BIOGENERATION OF LIPOPHENOLS BY LIPASES USING SELECTED SUBSTRATE MODELS

by

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Short Title

BIOGENERATION OF LIPOPHENOLS

ABSTRACT

M. Sc. Tamara Petel

The objective of the research was to carry out the biogeneration of lipophenols by enzymatic esterification of tricaprylin and caprylic acid with catechin and catechol in a model hexane system. Commercial lipases, including Lipase N from *Rhizopus niveus*, Lipozyme IM from *Mucor miehei* and Novozym 435 from *Candida antarctica* were used throughout this study. The effects of reaction time, incubation temperatures and agitation speeds on enzymatic hydrolytic activity were investigated to determine the optimal conditions for biocatalysis. The optimal temperatures for biocatalysis were determined to be 37.5°C for Lipase N, and 55°C for Lipozyme IM and Novozym 435; the optimum agitation speed was 100 rpm. Using Lipase N, maximum hydrolysis of 1.66 μmol free fatty acids/mL was obtained after 1.5 days of incubation, while with Lipozyme IM, maximum hydrolysis of 8.1 and 8.5 μmol free fatty acids/mL was obtained after 1 and 4 days, respectively. With Novozym 435, the highest hydrolysis of 4.0 and 6.1 μmol free fatty acids/mL were found after 2 and 9 days, respectively. The high-performance liquid chromatography (HPLC) analysis of the lipase-catalyzed reaction compounds indicated the absence of lipophenol production from tricaprylin and catechin as substrates with Lipase N as biocatalyst, where 47.8 and 37.3 relative % esterifications were observed using Lipozyme IM after 4 days and Novozym 435 after 8 days, respectively. The use of caprylic acid and catechin as substrates resulted in a relative % esterification of 44.0 after 3 days and 54.0 after 1 day using Lipozyme IM and Novozym 435, respectively. The relative % esterification of tricaprylin and catechol into lipophenols was 12.5 and 33.7% with Lipozyme IM and Novozym 435, respectively after 8 days of incubation while, the relative % esterifications of caprylic acid and catechol were 26.3 using Lipozyme IM after 1 day of incubation and 70.4 with Novozym 435 after 6 days. In conclusion, the relative % esterification varied according to the substrates used for the biogeneration of lipophenols by lipases in a model hexane medium.

RÉSUMÉ

M. Sc. Tamara Petel

L'objective de la recherche a était d'étudier la bio-génération de lipophénols par l'estérification enzymatique du tricaprylin ou de l'acide caprylique avec le catéchine ou le catéchol en milieu organique. Les lipases commerciales, incluant la Lipase N de *Rhizopus niveus*, la Lipozyme IM de *Mucor miehei* et la Novozym 435 de *Candida antarctica*, ont été utilisées dans cette étude. Les effets du temps réactionnel, de la température d'incubation et de la vitesse d'agitation sur l'activité spécifique ont été étudiés pour déterminer les conditions optimales pour la biocatalyse. Les résultats ont montré que les températures réactionnelles optimales pour la biocatalyse ont été de 37,5°C pour la Lipase N et de 55°C pour la Lipozyme IM et la Novozym 435, en utilisant une vitesse d'agitation de 100 rpm. En utilisant la Lipase N, une hydrolyse maximale de 1,66 µmol d'acides gras libres /mL a été obtenu après 1.5 jours de réaction, tandis qu'avec la Lipozyme IM, une hydrolyse maximale de 8.1 et 8.5 µmol d'acides gras libres/mL après 1 et 4 jours de réaction, respectivement. Avec la Novozym 435, une hydrolyse maximale de 4.0 et 6.1 µmol d'acides gras libres/mL après 2 et 9 jours de réactions, respectivement. Les réactions d'estérification enzymatiques, suivies par l'analyse en chromatographie liquide à haute performance (HPLC), ont montré qu'il n'y a pas formation de lipophénols, en utilisant le tricaprylin et le catéchine comme substrats et en présence de la Lipase N, tandis que les taux d'estérification de tricaprylin avec le catéchine de 47,8 et 37,3 % ont été atteint en présence de la Lipozyme IM après 4 jours et de Novozym 435 après 8 jours, respectivement. L'estérification de l'acide caprylique avec le catéchine a atteint un taux de 44,0 % après 3 jours et 54,0 % après 1 jour en présence de la Lipozyme IM et de la Novozym 435, respectivement. L 'estérification du tricaprylin avec le catéchol a atteint un taux d'estérification de 12,5 et 33,7 % en présence de la Lipozyme IM et de la Novozym 435, respectivement après 8 jours de réaction. L'estérification de l'acide caprylique avec le catéchol, a atteint un taux d'estérification de 26.3 en présence de la Lipozyme IM après 1 jour de réaction et 70,4 % en présence de la Novozym 435 après 6 jours.

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LIST OF ABBREVIATIONS

ω-3, n-3: Omega-3

ω-6, n-6: Omega-6

AA: Arachidonic acid

ALA: α-Linolenic acid

a_w: Water activity

BHA: Butylated hydroxyanisole

BHT: Butylated hydroxytoluene

BSA: Bovine serum albumin

CA: Caprylic acid

DHA: Docosahexaenoic acid

DPA: Decosapentaenoic acid

EPA: Eicosapentaenoic acid

FFAs: Free fatty acids

FID: Flame ionization detector

FTIR: Fourier transmission infrared

GC: Gas chromatography

GLA: γ-Linolenic acid

HDL: High-density lipoproteins

HPLC: High pressure liquid chromatography

LDL: Low-density lipoproteins

NMR: Nuclear magnetic resonance

PG: Propyl gallate

TBHQ: *tert*-Butylhydroquinone

TMS: Tetramethylsilane

UV-DAD: UV diode-array detector

1. INTRODUCTION

Lipids are a group of compounds that are generally soluble in organic solvents but display limited solubility in water. Dietary lipids have an important function in nutrition (Nawar, 1996) by supplying calories and essential fatty acids and by acting as vitamin carriers and increasing the palatability of foods. Lipids are composed of saturated and unsaturated fatty acids, usually in the form of mixed triacylglycerols (Stansby *et al.*, 1990), where the fatty acids can range from 4 to 24 carbons in length (Nawar, 1996). The health benefits of oils rich in polyunsaturated fatty acids, especially the omega-3 and omega-6 fatty acids, have been shown to play a role in the prevention and modulation of certain diseases (Stansby *et al.*, 1990; Connor, 2000).

Phenolic compounds make up another very important group of compounds. Phenols can range from simple molecules to complex macromolecular polymers. The characteristic structure of a phenolic compound consists of a hydroxyl group bonded to a benzene ring (Parr and Bolwell, 2000). Phenolic compounds act as anticarcinogens and antimutagens (Hollman, 2001) by decreasing the oxidative stress responsible for pathogenic mechanisms (Vinson *et al.*, 1998), which can lead to the development of degenerative diseases (Shahidi, 1997) due to oxidative damage to biomolecules (Hollman, 2001). Antioxidants such as phenolic compounds have been reported to reduce the formation of various off-flavors and other compounds resulting from oxidative processes (Shahidi, 1997). The chemical antioxidants available for food applications, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), are stable and efficient; however, these compounds are suspected of acting as promoters of carcinogenesis (Chen *et al.*, 1992; Ho, 1992; White and Xing, 1997). Consequently, there is an increasing interest in the recovery and use of natural phenolic compounds (Chen *et al.*, 1992; Ho, 1992; White and Xing, 1997) as antioxidants.

Since phenolic acids are hydrophilic, they exhibit little stability and solubility in different organic solvent systems and therefore have limited antioxidant efficiency in stabilizing oils and fats. (Stamatis *et al.*, 2001). Enzymatic esterification of phenolic

compounds with fatty alcohols, fatty acids or triacylglycerols could therefore be used to modify these physical properties by producing lipophenols with different solubility characteristics and making them more useful as food lipophilic antioxidants and emulsions (Buisman *et al.*, 1998). Lipophenols have the potential to become excellent nutraceutical products since they would possess the health benefits of the polyunsaturated fatty acids and the phenolic antioxidants.

Lipases (triacylglycerol acylhydrolase lipase, EC 3.1.1.3) are enzymes whose biological function is to catalyze the hydrolysis of triacylglycerides into monoacylglycerides, diacylglycerides, glycerol and free fatty acids (Kilara, 1985; Taipa *et al.*, 1992) at the water oil interface in aqueous media. Lipases may also act on a wide range of water-insoluble carboxylic esters. Lipases can be found in animal, plant and microbial sources (Verger *et al.*, 1990; Xu *et al.*, 2000a). Enzymatic biocatalysis in organic solvent media has certain advantages over that in aqueous media. In organic solvent medium, lipases can catalyze new reactions (Thomson *et al.*, 1999) such as ester synthesis, acidolysis and alcoholysis. Lipases, among other enzymes, can remain active in nearly anhydrous organic solvents, since the water needed for enzymatic activity remains tightly bound to the enzyme even when the bulk water is replaced with an organic solvent (Kermasha *et al.*, 1995). The occurrence of undesirable water-dependent side reactions, as well as product and substrate inhibitions, have been reported to be reduced in organic medium (Laane *et al.*, 1987) and easier product recovery is obtained from low boiling organic solvents (Frings *et al.*, 1999). The stability of several enzymes has also been reported to have improved in organic medium (Frings *et al.*, 1999).

The overall aim of this research was to produce lipophenols by enzymatic esterification of selected lipids and phenolic compounds in an organic solvent medium.

The specific objectives of this research were:

1. To optimize the reaction conditions for the biogeneration of lipophenols by Lipase N from *Rhizopus niveus*, Lipozyme IM from *Mucor meihei* and Novozym 435 lipase (B lipase) from *Candida antarctica*, with tricaprylin and caprylic acid as the lipid substrates, and catechin and catechol as the phenolic substrates.

2. To recover and separate the lipase-catalyzed lipophenolic compounds.

3. To characterize the lipophenols in terms of their chromatographic profiles and determine their formation rates.

2. LITERATURE REVIEW

2.1. Lipids

Lipids are natural fats, which are composed of a glycerol molecule and fatty acids. Fatty acids are any aliphatic monocarboxylic acids that can be released from the hydrolysis of natural fats. Lipids are a large group of compounds that are usually soluble in organic solvents and sparingly soluble in water (Nawar, 1996). Lipids are present in many biological systems, and are found in both plants and animals. They may occur as structural lipids such as phospholipids, sphingolipids and glycolipids or may be present as triacylglycerols (Gordon and Roedig-Penmann, 1999). Lipids are the main components of adipose tissue, along with proteins and carbohydrates. Glycerol esters of fatty acids compose up to 99% of the lipids of plant and animal origin (Potter and Hotchkiss, 1996). Fat is the main energy source of animals and plants (Potter and Hotchkiss, 1996). Differentiation between fats and oils is based on whether the lipid is solid or liquid at room temperature (Nawar, 1996). Dietary lipids play an important role in nutrition, supplying calories and essential fatty acids while, acting as vitamin carriers and increasing the palatability of food (Nawar, 1996).

2.1.1. Fatty Acids and Their Triacyglycerol Esters

Fatty acids range from 4 to 24 carbons in length. Three fatty acids combine with a glycerol molecule to form a triacylglycerol (Nawar, 1996). Short chain fatty acids include butyric (C4:0) and caproic acids (C6:0). Medium length fatty acids are composed of saturated 8 to 12 carbons chains. These are mainly composed of caprylic acid (C8:0), capric acid (C10:0) and lauric acid (C12:0), which resist oxidation and are stable at both low and elevated temperatures (Lee and Akoh, 1996; Willis *et al.*, 1998; Mogi *et al.*, 2000; Kim *et al.*, 2001). Medium chain triacyglycerols are usually liquid at room temperature and have low viscosities and melting points (Kim *et al.*, 2001). The free fatty acids from food, which are released during absorption, are more easily metabolized if they are of medium or short chain length (Xu *et al.*, 2000b). They are not formed into chylomirons for transport nor deposited in the adipose tissue. Unlike long chain fatty acids, medium chain fatty acids are metabolized in the portal vein of the liver for quick

energy (Lee and Akoh, 1996; Willis *et al.*, 1998; Jennings and Akoh, 1999; Paez *et al.*, 2002). Pancreatic lipase hydrolyzes esters at the *sn*-1 and *sn*-3 positions in the triacylglycerol molecule. Pancreatic lipase shows higher activity for the hydrolysis of medium chain fatty acids than long chain fatty acids (Paez *et al.*, 2002). Medium chain fatty acids may lower serum cholesterol, and are considered to be non-tumor promoting and anti-tumor fats (Lee and Akoh, 1996). Medium chain triacylglycerols have been shown to have health benefits, such as in the treatment of fat malabsorption in premature infants, and patients with cystic fibrosis (Jennings and Akoh, 1999), AIDS, anorexia (Willis *et al.*, 1998) and pancreatic deficiency (Mogi *et al.*, 2000; Yankah and Akoh, 2000). Medium chain triacylglycerols are used as a source of easily digestible high caloric food (Kennedy, 1991). Furthermore, improvements in patients affected with Crohn's disease, cystic fibrosis and colitis were determined when medium chain triacylglycerols were included in their diets. They have also been used in the production of cosmetics, toiletries and pharmaceuticals. Other uses of medium chain triacylglycerols include solvent carriers for dyes, flavors, vitamins and treatment of gallstones (Wong *et al.*, 2000). Long chain fatty acids are composed of 16 to 24 carbons in length, and may be saturated or unsaturated. Long chain saturated fatty acids have been associated with increased risk of coronary heart disease in adults, however, palmitic acid (C16:0) is an important source of energy for infants (Willis *et al.*, 1998). Long chain polyunsaturated fatty acids are important in human health due to their degree of unsaturation.

Important polyunsaturated fatty acids are the omega-3 (n-3, ω-3) and omega-6 (n-6, ω-6) fatty acids. Omega-3 fatty acids occur when the first double bond of the fatty acid is between the third and fourth carbon atom from the end of the chain. Omega-6 fatty acids occur when the first double bond lies between the sixth and seventh carbon atom. The ω-3 fatty acids have a higher degree of unsaturation than the ω-6 fatty acids (Hui, 1996). The ω-3 and ω-6 are important because they are precursors of hormone compounds such as eicosanoids, which are involved in many biological processes in the human body (Shahidi and Wanasundra, 1998). The ω-3 polyunsaturated fatty acids may be produced through a series of elongations and desaturations from α-linolenic acid (Shahidi and Wanasundra, 1998). They are structural components of the phospholipid

membranes in tissues that are found throughout the body, particularly in the retina, brain and spermatozoa. In these cases, docosahexanoic acid (C22:6ω-3, DHA) represents over 1/3 of total fatty acids (Connor, 2000). DHA is also the main material in the phospholipid of the retina and the gray matter of the brain (Tanaka *et al.*, 1993).

Omega-3 fatty acids have been shown to play a role in the prevention and modulation of certain diseases (Connor, 2000). Some diseases that can be prevented or alleviated with ω-3 are: coronary heart disease, stroke, essential fatty acid deficiencies in infancy including retina \and brain development, autoimmune disorders such as lupus and nephropathy, Crohn's disease, breast, colon and prostate cancers, mild hypertension and rheumatoid arthritis (Stansby *et al.*, 1990; Medina *et al.*, 1999; Connor, 2000). Dietary ω-3 fatty acids are able to prevent heart disease through the following behaviors. They can prevent arrhythmias (ventricular tachycardia and fibrillation), prostaglandin and leukotrieke precursors, inflamatory properties, inhibit the synthesis of cytokines and mitogens, stimulate endothelial-derived nitric oxide, are antithrombotic, have hypolipidemic properties with effects on triacylglycerols and very low-density lipoproteins (LDL) and can inhibit atherosclerosis (Connor, 2000; Xu *et al.*, 2000b). Fish oils, which are rich in ω-3 fatty acids, have the ability to slow down the growth of atherosclerotic plaque by inhibiting the cellular growth factors and the migration of monocytes (Xu *et al.*, 2000b).

Commercial sources of ω-3 polyunsaturated fatty acids can be found in fish oils, animal viscera and the fungus *Morteriella alpina* 1S-4. Some microalga have been found to contain oils that have polyunsaturated fatty acids in varying amounts. *Phaeodactylum tricornutum*, a fast growing microalga, produces eicosapentaenoic acid (C20:5ω-3, EPA), small amounts of DHA and arachidonic acid (AA). Recently, Poisson and Ergan (2001) used a strain of microalgal of *Isochrysis galbina* to produce DHA. Also, *Porphyridium cruentum* UTEX 161 produces AA and EPA (Medina *et al.*, 1999). EPA and DHA are plentiful in fish, shellfish and marine-mammals and are sparse or absent in land animals and plants. Phytoplankton, the water plants at the base of the food chain for marine life synthesize EPA and DHA. Land plants are also rich in ω-3, but are found in the form of

α-linolenic acid (ALA, 18:3 ω-3), which can synthesize EPA and DHA (Connor, 2000; Xu *et al.*, 2000b).

2.1.2. Lipid Composition of Selected Oils

The degree of saturation present in the lipid depends on the source. Vegetable oils are a good source of long chain unsaturated fatty acids, such as those extracted from canola, corn and olives. They are rich in oleic (C18:1), linoleic (C18:2) and linolenic (C18:3), as displayed in Table 1. Animal fats, such as butter, have a greater amount of short, medium and long chain saturated fatty acids. Marine oils, however, are an excellent source of polyunsaturated fatty acids. They contain 14-30% polyunsaturated fatty acids from the ω-3 family, including EPA and DHA (Rocquelin, 1990; Willis *et al.*, 1998; Jennings and Akoh, 1999). High levels of polyunsaturated fatty acids have the ability to decrease cholesterol in both low and high-density lipoproteins (LDL and HDL types). Nonetheless, oils with high degrees of unsaturation, such as linoleic acid (C18:2ω-6), are sensitive to oxidation and may produce products that contribute to atherosclerosis and carcinogenesis (Moussata and Akoh, 1997).

2.1.3. Autoxidation Reaction

The autoxidation reaction of lipids involves three steps: (1) initiation, (2) propagation and (3) termination (Fig. 1). The initiation step can be induced from the exposure of the lipid to light, heat, ionizing radiation, metal ions, metalloprotein catalysts or by the enzyme lipoxygenase (Shahidi and Naczk, 1995a; Gordon and Roedig-Penman, 1999). This reaction produces lipid free radicals, which can then react to produce lipid peroxy radicals. The propagation step can produce more lipid free radicals leading to lipid hydroperoxides, which then decompose to produce the secondary oxidation products, which are responsible for some off flavors in foods. The propagation may be terminated by the combination of two free radicals to produce a product that does not propagate. Termination reactions are limited by the low probability of the two free radicals colliding in the right orientation, even though two radicals have a low energy of activation (Gordon and Roedig-Penman, 1999).

Table 1. Fatty acid profiles of selected oils (g/100g).

Fatty Acids	Menhaden fish oil	Herring fish oil	Cod liver oil	Butter	Cocoa butter	Canola oil	Corn oil	Olive oil
Saturated								
4:0				2.6				
6:0				1.6				
8:0				0.9				
10:0				2.0				
12:0		0.2		2.3				
14:0	8.0	7.2	3.6	8.2	0.1			
16:0	15.1	11.7	10.6	21.3	25.4	4.0	10.9	11.0
18:0	3.8	0.8	2.8	9.8	33.2	1.8	1.8	2.2
20:0						0.7		
22:0						0.4		
24:0						0.2		
Monounsaturated								
16:1	10.5	9.6	8.3	1.8	0.2	0.2		0.8
18:1	14.5	12.0	20.7	20.4	32.6	56.1	24.2	72.5
20:1	1.3	13.6	10.4			1.7		0.3
22:1	0.4	20.6	7.3			0.6		
Polyunsaturated								
18:2 ω6	2.2	1.1	0.9	1.8	2.8	20.3	58.0	7.9
18:3 ω3	1.5	0.8	0.9	1.2	0.1	9.3	0.7	0.6
18:4	2.7	2.3	0.9					
20:4	1.2	0.3	0.9					
20:5 ω3	13.2	6.3	6.9					
22:5 ω3	4.9	0.6	0.9					
22:6 ω3	8.6	4.2	11.0					

Values compiled from USDA Nutrient Database for Standard Reference (2001)

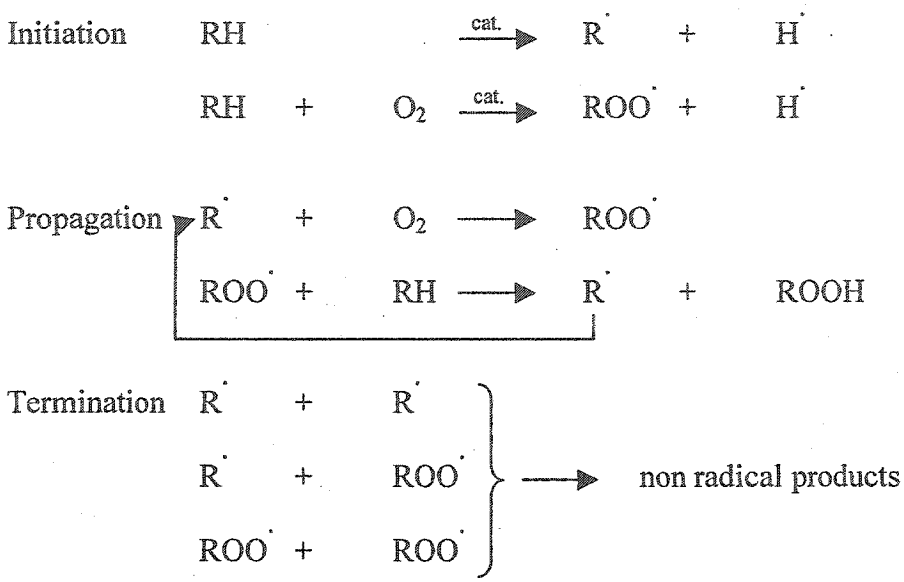

Figure 1. Autoxidation reaction, where RH is the unsaturated lipid, $R^{\cdot}$ is the lipid radical, $ROO^{\cdot}$ is the lipid peroxy radical and ROOH is the hydroperoxide.

The second group of molecules used in the biogeneration of lipophenols are the phenolic compounds, which are considered to be important antioxidants.

2.2. Antioxidants

Antioxidants are compounds that can delay, retard, or prevent the oxidative deterioration of lipids (Gordon and Roedig-Penman, 1999). Antioxidants are believed to reduce the risk of degenerative diseases by decreasing the concentration of free radicals and reactive oxygen species (Gordon and Roedig-Penman, 1999), therefore diminishing the chances of initiating autoxidation reactions (Fig. 2) (Parr and Bolwell, 2000). These degenerative diseases are due to the oxidants and free radicals, which are capable of mediating many disorders and reactive species such as triplet oxygen, water and unsaturated lipid molecules. In the human body, the production of oxygen radicals, especially the hydroxyl radicals, are capable of affecting lipid cell membranes, which can produce lipid peroxides. Reactive oxygen species are linked to many diseases, as well as the aging process (Shahidi, 1997). Oxidation of the LDL is a major step in the development of arteriosclerosis (Gordon and Roedig-Penman, 1999). In foods, free

radical reactions are involved in changes in flavor, texture, color, sensory attributes and decreasing nutritional value due to the destruction of essential fatty acids and lipid soluble vitamins (Shahidi, 1997).

Antioxidants may act as chain breakers or primary antioxidants, serving to scavenge non-lipid free radicals or reducing the rates of propagation by scavenging lipid free radicals (Fig. 2). An antioxidant free radical may be stabilized by kinetic factors, such as the bulky side chain and/or by thermodynamic factors such as resonance stabilization. Secondary antioxidants suppress the rate of the initiation reaction, thereby decreasing the rate of autoxidation. These types of inhibitors consist of: metal chelators, enzyme inhibitors, singlet oxygen scavengers, substances that prevent light or other radiation and reducing agents (Gordon and Roedig-Penman, 1999).

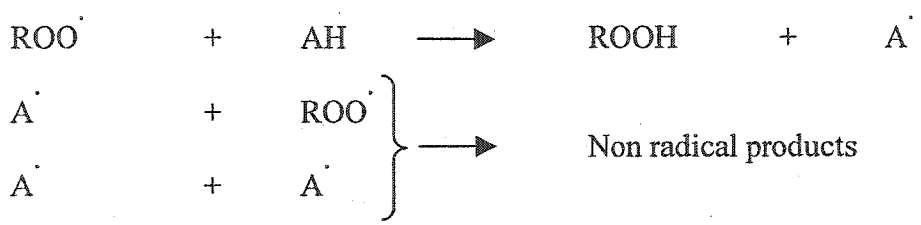

Figure 2. Primary antioxidants, where AH is the antioxidant, A· is the antioxidant radical and ROO· is the peroxy radical.

2.2.1. Natural Antioxidants

2.2.1.1. Phenolic Compounds

Plant phenolics range from simple to complex macromolecular polymers. They all possess a benzene ring with a hydroxy substituent within their structure (Parr and Bolwell, 2000). Phenolic compounds comprise one of the main classes of secondary metabolites in plants. They are polymeric and insoluble ligands that are found in all vascular plants (Shahidi and Naczk, 1995b).

In plants, phenolic compounds protect against tissue injuries, since they oxidize and combine with proteins and other components. The phenolic compounds may serve to inhibit growth of bacterial pathogens and decrease the palatability for herbivores

(Shahidi, 1997). The phenolic compounds have an important function in pollination of flowers. Red flowers containing anthocyanins are pollinated by birds, whereas the yellow flowers containing flavonoids or carotenoids are pollinated by insects (Parr and Bolewll, 2000). The by-products of photosynthesis produce high levels of oxygen, free radicals, and reactive oxygen species. The phenolic antioxidants act to counter these detrimental effects (Shahidi, 1997).

2.2.1.2. Phenolic Antioxidants

Phenolic compounds and their derivatives are potent antioxidants. Phenolic antioxidants are free radical terminators; they act by chelating catalytic metals and by acting as oxygen scavengers (Shahidi and Naczk, 1995a). They lower the oxidative stress resulting from free radicals, responsible for pathogenic mechanisms such as carcinogensis and arteriosclerosis (Vinson *et al.*, 1998). Phenolic antioxidants are good electron donors resulting in the phenoxyl radical intermediates being relatively stable due to resonance delocalization of unpaired π-electrons around the benzene ring, as well as lack of appropriate sites for an attack by molecular oxygen, as seen in Figure 3. The phenoxyl radical is formed by the reaction of a phenolic compound and a lipid radical. Exchanging the hydrogen atoms in the *ortho* or *para* positions with alkyl groups increases the electron density of the OH moiety through induction. This increases the reactivity towards the lipid radicals. The presence of an ethyl or *n*-butyl group at the *para* position, instead of a methyl group, would improve the activity of the antioxidant. Despite this, chain or branched alkyl groups in the *para* position will decrease the antioxidant activity. The phenoxyl radical is stabilized by bulky groups located at the *ortho* position due to increased steric hindrance in the vicinity of the radicals (Shahidi and Naczk, 1995a). Phenolic compounds with two hydroxyl groups in the *ortho* position are very good antioxidants (Parr and Bolwell, 2000). The phenoxyl radical, depending on its inhibitory structure, may have a lifetime of several seconds to several days. Phenoxyl radicals with free *ortho* or *para* position are able to form dimers (Gordon and Roedig-Penman, 1999).

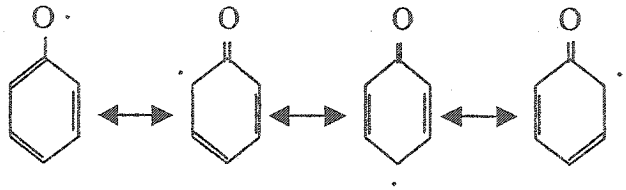

Figure 3. Phenoxyl radical stabilization.

There is an increasing interest for the recovery of natural polyphenolic compounds. Natural antioxidants in foods can be from endogenous compounds in one or more components of the food, and can be formed through reactions during processing. Conversely, they can be isolated from natural sources, such as fruits and vegetables (White and Xing, 1997). Phenolic compounds are associated with the sensory and nutritional quality of fresh and processed foods. In fruits, the sensory properties such as color, astringency, bitterness and aroma are directly related to the presence of phenolic compounds. The phenolic compounds play a role in the oxidation reactions during post harvest handling and processing of the fruits and vegetables (Lee, 1992). The amount of phenolic compounds present in the fruits or vegetables depends on the variety and season of cultivation. It has been known that fruits play an important role in diets and in the prevention of disease, which is illustrated by the age-old saying "an apple a day keeps the doctor away". There has been some research that shows that people who consume 110 grams or more apples per day have 49% lower risk of heart attacks than those who consume less than 18 grams of apples per day (Lu and Foo, 2000). Potatoes have been implicated as a source of water-soluble antioxidants, which act as free radical acceptors. Such polyphenols are glutathione, ascorbic acid, quercetin and chlorogenic acid (Al-Saikhan *et al.*, 1995). Dried tea leaves contain 10-20% (w/w) flavonoids, which accounts for its astringent properties (Sato *et al.*, 2002). Tea polyphenols are composed of (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechin gallate and (-)-epigallocatechin gallate which have antioxidant, antibacterial, antineoplastic and antiallergenic properties (Sato *et al.*, 2002). Sato and co-workers (2002) found that (-)-epicatechin gallate and (-)-epigallocatechin gallate had a protective effect against gastric mucosal lesions in rats.

Important natural antioxidants are ascorbic acid (vitamin C), α-tocopherol (vitamin E) and β-carotene (Vinson *et al.*, 1998). α-Tocopherol prevents lipid oxidation, including body lipids, polyunsaturated fatty acids, lipid components of cells and organelle membranes. Flavanones and cinnamic acid are able to act as free radical acceptors and chain breakers. Flavonols are able to chelate metal ions (Shahidi and Naczk, 1995a). Flavonoids are the most common and highly active antioxidants in food, acting as both primary and secondary antioxidants. Flavonoid glycosidase act in hydrophilic environments to scavenge radicals (Gordon and Roedig-Penman, 1999). In the food and cosmetic industries ascorbic acid and erythorbic acid are used as oxygen scavengers (Stamatis *et al.*, 1999). The action of some phenolic compounds have been identified, including isoflavonoids and phenolics interaction with the estrogen receptors in the impairment of hormone dependant cancers, such as breast cancer (Parr and Bolwell, 2000). The increased consumption of fruits and vegetables is linked to a reduced risk of degenerative diseases such as cancer, arteriosclerosis, rheumatoid arthritis, inflammatory bowel disease, cardiovascular disease, cataract formation, brain and immune dysfunction and stroke (Vinson *et al.*, 1998; Gordon and Roedig-Penman, 1999).

2.2.2. Synthetic Antioxidants

There are chemically synthesized antioxidants available, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), *tert*-butylhydroquinone (TBHQ) and propyl gallate (PG). They are highly stable, efficient and have a low cost. However, these antioxidants are suspected of being promoters of carcinogenesis (Chen *et al.*, 1992; Ho, 1992; White and Xing, 1997).

The aim of our research was to investigate the development of a biotechnological approach for the production of nutraceutical compounds. Hence, it is important to cover the literature on nutraceuticals as well as the status of the research.

