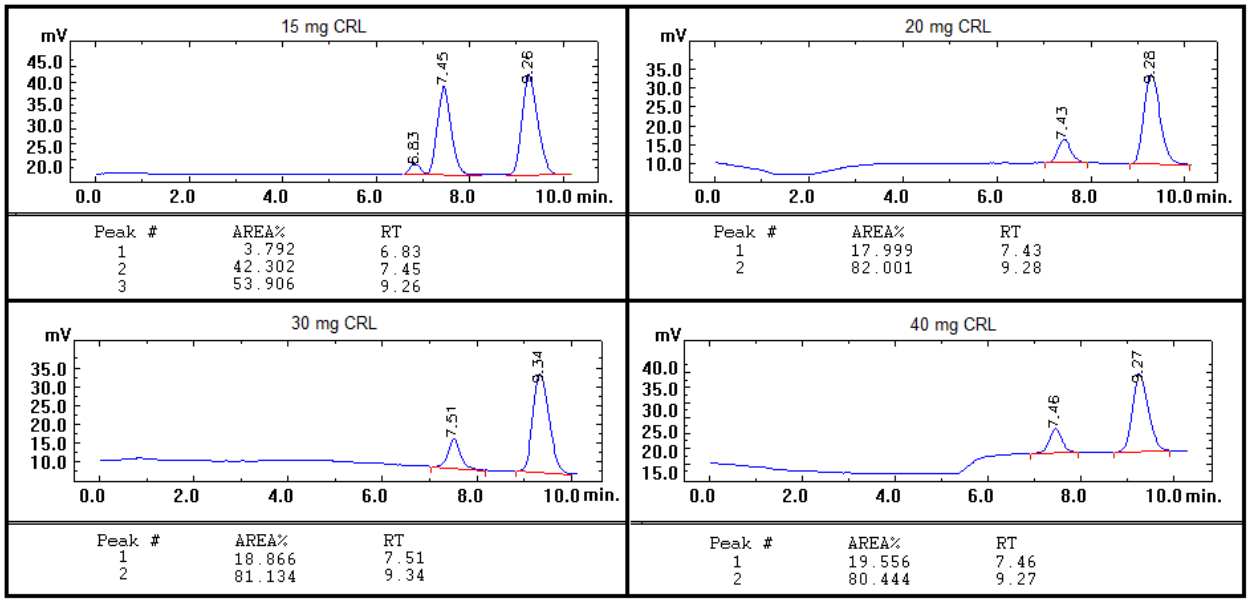


Figure 2.14 CRL Amount versus % Conversion in DKR Reactions of Racemic IME

2.4.6 Optimization of DKR Reactions with Respect to Temperature

Although the DKR reactions of racemic IME have been examined at 40 ºC by Chavez *et al.* [29], a couple of experiments were run at optimized conditions to monitor the temperature

effect on these reactions. Therefore, 0.95 mL of 0.5 M buffer solution of MOPS, 0.05 mL DMF, 10 µL (44.3 µmol) racemic IME, and 40 mg CRL were utilized. The pH of the reactions was measured before adding the lipase (7.97) and adjusted to 7.60 after adding the lipase. The aqueous mixtures were stirred for 72 hours at 30 or 45 ºC followed by chiral column HPLC analysis. At 30 ºC only 47% conversion was obtained showing that this temperature is low for a full conversion at optimized conditions. On the other hand, at 45 ºC a 77% conversion was achieved although 40 ºC gave the best results. Chiral column HPLC chromatographs of the DKR reactions in conversion of racemic IME to (*S*)-ibuprofen at optimal conditions with the two set of temperatures at 30 or 45 ºC are depicted in Figure 2.15.

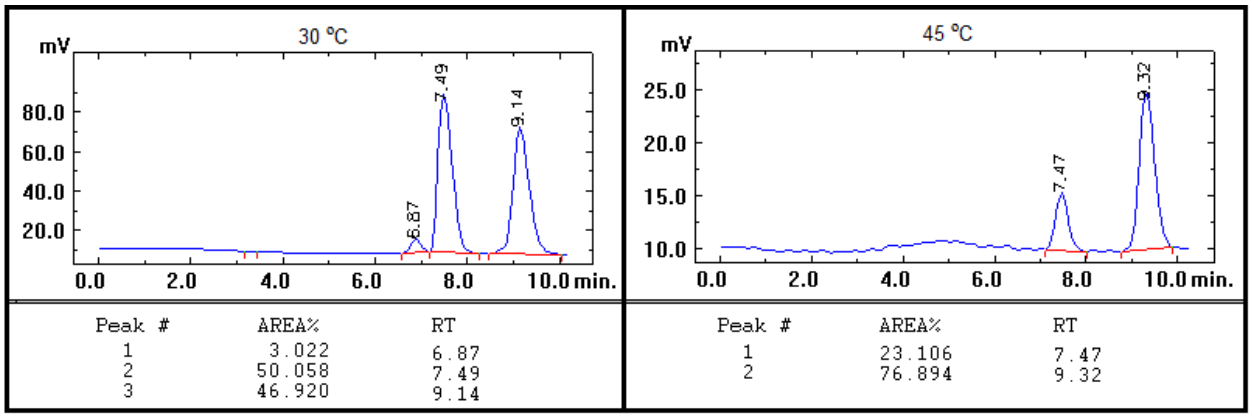


Figure 2.15 DKR Reactions of Racemic IME at 30 and 45 ºC

2.4.7 Optimization of DKR Reactions with Respect to Agitation Rate

Finally, a higher stir rate was examined to check the effect of a higher agitation rate at optimized conditions. Therefore, 0.95 mL of 0.5 M of a different buffer solution per experiment (bicine, HEPES, MOPS, phosphate, tricine, TES, tris, and triethanolamine), 0.05 mL DMF, 10 µL (44.3 µmol) racemic IME, and 40 mg CRL were utilized. The pH of the reactions was measured before adding the lipase and adjusted to 7.60 after adding the lipase. The aqueous mixtures were stirred at the highest possible rate of each individual magnetic stirrer for 72 hours (without opening the reaction caps) at 40 ºC followed by chiral column HPLC analysis.

Unlike liquid reactions in which reagents are mixed by diffusion control, because the lipase is only suspended in the aqueous media of DKR reactions, stirring was very important [66]. Chiral column HPLC chromatographs of one set of DKR reactions under optimal conditions at lower and higher stir rates with almost a 100% conversion (with MOPS, left and HEPES, right) for the latter are depicted in Figure 2.16.

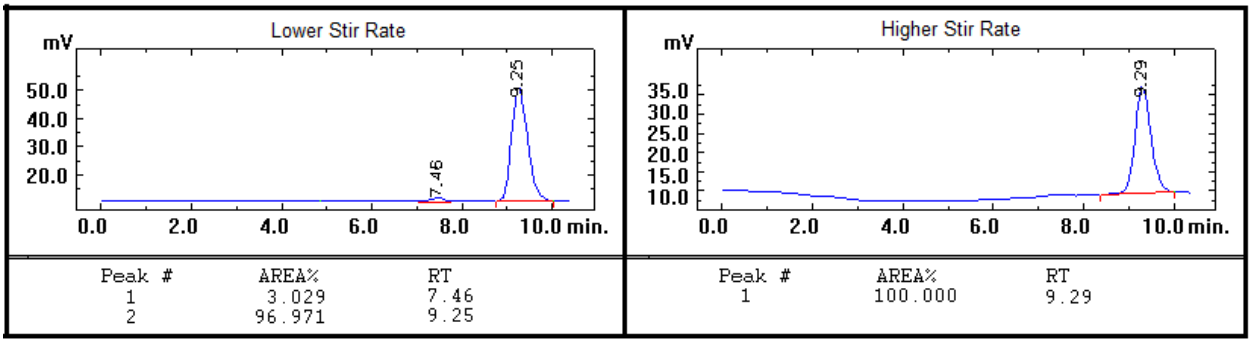


Figure 2.16 DKR of Racemic IME at Lower and Higher Stir Rates

2.5 Optimized Small Scale DKR Reactions

DKR reactions under optimal conditions were re-examined with 0.95 mL of 0.5 M buffer solution of HEPES, 0.05 mL DMF, 10 µL (44.3 µmol) racemic IME, and 40 mg CRL. The pH of the reactions was measured before adding the lipase and adjusted to 7.58, 7.63, and 7.68 after adding the lipase. The aqueous mixtures were stirred at the highest possible rate of each individual magnetic stirrer for 72 hours (without opening the reaction caps) at 40 ºC followed by chiral column HPLC analysis (Table 2.10).

Table 2.10 Optimized DKR of Racemic IME at pH Close to 7.6[a]

Exp #	pH before CRL	pH after CRL	Duration (hours)	pH	% Conversion
1	7.76	7.58	72	7.50	100
3	7.83	7.63	"	7.60	83
3	7.87	7.68	"	7.56	69

a 0.95 mL 0.5 M HEPES, 0.05 mL DMF, 44.3 µmol IME, 40 mg CRL

Figure 2.17 confirms that DKR reactions in conversion of racemic IME to (*S*)-ibuprofen under optimal conditions worked at the optimal pH of 7.6. Chiral column HPLC chromatographs of DKR reactions under optimal conditions at pH 7.58, 7.63, and 7.68, after adding the lipase, are depicted in Figures 2.16 (right chromatograph) and 2.18.

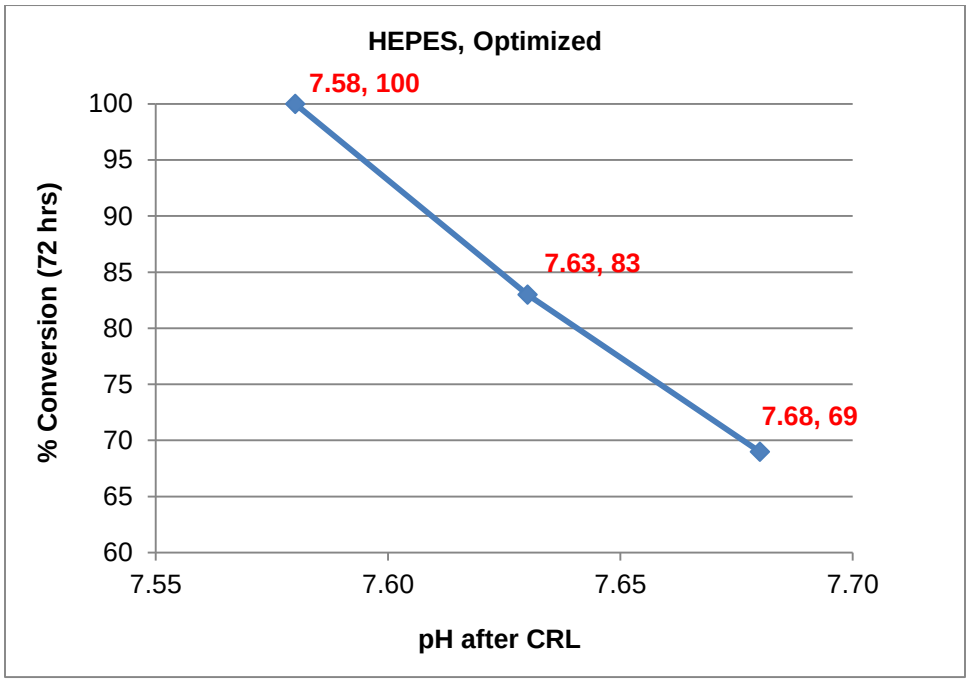


Figure 2.17 Optimized DKR of Racemic IME at pH Close to 7.6

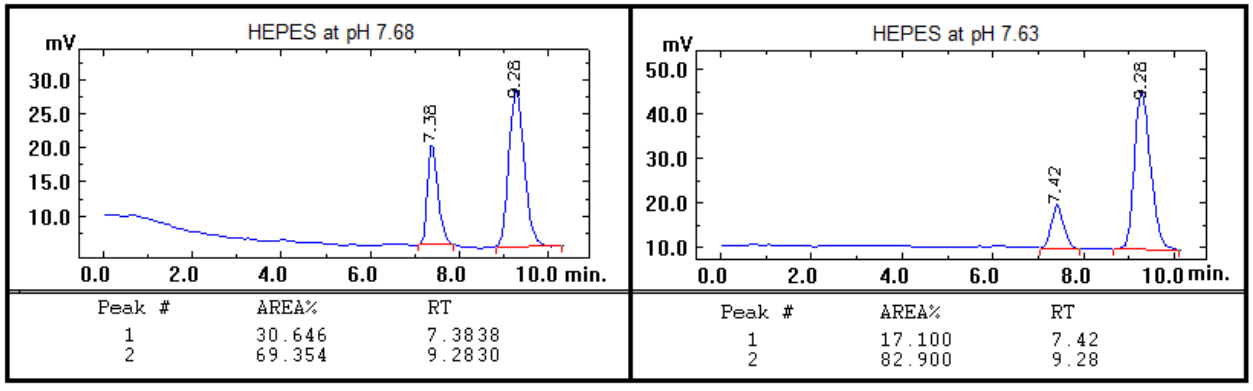


Figure 2.18 Optimized DKR of Racemic IME at pH Close to 7.6

Furthermore, DKR reactions in conversion of racemic IME to (*S*)-ibuprofen was re-examined in a set of reactions with all 8 different buffers at optimal conditions to confirm all the

achieved results. Therefore, in this set of experiments, 0.95 mL of 0.5 M of a different buffer solution per experiment (phosphate II, MOPS, TES, triethanolamine, HEPES, tris, tricine, and bicine), 0.05 mL DMF, 10 µL (44.3 µmol) racemic IME, and 40 mg CRL were utilized. The pH of the reactions was measured before adding the lipase (Table 2.11) and adjusted to 7.60 after adding the lipase. The aqueous mixtures were stirred at the highest possible rate of each individual magnetic stirrer for 72 hours (without opening the reaction caps) at 40 ºC followed by chiral column HPLC analysis.

Table 2.11 Optimized DKR Reactions in Conversion of Racemic IME to (*S*)-Ibuprofen[a]

Exp #	Buffer	pH before CRL	pH after CRL	Duration (hours)	pH	% Conversion
1	Phosphate II	7.70	7.60	72	7.54	92
2	MOPS	7.94	7.60	72	7.61	100
3	TES	7.82	7.60	72	7.45	97
4	Triethanolamine	7.69	7.60	72	7.35	95
5	HEPES	7.76	7.60	72	7.50	100
6	Tris	7.82	7.60	72	7.15	90
7	Tricine	7.90	7.60	72	7.28	92
8	Bicine	8.00	7.60	72	7.20	98

a 0.95 mL 0.5 M buffer, 0.05 mL DMF, 44.3 µmol IME, 40 mg CRL

DKR reactions in conversion of racemic IME to (*S*)-ibuprofen with 8 different buffers at fully optimized conditions resulted in yields above 90%, confirming that these reactions work with buffers with pKa range 7.20 to 8.35 at 0.5 M concentration, a pH of 7.6 (after adding CRL), using DMF instead of DMSO, increased stirring, and avoiding opening the reactions for 72 hours. Chiral column HPLC chromatographs of the DKR reactions with the 8 different buffers at fully optimized conditions are illustrated in Figure 2.19.

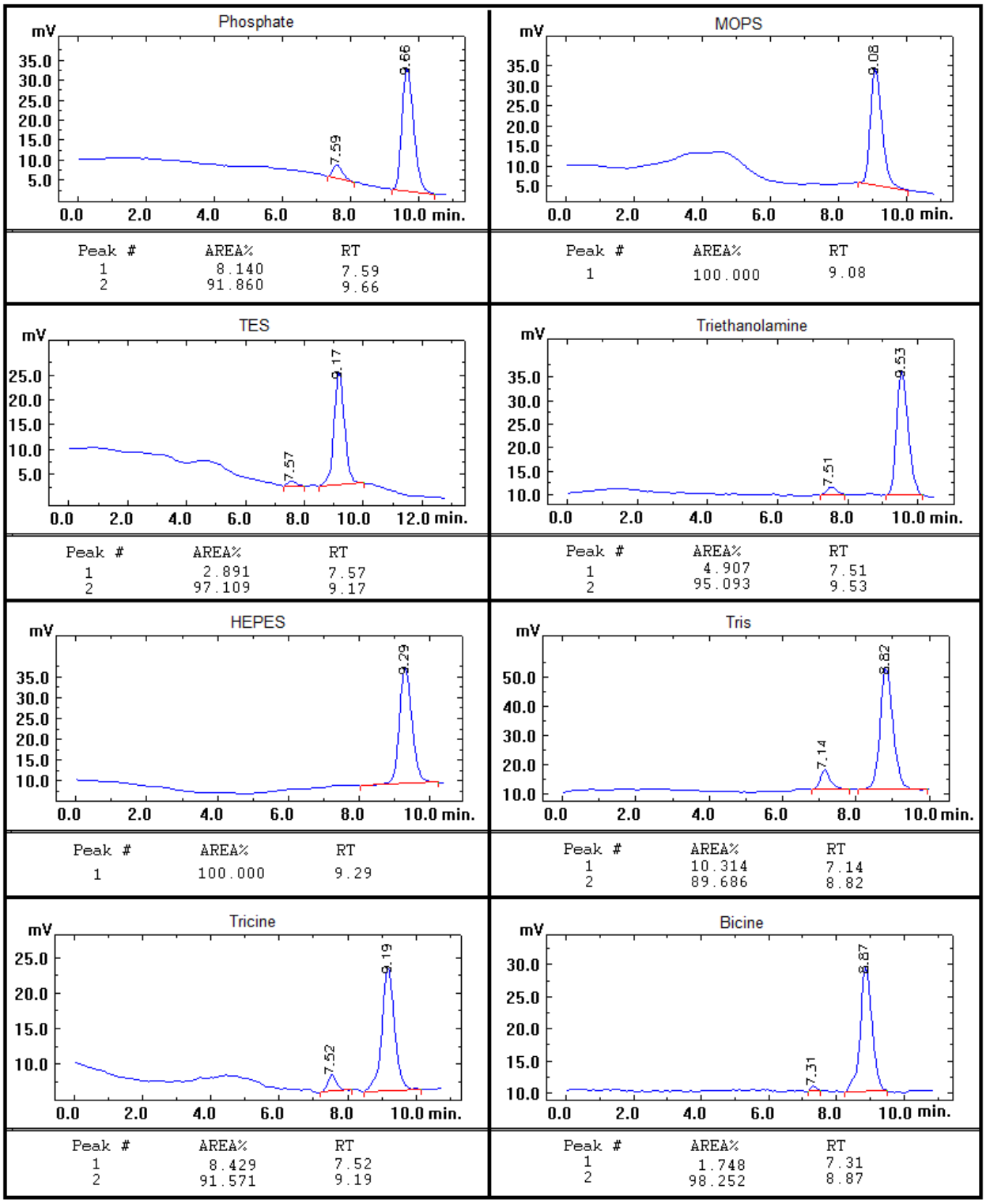


Figure 2.19 Optimized DKR Reactions with 8 Different Buffers

2.6 Optimized Large Scale DKR Reaction

An optimized large scale reaction was run to confirm that the DKR reaction is also viable at larger scale. Therefore, 48.0 mL of 0.20 M potassium phosphate buffer at pH 7.6, 2.0 mL (4% volume) of DMF, 1.00 g (4.54 mmol) of racemic IME, and 2.00 g CRL were used. The pH dropped to 7.3 upon adding the lipase, and by adding 0.1 M NaOH the pH was adjusted to 7.6. The reaction mixture was stirred at 300 rpm for 72 hours at 40 °C followed by chiral column HPLC analysis. (*S*)-ibuprofen was isolated from the reaction by first centrifuging the mixture to precipitate the enzyme, basifying with sodium bicarbonate to produce (*S*)-ibuprofen sodium salt, and extracting with cyclohexane to separate the unreacted (*R*)-IME from the salt. Next, the salt was acidified to make the carboxylic acid, extracted with cyclohexane, and the solvent was removed under reduced pressure to afford 93.6% yield (0.876 g, 4.25 mmol) of (*S*)-ibuprofen (96% ee). The determined optical rotation of $[\propto]_D^{20} = +57°$ (c 1, anhydrous ethanol, lit. +59.5°) and the melting point of 56-58 °C (lit. 55 °C) are in agreement with literature values [29, 112, 113]. A chiral column HPLC chromatograph of this DKR reaction is depicted in Figure 2.20; ^{1}H NMR, ^{13}C NMR, and IR spectra of (*S*)-ibuprofen are the same as racemic ibuprofen and are available in Appendix A, as Figures A.5 and A.6 (^{1}HNMR and IR) which matched literature values [104].

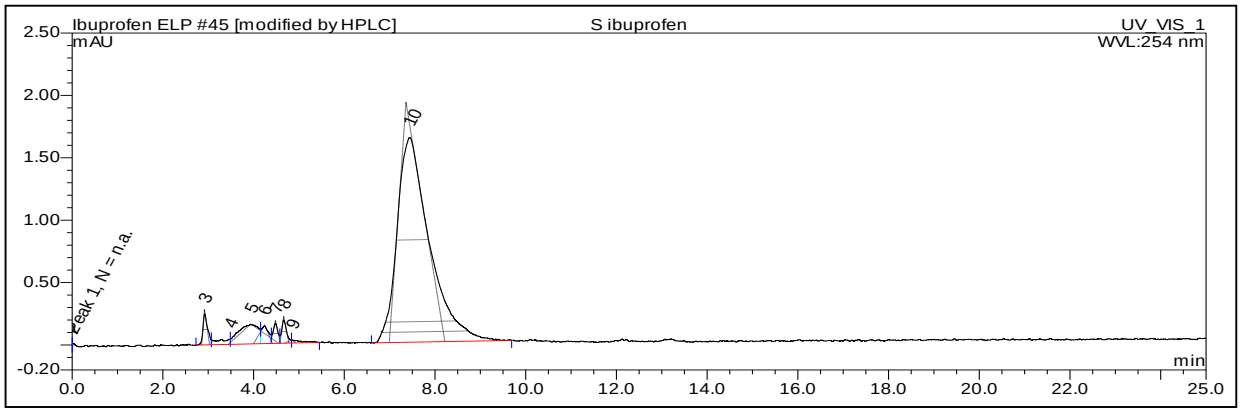
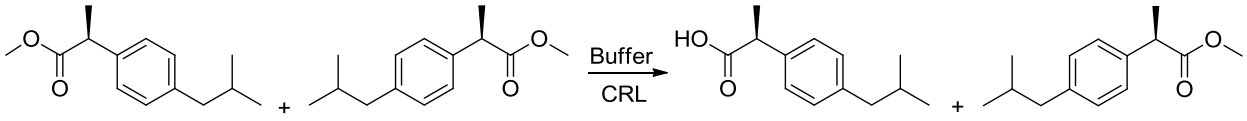


Figure 2.20 Optimized Large Scale DKR Reaction of Racemic IME to (*S*)-Ibuprofen

2.7 Resolution of (*R*)-Ibuprofen Methyl Ester, (*R*)-IME, by KR Reactions

In the process of optimizing DKR of racemic IME to (*S*)-ibuprofen, with respect to buffer, pH, substrate concentration, co-solvent, amount of enzyme, and stirring, (e.g., 0.95 mL of 0.5 M HEPES, 50 µL of 5% DMF, 10 µL or 44.3 µmol of racemic IME, and pH of 7.6 after adding 40 mg CRL), the most striking finding was that the pH of the buffer solution was greatly decreased by the addition of the acidic lipase-lactose mixture which might be due to the oxidation of the lactose filler to aldonic acids [98, 100]. This means that the racemization of (*R*)-IME to (*S*)-IME in the DKR reactions must be occurring at the optimal pH of 7.6. This is so close to neutral pH it seems unlikely that enolization is occurring in mildly basic buffer solutions as initially hypothesized [28, 29, 110]. To test this, KR [26] of racemic IME was employed to isolate (*R*)-IME [29], and subjected to the same pH and reaction conditions as DKR without lipase to observe whether or not racemization was occurring. The glycine buffer was used without using a base for adjusting the pH, and it was found to be a suitable buffer for running KR reactions because under acidic condition the pH did not change significantly. Even if the pH were to change it is still a KR reaction under acidic condition, and pH change does not matter. Therefore, 80.0 mL of the 1.0 M glycine buffer at pH 5.6, 4.0 mL (18 mmol) of racemic IME, and 3.20 g CRL were added respectively, and the mixture was stirred for 24 hours at 40 ºC followed by chiral column HPLC analysis. (*R*)-IME was isolated from the reaction by first centrifuging the mixture to precipitate the enzyme, acidifying with HCl to produce (*S*)-ibuprofen, and extracting with hexanes to isolate the unreacted (*R*)-IME from the acid. Next, the solvent was removed under reduced pressure to afford (*R*)-IME in 36% yield (1.42 g, 6.44 mmol) of the possible 50% theoretical yield of enriched (*R*)-IME (Scheme 2.3). Chiral column HPLC chromatographs of (*R*)-IME are shown in Figure

2.21; ^{1}H NMR, ^{13}C NMR, and IR spectra are available in Appendix A, Figures A.7, A.8, and A.9, which matched literature values [105].



