BIOSYNTHESIS OF PHENOLIC LIPID MODELS USING OLEYL ALCOHOL AND TRIOLEIN

by

Bena-Marie Lue

A thesis submitted to the Graduate and Post-Doctoral Studies Office in partial fulfillment of the requirements of the degree of Master of Science

Department of Food Science and Agricultural Chemistry

McGill University
Montreal, Quebec

May 2004

Your file Votre référence
ISBN: 0-612-98693-4
Our file Notre référence
ISBN: 0-612-98693-4

Short Title

BIOSYNTHESIS OF PHENOLIC LIPIDS

ABSTRACT

M. Sc. Bena-Marie Lue

The overall objective of this study was the optimization of a model enzymatic system in organic solvent media for the biosynthesis of selected phenolic lipid compounds. The model enzymatic system consisted of cinnamic acid and oleyl alcohol as substrates using commercial immobilized lipase (Novozym 435) from *Candida antarctica*. In order to optimize the initial enzymatic activity and bioconversion yield, selected parameters, including solvent hydrophobicity ($0.29 \leqslant \log P \leqslant 4.50$), initial water activity (a_w, 0.05 – 0.75), agitation speed (0 – 200 rpm), temperature (35 – 65°C) and substrate ratio (phenolic acid/fatty alcohol, 1.0:0.5 – 1.0:6.0), were investigated. The experimental findings showed that an increase in the hydrophobicity of the solvent mixture and a decrease in the a_w values of the reaction medium increased the initial enzymatic activity and bioconversion yield; the use of an iso-octane and butanone solvent mixture (85:15, v/v) and an initial a_w of 0.05 resulted in an initial enzymatic activity of 192.7 nmol product/g enzyme/min and a corresponding bioconversion yield of 95.3% after a 16-day reaction period. The results also showed that an agitation speed of 150 rpm and a reaction temperature of 55°C were optimal for the reaction system. The activation energy (E_a) of the esterification reaction was calculated as 43.6 kJ/mol. The optimal cinnamic acid to oleyl alcohol ratio was 1.0:6.0, with the absence of any enzymatic activity inhibition by alcohol within the concentrations investigated. Using the optimal conditions, the maximum initial enzymatic activity was 390.3 nmol product/g enzyme/min. In addition, analyses by high-performance liquid chromatography (HPLC), spectrophotometric scanning and electrospray ionization mass spectroscopy (ESI-MS) confirmed the biosynthesis of the phenolic lipid end product, cinnamyl oleyl. Application of the optimized model system with triolein as substrate was also investigated. Subsequent HPLC and ESI-MS analysis confirmed the formation of the hydrolysis products of triolein as well as two major end products, characterized as cinnamyl monoolein and cinnamyl diolein, and two minor ones.

RÉSUMÉ

M. Sc. Bena-Marie Lue

L'objective de cette étude est l'optimisation d'un système enzymatique modèle en milieux organiques en vue de la biosynthèse des lipides phénoliques sélectionnés. Le système enzymatique modèle établi consiste à l'estérification de l'acide cinnamique avec l'alcool oléique par la lipase immobilisée commercialle (Novozym 435) de *Candida antartica.* Afin d'optimiser l'activité enzymatique initiale et le rendement de bioconversion, des paramètres enzymatiques sélectionnés, incluant l'hydrophobicité des solvants organiques (0,29 $\leqslant$ log P $\leqslant$ 4,50), l'activité d'eau (a_w, 0,05 – 0,75), la vitesse d'agitation (0 – 200 rpm), la température (35 – 65°C) et le ratio des substrats (acide phénolique/alcool d'acide gras, 1,0:0,5 – 1,0:6,0), ont été étudiés. Les résultats expérimentaux ont montré que l'augmentation de l'hydrophobicité du mélange des solvants organiques et la diminution de l'a_w du système réactionnel ont augmenté l'activité enzymatique initiale et le rendement de la bioconversion. En effet, l'utilisation d'un mélange iso-octane et butanone (85:15, v/v) et d'une a_w initiale de 0,05 se traduit par une activité enzymatique initiale de 192,7 nmol produit/g enzyme/min et un rendement de bioconversion de 95,3% après 16 jours de réaction. Les résultats ont également montré que la vitesse d'agitation la plus appropriée et la température optimale sont respectivement de 150 rpm et 55°C. L'énergie d'activation (E_a) de la réaction d'estérification a été de 43,6 kJ/mol. Par ailleurs, le ratio optimal de l'acide cinnamique par rapport à l'alcool oléique a été de 1,0:6,0, sans la présence d'une inhibition enzymatique dans la gamme des concentrations utilisées. En utilisant les conditions optimales, l'activité enzymatique initiale maximale était 390,3 nmol produit/g enzyme/min. Les analyses par chromatographie liquide à haute performance (HPLC), par spectrophotométrie à balayage et par spectrométrie de masse à ionisation électrospray (ESI-MS) ont confirmées la biosynthèse d'un lipide phénolique, le cinnamate d'oléile. L'application du model enzymatique optimisé avec la trioléine comme substrat a également été étudiée. Les analyses par HPLC et par ESI-MS ont confirmé la formation des produits d'hydrolyse de la trioléine ainsi que de deux majeurs produits caractérisés comme le cinnamate monooléile et dioléile, et de deux autres produits mineurs.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Sélim Kermasha, for his support and guidance throughout my studies.

I would like to thank Dr. Salwa Karboune for advice throughout the course of this work. It was very much appreciated as was her encouragement and enthusiasm for the work. I would also like to thank Dr. Faustinus K. Yeboah for carrying out the ESI-MS structural analysis of end products.

Thank you to all my colleagues inside and outside the laboratory for their time, advice and friendship. I am especially appreciative to Abzal Hossain, Colin Hall and Kebba Sabally for their help and to Paula Arriagada for keeping me company in the basement laboratory.

Finally, I would especially like to thank my family for all their help, moral support and patience throughout my studies.

I would like to acknowledge CORPAQ, Government of Quebec for financial support and research funding.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ω-3: Omega-3

ω-6: Omega-6

ω-9: Omega-9

AA: Arachidonic acid

APCI-MS: Atmospheric pressure chemical ionization mass spectroscopy

a_w: Water activity

BHA: Butylated hydroxyanisole

BHT: Butylated hydroxytoluene

DHA: Docosahexaenoic acid

DPA: Docosapentaenoic acid

E_a: Activation energy

ELSD: Evaporative light scattering detector

EPA: Eicosapentaenoic acid

ESI-MS: Electrospray ionization mass spectroscopy

FTIR spectroscopy: Fourier transform infrared spectroscopy

GLC: Gas-liquid chromatography

HPLC: High-performance liquid chromatography

IR spectroscopy: Infrared spectroscopy

LC-MS: Liquid chromatography-mass spectroscopy

PG: Propyl gallate

PLU: Propyl laurate units

TBHQ: *tert*-Butylhydroquinone

TLC: Thin-layer chromatography

NMR: Nuclear magnetic resonance

UV: Ultraviolet

1. INTRODUCTION

Unsaturated lipids have been widely recognized for their role in the maintenance of human health. These lipids, including those from the ω-3 series, have been linked to inhibitory effects on atherosclerosis and cardiovascular diseases (Kennedy, 1991; de Deckere *et al.*, 1998; Hooper, 2001), tumour formation and promotion (Whelan, 1997), diabetes, inflammatory disorders and colon, breast and prostate cancers (Fernandes and Venkatraman, 1993; Bibus, 2001). Naturally present in many foods, phenolic compounds represent another important group which possesses antioxidant, antimicrobial (Friedman and Jürgens, 2000), anticarcinogenic (Hollman, 2001) and antimutagenic (Ho, 1992) properties in addition to sensory attributes including colour, astringency, bitterness and aroma (Andlauer *et al.*, 2000; Rodríguez-Delgado *et al.*, 2001).