2.3. Nutraceuticals

Nutraceuticals are bioactive compounds that deliver a health benefit and at the same time address a health issue that is a concern to the consumer (Witwer, 1999; Lachance, 2002). These products have physiological benefits and provide protection against chronic disease (Culhane, 1999). They may be in the form of dietary supplements that have some preventative, performance or therapeutic health benefit (Witwer, 1999). At the moment food manufactures are fortifying food products (Sloan, 1999), such as cereals, margarine, salad dressing, nutritional bars and calcium chews (Lachance, 2002). Certain biomolecules such as vitamins A, C, D, E, calcium and ω-3 are considered to be simple bioactive ingredients. Vitamin A is important in vision, growth development, reproduction and normal maintenance of epithelial tissues. Vitamin C is known to prevent colds. Vitamin D, cholecalciferol is widely present in fish and is responsible for antirachitic activity (Lall and Parazo, 1995). Vitamin E may reduce the risk of heart disease. Calcium is known to prevent osteoporosis. Folic acid reduces the risk of birth defects and may reduce the risk of heart disease (Witwer, 1999). Arachidonic acid is an essential fatty acid in human nutrition and a biogenic precursor of the biologically active prostaglandins and leucotrienes, which have significant functions in the circulatory system and central nervous systems. Arachidonic acid is also needed for visual acuity and improved cognitive development of infants after birth, therefore there is a potential for it to be incorporated as an ingredient in formulations of baby food (Medina *et al.*, 1999). There is also a connection of certain foods and a decrease in health risk such as: cranberry juice and urinary tract infections, broccoli or tomatoes and cancer, or soy and cholesterol and cancer (Sloan, 1999).

2.3.1. Nutraceutical Lipids

2.3.1.1. Benefits of Nutraceutical Lipids

Menhaden fish oil is considered a nutraceutical bio-ingredient. It has little functional value but it has medicinal and nutritional links due to its long chain polyunsaturated ω-3 fatty acids. It has received GRAS standing for the use in foods by the FDA in 1997. Supplements of ω-3 was recommended by substituting lipids commonly consumed with products rich in ω-3 fatty acids. Fish oil is commercially sold

as a nutraceutical product, without a medical prescription (Davidson *et al.*, 1991). These are available in the United States, Canada and European countries (Shahidi and Wanasundra, 1998). Capsules of fish oil that are rich in vitamins A and D are also commercially sold (Lall and Parazo, 1995). The supplement capsules usually contain fish liver oil, from cod or halibut. In order to receive the required dose there is a risk of vitamin A and D overdose, which may lead to toxicity and an increase in cholesterol and other saturated fatty acids; this has led to concentrated forms of ω-3 fatty acids being produced. These can be in the form of modified triacylglycerols, free fatty acids or as alkyl esters which could be either methyl or ethyl esters. These marine oil concentrates, which do not have saturated or monounsaturated fatty acids, are better than the original oil. This is due to the fact that they can keep the daily intake of total lipids low, which would make the concentrated forms of ω-3 ideal for pharmaceutical and food applications (Shahidi and Wanasundra, 1998), such as artificial milk formulas for infants (Poisson and Ergan, 2001). However, the polyunsaturated fatty acids are considered nutritionally more favorable than methyl esters or ethyl esters; experimental work showed that there was an altered intestinal absorption in laboratory animals with methyl and ethyl esters (Hamazaki *et al.*, 1982). Commercially, the triglycerides with polyunsaturated fatty acids rich in ω-3 are promoted as natural and the others as derivatives of fatty acids.

Medina *et al.* (1999) used polyunsaturated fatty acids from microalga to produce triglycerides that are rich in polyunsaturated fatty acids. The triglycerides from cod liver oil were rich in EPA and DHA, microalga *P. tricornutum* was rich in DHA whereas, *P. cruentum* was rich in DHA and AA. The lipase Novozym 435 easily synthesized triglycerides with high concentrations of EPA, DHA and AA, with yields of over 95%.

Evening primose oil is a commercial source of γ-linolenic acid (GLA, 18:3 ω-6), which is an essential fatty acid and a precursor to homo-γ-linolenic acid and AA (20:4 ω-6). GLA has the ability to increase the tissue biosynthesis of 1-series prostaglandins and plays an important role in the treatment of diabetes, hypertension, thromboembolic disease, atopic eczema and regulation of inflammatory response. The essential ω-6 fatty acids must come from the diet since it cannot be synthesized in the body. However, excess amounts of ω-6 fatty acids can cause vasoconstriction and platelet aggregation.

On the other hand, ω-3 polyunsaturated fatty acids compete with the ω-6 polyunsaturated fatty acid so that it may reverse some of the adverse effects of ω-6 (Akoh *et al.*, 1996). Akoh and co-workers (1996) used immobilized lipase SP 435 from *Candida antarctica* for the modification of the fatty acid composition of evening primose oil by incorporating EPA, through a transesterification reaction, which resulted in an 43% increase in EPA and a 32% reduction of linoleic acid (C18:2 ω-6) in the evening primose oil.

2.3.1.2. Structured Lipids

Structured lipids are triacylglycerols with specific fatty acids on fixed positions on the glycerol molecule (Iwasaki *et al.*, 1999). Lipid modification is used in improving the nutritional quality of fats and oils (Willis and Marangoni, 1999). Structured lipids are important from the textural point of view in the manufacturing of plastic fats, such as margarine, modified butter and shortening. Structured lipids are of nutritional and pharmacological importance due to the ability to synthesize triglycerols and phospholipids with a defined structure that can target a specific disorder or improve health (Marangoni *et al.*, 1993).

Structured lipids with medium chain fatty acids at the *sn*-1 and *sn*-3 position and polyunsaturated fatty acids at the *sn*-2 position are capable of improving nutritional and pharmaceutical properties. The *sn*-2 position on the monoacylglycerol is important since it can be absorbed through the intestines (Iwasaki *et al.*, 1999). Structured lipids have favorable effects on immune function, nitrogen balance and a better lipid clearance from the blood stream. The long chain fatty acid monoacylglycerols get absorbed directly. This means that the fatty acids at the *sn*-2 position are more efficiently used. These structured lipids are quickly hydrolyzed by the pancreatic lipase and absorbed into the mucosal cells. The structured lipids can then be used to treat lipid malabsorption (Xu *et al.*, 2000b). Structured lipids with DHA have beneficial effects on visual and auditory performance, brain, liver and adipose tissue lipid and fatty acid profiles (Xu *et al.*, 2000b).

Paez and colleagues (2002) used cod liver oil and caprylic acid to enzymatically produce structured lipids with immobilized Lipozyme IM (*Rhizomucor miehei*). Iwasaki

et al. (1999) used lipase from *R. miehei* and *Pseudomonas* sp. to catalyze the acidolysis of a single cell oil of DHA and decosapentaenoic acid (DPA) with caprylic acid; producing structured lipids containing caprylic acid at the *sn*-1 and *sn*-3 positions and DHA or DPA at the *sn*-2 position of the glycerol molecule. Kim and co-workers (2001) produced structured lipids by the esterification of tricaprylin and conjugated linoleic acid in *n*-hexane medium; of the three lipases tested, they found Novozym 435 (*C. antarctica*) to be the most effective, with 56.9% incorporation. Xu *et al.* (2000a) produced structured lipids from canola oil by enzymatic acidolysis with the use of 1,3 specific lipase, Lipozyme IM from *R. miehei*. Xu *et al.* (2000b) used menhaden oil and medium chain fatty acids to form structured lipids. Menhaden oil has high concentrations of EPA and DHA mostly on the sn-2 position of the triacyglycerol. The structured lipids were produced by enzymatic acidolysis with Lipozyme IM since it is *sn*-1,3 specific and it has weak activity on ω-3 long chain fatty acids.

Butterfat contains hypercholesterolemic fatty acids, such as myristic (C14:0) and palmitic (C16:0) at the *sn*-2 position. Esterification can improve the nutrition quality of butter fat by changing those fatty acids with oleate, a hypocholesterolemic fatty acid (Safari and Kermasha, 1994; Pabai *et al.*, 1995). Pabai *et al.* (1995) concluded that it was feasible to use *Aspergillus niger* for the interesterification of butter fat in a co-surfactant free or microemulsion system.

Caprenin, a commercially available structured lipid has half the calories of a naturally occurring triacylglycerol (McNeill and Sonnet, 1995). It has similar fat properties to cocoa butter with half the calories (Marangoni *et al.*, 1993). This is due to incomplete absorption during digestion. This structured lipid is composed of one molecule of behenic acid (C22) and two molecules of either caprylic (C8) or capric (C10) acids. The synthesis reaction consists of monobehenin and the free acids reacting at high temperatures or, a synthesis of monobehenin and a reactive anhydride form of the fatty acids reacting at lower temperatures. Caprenin can also be synthesized with monoerucin and caprylic acid as starting materials, which was then converted to caprenin by hydrogenation of the erucic acid esters (McNeill and Sonnet, 1995). McNeill and Sonnet (1995) tested three lipase preparations from nonspecific lipase *P. cepacia,* specific lipases

Geotrichum candidum and *C. rugosa*. The best one was *G. candidum*, which produced a small amount of transesterification of erucic acid, 75% yield of caprenin and 10% of interesterification products. Cocoa butter equivalents can also be produced through the interesterification of palm oil mid fraction. The interesterification reaction is used to add stearates, either in the form of a free acid or an ester into the palm oil mid fraction. The fatty acids at the *sn*-1,3 positions get interesterified with the use of a 1,3-specific lipase. The main component of the palm oil mid fraction is 1,3-dipalmitoyl-2-oleoyl-glycerol which undergoes interesterification to produce is 1-palmitoyl-2-oleoyl-3-steroyl-glycerol and 1,3-distearoyl-2-oleoyl-glycerol. This resulting fat has a triacylglycerol composition that is similar to that of cocoa butter, which can be used as a cocoa butter equivalent for the chocolate and confectionery industry (Bloomer *et al.*, 1990).

Salatrim is another low calorie fat that is produced by the interesterification of short chain (C2-4) and long chain (C16-22) fatty acids into a triacyglycerol. Salatrim may be composed of two short chain and one long chain triacylglycerol or one short chain and two long chain triacylglycerol. *Carica papaya latex* has short chain specificity and was used to interesterify triacetin with hydrogenated soybean oil to produce a low calorie triacylglycerol (Manogs *et al.*, 1999). Yankah and Akoh (2000) enzymatically produced structured lipids with stearic acid (C18:0) at the *sn*-2 site as a low calorie fat. The esterification of tristearin with caprylic acid or oleic acid with Lipozyme IM 60 from *R. meihei* in *n*-hexane medium incorporated caprylic acid at a higher rate than oleic acid. These low calorie fats can be used in baking, for chips coatings, dips, baked products or cocoa butter substitutes (Mangos *et al.*, 1999).

The fatty acid composition has an influence upon the stability and nutritional value of edible oils. Monounsaturates and polyunsaturates can replace the saturated fatty acids as part of a cholesterol lowering diet. Moussata and Akoh (1997) modified the fatty acid composition of melon seed oil through the incorporation of oleic acid (C18:1 ω-9) with the use of lipase PS30 (*Pseudomonas* sp.). This biocatalysis produced an increase in the oleic acid content, from 13.5 to 53% and a decrease in the linoleic acid (C18:2 ω-6) content from 65 to 33%. Therefore, the modification through the increase in oleic acid was used to increase its oxidative stability and nutritive value; since it is already rich in

linoleic acid, the addition of oleic acid will balance the fatty acid profile of the oil. Jennings and Akoh (1999) created a structured lipid consisting of fish oil and capric acid with the use of *R. miehei* as the biocatalyst; the resulting structured lipid was found to be nutritionally more beneficial than the original unmodified fish oil by combining the effects of high energy and health benefits.

Interesterification is also used as an alternative to hydrogenation for vegetable oil-based margarines, since interesterification does not produce the *trans* fatty acids, which are implicated in coronary heart disease, myocardial infraction and possibly breast cancer. *Trans* fatty acids seem to increase the LDL cholesterol and decrease the HDL cholesterol. Interesterification improves the melting and crystallization properties as well as providing a better texture, creaminess and stability of vegetable oils for the production of margarine (Marangoni *et al.*, 1993).

2.3.2. Lipophenols Obtained by Esterification of Lipids with Phenolic Compounds

Lipophenols are excellent nutraceutical products since they have the health effects of the fatty acids and the antioxidant activity of the phenolic compounds. Enzymatic esterification of hydrophobic and hydrophilic derivatives of phenolic acids with aliphatic alcohols can easily synthesize lipophilic antioxidants (Stamatis *et al.*, 2001). The enzymatic esterification depends on the phenolic structure; phenolic compounds that contain a primary hydroxyl group have a better enzymatic esterification than those with acid functional groups (Buisman *et al.*, 1998). The phenolic acids have low stability and solubility in different solvent systems, which reduces their antioxidant efficiency in stabilizing oils and fats. (Stamatis *et al.*, 2001). The esterification of the phenolic acids with fatty alcohols can change their solubility making them more useful as food antioxidants for oil based formulations and emulsions. Buisman and co-workers (1998) suggested that the esterification could be used as a tool to alter the physical properties, like solubility and miscibility in the formulation of antioxidant emulsions and food products. Phenolic compounds can be converted to esters by reactions with acid chlorides and acid anhydrides, but these reactions do not meet with the requirements of the food industry (McMurry, 1984).

Guyot *et al.* (1997) enzymatically esterified with lipase *C. antarctica*, phenolic acids and fatty alcohols. The yield of lipophenols depended on the time of reaction; high yields were observed with reaction times of two days or more, with yields varying from 3 to 98%. There was also a dependence on the electronic distribution of the phenolic acids, which affected the reactivity of the carboxilic moiety. In 2000, Guyot and co-workers synthesized fatty acid esters of 5-caffeoyl quinic acid by esterification catalyzed by *C. antarctica*; the esterification yields ranged from 40 to 75%, and were dependant on the carbon chain length of the fatty alcohols and the solvent. Buisman *et al.* (1998) reported that the esterification of polyphenols with octanoic acid after 15 hours by lipase *C. antarctica* resulted in the biosynthesis of lipophenolic antioxidants, which contain a primary hydroxy group and a aliphatic acid with a 85% yield. The product obtained was purified and used as a natural antioxidant in sunflower oil. Stamatis *et al.* (1999) esterified cinnamic acid derivatives and ascorbic acid with various chain length alcohols by lipase *C. antarctica* and *R. miehei*. The same authors reported that esterification of *p*-hydroxyphenylacetic acid catalyzed by *C. antarctica* had a 97% yield, whereas the esterification of cinnamic acid with *R. miehei* had a yield of 59% and a 65% yield of ascorbic fatty acid esters was produced after 24 h of incubation with *C. antarctica*. The reaction rate and the yield depended on the fatty acid chain length, the aromatic ring structure of the phenolic molecules and the molar ratio of the reactants. The resulting lipophenols had antioxidant activity in a micellar substrate composed of linoleic acid. In 2001, Stamatis and co-workers esterified natural phenolic antioxidants with aliphatic alcohols, monosacharides and alkylglucosides. The rate of reaction and the esterification yield appeared to be related to the structural characteristics of the substrates.

Compton and King (2001), used Novozym 435 to transesterify ethyl ferulate (ethyl 4-hydroxy-3-methoxy cinnamate) with triolein to produce monoferuloyl-monooleoyl-glycerol and feruloyl-dioeoyl-glycerol. These compounds are ultraviolet absorbing lipids, which are used in the production of all natural sunscreens. They absorb ultraviolet radiation at 290 to 375 nm due to the ferulyl moiety and the hydrophobicity of the lipid.

In order to demonstrate the bioconversion by lipases of lipids and phenolic compounds into the novel biomolecules, lipophenols, it is important to report on the methodologies used for their separation and characterization.

2.4. Characterization of Lipophenols

2.4.1. Thin Layer Chromatography

Thin layer chromatography separation is usually carried out on a glass plate with a layer of silica gel absorbent. A sample spot is applied to the gel slightly above where the level of the solvent will be. The lower edge of the plate is then immersed in the developing solvent, which is enclosed in a sealed glass tank. The sample fractions then migrate upwards by capillary action (Jänchen, 1984). The separated fractions may then be visualized by the eye if the spots are naturally colored or by UV if the gel contains phosphor that fluoresces at 254 nm. The plates may also be visualized by reacting them with reagents (Sherma, 1984), such as iodine vapours (Guyot *et al.*, 2000). Guyot *et al.* (1997) used TLC to visualize lipophenols on silica plates (60 F254) with a layer thickness of 0.25 mm. The migration solvents used were chloroform/methanol/acetic acid (63:2:1, v/v/v). Later, Guyot *et al.*, (2000) used methyl isobutyl ketone/formic acid/ water (3:1:2, v/v/v) as migration solvents. Buisman *et al.* (1998) used the same silica plates (60 F254, 0.25 mm) on aluminum to visualize the lipophenols with eluting solvent mixtures of diethyl ether/formic acid (4:1 v/v) or diethyl ether/PE (40-60)/formic acid (40:10:1). Stamatis *et al.* (1999) used the same TLC plates, but with solvent mixtures of chloroform/methanol/acetic acid (96:3:1, v/v/v) or chloroform/methanol/acetic acid/water (80:10:8:2, v/v/v/v), to visualize the phenol esters.

2.4.2. High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) uses a mobile solvent phase in order to pass the sample through a stationary phase in a column for separation. Non polar columns are used for lipid analysis. Such columns are polymer or carbon based (C18) and require polar solvents for the elution, such as water or methanol. This type of separation is good for samples with different functional groups rather than their chain lengths. The dominant interaction for this type of absorbant is the dispersion forces. The non polar molecules will be retained by the absorbant more than the polar molecules

(Swell, 1992). The wavelength of detection depends on the sample absorbance. Guyot *et al.* (1997) performed HPLC analysis with a RP-18 column and the phenolic acid esters of alcohols were detected at 280 nm. In 2000, Guyot and co-workers used the same column to separate 5-caffeoyl quinic esters (an ester of caffeic acid) of fatty acids which where detected at 324 nm. Conversely, when Stamatis *et al.* (1999; 2001) performed HPLC analysis with a Nucleosil C_{18} column for the separation of lipophilic antioxidants produced through the esterification of phenols and fatty acids, the esterification products were detected at 280 nm.

2.4.3. Gas Chromatography

Gas chromatography (GC) uses a mobile gas phase to pass the sample through a capillary column that contains a thin film of stationary liquid phase. Samples are usually methylated to increase the volatility. Separation occurs when the sample dissolves in the liquid phase and travels through the column by the carrier gas. Components that are more strongly attracted to the stationary liquid phase take a longer time to emerge from the column than those that are not as attracted to the liquid phase (Jennings, 1984). Detection may be carried out on a flame ionization detector (FID) or by mass spectrometry. Buisman *et al.* (1998) analyzed lipophenols by GC on a CP Sil CB-MS column (25 m x 0.25 mm i.d.) with a FID detector. The samples were either acetylated or methylated with distilled diazomethane to increase the volatility.

2.4.4. Fourier Transmission Infrared Spectrometry

Fourier transmission infrared (FTIR) spectrometer measures the interference pattern generated by a two-beam interferometer. The interferometer can split a beam of radiation into two separate paths and then recombine them after a certain path distance. The recombined beams may have interferance, found by a detector. The spectra is called an interferogram (Griffiths and de Haseth, 1986). Guyot *et al.* (1997; 2000) monitored the ester formation from the esterification of phenolic acids and fatty alcohols by FTIR spectrometry. The spectra was obtained by transmission on a KBr disk using a Mattson 3000 FTIR spectrometer for characterization. The IR spectum was different than that of the acid, and there was a formation of a new band. Stamatis *et al.* (2001) also used IR to determine the presence of the ester bond on the phenolic acid esters.

2.4.5. Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) measures the absorption of radio-frequency radiation by the nuclei of the sample that is positioned in a magnetic field. In a stable field, the absorbance depends on the nuclei frequency of radiation. The absorbance is based on the atomic characteristics and the position relative to other nuclei in the molecular structure or sample matrix (Horman, 1984). Buisman *et al.* (1998) used NMR to analyze the spectra of phenolic esters, with a 300 MHz spectrometer using tetramethylsilane (TMS) as internal standard in chloroform-*d*. Guyot *et al.* (2000) used NMR for identification of the synthesized products with a spectrometer, which was fitted with a proton carbon probe. Stamatis *et al.* (1999; 2001) used ^{13}C-NMR to determine the chemical structure of the phenol esters in a CD_3OD on a Varian-300 MHz spectrometer.

The biotechnological approach used for the generation of lipophenols is based on the use of lipases as biocatalysts. It is of great importance to describe the lipases in terms of their mode of action and specificity.

2.5. Lipase

2.5.1. Introduction

Lipases are enzymes that are capable of hydrolyzing triglycerides, diglycerides and monoglycerides (Kilara, 1985). Lipases are known as carboxylic ester hydrolases or glycerol ester hydrolases. The international name for lipases is triacylglycerol acylhydrolase lipase (EC 3.1.1.3) (Taipa *et al.*, 1992). They are water-soluble and act on water insoluble lipid substrates at the oil-water interface (Verger *et al.*, 1990; Lee and Akoh, 1996).

Lipases are essential for the metabolic reactions of lipids, since triglycerides are the main energy reservoirs for higher animals (Verger *et al*, 1990). Lipases are used in the production of cosmetics, detergents, leather and perfumes (Gandhi, 1997). They are also important in biotechnology, clinical medicine, pharmacology, nutrition and oil technology (Verger *et al*, 1990). The use of lipases in the food industry is increasing due to food related lipid modifications for the production of esters, biodegradable polyesters and fatty acids (Linko *et al.*, 1994). Lipases are gaining in popularity in the

pharmaceutical industry due to their ability to produce enatiomerically pure compounds (Indlekofer *et al.*, 1995).

Lipase-catalyzed reactions occur under milder reaction conditions of temperature, pressure and pH than chemical esterification (Chaplin and Bucke, 1990; Zhang and Haynes, 1999; Xu *et al.*, 2000a; Gunstone, 2001). The enzymes are capable of producing products that are very difficult to obtain by chemical methods (Roberts, 1990; Gunstone, 1999).

2.5.2. Sources of lipases

Lipases come from a wide variety of sources. They have been isolated from animals, plants and microorganisms (Xu *et al.*, 2000a; Verger *et al.*, 1990). Some examples of the lipases found in animals are lingual, gastric and pancreatic lipases. Plants such as wheat germ and castor beans also contain lipases. Microorganisms such as yeasts, molds and bacteria are also sources of lipase. Examples of microbial sources determined to contain lipases are the yeasts, *Candida*, *Torulopsis*, the molds *Rhizopus*, *Penicellium*, *Aspergillus*, and *Geotrichum* and bacteria such as, *Achromibacter* and *Staphylococcus*. Microbial lipases have been determined to be extracellular enzymes (Kilara, 1985).

2.5.3. Specificity of Lipases

There are two types of lipases, nonspecific and regiospecific. Lipases that have the ability to act in a random fashion are termed nonspecific lipases, as seen in Figure 4A (Thomson *et al.*, 1999). These lipases have no specificity towards the position on the glycerol molecule or the nature of the fatty acid released (Macrae, 1983). Regiospecific lipases attack specific sites on the triacylglycerol molecule, namely the *sn*-1,3 specific lipases as seen in Figure 4B (Thomson *et al.*, 1999). The *sn*-1,3 specificity seems to be due to the steric factors in the alcohol moiety. This causes the inability to access the *sn*-2 carbon ester to the active site of the lipase. For lipase reactions, the steric factors outweigh the electronic ones (Marangoni *et al.*, 1993). Lipase reactions are reversible; there is a hydrolysis and resynthesis of glycerides when lipases are incubated with oils and fats (Macrae, 1983).

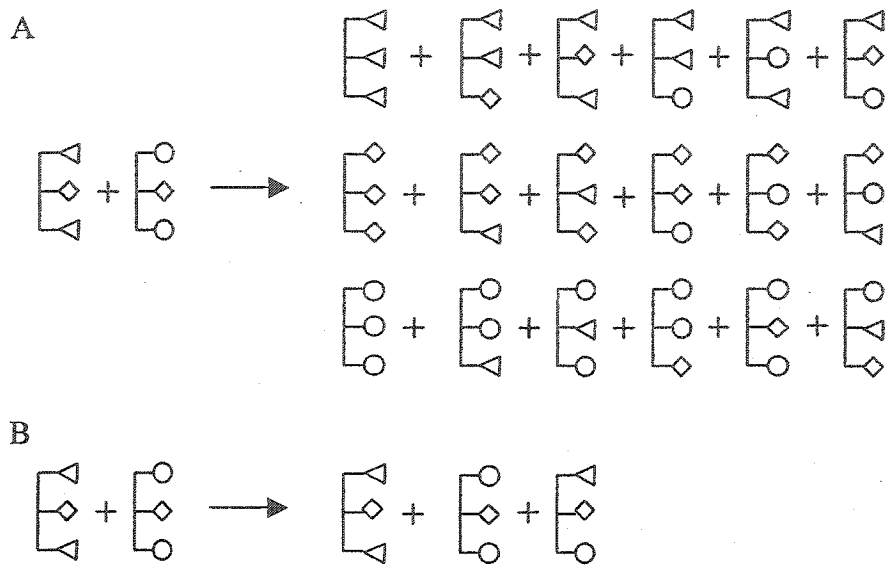

Figure 4. Interesterification of triglyceride molecules, (A) is the action of non specific lipase and (B) is a 1,3 specific lipase, where ◇ ,◁ ,○ , are three different types of fatty acid molecules.

These reactions also have stereoselectivity, where one stereoisomer or an enatiomeric pair of stereoisomers is formed or destroyed preferentially over all others (Brown, 1995). Lipase catalyzed reactions may have fatty acid substrate and hydrolytic positional specificity (Willis *et al.*, 1998; Zhang and Haynes, 1999; Xu *et al.*, 2000a), depending on the source of the lipase. The positional specificity remains even in organic media (Bloomer *et al.*, 1990). Reactions can be catalyzed without any side reactions therefore eliminating undesirable by-products. The generated product is in a pure form without any contamination (Chaplin and Bucke, 1990). Lipases use 'natural' reaction systems to produce 'natural' products (Xu *et al.*, 2000a).

2.5.4. Mechanisms

2.5.4.1. Hydrolysis

Lipases are used as catalysts in the hydrolysis of fats and oils to produce free fatty acids, monoglycerides, diglycerides and glycerols, as seen in Figure 5 (Macrae, 1983). Enzymatic hydrolysis takes place at ambient or moderate temperatures and pressure. This allows for the hydrolysis of fatty acids from unstable oils, such as conjugated or

polyunsaturated fatty acids (Gandhi, 1997). Lipases are able to hydrolyze large amounts of insoluble fatty acid esters with glycerides being the preferred substrate (Macrae, 1983). The hydrolysis reaction replaces the fatty acids with hydroxyl groups on the glycerol backbone. The hydrolysis reaction is preferred when there is excess water present (Willis and Marangoni, 1999).

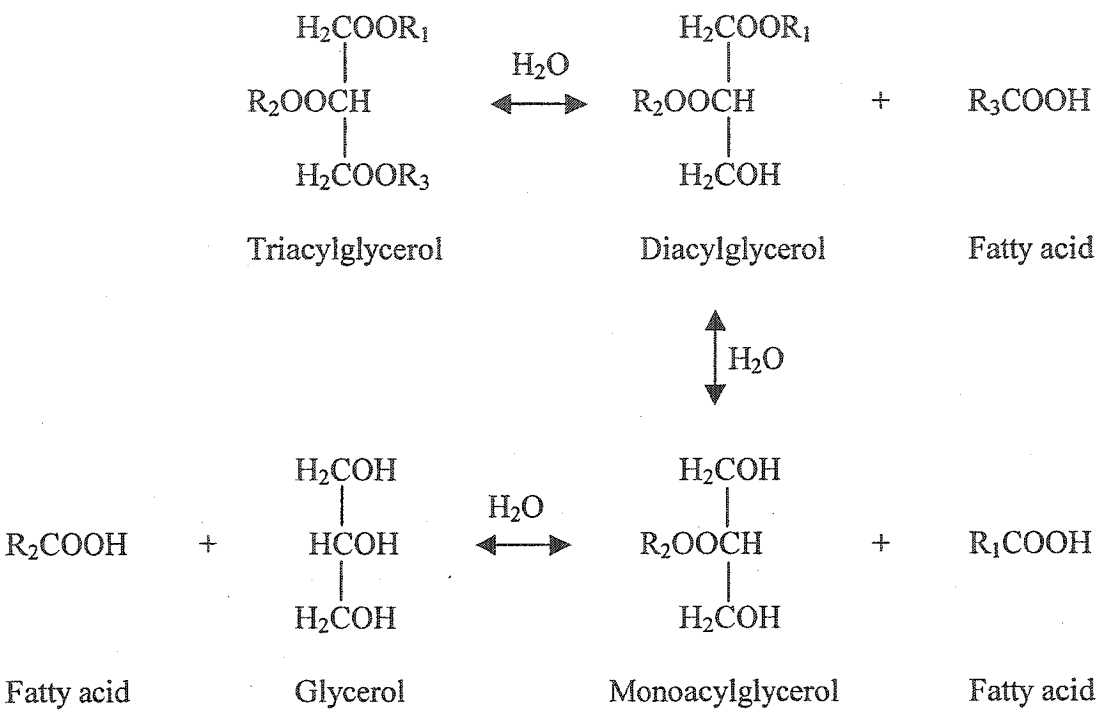

Figure 5. Enzymatic hydrolysis of a triacyglycerol.

2.5.4.2. Esterification

Esterification is the reverse reaction of hydrolysis (Willis and Marangoni, 1999), synthesizing the selected product (Gandhi, 1997). It is a reaction between an acid and alcohol, to produce an ester and water, as seen in Figure 6 (Gunstone, 1999). Esterification is dependent on the water present in the reaction system. A small quantity of water will promote esterification (Willis and Marangoni, 1999).

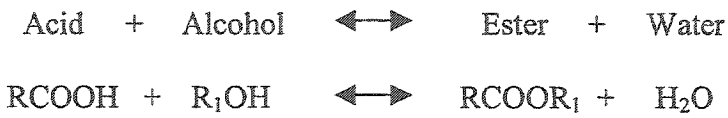

Figure 6. Reaction of esterification by lipase.

2.5.4.3. Interesterification

Interesterification is an acyl exchange between two molecules. The exchange occurs between an ester and an alcohol, and is also called alcoholysis (Linko *et al.*, 1994). It is a process of hydrolysis and esterification. The hydrolysis of the triacylglycerol molecule is followed by a resynthesis of the liberated fatty acids onto the glycerol molecule. There is a redistribution of free fatty acids between the glycerides. The interesterification reaction produces all possible combinations of glycerol and fatty acids (Sreenivasan, 1978). The random distribution of acyl residues on the glycerol molecule was displayed in Figure 4A. The reaction is dependant on the amount of water present in the system; as with esterification, a small quantity of water will promote interesterification (Macrae, 1983).

2.5.4.4. Transesterification

Transesterification occurs when an acyl moiety is exchanged. The exchange may occur between an ester and an acid (Fig. 7) called acidolysis. Likewise, there may be an exchange between an ester and an alcohol called alcoholysis. Ester synthesis is favored with low water activity (Linko *et al.*, 1994).

Most enzyme catalysis are reported to act in aqueous media. However, the biocatalysis of enzymes in non-conventional media, particularly organic solvent, represents a novel approach for their use in industrial applications. It is therefore important to describe the approach as well as the technologies used for the enzyme biocatalysis in organic solvent media.

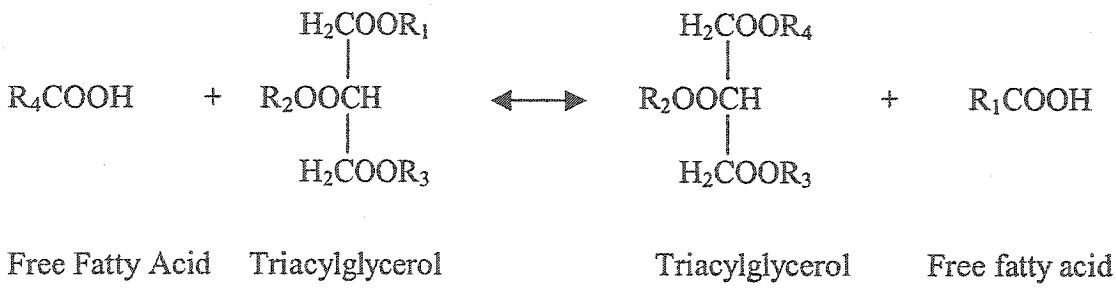

Figure 7. Transesterification reaction catalyzed by lipase.