Scheme 2.3 KR of Racemic IME to (*S*)-Ibuprofen and Unreacted (*R*)-IME

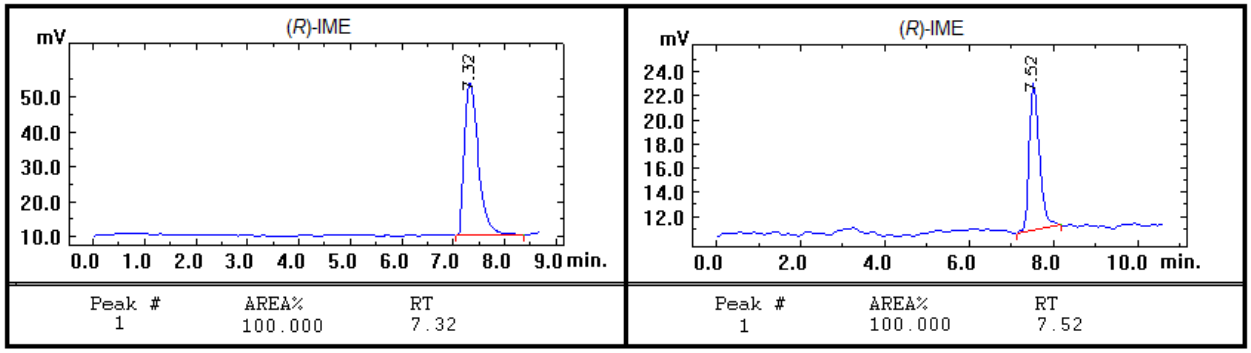


Figure 2.21 Chromatographs of (*R*)-IME after KR Reactions

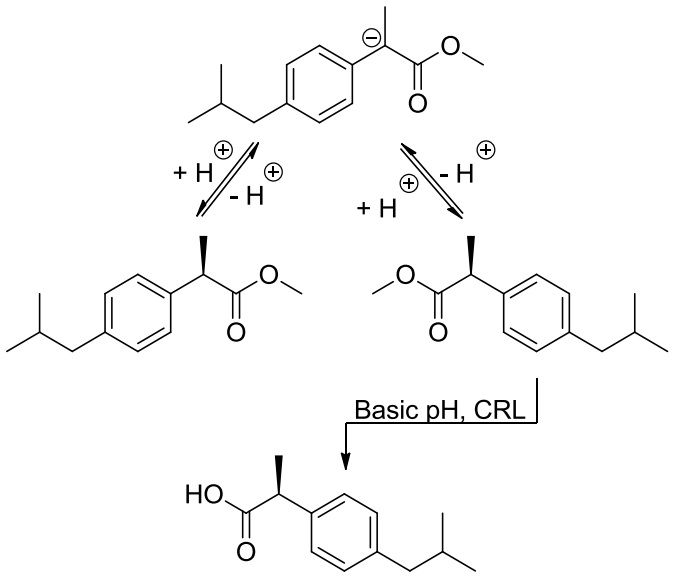
2.8 Attempted Racemization of (*R*)-IME without CRL

Using KR of racemic IME, (*R*)-IME was isolated and subjected to the same pH and reaction conditions as optimized DKR, (e.g., 0.95 mL of 0.5 M HEPES, 50 µL of 5% DMF, 10 µL or 44.3 µmol of (*R*)-IME, and pH of 7.6 in 144 hours), without the lipase to observe whether or not racemization was occurring based on the enolization hypothesis (Scheme 2.4). Also, different volumes of different buffers at different pHs and different ratios of reagents without the lipase were tested to observe whether or not racemization was occurring (Table 2.12).

Table 2.12 Attempted Racemization of (*R*)-IME without CRL at Optimal and various pH[a]

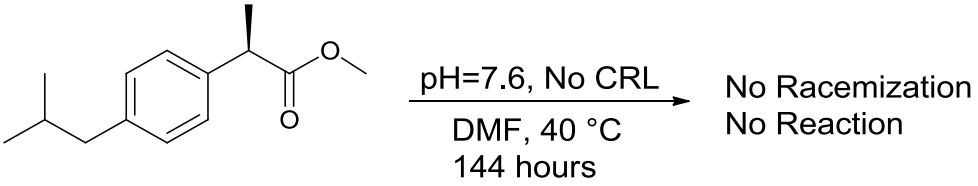
Exp #	Reaction Conditions	pH	% Conversion
1	50 µL (R)-IME, 100 µL DMSO, 400 µL Sodium Phosphate 0.25 M	6.60	0
2	"	7.00	0
3	50 µL (R)-IME, 100 µL DMSO, 400 µL Tricine 0.5 M	8.40	0
4	50 µL (R)-IME, 100 µL DMSO, 400 µL Ethanolamine 0.5 M	9.20	0
5	25 µL (R)-IME, 100 µL DMSO, 400 µL Sodium Bicarbonate 1.0 M	8.90	0
6	"	9.10	0
7	"	9.30	0
8	"	9.50	0
9	80 µL (R)-IME, 200 µL DMSO, 750 µL Sodium Bicarbonate 1.0 M	9.03	0
10	"	9.68	0
11	50 µL (R)-IME, 100 µL DMSO, 400 µL Bicine 0.5 M	6.57	0
12	"	7.23	0
13	"	7.46	0
14	25 µL (R)-IME, 100 µL DMSO, 400 µL Bicine 0.5 M	7.61	0
15	25 µL (R)-IME, 50 µL DMSO, 200 µL Bicine 0.5 M	7.61	0
16	"	7.64	0
17	50 µL (R)-IME, 100 µL DMSO, 400 µL Bicine 0.5 M	8.58	0
18	"	8.80	0
19	50 µL (R)-IME, 100 µL DMSO, 400 µL MOPS 0.5 M	7.34	0
20	"	7.61	0
21	same except MOPS 1.0 M	7.60	0
22	25 µL (R)-IME, 50 µL DMSO, 200 µL MOPS 0.5 M	7.55	0
23	"	7.57	0
24	25 µL (R)-IME, 100 µL DMSO, 400 µL MOPS 1.0 M	7.61	0
25	25 µL (R)-IME, 25 µL DMF, 475 µL MOPS 1.0 M	7.61	0
26	10 µL (R)-IME, 50 µL DMF, 950 µL MOPS 0.5 M (DKR Optimized)	7.60	0
27	"	7.60	0
28	"	7.60	0
29	50 µL (R)-IME, 100 µL DMSO, 400 µL HEPES 1.0 M	7.76	0
30	same except HEPES 0.5 M	8.05	0
31	10 µL (R)-IME, 50 µL DMF, 950 µL HEPES 0.5 M	7.68	0
32	"	7.63	0
33	" (DKR Optimized)	7.60	0
34	"	7.60	0
35	"	7.60	0

a The color code illustrates the type of buffer used.



Scheme 2.4 Previously Proposed DKR Mechanism of Racemic IME to (*S*)-Ibuprofen

As shown in Table 2.12, no racemization or hydrolysis of (*R*)-IME was observed by chiral column HPLC analysis over the course of 144 hours (Scheme 2.5). This experiment was repeated many times with different buffers at different conditions and over 10 times under the above-stated conditions with HEPES and MOPS with the same result of no racemization and no reaction.



Scheme 2.5 Attempted Racemization of (*R*)-IME without CRL

2.9 DKR Reactions without Co-solvent

Once all the parameters were optimized, a few reactions at optimal conditions were performed without the presence of co-solvent (e.g., 1.0 mL of 0.5 M HEPES, 10 µL or 44.3 µmol of racemic IME, and pH of 7.6 after adding 40 mg CRL) for 72 hours at 40 ˚C, to examine the absence of co-solvent at optimal conditions.

Figure 2.22 shows that although all racemic IME was converted to ibuprofen in 94 hours, the enantio-selectivity was reduced without the co-solvent. In particular, the enantiomeric excess of the product was only 68%, 84% (*S*) to 16% (*R*)-ibuprofen. In both chromatographs (*R*)-ibuprofen was present showing that without co-solvent the lipase enantio-selectivity was reduced. More important, under optimal conditions enzymatic hydrolysis can proceed without a co-solvent to more than 50% conversion; however a co-solvent does increase the enzyme enantio-selectivity.

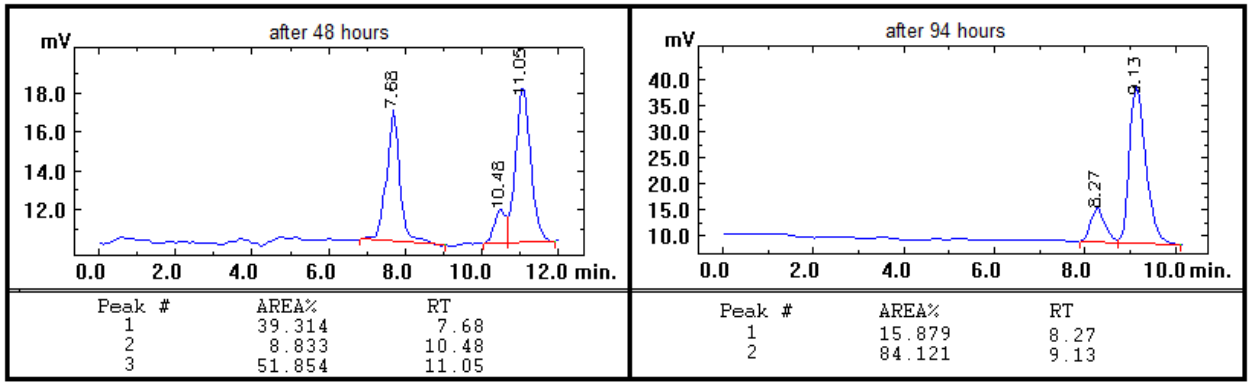


Figure 2.22 DKR of Racemic IME without Co-solvent after 48 and 94 Hours

2.10 Product Precipitation from DKR Reactions

After conversion of racemic IME to the *(S)*-ibuprofen (Figure 2.23.a), if the reaction was allowed to cool to room temperature (22 ℃) for 20 minutes or so, there was an apparent increase in the amount of starting ester by chiral column HPLC analysis as shown in Figure 2.23.b. This phenomenon was observed multiple times. If these room temperature samples were warmed to 40 ℃, the original analysis of only one strong peak was observed. This indicates that (*S*)-ibuprofen was precipitating out of the buffer solution at room temperature but a small amount of the original ester substrate was not precipitating. Thus, care must be taken to quickly sample each reaction close to its reaction temperature.

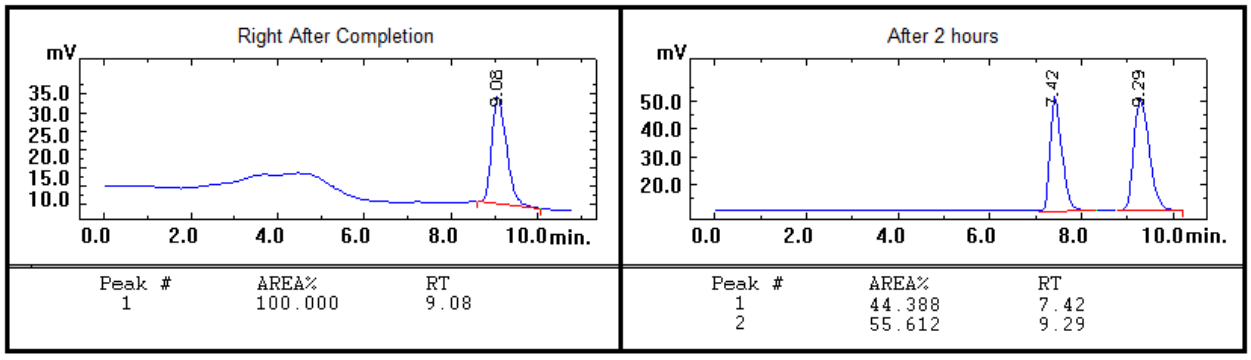


Figure 2.23.a) DKR after Completion b) after 2 Hours at Room Temperature

2.11 Naphthalene as Inhibitor of KR Reactions

Prior to understanding this precipitation phenomenon, several compounds were tested as concentration standards for KR reactions. Among these were phenol, catechol, phenyl acetic acid, acetone, and naphthalene. KR of racemic IME with all the standards (e.g., 4.3 mL of 0.5 M sodium bicarbonate buffer, 0.9 mmol racemic IME, 3 mg naphthalene, and 220 mg CRL) was performed. The retention time of naphthalene was 8.92 (100%) with no other peak present (1 mg naphthalene was dissolved in 0.23 mL hexanes). Once added to KR reactions, none of these compounds could be observed by HPLC analysis except naphthalene (Figure 2.24, the second peak). Naphthalene completely inhibited the conversion of racemic ibuprofen to (*S*)-ibuprofen and only the first peak of racemic IME was observed in all the trials with naphthalene.

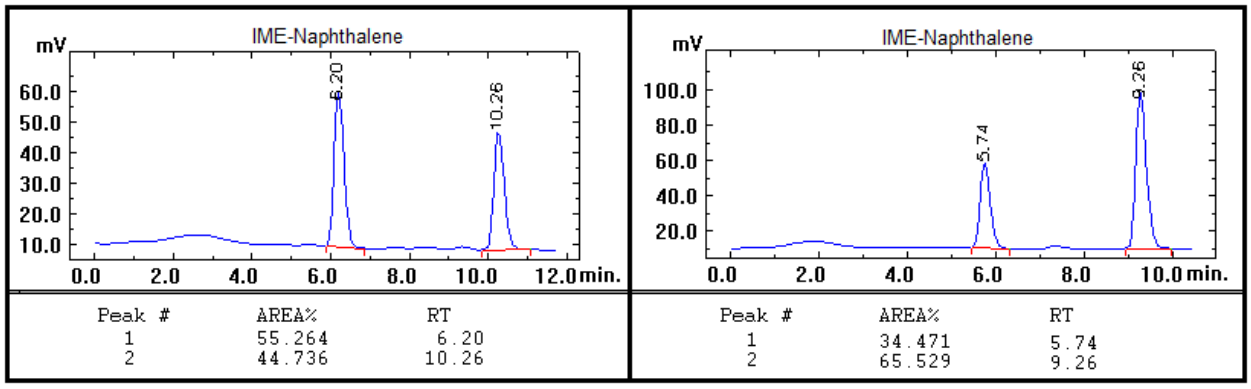


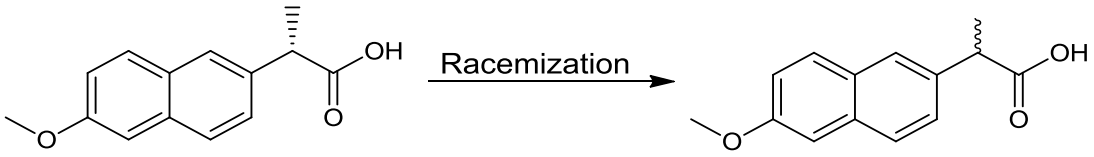
Figure 2.24 Naphthalene as Inhibitor of KR Reactions of Racemic IME

2.12 Conversion of Racemic Naproxen to (*S*)-Naproxen

The DKR reaction of the naproxen methyl ester (NME) to (*S*)-naproxen was examined. The goal was to synthesize racemic naproxen from commercial (*S*)-naproxen, and to convert it back to (*S*)-naproxen as test model of the more generic process for other profens. (*S*)-naproxen was isolated from (*S*)-naproxen sodium salt tablets for esterification, but a shorter procedure was established using direct Fischer esterification of naproxen sodium salt to make (*S*)-NME. Buffer solutions of HEPES or MOPS and lauric acid as surfactant were utilized for testing DKR reactions that only ended up as hydrolysis reactions. Then, racemization of (*S*)-NME at pHs close to 7.6 was attempted.

2.12.1 Isolation of (*S*)-Naproxen form (*S*)-Naproxen Sodium Tablets

Inexpensive (*S*)-naproxen was isolated from commercial (*S*)-naproxen sodium tablets (220 mg/tablet) by water extraction (1 tablet/5 mL), vacuum filtration, addition of HCl to the filtrate up to pH 5, extraction with dichloromethane, and solvent removal under reduced pressure to give 2.156 g with a 98% yield of pure naproxen carboxylic acid. A substantial savings over naproxen purchased from chemical suppliers was achieved. Chiral column HPLC chromatographs of naproxen are shown in Figure 2.25.a. By adding more acid in the above process, racemization of naproxen was observed with no reflux and in only a few seconds (Scheme 2.6 and Figure 2.25.b). Different solvents such as DMSO and DMF were tested for solubility of the tablets, but naproxen sodium was more soluble in ethanol. In the process of extraction naproxen was not soluble in hexanes, so dichloromethane was utilized to extract naproxen from the aqueous layer. ^{1}H NMR and ^{13}C NMR spectra of (*S*)-naproxen are available in Appendix A, Figures A.10 and A.11.



Scheme 2.6 Racemization of (*S*)-Naproxen

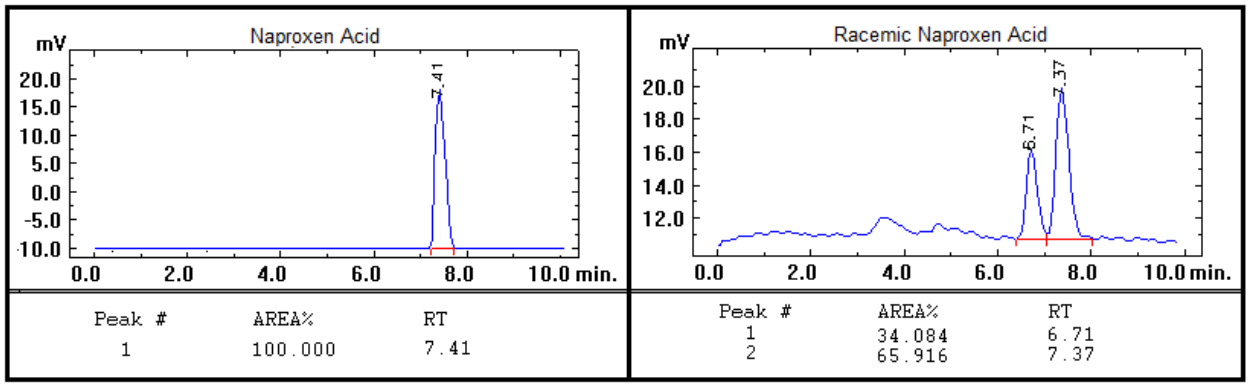
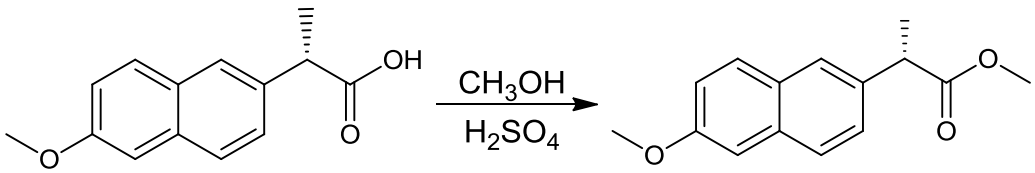


Figure 2.25.a) Extracted Naproxen from Tablets b) Racemization of Naproxen

2.12.2 Conversion of (*S*)-Naproxen Sodium to (*S*)-Naproxen Methyl Ester (NME)

Direct Fischer Esterification was performed with 10 naproxen sodium tablets (2.2 g, 8.7 mmol), excess methanol and acid catalysis (Scheme 2.7). The (*S*)-NME was extracted with hexanes several times and the solvent was removed under reduced pressure, and the ester was further distilled bulb to bulb by horizontal distillation to give 2.00 g (94% yield) of (*S*)-NME as a white crystalline solid. A chiral column HPLC chromatograph of (*S*)-NME is shown in Figure 2.26. ^{1}H NMR and ^{13}C NMR spectra of (*S*)-NME are available in Appendix A, Figures A.12 and A.13.



Scheme 2.7 Fischer Esterification of (*S*)-Naproxen to (*S*)-NME

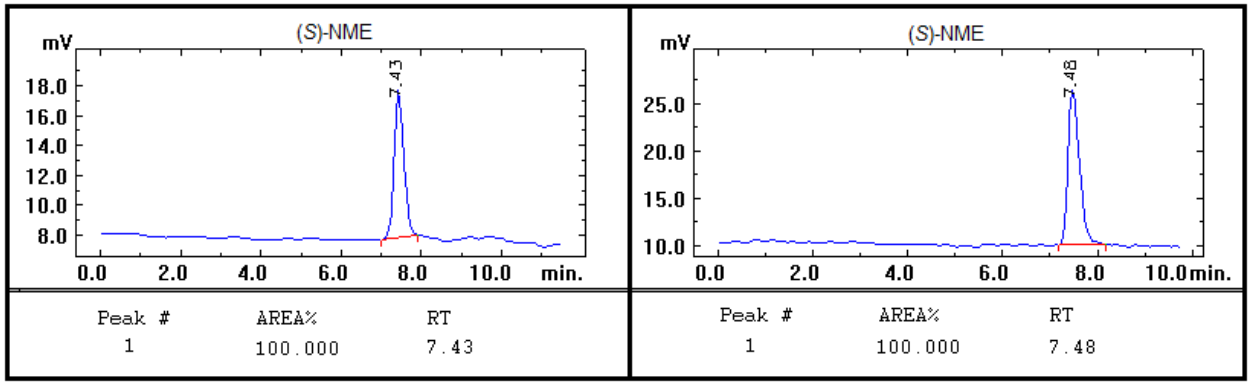


Figure 2.26 (*S*)-NME from Direct Fischer Esterification

2.12.3 Hydrolysis of (*S*)-Naproxen Methyl Ester to (*S*)-Naproxen

The obtained (*S*)-NME was subjected to enzymatic hydrolysis conditions (Table 2.13) to test whether the less sensitive hydrolysis reaction would occur to convert the more insoluble ester to (*S*)-Naproxen.

Table 2.13 Hydrolysis of (*S*)-NME to (*S*)-Naproxen under Different Conditions

Exp #	40 mg CRL (*S*)-NME (mg), Lauric Acid (mg) DMF (µL)	pH Before CRL	pH After CRL	Duration (hours)	pH	% Conversion
1	10, 10, 50, 950 µL MOPS 1.0 M	7.78	7.64	144	7.43	46
2	10, 10, 50, 950 µL MOPS 0.5 M	7.94	7.60	72	7.42	12
3	5, 5, 75, 1425 µL HEPES 0.5 M	7.78	7.64	72	7.51	13
4	5, 5, 75, 1425 µL HEPES 0.5 M	7.80	7.68	72	7.50	52
5	10, 5, 50, 950 µL HEPES 0.5 M	7.78	7.64	144	7.37	46
6	5, 5, 50, 950 µL HEPES 0.5 M	7.81	7.64	144	7.39	5

The hydrolyses did not go to full completion after 144 hours and ceased at about 50% conversion (Figure 2.27 and Table 2.13, experiments # 1 and 4) due to the same concern with racemic IME of products inhibition and the methanol byproduct that inactivated the lipase [1, 8, 28, 59, 66, 69]. On top of the inhibition and inactivation, the solubility of the (*S*)-NME was a concern that prevented the hydrolysis to proceed. Indeed, the solubility of (*S*)-NME was a far greater issue than for racemic IME. Although lauric acid (LA, dodecanoic acid) was added as a surfactant to improve the solubility, (*S*)-NME was never completely dissolved (Table 2.13).