Due to their potential action as promoters of carcinogenesis, the overall demand for synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) continues to decrease (Shahidi and Naczk, 1995*a*; Cuppett *et al.*, 1997; Buisman *et al.*, 1998; Stamatis *et al.*, 1999). As a result, research has shifted to focus more on the development and utilization of antioxidants from natural sources (Cuppett *et al.*, 1997). Natural plant phenolics are perceived by many to be potential replacements for these already established, albeit controversial synthetic antioxidants; however, a major drawback in their use in practical food applications lies in their hydrophilic nature (Buisman *et al.*, 1998). Specifically, the hydrophilic nature of phenolic compounds reduces their effectiveness in oil based formulae and emulsions (Stamatis *et al.*, 1999). Altering solubility and miscibility properties of phenolic compounds through the addition of aliphatic side-chain groups could result in their more widespread application as antioxidants in emulsion systems (Kontogianni *et al.*, 2001). Moreover, both the functional and nutritional properties of these novel phenolic antioxidants could be further enhanced through incorporation of unsaturated lipids into their structures (Petel, 2003). While most research to date has concentrated on the biosynthesis of phenolic compounds with short and medium chain aliphatic groups (Guyot *et al.*, 1997; Buisman *et al.*, 1998; Stamatis *et al.*, 1999 and 2001), the present work will attempt to

focus on the use of long-chain monounsaturated lipids and derivatives to generate phenolic lipid compounds with a view towards the eventual use of ω-3 polyunsaturated lipids.

Production of phenolic lipids will be investigated using an approach that considers the ability of lipase to catalyze both esterification of fatty alcohols (Stamatis *et al.*, 1999) and transesterification of triacylglycerols, with phenolic acids (Compton *et al.*, 2000). Advantages associated with enzyme-catalyzed reactions include the use of milder reaction conditions as compared to chemical methods (Willis *et al.*, 1998; Osório *et al.*, 2001), the reduction of overall reaction times, a decrease in side reactions and an increase in selectivity (Schuchardt *et al.*, 1998; Gunstone, 1999). However, due to the nature of the biosynthesis reactions, a monophasic organic solvent system will be used; advantages of organic media include its favourable equilibria towards synthesis rather than hydrolysis, an increase in the solubility of hydrophobic substrates as well as the stability of the lipase (Kvittingen, 1994; Klibanov, 1997).

The overall aim of this research was the optimization of a model enzymatic system in organic solvent media for the production and characterization of subsequent phenolic lipid compounds.

The specific objectives of this research were:

1. The optimization of an enzymatic system for the biosynthesis of phenolic lipid compounds using *t*-cinnamic acid and oleyl alcohol as model phenolic and lipid substrates, respectively, with immobilized Novozym 435 lipase from *Candida antarctica*.

2. The application of this optimized model enzymatic system for the biosynthesis of phenolic lipid products using *t*-cinnamic acid and a triacylglycerol, triolein, as model phenolic and lipid substrates, respectively.

3. The recovery, separation and characterization of the model phenolic lipid end products.

2. LITERATURE REVIEW

2.1. Unsaturated Lipids

The term lipid is often used to describe a chemically varied group of fatty substances that are either insoluble or poorly soluble in water, but soluble in organic solvents such as chloroform, hydrocarbons, alcohols or ethers (Gurr, 1992). Due to their largely heterogeneous nature, the classification of lipids is somewhat difficult. Most often, lipids are divided into four separate groups as suggested by Bloor in 1925 (Pomeranz and Meloan, 1994); simple lipids, composite lipids, sphingolipids and derived lipids. In the present context, the unsaturated lipids of interest belong to either the simple or derived lipid groups. Simple lipids may be defined as compounds containing two kinds of structural moieties (i.e. an alcohol and a fatty acid) and include esters of glycerol and fatty acids such as mono-, di- and triacylglycerols. Derived lipids may be described as compounds containing a single structural moiety that occur as such or are released from other lipids by hydrolysis and include both fatty acids and fatty alcohols (Pomeranz and Meloan, 1994).

2.1.1. Structure

Derived lipids such as fatty acids or fatty alcohols are composed of a basic hydrocarbon chain attached to either a carboxylic or alcohol functional group and may be distinguished from one another according to chain length, number and positioning of double bonds within the molecule (Willis *et al.*, 1998). In general, fatty acids with only one carbon-carbon double bond are referred to as monounsaturated while those with multiple carbon-carbon double bonds are termed polyunsaturated in nature. Simple triacylglycerols and their partial glycerides may consist of one or more fatty acids with varying degrees of unsaturation linked to a molecule of glycerol by an ester bond (Nawar, 1996).

Unsaturated lipids are commonly differentiated from one another based on the position of the first double bond from the methyl end of the fatty acid chain. Three distinct groups include the ω-3, ω-6 and ω-9 series whereby the first double bond is positioned between the third and fourth, sixth and seventh or ninth and tenth carbon

atoms, respectively (Bibus, 2001). Examples of unsaturated lipids belonging to each group include the long chain monounsaturated oleic acid (C18:1, ω-9), polyunsaturated linoleic acid possessing two double bonds (C18:2, ω-6) and linolenic acid possessing a total of three carbon-carbon double bonds (C18:3, ω-3). In general, double bonds may be either *cis-* or *trans-* in configuration.

2.1.2. Sources

Major sources of unsaturated lipids include fish and marine oils (Calder, 2001; Haraldsson and Hjaltason, 2001), plant seed and specialty oils (Whelan, 1997; Ayorinde *et al.*, 1999, Gunstone, 2001*a*, *b*) as well as selected microorganisms (Ratledge, 2001).

Fish and marine oils represent an important source of *cis-* unsaturated long chain fatty acids from the ω-3 family (Stansby *et al.*, 1990; Shahidi and Wanasundara, 1998). In particular, eicosapentaenoic acid (EPA, C20:5), docosapentaenoic acid (DPA, C22:5) and docosahexaenoic acid (DHA, C22:6) are abundant in menhaden and anchovy type fish species (Opstvedt *et al.*, 1990), typically comprising between 15 – 30% of the fatty acids present in the oil (Calder, 2001). Consistent with this range, studies by Perona and Ruiz-Gutiérrez (1999) on sardine oil yielded values for EPA, DPA and DHA of 14.8, 3.0 and 13.8%, respectively, while studies on sardine cannery effluents (Schmitt-Rozieres *et al.*, 2000) yielded values for EPA and DHA of 11.9 and 9.5%. The species, both season and geographical location of the catch as well as the fat content of the food ingested by the fish prior to being caught are all factors which influence specific fatty acid profiles of fish oils (Gurr, 1992; Opstvedt *et al.*, 1990; Sargent, 1997).