2.6. Biocatalysis in Organic Solvent Media

Organic solvent media has an increased stability over an aqueous media. Organic solvents have the ability to lower the inhibition effects due to a reduced product concentration around the enzyme, resulting in a better solubility of the product in the bulk organic phase rather than the hydrophilic microenvironment around the enzyme. Molecules that are insoluble in water are easily catalyzed in organic solvents (Frings *et al.*, 1999).

The advantages of using organic solvents as the biocatalysis media include increased solubility of many non-polar compounds in organic media. The synthesis reactions are dominant in non-aqueous media. There is a reduction in undesirable water dependent side reactions as well as product and substrate inhibition in organic media (Laane *et al.*, 1987). Ease of recovery of both product and biocatalyst from organic phase systems is also an advantage. There is an elimination of microbial contamination in organic media that occurs in aqueous media (Dordick, 1989). Organic solvent media is useful when the reactants or products of the reaction are hydrophilic compounds. Also, when low water content in the reaction media is preferred, as in the case of esterification (Kermasha *et al.*, 1995), it shifts the thermodynamic equilibrium towards the esterification reaction (Tweddell *et al.*, 1998). The main disadvantages are that the biocatalyst can be deactivated by protein denaturation or inhibited by the organic solvent (Brink *et al.*, 1988; Isono *et al.*, 1995). The organic media reaction system is more complex than the aqueous one (Brink *et al.*, 1988).

2.6.1. Effect of the Environment on the Activity of the Lipase

Normally, most enzymes are water-soluble proteins that are found in intracellular environments comprised of aqueous media at physiological temperature and pH (Shield *et al*, 1986). The environment of many enzymes or multienzyme complexes also function in hydrophobic media, such as lipases, esterases, dehydrogenases and those that are responsible for xenobiotic metabolism. They act in the presence of a membrane and can be catalytically active in organic media (Dordick, 1989). Some lipases can maintain their stability and high activity in organic solvents (Nakamura *et al.*, 1995).

2.6.1.1. Enzyme Stability

The stability of the enzyme in a hydrophobic system is important. Some molecular characteristics contribute to its conformational stability. These characteristics include disulfide bridges, hydrophobic interactions, hydrogen bonding and electrostatic interactions. Not all of these forces contribute to the conformational stability in aqueous solutions as they would in non-aqueous solvents. The decrease in activity may be due to minute changes in the structure of the active site (Vazquez-Duhalt *et al.*, 1992). Enzymes act as biocatalysts in nearly all anhydrous organic solvents. The water needed for enzymatic activity is bound tightly to the enzyme molecule and remains bound even when the bulk water is replaced with organic solvent (Kermasha *et al.*, 1995). A thin monolayer of water around the enzyme is necessary to preserve its three dimensional shape which will allow it to retain its catalytic activity. The water present may be measured as water concentration and as thermodynamic water activity (a_w) in the organic phase. Water concentration or content is the total water present in the system. The water may be present in several different phases; it may be bound to the enzyme molecule, dissolved in the organic media phase, adsorbed by the solid components, such as the immobilization support materials or in the vapor phase (Bell *et al.*, 1997). The thermodynamic a_w is defined as the water vapor pressure over the solution divided by the water vapor pressure over pure water. When all the phases, (such as reaction medium, enzyme preparation and headspace in the reactor) are in equilibrium, the a_w is the same in all the phases (Fureby, 1995). Therefore, water bound to the enzyme or the hydration of the system is best described by the thermodynamic a_w. Each enzyme has a unique water

requirement (Dordick, 1989; Gandhi *et al.*, 2000), necessary for its active conformation (Wehtje *et al.*, 1997). In general, catalytic activity increases with increased a_w, since water increases protein flexibility by the creation of multiple hydrogen bonds with the enzyme in organic solvent systems (Persson *et al.*, 2002). Some enzymes show an increase in activity as the a_w increases, while others may have bell shaped water activity profiles (Persson *et al.*, 2002). However, a small amount of water may increase the a_w of the enzyme to favour the hydrolysis reaction (Chamouleau *et al.*, 2001). Reactants or products present in significant amounts may also have a role in the solvation of water and on the a_w, especially for non-polar solvents (Voutsas *et al.*, 2001). Non-polar solvents like hexane and isooctane cannot contain large amounts of water and are therefore unable to strip the water from the enzyme (Gorman and Dordick, 1992). Partridge *et al.* (1998) suggested that the solvents do not directly affect the enzyme water interactions at a fixed a_w.

2.6.1.2. Nature of the Organic Solvent

The organic solvents can have an effect on the enzymatic activity and the stereoselectivity of the reaction. The bulk solvent has an effect on the polarity or hydrophobicity. The enzymatic activity of non-aqueous systems correlates with the hydrophobicity index, the log P value ($P = [Solute]_{octanol}/[Solute]_{water}$). The log P is the logarithm of the partition coefficient of solvent in 1-octanol and water systems. Solvents with a log P value of less than 2 are unfavorable enzymatic systems due to the strong distortion of the water-enzyme interaction, which is fundamental for activity. Solvents with a log P value between 2 and 4 have the ability to distort the water-enzyme interactions and affect the enzymes in an unpredictable way. However, solvents with log P values over 4 are unable to distort the water-enzyme interactions, indicating the enzyme is in an active form available for ester synthesis (Laane *et al.*, 1987; Vazquez-Duhalt *et al.*, 1992; Yang *et al.*, 1994).

The structure of the solvent influences the activity and the stereoselectivity of the lipase. The solvent can have enantioselective inhibition on the lipase depending on the shape of the solvent molecule (Nakamura *et al.*, 1995). Nakamura *et al.* (1995) discovered that the enantioselectivity of a solvent is higher in a structurally linear solvent

than a branched chain solvent. The enantioselectivity decreases with the increasing ring size of the solvent molecule. The lipase can recognize the structure of its substrate and the solvent.

2.6.2. Selected Systems Using Organic Medium

2.6.2.1. Monophasic

Monophasic organic solvents systems lack a distinct aqueous phase and involve insoluble enzyme preparations in nearly anhydrous media or in organic aqueous systems where the main component is the organic phase. In a monophasic organic system the enzymes are not in direct contact with the bulk water phase. There are several applications for monophasic systems, such as solubility improvements, suppression of hydrolysis, improved thermal stability, and a change in substrate specificity (Dordick, 1989).

2.6.2.2. Biphasic

Biphasic systems consist of water and water immiscible solvent, usually in the form of reverse micelles or microaqueous systems. In a biphasic or reverse micellar system, the enzyme is solubilized in water, consisting of the aqueous phase. In a microaqueous media the enzyme is solubilized in the organic solvent. The lipase activity is dependent on the type of organic media (Tweddell *et al.*, 1997). The biphasic system provides a better media for the lipase since it has a water/solvent interphase that allows for the enzyme to be in the proper conformation. Biphasic systems of 10-20% water allow for better interesterification rates (Tweddell *et al.*, 1998).

2.6.2.3. Microemulsion System

A microemulsion is a monophasic dispersed system consisting of a liquid phase of oil, water and a surfactant. It is transparent, stable and isotropic, both thermodynamically and kinetically (Abe *et al.*, 1986; Larsson, 1994). The system can contain any phase volume ratio of two immiscible liquids. The microemulsion forms spontaneously with the introduction of the two components (Abe *et al.*, 1986). The microemulsion system has a large interfacial area and a very low interfacial tension (Kermasha *et al.*, 1995).

Microemulsions have droplet diameters in the order of 10 to 250 nanometers in size (Attwood and Florence, 1983).

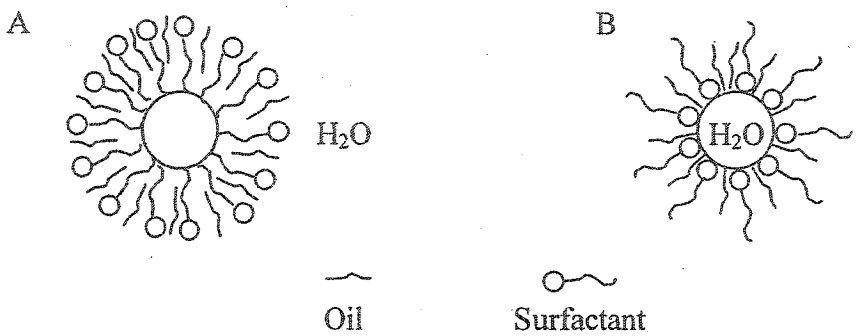

Figure 8. Microemulsion structure in a (A) microemulsion, and a (B) reverse microemulsion.

Microemulsions can be of the oil in water (microemulsion) or water in oil (reverse microemulsion) type, both have low viscosities (Fig. 8). The dispersed phase usually has characteristic lengths of 10^2 Å (Abe *et al.*, 1986). The microemulsion system has an interfacial film. This separates the aqueous phase from the oil phase, which is where the lipases act (Bellocq, 1996).

2.6.2.4. Reverse Micellar System

Reverse micellar systems are homogeneous, thermodynamically stable and optically transparent. The reverse micellar system is formed spontaneously when dissolved surfactants and a small quantity of water is present in an apolar solvent. The reverse micellar stucture has small water droplets, in the order of nanometers that are stabilized by a monolayer of surfactant molecules. The polar heads of the surfactant molecules are facing the interior water pool and the hydrophobic tails of the surfactant are in the continuous organic phase (Laane *et al.*, 1987; Walde and Luisi, 1990). This environment allows for an aqueous phase for the hydrophilic enzyme, an interface for the surface-active enzyme and an organic phase for the hydrophobic substrates or products, as seen in Figure 9 (Creagh *et al.*, 1993). This means that the enzymes are protected from the nonpolar environment by a layer of water and the surfactant shell (Han and Rhee, 1986). This system contains less than 5% water (Walde and Luisi, 1990). Within the 5% of water in this system, it is still possible to solubilize hydrophilic molecules, like

enzymes (Kermasha *et al.*, 1995). Some of the water solubilizes the hydrophilic head groups of the surfactant and this allows for the water to be tightly bound to the enzyme. The water in this intracellular environment is less mobile than the bulk water (Shield *et al.*, 1986).

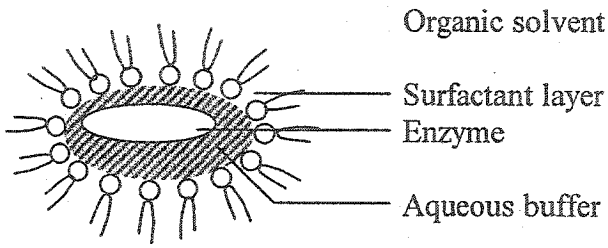

Figure 9. Enzyme in a reversed micelle.

The reversed micellar system allows for proteins to remain in their preferred conformation. Enzymes with hydrophobic regions can position themselves in the surfactant layer, so that they are in their native conformation. Hydrophilic enzymes can be centered in the hydrophilic microenvironment, without the bulk organic layer affecting them (Shield *et al.*, 1986).

3. THESIS RATIONAL

There is an increasing interest in the production of natural products. The food industry can proceed with biotechnological conversion of endogenous products and by-products of the food industry into bio-ingredients and value-added nutraceutical products.

Almost 60% of the fish weight is rejected during processing, of which 15% corresponds to low quality fish oil (Nadeau, 1992). The low quality oil is already separated by the fish processing plants and is easily obtained. This oil is usually sold at a low price, which can be directly exploited for the production of high value–added natural bioingredients by enzymatic bioconversion. Fish oil contains 14-30% of important polyunsaturated fatty acids from the omega-3 family, namely eicosapentaenoic (C20:5ω3) and docosahexaenoic (C22:6ω3) acids (Rocquelin, 1990; Willis *et al.*, 1998; Jennings and Akoh, 1999).

In addition, Quebec apple industry generates about 8,000 to 9,000 tones of wastes per year (Anonymous 1, 1999) whereas that of potato underutilizes 5-20% of the total production, which is estimated at 350 000 tones, where a small portion (5%) of these waste materials is used for animal feed (Anonymous 2, 1999). The fruit and vegetable wastes contain many phenolic compounds that may act as antioxidants, which are linked to a lower risk of degenerative diseases (Vinson *et al.*, 1998).

Research work (Guyot *et al.* 1997; Buisman *et al.*, 1998; Stamatis *et al.*, 2001) has been conducted to produce lipophenols through the lipase-catalyzed esterification of alcohol esters of fatty acids with phenolic acids. However, little research work has been reported with the use of fatty acids and their acylglycerol esters for the production of lipophenols. This is a novel approach for the use of more practical substrates for the production of lipophenols, which would be considered as nutraceutical products since they provide the health benefits of the selected ω-3 polyunsaturated lipids as well as the antioxidant activity of the phenolic compounds.

This research was aimed at the investigation and development of a biotechnological approach for the production of nutraceutical products using a model system. The specific objective of the present research work was to address in a model system the production of lipophenols by lipase-catalyzed esterification. The obtained results could be used as a prelude for the enzymatic biocatalysis in organic solvent medium for the bioconversion of endogeneous oil and phenolic compounds obtained from fish as well as apple and potato industry wastes into value-added bio-ingredients.

The research approach is based on the use of a model system for the bioconversion in organic solvent media of selected fatty acid and its triacylglycerol ester, as well as phenolic compounds into natural nutraceutical compounds possessing nutritional benefits.

4. MATERIALS AND METHODS

4.1. Materials

Commercial Lipase N from *Rhizopus niveus* was obtained from Amano Pharmaceutical Co. LTD (Nagoya, Japan) while immobilized Lipozyme IM from *Mucor meihei* and Novozym 435 (B lipase) from *Candida antarctica* were from Novo Nordisk A/S (Bagsvaerd, Denmark). (+)-Catechin, catechol (pyrocatechol), bovine serum albumin (BSA), Folin-ciocalteau (phenol reagent), tricaprylin and caprylic acid were purchased from Sigma Chemical Co. (St-Louis, MO). Dicaprylin, monocaprylin and caprylic acid methyl ester were purchased from Nu-Chek-Prep, Inc. (Elysian, MN). Chloroform, hexane, pentane, heptane, methanol and acetonitrile were purchased from Fisher Scientific (Fair Lawn, N.J.) as well as sodium phosphate monobasic, sodium phosphate dibasic, sodium hydroxide, sodium carbonate, potassium sodium tartarate, cupric sulfate, hydrochloric acid, sodium sulfate anhydrous, sodium tartrate and aqualine complete 1.

4.2. Methods

4.2.1. Protein Determination

Determination of protein concentration of the lipases was performed using the method described by Hartree (1972). BSA was used as the protein standard.

4.2.2. Lipase-Catalyzed Esterification

Esterification of lipids and phenols to produce lipophenols was performed in accordance to a modification of the procedure described by Kermasha *et al.* (1995). The reaction medium was composed of 9 mL of hexane to which the selected lipid, tricaprylin (50 µL) or caprylic acid (16 µL) was directly added at a concentration of 100 µM after which 25 µL of the selected phenol stock solution (0.86 mM in methanol), catechin or catechol, was added at a final concentration of 21.5 µM. The enzyme assay was initiated by the addition of either 25 µL of the Lipase N enzyme suspension (29.1 mg protein in 1 mL of sodium phosphate buffer solution, 0.1 M, pH 7) or 10 mg of the immobilized

enzymes consisting of either Lipozyme IM (1.56 mg protein) or Novozym 435 (0.67 mg protein) in 25 μL sodium phosphate butter solution (0.1 M, pH 7).

The enzymatic reactions were carried out in 50 mL Erlenmeyer flasks under vacuum at 37.5°C and 100 rpm in an orbital shaker (Lab-Line Instruments, Inc., Melrose Park, IL). Triplicate enzyme and control assays were conducted in tandem, where the control assays did not contain the enzyme extract. The enzymatic reactions were halted by the addition of 4 mL of chloroform.

4.2.3. Determination of Lipase Activity

The concentration of free fatty acids was measured and used as an indication of the hydrolytic and esterifying activities of the lipases. Twenty mL of deionized water was added to the reaction medium and the free fatty acids were titrated with a standardized solution of NaOH (0.01 M) using the Mettler DL 58 automated titrator (Mettler Toledo AG, Sonnenbergstrasse, Switzerland) equipped with a sample changer (Mettler ST20 A) and computerized data handling (DLWin V3.0). The specific activity of lipase was defined as micromoles of free fatty acid released per mg protein per min.

4.2.4. Effect of Selected Parameters on Enzyme Activity

4.2.4.1. Determination of Lipase Activity with Time

The lipase hydrolytic activity was investigated over an incubation period of 6 to 15 days at 37°C and 100 rpm.

4.2.4.2. Determination of pH Change of Reaction Medium with Time

The pH changes in the reaction medium with respect to time were investigated at 37°C and 100 rpm. After the addition of twenty mL of deionized water to the reaction medium, the pH was measured with the DG 101-SC electrode.

4.2.4.3. Determination of Optimum Temperature

The optimum temperature for lipase activity was investigated by incubation of the reaction medium at different temperatures ranging from 30 to 55°C and at 100 rpm, using standard assay conditions. The optimal incubation times used were 1.5, 1 and 2 days for the enzymatic assays with Lipase N, Lipozyme IM and Novozym 435, respectively.

4.2.4.4. Determination of Optimum Agitation Speed

The optimum agitation speed for lipase activity was investigated by agitating the reaction medium at different speeds ranging from 0 to 175 rpm. The assays were conducted at 37.5°C with an incubation time of 1.5 days for Lipase N and at 55°C with incubation times of 1 and 2 days for Lipozyme IM and Novozym 435, respectively.

4.2.4.5. Determination of Water Activity

The variations of water activity (a_w) of the reaction medium over time was measured using the Novasina Aw Sprint TH-500 system (Axair Ltd., Pfäffikon, Switzerland) at 25°C (Guyot *et al.* 1997; Selmi *et al.* 1997) throughout the incubation periods. The a_w sensor was protected from organic solvents by an eVc-26 universal filter (Novasina) and the Aw Sprint was calibrated with standard saturated salt solutions (Novasina humidity sources) of LiCl, $Mg(Cl)_2 \cdot 6H_2O$, $Mg(CO_3)_2 \cdot 6H_2O$, NaCl, $Ba(Cl)_2 \cdot 2H_2O$ and $K_2Cr_2O_7$ exhibiting a_w of 0.113, 0.328, 0.529, 0.753, 0.901 and 0.980, respectively.

4.2.4.6. Determination of Water Content

The water content was determined by titration using the Mettler DL 58 automated titrator equipped with a Karl Fisher titration system (Mettler DV 705) and computerized data handling (Mettler Toledo AG, Sonnenbergstrasse, Switzerland). The variations of water content in the reaction medium was investigated throughout the incubation periods by injecting the organic reaction medium (100 µL) into the Karl Fisher cell containing 40 mL of methanol, followed by titration with the aqualine complete 1 Karl Fisher reagent (Selmi *et al.*, 1997).

4.2.5. Characterization of Lipohenols

4.2.5.1. Lipophenol Extraction

The lipophenols, produced by the lipase-catalyzed esterification or interesterification reactions, were extracted from the reaction assays using a modification of the procedure described by Humeau *et al.* (1998). The hexane layer was recovered and the erleynmyer flask was washed with 5 mL of hexane to remove any remaining lipophenols. The hexane extract was subsequently treated with anhydrous sodium sulfate

to remove any traces of water and then evaporated using the Automatic Environmental Speed Vac system (AES 1010, Savant Instruments Inc., Holbrook, N.Y.). The concentrated phenolic esters were subsequently resolubilized in acetonitrile for further analysis.

4.2.5.2. HPLC Separation of Acylglycerols, Caprylic Acid and Lipophenols

Separation of the acylglycerols, free fatty acids and lipophenolic compounds of the lipase reaction medium was carried out according to a modified procedure described by Guyot *et al.* (1997) and Stamatis *et al.* (1999). Separation was performed on an Agilent Zorbax SB-C18 reverse phase column (250 x 4.6 mm, 5 μm) (Fisher Scientific) using a Beckman HPLC Gold system (Model 126, Beckman Instruments Inc., San Ramon, CA) equipped with an autosampler (Model 507) and a UV diode-array (UV-DAD) detector (Model 168) with computerized data handling and integration analysis. A 50 μL injection was performed and detection of the reaction products was carried out at 205 and 280 nm with scanning from 190 to 400 nm at 1-second intervals. The flow rate was 1 mL/min for 25 min. The mobile phase, consisting of deionized water and acetonitrile, was at a flow rate of 1mL/min beginning with 15% water and 85% acetonitrile for 5 min followed by an increase in acetonitrile to 100% in 15 min, where it was held for 5 min.

Standards of tricaprylin, dicaprylin, monocaprylin, caprylic acid, catechin and catechol were analyzed spectrophotometrically (UV 650 Spectrophotometer, Beckman Instruments, Inc., Fullerton, CA) to determine their linear range of concentration for quantification. In addition, each standard was subjected to HPLC analysis to characterize its chromatographic profile and to establish a calibration curve.

4.2.6. Statistical Analyses

The mean ($\overline{x}$), the standard deviation (s) and relative standard deviation (r) were calculated as follows:

$$\overline{x} = \Sigma x / n$$

$$s = \sqrt{\frac{\Sigma (x - \overline{x})^2}{n - 1}}$$

$$r = (s / x) \cdot 100$$

where, x is one data point and n is the number of samples in a given set (Freund, 1984).

The mean and standard deviation values of a given set of data were calculated for all samples and controls for the lipase-catalyzed reactions, which were carried out in triplicates. The standard deviation for the lipase activity was calculated on the basis of the difference between replicates of the sample and the control, expressed as micromoles of free fatty acid per mL or micromoles of free fatty acid released per mg protein per min. The mean and standard deviation values were calculated for the pH of the reaction medium, as well as for the water activity and water content based on triplicates. The mean and relative percent standard deviation values were analyzed for the peak areas of the HPLC chromatograms based on duplicates.

5. RESULTS

5.1. Tricaprylin as Model Lipid Substrate

5.1.1. Interesterification of Tricaprylin and Catechin

5.1.1.1. Lipase Activity and pH of the Reaction Medium with Time

The concentration of free fatty acids (FFAs) in the hexane reaction medium containing tricaprylin and catechin as substrates was used as an indication of the hydrolytic and esterifying activities of lipase in order to determine the optimal conditions for biocatalysis. The results indicate that for all three enzymes, Lipase N, Lipozyme IM and Novozym 435, there was an increase in the concentration of FFAs during the lipase-catalyzed reaction (Fig. 10A) and a corresponding decrease in the pH of the reaction medium (Fig. 10B). Biocatalysis with Lipase N showed a maximum FFA concentration of 1.66 (±0.19) μmol/mL during hydrolysis of tricaprylin after 1.5 days of incubation during which a rapid pH decrease from 6.5 (±0.024) to 5.5 (±0.096) of the reaction medium occurred, followed by a decrease in FFA concentration and subsequent increase to a pH of 6 (±0.19) during the next 4 days (Fig. 10B). The reaction catalyzed by Lipozyme IM showed an increase in FFA concentration during the first 8 days of incubation reaching a maximum of 10.8 (±0.0031) μmol/mL followed by a decrease, while the pH of the reaction medium decreased sharply from 6 (±0.011) to 4.4 (±0.078) during the first 6 h of incubation and then remained at a constant pH of 4.4 to 4.5 throughout the entire 15-day incubation period. The enzymatic reaction catalyzed by Novozym 435 reached a maximum increase in FFAs of 7.48 (±0.73) μmol/mL only after 15 days of incubation during which a concomitant decrease in pH from 6.6 (±0.076) to 4.9 (±0.0070) was observed.

The overall findings (Fig. 10) show that depending on the enzymes used, the concentration of FFAs varied with time where Lipozyme IM had the highest degree of hydrolysis in the hexane reaction medium followed by Novozym 435 and then by Lipase N.

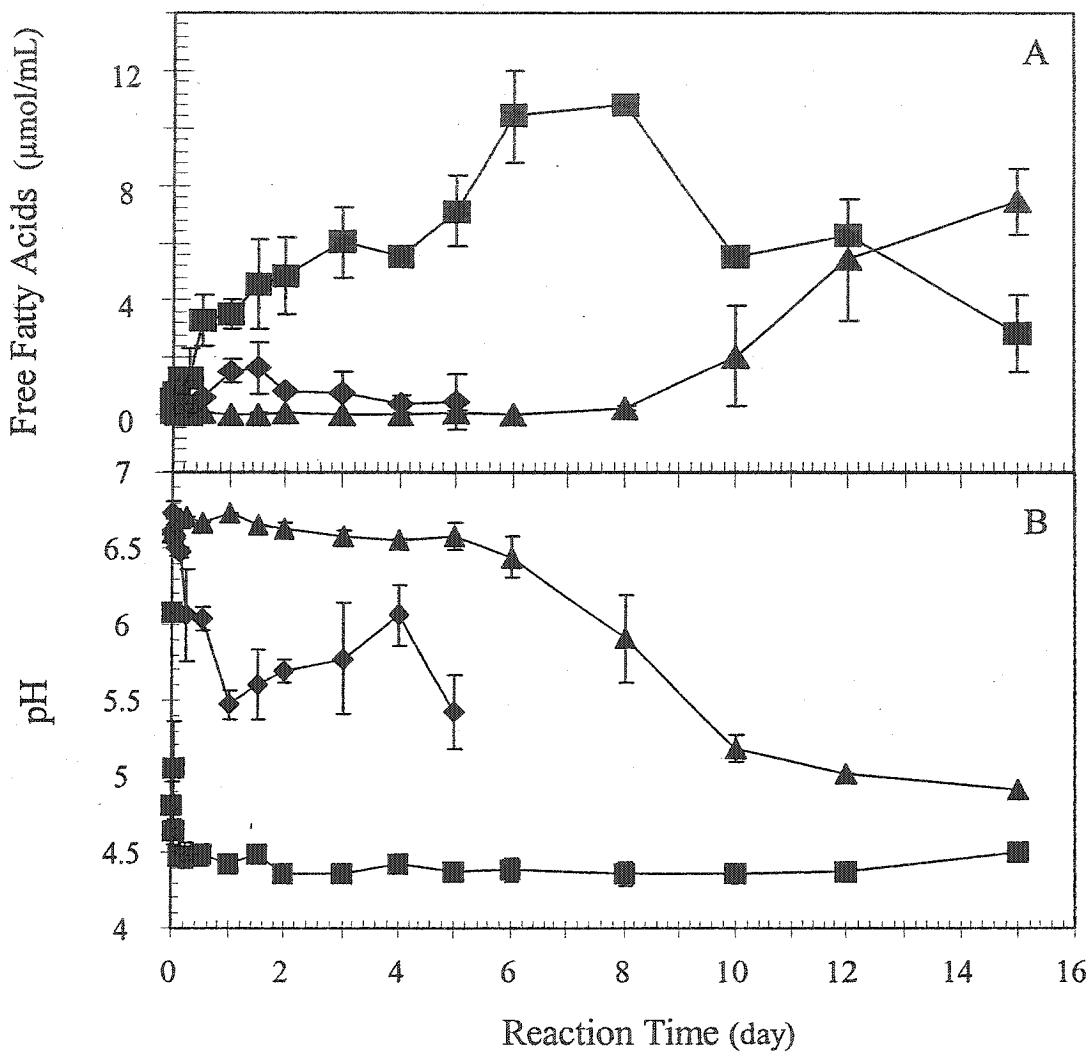

Figure 10. Effect of reaction time on enzyme activity (A) and pH changes (B) in the hexane reaction medium with Lipase N from *Rhizopus niveus* (◆), Lipozyme IM from *Mucor meihei* (■) and Novozym 435 (B lipase) from *Candida antarctica* (▲) using tricaprylin and catechin as substrates. The error bars (I) represent the standard deviation values.

5.1.1.2. Effect of Temperature on Lipase Activity

The effect of temperature on enzymatic activity was investigated for each of the lipases in the range of 30 to 55°C (Fig. 11A). Lipase N was found to have maximum specific activity at 37.5°C, whereas Lipozyme IM showed 4.3 times higher specific activity at 55°C than that observed at 45°C, while Novozym 435 showed 16.6 times higher specific activity at 55°C than that observed at 50°C.

Figure 11B shows the specific activity of the lipase reactions as a function of temperature, described by the Arrhenius law ($V=A_i e^{-(Ea/RT)}$). The activation energies of the selected enzymes (Table 2) indicated that there was a higher sensitivity of the enzymatic reaction rate to temperature when Novozym 435 acted as biocatalyst, followed by Lipozyme IM, compared to that observed with the free Lipase N.

On the basis of these findings, all further studies were performed at the temperatures of 37.5, 55 and 55°C for the enzymes of Lipase N, Lipozyme IM and Novozym 435, respectively.

Biocatalysis of the selected lipases of Lipozyme IM and Novozym 435 was subsequently investigated at the maximal temperature of 55°C. The variations of the FFA concentrations and the pH of the reaction medium with respect to reaction time are shown in Figure 12. Biocatalysis with Lipozyme IM showed an increase in the released FFA concentration to 8.1 (±0.81) μmol /mL after 1 day of reaction time with a concomitant decrease in the pH of the reaction medium, from 6.6 (±0.033) to 4.5 (±0.058). The released FFA concentration then decreased until day 4 where it increased to 8.5 (±0.82) μmol /mL and then gradually decreased. The FFA concentration of the reaction medium containing Novozym 435 as biocatalyst showed two increases of 4.0 (±0.39) and 6.0 (±0.20) μmol /mL at 2 and 9 days, respectively. As the concentration of FFA increased during this time, the pH of the reaction medium declined from 6.6 (±0.033) to 5.1 (±0.14) at day 2 and then remained fairly stable over the next few days. At day 9, a small decrease in the pH of the reaction medium to 5.0 (±0.025) was observed followed by a pH increase to 5.8 (±0.28) by day 14.

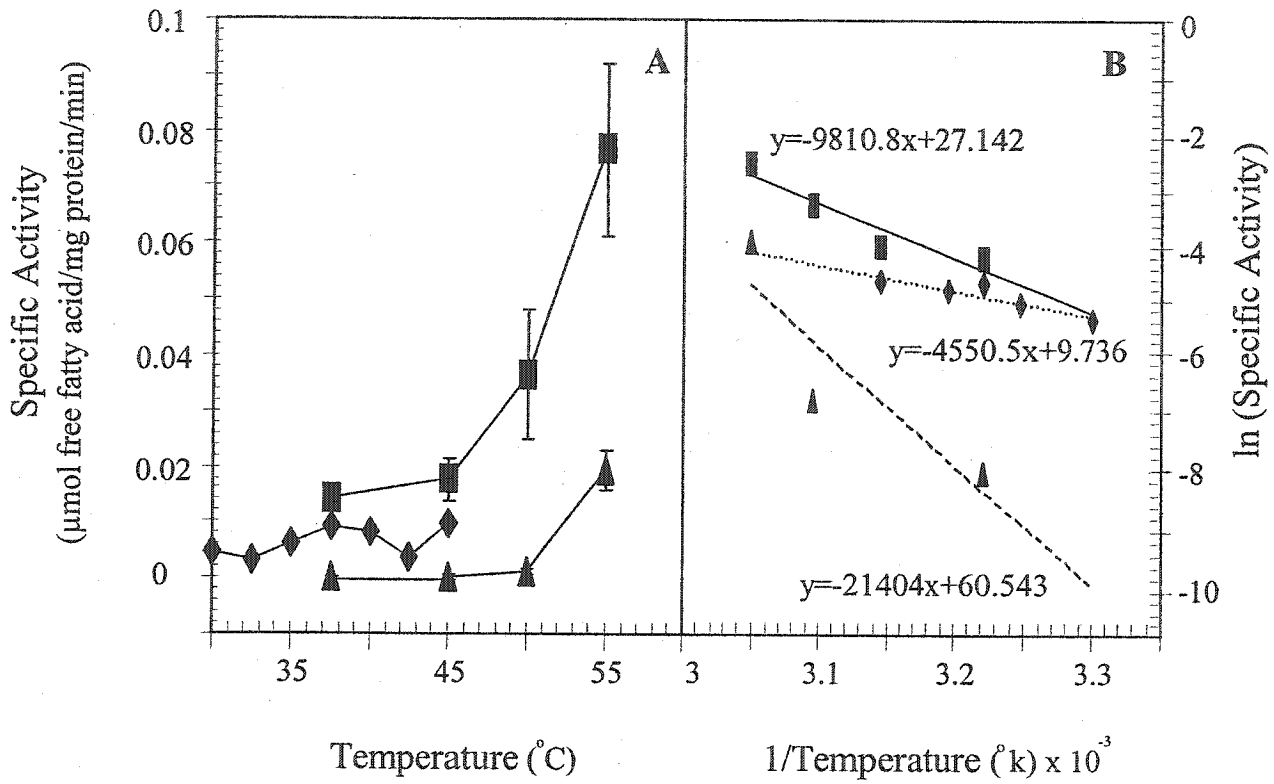

Figure 11. Effect of temperature on enzyme activity (A) and Arrhenius plots (B) of Lipase N from *Rhizopus niveus* (◆), Lipozyme IM from *Mucor meihei* (■) and Novozym 435 (B lipase) from *Candida antarctica* (▲) in hexane medium, using tricaprylin and catechin as substrates. The error bars (I) represent the standard deviation values.