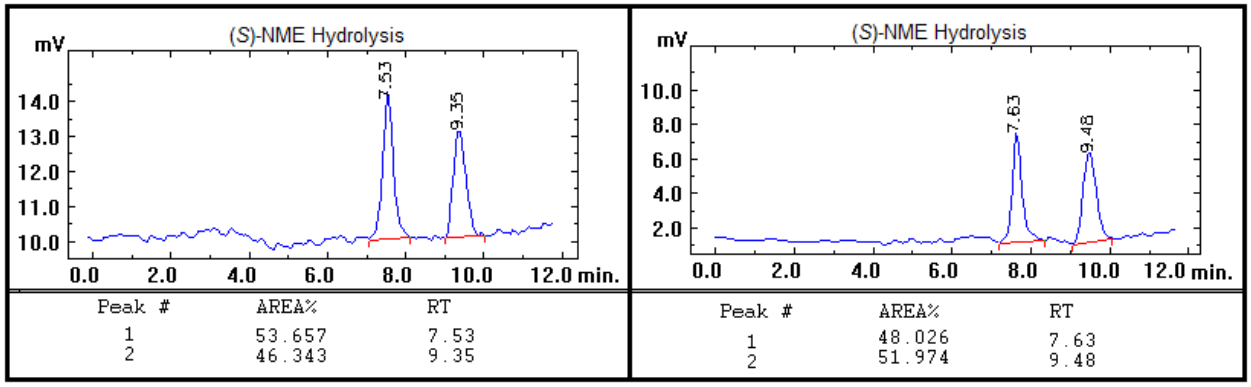


Figure 2.27 Hydrolysis of (*S*)-NME to (*S*)-Naproxen

The same phenomenon of product precipitation that was observed with (*S*)-ibuprofen was also observed with (*S*)-Naproxen to a greater extent (Figures 2.28.a, 2.28.b, and Table 2.13, experiment # 5). Upon keeping the reaction at room temperature for about 20 minutes, analysis of an aliquot showed a greater ratio of ester to carboxylic acid than when the aliquot was removed from the warm reaction.

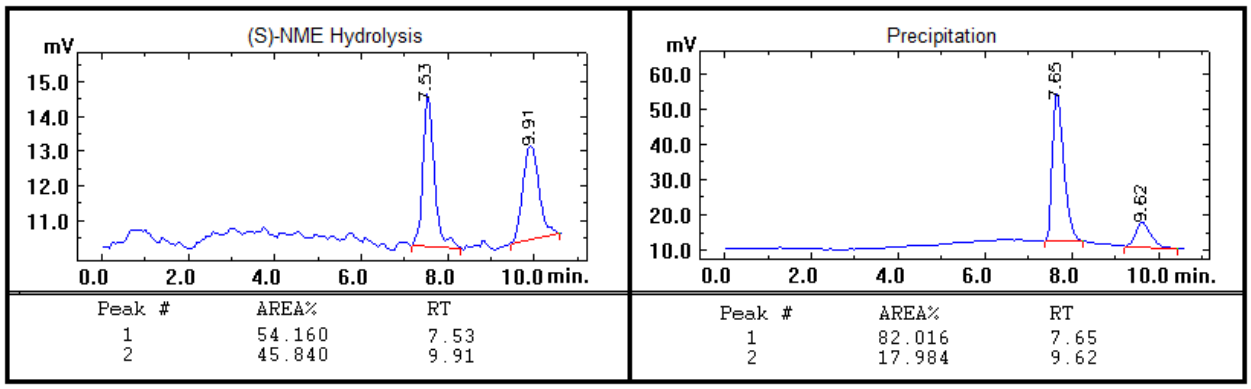


Figure 2.28.a) Hydrolysis of (*S*)-NME b) (*S*)-Naproxen Precipitation after 20 minutes

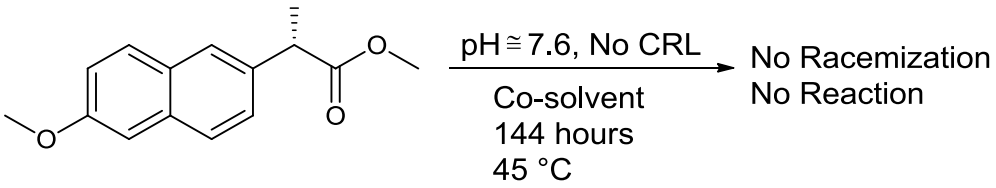
2.12.4 Attempted Racemization of (*S*)-Naproxen Methyl Ester without CRL

Using direct Fischer esterification on naproxen sodium salt tablets, (*S*)-NME was obtained and subjected to racemization reaction conditions without the lipase over 144 hours at a pH mostly close to 7.6 to observe whether or not racemization was occurring (Table 2.14).

Table 2.14 Attempted Racemization of (*S*)-NME

Exp #	(*S*)-NME (mg), DMSO (µL), Buffer (µL)	Temperature (°C)	pH	% Conversion
1	27, 100, 400, 0.5 M MOPS	45	7.29	0
2	27, 100, 400, "	45	7.40	0
3	27, 100, 400, "	45	7.52	0
4	27, 100, 400, "	45	7.45	0
5	13, 100, 400, "	45	7.65	0
6	10, 200, 800, 5 mg LA, 1.0 M MOPS	45	7.60	0
7	10, 200, 800, "	45	7.66	0
8	10, 50 DMF, 950, "	45	7.70	0
9	10, 50 DMF, 950, 5 mg LA, "	45	7.65	0
10	5, 75 DMF, 1425, 5 mg LA, 0.5 M HEPES	40	7.70	0
11	5, 50 DMF, 950, 5 mg LA, "	40	7.57	0

No racemization or hydrolysis of (*S*)-NME was observed by chiral column HPLC analysis over the course of 144 hours (Scheme 2.8 and Figure 2.29). This experiment was repeated with 0.5 or 1.0 M MOPS and HEPES buffers at different conditions and no racemization was observed by chiral column HPLC analysis. Solubility was a main issue in racemization of (*S*)-NME. Therefore, lauric acid was added to the reaction mixtures, yet no racemization was observed. To the same reaction mixtures (Table 2.14, experiments 1-5) with DMSO as co-solvent and after 144 hours an additional 50 µL DMSO was added to each reaction vial, and were stirred at 45 °C. After the second 144 hours no racemization was observed. To the same reaction mixtures (Table 2.14, experiments 1-5) with the additional DMSO, 10 mg of lauric acid was added to each reaction vial, and were stirred at 45 °C. After the third 144 hours no racemization was observed by chiral column HPLC analysis.



Scheme 2.8 Attempted Racemization of (*S*)-NME without CRL

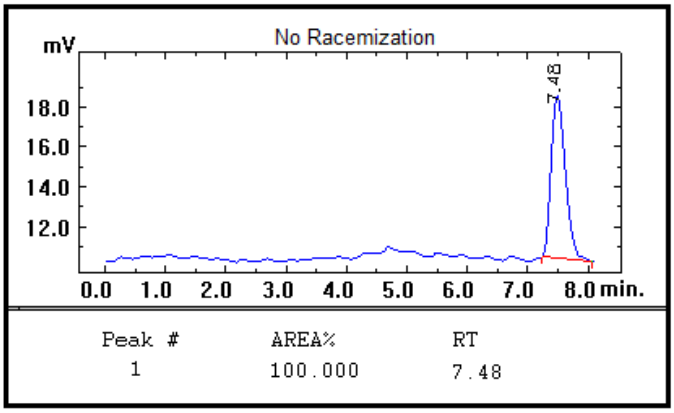


Figure 2.29 Chromatograph of (*S*)-NME with No Racemization

Chapter 3 : Conclusions

In the process of isolation of racemic ibuprofen from tablets, a substantial monetary saving over ibuprofen purchased from chemical suppliers was achieved, and pure racemic ibuprofen with no traces of residual impurities was obtained in all the experimental runs. Conversion of racemic ibuprofen to racemic ibuprofen methyl ester (IME) was performed several times and the same pure product was obtained in all the experiments. A higher yield of racemic IME was achieved by increasing the number of hexanes extractions after Fischer esterification; therefore, a recommended optimization of this step would be to implement a continuous extraction instead of multiple manual extractions.

One of the goals of this research was to optimize the Dynamic Kinetic Resolution (DKR) reaction parameters (buffer, pH, substrate concentration, co-solvent, amount of enzyme, temperature, and agitation rate) in conversion of racemic IME to (*S*)-ibuprofen, assuming that (*R*)-IME was converted to (*S*)-IME via enolization in basic aqueous solutions. The result was that the DKR reactions worked with 8 different buffers at 0.2-0.5 M concentration (for large and small scale reactions, respectively) with a pKa range of 7.20 to 8.35. A higher buffer concentration prevented DKR reaction progress by disturbing the enzymatic structure. These buffers were adjusted to a pH of 7.6 after adding *Candida rugosa* lipase (CRL) and this mildly basic pH was the optimal pH for running these reactions. The best buffers among the 8 different buffers for performing these reactions were 0.5 M buffers of MOPS, TES, and HEPES with a pKa close to 7.5 because the optimal operating pH was close to 7.6.

An observation that, to our knowledge, has never been reported in the literature is the acidity of the commercial CRL mixture. Only one reference [29] reports a pH of 9.8 for DKR reactions and does not specify the pH drop after adding the acidic CRL, which in this work was

significant (e.g., 9.23 to 7.73), and assumes that the pH remained the same before and after adding the lipase. Indeed, others [28, 56-63] only report the concentration of a base for these reactions, a recipe, without discussing the pH at which these reactions take place. This research demonstrated that for all buffers the pH decreased dramatically after adding the enzyme. Therefore, the lipase behaved as an acidic reagent by adding it to the reaction mixtures. As previously stated, this could be due to oxidation of the lipase lactose filler to organic acids, i.e. aldonic acids [98, 100]. The mechanism of how this oxidation occurs should be studied. In summary, the optimal pH for DKR reactions after the addition of acidic CRL was measured to be 7.6, not 9.8.

Another important finding of this work was that a lower concentration of racemic IME not only guaranteed a full conversion to the pure (*S*)-ibuprofen, but also provided the shortest reaction time that has ever been reported for this reaction, which was 72 hours. Therefore, the rate of DKR reactions depends on the substrate concentration, and the higher the IME concentration, the slower the rate of the reactions. Indeed many DKR reactions at optimized conditions were run with only 50% conversion of racemic IME to (*S*)-Ibuprofen simply because the substrate concentration was too high (i.e., 222.0 μmol versus the optimum 44.3 μmol). Therefore, a low substrate concentration (racemic IME) was very important for a high conversion in these reactions. The fact that a lower substrate concentration leads to more conversion is due to the products of methanol, (*S*)-ibuprofen, and acid in general, inhibiting these reactions [1, 8, 28, 59, 66, 69] because the products bind to the active site of the enzyme and inhibit the enzyme to further catalyze these reactions, as demonstrated by the naphthalene inhibition of KR reactions in this research. Consequently, a smaller ratio of racemic IME to a fixed amount of lipase decreased product inhibition of the enzyme.

The use of DMSO and DMF as co-solvents was investigated. After adding DMF to all the buffers a lesser change of pH was observed as opposed to adding DMSO. Furthermore, upon adding 5% DMF or 20% DMSO, the solubility and the enantio-selectivity of the ester increased. These solvents were also theorized to increase the rate of enolization of racemic IME at higher pH [28, 29, 110]. In addition to observing a greater rate of reaction for DMF over DMSO, the pH of the reactions with the former co-solvent were more stable whereas the pH with the latter co-solvent always decreased over time. Therefore, DMF maintained the pH better throughout the course of the reaction. This indicates that DMSO, a reagent in Swern oxidations [111], is either aiding oxidation of the lipase-lactose filler in oxygen or promoting the formation of the open form of lactose, an aldehyde which can more easily be oxidized with oxygen from the air (auto oxidation of aldehydes) to carboxylic acids [98]. Thus DMSO should be avoided for DKR reactions due to lowering the pH, whereas DMF was found to be the best co-solvent for these reactions as it was reported for KR [17]. The mechanism of oxidation of lactose to its aldonic acid and how to prevent this oxidation should be studied. In particular, did the amount of DMF versus DMSO used contribute to their observed differences in pH stability and would an anaerobic nitrogen atmosphere prevent oxidation and stabilize the pH with any co-solvent?

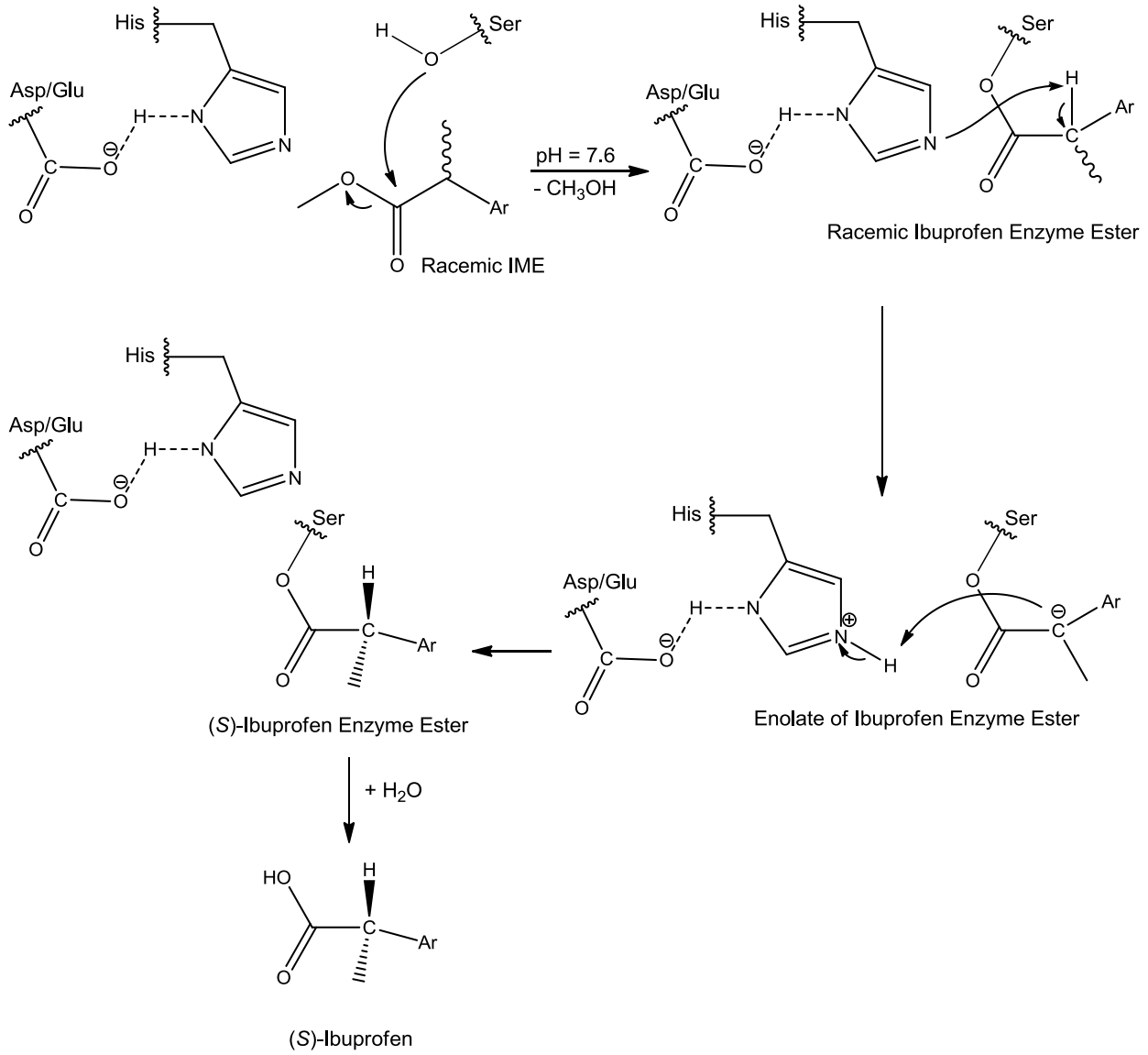
Another finding of this work was that increasing the amount of CRL above 20 mg per mL buffer did not increase the yield of product in 72 hours. Therefore using either 40 or 20 mg of CRL did not make a significant difference in the yield of the product except that working with higher amounts of enzyme in larger scale reactions made the work up and the product isolation cumbersome because of emulsions. To explain this finding, it may be that the capacity of the buffer to maintain a pH of 7.6 is more important than the amount of lipase above a certain amount.

By applying all the optimized parameters including using the right temperature of 40 ºC, increased stirring (higher collision of substrate-enzyme), and avoiding opening the reaction, the DKR reactions worked with a variety of buffers and resulted in yields above 90% after 72 hours. Combined, these conditions decreased the time required to convert racemic IME to (*S*)-ibuprofen to half the time than previously reported. The temperature is crucial because an increased temperature increases the rate of most reactions but can also deactivate enzymes. Higher stirring is best because these reactions are suspensions. Avoiding opening the container over the course of small scale reactions is important because they dry out quickly and can absorb oxygen (auto-oxidation of aldehydes) or carbon dioxide to create more acid.

Because enolization of (*R*)-IME to (*S*)-IME was unlikely in mildly basic solutions, (*R*)-IME was isolated by Kinetic Resolution (KR) of racemic IME under acidic conditions. After several attempts under the same reaction conditions as DKR excluding CRL, racemization of (*R*)-IME was not observed at a pH of 7.6. Therefore, it was concluded that the conversion of racemic IME to (*S*)-ibuprofen must be catalyzed by *Candida rugosa* lipase, showing that the previous enolization hypothesis as to how this conversion occurs is not valid (Scheme 1.9). This leads to the conclusion that a slightly elevated pH of 7.6 is needed for CRL to exhibit isomerase activity and catalyze DKR reactions over just KR reactions.

Because the mechanism of DKR was shown not to be base-catalyzed racemization of (*R*)-IME as previously conjectured, we hypothesize that conversion of (*R*)-IME to (*S*)-ibuprofen must occur within the enzyme's active site as shown in Scheme 3.1. Since the imidazole moiety of Histidine has a pKa of 6.9, it is likely that, after acylation of racemic IME by the Serine moiety and release of methanol, the basic nitrogen on the imidazole at pH 7.6 deprotonates the chiral center of the acyl-enzyme complex to form an ibuprofen enzyme ester enolate. Re-protonation of

the enolate to form the (*S*)-acyl enzyme complex is preferred. Then, upon the addition of water, the ester complex is hydrolyzed to (*S*)-ibuprofen and the free Serine moiety.



Scheme 3.1 Working Hypothesis of Conversion of Racemic IME to (*S*)-Ibuprofen

As evidence that this mechanism is feasible, Figure 3.1 shows an X-ray structure of the CRL active site with methyl[1-(methoxy)phenylmethyl]phosphonyl (spheres) bound to the catalytic Serine [114]. The distance between a Histidine nitrogen and the hydrogen of the chiral center that should be deprotonated (both in turquoise) if ibuprofen were bound is only 2.68 Å.

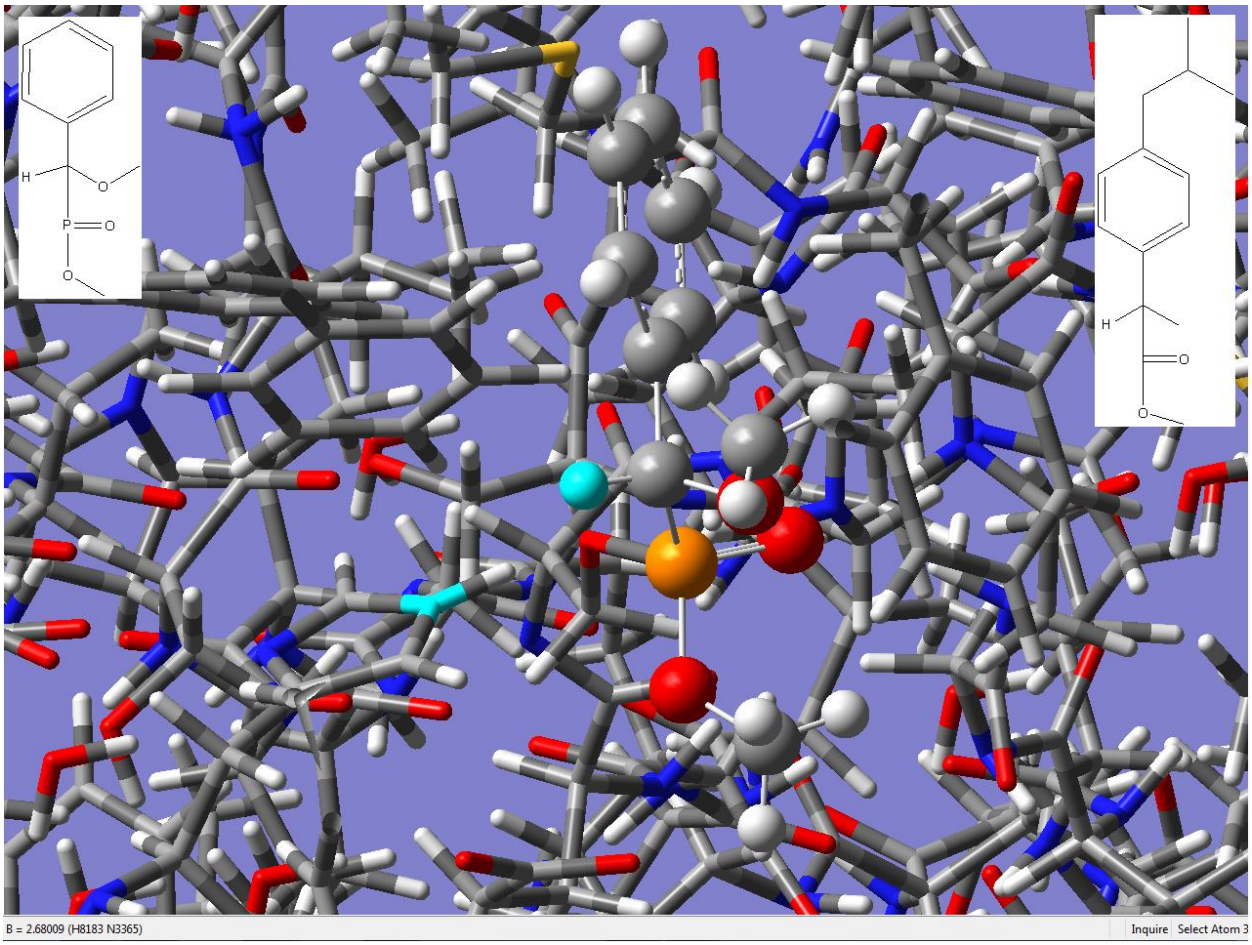
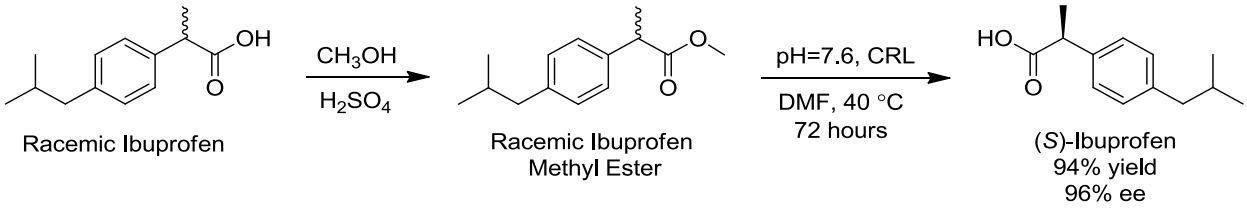


Figure 3.1 Methyl[1-(methoxy)phenylmethyl]phosphonyl bound to the catalytic Serine of CRL [114]

Because this mechanism requires a deprotonated Histidine to enolize the acyl-enzyme complex, the importance of a stable pH cannot be overstated and is probably a major reason why DKR reactions are inhibited by higher IME concentrations producing more acid (ibuprofen) and why the conversion does not correlate well to the amount of CRL. Future work in this field should attempt to increase the buffer capacity in DKR reaction without deactivating CRL, e.g., separating CRL from the buffer salts with a membrane.