According to Opstvedt *et al.* (1990), the greatest difference between the three major varieties of commercial fish oils, defined as anchovy type, herring type and menhaden, lies in their monounsaturated fatty acid content which ranges from 26% of the total fatty acid in menhaden oil up to 51% total fatty acid content in the herring type oil. In the case of each oil type, oleic acid (C18:1, ω-9) is present in considerable quantities, ranging from 11 – 12% and representing between 24 – 42% of the overall monounsaturated fatty acids present. In addition, both gadoleic acid (C20:1) and erucic

acid (C22:1) are also present in high quantities in the herring type oil only, representing around 14 and 16% of total fatty acid content, respectively.

Plant seed and specialty oils also represent an important source of *cis*- long chain unsaturated fatty acids such as oleic (C18:1, ω-9), linoleic (C18:2, ω-6) and linolenic acids (C18:3, ω-3) (Willis *et al.*, 1998; Bibus, 2001). Specifically, the oils of hazelnut (82 – 86%, Gunstone, 2001*b*), high-oleic sunflower (82%), olive (78%), high-oleic safflower (74%), almond (61%) and rapeseed (56%) all contain high amounts of oleic acid (Gunstone, 2001*a*). Linoleic acid (C18:2, ω-6) may be found in abundance in crops such as corn (59%) and soybean (52%) (Kennedy, 1991; Gunstone, 2001*b*), grapeseed (66 – 72%, Ayorinde *et al.*, 1999) and evening primrose (65 – 80%, Clough, 2001) while the corresponding γ-linolenic acid (C18:3, ω-6) is present in high quantities in borage (22 – 23%) and black current (14 – 20%) oils (Clough, 2001). Alpha-linolenic acid (C18:3, ω-3) predominates in flaxseed (55%, Bibus, 2001) and specialty oils such as those from

Table 1. Major fatty acid composition of selected vegetable oils used in foods.

Fatty Acid[a]	Canola[b]	Soybean[b]	Olive[c]	Corn[c]
		%[d]		
16:0	5	12	12	14
18:0	2	4	2	2
20:0	1	< 1	< 1	< 1
16:1	< 1	< 1	1	< 1
18:1	59	21	72	30
20:1	2	< 1	0	0
18:2	21	53	11	50
18:3	10	8	1	2

[a]Fatty acids present at low levels were omitted.
[b]Adapted from Willis *et al.* (1998).
[c]Adapted from Gurr (1992).
[d]Percent fatty acid composition was given as g/100g^{-1} total fatty acids.

perilla (57 – 64%) and basil seed (44 – 65%) (Gunstone, 2001*a*). Fatty acid profiles of selected vegetable oils possessing considerable amounts of unsaturated lipids are detailed more fully in Table 1.

It should also be noted that while unsaturated lipid contents were discussed in terms of fatty acids present, most lipids in fish and marine mammals as well as in seed oils exist in the form of triacylglycerols (Gurr, 1992). Additional lipid types are present in much smaller quantities, including fatty alcohols such as oleyl alcohol (Liebert, 1985).

Microorganisms, mainly from algae and fungi sources, have also been used for the production of unsaturated fatty acids such as arachidonic acid (AA), EPA and DHA. Examples include the fungi *Mucor circinelloides* and *Mortierella alpina* as well as microalgae *Chlorella vulgaris*, *Crypthecodium cohnii*, *Phaeodactylum tricornutum* and *Porphyridium cruentum* (Willis *et al.*, 1998; Ratledge, 2001).

2.1.3. Nutritional and Functional Properties

Proven nutritional benefits result from inclusion of a range of unsaturated lipids in the diet. First and foremost, linoleic (C18:2, ω-6) and linolenic (C18:3, ω-3) acids are considered as essential fatty acids since they cannot be synthesized by precursors in the body. Elongation and desaturation of essential linoleic acid ingested in the diet yields AA (C20:4, ω-6) which can be incorporated directly into membrane phospholipids or further metabolized through the lipoxygenase or cycloxygenase pathways to yield leukotrienes (i.e. A_4) or prostaglandins (i.e. PGE_2). Linolenic acid (C18:3, ω-3) is metabolized in much the same way to yield families of leukotrienes (i.e. A_5) and prostaglandins (i.e. PGE_3) different from those generated by the ω-6 series. Moreover, the initial desaturation of linoleic and linolenic acids are influenced by competition for the rate-limiting Δ6 desaturase enzyme (Opara and Hubbard, 1993; Jones and Kubow, 1999; Willis *et al.*, 1998; Williams and Roche, 2001).

Metabolites produced through these pathways possess hormone-like activity with resulting inhibitory effects, particularly in the case of the ω-3 series, on conditions such as atherosclerosis and cardiovascular disease (Kennedy, 1991; de Deckere *et al.*, 1998;

Hooper, 2001), tumour formation and promotion (Whelan, 1997), diabetes, inflammatory disorders, particular cancers including colon, breast and prostate, in addition to a range of psychiatric disorders (Fernandes and Venkatraman, 1993; Bibus, 2001). In contrast, essential fatty acid deficiency leads to dermatosis and increased membrane permeability, impaired vision, growth retardation and reproductive failure (Gurr, 1992; Willis *et al.*, 1998; Bibus, 2001). To prevent this, linoleic acid (C18:2, ω-6) is needed in the diet at levels between 0.5 and 2.0% of total energy while linolenic acid (C18:3, ω-3) is required at levels around 0.5% (Willis *et al.*, 1998). It should be stressed that optimal health depends on the levels of both the ω-6 and ω-3 series of acids in the body.

Structured triacylglycerols have been defined as any fats that are modified or restructured from natural oils and fats, having special functionality or nutritional properties for edible or pharmaceutical purposes (Høy and Xu, 2001). Omega-3, 6 and 9 fatty acids are often incorporated into structured lipids to promote health and nutrition. Kennedy (1991) once noted optimum levels of ω-fatty acids for structured lipids as between 2 – 5 % ω-3 fatty acids and between 3 – 4 % ω-6 fatty acids, with ω-9 monounsaturated fatty acids present for balance. Presently, it is becoming more and more clear due to both epidemiological and experimental studies that *cis*-monounsaturated fatty acids, including oleic acid (C18:1, ω-9) are in themselves beneficial to human health. In particular, *cis*-monounsaturated fatty acids have been linked to beneficial effects on immune and inflammatory systems, blood coagulation and to a lesser extent on coronary heart disease (Moreno and Mitjavila, 2003). Thus, replacement of saturated fats with high levels of *cis*-monounsaturated fatty acids, typical of the traditional Mediterranean diet, could vastly improve overall health (Williams and Roche, 2001) through such means as the modulation of cellular oxidative stress, the modulation of lipoproteins (i.e. by enhancement of high-density lipoprotein-cholesterol levels as observed using polyunsaturated acids) and the down-regulation of inflammatory mediators (Moreno and Mitjavila, 2003).