Table 2. Temperature optima and activation energy parameters for enzyme activity of selected lipases with tricaprylin and catechin as substrates in hexane medium.

Enzyme	Maximum Temperature (°C)	Ea[a] (kJ/mole)
Lipase N[b]	37.5	37.8
Lipozyme IM[c]	55.0	81.6
Novozym 435[d]	55.0	177.9

[a] Ea is the activation energy as expressed by Arrhenius law ($V=A_i e^{-(Ea/RT)}$), where V is the rate constant at a specific temperature; A_i is a pre-exponental constant; E_a is the activation energy defined as kJ/mole; R is the gas constant (8.314 mol °k/J) and T is the temperature in Kelvin (k).

[b] Activity of the enzyme preparation from *Rhizopus niveus* (Amano) after 1.5 days reaction time and 100 rpm.

[c] Activity of the enzyme preparation from *Mucor meihei* (Novo) after1 day reaction time and 100 rpm.

[d] Activity of the enzyme preparation from *Candida antarctica* (Novo) after 2 days reaction time and 100 rpm.

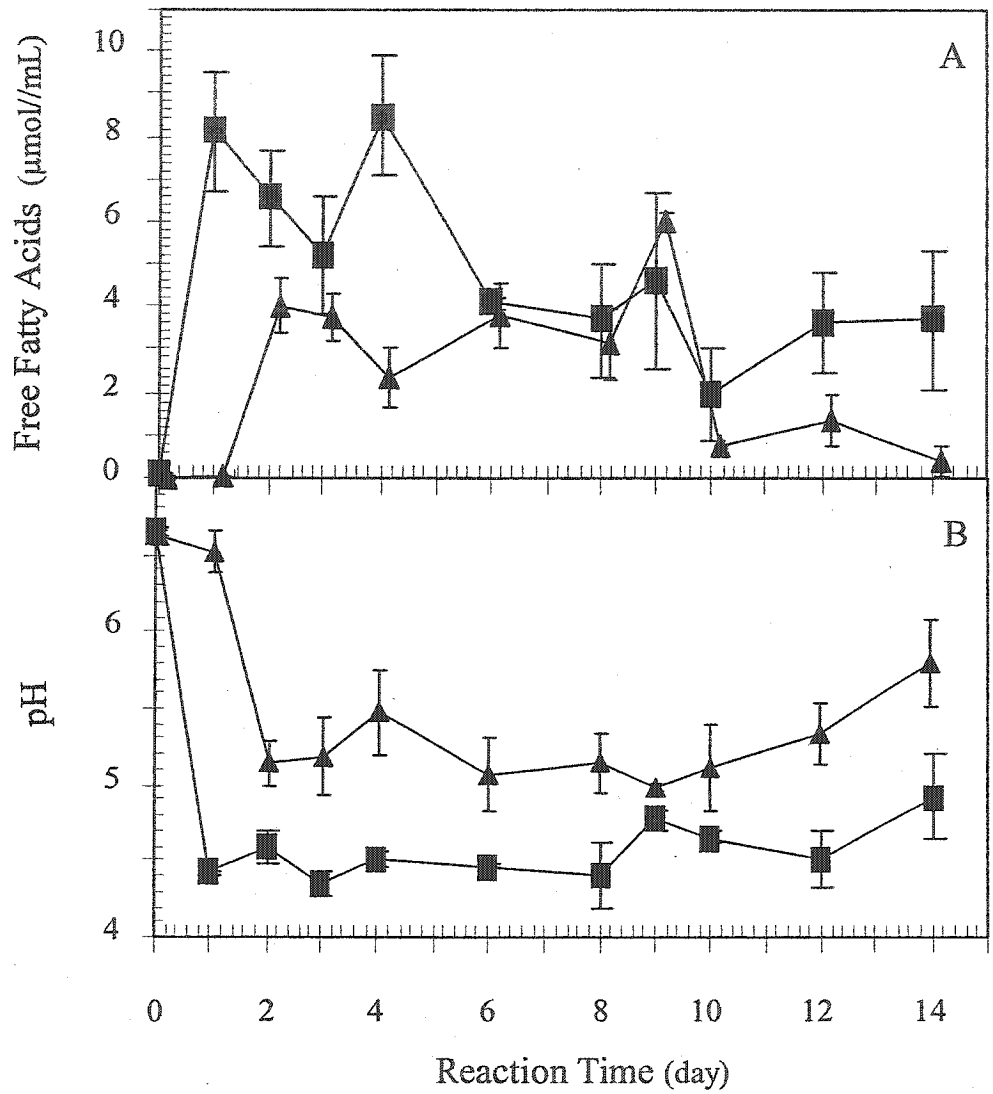

Figure 12. Effect of reaction time on enzyme activity (A) and pH changes (B) in the hexane reaction medium with Lipozyme IM from *Mucor meihei* (■) and Novozym 435 (B lipase) from *Candida antarctica* (▲) using tricaprylin and catechin as substrates. The error bars (I) represent the standard deviation values.

The overall findings (Fig. 12) show that the increase in the temperature of the reaction medium from 37.5 to 55°C increased the enzymatic hydrolysis rates, so that the concentration of FFAs varied with time where Lipozyme IM had the highest degree of hydrolysis in the hexane reaction medium followed by Novozym 435.

5.1.1.3. Effect of Agitation Speed on Lipase Activity

Enzymatic activity of the lipases was investigated at various agitation speeds, ranging from 0 to 175 rpm with Lipase N and from 75 to 150 rpm with Lipozyme IM and Novozym 435 (Fig. 13). The specific activity of Lipase N increased linearly with 4.4 times more specific activity as the agitation speed increased to 100 rpm, and then decreased at higher agitation speeds. The specific activity of Lipozyme IM as biocatalyst increased with agitation speed up to 100 rpm and remained constant up to 125 rpm, after which a 9.9-fold decrease in specific activity occurred at 150 rpm. When Novozym 435 was used as a biocatalyst, the specific activity increased 7.9 times more with the use of agitation speeds ranging from 75 to 100 rpm, followed by a decrease in specific activity to 0.0016 (±0.00015) μmol FFA/mg protein/min at 125 rpm.

On the basis of these findings, all further studies were performed at agitation speeds of 100 rpm for the enzymes of Lipase N, Lipozyme IM and Novozym 435.

5.1.1.4. Effect of Water Activity and Water Content on the Lipase Reaction

Figure 14 shows that the water activity (a_w) of the reaction medium remained fairly stable over time for the three lipases of Lipase N, Lipozyme IM and Novozym 435 while the water content of the reaction medium generally tended to increase slightly. The theoretical water content (v/v) of the hexane reaction medium containing sodium phosphate buffer was 0.2747%, whereas the initial experimental water content of the reaction medium was found to be slightly lower at 0.203% (± 0.010). The a_w of the reaction medium containing Lipase N as biocatalyst remained unchanged compared to the control assay with an average value of 0.179 (± 0.018) a_w, while the water content (v/v) increased slightly during 2 days reaching a maximum of 0.22% (± 0.023) (Fig. 14A). The a_w of the reaction medium containing Lipozyme IM and Novozym 435 (Fig. 14B and 14C) were similar to that of the control assays and remained fairly stable from 0.158 (±

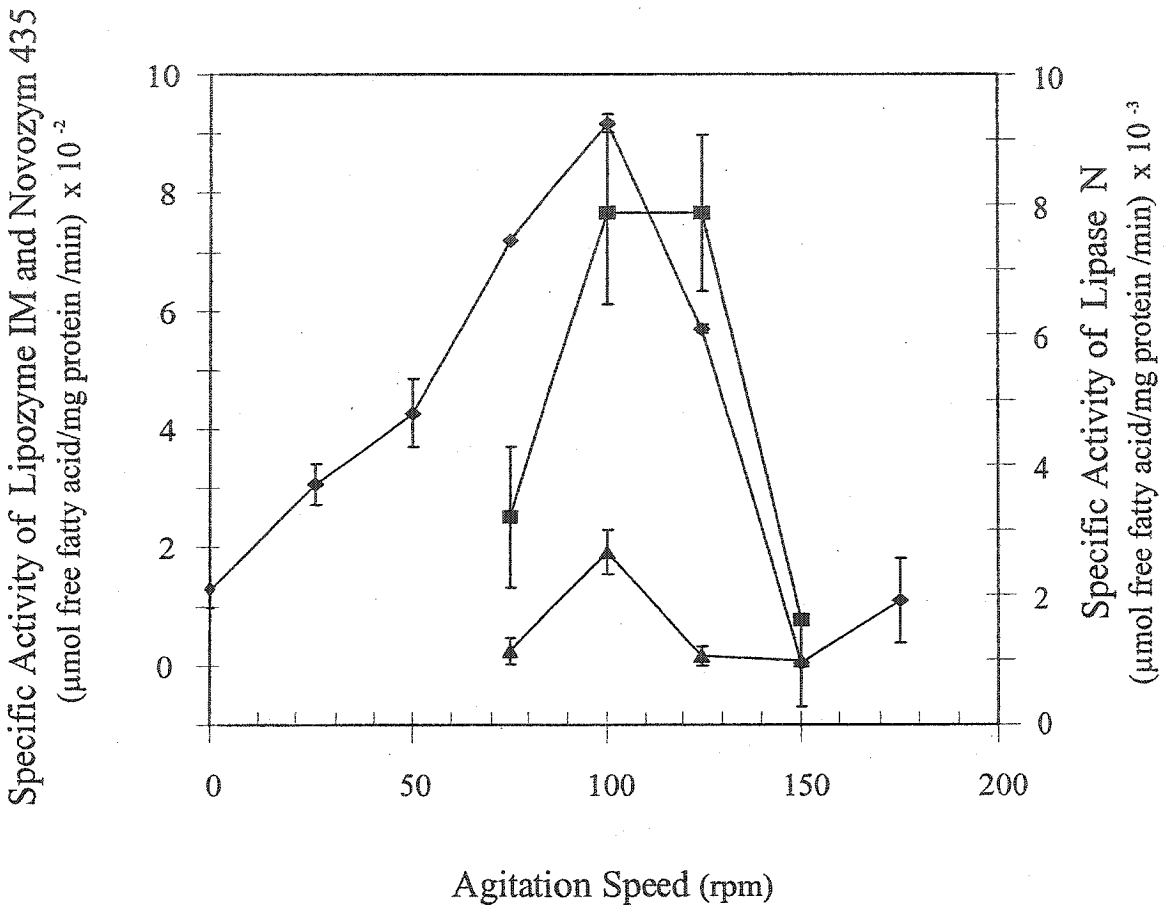

Figure 13. Effect of agitation speed on the hydrolytic activity of Lipase N from *Rhizopus niveus* (◆), Lipozyme IM from *Mucor meihei* (■) and Novozym 435 (B lipase) from *Candida antarctica* (▲) in hexane medium using tricaprylin and catechin as substrates. The error bars (I) represent the standard deviation values.

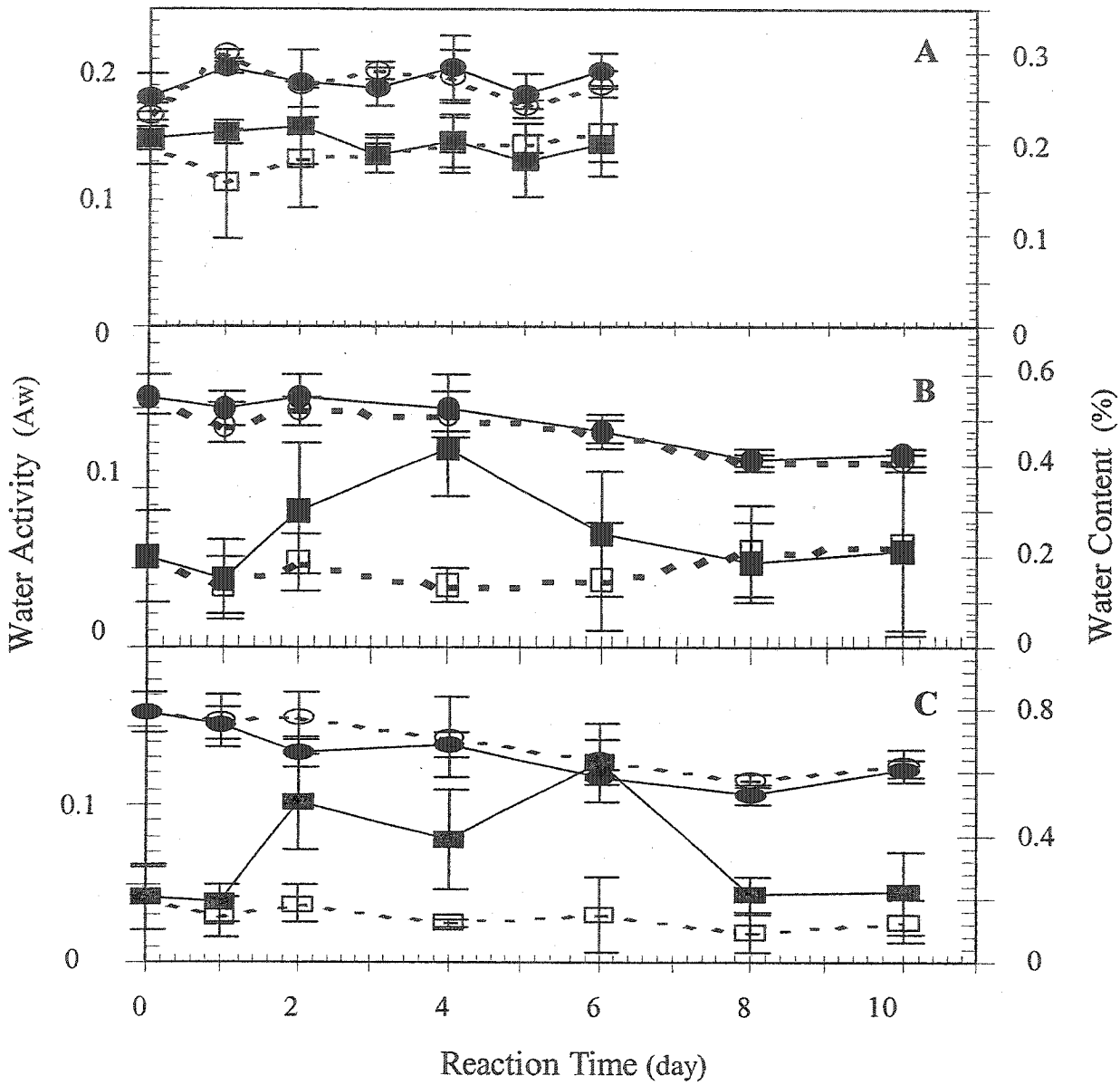

Figure 14. Changes in the water activity of the sample (●) and control (○) assays and changes in the water content of the sample (■) and control (□) assays in the hexane reaction medium of Lipase N from *Rhizopus niveus* (A), Lipozyme IM lipase from *Mucor meihei* (B) and Novozym 435 lipase (B lipase) from *Candida antarctica* (C), using tricaprylin and catechin as substrates. The error bars (I) represent the standard deviation values.

0.013) a_w to 0.122 (± 0.0069) a_w over 10 days. The results also show that the water content (v/v) of the reaction medium containing Lipozyme IM increased to 0.437% (± 0.052) at day 4, followed by a decrease (Fig. 14B). The water content (v/v) of the reaction medium with Novozym 435 as biocatalyst increased to 0.510% (± 0.057) after 2 days, followed by a slight drop to 0.389% (± 0.04) at day 4 and then another increase to 0.637% (± 0.071) at day 6, followed by a decrease at day 8.

The overall findings (Fig. 14) indicate slight variations in the a_w and water contents of the hexane reaction medium containing the lipases of Lipase N, Lipozyme IM and Novozym 435.

5.1.1.5. HPLC Analysis of Acylglycerols and Lipophenols

HPLC analysis of the lipase-catalyzed reaction between tricaprylin and catechin in hexane medium was performed to investigate the occurance of lipophenol production. The results show that with the use of Lipozyme IM (Fig. 15B and 16B) and Novozym 435 (Fig. 15C and 16C) enzymes, there was a production of three novel end products at 205nm and two of these end products (peaks 4 and 5) also exhibited a limited absorbance at 280 nm. These three unknown peaks (peaks 4, 5 and 6) had elution times of 4.9, 5.6 and 8.8 min, respectively. In contrast, the use of the Lipase N preparation in the enzyme assays showed no hydrolysis products or novel products, as indicated by the similarity of the peaks obtained to the chromatograms for the control assays (Fig. 15A and 16A) throughout the 8-day period.

The general trend observed for the lipase-catalyzed reactions between tricaprylin and catechin involved the hydrolysis of tricaprylin, followed by esterification of the hydrolysis products with catechin. The relative % hydrolysis of tricaprylin by Lipozyme IM increased to 39.7 at day 1 and generally tended to decrease except for a small increase at day 3 (Table 3). Dicaprylin (Fig. 17A) was also present on days 1 and 4 in significant concentrations whereas monocaprylin was present in similar concentrations, from day 2 to 3 and on the 8^{th} day. In addition, there was an increase in caprylic acid (CA) concentration at day 1 followed by a decrease. Table 3 shows that unknown 1 had a small continuous presence with small peak areas throughout the 8 days. Unknown 2

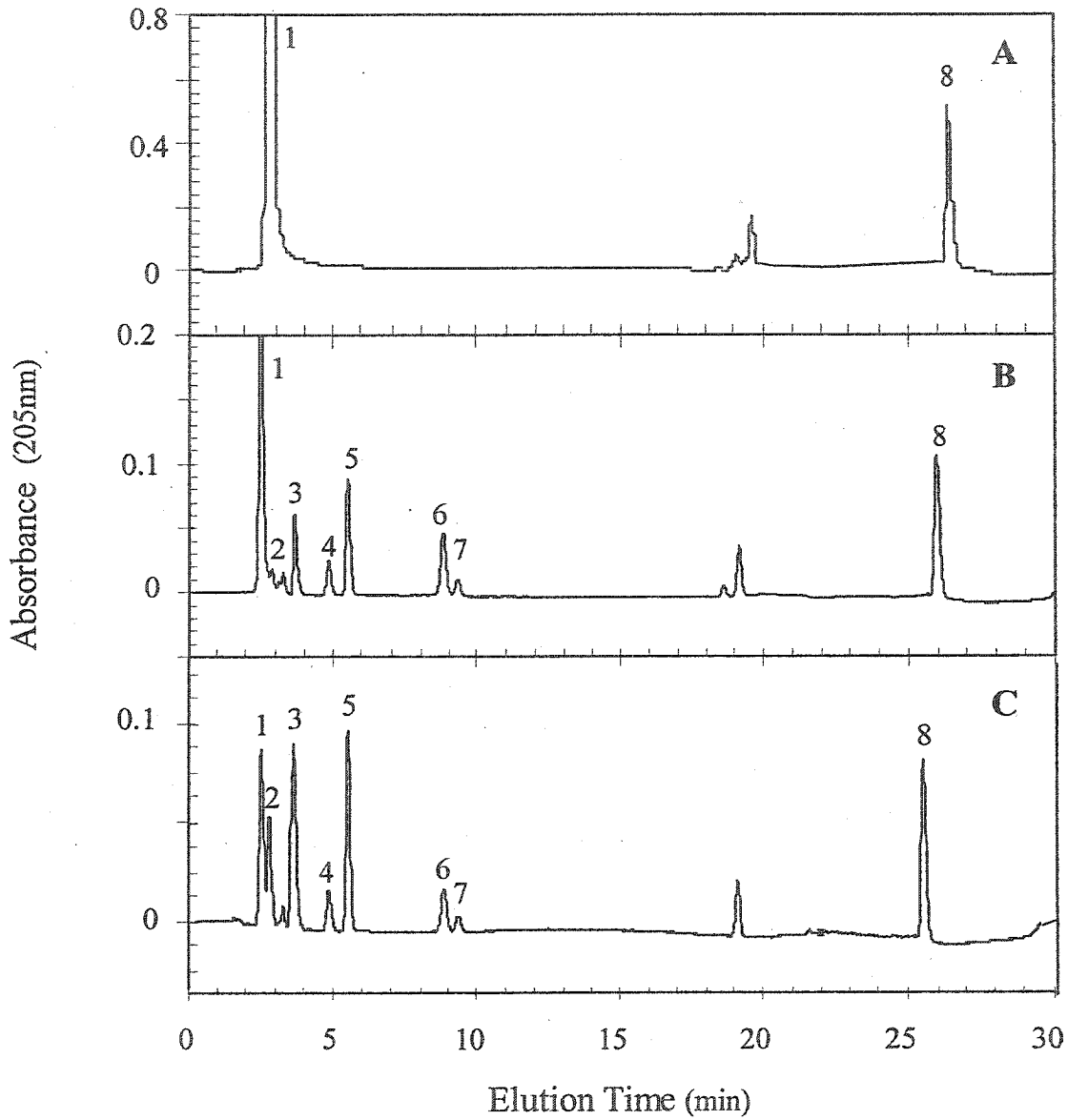

Figure 15. HPLC chromatograms of the reaction assay components of (A) the control, (B) Lipozyme IM lipase from *Mucor meihei* and (C) Novozym 435 lipase (B lipase) from *Candida antarctica* in hexane medium with tricaprylin and catechin as substrates, after 4 days of incubation where the products were identified to be (1) catechin, (2) monocaprylin, (3) caprylic acid, (4) unknown 1, (5) unknown 2, (6) unknown 3, (7) dicaprylin and (8) tricaprylin.

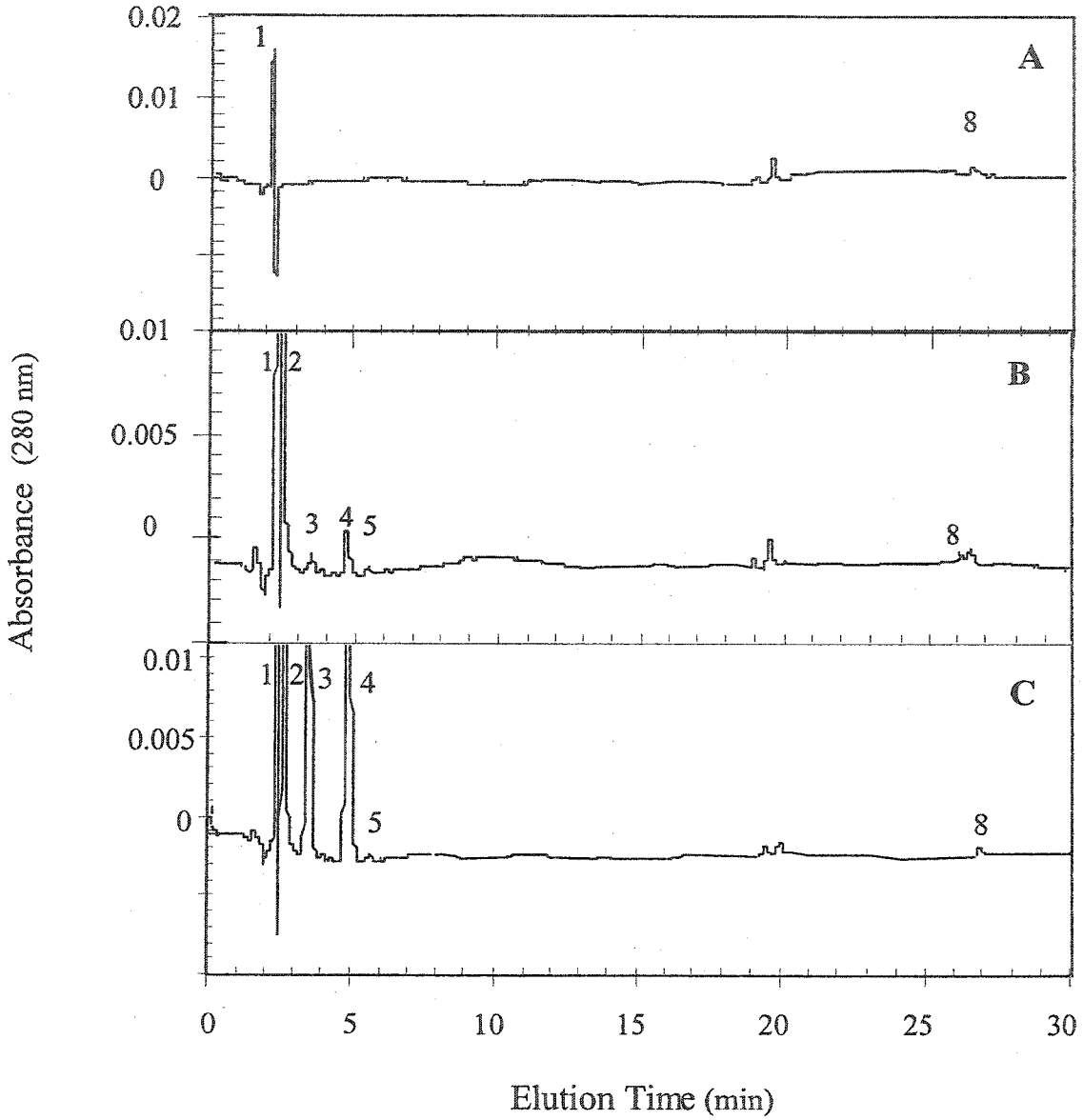

Figure 16. HPLC chromatograms of the reaction assay components of (A) the control, (B) Lipozyme IM lipase from *Mucor meihei* and (C) Novozym 435 lipase (B lipase) from *Candida antarctica* in hexane medium with tricaprylin and catechin as substrates, after 4 days of incubation where the products were identified to be (1) catechin, (2) monocaprylin, (3) caprylic acid, (4) unknown 1, (5) unknown 2 and (8) tricaprylin.

Table 3. Comparison of peak areas from the HPLC analyses of the lipase-catalyzed reaction between tricaprylin and catechin in hexane medium, using different enzyme preparations.

	Reaction Time (Days)	Lipid Products (Peak areas at 205 nm)			Relative Hydrolysis[d] (%)	Lipophenol Products[e] (Peak areas at 205 nm)				Relative Esterification[g] (%)
		Tricaprylin[a]	Hydrolysis[b]	Total Lipid[c]		Unknown 1	Unknown 2	Unknown 3	Total Lipophenols[f]	
Lipozyme IM[i]	0	71.2 (±4.97%)[h]	0	71.2	0	0	0	0	0	0
	1	24.6 (±0.43%)[h]	28.3	52.9	39.7	4.3 (±1.81%)[h]	3.0 (±1.98%)[h]	3.8 (±1.61%)[h]	11.2	17.5
	2	28.5 (±6.37%)[h]	16.5	45.0	23.2	2.4 (±5.02%)[h]	7.1 (±6.21%)[h]	6.6 (±5.56%)[h]	16.1	26.4
	3	38.1 (±2.78%)[h]	20.6	58.7	28.9	3.2 (±4.74%)[h]	11.4 (±2.17%)[h]	0	14.5	19.8
	4	20.1 (±4.04%)[h]	9.8	29.9	13.8	4.4 (±6.92%)[h]	13.4 (±4.42%)[h]	9.7 (±4.04%)[h]	27.4	47.8
	6	34.5 (±0.83%)[h]	3.7	38.2	5.1	2.0 (±2.77%)[h]	0.5 (5.65%)[h]	5.5 (±4.12%)[h]	8.0	17.4
	8	36.9 (±7.33%)[h]	6	42.9	8.4	2.5 (±5.63%)[h]	2.5 (±7.07%)[h]	6.1 (±6.51%)[h]	11.2	20.7
Novozym 435[j]	0	71.2 (±4.97%)[h]	0	71.2	0	0	0	0	0	0
	1	47.1 (±3.87%)[h]	4.5	51.6	6.3	2.4 (±1.11%)[h]	2.9 (±4.98%)[h]	0	5.3	9.2
	2	42.2 (±2.47%)[h]	40.2	82.4	56.5	3.6 (±3.35%)[h]	10.8 (±2.54%)[h]	0	14.4	14.9
	3	36.0 (±5.07%)[h]	28.3	64.3	39.7	5.9 (±4.66%)[h]	1.2 (±4.87%)[h]	12.2 (±4.70%)[h]	19.2	23.0
	4	21.7 (±3.83%)[h]	33.6	55.3	47.2	4.4 (±4.01%)[h]	19.3 (±4.16%)[h]	5.6 (±3.62%)[h]	29.3	34.6
	6	39.7 (±4.44%)[h]	10.0	49.7	14.0	6.6 (±4.55%)[h]	2.2 (±1.83%)[h]	11.2 (±4.34%)[h]	20.0	28.7
	8	25.3 (±6.87%)[h]	27.3	52.6	38.3	7.3 (±6.70%)[h]	13.2 (±6.96%)[h]	10.8 (±4.78%)[h]	31.3	37.3

[a] Peak area of tricaprylin from HPLC analysis.

[b] The sum of the peak areas corresponding to caprylic acid, monocaprylin and dicaprylin, resulting from the hydrolysis of tricaprylin.

[c] The sum of the peak areas corresponding to tricaprylin, dicaprylin, monocaprylin and caprylic acid.

[d] The hydrolysis peaks divided by the initial peak area of tricaprylin of 71.2 at time 0, multiplied by 100.

[e] Unknown peaks in the HPLC chromatogram representing lipophenols.

[f] The sum of lipophenol peak areas.

[g] The total lipophenols divided by the sum of the total lipid and total lipophenols, multiplied by 100.

[h] Relative percent standard deviation calculated by dividing the standard diviation by the mean, multiplied by 100.

[i] Enzyme preparation from *Mucor meihei*.

[j] Enzyme preparation from *Candida antarctica*.