This new hypothesis of overall deracemization of ibuprofen to the desired (*S*)-ibuprofen by CRL (Scheme 3.2), contradicts the previous literature mechanism that DKR occurs through enolization outside the lipase in highly basic conditions (Scheme 2.4). The significance of this

conclusion is that no one has reported that enolization in DKR reactions is enzymatic and not purely chemical. Considering that other ibuprofen isomerases involve thio-ester activation [115, 116], this new mechanism of isomerization needs to be further studied. It is interesting to consider that the catalytic triad (Serine, Histidine, and Glutamic or Aspartic acids) found in CRL and ubiquitous in lipase, hydrolase, esterase, protease, etc. not only may prefer one enantiomer over the other, but as shown in this work, may actively convert an undesired compound to its enantiomer (e.g., alpha-amino acids), a form of repair of stereo-chemistry.



Scheme 3.2 Deracemization of Ibuprofen to (S)-Ibuprofen

Chapter 4 : Experimental

4.1 Materials

All buffers were prepared using Millipore filtered water at various concentrations from 0.1.0 M to 1.0 M. Adjusters for different buffers were mainly HCl and NaOH. Buffers and adjusters, DMSO and DMF as co-solvents, *Candida rugosa* lipase (CRL, 700 units/mg of solid), HPLC grade solvents, hexanes (98.5% CHROMASOLV), 2-propanol, and methanol (99.9% CHROMASOLV), were purchased from the Sigma-Aldrich Chemical Company, and CRL was used without any treatment. Racemic ibuprofen (200 mg/tablet) was isolated from inexpensive commercial tablets purchased from Walmart. All other chemicals and analytical grade reagents were from commercial sources and were used without further purification.

4.2 Equipment

4.2.1 Solvent Evaporation

A Buchi R-110 Rotovapor rotary evaporator (from BUCHI, LABORATORLUMS-TECKNIK AG, 70 Switzerland) was utilized to remove volatile organic solvents from different reaction mixtures (Figure 4.1).

Figure 4.1 Rotary Evaporator for Removing Organic Solvents

4.2.2 Horizontal Bulb to Bulb Distillation

A Kuelgrohr apparatus (Sigma Aldrich Chemical Company, Inc.) was used to further purify compounds after rotary evaporation by distillation at higher vacuum and by removal of high-boiling point solvents. An ice water bath was used to trap distillates in the bulb and liquid nitrogen was used to trap volatiles before the vacuum pump (Figure 4.2).

Figure 4.2 Kuelgrohr Horizontal Distillation for Purifying Compounds

4.2.3 Chiral Column High Performance Liquid Chromatography

Chiral Column High Performance Liquid Chromatography (Figure 4.3) with a Spectra-Physics Spectra System P1500 gradient pump (Figure 4.4.a-left), a UV2000 detector set at 256 nm (Figure 4.4.a-right), a Chiralcel OJ column (Diacel Chemical Industries, Inc., Figure 4.4.b), and Winner for Windows Software were employed to quantify, identify, and monitor all reactions at room temperature. The column is capable of resolving both racemic ibuprofen and the less polar racemic ibuprofen methyl ester with a utilized mobile phase of hexanes/2-propanol (98/2, v/v), a flow rate of 1 mL/min, and helium solvent degassing. Analytes of 1.0-2.0 μL were introduced into the pump injector at room temperature (22 ℃).

Figure 4.3 Chiral Column High Performance Liquid Chromatography Setup

Figure 4.4.a) Gradient Pump (left), UV Detector (right) b) Chiralcel OJ Column

4.2.4 pH Measurements

An Oakton bench pH/Ion Meter (pH 1100 series) was used to measure the pH of different buffer solutions and of the reaction mixtures before and after adding the lipase (Figure 4.5).

Figure 4.5 pH/Ion Meter for Measuring the pH of Buffer Solutions

4.2.5 Nuclear Magnetic Resonance (NMR) Spectrometry

Proton Nuclear Magnetic Resonance (^{1}H NMR) and carbon-13 (^{13}C) NMR spectra of crude reagents and isolated products were recorded on a JEOL 600 MHz spectrometer (Figure 4.6) and a Bruker-Spectrospin 300 MHz spectrometer (Figure 4.7) with deuterated chloroform ($CDCl_3$) as the solvent and tetramethylsilane (0.03% v/v) as internal standard.

Figure 4.6 JEOL 600 MHz Nuclear Magnetic Resonance Spectrometer

Figure 4.7 Bruker-Spectrospin 300 MHz Nuclear Magnetic Resonance Spectrometer

4.2.6 Infrared Spectrometry

Infrared spectra of isolated products were recorded on a Perkin-Elmer Spectrum 100 FT-IR spectrometer (Figure 4.8).

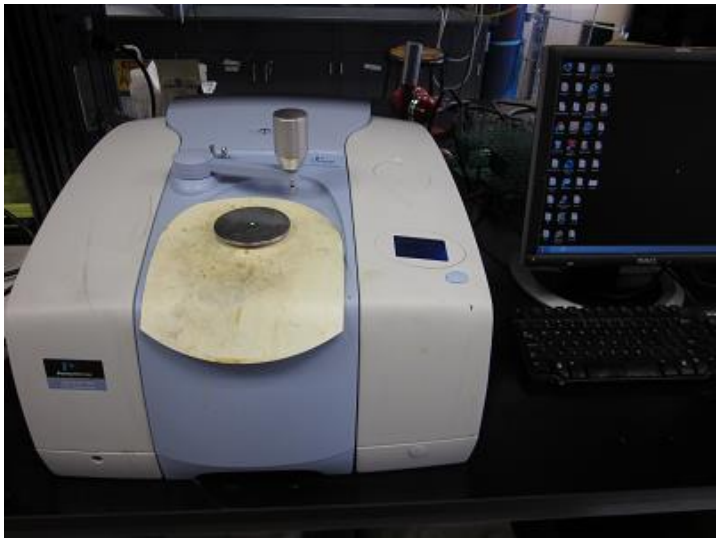


Figure 4.8 Perkin-Elmer Spectrum 100 FT- Infrared Spectrometer

4.2.7 Polarimetry

A Polax-2L Atago Polarimeter (Figure 4.9) was used to measure the optical rotation of the isolated product, (*S*)-ibuprofen, at 20 °C and 589 nm wavelength with sample concentration of 1g/100 mL (c 1) in anhydrous ethanol.

Figure 4.9 Polarimeter for Measuring Optical Rotation [117]

4.2.8 Thin Layer Chromatography (TLC)

A UVGL-15 Mineralight multiband UV 254/366 nm (115 V, 60 Hz, 0.16 A) lamp was used to visualize sample spot movement on TLC plates to monitor reactions (Figure 4.10). TLC plates were silica gel on aluminum sheets (size 2.5 cm x 7 cm).

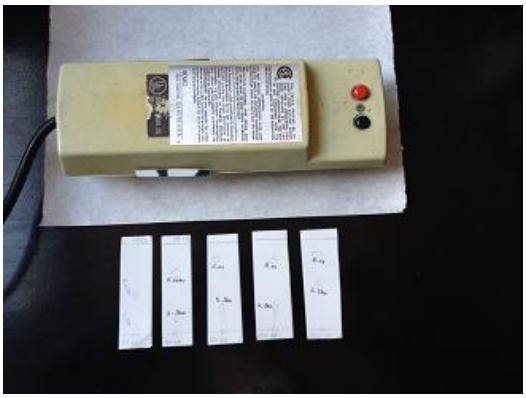


Figure 4.10 UV Lamp for Visualizing Sample Spots

4.2.9 Radial Chromatography

A Chromatotron 7924 T (Figure 4.11) with silica gel plate (4 mm thickness) and a mobile phase of 15 to 25% v/v ethyl acetate/hexanes was used to moisten the plate and to separate the ibuprofen methyl ester (IME) from (*S*)-ibuprofen. An aliquot of the sample (about 1 g) was introduced into the sorbent rotor silica plate as it rotated. A UV lamp was utilized to observe the bands as they eluded from the center to the outside of the plate. Afterward a final wash with methanol of the plate was performed to remove impurities.

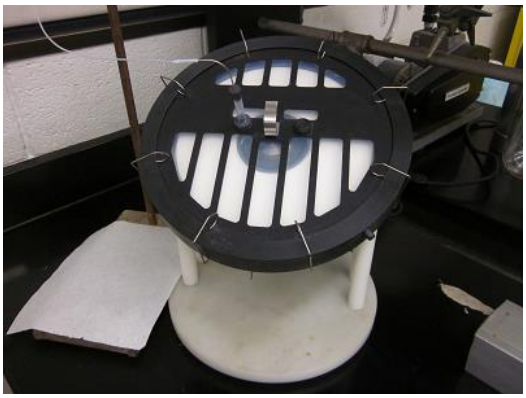


Figure 4.11 Chromatotron 7924 T for Separating Compounds

4.2.10 Melting-Point Determination

A DigiMelt MPA 160 (Stanford Research System, SRS) melting point apparatus (Figure 4.12) with built in thermometer was used to determine the melting point of crude and isolated products packed at the bottom of melting point tubes to a height of 1-2 mm.

Figure 4.12 Melting Point Apparatus

4.2.11 Heating and Stirring

Heating wells of various sizes filled with sands along with electrical variable autotransformers (0-140 V, 10 A, 50/60 Hz) were utilized as heating source to warm up all the reactions to 40 °C. Various types of magnetic stirrers were used to mix all the reactions while heating. Typical setups of sand bath heating sources are illustrated in Figure 4.13.

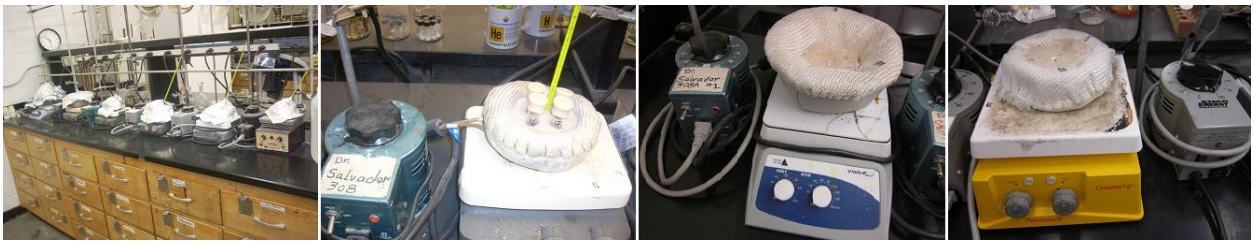


Figure 4.13 Different Heating Sources and Stirrers

4.2.12 Mass Measurement

A Sartorius Basic balance (Figure 4.14) with up to 4 digit accuracy was utilized for all mass measurements.

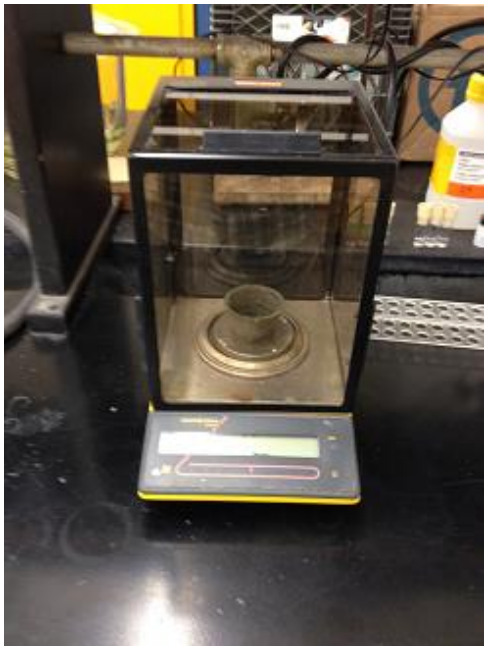


Figure 4.14 Sartorius Balance

4.2.13 Drying

A VWR laboratory oven model 1350 (15 A) was employed to dry glassware (Figure 4.15).

Figure 4.15 Laboratory Oven

4. 3 Experimental

4.3.1 Isolation of Racemic Ibuprofen from Tablets

Isolation of 2-[4-(2-methylpropyl)phenyl]propanoic acid (racemic ibuprofen) from commercial tablets (200 mg/tablet) was done (Figure 4.16) according to Chavez *et al.* [29]. One hundred tablets were broken in half and suspended in 500 mL acetone for 30 minutes (5 mL/tablet) inside a clean and dry 1 L Erlenmeyer flask. A beaker for keeping the suspension was avoided because some of the mixture might spill around when transferring into a Buchner funnel. Vacuum filtration was done to separate the coating and the filler from the filtrate containing the dissolved racemic ibuprofen in acetone. The solvent was removed by vacuum distillation on the rotary evaporator, and the remaining white powder was further distilled by Kuelgrohr horizontal distillation (bp 149 °C, 0.4 Torr) to afford the pure product. Mass measurement, melting point determination, Chiral column High Performance Liquid Chromatography (HPLC), Proton Nuclear Magnetic Resonance (^{1}H NMR), and Carbon-13 (^{13}C) NMR were performed on the pure racemic ibuprofen distillate for identification.

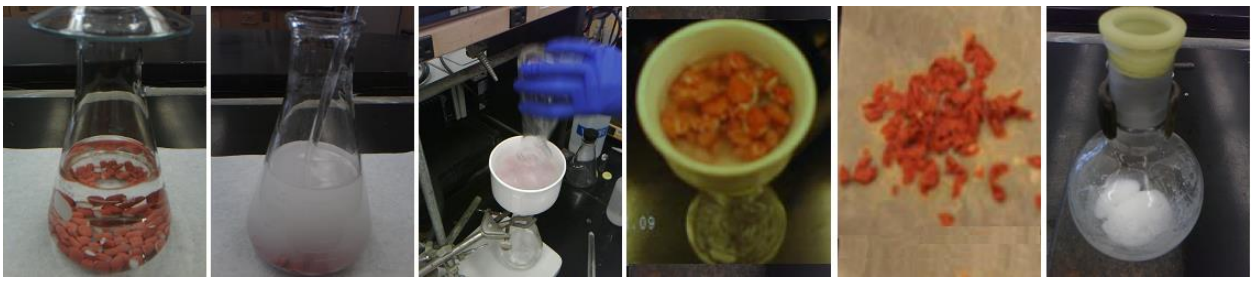


Figure 4.16 Isolation of Racemic Ibuprofen form Commercial Tablets

4.3.2 Conversion of Racemic Ibuprofen to Racemic IME

The Fischer esterification of racemic ibuprofen to make racemic ibuprofen methyl ester (IME) was performed according to Chavez *et al.* [29]. To a clean and dry 200 mL round bottom flask with a magnetic stir bar was added 81.0 mL (2.00 mol) of HPLC grade methanol, 4.12 g (20.0 mmol) of pure racemic ibuprofen, and 1.0 mL (18 mmol) of conc. H_2SO_4 as catalyst. The

reaction mixture was stirred on top of a stirrer inside a sand bath heating well to create a homogenous heat at 40 °C for 12 hours. The resulting racemic IME was extracted from excess methanol and acid with 20 x 10 mL hexanes using a clean and dry 250 mL separatory funnel. The volatile solvents containing the ester were removed under reduced pressure by rotary evaporation, and the remaining clear oil was further distilled by Kuelgrohr horizontal distillation (bp 96 °C, 0.5 Torr [106]) to afford the pure product. Mass measurement, chiral column HPLC, ^{1}H NMR, and ^{13}C NMR were performed on the pure racemic methyl 2-[4-(2-methylpropyl)phenyl]propanoate (racemic IME) distillate for identification.

4.3.3 Buffer Preparation

Various types of buffers including bicine, glycine, HEPES, MOPS, phosphate, tricine TES, tris, and triethanolamine mainly at 0.5 M or 1.0 M concentrations were prepared [109] and used as aqueous solvents for conducting Kinetic Resolution (KR) and Dynamic Kinetic Resolution (DKR) reactions of racemic IME.

4.3.3.1 Preparation of 0.5 and 1.0 M Bicine Buffer

To a clean and dry 100 mL volumetric flask, 8.160 g of bicine was added; the flask was filled to the meniscus with Millipore filtered water and shaken to dissolve the solid. The pH of this 0.5 M bicine buffer solution was determined to be 5.0. To a clean and dry 100 mL volumetric flask 4.0 g of NaOH pellets were added; the flask was filled to the meniscus with Millipore filtered water, and shaken to dissolve the pellets. This 1.0 M NaOH solution was used as an adjuster to raise the pH of the 0.5 M bicine buffer to the desired level. For small scale reactions, 0.8 or 0.95 mL of the bicine buffer was consumed. Thus, a 0.5 mL aliquot of the 0.5 M bicine buffer solution was transferred to a 5 mL vial, and 1.0 M NaOH was added drop wise along with more drops of

the buffer if needed to reach the desired volume and an adjusted pH at 8.1 to make 0.5 M bicine buffer.

Similarly, to a clean and dry 10 mL volumetric flask, 1.632 g of bicine was added; the flask was filled to the meniscus with Millipore filtered water, and shaken to dissolve the solid. The pH of this 1.0 M bicine buffer solution was determined to be 4.5. The 1.0 M NaOH solution was used as an adjuster to raise the pH of 1.0 M bicine buffer to the desired level. For small scale reactions 0.8 mL of the bicine buffer was consumed. Thus, a 0.5 mL aliquot of the 1.0 M bicine buffer solution was transferred inside a 5 mL vial, and 1.0 M NaOH was added drop wise along with more drops of the buffer if needed to reach to the desired volume and an adjusted pH at 7.8 to make 1.0 M bicine buffer.

4.3.3.2 Preparation of 0.5 and 1.0 M Glycine Buffer

To a clean and dry 10 mL volumetric flask, 0.375 g of glycine was added; the flask was filled to the meniscus with Millipore filtered water and shaken to dissolve the solid. The pH of this 0.5 M glycine buffer solution was determined to be 6.1. The 1.0 M NaOH solution was used as an adjuster to raise the pH of 0.5 M glycine buffer to the desired level. For small scale reactions, 1 mL of the glycine buffer was consumed. Thus, a 0.5 mL aliquot of the 0.5 M glycine buffer solution was transferred inside a 5 mL vial, and 1.0 M NaOH was added drop wise along with more drops of the buffer if needed to reach the desired volume and an adjusted pH at 8.7 to make 0.5 M glycine buffer.

Similarly, to a clean and dry 100 mL volumetric flask, 7.507 g of glycine was added; the flask was filled to the meniscus with Millipore filtered water and shaken to dissolve the solid. The pH of this 1.0 M glycine buffer solution was determined to be 5.6. This buffer directly was consumed without adding any adjuster for large scale reactions to isolate (*R*)-IME; also for testing

small scale KR reactions, the 1.0 M NaOH solution was used as an adjuster to raise the pH of 1.0 M glycine buffer to the desired level. For small scale KR reactions 2 mL of the glycine buffer was consumed. Thus, a 1 mL aliquot of the 1.0 M glycine buffer solution was transferred inside a 5 mL vial, and 1.0 M NaOH was added drop wise along with more drops of the buffer if needed to reach to the desired volume and the desired basic pH such as 7.2 and 8.0 to make 1.0 M glycine buffer.

4.3.3.3 Preparation of 0.5 and 1.0 M HEPES Buffer

To a clean and dry 100 mL volumetric flask, 11.915 g of HEPES was added; the flask was filled to the meniscus with Millipore filtered water and shaken to dissolve the solid. The pH of this 0.5 M HEPES buffer solution was determined to be 5.1. The 1.0 M NaOH solution was used as an adjuster to raise the pH of the 0.5 M HEPES buffer to the desired level. For small scale reactions, 0.8 or 0.95 mL of the HEPES buffer was consumed. Thus, a 0.5 mL aliquot of the 0.5 M buffer solution was transferred to a 5 mL vial, and 1.0 M NaOH was added drop wise along with more drops of the buffer if needed to reach the desired volume and an adjusted pH at 7.8 to make 0.5 M HEPES buffer. For large scale DKR with this buffer, the same routine as the small scale HEPES buffer preparation was employed.

Similarly, to a clean and dry 100 mL volumetric flask, 23.830 g of HEPES was added; the flask was filled to the meniscus with Millipore filtered water and shaken to dissolve the solid. The pH of this 1.0 M HEPES buffer solution was determined to be 4.8. The 1.0 M NaOH solution was used as an adjuster to raise the pH of 1.0 M HEPES buffer to the desired level. For small scale reactions, 0.8 or 0.95 mL of the HEPES buffer was consumed. Thus, a 0.5 mL aliquot of the 1.0 M HEPES buffer solution was transferred inside a 5 mL vial and 1.0 M NaOH was added drop

wise along with more drops of the buffer if needed to reach to the desired volume and an adjusted pH at 7.7 to make 1.0 M HEPES buffer.

4.3.3.4 Preparation of 0.5 and 1.0 M MOPS Buffer

To a clean and dry 100 mL volumetric flask, 10.465 g of MOPS was added; the flask was filled to the meniscus with Millipore filtered water and shaken to dissolve the solid. The pH of this 0.5 M MOPS buffer solution was determined to be 3.9. The 1.0 M NaOH solution was used as an adjuster to raise the pH of the 0.5 M MOPS buffer to the desired level. For small scale reactions, 0.8 or 0.95 mL of the MOPS buffer was consumed. Thus, a 0.5 mL aliquot of the 0.5 M buffer solution was transferred to a 5 mL vial and 1.0 M NaOH was added drop wise along with more drops of the buffer if needed to reach the desired volume and an adjusted pH at 7.9 to make 0.5 M MOPS buffer.

Similarly, to a clean and dry 100 mL volumetric flask, 20.930 g of MOPS were added; the flask was filled to the meniscus with Millipore filtered water and shaken to dissolve the solid. The pH of this 1.0 M MOPS buffer solution was determined to be 3.2. The 1.0 M NaOH solution was used as an adjuster to raise the pH of 1.0 M MOPS buffer to the desired level. For small scale reactions, 0.8 or 0.95 mL of the MOPS buffer was consumed. Thus, a 0.5 mL aliquot of the 1.0 M MOPS buffer solution was transferred inside a 5 mL vial and 1.0 M NaOH was added drop wise along with more drops of the buffer if needed to reach to the desired volume and an adjusted pH at 7.7 to make 1.0 M MOPS buffer.

4.3.3.5 Preparation of 0.2 and 0.5 M Phosphate Buffer

To a clean and dry 10 mL volumetric flask, 0.710 g of sodium phosphate dibasic was added; the flask was filled to the meniscus with Millipore filtered water and shaken to dissolve the solid. The pH of this 0.5 M sodium phosphate dibasic buffer solution was determined to be 9.3. To a

clean and dry 10 mL volumetric flask, 0.510 g of sodium phosphate monobasic was added; the flask was filled to the meniscus with Millipore filtered water, and shaken to dissolve the solid. The pH of this 0.5 M sodium phosphate monobasic buffer solution was determined to be 4.3. The 0.5 M sodium phosphate monobasic solution was used as an adjuster to decrease the pH of the 0.5 M sodium phosphate dibasic buffer to the desired level. For small scale reactions, 0.8 or 0.95 mL of the phosphate buffer was consumed. Thus, a 0.5 mL aliquot of the 0.5 M dibasic buffer solution was transferred to a 5 mL vial and 0.5 M monobasic was added drop wise along with more drops of the buffer if needed to reach the desired volume and an adjusted pH about 7.8 to make 0.5 M phosphate buffer.

Similarly, to a clean and dry 100 mL volumetric flask, 3.484 g of potassium phosphate dibasic was added; the flask was filled to the meniscus with Millipore filtered water, and shaken to dissolve the solid to make 0.2 M potassium phosphate dibasic. To a clean and dry 100 mL volumetric flask, 2.722 g of potassium phosphate monobasic was added; the flask was filled to the meniscus with Millipore filtered water and shaken to dissolve the solid. The 0.2 M potassium phosphate monobasic solution was used as an adjuster to decrease the pH of the 0.5 M potassium phosphate dibasic buffer to the desired level. This buffer was used for the large scale DKR reaction of conversion of racemic IME to (S)-ibuprofen.