Additional functional uses of unsaturated lipids include food-grade emulsifiers or surfactants, found as mono- and diacylglycerols (Krog, 1997; Gunstone, 1999), as well as a general ability to alter organoleptic properties of foods due to their fatty nature (Gurr,

1992). While many fat flavours are desirable in nature, it should be noted that overall stability and resistance to oxidation also decrease with increasing unsaturation, eventually producing secondary products such as volatile aldehydes in foodstuff (Lin, 1994). For this reason, manufacturers must be weary of oxidative processes and take the necessary steps to maintain the quality of their products. Lastly, long-chain unsaturated alcohols have also been readily employed in cosmetics and toiletries as an emulsifier, emollient and antifoaming agent (Liebert, 1985) as well as in topical ointments as a means of transdermal drug delivery (Kanikkannan and Singh, 2002).

2.2. Phenolic Compounds

Due in part to a desire to broaden the understanding of potentially bioactive or otherwise functional compounds and an ever increasing demand for more "natural" food products (Iwasaki and Yamane, 2000), scientists continue to investigate the structures, sources and corresponding properties of naturally occurring phenolic compounds.

2.2.1. Structure

While the characteristic structure of a phenol consists of a hydroxyl group bonded directly to a benzene ring, more complex phenolic structures with additional rings and other substituents, including functional derivatives such as esters, methyl esters and glycosides, are quite common (Ho, 1992; Shahidi and Naczk, 1995*b*).

Most plant phenolics share a common origin based on the initial deamination of the amino acid phenylalanine to cinnamic acid before entering the phenylpropanoid pathway. Introduction of one or more hydroxy groups to this phenylpropanoid unit ($C_6 - C_3$) results in the biosynthesis of a wide variety of plant phenols, including cinnamic acids ($C_6 - C_3$), benzoic acids ($C_6 - C_3$ or $C_6 - C_1$), flavonoids ($C_6 - C_3 - C_6$), proanthocyanidins ($(C_6 - C_3 - C_6)_n$), stilbenes ($C_6 - C_2 - C_6$), coumarins ($C_6 - C_3$), lignans ($C_6 - C_3 - C_3 - C_6$) and lignins ($(C_6 - C_3)_n$). Subclasses exist within families to further classify each group according to structure (Hollman, 2001). According to Ho (1992), phenolic compounds occurring commonly in foods may be classified into three major groups: simple phenols and phenolic acids, hydroxycinnamic acid derivatives and flavonoids.

Simple phenols and phenolic acids include monophenols such as *p*-cresol, diphenols such as hydroquinone in addition to triphenols such as gallic acid (Ho, 1992). Distinction between the structures of the above-mentioned compounds lies in the number of hydroxyl groups attached to the aromatic ring. Hydroxycinnamic acids and their derivatives include phenolics such as *p*-coumaric, caffeic and ferulic acids. These compounds differ from hydroxy derivatives of benzoic acid such as *p*-hydroxybenzoic and dihydroxybenzoic acid in that they all contain a carbon-carbon double bond conjugated to their ring structure. Flavonoids consist of catechins, proanthocyanins,

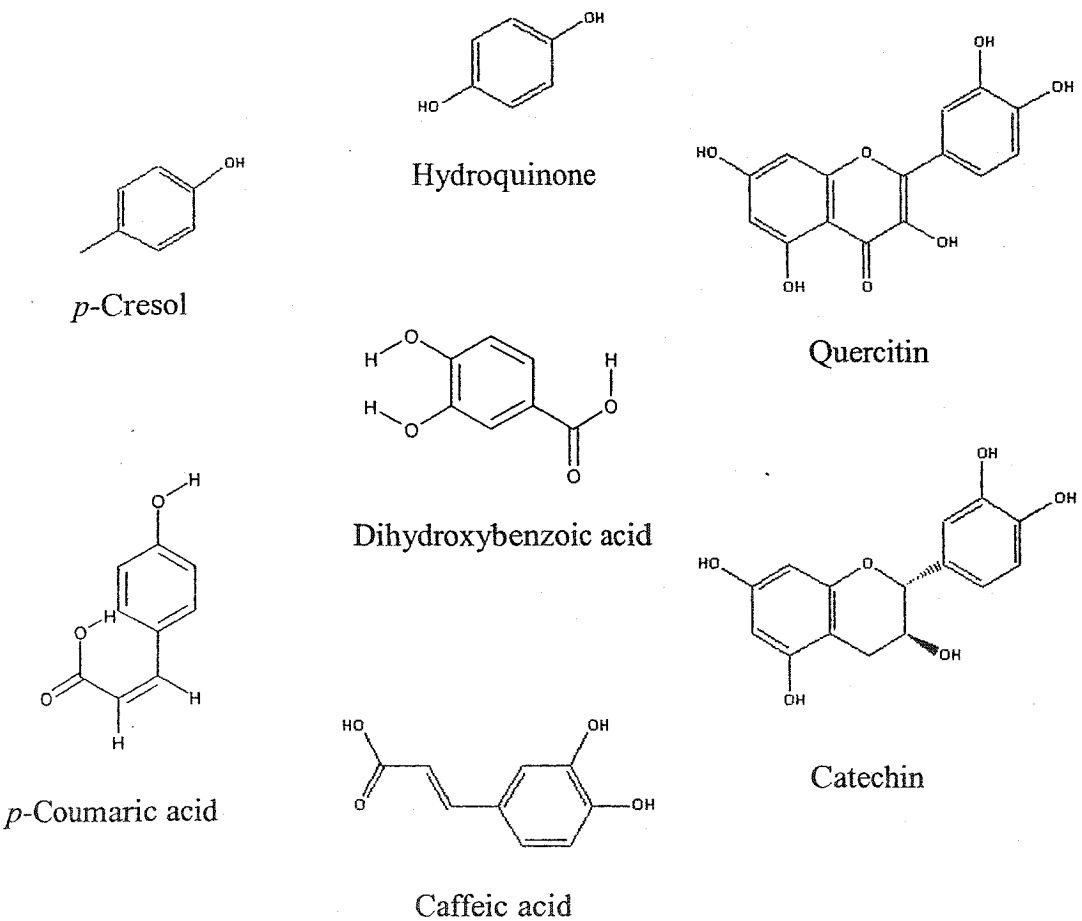


Figure 1. Structures of selected phenolic compounds commonly found in foods.

anthocyanidins, flavons, flavonols and their glycosides (Ho, 1992; Cuppett *et al.*, 1997). Chemical structures of some of the most common phenols found in food are displayed in Figure 1.