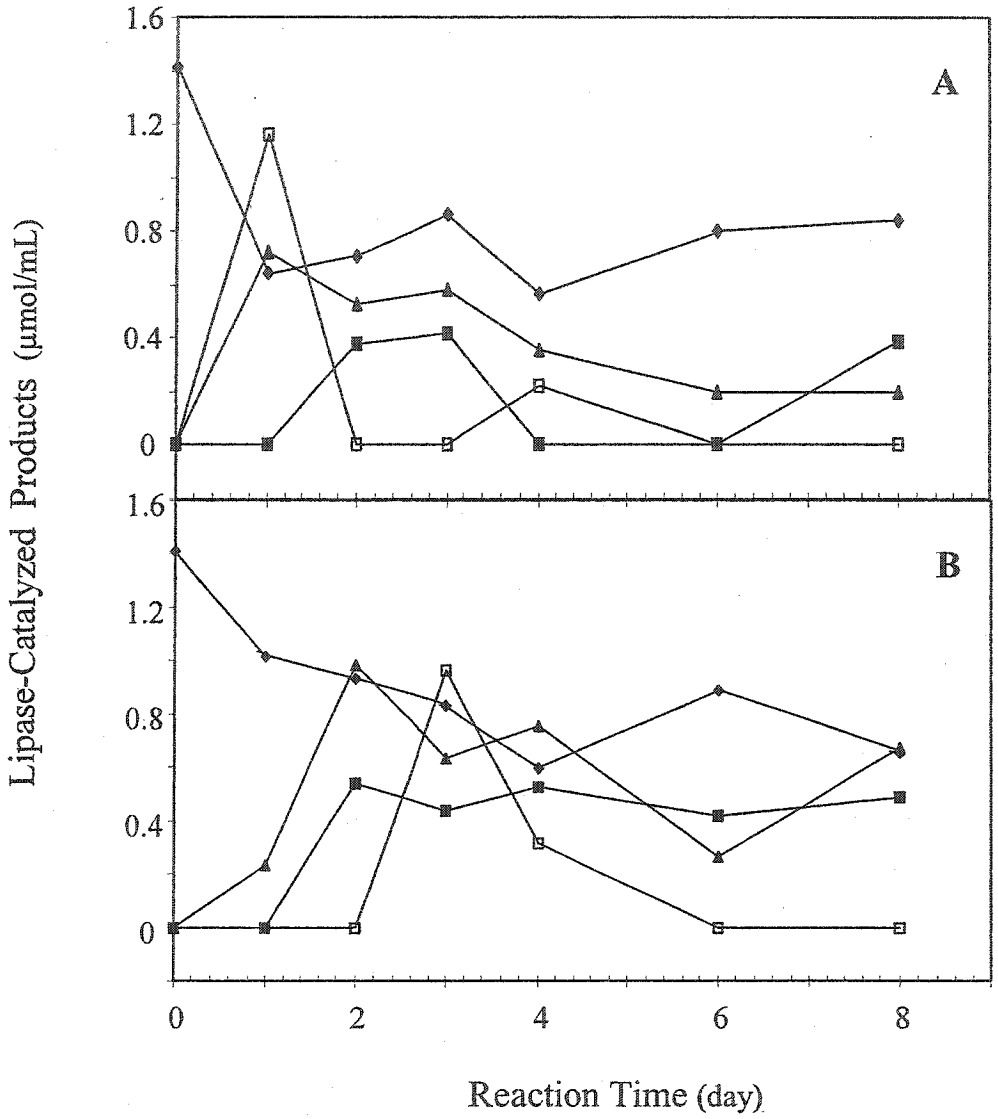

Figure 17. Concentrations of lipase-catalyzed products, including tricaprylin (◆), dicaprylin (□), monocaprylin (■) and caprylic acid (▲), obtained from HPLC analyses, with (A) Lipozyme IM from *Mucor meihei* and (B) Novozym 435 (B lipase) from *Candida antarctica* in hexane medium with tricaprylin and catechin as substrates.

increased linearly in peak area until day 4, and then decreased up to day 6 with a small increase at day 8. Unknown 3 increased in peak area from day 1 to 2 and then was not present at day 3, followed by a large appearance at day 4, and tending to decrease until day 8.

There was a maximum of 56.5% hydrolysis of tricaprylin by Novozym 435 by day 2 of incubation (Table 3). The dicaprylin concentration increased at day 3 and declined by day 6, whereas the monocaprylin concentration increased up to day 2 and then remained stable. The CA concentration increased until day 2 and then tended to decrease. Table 3 shows that unknown 1 had a small general positive trend in peak area from day 1 to day 8. Unknown 2 increased in peak area until day 2, followed by a large decrease at day 3 and then an increase with twice as much peak area on day 4 compared to day 2, followed by another large decrease on day 6, and a subsequent increase at day 8. Unknown 3 increased by day 3, decreased at day 4, and then returned to the peak areas observed at day 3 for days 6 and 8.

The overall findings (Table 3) show that depending on the enzymes used, the relative % esterification differed with time, where Lipozyme IM had the highest relative % esterification of 47.8 on day 4 in the hexane reaction medium due to a large presence of unknown 2, which coincided with a decrease in the concentrations of tricaprylin, monocaprylin and CA. This was followed by the reaction catalyzed by Novozym 435 which had a maximum of 34.6% relative esterification at day 4 due to a large presence of unknown 2 mirrored by decreasing concentrations of tricaprylin, dicaprylin and CA. The reaction catalyzed by Novozym 435 showed a similar relative % esterification at day 8 of 37.3 due to the large presence of all three unknowns, which coincided with a decrease in the concentrations of tricaprylin and dicaprylin. In contrast, the use of the lipase preparation of Lipase N showed no hydrolytic or esterifying activity with the HPLC analyses.

On the basis of these findings, all further studies were conducted with the enzymes of Lipozyme IM and Novozym 435.

5.1.2. Interesterification of Tricaprylin and Catechol

5.1.2.1. Lipase Activity and pH of the Reaction Medium with Time

Since the lipase reaction involves the initial hydrolysis of tricaprylin followed by subsequent esterification with catechol, the concentration of FFAs in the reaction medium was monitored over time and used as an indication of the enzymatic reaction between tricaprylin and catechol in hexane medium. The results (Fig. 18A) showed that there were increases of 2.18 (±0.22) and 1.68 (±0.17) μmol/mL in FFA concentrations in the hexane reaction medium at day 1 of incubation with Lipozyme IM and Novozym 435, respectively, followed by a decrease at day 2 (Fig. 18A). Further biocatalysis with Lipozyme IM increased the FFA concentration to 1.88 (±0.19) μmol/mL at day 3 followed by a decrease until day 8. The reaction catalyzed by Novozym 435 showed an increase in FFA concentration up to 5.8 (±0.11) μmol/mL by day 4, followed by a 33% decrease by day 6 and then subsequent increase to a final concentration of 6.1 (±0.67) μmol FFA/mL by day 8.

Increases in FFA concentration were monitored by measuring the corresponding decreases in the pH profiles of the reaction medium (Fig. 18B). The pH of the reaction medium with Lipozyme IM declined from 6.8 (±0.011) to 4.2 (±0.024) and reached a pH of 4.5 (±0.038) during the 8 days. The pH of the reaction medium containing Novozym 435 decreased from 6.8 (±0.11) to 5.5 (±0.029) at day 1, and then increased to 6.1 (±0.13) at day 2 followed by a decrease to 4.9 (±0.21) by day 4. These variations in the pH profile of the reaction medium do not reflect the respective variations in the concentrations of FFAs.

The overall findings (Fig. 18) show that, depending on the enzymes used, the concentration of FFAs varied with time where Novozym 435 had the highest degree of hydrolysis in the hexane reaction medium with tricaprylin and catechol as substrates followed by Lipozyme IM.

5.1.2.2. HPLC Analysis of Acylglycerols and Lipophenols

The HPLC analysis of the lipase-catalyzed reaction between tricaprylin and catechol in the hexane medium using Lipozyme IM (Fig. 19B and 20B) and Novozym 435 (Fig. 19C and 20C) revealed that there were three novel end products at 205 nm and

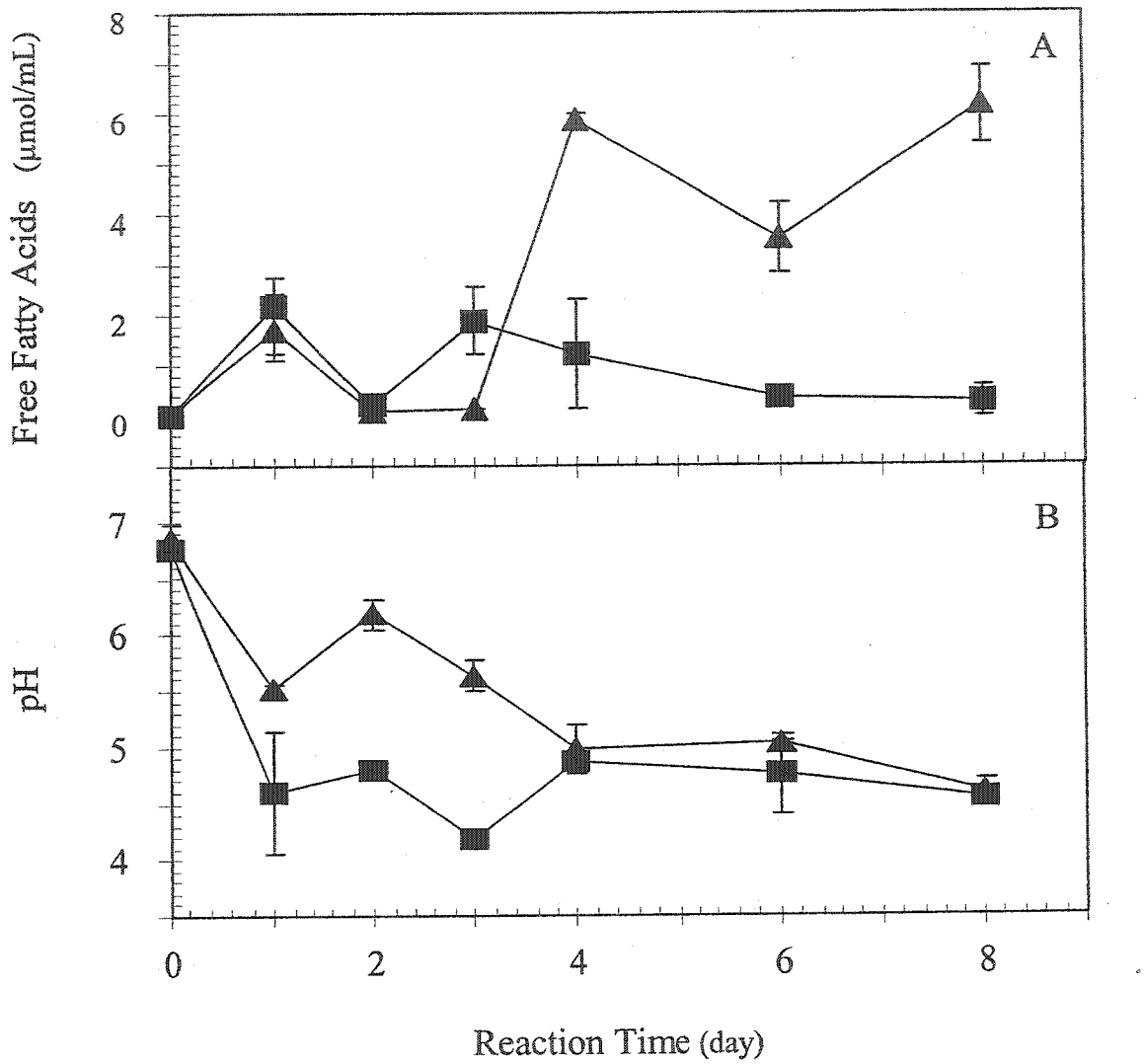

Figure 18. Effect of time on enzyme activity (A) and pH changes in the hexane reaction medium (B) with Lipozyme IM from *Mucor meihei* (■) and Novozym 435 (B lipase) from *Candida antarctica* (▲) using tricaprylin and catechol as substrates. The error bars (I) represent the standard deviation values.

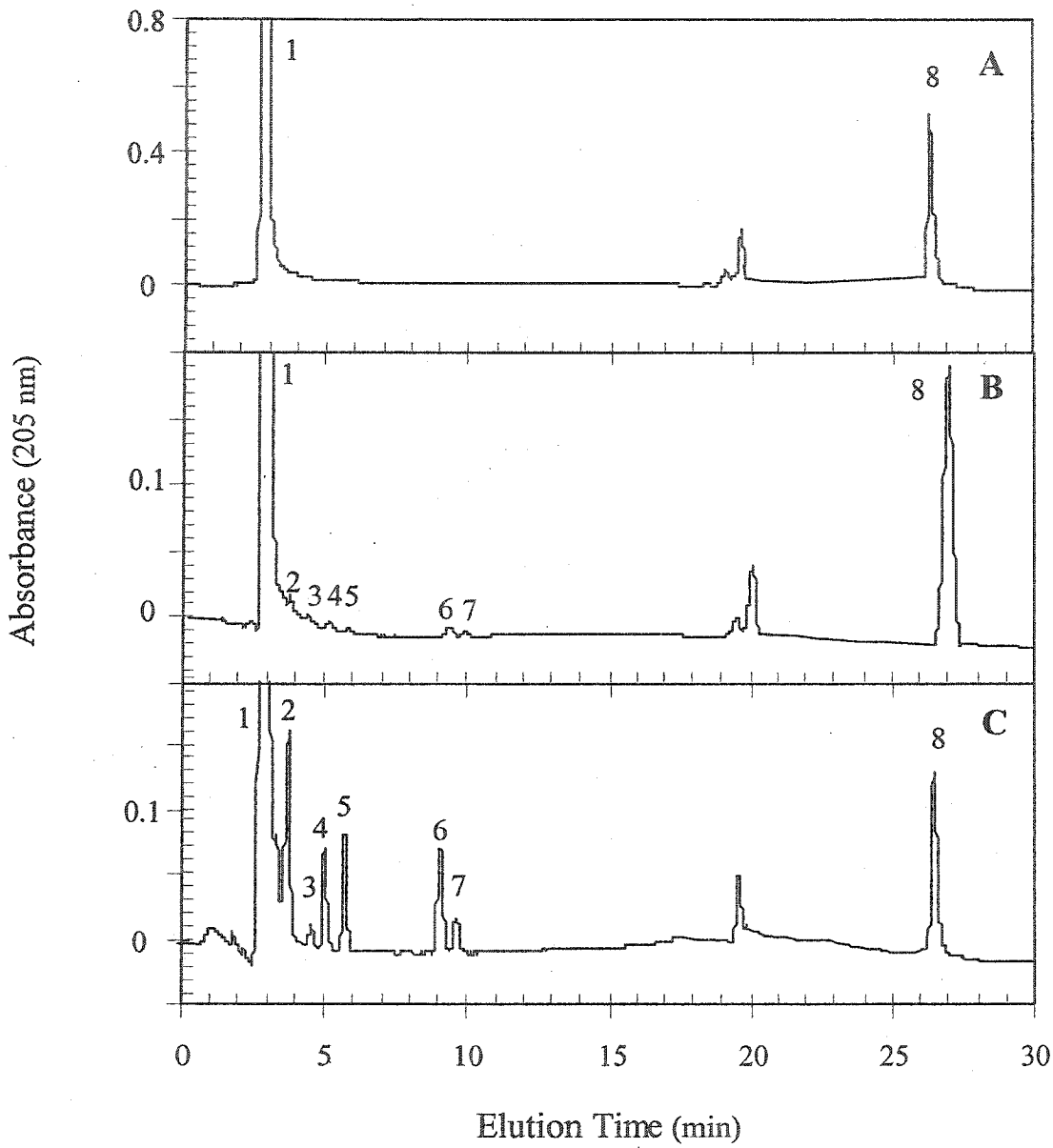

Figure 19. HPLC chromatograms of the reaction assay components of (A) the control, (B) Lipozyme IM lipase from *Mucor meihei* and (C) Novozym 435 lipase (B lipase) from *Candida antarctica* in hexane medium with tricaprylin and catechol as substrates, after 8 days of incubation where the products were identified to be (1) catechol, (2) monocaprylin, (3) caprylic acid, (4) unknown 1, (5) unknown 2, (6) unknown 3, (7) dicaprylin and (8) tricaprylin.

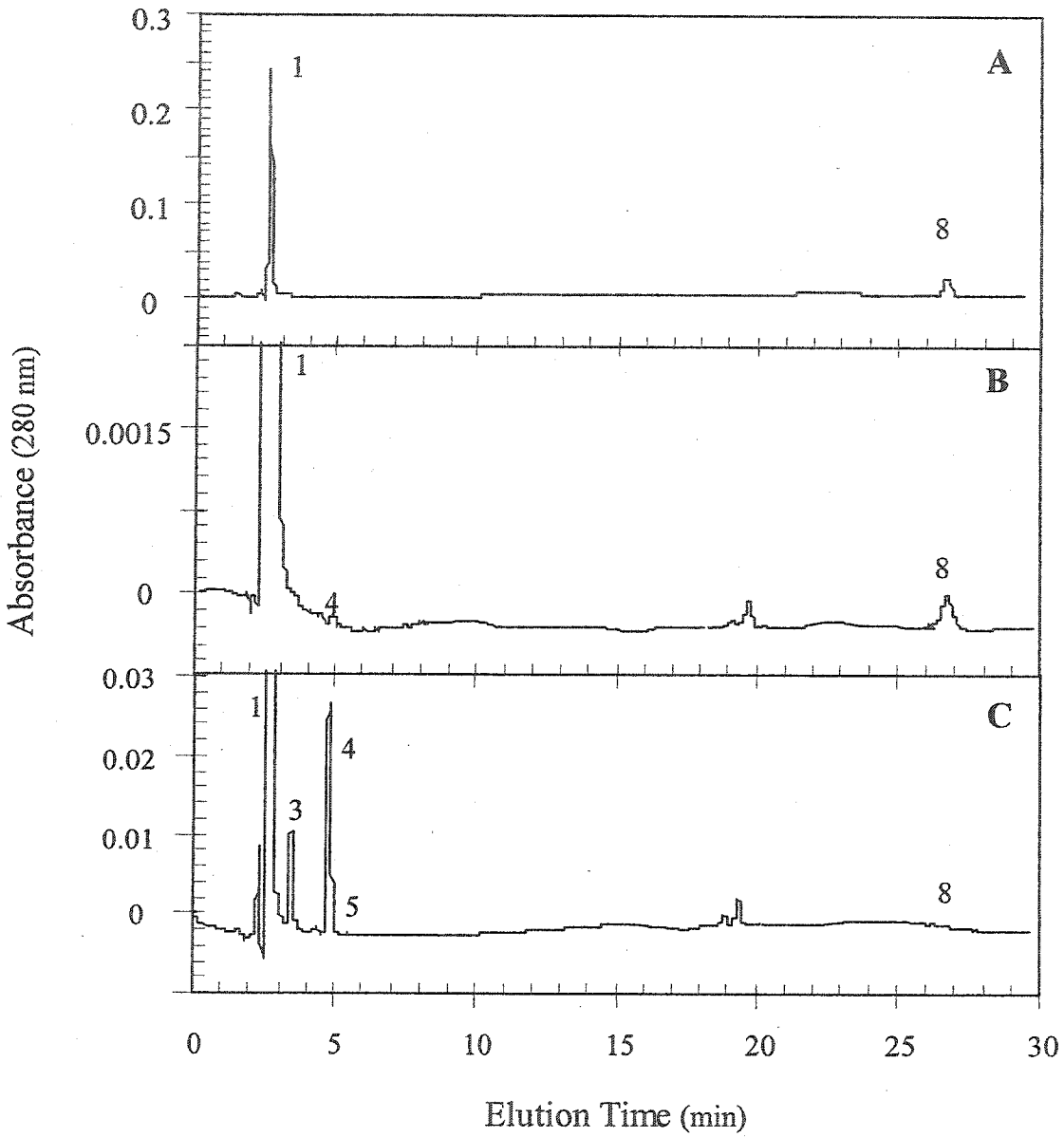

Figure 20. HPLC chromatograms of the reaction assay components of (A) the control, (B) Lipozyme IM lipase from *Mucor meihei* and (C) Novozym 435 lipase (B lipase) from *Candida antarctica* in hexane medium with tricaprylin and catechol as substrates, after 8 days of incubation where the products were identified to be (1) catechol, (3) caprylic acid, (4) unknown 1, (5) unknown 2 and (8) tricaprylin.

that two of these end products (peaks 4 and 5) also showed a limited absorbance at 280 nm. These three unknown peaks (peaks 4, 5 and 6) had elution times of 5.0, 5.7 and 9.1 min, respectively.

Table 4 shows the lipase-catalyzed reaction between tricaprylin and catechol, which involved the hydrolysis of tricaprylin followed by esterification of the hydrolysis products with catechol. Maximum hydrolysis of tricaprylin by Lipozyme IM was 14.2 % at day 1 (Table 4), after which it decreased and then remained stable (Fig. 21A). The dicaprylin concentration increased at day 1, then decreased at day 2 followed by a subsequent increase at day 3, after which it remained constant. There was no formation of monocaprylin throughout the 8-day period, while the CA concentration increased at day 1 and remained constant until day 4 where it started to decrease. Table 4 also shows that unknown 1 had larger peak areas at days 1 and 4, while for the remaining days, there were lower peak areas. Unknown 2 increased in peak area up to day 2, after which it decreased up to day 6. Unknown 3 increased in peak area at day 2 and then remained constant until day 6, followed by an increase at day 8.

Maximum hydrolysis of tricaprylin by Novozym 435 was 80% at day 6 (Table 4). Dicaprylin was present at day 8 at a significant concentration, whereas the monocaprylin concentration increased at day 4 and remained constant. The CA concentration increased up to day 6 and then decreased by day 8. Unknown 1 increased in peak area at day 2, followed by a decrease at day 3 and then increased at day 8. The peak areas for unknown 2 increased linearly, reaching a maximum at day 6 and then decreased. Unknown 3 appeared at days 2, 4 and 8 at increasing peak areas.

The overall findings (Table 4) show that the relative esterification differed with tricaprylin and catechol as substrates in the hexane reaction medium depending on the enzymes used. The maximum relative % esterification of 12.5 by Lipozyme IM was observed at day 8 due to the presence of the 3 unknowns of which there was a significant amount of unknown 3, and decreases in the concentrations of tricaprylin and CA. However, Novozym 435 showed a higher relative % esterification compared to Lipozyme

Table 4. Comparison of peak areas from the HPLC analyses of the lipase-catalyzed reaction between tricaprylin and catechol in hexane medium, using different enzyme preparations.

	Reaction Time (Days)	Lipid Products (Peak areas at 205 nm)			Relative Hydrolysis[d] (%)	Lipophenol Products[e] (Peak areas at 205 nm)				Relative Esterification[g] (%)
		Tricaprylin[a]	Hydrolysis[b]	Total lipid[c]		Unknown 1	Unknown 2	Unknown 3	Total Lipophenols[f]	
Lipozyme IM[i]	0	84.7 (± 2.73%)[h]	0	84.7	0	0	0	0	0	0
	1	68.8 (± 5.13%)[h]	12.1	80.9	14.2	2.2 (±7.76%)[h]	1.3(±9.21%)[h]	0	3.5	4.1
	2	70.3 (± 5.61%)[h]	8.1	78.4	9.5	1.2 (±4.60%)[h]	1.6 (±6.45%)[h]	0	2.8	3.4
	3	78.8 (± 4.31%)[h]	8.6	87.4	10.1	1.4 (±7.17%)[h]	1.4 (±1.02%)[h]	0.7 (±2.97%)[h]	3.5	3.8
	4	59.6 (± 4.58%)[h]	9.1	68.7	10.7	1.9 (±9.74%)[h]	0.5 (±0.25%)[h]	0.7 (±5.04%)[h]	3.1	4.3
	6	67.7 (± 1.12%)[h]	6.2	73.9	7.3	1.3 (±5.89%)[h]	0.4 (±4.61%)[h]	0.9 (±7.27%)[h]	2.5	3.2
	8	54.9 (± 3.49%)[h]	5.4	60.3	6.3	1.2 (±7.67%)[h]	0.8 (±5.29%)[h]	6.6 (±2.83%)[h]	8.6	12.5
Novozym 435[j]	0	84.7 (± 2.73%)[h]	0	84.7	0	0	0	0	0	0
	1	44.3 (± 0.84%)[h]	19.2	63.5	22.7	0	0	0	0	0
	2	37.1 (± 3.62%)[h]	41.6	78.7	49.1	4.2 (±5.01%)[h]	9.3 (±2.54%)[h]	4.9 (±3.62%)[h]	18.4	18.9
	3	40.7 (± 2.20%)[h]	26.2	66.9	30.9	0	9.4 (±3.62%)[h]	0	9.4	12.3
	4	42.9 (± 0.70%)[h]	49.9	92.8	58.9	8.9 (±3.92%)[h]	12.8 (±4.16%)[h]	11.4 (±3.62%)[h]	33.1	26.3
	6	42.3 (± 7.88%)[h]	68.4	110.7	80.7	6.7 (±5.95%)[h]	26.3 (±3.62%)[h]	0	33.0	22.9
	8	33.1 (± 3.13%)[h]	57.1	90.2	67.4	13.4 (±6.23%)[h]	14.5 (±6.96%)[h]	18.0 (±4.78%)[h]	45.9	33.7

[a] Peak area of tricaprylin from HPLC analysis.

[b] The sum of the peak areas corresponding to caprylic acid, monocaprylin and dicaprylin, resulting from the hydrolysis of tricaprylin.

[c] The sum of the peak areas corresponding to tricaprylin, dicaprylin, monocaprylin and caprylic acid

[d] The hydrolysis peaks divided by the initial peak area of tricaprylin of 84.7 at time 0, multiplied by 100.

[e] Unknown peaks in the HPLC chromatogram representing lipophenols.

[f] The sum of lipophenol peak areas.

[g] The total lipophenols divided by the sum of the total lipid and total lipophenols, multiplied by 100.

[h] Relative percent standard deviation calculated by dividing the standard deviation by the mean, multiplied by 100.

[i] Enzyme preparation from *Mucor meihei*.

[j] Enzyme preparation from *Candida antarctica*.

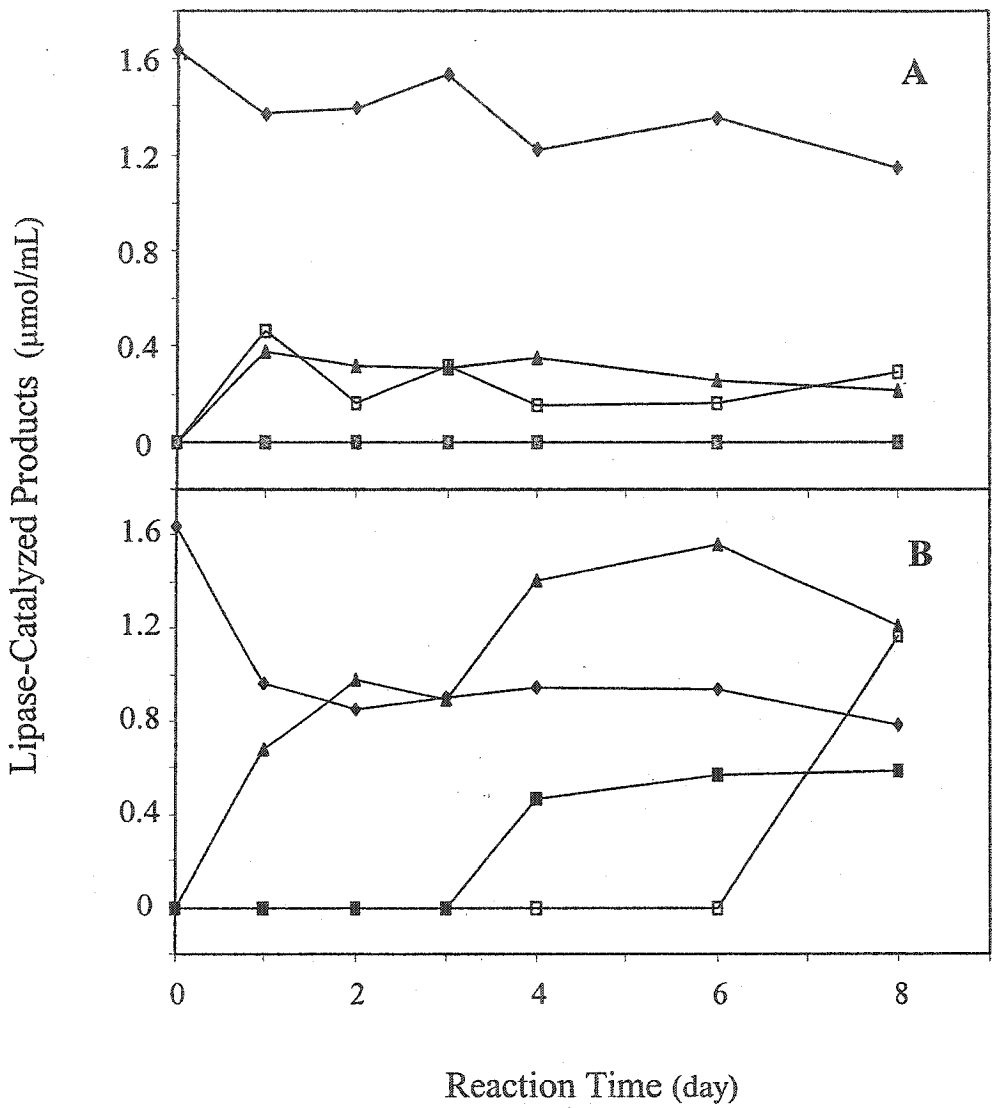

Figure 21. Concentrations of lipase-catalyzed products, including tricaprylin (◆), dicaprylin (□), monocaprylin (■) and caprylic acid (▲) obtained from the HPLC analysis, with (A) Lipozyme IM from *Mucor meihei* and (B) Novozym 435 (B lipase) from *Candida antarctica* in hexane medium with tricaprylin and catechol as substrates.

IM with a maximum of 33.7 at day 8 due to the presence of large amounts of the 3 unknowns, and decreasing concentrations of tricaprylin and CA.

5.2. Caprylic Acid as Model Fatty Acid Substrate

5.2.1. Esterification of Caprylic Acid and Catechin

5.2.1.1. Esterification Reaction and pH of the Reaction Medium with Time

Figure 22A shows that for the lipase reaction medium using CA and catechin as substrates, there was an increase in the consumed concentration of CA thereby suggesting the esterification of CA with catechin to produce lipophenols. Increases in the pH of the reaction medium (Fig. 22B) may also be due to decreases in CA concentrations as a result of subsequent esterification with catechin. The results show that during Lipozyme IM biocatalysis in the reaction medium containing CA and catechin as substrates, a linear consumption of CA occurred with respect to reaction time reaching a concentration of 11.03 (±0.10) μmol CA/mL after 3 days of incubation time, followed by a sharp decrease to 4.12 (±0.18) μmol CA/mL at day 4 and then a subsequent increase in concentration to 8.59 (±0.19) μmol CA/mL after 6 days of incubation time. The pH of the reaction medium (Fig. 22B) remained stable at 4.5 throughout the 10-day period except for a slight increase to 4.7 (±0.083) at day 3. With Novozym 435 as biocatalyst, a higher CA consumption of 12.01 (±0.41) μmol CA/mL was observed after 1 day of incubation time and then remained constant while the pH of the reaction medium showed a large increase from 4.9 (±0.039) to 6 (±0.14) by day 1 and remained fairly stable.

The overall findings (Fig. 22) show that depending on the enzymes used, the concentration of FFAs varied with time where Novozym 435 had the highest degree of esterification in the hexane reaction medium with CA and catechin as substrates, followed by Lipozyme IM.