4.3.3.6 Preparation of 0.5 M Tricine Buffer

To a clean and dry 50 mL volumetric flask, 4.480 g of tricine was added; the flask was filled to the meniscus with Millipore filtered water and shaken to dissolve the solid. The pH of this 0.5 M tricine buffer solution was determined to be 4.5. The 1.0 M NaOH solution was used as an adjuster to raise the pH of the 0.5 M tricine buffer to the desired level. For small scale reactions, 0.8 or 0.95 mL of the tricine buffer was consumed. Thus, a 0.5 mL aliquot of the 0.5 M

tricine buffer solution was transferred to a 5 mL vial and 1.0 M NaOH was added drop wise along with more drops of the buffer if needed to reach the desired volume and an adjusted pH at 7.8 to make 0.5 M tricine buffer.

4.3.3.7 Preparation of 0.5 M TES, Tris, and Triethanolamine Buffers

To separate clean and dry 10 mL volumetric flasks, 1.146 g TES, 0.606 g tris, and 0.746 g triethanolamine were added; the flasks were filled to the meniscus with Millipore filtered water and shaken to dissolve the two solids and the liquid triethanolamine. The pH of 0.5 M TES, tris, and triethanolamine buffer solutions were determined to be 2.9, 10.1, and 10.7 respectively. The 1.0 M NaOH solution was used as an adjuster to raise the pH of the 0.5 M TES buffer to the desired level. The 1.0 M HCl solution was used as an adjuster to decrease the pH of the 0.5 M tris and triethanolamine buffers to the desired level. For small scale reactions, 0.8 or 0.95 mL of the TES, tris, and triethanolamine buffers were consumed. Thus, a 0.5 mL aliquot of the 0.5 M buffer solutions were transferred to 5 mL vials, and 1.0 M NaOH (TES) 1.0 M HCl (tris and triethanolamine) were added drop wise along with more drops of the buffers if needed to reach the desired volumes to make 0.5 M TES, tris, and triethanolamine buffers at adjusted pH of 7.8, 7.8, and 7.7, respectively.

4.3.4 Optimization of DKR Reaction Parameters

DKR reaction parameters in conversion of racemic IME to (*S*)-Ibuprofen were optimized with respect to buffers, pH, substrate concentration, co-solvent, amount of enzyme, temperature, and agitation rate.

4.3.4.1 Optimization of DKR Reactions with Respect to Buffers

To separate clean and dry 5 mL vials with magnetic stir bars, 0.8 mL of 0.5 or 1.0 M of a different buffer solution per experiment (imidazole, sodium phosphate II, MOPS, TES, HEPES,

triethanolamine, tricine, tris, bicine, ethanolamine, glycine, and sodium bicarbonate), 0.2 mL DMSO, 50 µL (222.0 µmol) racemic IME, and 40 mg CRL were added. The pH of the reactions was measured before and after adding CRL (Table 2.3). The aqueous mixtures were stirred for 144 hours at 40 ºC and monitored by chiral column HPLC by removing a 0.2 mL aliquot of the sample, acidifying it with 1.0 M HCl to pH 5, and extracting the organic compounds into 0.3 mL hexanes for injection into the HPLC (Figure 4.17). Other combinations of reagents were tried such as the above scale with 50 mg CRL or 1.0 mL of buffer, 0.25 mL DMSO, 50 µL (222.0 µmol) racemic IME and 40 mg CRL.

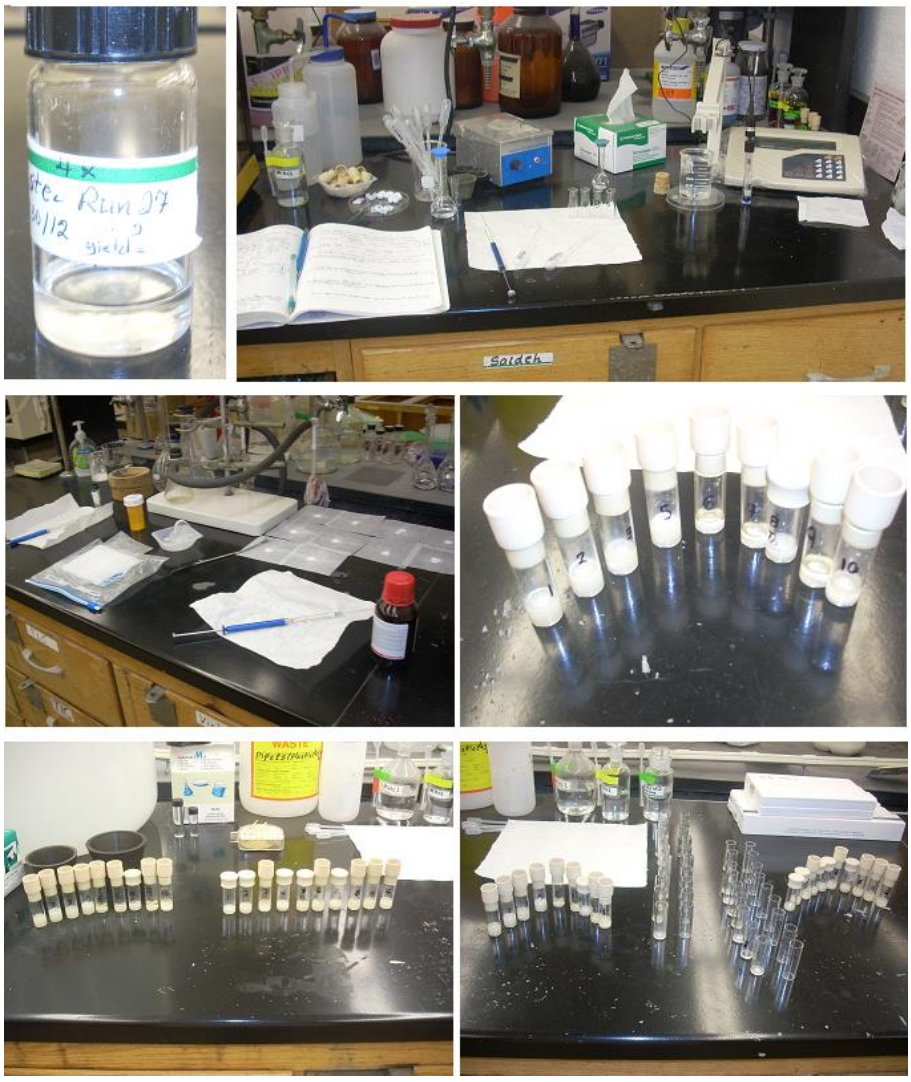


Figure 4.17 Typical DKR Reactions (Preparation and Monitoring by Chiral HPLC)

4.3.4.2 Optimization of KR and DKR Reactions with Respect to pH

Several enzymatic hydrolyses in conversion of racemic IME to (*S*)-ibuprofen were examined with different buffers in acidic, neutral, and basic pH up to 8.7 (after adding CRL) to investigate the pH at which the hydrolysis occurs. In the first set of reactions to separate clean and dry 5 mL vials with magnetic stir bars, 1.0 mL of 1.0 M of a different buffer solution per experiment (imidazole, MOPS, TES, HEPES, tris, tricine, or bicine), 50 µL (222.0 µmol) racemic IME, and 50 mg CRL were added. The pH of the reactions was measured before and after adding CRL (Table 2.4, experiments 1-7). The aqueous mixtures were stirred for 48 hours at 40 ºC and monitored by chiral column HPLC by removing a 0.2 mL aliquot of the sample, acidifying it with 1.0 M HCl to pH 5, and extracting the organic compounds into 0.3 mL hexanes for injection into the HPLC.

In a second set of reactions under the same reaction conditions using the same buffers, enzymatic hydrolysis was repeated at a lower pH of 7.6 (after adding CRL) and a shorter reaction duration of 36 hours (Table 2.4, experiments 8-14). In a third set of reactions under the same reaction condition using glycine buffer, the experiment was repeated at acidic, neutral, and basic pH of 5.77, 7.21, and 8.0 (after adding CRL), respectively with a 24 hour reaction duration (Table 2.4, experiments 15-17).

Several DKR reactions were performed to investigate the effect of pH with all the 12 different buffer solutions. The following demonstrates 5 sets of these reactions. To separate clean and dry 5 mL vials with magnetic stir bars, 0.8 mL of 0.5 M buffer solution of bicine, 0.2 mL DMSO, 50 µL (222.0 µmol) racemic IME, and 40 mg CRL were added. The pH of the reactions was measured before and after adding the lipase and was adjusted after adding CRL (Table 2.5, experiments 1-8). In the second set of experiments, 7 reactions with the same scale except 50 mg

CRL with 0.5 M bicine were performed, and the pH was adjusted after adding CRL (Table 2.5, experiments 9-15).

In a third set of experiments, to separate clean and dry 5 mL vials with magnetic stir bars, 0.8 mL of 0.5 M buffer solution of HEPES, 0.2 mL DMSO, 50 µL (222.0 µmol) racemic IME, and 40 mg CRL were added. The pH of the reactions was measured before and after adding the lipase, and was adjusted after adding CRL (Table 2.6, experiments 1-6). In a fourth set of experiments, 6 reactions with the same scale, except 50 mg CRL with 0.5 M HEPES, were performed, and the pH was adjusted after adding CRL (Table 2.6, experiments 7-12). In a fifth set of experiments, to separate clean and dry 5 mL vials with magnetic stir bars, 1.0 mL of 0.5 M buffer solution of MOPS, 0.25 mL DMSO, 50 µL (222.0 µmol) racemic IME, and 40 mg CRL were added. The pH of the reactions was measured before and after adding CRL (Table 2.7). The aqueous mixtures were stirred for 120 hours for the first, second, and third set and 144 hours for the fourth and fifth set at 40 ºC, and they were monitored by chiral column HPLC by removing a 0.2 mL aliquot of the sample, acidifying it with 1.0 M HCl to pH 5, and extracting the organic compounds into 0.3 mL hexanes for injection into the HPLC.

4.3.4.3 Optimization of DKR Reactions with Respect to Substrate Concentration

To separate clean and dry 5 mL vials with magnetic stir bars, 0.8 mL of 0.5 M buffer solutions of MOPS, 0.2 mL DMSO, a different amount of racemic IME per experiment 50 (222.0 µmol), 40 (177.2 µmol), 30 (133.0 µmol), 25 (110.8 µmol), 12 (53.2 µmol), and 10 µL (44.3 µmol), plus 40 mg CRL (Table 2.8) were added. In a second set under the same reaction conditions, 0.95 mL MOPS 0.5 M, and 0.05 mL DMF, as opposed to DMSO, was used. In a third set under the same reaction conditions, 0.95 mL HEPES 0.5 M and 0.05 mL DMF were used. The pH of the reactions was measured before adding the lipase and adjusted to 7.6 after adding CRL. The

aqueous mixtures were stirred for 144 hours for the first set, 72 hours for the second set, 120 and 72 hours for the third set at 40 ºC, and they were monitored by chiral column HPLC by removing a 0.2 mL aliquot of the sample, acidifying it with 1.0 M HCl to pH 5, and extracting the organic compounds into 0.3 mL hexanes for injection into the HPLC.

4.3.4.4 Optimization of DKR Reactions with Respect to Co-solvent

To separate clean and dry 5 mL vials with magnetic stir bars, 0.8 mL of 0.5 M of a different buffer solution per experiment (bicine, HEPES, MOPS, phosphate, tricine, TES, tris, or triethanolamine), 0.2 mL DMSO, 10 µL (44.3 µmol) racemic IME, and 40 mg CRL were added. Similarly, the same reaction conditions with the same buffers were repeated except with 0.95 mL of 0.5 M of the buffers. The pH of the reactions was measured before adding the lipase and adjusted to 7.6 after adding CRL. The aqueous mixtures were stirred for 72 hours at 40 ºC, and they were monitored by chiral column HPLC by removing a 0.2 mL aliquot of the sample, acidifying it with 1.0 M HCl to pH 5, and extracting the organic compounds into 0.3 mL hexanes for injection into the HPLC.

4.3.4.5 Optimization of DKR Reactions with Respect to Mass of CRL Enzyme

To separate clean and dry 5 mL vials with magnetic stir bars, 0.95 mL of 0.5 M buffer solutions of HEPES or MOPS, 0.05 mL DMF, 10 µL (44.3 µmol) racemic IME, and different amounts of CRL (50, 40, 30, 20, 15, and 0 mg) were added. The pH of the reactions was measured before adding the lipase and adjusted to 7.6 after adding CRL. The aqueous mixtures were stirred for 72 hours at 40 ºC, and they were monitored by chiral column HPLC by removing a 0.2 mL aliquot of the sample, acidifying it with 1.0 M HCl to pH 5, and extracting the organic compounds into 0.3 mL hexanes for injection into the HPLC.

4.3.4.6 Optimization of DKR Reactions with Respect to Temperature

To separate clean and dry 5 mL vials with magnetic stir bars, 0.95 mL of 0.5 M buffer solution of MOPS, 0.05 mL DMF, 10 µL (44.3 µmol) racemic IME, and 40 mg CRL were added. The pH of the reactions was measured before adding the lipase (7.97) and adjusted to 7.6 after adding CRL. The aqueous mixtures were stirred for 72 hours at 30 of 45 ºC, and they were monitored by chiral column HPLC by removing a 0.2 mL aliquot of the sample, acidifying it with 1.0 M HCl to pH 5, and extracting the organic compounds into 0.3 mL hexanes for injection into the HPLC.

4.3.4.7 Optimization of DKR Reactions with Respect to Agitation Rate

To separate clean and dry 5 mL vials with magnetic stir bars, 0.95 mL of 0.5 M of a different buffer solution per experiment (bicine, HEPES, MOPS, phosphate, tricine, TES, tris, and triethanolamine), 0.05 mL DMF, 10 µL (44.3 µmol) racemic IME, and 40 mg CRL were added. The pH of the reactions was measured before adding the lipase and adjusted to 7.6 after adding CRL. The aqueous mixtures were stirred at the highest rate of each individual magnetic stirrer for 72 hours at 40 ºC, and they were monitored by chiral column HPLC by removing a 0.2 mL aliquot of the sample, acidifying it with 1.0 M HCl to pH 5, and extracting the organic compounds into 0.3 mL hexanes for injection into the HPLC.

4.3.5 Optimized Large Scale DKR Reaction

To a clean and dry 100 mL round bottom flask with a magnetic stir bar, 48.0 mL of 0.20 M potassium phosphate buffer at pH 7.6, 2.0 mL (4% volume) of DMF, 1.00 g (4.54 mmol) of racemic IME, and 2.00 g CRL was added. The pH dropped to 7.3 upon adding the lipase, and by adding 0.1 M NaOH, the pH was adjusted to 7.6. The reaction mixture was stirred at 300 rpm and 40 °C on top of a stirrer inside a sand bath heating well for 72 hours (Figure 4.18). Then the content was

transferred to 100 mL Falcon tubes and centrifuged at 400 rpm and 4 ºC to precipitate the enzyme. The top clear lipase free aqueous layer containing the (*S*)-ibuprofen was decanted into a clean and dry 250 mL separatory funnel and basified with 10% (v/v) $NaHCO_3$ (to avoid racemization) to make the (*S*)-ibuprofen sodium salt in the basic aqueous solution. The basic solution containing (*S*)-ibuprofen sodium salt and traces of (*R*)-IME was extracted with 3 x 40 mL of cyclohexane using a clean and dry 250 mL separatory funnel to extract the unreacted (*R*)-IME from the basic aqueous solution into the organic layer. The bottom basic layer was drained into a clean and dry 250 mL beaker. The top organic layer containing (*R*)-IME inside the separatory funnel remained as trace byproduct. The basic aqueous (*S*)-ibuprofen sodium salt was acidified with 0.1 M HCl and extracted with 3 x 40 mL cyclohexane using a clean and dry 250 mL separatory funnel. The top organic layer containing the (*S*)-ibuprofen inside the separatory funnel was transferred into a clean and dry 250 mL round bottom flask. The solvent was removed under reduced pressure to afford the desired (*S*)-ibuprofen (Figure 4.18). Mass measurement, chiral column HPLC, and ^1H NMR were performed on the (*S*)-ibuprofen for identification. Also, optical rotation and the melting point of the pure enantiomer were determined.

Figure 4.18 Large Scale DKR Reaction and Solid (*S*)-ibuprofen

4.3.6 Resolution of (*R*)-IME by KR Reactions

To a clean and dry 200 mL round bottom flask with a magnetic stir bar, 80.0 mL of the 1.0 M glycine buffer at pH 5.6, 4.0 mL (18 mmol) of racemic IME, and 3.20 g CRL were added. The pH did not change upon adding the lipase. The reaction mixture was stirred at 40 ºC on top of a stirrer inside a sand bath heating well for 24 hours so that chiral column HPLC analysis showed close to 50% (*R*)-IME and 50% (*S*)-ibuprofen (Figure 2.7). The resulting mixture of unreacted (*R*)-IME and (*S*)-ibuprofen along with the lipase was acidified to pH 5 with 1.0 M HCl, transferred to test tubes (Falcon tubes), and centrifuged for 20 minutes to precipitate the enzyme. The top clear lipase free aqueous layer containing (*R*)-IME and (*S*)-ibuprofen was decanted into a clean and dry 250 mL separatory funnel and extracted with 20 × 10 mL of hexanes.

4.3.7 Isolation of (*R*)-IME by Acid/Base Extraction

The organic layer from the previous section was basified with 20.0 mL (10 mmol) of 0.5 M NaOH to extract the (*S*)-ibuprofen sodium salt into the basic aqueous layer in the separatory funnel, and the bottom basic layer was drained into a clean and dry 150 mL beaker for further treatment to obtain (*S*)-ibuprofen. Then the top hexane layer containing the (*R*)-IME inside the separatory funnel was transferred into a clean and dry 250 mL round bottom flask. The solvent was evaporated under reduced pressure, and the resulting (*R*)-IME oil was further purified by horizontal distillation (bp 88 °C, 0.25 Torr). Mass measurement, chiral column HPLC, ^{1}H NMR, ^{13}C NMR, and IR were performed on the product for identification.

Prior to the above described extraction of (*R*)-IME and (*S*)-ibuprofen from water with hexanes, the continuous extraction setup pictured in Figure 4.19 was attempted to reduce the number of extractions performed. This procedure was not effective to isolate as much mixture and, thus, was not used further.

Figure 4.19 Continuous Extraction of KR Reaction Products with Hexanes

4.3.8 Thin Layer Chromatography (TLC) of (*R*)-IME and (*S*)-Ibuprofen

Silica gel on aluminum plates (size 2.5 cm x 7 cm) were used to run TLC in order to observe the order of spots of (*R*)-IME/(*S*)-ibuprofen mixtures from section 4.3.6 on TLC plates. Prior to utilizing radial chromatography, TLC was performed to obtain the appropriate ratio of the mobile phase needed for separating (*R*)-IME from (*S*)-ibuprofen via radial chromatography. The utilized mobile phase for radial chromatography should match the R_f values close or above to 25% of the more polar compound, (*S*)-ibuprofen, so that the more polar compound that eludes later comes quicker. Therefore, the sample spots of (*R*)-IME/(*S*)-ibuprofen mixtures were developed on TLC plates using different concentrations of ethyl acetate (polar solvent) in hexanes (non-polar solvent). To do so, into 5 clean and dry flasks were added 5/95, 10/90, 15/85, 20/80, and 25/75 mL/mL of ethyl acetate and hexanes. Into 5 clean and dry 100 mL beakers were added about 2 mL of each of the prepared mobile phase solutions, and 5 TLC plates already spotted with the product of a KR reaction, a (*R*)-IME/(*S*)-ibuprofen mixture, were immersed into the mobile phase right below the

solvent level in the beakers. The beakers were covered with aluminum foil and monitored until the solvent front reached 1.5 cm below the TLC plate top edge. After the plates were removed from the beaker and dried, a UV lamp was utilized to monitor the spots. Retention factors (R_f values) were calculated for each compound (Table 4.1).

Table 4.1 Retention Factors of KR Products, (*R*)-IME/(*S*)-Ibuprofen Mixtures

Set 1 Exp #	Ethyl Acetate/ Hexanes	(*R*)-IME R_f (%)	(S)-Ibuprofen R_f (%)	Set 2 Exp #	(*R*)-IME R_f (%)	(*S*)-Ibuprofen R_f (%)
1	5/95	42	6	1	76	32
2	10/90	65	12	2	80	33
3	15/85	75	19	3	84	36
4	20/80	81	21	4	85	45
5	25//75	82	49	5	93	64

As depicted in Table 4.1 and Figure 4.20, (*R*)-IME had a higher R_f value in both sets of experiments and in the 5 different sets of solutions of ethyl acetate/ hexanes, verifying that it is less polar than (*S*)-ibuprofen. Table 4.1 shows that the more polar the mobile phase, the higher the R_f value; however, the polarity of (*R*)-IME compared to (*S*)-ibuprofen was always lower no matter how polar the mobile phase was. Therefore, for separating (*R*)-IME/(*S*)-ibuprofen mixtures, the less polar (*R*)-IME will be eluded first followed by (*S*)-ibuprofen.

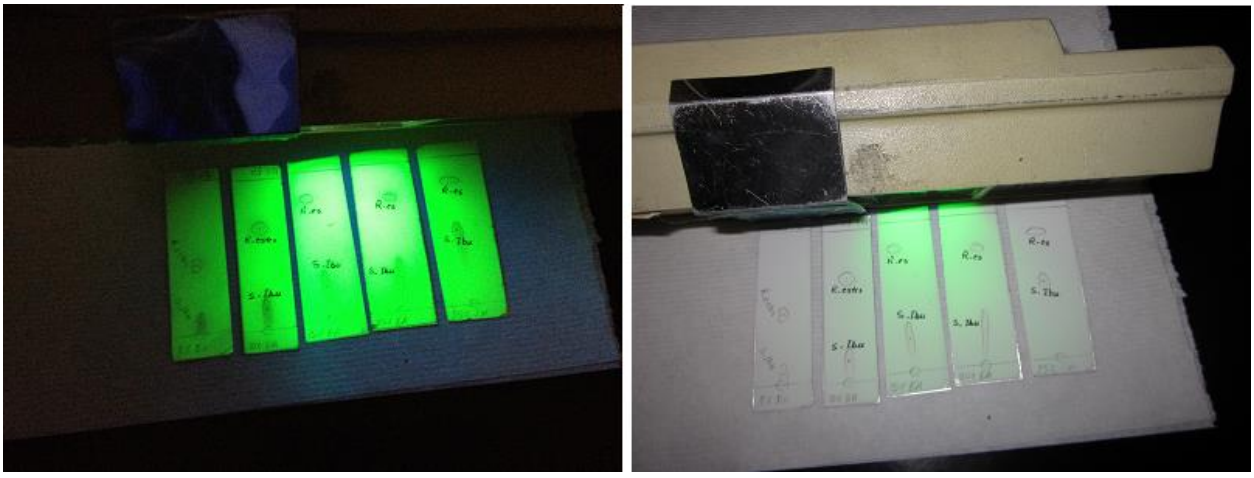


Figure 4.20 Spotted TLC Plates of (*R*)-IME/(*S*)-Ibuprofen Mixtures under UV Lamp

4.3.9 Radial Chromatography of (*R*)-IME and (*S*)-Ibuprofen

Radial chromatography is a separation technique that has been employed for analysis and resolution of (*S*)-ibuprofen from its esters [7]. In order to run a radial chromatography (sorbent rotor plate with 4 mm thickness), first the product of KR reaction (section 4.3.6, a 50/50 mixture of (*R*)-IME/(*S*)-ibuprofen) was obtained. The hexanes was evaporated under reduced pressure to afford the mixture of (*R*)-IME and (*S*)-ibuprofen.