2.2.2. Sources

As secondary plant metabolites, phenolic compounds are numerous and widespread in nature (Friedman and Jürgens, 2000). In particular, a wide variety of structurally diverse phenols may be found in foods such as black tea (Mattila *et al.*, 2000), lentils and green beans (Escarpa and González, 2000), grapes (Revilla and Ryan; 2000), potatoes (Friedman and Jürgens, 2000), cauliflower and brussel sprouts (Llorach *et al.*, 2002), rice hull (Kikuzaki *et al.*, 2002) and beer (Cui *et al.*, 1999).

Simple phenols such as *p*-cresol have previously been isolated from raspberries and blackberries while others have been identified in cocoa beans, sesame oil and red wine pomace (Ho, 1992; Escarpa and González, 2000). Hydroxycinnamic acids and derivatives, particularly *p*-coumaric, caffeic and ferulic acids, have been found in citrus fruits, plums, corn flour and *Brassica* oilseed (Ho, 1992). Further, cinnamic acid and its derivatives, including *p*-coumaric and ferulic acids were isolated from pineapple stems (*Ananas comosus* var. Cayenne; Tawata *et al.*, 1996) while naturally occurring ferulic acid esters of varying lengths were extracted and characterized from the stem bark of *Pavetta owariensis* (Rubiaceae), *Pinus roxburghii* (Pinaceae) and *Tecomella undulata* (Bignoniaceae) (Baldé *et al.*, 1991). Flavonoid subclasses are also extremely widespread and have been extracted from tea leaves, onions, broccoli, apples, oranges and parsley (Mattila *et al.*, 2000). Berries, including highbush blueberries (Wang *et al.*, 2000), strawberries, raspberries, chokeberries and bilberries are also rich sources of phenolic compounds, particularly with respect to flavonoids such as anthocyanins, pigments responsible for the vivid colours present in many fruits and vegetables (Häkkinen and Auriola, 1998).

Regarding actual quantities, chlorogenic acid, phloridzin and epicatechin were the main phenolic compounds found in apple juice, present in commercial juice at levels of 130, 24 and 12 mg/L, respectively. Chlorogenic acid was also found to be the major

phenolic constituent of pear juice and was found at levels ranging from 15 – 21 mg/kg fresh weight (Schieber *et al.*, 2001). Following the analysis of cranberry juice, Chen *et al.* (2001) concluded that a total of 400 mg of total phenolics per litre of sample was found in freshly squeezed cranberry juice, having a distribution of approximately 44% as phenolic acids and 56% as flavonoids. Moreover, as the major phenolic acid detected, benzoic acid was present in quantities (34 – 41 mg/L of juice) comparable to those used in the preservation of most perishable food products. Catechins, primarily composed of epicatechin, were also found in tea leaves where they could constitute up to 30% of dry leaf weight (Ho, 1992). Overall, specific quantities and types of phenolic compounds present in foods were variable, as seen through the analysis of Spanish virgin olive oil, whereby concentrations of hydroxytyrosol, tyrosol and luteolin increased as the olive fruits matured with a concurrent decrease in glucoside aglycons (Brenes *et al.*, 1999).

2.2.3. Antioxidant Properties

Antioxidants are added to foods in order to prevent the formation of various off-flavours and other objectionable compounds resulting from the oxidation process. Most natural antioxidants, such as those found in plants, are phenolic in nature and possess varying degrees of antioxidant activity depending on their individual structures (Ho, 1992; Shahidi and Naczk, 1995*a*).

It is a well established fact that the autooxidation of food lipids takes place in a three step process involving initiation (I), propagation (II) and termination (III) steps. During initiation, free radical species are formed which react with atmospheric oxygen and each other to produce a chain reaction. Propagation of this chain reaction continues indefinitely as long as there remains an abundance of free radical species (Nawar, 1996). The last step, termination, occurs when free radicals react with each other to form non-radical species, completing one cycle of lipid autooxidation. Individual steps of the autooxidation process are detailed more fully in Figure 2. It should be noted that re-initiation often causes the cycle to repeat itself indefinitely or until oxidizable substrate is depleted (Cuppett *et al.*, 1997).

I. Initiation	RH				→	R•	+	H•
	RH	+	O_2		→	ROO•	+	H•
II. Propagation	R•	+	O_2		→	ROO•		
	ROO•	+	RH		→	R•	+	ROOH
III. Termination	R•	+	R•		→	RR		
	R•	+	ROO•		→	ROOR		
	ROO•	+	ROO•		→	ROOR	+	O_2

Figure 2. The three reaction steps of autoxidation. Identification of species include lipid (RH) and oxygen (O_2) as initial reactants, free (R•, H•) and peroxy (ROO•) radical species and non-radical products (RR, ROOR), including hydroperoxides (ROOH). Adapted from Cuppett *et al.* (1997).

The primary mechanism by which a phenol acts to inhibit lipid oxidation includes the trapping of peroxy radicals generated during initiation or propagation steps. This may be accomplished in two different ways. In the first mechanism, the peroxy radical abstracts a hydrogen proton from the antioxidant to yield an aroxyl radical and a hydroperoxide. In the second mechanism, a peroxy and an aroxyl radical react by radical-radical coupling to form a non-radical product (Cuppett *et al.*, 1997; Botía *et al.*, 2001). The resulting aroxyl radical formed after abstraction of hydrogen is stabilized by resonance delocalization of the unpaired electron around the aromatic ring structure. Moreover, the high stability of these radical intermediates and lack of a position suitable for attack by molecular oxygen are key factors influencing antioxidant activity (Nawar, 1996). Other mechanisms of phenolic antioxidant action include chelation of transition metals involved in free radical generation as well as the modulation of selected enzymatic activities (Mauri *et al.*, 1999).

Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tertiary-butylhydroquinone (TBHQ) are used individually and as mixtures to extend the shelf life and quality of foods (Shahidi and Naczk, 1995*a*). While they have been proven effective in a variety of food systems,

possess high stability and are low in cost (Ho, 1992), overall demand for these additives in food has been dropping steadily due to their suspected action as promoters of carcinogenesis (Cuppett *et al.,* 1997; Buisman *et al.*, 1998; Stamatis *et al.*, 1999). As a result of these findings and of a general rejection of synthetic food additives by consumers, research has shifted to focus more on the development and utilization of antioxidants from natural sources (Cuppett *et al.,* 1997). Endogenous to foods and thus considered safe (Stamatis *et al.*, 1999), phenolic compounds encompass a wide range of structurally diverse compounds whose proven antioxidant properties certainly warrant further investigations. Additional advantages of phenolic compounds relate to their antimicrobial (Friedman and Jürgens, 2000), anticarcinogenic (Hollman, 2001) and antimutagenic (Ho, 1992) effects as well as their role in sensory attributes including colour, astringency, bitterness and aroma (Andlauer *et al.*, 2000; Rodríguez-Delgado *et al.*, 2001).