5.2.1.2. Effect of Water Activity and Water Content on the Lipase Reaction

Figure 23 indicates that the reaction medium had constant a_w values over time for the lipases of Lipozyme IM and Novozym 435. The theoretical water content in the reaction medium containing sodium phosphate buffer was 0.2756% whereas the actual experimental water contents were 0.310% (± 0.037) and 0.195% (± 0.021) for the reaction

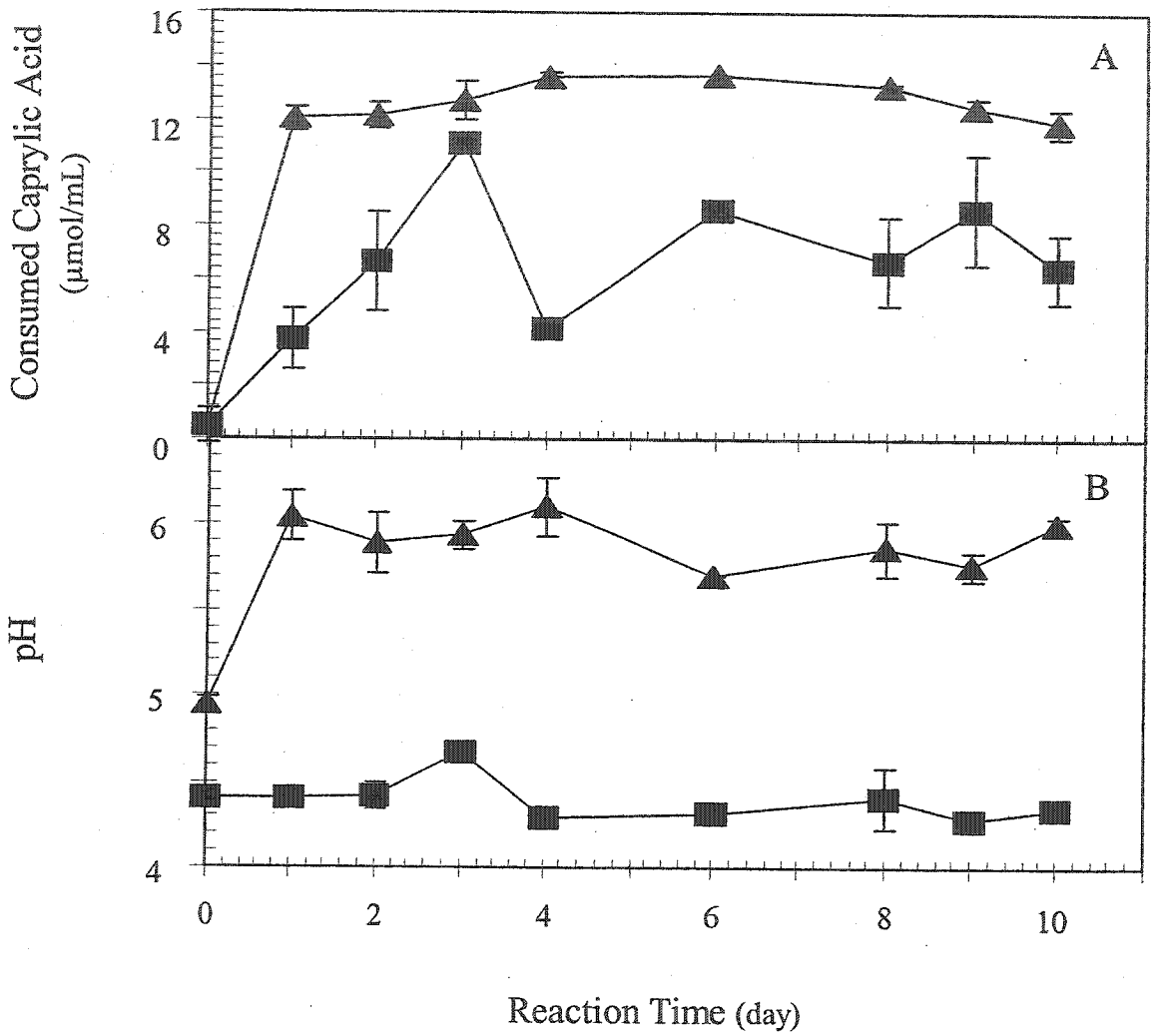

Figure 22. Effect of reaction time on enzyme activity (A) and pH of the hexane reaction medium (B) of Lipozyme IM from *Mucor meihei* (■) and Novozym 435 (B lipase) from *Candida antarctica* (▲) using caprylic acid and catechin as substrates. The error bars (I) represent the standard deviation values.

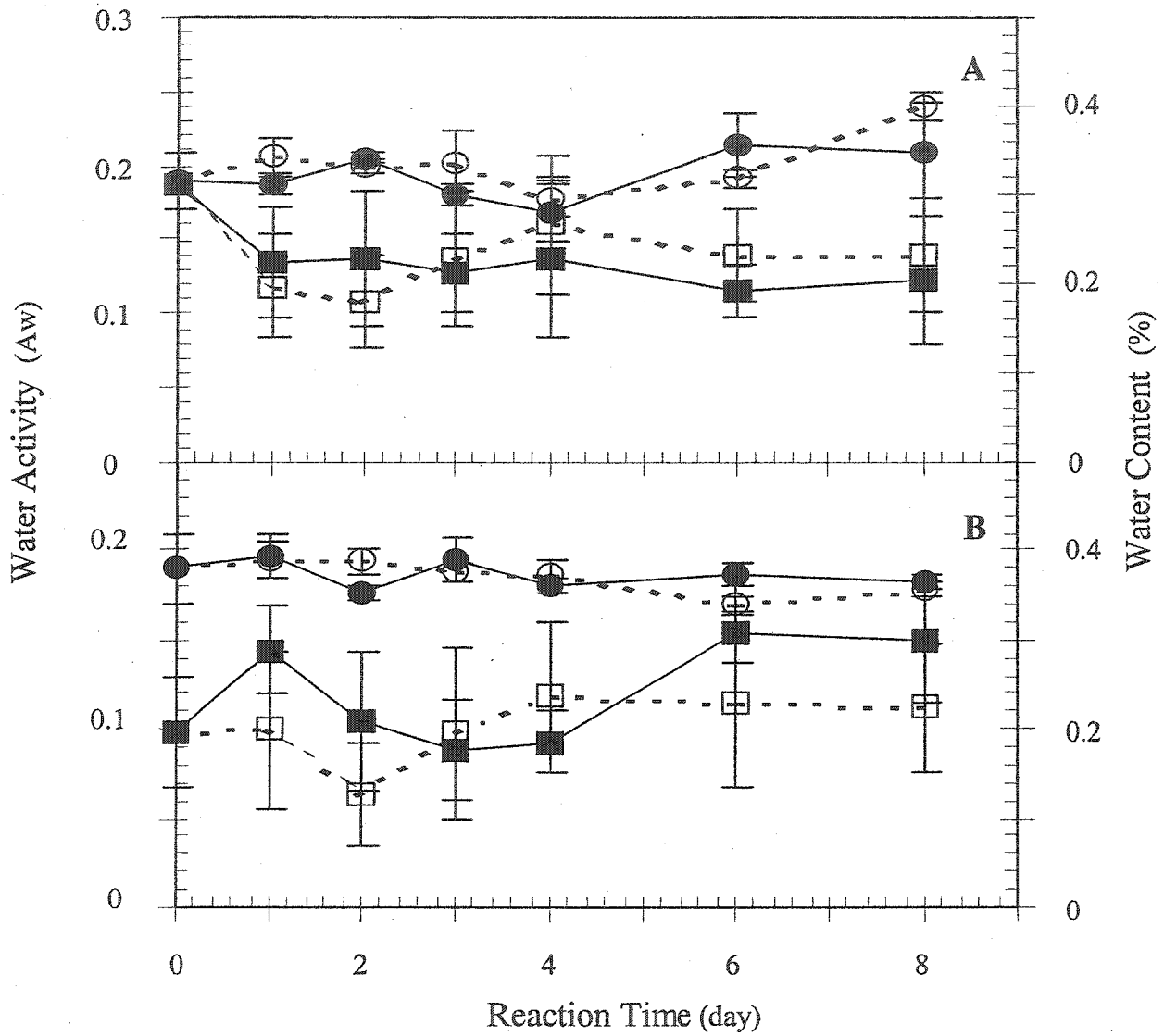

Figure 23. Changes in the water activity of the sample (●) and control (○) assays and changes in the water content of the sample (■) and control (□) assays in the hexane reaction medium of Lipozyme IM lipase from *Mucor meihei* (A) and Novozym 435 lipase (B lipase) from *Candida antarctica* (B) using caprylic acid and catechin as substrates. The error bars (I) represent the standard deviation values.

medium with the Lipozyme IM and Novozym 435 lipases, respectively. The a_w value of the reaction medium containing Lipozyme IM remained fairly stable from a_w 0.168 (± 0.018) a_w to 0.213 (± 0.021) (Fig. 23A). The water content of the reaction medium containing Lipozyme IM decreased from 0.310% (± 0.037) to 0.228% (± 0.027) at day 1 and then remained fairly constant at 0.204% (± 0.022) by day 6. The a_w of the reaction medium containing Novozym 435 remained somewhat constant between the range of 0.177 (± 0.0043) to 0.197 (± 0.012) a_w. The water content of the reaction medium containing Novozym 435 increased from 0.195% (± 0.021) to 0.284% (± 0.032) during day 1 of incubation followed by a decrease to 0.177% (± 0.019) after 3 days and then an increase to 0.306% (± 0.032) by day 6 (Fig. 23B).

The general findings (Fig. 23) indicate very little overall variations in the a_w and water contents in the hexane reaction medium containing Lipozyme IM and Novozym 435.

5.2.1.3. HPLC Analysis of Caprylic Acid and Lipophenols

The HPLC analyses of the esterification reaction between CA and catechin in hexane medium using Lipozyme IM (Fig. 24B and 25B) and Novozym 435 (Fig. 24C and 25C) demonstrated the production of only one novel molecule (unknown 2) at 205 and 280 nm with a retention time of 5.6 min. An absorbance scan of the novel molecule between 190 to 400 nm was similar to unknown 2 (Fig. 15B, 15C, 16B and 16B) that was present in the lipase reactions using tricaprylin and catechin as substrates. These findings confirm that unknown 2 resulted from the esterification of CA and catechin.

The overall results (Table 5) shows that the relative % esterification of CA with catechin using Lipozyme IM showed a maximum of 44.0 at day 3 and decreased by half by day 8, whereas Novozym 435 showed a maximum relative % esterification of 54.0 by day 1 and remained fairly stable over the next 8 days, except for a small decrease to 30.6 by day 4.

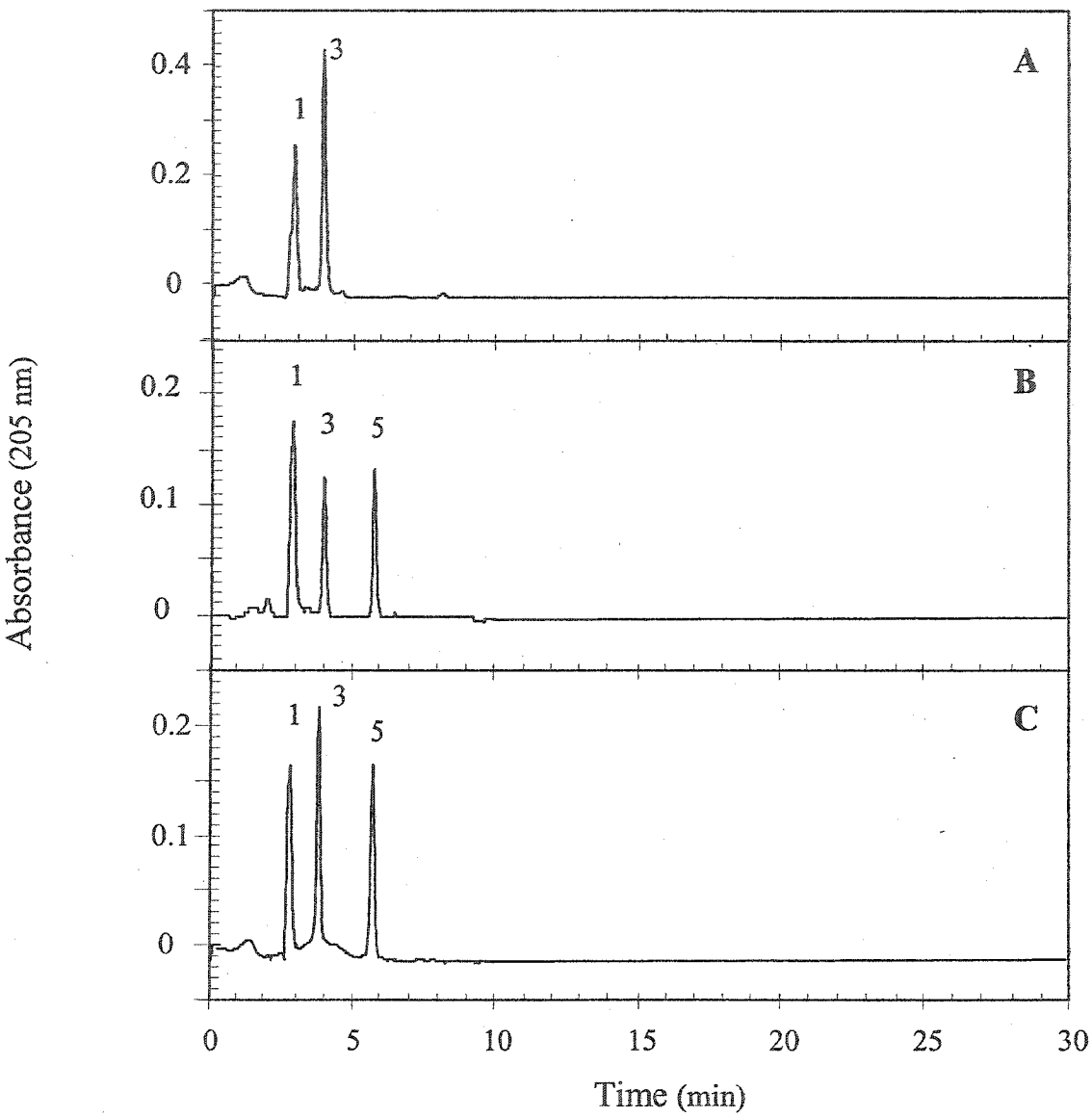

Figure 24. HPLC chromatograms of the reaction assay components of (A) the control, (B) Lipozyme IM lipase from *Mucor meihei* and (C) Novozym 435 lipase (B lipase) from *Candida antarctica* in hexane medium with caprylic acid and catechin as substrates, after 8 days of incubation where the products were identified to be (1) catechin, (3) caprylic acid and (5) unknown 2.

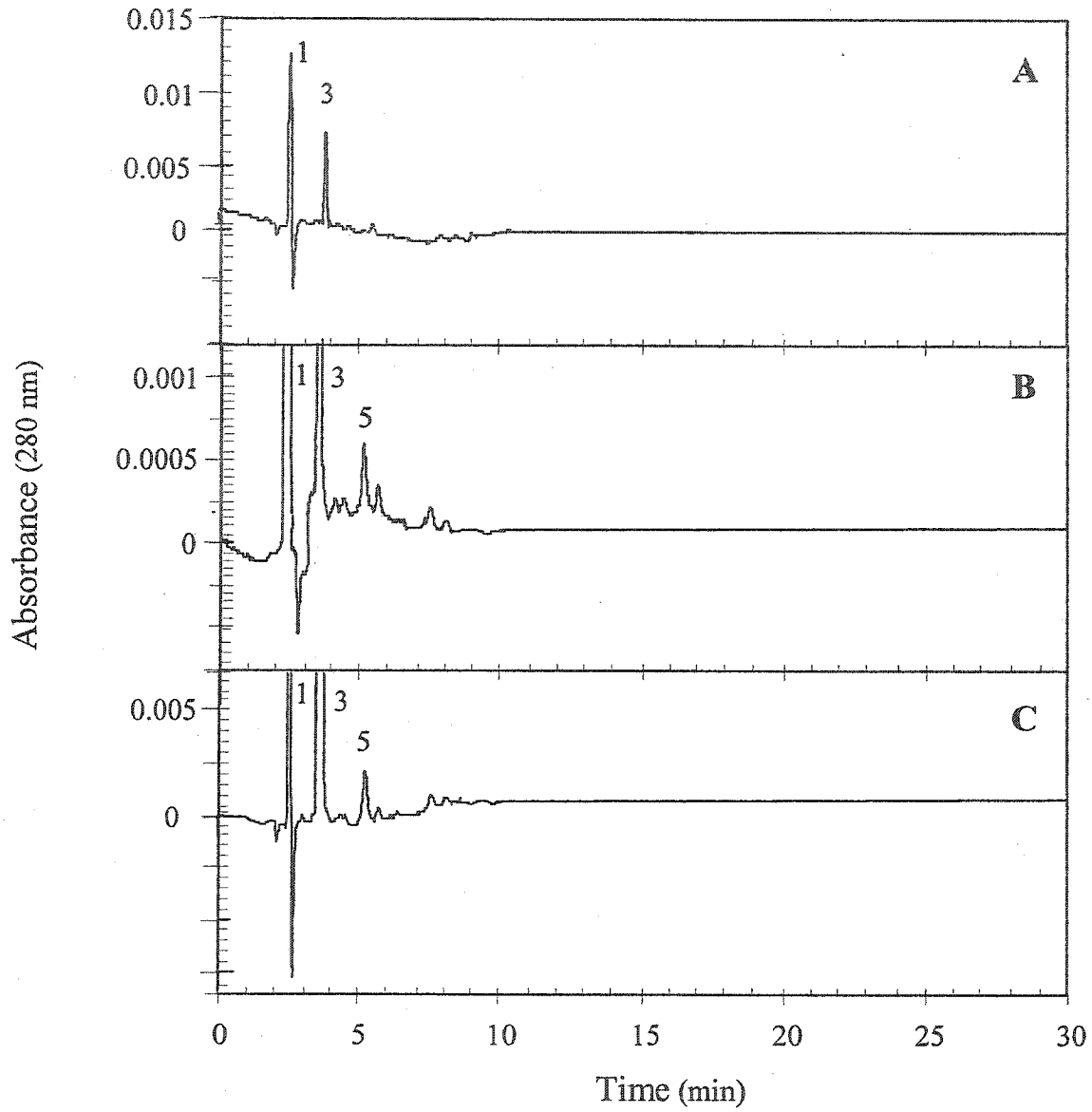

Figure 25. HPLC chromatograms of the reaction assay components of (A) the control, (B) Lipozyme IM lipase from *Mucor meihei* and (C) Novozym 435 lipase (B lipase) from *Candida antarctica* in hexane medium with caprylic acid and catechin as substrates, after 8 days of incubation where the products were identified to be (1) catechin, (3) caprylic acid and (5) unknown 2.

Table 5. Comparison of the consumed peak areas from the HPLC analyses of the esterification reaction between caprylic acid and catechin in hexane medium, using different enzyme preparations.

Enzyme Preparation	Reaction Time (days)	Caprylic Acid	
		Peak area Consumed[a]	Relative Esterification[b] (%)
Lipozyme IM[d]	0	0	0
	1	5.9 (±5.66)[c]	14.9
	2	5.0 (±0.91)[c]	12.7
	3	17.5 (±4.09)[c]	44.0
	4	15.8 (±0.31)[c]	40.0
	6	15.4 (±4.50)[c]	38.7
	8	7.3 (±3.53)[c]	18.3
Novozym 435[e]	0	0	0
	1	21.5 (±4.08)[c]	54.0
	2	19.0 (±2.72)[c]	48.0
	3	16.0 (±3.89)[c]	40.4
	4	12.1 (±4.57)[c]	30.6
	6	19.0 (±6.05)[c]	47.8
	8	17.3 (±4.39)[c]	43.7

[a] The peak area consumed is the difference in CA peak areas from the samples and the controls.
[b] The peak area consumed divided by the initial peak area of 39.7 at time 0, multiplied by 100.
[c] Relative standard deviation calculated by dividing the standard deviation by the mean, multiplied by 100.
[d] Enzyme preparation from *Mucor meihei*.
[e] Enzyme preparation from *Candida antarctica*.

5.2.2. Esterification of Caprylic Acid and Catechol

5.2.2.1. Esterification reaction and pH of the reaction medium with time

Figure 26A shows that for the lipase reaction using CA and catechol as substrates, there was an increase in the consumed concentration of CA, thereby suggesting the esterification of CA with catechol. Increases in the pH of the reaction medium may also be due to the increases in consumed CA concentration as a result of esterification (Fig. 26B). The reaction catalyzed by Lipozyme IM showed a linear consumption of CA with time, with a maximum concentration of 2.66 (±0.00078) µmol CA/mL at day 3 of incubation, followed by a decrease in concentration to 0.59 (±0.061) µmol CA/mL by day 4, and then a subsequent increase to 2.39 (±0.040) µmol CA/mL at day 8. The pH of the reaction medium containing Lipozyme IM decreased from 4.5 (±0.011) to 4 (±0.0035) by day 4 and then remained constant (Fig. 26B). The reaction catalyzed by Novozym 435 showed a large concentration of consumed CA of 13.22 (±0.010) µmol CA/mL at day 1, after which it was stable. The pH of the reaction medium containing Novozym 435 also increased from 5 (±0.28) to 5.7 (±0.13) over a 2-day period and then remained constant.

The overall findings (Fig. 26) show that depending on the enzymes used, the FFA concentrations varied with time where Novozym 435 had a much higher degree of esterification in the hexane reaction medium with CA and catechol as substrates compared to Lipozyme IM.

5.2.2.1. HPLC Analysis of Caprylic Acid and Lipophenols

The HPLC analysis of the esterification reaction between CA and catechol in hexane medium using Lipozyme IM (Fig. 27B and 28B) and Novozym 435 (Fig. 27C and 28C) demonstrated the production of one novel product at 205 and 280 nm that was not present in the control assays (Fig. 27A and 28A). The unknown had a retention time of 5.49 min. An absorbance scan of the novel product between 190 to 400 nm was similar to unknown 2 (Fig. 19B, 19C, 20B and 20C) obtained during the lipase reaction with tricaprylin and catechol as substrates, thereby confirming that unknown 2 resulted due to esterification of CA and catechol.

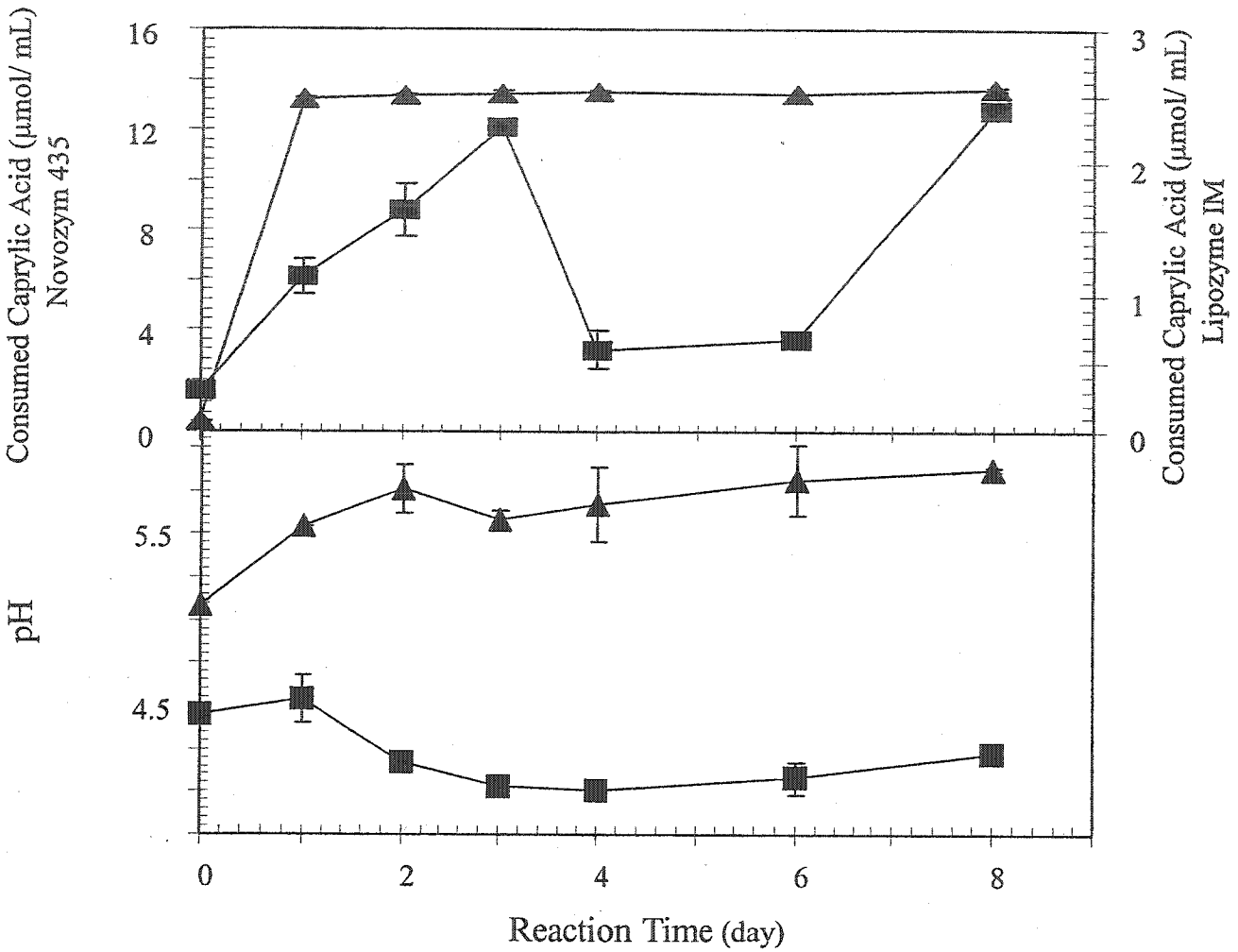

Figure 26. Effect of reaction time on enzyme activity (A) and pH of the hexane reaction medium using catechol and caprylic acid as substrates, with Lipozyme IM from *Mucor meihei* (■) and Novozym 435 (B lipase) from *Candida antarctica* (▲). The error bars (I) represent the standard deviation values.

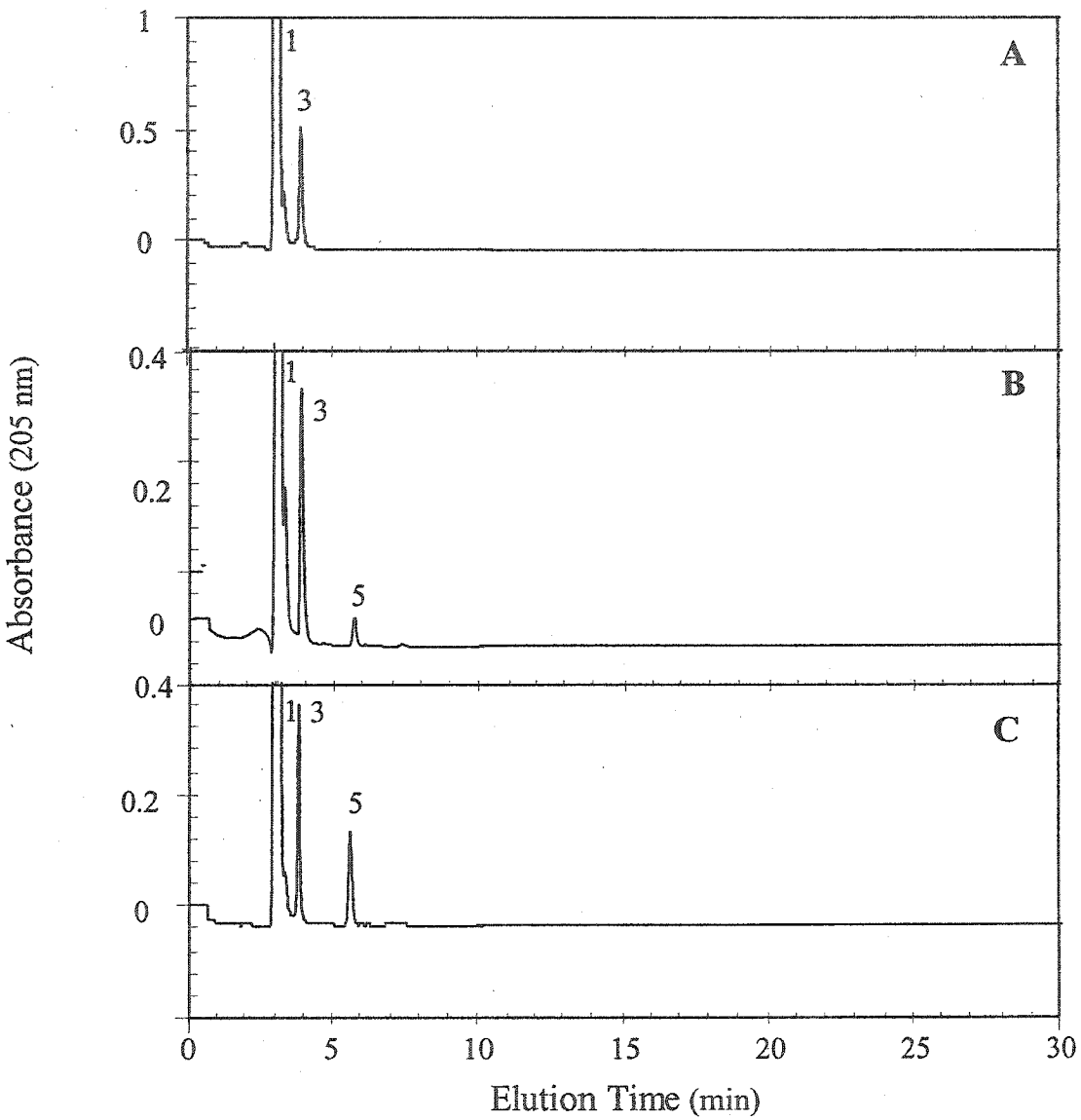

Figure 27. HPLC chromatograms of the reaction assay components of (A) the control, (B) Lipozyme IM lipase from *Mucor meihei* and (C) Novozym 435 lipase (B lipase) from *Candida antarctica* in hexane medium using caprylic acid and catechol as substrates, after 3 days of incubation where the products were identified to be (1) catechol, (3) caprylic acid and (5) unknown 2.

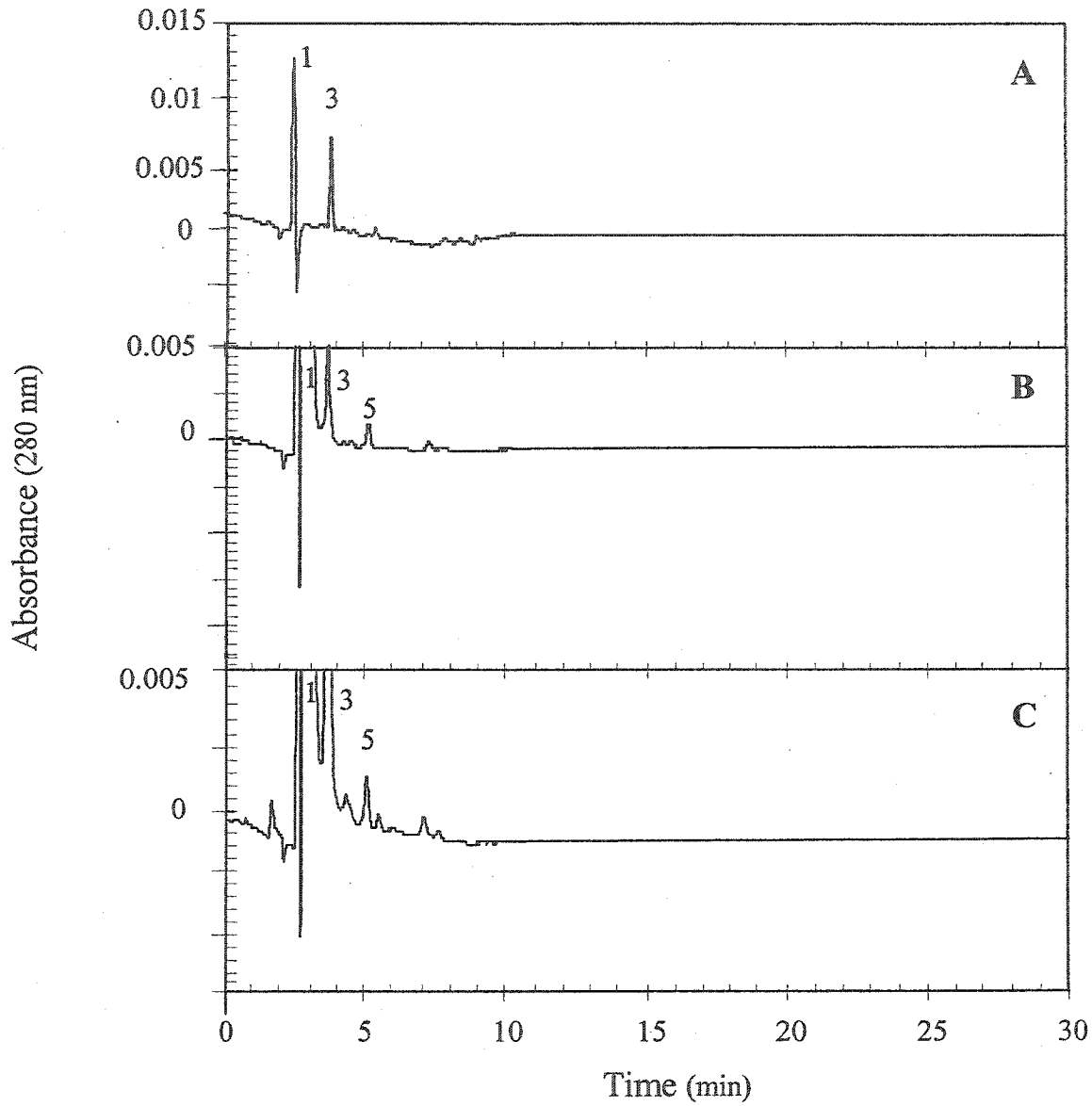

Figure 28. HPLC chromatograms of the reaction assay components of (A) the control, (B) Lipozyme IM lipase from *Mucor meihei* and (C) Novozym 435 lipase (B lipase) from *Candida antarctica* in hexane medium with caprylic acid and catechol as substrates, after 3 days of incubation where the products were identified to be (1) catechin, (3) caprylic acid and (5) unknown 2.