The developing solvent based on the TLC experiment (200 mL of 15/85 ethyl acetate/hexanes) was added to the plastic funnel of the radial chromatography unit. The UV lamp was attached to the Chromatotron by a rubber band so that the light was on the plate. The stopcock of the funnel was opened for the solvent to run through the silica plate (while the UV lamp was on and the lab was dark) until the solvent began to drip from the outlet tube. Then with a glass pipet a 488 mg sample was deposited into the solvent inlet along with more washing of the pipet and the solvent inlet. The solvent was run through the stainless steel capillary ring to deliver solvent into the glass pipet and the inlet so that all the sample would end up in the sorbent rotor plate. While the solvent was running, a clean and dry test tube was underneath the outlet tube; wide purple bands expanded from the center of the sorbent rotor plate to its edge, so the test tube was changed to collect the nonpolar (*R*)-IME that came first. An additional 50 mL of 20/80 ethyl acetate/hexanes was utilized to increase the mobile phase polarity so that the more polar (*S*)-ibuprofen eluded from the center to the outside of the sorbent rotor plate. When the second purple band approached the edge, again the test tube was changed to collect the (*S*)-ibuprofen. Afterward a final wash with 100 mL of methanol (for 4 mm sorbent rotor plate) was performed to remove impurities, the unit was unplugged, the UV lamp and the Teflon lid were removed, and a plastic cover was placed on top of the Chromatotron so that it was covered yet allowed air to flow for the

remaining methanol to evaporate. The content of each test tube was transferred into 5 clean and dry 100 mL round bottom flasks, and the solvent was removed under reduced pressure.

Once the compounds were collected in different beakers, chiral column HPLC were run to examine the content and purity of each beaker. The beakers with the same product were combined, and the solvent was removed under reduced pressure to obtain (*R*)-IME and (*S*)-ibuprofen followed by chiral column HPLC analysis and TLC to assure the purity of the products (Figures 4.21.a and 4.21.b). The resolved (*R*)-IME (132 mg, 0.60 mmol) was 27% of the possible 50% theoretical (*R*)-IME (0.6 mmol (*R*)-IME / 2.22 mmol, theoretical mmol of racemic IME) and was used for attempted racemization reactions. The resolved (*S*)-ibuprofen (160.3 mg, 0.778 mmol) was 35% of the possible 50% theoretical (*S*)-ibuprofen (0.778 mmol (*S*)-ibuprofen / 2.22 mmol, theoretical mmol of (*S*)-ibuprofen).

Radial chromatography is an efficient method for resolution of KR products, (*S*)-ibuprofen, and its esters, because bands were clearly distinguished on silica sorbent rotor plate, and this technique allows 100% separation (Figures 4.21.a and 4.21.b). However, the limitation is the resolution of only 1 g of sample per run with 4 mm thickness for silica sorbent rotor plate.

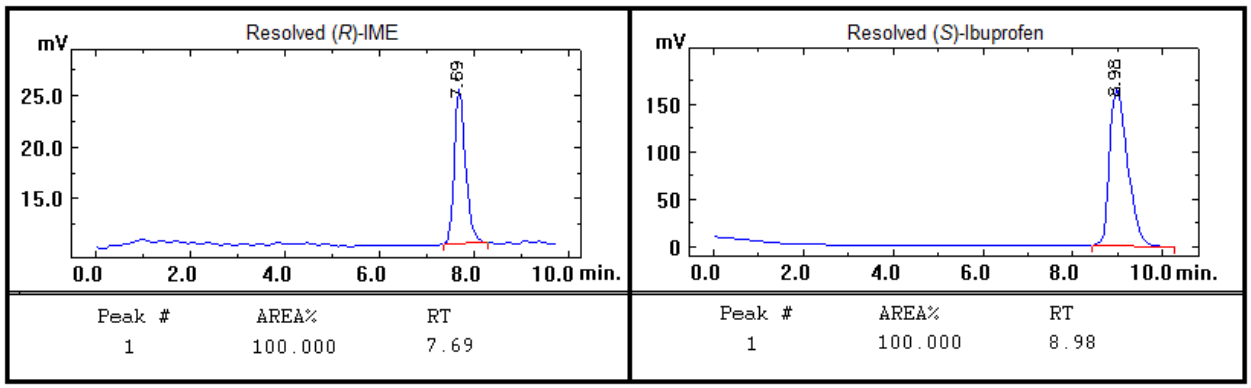


Figure 4.21.a) (*R*)-IME b) (*S*)-Ibuprofen Isolated by Radial Chromatography

4.3.10 Attempted Racemization of (*R*)-IME without CRL

To separate clean and dry 5 mL vials with magnetic stir bars, 0.95 mL of 0.5 M of a different buffer solution per experiment (bicine, HEPES or MOPS) at pH 7.6, 0.05 mL DMF, and 10 µL (44.3 µmol) of (*R*)-IME were added (Table 2.12). The aqueous mixtures were stirred for 144 hours at 40 ºC, and they were monitored daily by chiral column HPLC by removing a 0.1 mL aliquot of the sample and extracting the organic compounds into 0.2 mL hexanes for injection into the HPLC.

To separate clean and dry 5 mL vials with magnetic stir bars, various volumes of 0.25-1.0 M of a different buffer solution per experiment (phosphate, tricine, ethanolamine, sodium bicarbonate, bicine, MOPS, or HEPES) at different pHs (below, at, or above 7.6) with different volumes of DMSO or DMF and different volumes of (*R*)-IME were added (Table 2.12). The aqueous mixtures were stirred for 144 hours at 40 ºC, and they were monitored by chiral column HPLC daily over the time by removing a 0.1 mL aliquot of the sample and extracting the organic compounds into 0.2 mL hexanes for injection into the HPLC (Figure 4.22).

Figure 4.22 Typical Preparations on Attempted Racemization Reactions of Racemic IME

4.3.11 Isolation of (*S*)-Naproxen form (*S*)-Naproxen Sodium Tablets

To a clean and dry 25 mL beaker with a magnetic stir bar, 5 mL of Millipore filtered water and one naproxen sodium tablet (220 mg/tablet) split in half were added, and the mixture was stirred for 30 minutes. Vacuum filtration was done to separate the coating and the filler from the filtrate containing the dissolved naproxen sodium in water. The filtrate was acidified to pH 5 with 1.0 M HCl, and transferred into a clean and dry 100 mL separatory funnel and extracted with 5×5 mL of dichloromethane (Figure 4.23). The solvent was removed by vacuum distillation on the rotary evaporator to give naproxen as a white powder. Mass measurement, chiral column HPLC, ^{1}H NMR, and ^{13}C NMR were performed. By implementing this technique a substantial savings over naproxen purchased from chemical suppliers was achieved.

Figure 4.23 Naproxen Filtrate in Dichloromethane

4.3.12 Conversion of (*S*)-Naproxen Sodium to (*S*)-Naproxen Methyl Ester (NME)

A direct Fischer esterification of naproxen sodium salt was performed to make (*S*)-NME. To a clean and dry 100 mL round bottom flask with a magnetic stir bar, 40.5 mL (1.00 mol) of HPLC grade methanol, 10 naproxen tablets (2.2 g, 8.7 mmol), and 2.0 mL (36 mmol) of conc. H_2SO_4 as catalyst were added. The reaction mixture was stirred and refluxed on top of a stirrer

inside a sand bath heating well at 40 °C for 12 hours. The resulting NME was extracted from excess methanol and acid with 10×10 mL hexanes using a clean and dry 250 mL separatory funnel. The volatile solvents containing the ester were removed under reduced pressure by rotary evaporation, and the remaining white powder was further distilled bulb to bulb by Kuelgrohr apparatus (bp 145 °C, 0.4 Torr) to afford the pure product (Figure 4.24). Mass measurement, chiral column HPLC, ^{1}H NMR, and ^{13}C NMR were performed on the pure (*S*)-NME distillate for identification.

Figure 4.24 Naproxen Methyl Ester in Hexanes (left) and after Horizontal Distillation (right)

4.3.13 Hydrolysis of (*S*)-Naproxen Methyl Ester to (*S*)-Naproxen

To separate clean and dry 5 mL vials with magnetic stir bars, 0.95 mL of 0.5 M buffer solutions of HEPES or MOPS, 0.05 mL DMF, 10 mg (41.0 µmol) (*S*)-NME, 10 mg of lauric acid (surfactant), and 40 mg CRL were added (Table 2.13, experiment 1). The pH of the reactions was measured before adding the lipase and adjusted to 7.6 after adding CRL. The aqueous mixtures were stirred for 72 or 144 hours at 40 ºC, and they were monitored by chiral column HPLC by removing a 0.2 mL aliquot of the sample and extracting the organic compounds into 0.3 mL hexanes for injection into the HPLC. The hydrolysis of (*S*)-NME to (*S*)-naproxen was performed with different ratios of the reagents at pH 7.6 and above (Table 2.13).

4.3.14 Attempted Racemization of (*S*)-Naproxen Methyl Ester without CRL

To separate clean and dry 5 mL vials with magnetic stir bars, 0.40 mL of 0.5 M buffer solutions of MOPS at pH 7.29, 0.10 mL DMSO, and 27 mg (110.7 μmol) of (*S*)-NME were added (Table 2.14, experiment 1). The aqueous mixtures were stirred for 144 hours at mostly 45 ºC, and they were monitored by chiral column HPLC daily by removing a 0.1 mL aliquot of the sample and extracting the organic compounds into 0.2 mL hexanes for injection into the HPLC. Racemization reactions of (*S*)-NME was attempted with different buffers at different concentrations, along with different ratios of the reagents including lauric acid at pH close to 7.6 (Table 2.14).

Appendix A

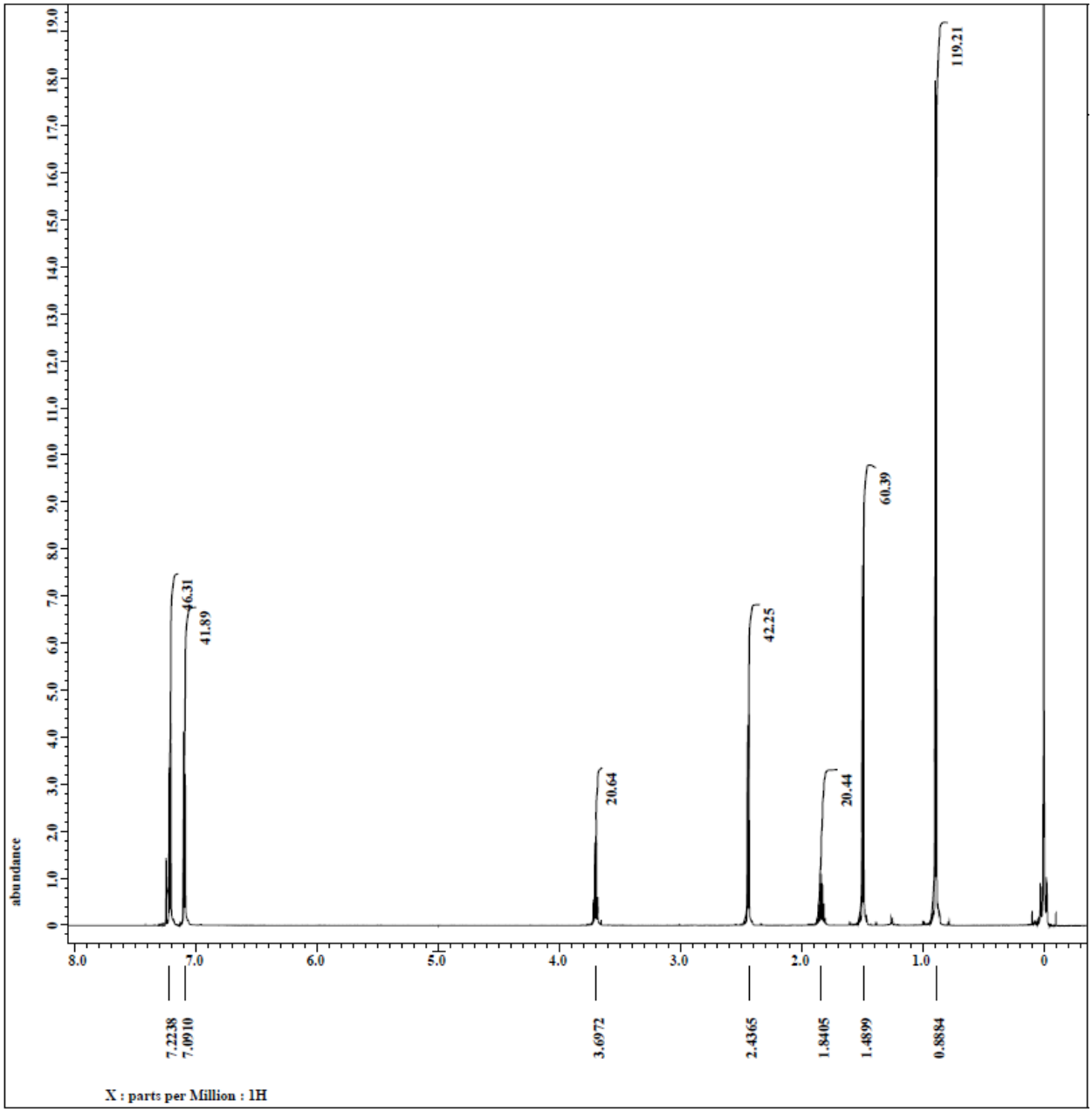


Figure A.1 Proton Nuclear Magnetic Resonance (^{1}H NMR) Spectrum of Racemic Ibuprofen