2.3. Phenolic Lipids

Based on their properties, plant phenolics are perceived by many to be potential replacements for already established, though less desirable synthetic antioxidants; however, one major drawback in their widespread use in practical food applications lies in their hydrophilic nature (Buisman *et al.*, 1998). Specifically, the hydrophilic nature of phenolic compounds reduces their effectiveness in the stabilization of fats and oils when an aqueous phase is also present (Stamatis *et al.*, 1999). Whereas ideal antioxidants in bulk lipid systems remain at the air-bulk lipid interface where they are most effective in preventing oxidation, those in emulsion systems (i.e. oil-in-water) should be solubilized with active surfaces oriented to the oil-water interface to facilitate access to peroxy radical sites (Nawar, 1996). Altering solubility and miscibility properties of phenolic compounds through the addition of aliphatic side-chain groups could result in their more widespread application as antioxidants in emulsion systems. While most research to date has concentrated on synthesis of phenolic compounds with short and medium chain aliphatic groups, an attempt has been made to focus on the use of long chain aliphatic groups to synthesize phenolic lipids.

Another concept worth mentioning involves the modification of lipids in order to improve associated nutritional and/or functional properties. Much research has been published regarding the introduction of medium chain, such as caprylic (Paez *et al.*, 2003) and polyunsaturated fatty acids such as EPA, DHA and DPA (Jennings and Akoh, 1999; Iwasaki *et al.*, 1999; Haraldsson *et al.*, 2000; Kim *et al.*, 2001) into structured lipids with noted health benefits (Høy and Xu, 2001). Research into novel fats containing reduced or even zero energy is also ongoing with commercial products including Salatrim (Smith *et al.*, 1994), caprenin, bohenin (Auerbach *et al.*, 2001) and olestra (Yankah and Akoh, 2001) leading the way. This overwhelming interest is based largely on consumers' desire to maintain overall well-being with minimal effort and an industries' ability to respond to this need. Furthermore, with the consumption of manufactured foods continually on the rise, there is a distinct advantage to providing more healthful choices for consumers. The concept of a natural lipophilic antioxidant composed of a long-chain aliphatic and phenolic moiety readily fits this mould, particularly since the inclusion of unsaturated lipids into these compounds could result in additional nutritional benefits (Petel, 2003).

2.4. Lipases

Defined as glycerol ester hydrolases (E.C.3.1.1.3), lipases naturally catalyze the hydrolysis reaction of triacylglycerols as well as other carboxylic esters into their corresponding components (Whitaker, 1996). Lipases are spontaneously soluble in water as a result of their globular protein nature. In contrast, their natural substrates (i.e. lipids) do not readily dissolve in aqueous solution. As a result, reactions involving lipase occur at a lipid-water interface where enzyme and substrate make contact (Brockman, 1984).

Lipases originate from a wide variety of sources including animals, plants and microorganisms. Examples from animal sources include pancreatic and pregastric lipases. As well, plant lipases from germinating oilseeds have previously provided a cheap alternative enzyme source. Finally, microorganisms including yeasts, fungi and bacteria, produce lipases in both endogenous and exogenous forms and are highly regarded from an industrial and economic standpoint. Hence, in spite of the fact that only a small

number of lipases are supplied commercially, a vast number derived from a variety of sources are known (Quinlan and Moore, 1993).

Advantages of lipase-catalyzed reactions include high enzyme selectivity, lower overall reaction time and fewer side reactions when compared to chemical methods (Willis *et al.*, 1998; Osório *et al.*, 2001). Specifically, because lipase-catalyzed reactions are carried out under milder conditions of temperature (i.e. < 70° C) and pressure (i.e. atmospheric), fewer undesirable bi-products are formed (Gunstone, 1999; Schuchardt *et al.*, 1998). This makes purification of the final product as well as waste disposal less of a problem. Furthermore, these reactions require less energy and are conducted in equipment of lower capital cost than many chemical processes, making the use of lipases as biocatalysts very desirable as an alternative to chemical methods (Gunstone, 1999). Moreover, one unique advantage associated with enzymatic processes is the ability to market naturally produced products to consumers as opposed to chemically produced ones. Potential uses for lipases extend throughout the food, cosmetic and pharmaceutical industries and include the biosynthesis of phenolic lipid compounds.

2.4.1. Mechanism of Action

Lipase-catalyzed reactions have been the subject of increasing interest and importance over the last ten to fifteen years. According to Gunstone (1999), the major reason for this is connected to the realization that under appropriate conditions, a lipase can promote either ester formation or ester hydrolysis. Moreover, careful choice of lipase, reaction conditions and substrates may allow for controlled acylation and deacylation to produce specific fatty acids and triacylglycerols (i.e. structured lipids). Classes of reactions catalyzed by lipases include hydrolysis reactions, as well as synthesis such as esterification and transesterification.

2.4.1.1. Hydrolysis

Lipid hydrolysis refers to the splitting of fat into its constituent acids and alcohols in the presence of water (Gandhi, 1997); the natural function of lipase is to catalyze this fat hydrolysis reaction (Quinlan and Moore, 1993). Furthermore, lipase-catalyzed hydrolysis reactions proceed only in the presence of adequate amounts of water. This is

due to the fact that water molecules participate in the breaking of covalent bonds in the substrate as well as subsequent incorporation of their elements into these bonds to form reaction products (Whitaker, 1996), as seen in Figure 3. Thus, an environment with a high water activity actually promotes triacylglycerol hydrolysis reactions (Osório *et al.*, 2001).

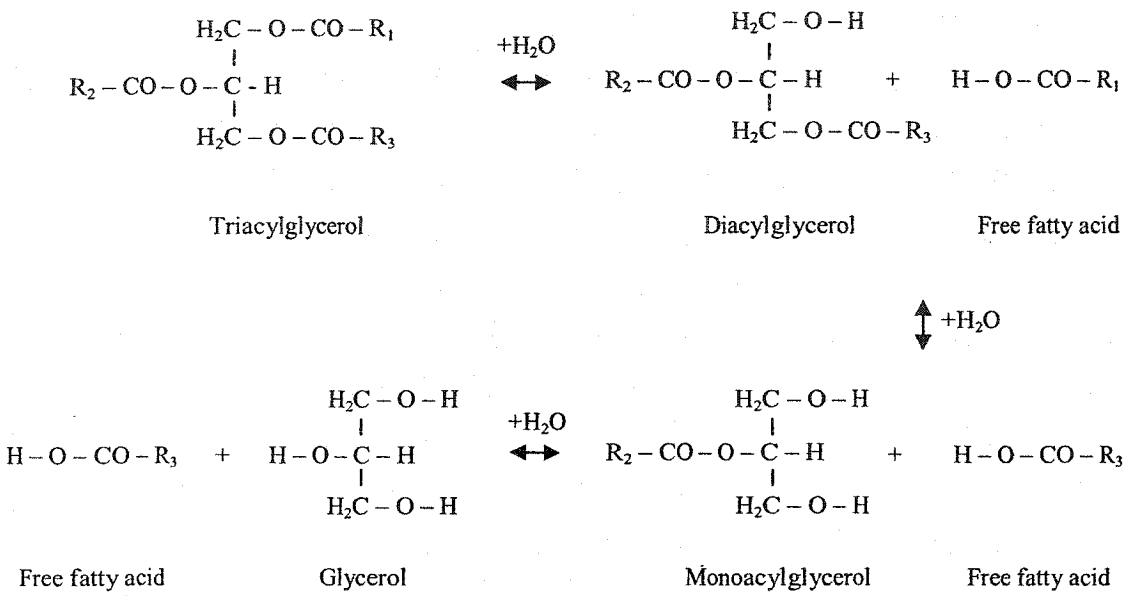


Figure 3. Forward reaction denotes the enzymatic hydrolysis of a triacylglycerol molecule. The reverse reaction corresponds to synthesis by esterification.