The overall results (Table 6) show that the relative % esterification using Lipozyme IM showed a maximum of 26.3 at day 1 of reaction time and then decreased over time. The relative % esterification with Novozym 435 increased over the 6-day period with a maximum of 70.4 at day 6 followed by a sharp decrease to 9.0 over the next 2-day period.

Table 6. Comparison of the consumed peak areas from the HPLC analyses of the esterification reaction between caprylic acid and catechol in hexane medium, using different enzyme preparations.

Enzyme Preparation	Reaction Time (Days)	Caprylic Acid	
		Peak Area Consumed[a]	Relative Esterification[b] (%)
Lipozyme IM[d]	0	0	0
	1	15.7 (±4.00)[c]	26.3
	2	11.7 (±6.00)[c]	19.7
	3	7.3 (±4.32)[c]	12.3
	4	8.9 (±6.40)[c]	15.0
	6	5.6 (±4.50)[c]	9.4
	8	7.2 (±3.34)[c]	12.2
Novozym 435[e]	0	0	0
	1	2.2 (±6.46)[c]	3.7
	2	2.8 (±4.33)[c]	4.7
	3	25.0 (±5.79)[c]	42.0
	4	30.2 (±3.83)[c]	50.7
	6	41.9 (±2.94)[c]	70.4
	8	5.4 (±4.57)[c]	9.0

[a] The peak area consumed is the difference in CA peak areas from the samples and controls.
[b] The peak area consumed divided by the initial peak area of 59.6 at time is 0, multipilied by 100.
[c] Relative standard deviation calculated by dividing the standard deviation by the mean, multiplied by 100.
[d] Enzyme preparation from *Mucor meihei*.
[e] Enzyme preparation from *Candida antarctica*.

6. DISCUSSION

6.1. Tricaprylin as Model Lipid Substrate

6.1.1. Interesterification of Tricaprylin and Catechin

The overall findings (Fig. 10) show that depending on the enzymes used, the concentration of released FFAs varied with time where Lipozyme IM had the highest degree of hydrolysis with a FFA concentration of 10.8 μmol/mL in the hexane reaction medium, followed by Novozym 435, and then by Lipase N with FFA concentrations of 7.48 and 1.66 μmol/mL, repectively. Bloomer *et al.* (1990) reported that out of 12 lipases tested, the highest specific activity was obtained with lipase extracts from *R. arrhizus*, and the lowest with enzyme preparations from *R. niveus* (Amano N) in titration studies of FFA using sunflower seed oil as substrate. Lai *et al.* (2000) investigated the transesterification of palm stearin with anhydrous milkfat in a solvent free system and reported that the degree of hydrolysis (% FFA) varied slightly depending on the selected lipase preparations, including *R. niveus* (1.58%), *A. niger* (1.79%), *C. rugosa* (2.10%), *Alcligenes* sp. (2.56%), *Pseudononas* sp. (2.61%) and immobilized *R. meihei* (Lipozyme IM 60) (2.18%).

Decreases in the pH of the reaction medium (Fig. 10B) may be due to the hydrolysis of the substrate tricaprylin by lipase resulting in the formation of FFAs (Fig. 10A). These pH changes in the reaction medium may produce variations in the hydrolytic and esterification activities of the lipase as a result of changes in the pH of the microenvironment of the enzyme, thereby altering its ionization state. In an aqueous system and at different pH environments, the ionization state of an enzyme can affect its catalytic activity and stability (Harper *et al.*, 2000 and 2001). Harper *et al.* (2000) observed that for a subtilisin Carlsberg catalyzed transesterification reaction in toluene, a minor amount of acid was produced as a by-product of hydrolysis, which may have led to the protonation of the enzyme, thereby resulting in a decrease in its activity. These authors reported that the addition of solid state buffers to the reaction medium was able to prevent protonation of the enzyme, thereby shortening reaction times and improving yields.

The optimum temperature for enzyme activity is influenced by the conditions of the reaction, such as the immobilization supports used, chemical enzyme modifications as well as pH and presence of organic solvents (Dordick, 1989). The Lipase N preparation had optimal activity at 37.5°C, similar to that reported by Safari *et al.* (1994) and Kermasha *et al.* (1995) for the interesterification of butter fat with Lipase N. Tweddell *et al.* (1999) compared the lipase from *R. niveus* (Lipase N) in its free and immobilized states at a slightly higher temperature of 40°C, and found that the hydrolytic activity increased when Duolite XAD 761 support was used. Recent research has shown that Lipozyme IM and Novozym 435 are thermostable and can be active over a broad temperature range of 30-70°C (Goma-Doncescu and Legoy, 1997) and 40-60°C (Novo Nordisk A/S, 2000), respectively. The maximal temperature of 55°C for lipase activity for both Lipozyme IM and Novozym 435 found in this study, is within the temperatures ranging from 30°C to 65°C reported in the literature (Guyot *et al.* 1997; Stamatis *et al.*, 1999, 2000 and 2001; Shimada *et al.*, 1999; Zhang and Hayes, 1999). Senanayake and Shahidi (1999) observed that the enzyme reaction involving Lipozyme IM and Novozym 435 showed constant activity in the temperature range of 37-55°C in hexane medium.

The activation energy values reported in Table 2 are within the range of 40 to 200 kJ/mole reported in the literature for enzyme-catalyzed reactions. The activation energy for the esterification of geranyl butyrate with Novozym 435 was estimated to be 62.3 kJ/mole (14.9 kcal/mole) in a solvent free medium (Oguntimein *et al.*, 1995), which was lower than 177.9 kJ/mole obtained for Novozym 435 in this study. The free and immobilized enzymes from *Burkholderia cepica* showed activation energies of 21.3 and 20.7 kJ/mole, respectively, for the hydrolysis of *p*-nitrophenyl palmitate in *n*-heptane (Pencreac'h and Baratti, 1999). Differences in the activation energies reported may be due to the different substrates used, the water activity (a_w) of the reaction medium, or the medium itself in which hydrolysis occured.

The use of the maximal temperature of 55°C (Fig 12) for biocatalysis resulted in an increase in the hydrolysis rate, thereby suggesting that the subsequent esterification reactions could take place in a shorter time. The results (Fig. 10A) show that at the higher temperature of 55°C, the hydrolysis reaction (Fig. 12A) reached a maximum at

day 1 and 4 with Lipozyme IM, and at day 2 with Novozym 435 compared to the activity shown previously at 37.5°C. Bilyk *et al.* (1991) reported that an increase of 25°C in temperature decreased the reaction time from 20 to 6 hours for the hydrolysis of tallow in hexane by enzyme preparations from *R. meihei*. Zhang *et al.* (2000) noted a slight increase in the FFA content with increasing reaction temperatures for the interesterification of palm stearin and coconut oil by Lipozyme IM; similar observations were noticed when the same reaction was catalyzed by Lipozyme TL IM (*Thermomyces lanuginosa*) (Zhang *et al.*, 2001). Pencreac'h and Baratti (1999) noted that the reaction rate for hydrolysis of *p*-nitrophenyl palmitate increased with temperatures from 25 to 65°C with free and immobilized enzyme prepatations of *Bukholderia cepica* in *n*-heptane.

The specific activities of the three enzymes with respect to agitation speed (Fig 13) showed a maximum at 100 rpm, which was different from those reported in the literature and may be due to the nature of the hydrolysis and esterification reactions taking place. Safari *et al.* (1994) and Kermasha *et al.* (1995) used higher agitation speeds of 145 rpm for the interesterification of butterfat by Lipase N. Medina *et al.* (1999) investigated the esterification of poluyunsaturated fatty acids with glycerol by Lipozyme IM and Novozym 435, and reported that higher rates were observed at 200 rpm compared to those obtained at 175 and 100 rpm in hexane medium. Yang et al. (2001) noted that the transesterification rate of triacetin and steric acid by Chirazyme L-2 (*C. antarctica*) increased as the shaking speed increased from 200 to 400 and 600 rpm in a solvent free system.

The first step in the lipase reaction was the hydrolysis of tricaprylin to produce FFAs, dicaprylin and water; however, at any step during the enzyme assay, any number of a series of reactions could also occur including further hydrolysis of tricaprylin, transesterification or esterification between CA and catechin. The hydrolysis of tricaprylin with Lipase N, Lipozyme IM and Novozym 435 was paralleled by a slight increase in water content (Fig 14A) of the reaction medium. The a_w of the reaction medium is the equilibrium of water between all phases in the reaction system and is an indication of enzyme hydration (Partridge *et al.*, 1998). Since water is distributed between all phases in a reaction system (Partridge *et al.*, 1998), at certain water contents,

the water associated with each phase will differ depending on the nature of the organic solvent. In addition, the a_w and water content required for optimal enzyme activity in water immiscible solvent systems depends on the source of enzyme, the solvent polarity and the type of support used (Dordick, 1989; Gandhi *et al.*, 2000). The different support materials used for the immobilization of Lipozyme IM and Novozym 435 may also have had an effect on the partitioning of water (Oguntimein *et al.*, 1995), as Lipozyme IM was immobilized on macroporus anion exchange resin and Novozym 435 was immobilized on an acrylic resin.

Tweddell *et al.* (1998; 1999) noted that Lipase N showed a slightly better activity at 0.75 a_w in *n*-hexane for the esterification of oleic acid and ethanol. The same authors also reported that when Lipase N was immobilized onto a Duolite XAD 761 support, there were no significant changes for the enzymatic esterification of oleic acid and ethanol between 0.11-0.75 a_w. The a_w values reported by Tweddell *et al.* (1998; 1999) are higher than the a_w of 0.179 for the hexane reaction medium used for the reaction of tricaprylin and catechin with Lipase N (Fig. 14A). Zang *et al.* (2000) interesterified palm stearin and coconut oil using Lipozyme IM and observed a stable degree of interesterification when no water was added to the solvent free reaction system. Li and Ward (1993) used Lipozyme IM 60 from *M. miehei* for the esterification of glycerol with a *n*-3 fatty acid concentrate of cod liver oil in hexane medium and observed an 80% esterification yield with 0% water. Cerdán *et al.* (1998) reported that an initial water content between 0 and 2% did not alter the degree of esterification of glycerol with *n*-3 polyunsaturated fatty acid concentrates from cod liver oil in a hexane medium using an enzyme preparation of Novozym 435. Medina *et al.* (1999) demonstrated that the highest production of triacyglycerols from glycerol and concentrated polyunsaturated fatty acids from cod liver oil was obtained with the use of dehydrated Novozym 435, with no additional water in a hexane reaction medium. These findings reported in the literature suggest that little water was required to maintain activity with Lipozyme IM and Novozym 435, and the water contents used were lower than the actual experimental water content (v/v) of 0.203% and a_w value of 0.158 used in our study in the hexane reaction medium with tricaprylin and catechin as substrates (Fig. 14B and 14C).

The overall findings from the HPLC analysis (Fig. 15, 16 and Table 3) show that the enzymes of Lipozyme IM and Novozym 435 produced three unknowns from tricaprylin and catechin. The findings based on the appearance of the unknown peaks and the presence of the hydrolysis products (Fig. 17) suggest that unknown 1 may be due to the esterification of monocaprylin and catechin, while unknown 2 may be due to the esterification of CA and catechin, and unknown 3 could be due to the esterification of dicaprylin and catechin.

The concentration of FFAs observed from the titration analysis (Fig. 12A) and the pH of the reaction medium (Fig. 12B) may reflect the % hydrolysis of tricaprylin determined by HPLC. The HPLC analysis for the reaction between tricaprylin and catechin catalyzed by Lipozyme IM showed a decreasing trend in the concentration of FFAs over time (Fig. 17B), similar to that observed for the titration analysis of the FFAs (Fig. 12A). Conversely, the HPLC analysis of the reaction between tricaprylin and catechin catalyzed by Novozym 435 showed slightly different profiles, but were in agreement until day 4 of incubation.

The hydrolysis of tricaprylin should have consumed water, while the esterification of lipophenols should have produced water during the continuous hydrolysis/ esterification process. The reaction catalyzed by Lipozyme IM showed a high degree of hydrolysis of tricaprylin to produce dicaprylin and CA on day 1 (Fig. 17B) and a small biogeneration of lipophenols, resulting in little change in a_w and no change in water content (Fig. 14A). The reaction catalyzed by Novozym 435 showed decreases in the concentrations of tricaprylin, dicaprylin, monocaprylin and CA, and an increase in unknown 2 was observed on day 4 along with little change in water content and a_w (Fig. 14B). The reaction rates may be different for each of the products as well as substrates, and could in turn influence the presence and disappearance of some unknown peaks.

The esterification and transesterification rates for Lipase N reported in the literature indicated low enzyme activity or inactivity. These findings were similar to our experimental results observed with Lipase N, which showed no significant activity (Fig. 10A) and no esterification products throughout the incubation period with tricaprylin and

catechin as substrates. Chopineau *et al.* (1988) screened lipase extracts from *R. niveus* for the transesterification of triolein and sugar alcohol (sorbitol) in pyridine and reported the absence of enzyme activity. Wang *et al.* (2002) used enzyme preparations from *Rhizopus* sp. (Amano N) for the esterification of *dl*-methanol with valeric acid in cyclohexane to produce a menthyl ester and noted that there was hydrolytic activity but no esterification after 120 h. Lai *et al.* (2000) transesterified palm stearin with anhydrous milkfat in a solvent free system, and the degree of transesterification (%) varied with selected lipase preparations from *A. niger* (10.3%), *Alcligenes* sp. (12.2%), *Pseudononas* sp. (33.9%), *C. rugosa* (8.0%), immobilized *R. meihei* (Lipozyme IM 60) (32.3%) and *R. niveus* (6.8%) with the lowest rate.

Many transesterification reactions of fatty acids and triacyglyercols using Lipozyme IM and Novozym 435 are reported in the literature. Akoh *et al.* (1996) incorporated EPA into evening primose oil using enzymes from Novozym 435 in a hexane medium, and obtained 43% incorporation of EPA after 24 h of incubation, followed by a decrease after 48 h. Senanayake and Shahidi (1999) determined that there was a higher incorporation of DHA into borage oil in hexane medium when Lipozyme IM, Novozym 435 and *Pseudononas* sp. (PS-30) were used compared to other enzyme extracts, with an incorporation of 13.1, 25.8 and 16.8%, respectively after 24 hours of incubation. Kim *et al.* (2001) transesterified tricaprylin and conjugated linoleic acid ethyl ester, observing an incorporation of 53.8, 56.9 and 41.4% after 24 h of incubation in hexane medium using enzymes of Lipozyme IM, Novozym 435 and Lipase PS-C (*P. cepacia*), respectively. Recently, Compton and King (2001) used supercritical CO_2 for the transesterification of triolein and ethyl ferulate (ethyl 4-hydroxy-3-methyl cinnamate) to produce ferulyl glycerol, monoferuloyl-monooleyl-glycerol and feruloyl-dioleoyl-glycerol, and reported a combined yield of monoferuloyl-monooleyl-glycerol and feruloyl-dioleoyl-glycerol of 74% after 2 days of incubation with Novozym 435. The same authors reported that when transesterification was conducted in a toluene environment, there was a 46% equilibrium yield after 3 days, whereas in a solvent free medium, there was a 77% equilibrium yield after 6 days. These literature findings occurred using shorter incubation times than those observed for the lipase-catalyzed

reaction of tricaprylin and catechin where the relative % esterification (Table 3) was 47.8 and 37.3 with Lipozyme IM after 4 days and Novozym 435 after 8 days, respectively.

6.1.2. Interesterification of Tricaprylin and Catechol

The overall results show that the lipase-catalyzed reaction between tricaprylin and catechol (Fig. 18A) with Lipozyme IM released high FFA concentrations at day 1 and 3 of incubation, compared to an increase in the concentration of released FFAs at day 1 and 4 with tricaprylin and catechin as substrates (Fig. 12A). The overall results show that the reaction medium with Lipozyme IM had 4 times less FFAs with catechol as the phenolic substrate with tricaprylin compared to that observed with catechin as the phenolic substrate. These findings suggest that the enzyme may be inhibited by catechol compared to catechin. The reaction medium containing Novozym 435 showed high FFA concentrations at day 1, 4 and 8 with catechol and tricaprylin as substrates; however, when catechin was the phenolic substrate, the FFA concentrations increased at day 2 and 9 (Fig. 12B). The overall findings indicate that biocatalysis with Novozym 435 showed similar FFA concentrations released during tricaprylin hydrolysis, regardless of the phenolic substrate used.

The pH profiles of the reaction medium containing Lipozyme IM and Novozym 435 were different with catechol (Fig. 18B) and catechin (Fig. 12B) as phenolic substrates. The pH of the reaction medium with catechol as the phenolic substrate and tricaprylin had an initial pH of 6.5. In the presence of Lipozyme IM, a pH decrease of 2.2 units was observed in 1 day, whereas with Novozym 435, more than 4 days were required to reach the same decrease in pH. When catechin was the phenolic substrate with tricaprylin, there was a larger pH decrease for both enzymes (Fig. 12B). These variations in pH may be due to the presence of different FFA concentrations in the reaction medium. The pH of the reaction medium may also change the microenvironment of the enzyme thereby changing its ionization state during the lipase-catalyzed reaction and subsequently affecting its activity and specificity (Harper *et al.*, 2000 and 2001).

The findings obtained from the HPLC analysis (Fig. 19, 20 and Table 4) suggest that the enzymes, Lipozyme IM and Novozym 435 produced the same unknowns in the presence of tricaprylin and catechol. The findings based on the appearance of the unknown peaks and the presence of the hydrolysis products (Fig. 21) suggest that unknown 1 may be due to the esterification of monocaprylin and catechol, while unknown 2 may be due to the esterification of CA and catechol, and unknown 3 could be due to the esterification of dicaprylin and catechol.

The concentration of FFAs observed from the titration analysis during the hydrolysis of tricaprylin (Fig. 18A) was similar to the % hydrolysis of tricaprylin determined by HPLC. The HPLC analysis for the lipase reaction between tricaprylin and catechol with Lipozyme IM (Fig. 21A) as catalyst showed a small initial increase in CA concentration and then remained fairly constant with a small decreasing trend; similarly, this same general trend was observed during the titration of FFAs (Fig. 18A). The overall findings obtained using Novozym 435 as catalyst showed a general increase in FFA concentration over the 8 day incubation period with both the titration analysis (Fig. 18A) and the HPLC analysis (Fig. 21B).

These overall findings (Table 4) suggest that there was 2.3 times more relative % esterification of tricaprylin with catechol as the phenolic substrate using Novozym 435 compared to that obtained with Lipozyme IM. This may suggest that Lipozyme IM had less affinity to catechol as phenolic substrate compared to Novozym 435.

6.2. Caprylic Acid as Model Fatty Acid Substrate

6.2.1. Esterification of Caprylic Acid and Catechin

The lipase-catalyzed reactions using Lipozyme IM and Novozym 435 as biocatalysts required 3 days and 1 day of incubation, respectively for maximal activity as indicated by increases in consumed CA concentrations (Fig. 22A). These esterification rates for Lipozyme IM and Novozym 435 are slower than those observed in the investigations by Wong *et al.* (2000) for the esterification of capric acid and glycerol in hexane medium using lipase preparations from *C. rugosa*, where the esterification rate

increased during 0 to 16 h after which it was slower. Zhang and Hayes (1999) noted that the lipase-catalyzed esterification of *t*-butanol and fructose increased over a 24 h period.

The results (Fig. 22B) show that the initial pH of the reaction medium containing the two enzymes differed by 0.5 pH units. The reaction medium with Lipozyme IM had a lower initial pH value than that with Novozym 435, which may be due to the influence of the different immobilization supports used for the enzymes, as Lipozyme IM and Novozym 435 were immobilized on a macroporus anion exchange and an acrylic resin, respectively. The lower initial pH value of the reaction medium containing Lipozyme IM may therefore be due to the anion exchange immobilization support used which may have repelled a higher concentration of positively charged hydrogen ions (H^+) around the enzyme preparation (Bickerstaff, 1997), thereby decreasing the pH of the reaction medium. The immobilization supports may also have influenced the pH of the reaction medium during the course of the reaction, since the medium pH remained stable for Lipozyme IM, although the concentration of consumed CA increased. The overall results (Fig. 12B) indicate that the reaction medium containing Lipozyme IM and the substrates tricaprylin and catechin had a higher initial pH of 6.5, followed by a decrease to 4.5, which was the same pH as that observed with CA and catechin as substrates. The reaction medium containing Novozym 435 and the substrates tricaprylin and catechin showed an initial pH of 6.6 that subsequently decreased to a pH of 5, whereas with CA and catechin as substrates, the initial pH was 5 and then increased to 6.

The overall results showed that the initial water contents of the reaction medium with Lipozyme IM and Novozym 435 as catalysts differed by 0.1%, while the a_w values remained the same. The overall results also show that initially there was a lower water content in the reaction medium with Novozym 435 compared to that with Lipozyme IM. This initial decrease in the water content may be due to increased binding of the water to the support materials of the immobilized enzymes, the substrates and/or the lipophenolic compounds. The esterification reaction between CA and catechin should have produced an increase in a_w and water content. The overall results show that with Lipozyme IM as biocatalyst, the a_w of the reaction medium remained fairly constant with a slight decrease at day 4 corresponding to a decrease in the consumption of CA with constant water

activity. The a_w values for the reaction medium containing Novozym 435 remained rather constant throughout the 8-day period, while the water content of the reaction medium slightly increased by day 1, coinciding with a maximum consumption of CA (Fig. 22A).

The a_w values measured the water that was in equilibrium at all phases and may therefore be used to as an indication of a higher degree of enzyme hydration (Partridge *et al.*, 1998). However, low a_w values were required, as reported in the literature, to maintain the catalytic activity of Lipozyme IM and Novozym 435. Valivety and co-workers (1992) noted that for the esterification of dodecanol and decanoic acid in hexane medium, the immobilized enzymes from *R. miehei* (Lipozyme IM 20) had a 30% higher reaction rate in an almost anhydrous environment possessing an a_w value of less than 0.0001 compared to that with an a_w of 0.55. Zhang and Hayes (1999) used Lipozyme IM for the esterification of fructose and oleic acid in a *tert*-butanol medium, without any additional water or dehydrating the enzyme and substrates and reported a 80% conversion of the fructose over a 24 h period. Chamouleau and co-workers (2001) observed that the lower the water activity, the higher the synthetic activity of the immobilized enzyme from Novozym 435, where the highest esterification of fructose monopalmitate of 28.5% in 2-methyl-2-butanol was achieved with an initial a_w of less than 0.07. These findings reported in the literature for the enzymes Lipozyme IM and Novozym 435 were lower than the initial actual a_w value of 0.190 used in the hexane reaction medium with CA and catechin as substrates (Fig. 23A and 23B).

The HPLC analysis results (Fig. 24, 25 and Table 5) produced the same unknown 2 that was present in the reaction between tricaprylin and catechin, thereby indicating that the biogeneration of unknown 2 may be due to the esterification of CA and catechin.

The overall findings indicate that the CA concentration obtained by the titration analyses (Fig. 22A), thus producing an increase in the pH of the reaction medium (Fig. 22B), was similar to those analyzed by HPLC (Table 5) over time. The results show that biocatalysis with Lipozyme IM reached the highest consumed CA peak area of 17.5 by day 3 of reaction time, corresponding to the maximum consumed CA and a small pH

increase in the reaction medium. Biocatalysis using Novozym 435 in the same reaction medium showed maximum consumed CA and consumed peak area of CA at day 1 of incubation with a corresponding decrease in the pH of the reaction medium.

Some esterification reactions, reported in the literature between alcohols and phenolic compounds, required longer incubation times than those for the lipase-catalyzed reactions between CA and catechin in hexane medium where the relative % esterification (Table 5) were 44.0 and 54.0 with Lipozyme IM after 3 days and Novozym 435 after 1 day, respectively. Stamatis *et al.* (2001) used enzymes from Lipozyme IM and Novozym 435 to catalyze the reaction between 1-octanol and cinnamic acid and reported 59 and 82% conversion, respectively after 12 days of incubation. These authors reported that when *p*-hydroxyphenylpropionic acid was used with 1-octanol as substrates, there was an esterification of 57 and 97% after 12 days of incubation using Lipozyme IM and Novozym 435, respectively. These same authors reported that the esterification of methoxylated (ferulic acid) or hydroxylated (*p*-coumaric acid) derivatives of cinnamic acid with 1-octanol had lower conversions of 30 and 22% with Lipozyme IM, respectively and 11 and 25 % with Novozym 435, respectively after 12 days of incubation. Guyot *et al.* (1997) esterified 1-butanol with cinnamic acid using Novozym 435 and obtained a 97% esterification after 15 days. Later, Guyot *et al.* (2000) used Novozym 435 for the esterification of 5-caffeoyl quinic acid with fatty alcohols in a solvent free medium, and reported 60 and 40% esterification with C8 and C12 to C18, respectively after 30 days of incubation. Busiman *et al.* (1998) used Novozym 435 for the esterification of butyl, hexyl and dodecyl esters of cinnamic acid in *n*-pentane and obtained 85, 70 and 68% yields after 5 days. These authors reported that hydroxytyrosol (3,4-DHPEA) containing an ethanolic hydroxy group was esterified to caprylic acid in diethyl ether with a 85% yield after 15 h; however, when diethyl ether was replaced with non-polar solvents, *n*-pentane and *n*-hexane, the ester yields were in the range of 70-80%. These same authors noted shorter incubation times were required for the esterification of 3,5-di-*t*-butyl-4-hydroxybenzylalcohol with caprylic acid in cyclohexane, to produce a 98% conversion within 2.5 h of incubation (Busiman *et al.*, 1998). Otto *et al.* (1998) used Novozym 435 for the esterification of glucuronic acid with *n*-butanol and obtained

22% esterification after 30 h of incubation. However, when *n*-butanol was replaced with cinnamic alcohol, there was only 15% esterification.

6.2.2. Esterification of Caprylic Acid and Catechol

The results show that the CA concentrations in the reaction medium containing Lipozyme IM were similar with respect to incubation time for both phenolic substrates, catechin (Fig. 22A) and catechol (Fig. 26A), up to day 4 where 5 times more CA with catechin was consumed compared to that obtained with catechol by day 3 of incubation, thereby suggesting substrate inhibition of enzyme activity by catechol. The CA concentration profiles for Novozym 435 with both the phenolic substrates, catechin (Fig. 22A) and catechol, indicated a sharp consumption at day 1 of incubation, after which the CA concentration remained constant. The pH profiles of the reaction medium were similar for CA with both phenolic substrates with respect to incubation time.

The results (Fig. 26B) also show that the initial pH value of the reaction medium of the two enzymes differed by 0.5 pH units. Lipozyme IM had a lower initial pH value than Novozym 435, which may be due to the immobilization supports of the enzymes. The differences in pH are the same as those observed with CA and catechin (Fig 26B). The overall results also show that the pH of the reaction medium with Lipozyme IM as biocatalyst with CA and catechin as substrates had a constant pH of approximately 4.5 (Fig. 22B), whereas when catechol was the phenolic substrate with CA (Fig. 26B), the initial pH was also 4.5 but decreased slightly by 0.5 pH units during reaction time. The pH of the reaction medium with Novozym 435 as biocatalyst with CA and catechin as substrates increased 1.1 pH units over day 1 to a pH of 6, whereas when CA and catechol were the substrates, similar changes in pH was observed, and the pH increased to 5.7 over a 2 day period.

The HPLC analysis results (Fig. 27, 28 and Table 6) produced the same unknown 2 that was present in the reaction between tricaprylin and catechol, thereby indicating that the biogeneration of unknown 2 may be due to the esterification of CA and catechol.

The CA concentration obtained by the titration analyses (Fig. 26A) and an increase in the pH of the reaction medium (Fig. 26B) may be related to the consumption of CA as analyzed by HPLC (Table 6) over time. The results show that biocatalysis with Lipozyme IM reached a maximum consumed CA peak area at day 1, whereas the CA concentration increased up to day 3 followed by a decrease, and a slight pH decrease of the reaction medium. Biocatalysis with Novozym 435 showed an increase in consumed CA peak area up to day 6 however, the CA concentration increased at day 1 with a corresponding slight increase in the pH of the reaction medium over time.

The esterification reaction between CA and catechol using Novozym 435 as biocatalyst showed 2.7 times higher esterification than that observed with Lipozyme IM (Table 6). This may suggest that Lipozyme IM had less affinity to catechol as phenolic substrate compared to Novozym 435. Similar esterifications of fatty alcohols with phenolic compounds were described in the literature (Guyot *et al.*, 1997 and 2000; Busiman *et al.*, 1998; Otto *et al.*, 1998 and Stamatis *et al.*, 2001).

7. CONCLUSION

The results obtained in this study showed the production of three unknown novel biomolecules, from the lipase-catalyzed reaction of tricaprylin and either catechin or catechol as substrates in hexane medium. The lipase-catalyzed reaction with caprylic acid and either catechin or catechol as substrates produced one unknown peak in the HPLC analysis. The esterification reaction is dependant on the phenolic substrates and enzyme preparations used. The relative % esterification of the lipase-catalyzed reaction of tricaprylin and catechin as substrates was greater with Lipozyme IM compared to Novozym 435, whereas with tricaprylin and catechol as substrates, there was a higher relative % esterification using Novozym 435 than with Lipozyme IM. The lipase-catalyzed reaction with caprylic acid and catechin or catechol as substrates showed a better relative % esterification with Novozym 435 than with Lipozyme IM. The enzyme preparations of Lipozyme IM were greatly inhibited by the phenolic substrate catechol when caprylic acid and tricaprylin were used as the lipid substrates.

The overall results indicate the potential use of the lipase-catalyzed reaction for the esterification of lipids and phenolic compounds by Lipozyme IM and Novozym 435 to produce novel biomolecules that possess altered physical properties from the original substrates.

8. REFERENCES

Abe, M., Schechter, D., Schechter, R.S., Wade, W.H., Weerasooriya, U. and Yiv, S. (1986). Microemulsion formation with branched tail polyoxyethylene sulfonate surfactants. *J. Colloid Interface Sci.* **114**, 342-356.

Acros, J.A., Otero, C. and Hill, C.G.Jr. (1998). Rapid enzymatic production of acylglycerols from conjugated linoleic acid and glycerol in a solvent free system. *Biotechnol. Lett.* **20**, 617-621.