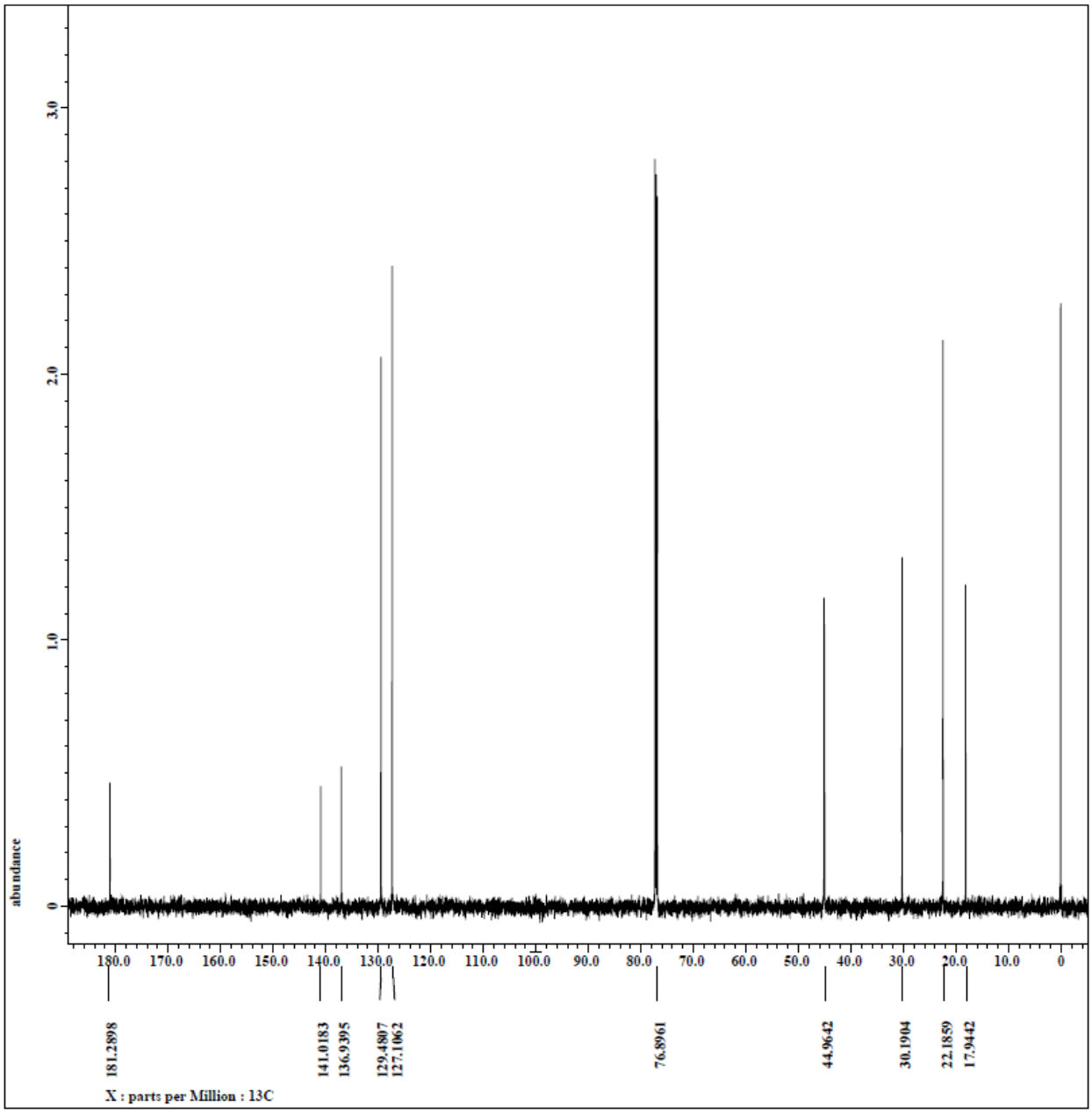


Figure A.2 Carbon-13 (^{13}C) NMR Spectrum of Racemic Ibuprofen

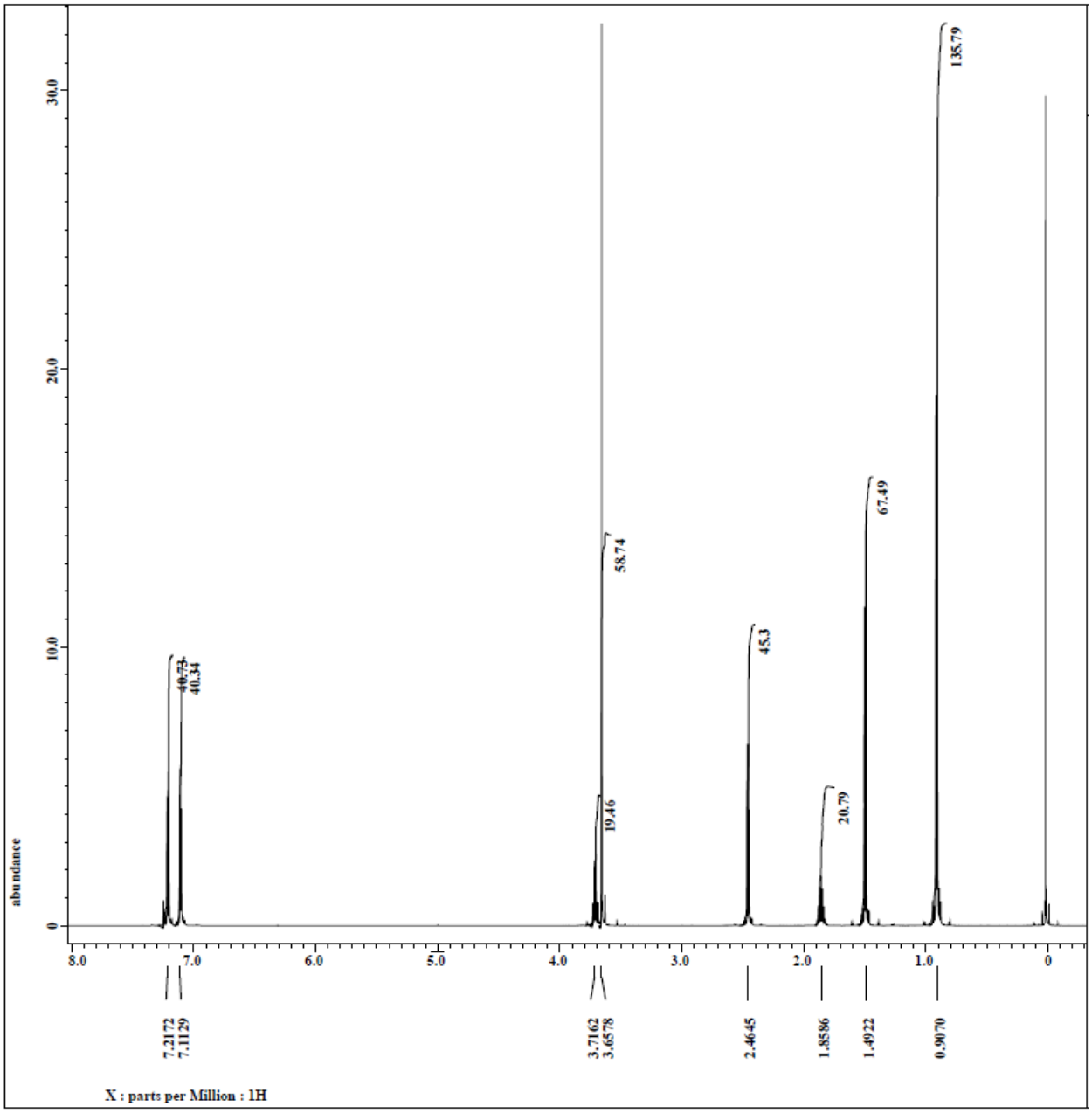


Figure A.3 Proton Nuclear Magnetic Resonance (^{1}H NMR) Spectrum of Racemic IME

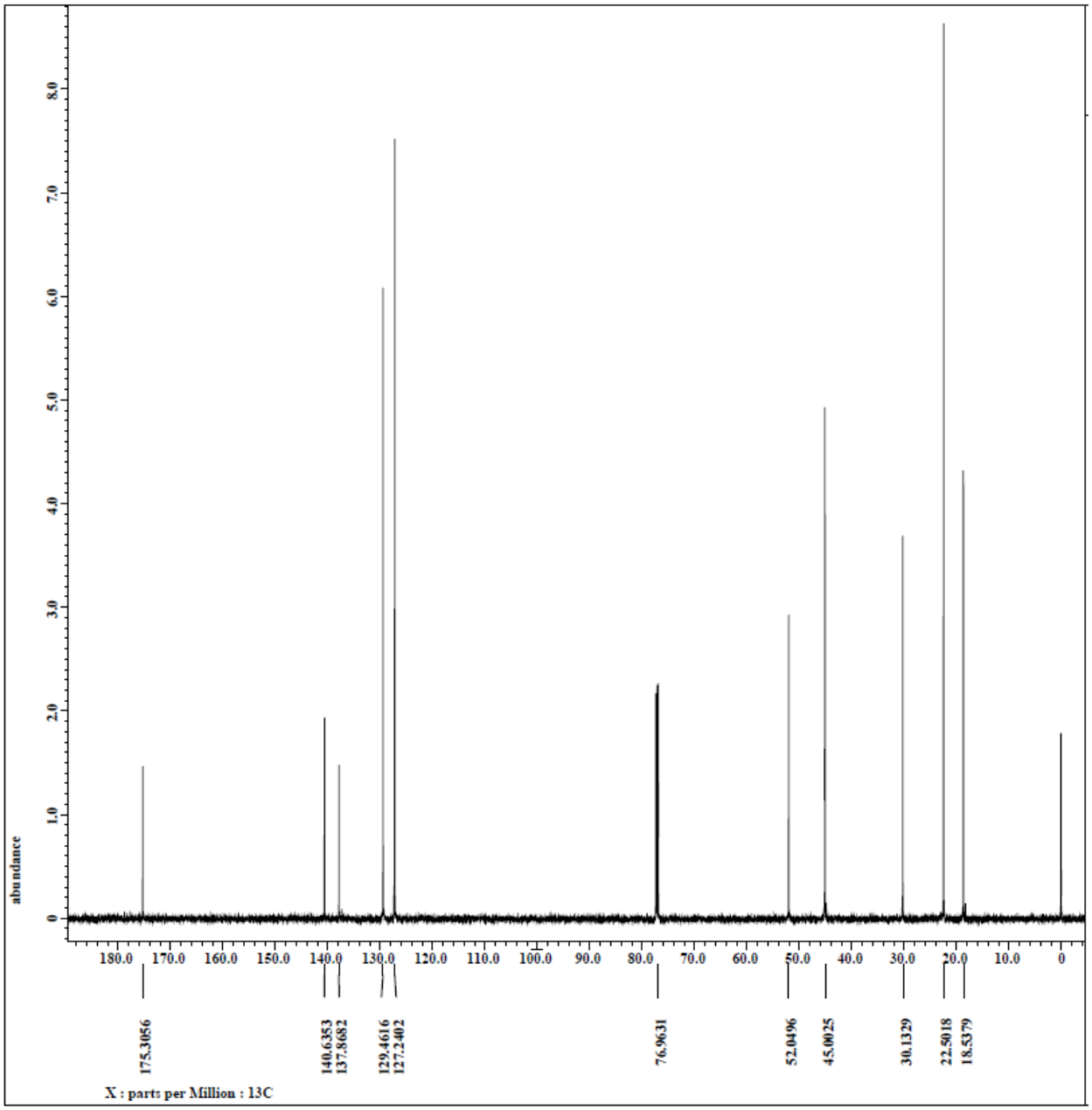


Figure A.4 Carbon-13 (^{13}C) NMR Spectrum of Racemic IME

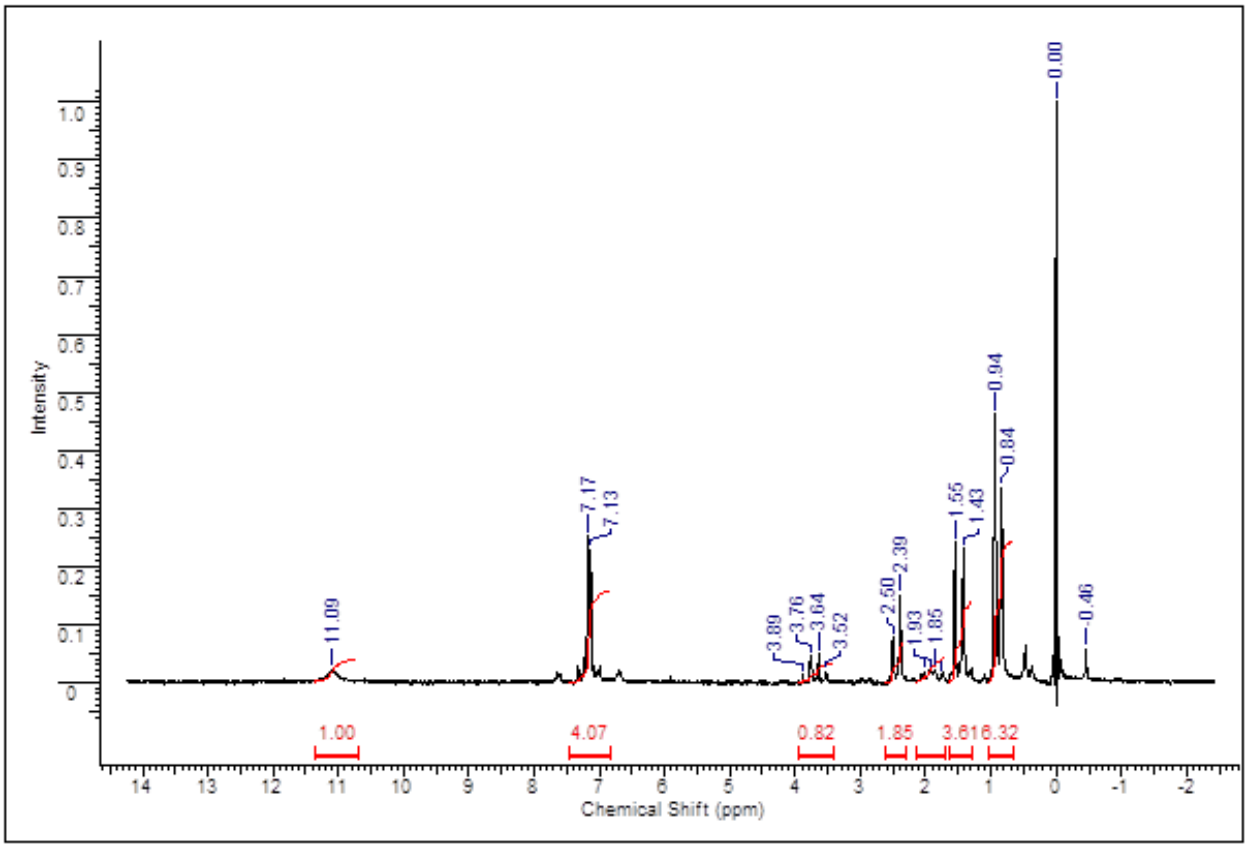


Figure A.5 Proton Nuclear Magnetic Resonance (^{1}H NMR) Spectrum of (*S*)-Ibuprofen

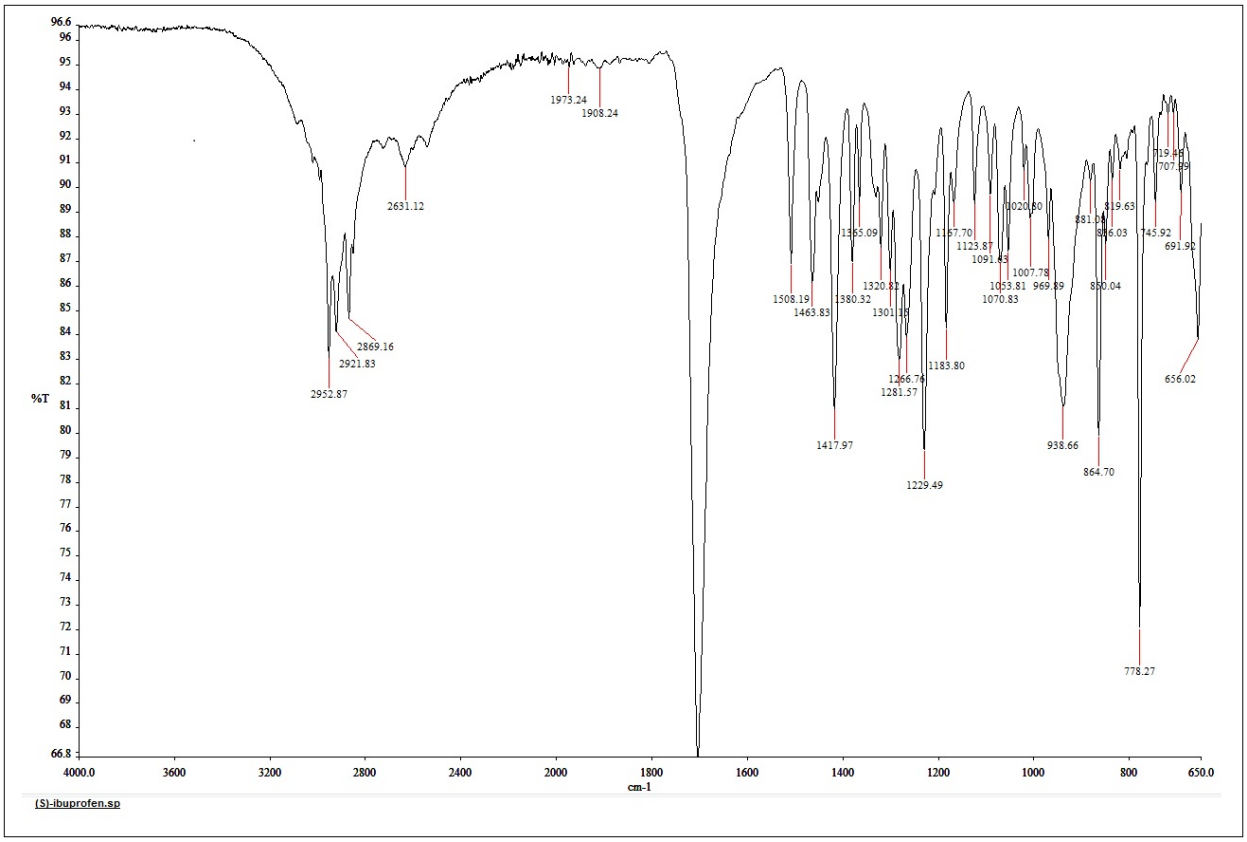


Figure A.6 Infrared (IR) Spectrum of (*S*)-Ibuprofen

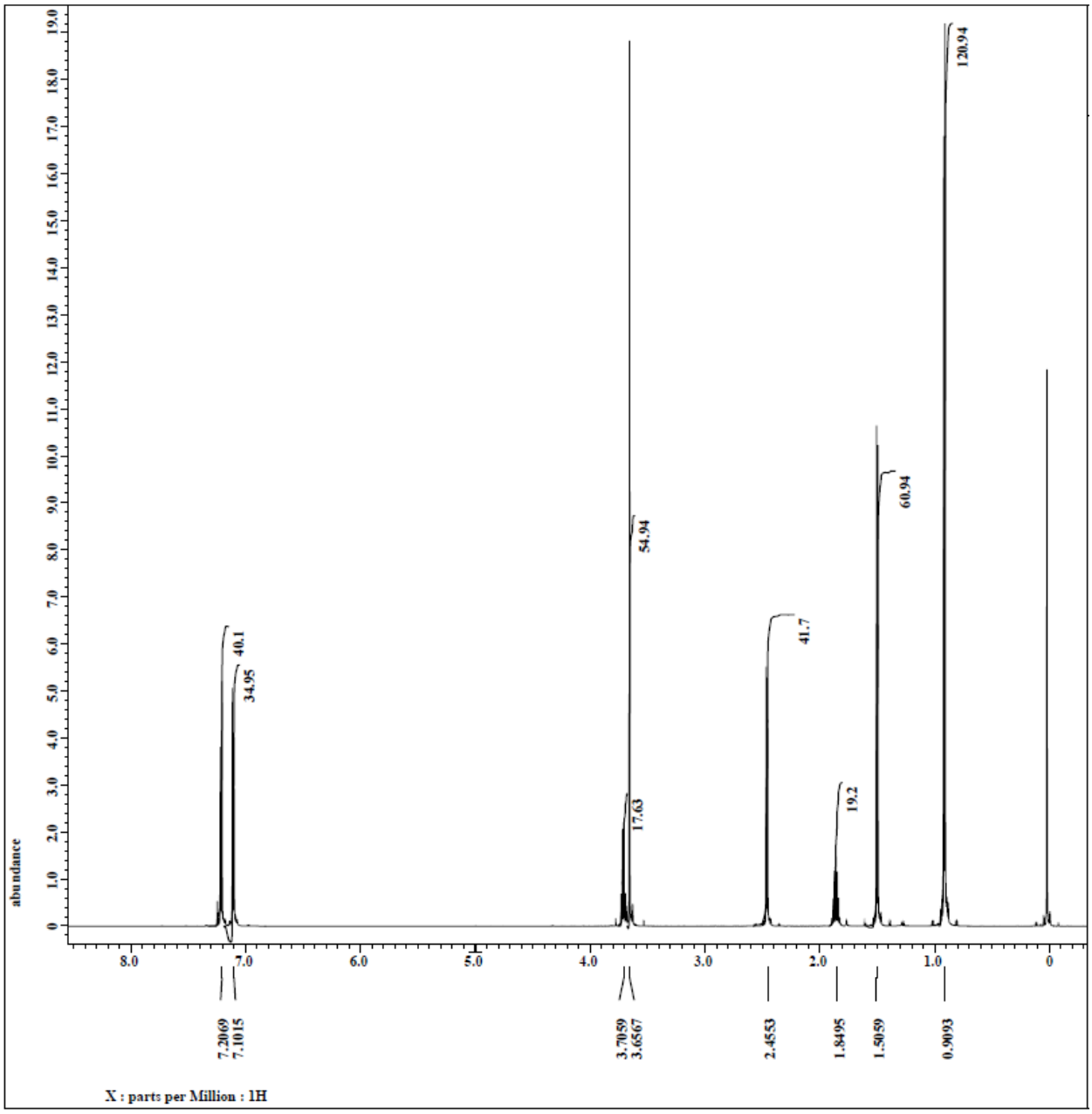


Figure A.7 Proton Nuclear Magnetic Resonance (^{1}H NMR) Spectrum of (*R*)-IME

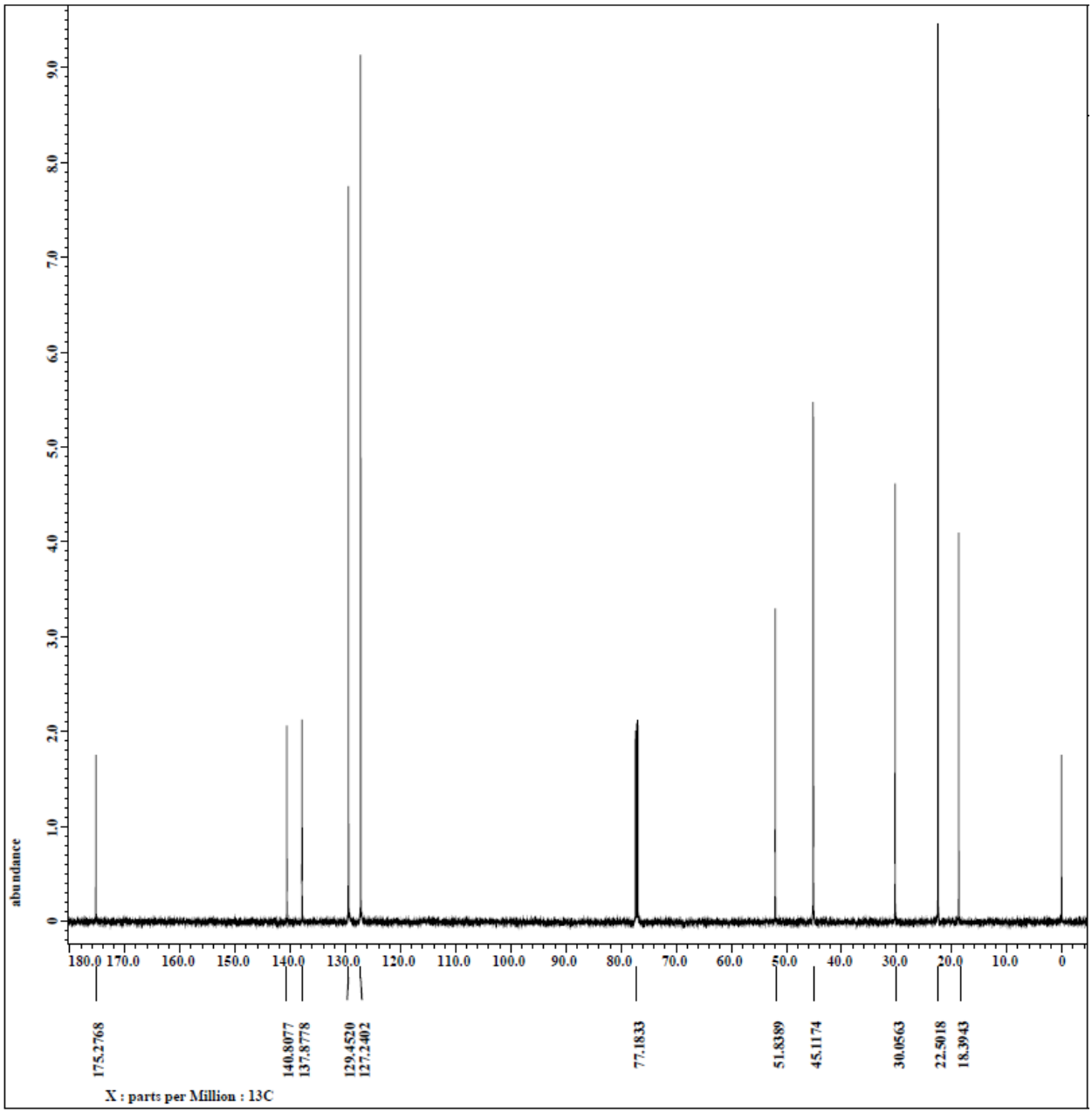


Figure A.8 Carbon-13 (^{13}C) NMR Spectrum of (*R*)-IME

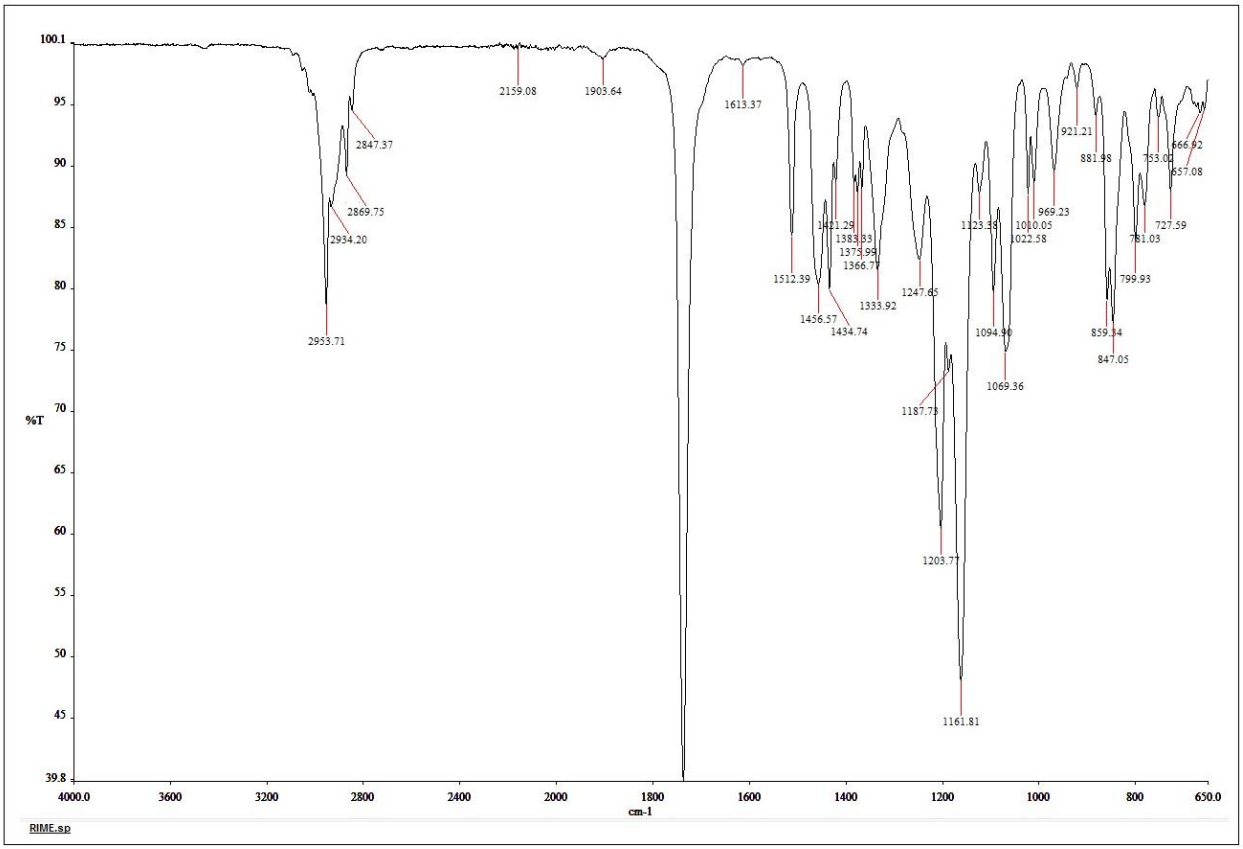


Figure A.9 Infrared (IR) Spectrum of (*R*)-IME

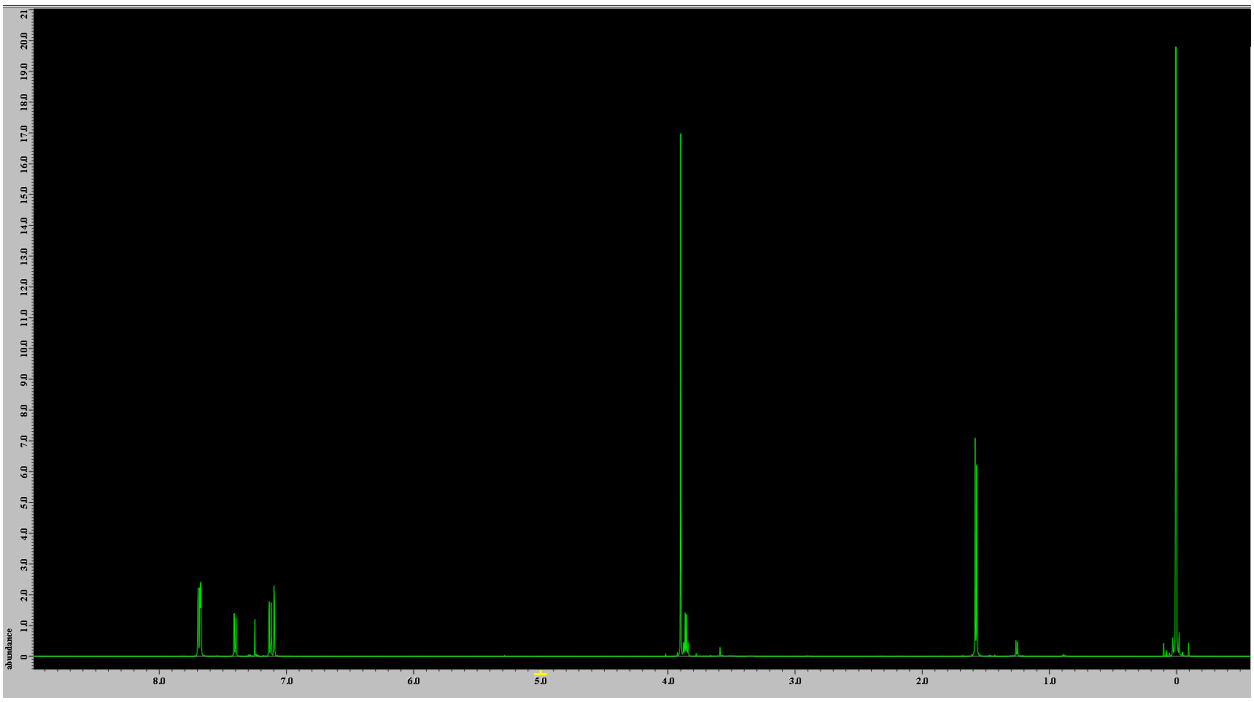


Figure A.10 Proton Nuclear Magnetic Resonance (^{1}H NMR) Spectrum of (*S*)-Naproxen