Specific reaction products are determined according to the extent of the hydrolysis reaction. Mixtures of diacylglycerols, monoacylglycerols and free fatty acids are common; however, the more complete the hydrolysis, the higher the concentration of free fatty acids present in the final mixture. Additionally, the overall sequence of hydrolysis depends largely on the specificity of the enzyme employed with non-specific lipases hydrolyzing the *sn*-1, 2 and 3 positions of triacylglycerol molecules in a random order as opposed to positional, substrate and stereospecific lipases. The issue of acyl-migration should also be considered when targeting particular molecular structures (Jensen *et al.*, 1983).

Lipase-catalyzed hydrolysis reactions are ideal for the removal of fatty acids from unstable oils, including conjugated or highly unsaturated fatty acids, owing to mild conditions which effectively reduce unwanted oxidation reactions (Gandhi, 1997). Gunstone (1999) also noted the use of lipase-catalyzed hydrolysis reactions to produce glycerol esters enriched in ω-3 fatty acids from seal blubber and menhaden oil. Because natural fish oils do not contain more than about one-third of their fatty acids from the ω-3 family, hydrolysis reactions are particularly helpful for the purpose of concentration (Opstvedt *et al.*, 1990).

2.4.1.2. Esterification

Though designed by nature to effect hydrolysis of lipids, lipases also promote the synthesis of ester bonds under appropriate reaction conditions (Willis *et al.*, 1998; Gunstone, 1999). Esterification is but one such synthesis reaction that may be defined in several ways; the most straightforward definition being the reaction of acids and alcohols to produce fats and water. Also referred to in Figure 3, an alternative description for esterification may include the reverse reaction of hydrolysis (Gandhi, 1997).

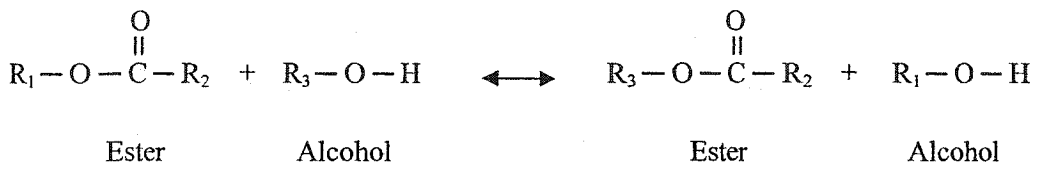
This being the case, the water content of a reaction system strongly affects the activity of lipases in terms of their ability to undergo esterification reactions. Low water content actually shifts the equilibrium of these reversible reactions to favour the synthesis of lipids. For this reason, researchers typically work with organic solvents when synthesis reactions are required (Osório *et al.*, 2001). Additional techniques used to drive synthesis reactions include continual removal of molecules such as water that are formed during the process by azeotropic distillation (Yan *et al.*, 2002) or evaporation under reduced pressure. One example of an esterification reaction is the reaction of cinnamic acid with oleyl alcohol to yield a model phenolic lipid ester and a molecule of water.

An important point to note regarding lipase-catalyzed synthesis reactions is that while they are often described in terms of reverse hydrolysis reactions, there is no clear relationship between the hydrolytic and synthetic activities of a given lipase. Thus, a lipase with a high hydrolytic activity may demonstrate very high to absolutely no

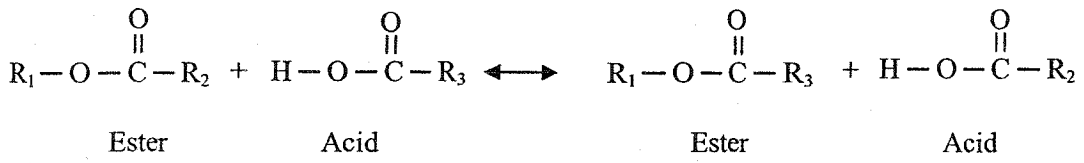
synthetic activity in other applications, depending on the given enzyme. Important implications include the absence of the possibility of making theoretical predictions for synthesis based on hydrolytic reaction rates (Wu *et al.*, 1996).

2.4.1.3. Transesterification

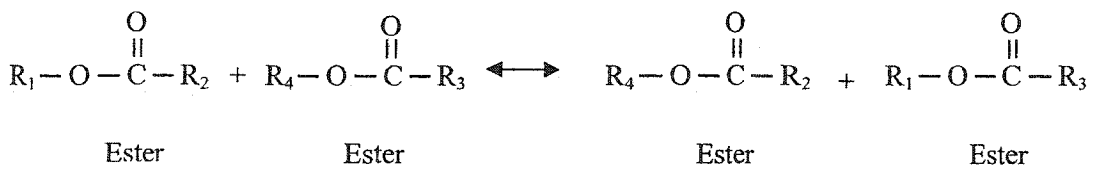
While ester synthesis via esterification reactions produce water as a bi-product, synthesis by way of transesterification processes, including alcoholysis, acidolysis and interesterification, give rise to alcohol, acid or ester containing compounds instead (Gandhi, 1997). Lipase-catalyzed alcoholysis, acidolysis and interesterification reactions are described more clearly in Figure 4.



(i) Alcoholysis



(ii) Acidolysis



(iii) Interesterification

Figure 4. Lipase-catalyzed transesterification: Reactions may be divided into *(i)* alcoholysis, *(ii)* acidolysis and *(iii)* interesterification.

The first reaction illustrated in Figure 4, alcoholysis (*i*), is the reaction of an ester with an alcohol to produce an ester with a different alkyl group (Gunstone, 1999). In cases like this, the alcohol substrates may be either monohydric (i.e. ethanol) or polyhydric (i.e. glycerol) in nature. The second reaction, acidolysis *(ii)*, is the reaction of an ester with an acid leading to exchange of acyl groups. Willis *et al.* (1998) described acidolysis as the transfer of an acyl group between an acid and an ester often used to incorporate novel free fatty acids into triacylglycerols. The last synthesis reaction illustrated is that of interesterification (*iii*) which Gunstone (1999) described as the reaction of one ester with another leading to randomization of acyl and alcohol moieties. Opstvedt *et al.* (1990) further described interesterification as a process whereby the functional qualities of fats could be altered and improved.

Sequentially, the first stage of interesterification actually involves the hydrolysis of triacylglycerols with consumption of water to yield diacylglycerols, monoacylglycerols and free fatty acids. Under appropriate conditions, subsequent resynthesis of the liberated fatty acids onto glycerol molecules occurs to produce combinations of glycerol and fatty acids. As well, while hydrolysis products will continue to accumulate until equilibrium is reached, rates of these multi-substrate reactions may be limited by either the acylation or deacylation steps of the reaction (Willis *et al.*, 1998).