Akoh, C.C., Jennings, B.H. and Lillard, D.A. (1996). Enzymatic modification of evening primose oil: Incorporation of *n*-3 polyunsaturated fatty acids. *J. Am. Oil Chem. Soc.* **73**, 1059-1062.

Al-Saikhan, M.S., Howard, L.R. and Miller, J.C.Jr. (1995). Antioxidant activity and total phenolics in different genotypes (*Solanum tuberosumn L.*). *J. Food Sci.* **60**, 341-343, 347.

Anonymous 1. (1999). Information obtained from the Company A. Lassonde, Inc., Quebec.

Anonymous 2. (1999). Information obtained from the Potato Producers Association of Quebec.

Attwood, D. and Florence, A.T. (1983). Emulsions. In *Surfactant Systems, Their Chemistry, Pharmacy and Biology*, Chapman and Hall, New York., N.Y., pp. 469-566.

Badings, H.T. and De Jong, C. (1983). Glass capillary gas chromatography of fatty acid methyl esters. A study for the quantitative analysis of short and long chain fatty acids in lipids. *J. Chromatogr.* **279**, 493-506.

Bell, G., Janssen, A.E.M. and Halling, P.J. (1997). Water activity fails to predict critical hydration level for enzyme activity in polar organic solvents: Interconversion of water concentrations and activities. *Enz. Microb. Technol.* **20**, 471-477.

Bellocq, A.-M. (1996). Flexible surfactant films: Phase, behavior, structure, and applications. In *Emulsions and Emulsion Stability*, J. Sjöblom (Ed.), Surfactant Science Series Volume 61, Marcel Dekker, Inc., New York, N.Y., pp. 181-230.

Bickerstaff, G.F. (1997). Immobilization of enzymes and cells: Some practical considerations. In *Immobilization of Enzymes and Cells*, G.F. Bickerstaff (Ed.), Humana Press, Totowa, N.J., pp. 1-11.

Bilyk, A., Bistline, R.G.Jr., Haas, M.J. and Feairheller, S.H. (1991). Lipase-catalyzed triglyceride hydrolysis in organic solvent. *J. Am. Oil Chem. Soc.* **68**, 320-323.

Bloomer, S., Adlercreutz, P. and Mattiasson, B. (1990). Triglyceride interesterification by lipases. 1. Cocoa butter equivalents from a fraction of palm oil. *J. Am. Oil Chem. Soc.* **67**, 519-524.

Brink, L.E.S., Tramper, J., Luyben, K.C.A.M. and Van't Riet, K. (1988). Biocatalysis in organic media. *Enz. Microb. Technol.* **10**, 736-743.

Brown, W.H. (1995). Chirality. In *Organic Chemistry*, W.H. Brown (Ed.), Harcourt Brace College Publishers, Montreal, QC, pp. 256-310.

Buisman, G.J.H., van Helteren, C.T.W., Kramer, G.F.H., Veldsink, J.W., Derksen, J.T.P. and Cuperus, F.P. (1998). Enzymatic esterification of functionalized phenols for the synthesis of lipophilic antioxidants. *Biotechnol. Lett.* **20**, 131-136.

Ceridán, L.E., Medina, A.R., Giménez, A.G., González, M.J.I. and Grima, E.M. (1998). Synthesis of polyunsaturated fatty acid-enriched triglycerides by lipase-catalyzed estertification. *J. Am. Oil Chem. Soc.* **75**, 1329-1337.

Chamouleau, F., Coulon, D., Girardin, M. and Goul, M. (2001). Influence of water activity on sugar esters lipase-catalyzed synthesis in organic media. *J. Mol. Cat. B: Enz.* **11**, 949-954.

Chaplin, M.F. and Bucke, C. (1990). Fundamentals of enzyme kinetics. In *Enzyme Technology*, Cambridge University Press, New York, N.Y., pp. 1-38.

Chen, Q., Shi, H. and Ho, C.-T. (1992). Effects of rosemary extracts and major constitutes in lipid oxidation and soybean lipoxygenase activity. *J. Am. Oil Chem. Soc.* **69**, 999-1002.

Chopineau, J., McCafferty, F.D., Therisod, M. and Klibanov, A.M. (1988). Production of biosurfactant from sugar alcohols and vegetable oils catalyzed by lipases in nonaqueous medium. *Biotechnol. Bioengineer.* **31**, 208-214.

Compton, D.L. and King, J.W. (2001). Lipase-catalyzed synthesis of triolein-based sunscreens in supercritical CO_2. *J. Am. Oil Chem. Soc.* **78**, 43-47.

Connor, W.E. (2000). Importance of n-3 fatty acids in health and disease. *Am. J. Clin. Nutr.* **71**, 171S-175S.

Creagh, A.L., Prausnitz, J.M. and Blanch, H.W. (1993). Structural catalytic properties of enzymes in reverse micelles. *Enz. Microb. Technol.* **15**, 383-392.

Culhane, C.T. (1999). Resounding roar. *Food in Canada,* June, pp. 16-19, 21.

Davidson, M.H., Burns, J.H., Subbaiah, P.V., Conn, M.E. and Drennan, K.B. (1991). Marine oil capsule therapy for the treatment of hyperlipidemia. *Arch. Intern. Med.* **151**, 1732-1740.

Dordick, J.S. (1989). Enzymatic catalysis in monophasic organic solvents. *Enz. Microb. Technol.* **11**, 194-211.

Freund, J.E. (1984). Summearizing data: statistical descriptions. In *Modern Elementary Statistics*, 6th edition, Prentice-Hall, Inc., Englewood Cliffs, N.J., pp. 37-80.

Frings, K., Koch, M. and Hartmeier, W. (1999). Kinetic resolution of 1-phenyl ethanol with high enantioselectivity with native and immobilized lipase in organic solvents. *Enz. Microb. Technol.* **25**, 303-309.

Fureby, A.M. (1995). Bioorganic synthesis. In *Aspects on Lipase Catalyzed-Preparation of Partial Glycerides*. Department of Biotechnology, Lund University, Sweden, pp. 19-23.

Gandhi, N.N. (1997). Applications of lipase. *J. Am. Oil Chem. Soc.* **74**, 621-634.

Gandhi, N.N., Sawant, S.B., Joshi, J.B. and Mukesh, D. (1995). Lipozyme deactivation by butanol and temperature. *Enz. Microb. Technol.* **17**, 373-380.

Ganhdi, N.N., Patil, N.S., Sawant, S.B. and Joshi, J.B. (2000). Lipase-catalyzed esterification. *Catal. Rev. -Sci. Eng.* **42**, 439-480.

Goma-Doncescu, N. and Legoy, M.D. (1997). An organic transesterification route for fatty acid ester production from vegetable oils in a solvent-free system. *J. Am. Oil Chem. Soc.* **74**, 1137-1143.

Gordon, M.H. and Roedig-Penmann, A. (1999). Antioxidant properties of flavonols. In *Lipids in Health and Nutrition,* J.H.P. Tyman (Ed.), The Royal Society of Chemistry, Cambridge, UK, pp. 47-64.

Gorman, L.S. and Dordick, J.S. (1992). Organic solvents strip water of enzymes. *Biotechnol. Bioengineer.* **39**, 392-397.

Griffiths, P.R. and de Haseth, J.A. (1986). The michelson interferometer. In *Fourier Transform Infrared Spectrometry*, John Wiley & Sons, New York, N.Y., pp.1-55.

Gunstone, F.D. (1999). Review: Enzymes as biocatalysts in the modification of natural lipids. *J. Sci. Food Agric.* **79**, 1535-1549.

Gunstone, F.D. (2001). Procedures used for lipid modification. In *Structured and Modified Lipids*, F.D. Gunstone (Ed.), Marcel Dekker Inc., New York, N.Y., pp. 11-35.

Guyot, B., Bosquette, B., Pina, M. and Graille, J. (1997). Esterification of phenolic acids from green coffee with immobilized lipase from *Candida antartica* in solvent free medium. *Biotechnol. Lett.* **19**, 529-532.

Guyot, B., Gueule, D., Pina, J.M., Graille, V.F. and Farines, M. (2000). Enzymatic synthesis of fatty esters in 5-caffeoyl quinic acid. *Eur. J. Lipid Sci. Technol.* **102**, 93-96.

Halling, P.J. (2000). Biocatalysts in low-water media: understanding the effects of reaction conditions. *Current Opinion Chem. Biol.* **4**, 74-84.

Hamazaki, A., Hirai, A., Terano, T., Sajiki, J., Kondo, S., Fujita, T., Tamura, Y. and Kumagai, A. (1982). Effects of orally administered ethyl esters of eicosapentaenoic acid (EPA, C20:5, ω-3) on PGI_2-like substance production on rat aeorta. *Prostaglandins* **23**, 557-567.

Han, D. and Rhee, J.S. (1986). Characteristics of lipase catalyzed hydrolysis of olive oil in AOT-isooctane reversed micelles. *Biotechnol. Bioengineer.* **28**, 1250-1255.

Harper, N., Dolman, M., Moore, B.D. and Halling, P.J. (2000). Acid-base control for biocatalysis in organic media: New solid state proton/cation buffers and an indicatior. *Chem. Eur. J.* **6**, 1923-1929.

Harper, N., Dolman, M., Moore, B.D. and Halling, P.J. (2001). Effect of water activity on the rate profile of subtilisin Carlsberg in toluene in the presence of organo-soluble acid-base buffer. *Enz. Microb. Technol.* **29**, 1413-416.

Hartree, E.P. (1972). Determination of proteins: A modification of the Lowry method that gives a linear photometric response. *Anal. Biochem.* 48, 422-427.

Ho, C.-T. (1992). Phenolic compounds in food, an overview. In *Phenolic Compounds in Food and Their Effects on Health I Analysis, Occurrence & Chemistry,* C.-T. Ho, C.Y. Lee and M.-T. Huang (Eds.), ACS Symposium 506, American Oil Chemists' Society, Washington, D.C., pp. 2-7.

Hollman, P.C.H. (2001). Evidance for health benefits of plant phenols: local or systemic effects? *J. Sci. Food Agric.* **81**, 842-852.

Horman, I. (1984). NMR spectroscopy. In *Analysis of Foods and Beverages: Modern Techniques,* G. Charalambous (Ed.), Academic Press, Inc., New York, N.Y., pp. 205-264.

Hui, Y.H. (1996). Marine Oils. In *Bailey's Industrial Oil & Fat Products, Edible Oil & Fat Products: Products and Application Technology,* 5th edition, Volume 3, John Wiley & Sons, Inc., New York, N.Y., pp. 225-253.

Humeau, C., Girardin, M., Coulon, M. and Miclo, A. (1995). Synthesis of 6-o-palmitol L-ascorbic acid by *Candida antartica. Biotechnol. Lett.* 17, 1091-1094.

Humeau, C., Girardin, M., Rovel, B. and Miclo, A. (1998). Enzymatic synthesis of fatty acid ascorbyl esters. *J. Mol. Cat. B: Enz.* 5, SI 19-23.

Indlekofer, M., Funke, M., Claassen, W. and Reus, M. (1995). Continuous enantioselective transesterification in organic solvents. Use of suspended lipase preparations in a microfiltration membrane reactor. *Biotechnol. Prog.* 11, 436-442.

Irimescu, R., Furihata, K., Hata, K., Iwasaki, Y. and Yamane, T. (2001). Utilization of reaction medium-dependant regiospecificity of *Candida antarctica* lipase (Novozym 435) for the synthesis of 1,3-dicapryloyl-2-docosahexaenoyl (or eicosapentaenoyl) glycerol. *J. Am. Oil Chem. Soc.* 78, 285-289.

Isono, Y., Nabetani, H. and Nakajima, M. (1995). Lipase-surfactant complex as catalyst of interesterification and esterification in organic media. *J. Ferment. Bioengineer.* **80**, 170-175.

Iwasaki, Y., Han, J.J., Narita, M., Rosu, R. and Yamane, T. (1999). Enzymatic synthesis of structured lipids from single cell oil of high docosahexaenoic acid content. *J. Am. Oil Chem. Soc.* **76**, 563-569.

Jänchen, D.E. (1984). Quantitative thin-layer chromatography of foods and beverages. In *Analysis of Foods and Beverages: Modern Techniques,* G. Charalambous (Ed.), Academic Press Inc., New York, N.Y., pp. 69-91.

Jennings, B.H. and Akoh, C.C. (1999). Enzymatic modification of triacylglycerols of high eicosapentaenoic and docosahexaenoic acid content to produce structured lipids. *J. Am. Oil Chem. Soc.* **76**, 1133-1137.

Jennings, W. (1984). Gas chromatography. In *Food analysis: Principles and Techniques, vol. 4 Separation Techniques,* D.W. Gruenwedel and J.R. Whitaker (Eds.), Marcel Dekker Inc., New York, N.Y., pp. 409-444.

Kennedy, J.P. (1991). Structured lipids: Fats of the future. *Food Techhnol.* **45**, 76, 78, 80, 83.

Kermasha, S. and Bisakowski, B. (1998). The effect of immobilization on the hydrolytic/ interesterification activity of lipase from *Rhizopus niveus*. *J. Am. Oil Chem. Soc.* **75**, 1791-1799.

Kermasha, S., Safari, M. and Geotghebeur, M. (1995). Interesterification of butter fat by lipase from *Rhizopus niveus* in cosurfactant-free microemulsion system. *Appl. Biochem. Biotechnol.* **53**, 229-244.

Kilara, A. (1985). Enzyme-modified lipid food ingredients. *Process Biochem.* **20**, 35-45.

Kim, H.I., Yoon, C.S. and Lee, K.W. (2001). Transesterification of conjugated linoleic acid and tricaprylin by lipase in organic solvents. *Food Res. Int.* **34**, 301-306.

Krishna, S., Divakar, S., Prapulla, S.G. and Karanth, N.G. (2001). Enzymatic synthesis of isoamyl acetate using immobilized lipase from *Rhizomucor miehei. J. Biotechnol.* **87**, 193-201.

Laane, C., Boeren, S., Vos, K. and Veeger, C. (1987). Rules for optimization of biocatalysis in organic solvents. *Biotech. Bioengineer.* **30**, 81-87.

Lachance, P.A. (2002). Nutraceuticals, for real. *Food Technol.* **56**, 20.

Lai, O.M., Ghazali, H.M., Cho, F. and Chong, C.L. (2000). Enzymatic transesterification of palm stearin:anhydrous milk fat mixtures using 1,3-specific and non specific lipases. *Food Chem.* **70**, 221-225.

Lall, S.P. and Parazo, M.P. (1995). Vitamins in fish and shellfish. In *Fish and Fishery Products Composition, Nutritive Properties and Stability*, A. Ruiter (Ed.), CAB International, Wallingford, UK, pp. 157-213.

Larsson, K. (1994). The liquid state. In *Lipids-Molecular Organization, Physical Functions and Technical Applications.* The Oily Press, Ltd., Dundee, Scotland, pp. 75-80.

Lee, C.Y. (1992). Enzymatic oxidation of phenolic compounds in fruits. In *Phenolic Compounds in Food and Their Effects on Health I Analysis, Occurrence, & Chemistry*, C.-T. Ho, C.Y. Lee and M.-T. Huang (Eds.), American Oil Chemists' Society, Washington, D.C., pp 305-317.

Lee, K.-T. and Akoh, C.C. (1996). Immobilized lipase-catalyzed production of structured lipids with eicosapentaenoic acid at specific positions. *J. Am. Oil Chem. Soc.* **73**, 611-615.

Li, A.-Y. and Ward, O.P. (1993). Lipase-catalyzed esterification of glycerol and n-3 polyunsaturated fatty acid concentrate in organic solvent. *J. Am. Oil Chem. Soc.* **70**, 745-748.

Linko, Y.-Y., Lämsä, M., Huhtala, A. and Linko P. (1994). Lipase-catalyzed transesterification of rapeseed oil and 2-ehyl-1-hexanol. *J. Am. Oil Chem. Soc.* **71**, 1411-1414.

Lu, Y. and Foo, L. (2000). Antioxidant and radical scavenging activities of polyphenols from apple pomace. *Food Chem.* **68**, 81-85.

Macrae, A.R. (1983). Lipase-catalyzed interesterification of oils and fats. *J. Am. Oil Chem. Soc.* **60**, 243A-246A.

Mangos, T.J., Jones, K.C. and Foglia, T.A. (1999). Lipase-catalyzed synthesis of structured low-calorie triacylglycerols. *J. Am. Oil Chem. Soc.* **76**, 1127-1132.

Marangoni, A.G., McCurdy, R.D. and Brown, E.D. (1993). Enzymatic esterification of triolein with tripalmitin in canola lecithin-hexane reverse micelles. *J. Am. Oil Chem. Soc.* **70**, 737-744.

McMurry, J. (1984). Acrylimides and polyphenols. In *Organic Chemistry*, Brooks/Cole Publishing Company, Monterey, CA, p. 980.

McNeill, G.P. and Sonnet, P.E. (1995). Low-calorie synthesis by lipase-catalyzed esterification of monoglycerides. *J. Am. Oil Chem. Soc.* **72**, 1301-1307.

Medina, A.R., Cerdán, L.E., Giménez, A.G., Páez, B.C., González, M.J.I. and Grima, E.M. (1999). Lipase-catalyzed esterification of glycerol and polyunsaturated fatty acids from fish and microalgea oils. *J. Biotechnol.* **70**, 379-391.

Mogi, K., Nakajima, M. and Mukataka, S. (2000). Transesterification reaction between medium- and long-chain fatty acid triglycerides using surfactant-modified lipase. *Biotechnol. Bioengineer.* **67**, 513-519.

Moussata, C.O. and Akoh, C.C. (1997). Lipase-catalyzed incorporation of oleic acid into melon seed oil. *J. Am. Oil Chem. Soc.* **74**, 117-179.

Nadeau, M. (1992). Fiches techniques, Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec (MAPAQ).

Nakamura, K., Kinoshita, M. and Atsuyoshi, O. (1995). Structure of solvent affects enantioselectivity of lipase-catalyzed transesterification. *Tetrahedron* **51**, 8799-8808.

Nawar, W.W. (1996). Lipids. In *Food Chemistry,* 3rd edition. O.R. Fennema (Ed.), Marcel Dekker, Inc., New York, N.Y., pp. 225-319.

Novo Nordisk A/S (2000). Technical Data Sheet Novozym 435 B606-d-GB, Bagsvaerd, Denmark, pp.1-2.

Oguntimein, G.B., Anderson, W.A. and Moo-Young, M. (1995). Synthesis of geraniol esters in a solvent-free system catalyzed by *Candida antarctica* lipase. *Biotechnol. Lett.* **17**, 77-82.

Otto, R.T., Bornscheuer, U.T., Scheib, H., Pleiss, J., Syldatk, C. and Schmid, R.D. (1998). Lipase-catalyzed esterification of unusual substrates: Synthesis of glucuronic acid and ascorbic acid (vitamin C) esters. *Biotechnol. Lett.* **20**, 1091-1094.

Pabai, F., Kermasha, S. and Morin, A. (1995). Interesterification of butterfat by partially purified extracellular lipases from *Pseudomonas putida*, *Aspergillus niger* and *Rhizopus oryzae*. *J. Microbiol. Biotechnol.* **11**, 669-677.

Paez, B.C., Medina, A.R., Rubio, F.C., Moreno, P.G. and Grima, E.M. (2002). Production of structured triglycerides rich in n-3 polyunsaturated fatty acids by acidolysis of cod liver oil and caprylic acid in a packed bed reactor: Equilibrium and kinetics. *Chem. Engineer. Sci.* **57**, 1237-1249.

Parr, A.J. and Bolwell, G.P. (2000). Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content profile. *J. Sci. Food Agric.* **80**, 5985-1012.

Partridge, J., Dennison, P.R., Moore, B.D. and Halling, P.J. (1998). Activity and mobility of subtilisin in low water organic media: Hydration is more important than solvent dielectric. *Biochim. Biophy. Acta* **1386**, 79-89.

Partridge, J., Halling, P.J. and Moore, B.D. (2000). Solid state proton/sodium buffers: "Chemical pH stats" for biocatalysts in organic solvents. *J. Chem. Soc., Perkin Trans 2*, 465-471.

Pencreac'h, G. and Baratti, J.C. (1996). Hydrolysis of p-nitrophenyl palmitate in n-heptane by the *Pseudomonas cepacia* lipase: A simple test for the determination of lipase activity in organic media. *Enz. Microb. Technol.* **18**, 417-422.

Pencreac'h, G. and Baratti, J.C. (1999). Properties of free and immobilized lipase from *Burkholderia cepacia* in organic media. *Appl. Microbiol. Biotechnol.* **52**, 276-280.

Persson, M., Wehtje, E. and Adlercreutz, P. (2002). Factors governing the activity of lyophilized and immobilized lipase preparations in organic solvents. *ChemBioChem* **3**, 566-571.

Poisson, L. and Ergan, F. (2001). Docosahexaenoic acid ethyl esters from *Isochrysis galbana*. *J. Biotechnol.* **91**, 75-81.

Potter, N.N. and Hotchkiss, J.H. (1996). Constituents of foods: Properties and significance. In *Food Science*, 5th edition. N.N. Potter and J.H. Hotchkiss (Eds.), Chapmann & Hall, New York, N.Y., pp. 24-45.

Roberts, S.M. (1990). Enzymes in organic synthesis. In *Microbial Enzymes and Biotechnology*, 2nd edition. W.M. Fogarty and C.T. Kelly (Eds.), Elsevier Applied Science, New York, N.Y., pp. 395-424

Rocquelin, G. (1990) How can the processessing of fats and oils meet tomorrow's demand for food of higher nutritive value? A European perspective. In *World Conference Proceedings Edible Fats and Oils Processing: Basic Principles and Modern Practices*, D.R. Erickson (Ed.), American Oils Chemists' Society Champaign, IL., pp. 166-172.

Safari, M. and Kermasha, S. (1994). Interesteification of butterfat by commercial microbial lipases in a cosurfactant free microemulsion system. *J. Am. Oil Chem. Soc.* **71**, 969-973.

Sato, H., Matsui, T. and Arakawa, Y. (2002). The protective effect of catechin on gastric mucosal lesions in rats. *J. Gastroenterol.* **37**, 106-111.

Screenivasan, B.J. (1978). Interesterification of fats. *J. Am. Oil Chem. Soc.* **55**, 796-805.

Selmi, B., Gontier, E., Ergan, F., Barbotin, J.N. and Thomas, D. (1997). Lipase-catalyzed synthesis of tricaprylin in a medium solely composed of substrates. Water production and elimination. *Enz. Microb. Technol.* **20**, 322-325.

Senanayake, S.P.J.N. and Shahidi, F. (1999). Enzymatic incorporation of docosahexanoic acid into borage oil. *J. Am. Oil Chem. Soc.* **76**, 1009-1015.

Seriburi, V. and Akoh C.C. (1998). Enzymatic esterification of lard and high-oleic sunflower oil with *Candida antarctica* lipase to produce plastic fats. *J. Am. Oil Chem. Soc.* **75**, 1339-1345.

Sewell, P.A. (1992). High pressure liquid chromatography. In *Lipid Analysis: A Practical Approach,* R.J. Hamilton and S. Hamilton (Eds.), IRL Press, New York, N.Y., pp. 153-203.

Shahidi, F. (1997). Natural antioxidant: An overview. In *Natural Antioxidants: Chemistry Health Effects and Applications*, F. Shahidi (Ed.), AOCS Press, Champaign, IL, pp. 1-11.

Shahidi, F. and Naczk, M. (1995a). Antioxidant properties of food phenolics. In *Food Phenolics Sources Chemistry Effects Applications,* Technomic Publishing Co., Inc., Lancaster, PA, pp. 235-277.

Shahidi, F. and Naczk, M. (1995b). Food phenolics: An overview. In *Food Phenolics Sources Chemistry Effects Applications,* Technomic Publishing Co., Inc., Lancaster, PA, pp. 1-5.

Shahidi, F. and Wanasundra, N. (1998). Omega-3 fatty acid concentrates: Nutritional aspects and production technologies. *Trends Food Sci. Technol.* **9**, 230-240.

Sherma, J. (1984). Thin-layer chromatography. In *Food Analysis: Principles and Techniques, Vol. 4, Separation Techniques,* D.W. Gruenwedel and J.R. Whitaker (Eds.), Marcel Dekker, Inc., New York, N.Y., pp. 297-346.

Shield, J.W., Ferguson, H.D., Bommarius, A.S. and Hatton, T.A. (1986). Enzymes in reversed micelles as catalysts for organic-phase synthesis reactions. *Ind. Eng. Chem. Fundam.* **25**, 603-612.

Sloan, A.E. (1999). The new market: Foods for the not-so-healthy. *Food Technol.* **53**, 54-58, 60.

Stamatis, H., Sereti, V. and Kolisis, F.N. (1999). Studies on the enzymatic synthesis of lipophilic derivatives of natural antioxidants. *J. Am. Oil Chem. Soc.* **76**, 1505-1510.

Stamatis, H., Sereti, V. and Kolisis, F.N. (2001). Enzymatic synthesis of hydrophilic and hydrophobic derivatives of natural phenolic acids in organic media. *J. Mol. Catal. B: Enz.* **11**, 323-328.

Stansby, M.E., Schlenk, H. and Gruger, E.H. (1990). Fatty acid composition of fish. In *Fish Oils in Nutrition*, M.E. Stansby (Ed.), An AVI book, New York, N.Y., pp. 6-39.

Steinke, G., Weitkamp. P., Klein, E. and Mukherjee, K.D. (2001). High-yeild preparation of wax esters via lipase-catalyzed esterification using fatty acids and alcohols from crambe and camelina oil. *J. Agric Food Chem.* **49**, 647-651.

Taipa, M.A., Aires-Barros, M.R. and Cabral, J.M.S. (1992). Purification of lipases. *J. Biotechnol.* **26**, 111-142.

Tanaka, Y., Fundara, T., Hirano, J. and Hashizume, R. (1993). Triglyceride specificity of *Candida cylindracea* lipase: Effect of docohexanoic acid on resistance of triglyceride to lipase. *J. Am. Oil Chem. Soc.* **70**, 1031-1034.

Thomson, C.A., Delaquis, P.J. and Mazza, G. (1999). Detection and measurement of microbial lipase activity: A review. *Crit. Review. Food Sci. Nutr.* **39**, 165-187.

Tweddell, R.J., Kermasha, S., Combes, D. and Marty A. (1997). Optimization of the reaction medium for esterification catalyzed by a lipase from *Pseudomonas fluorescens*. *Biotechnol. Lett.* **19**, 939-942.

Tweddell, R.J., Kermasha, S., Combes, D. and Marty, A. (1998). Esterification and interesterification activities of lipase from *Rhizopus niveus* and *Mucor meihei* in three different types of organic media: A comparative study. *Enz. Microb. Technol.* **22**, 439-445.

Tweddell, R.J., Kermasha, S., Combes, D. and Marty, A. (1999). Immobilization of lipase from *Rhizopus niveus*: A way to enhance its synthetic activity in organic solvent. *Biocatal. Biotransform.* **16**, 411-426.

USDA Nutrient Database for Standard Reference, Release 14 (2001). Agriculture and Research Service. <www.nal.usda.gov/fnic/foodcomp/index.html>

Valivety, R.H., Hailling, P.J. and Macrae, A.R. (1992). *Rhizomucor miehei* lipase remains highly active at water activity below 0.0001. *FEBS Lett.* **301**, 258-260.

Vazquez-Dehalt, R., Fedorak, P.M. and Westlake, D.W.S. (1992). Role of enzyme hydrophobicity in biocatalysis in organic solvents. *Enz. Microb. Technol.* **14**, 837-841.

Verger, R., Rivière, C., Moreau, H., Gargouri, Y., Rogalska, E., Nury, S., Moulin, A., Ferrato, F., Ransac, S., Carrière, F. and Trétout, N. (1990). Enzyme kinetics of lypolysis lipase inhibition by proteins. In *Lipases: Structure, Mechanism and Genetic Enginnering*, L. Alberghina, R.D. Schmid and R. Verger (Eds.), VCH Publishers, Inc., New York, N.Y., pp. 105-115.

Vinson, J.A., Hao, H., Su, X. and Zubik, L. (1998). Phenol antioxidant quantity and quality in foods: Vegetables. *J. Agric. Food Chem.* **46**, 3630-3634.

Voutsas, E.C., Spiliotis, N.M. and Tassios, D.P. (2001). Enzymatic reactions in non-conventional media: Prediction of solvent water content for optimum water activity. *Biocatal. Biotransform.* **19**, 99-118.

Walde, P. and Luisi, P.L. (1990). Lipases in reverse micelles. In *Lipases: Structure, Mechanism and Genetic Enginnering*, L. Alberghina, R. D. Schmid and R. Verger (Eds.), VCH Publishers, Inc., New York, N.Y., pp. 155-158.

Wehtje, E., Kaur, J., Adlercreutz, P., Chand, S. and Mattiasson, B. (1997). Water activity control in enzymatic esterification processes. *Enzyme Microb. Technol.* **21**, 502-510.

Whang, D.-L., Nag, A., Lee, G.-C. and Shaw, J.-F. (2002). Factors affecting the resolution of *dl*-menthol by immobilized lipase-catalyzed esterification in organic solvent. *J. Agric. Food Chem.* **50**, 262-265.

White, P.J. and Xing, Y. (1997). Antioxidants from cereals and legumes. In *Natural Antioxidants: Chemistry Health Effects and Applications,* F. Shahidi (Ed.), AOCS Press, Champaign, IL, pp. 25-63.

Willis, W.M. and Marangoni, A.G. (1999). Assessment of lipase-and chemically catalyzed lipid modification strategies for the production of structured lipids. *J. Am. Oil Chem. Soc.* **76**, 443-450.

Willis, W.M., Lencki, R.W. and Marangoni, A.G. (1998). Lipid modification strategies in the production of nutritionally functional fats and oils. *Crit. Rev. Food Sci. Nutr.* **38**, 639-674.

Witwer, R.S. (1999). Marketing of bioactive ingredients in food products. *Food Technol.* **53**, 50-53.

Wong, W.C., Basri, M., Razak, C.N.A. and Salleh, A.B. (2000). Synthesis of medium-chain glycerides using lipase from *Candida rugosa*. *J. Am. Oil Chem. Soc.* **77**, 85-88.

Xu, X., Fomuso, L.B. and Akoh, C.C. (2000a). Synthesis of structured triacylglycerols by lipase-catalyzed acidolysis in a packed bed bioreactor. *J. Agric. Food Chem.* **48**, 3-10.

Xu, X., Fomuso, L.B. and Akoh, C.C. (2000b). Modification of Menhaden oil by enzymatic acidolysis to produce structured lipids: Optimization response surface design in a packed bed reactor. *J. Am. Oil Chem. Soc.* **77**, 171-176.

Yang, T.H., Jang, Y., Han, J.J. and Rhee, J.S. (2001). Enzymatic synthesis of low-calorie structured lipids in a solvent free medium. *J. Am. Oil Chem. Soc.* **78**, 291-296.

Yankah, V.V. and Akoh, C.C. (2000). Lipase-catalyzed acidolysis of tristearin with oleic or caprylic acids to produce structured lipids. *J. Am. Oil Chem. Soc.* **77**, 495-500.

Zhang, H., Xu, X., Mu, H., Nilsson, J., Adler-Nissen, J. and Høy, C.-E. (2000). Lipozyme IM-catalyzed interesterification for the production of margarine fats in a 1 kg scale stirred tank reactor. *Eur. J. Lipid Sci. Technol.* **102**, 411-418.

Zhang, H., Xu, X., Mu, H., Nilsson, J., Mu, H., Adler-Nissen, J. and Høy, C.-E. (2001). Production of margarine fats by enzymatic interesterification with silica-granulated *Thermomyces lanuginose* lipase in a large-scale study. *J. Am. Oil Chem. Soc.* 78, 57-64.

Zhang, X. and Hayes, D.G. (1999). Increased rate of lipase-catalyzed saccharide-fatty acid esterification by control of reaction medium. *J. Am. Oil Chem. Soc.* 76, 1495-1500.