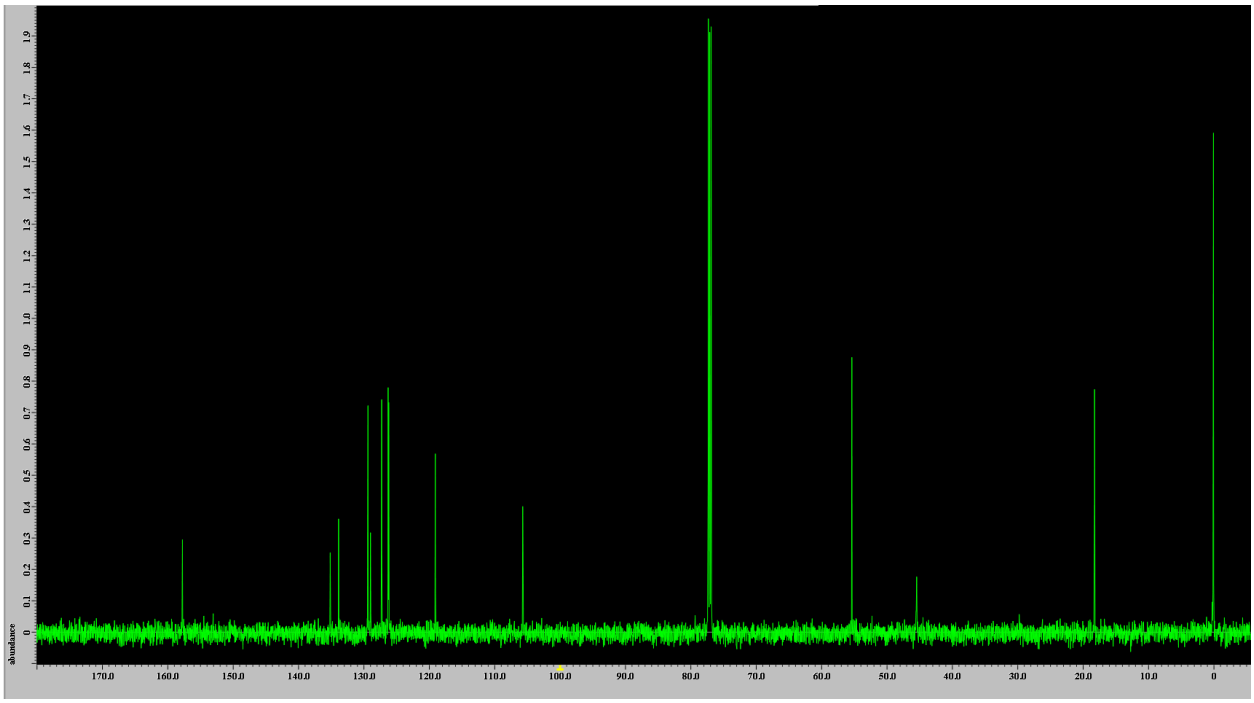


Figure A.11 Carbon-13 (^{13}C) NMR Spectrum of (*S*)-Naproxen

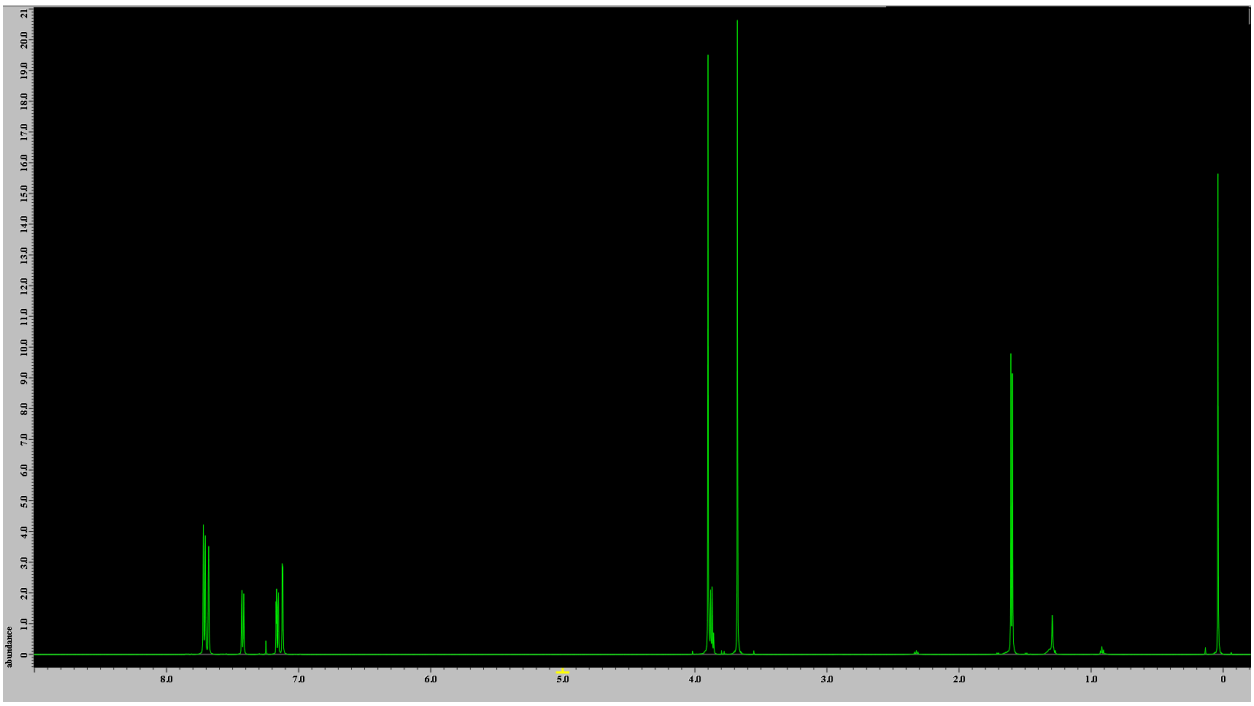


Figure A.12 Proton Nuclear Magnetic Resonance (^{1}H NMR) Spectrum of Naproxen Methyl Ester

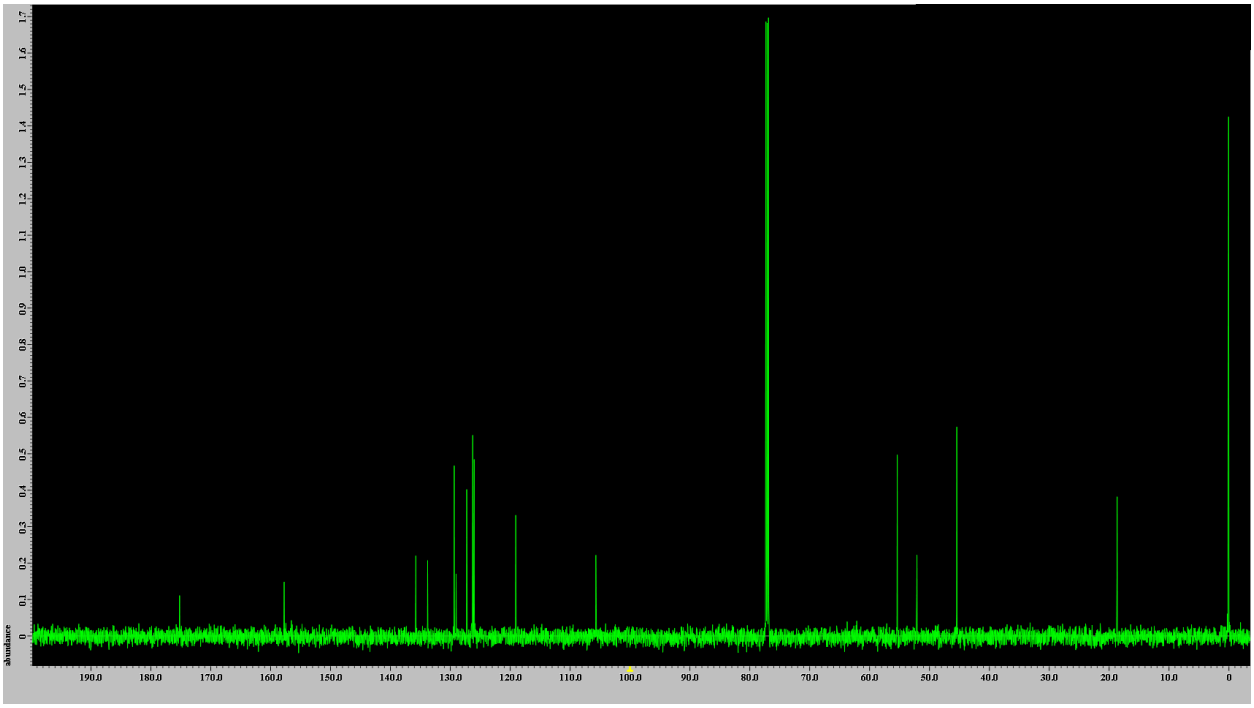


Figure A.13 Carbon-13 (^{13}C) NMR Spectrum of Naproxen Methyl Ester

Appendix B

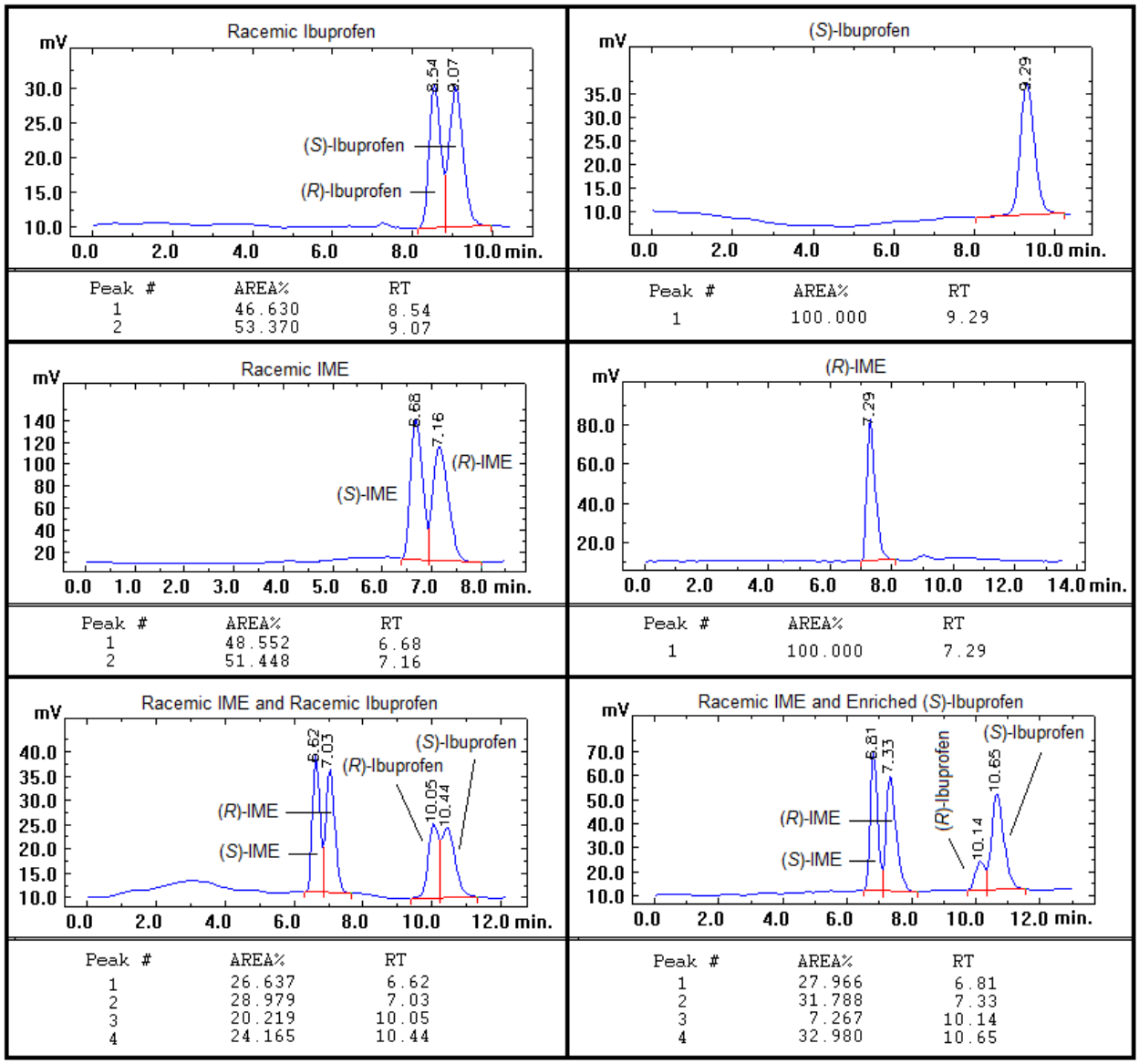


Figure B.1 Chiral Column HPLC Chromatographs of Racemic Ibuprofen, (*S*)-Ibuprofen, Racemic IME, (*R*)-IME, a Mixture of Racemic IME and Racemic Ibuprofen, and a Mixture of Racemic IME plus Enriched (*S*)-ibuprofen
Panel Order: left to right-top to bottom

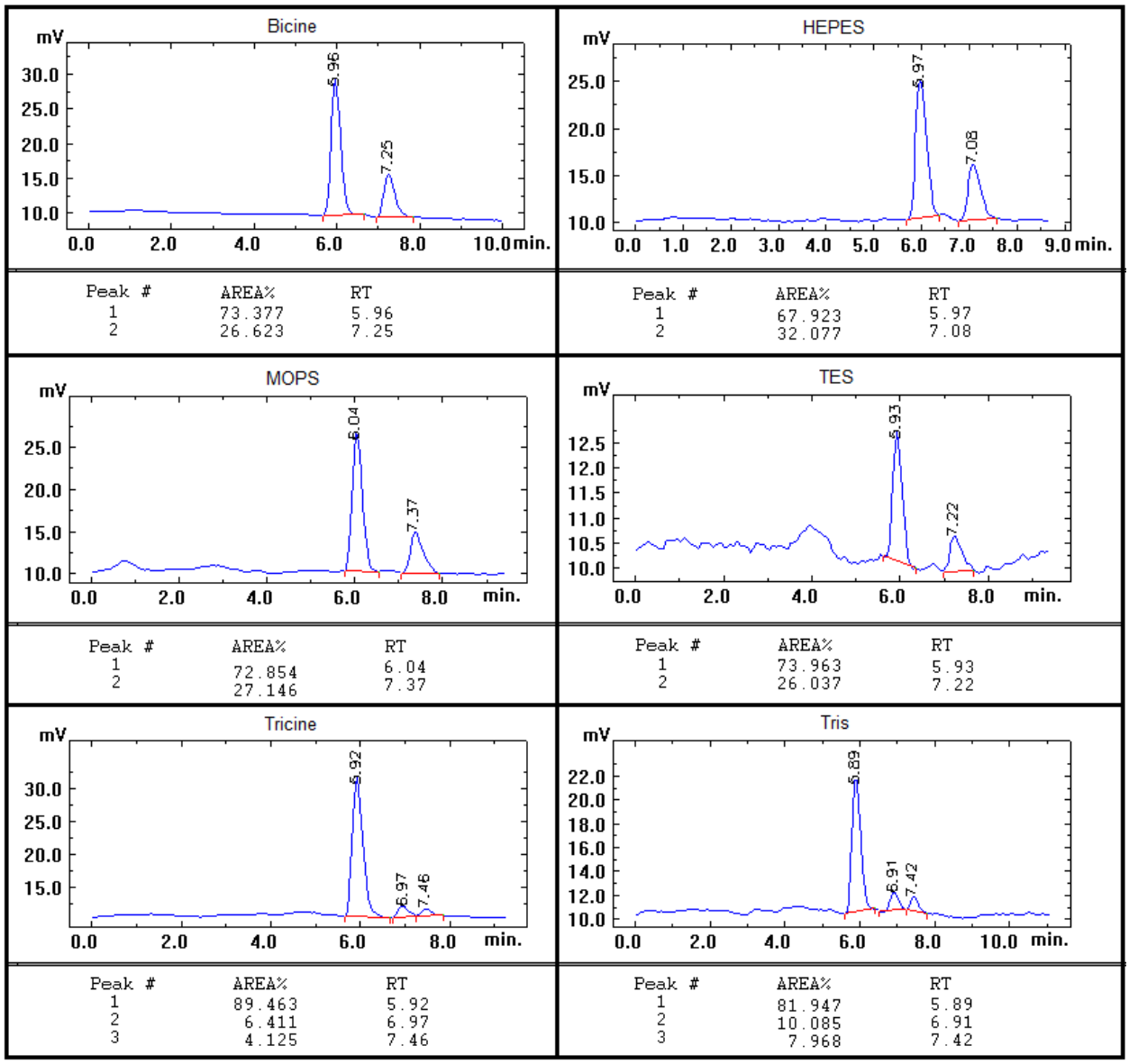


Figure B.2 Chiral Column HPLC Chromatographs of Lower Conversions above pH 7.6 of KR Reactions of Racemic IME after 48 hours with Different Buffers

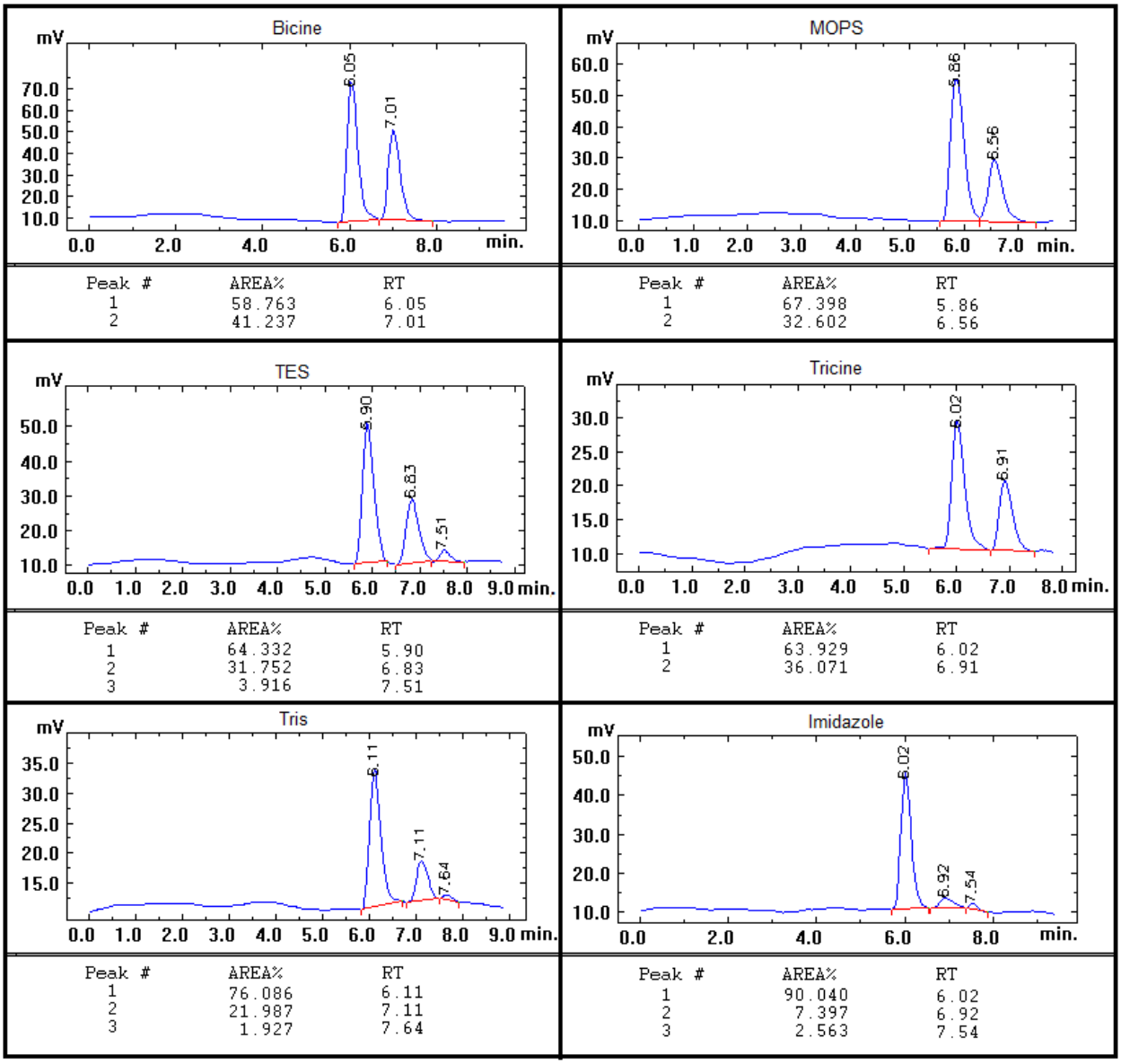


Figure B.3 Chiral Column HPLC Chromatographs of Increased Conversion at pH 7.6 of KR Reactions of Racemic IME after 36 hours with Different Buffers

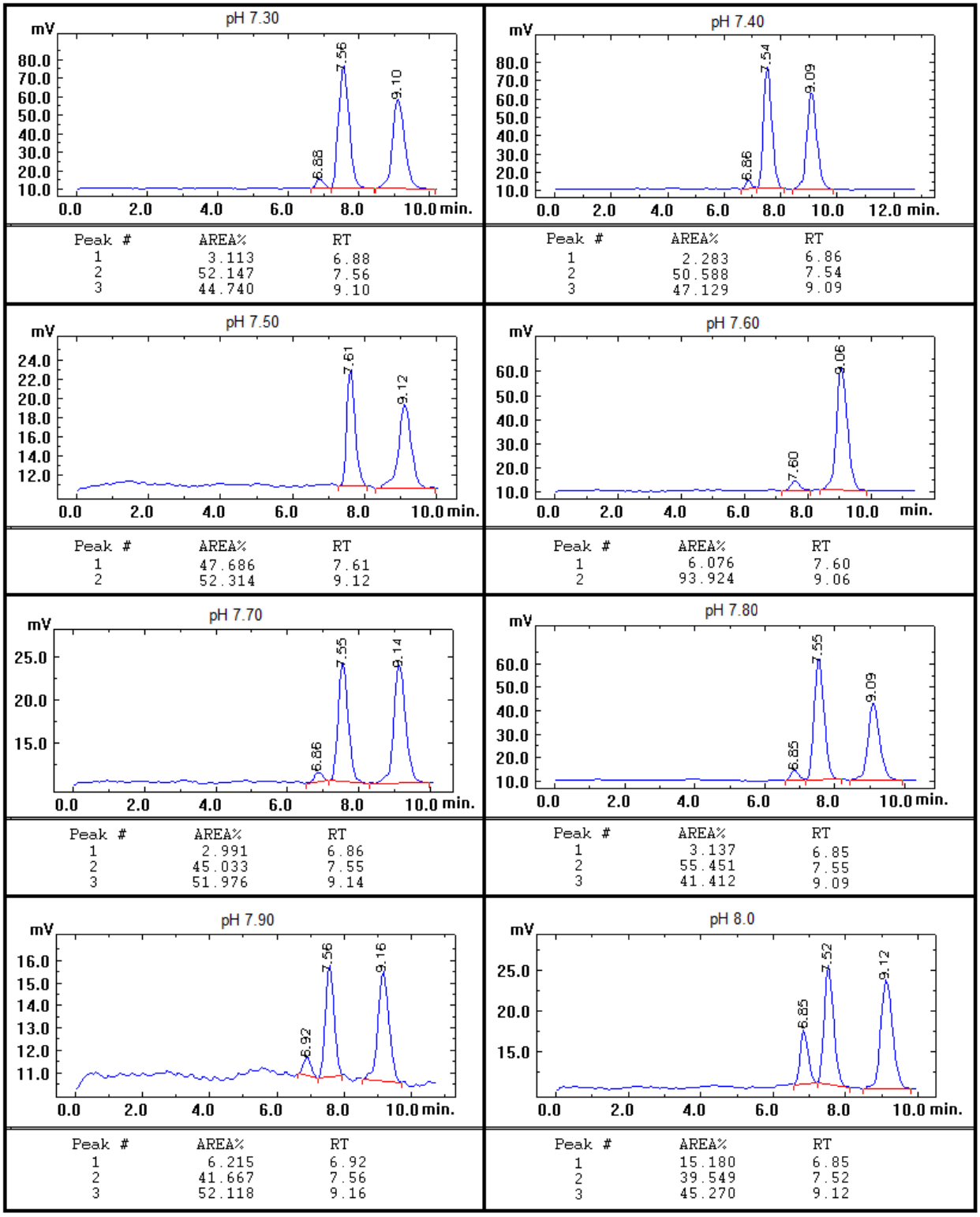


Figure B.4 Chiral Column HPLC Chromatographs of pH Effect on DKR of Racemic IME of 0.5 M Bicine buffer at pH range 7.30-8.0 at 40 ºC after 120 hours
Panel Order: left to right-top to bottom

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Curriculum Vitae

Saideh Sadat Mortazavi was born in Tehran, Iran to Hosein Mortazavi and Nahid Rahmani. She graduated with a Bachelor of Science degree in chemistry from the University of Shahid Beheshty (Tehran) in 1994. From 1998 to 2007, she held an administrative and teaching position at Tehran International School. She started her Master's in chemistry at the University of Texas at El Paso (UTEP) in August of 2007 and joined Dr. James M. Salvador's research group in December of 2007. While pursuing her Master's, she worked as a teaching assistant for the department of chemistry. She received her Master's of Science degree in chemistry in December of 2009. In the spring of 2010 she started the doctoral program in chemistry at UTEP and continued to work as an assistant instructor for organic chemistry laboratories.

Saideh has been the recipient of awards and honors including the University of Texas at El Paso Graduate School Research Award (Spring 2009 and Spring 2011), the Science, Technology, Engineering, Mathematics (STEM)" scholarship (Fall 2009), the Lanward Graduate Scholarship (Fall 2011), and an Outstanding Graduate Student in Chemistry Award (Fall 2009). She has presented her research at national, regional, and local conferences such as at the Society for the Advancement of Chicanos and Native Americans in Science (SACNAS, 2009, 2010, 2013), the American Chemical Society's 65th Southwestern Regional Meeting (SWRM, 2009), the American Institute of Chemical Engineers (AIChE, 2013), and the 247th American Chemical Society National Meeting (ACS, 2014). Her current goal is to publish her master's and doctoral research work, pursue a postdoctoral position, and teach.

This dissertation has been typed by Saideh Sadat Mortazavi.