2.4.2. Kinetics of a Multi-Substrate Reaction

Multi-substrate reactions, such as transfer reactions, are often composed of two substrates and two products (bi-bi). Moreover, while these two substrate two product (bi-bi) reactions may follow any one of a number of sequential or non-sequential mechanisms, including ping-pong, random or compulsory order (Garcia *et al.*, 1999), focus will be placed on the ping-pong bi-bi mechanism of enzyme action (Palmer, 1995).

The ping-pong bi-bi or double displacement mechanism is a non-sequential mechanism, defined as such because it is not necessary for both substrates to bind to the enzyme to form a ternary complex before the first product is produced. The actual sequence of events begins with the substrate binding with the enzyme to form a binary complex. Next, intramolecular reorganization of bonds take place before the first product

is released. The second substrate then binds with the newly modified enzyme before another intramolecular bond rearrangement occurs. Finally, the second product is released leaving the enzyme intact in its original form. This basic mechanism of enzyme action is represented by the diagram displayed in Figure 5, devised by Cleland in addition to a simplified rate equation associated with the ping-pong bi-bi mechanistic model derived by Alberty (Palmer, 1995; Biselli *et al.*, 2002).

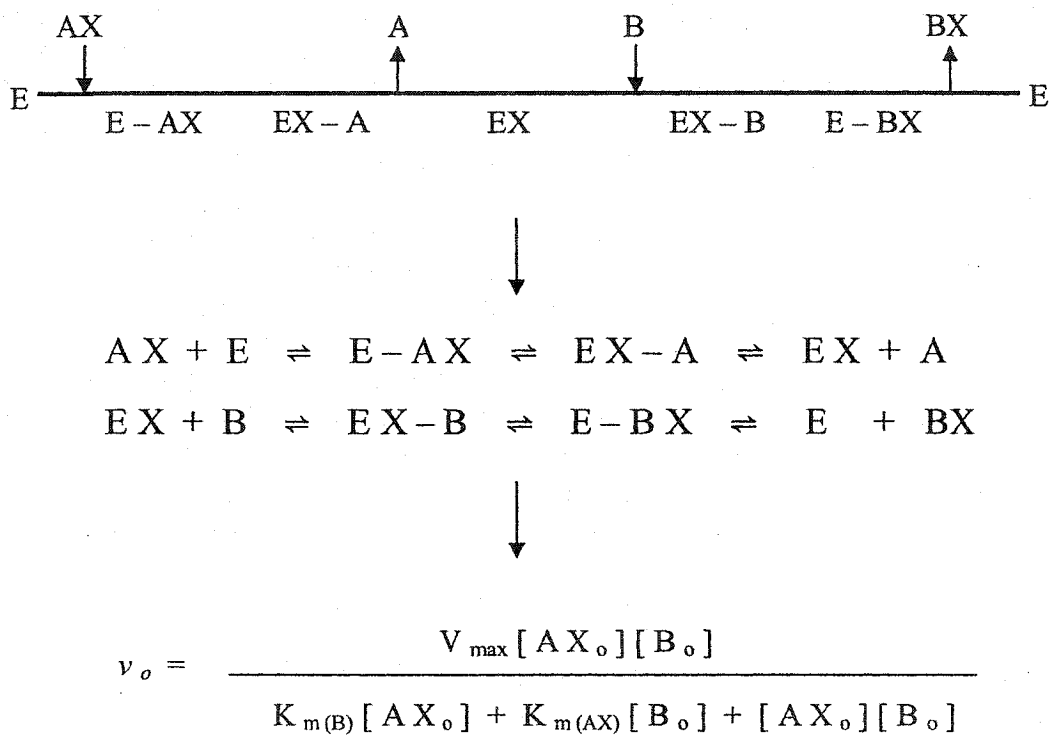


Figure 5. Ping-pong bi-bi mechanism of enzyme action and corresponding rate equation. Identification of reaction species include free enzyme (E), substrates (AX, B), binary complexes before (E-AX, EX-B) and after (EX-A, E-BX) intramolecular reorganizations, modified enzyme complex (EX) and products of the reaction (A, BX). Identification of values in rate equation include initial velocity (v_o) and concentrations of substrates (AX_o, B), maximum velocity when substrates are saturating (V_{max}), concentration of AX which gives $\frac{1}{2}V_{max}$ when B is saturating ($K_{m(AX)}$) and concentration of B which gives $\frac{1}{2}V_{max}$ when AX is saturating ($K_{m(B)}$). Adapted from Palmer (1995).

The three-dimensional structure of lipase includes an active site composed of three specific amino acid residues, serine (Ser), histidine (His) and either aspartate (Asp) or glutamate (Glu), which are collectively referred to as a catalytic triad. Other typical distinguishing features include an oxyanion hole and a lid structure formed by an α-helix which covers over the active site of the lipase. In order for catalysis to occur, this lid structure must be displaced through interfacial activation to expose the active site. Regarding esterification according to the ping-pong model, catalysis begins with the nucleophilic attack of the serine residue of the catalytic triad on the acid substrate, followed by the release of a molecule of water from the intermediate and the formation of an acyl-enzyme complex. A further nucleophilic attack by the alcohol substrate on the complex results in a second tetrahedral intermediate which subsequently releases an ester molecule and the lipase in its native form (Gandhi *et al.*, 2000; Martinelle and Hult, 1994). Esterification reactions reported to follow ping-pong bi-bi kinetics include that of oleic acid with methanol (Ramamurthi and McCurdy, 1994) or ethanol (Hazarika *et al.*, 2002) as well as isoamyl alcohol with butyric acid (Hari Krishna and Karanth, 2001).

2.5. Biocatalysis in Organic Solvent Media

In the past, enzymatic reactions were carried out in aqueous media whilst organic solvents were considered as denaturants; presently, it is now a well established fact that many enzymes remain active even in organic media (Kvittingen, 1994; Sellek and Chaudhuri, 1999). Moreover, it is now known that there is enormous industrial potential for exploitation of properties, including retained and occasionally improved chemo-, regio- and stereoselectivities of enzymes in these systems (Lortie, 1997; Schmid *et al.*, 2001; Hari Krishna, 2002; Secundo and Carrea, 2003). Organic solvent systems employed alone, or in conjunction with water or water immiscible solvents include monophasic, biphasic, microemulsion and reverse micellar systems (Secundo and Carrea, 2003).

Globular proteins such as lipase are stabilized in aqueous solution by a series of weak interactions composed of hydrogen bonding, ionic, hydrophobic and van der Waals' interactions in addition to covalent disulfide bridges which act collectively to create a strong stabilizing force (Lehninger *et al.*, 1993). Because this stabilizing force

includes significant interactions with the water molecules of the aqueous phase, disruption through increasing temperatures destabilize the protein by unfolding of the tertiary structure and can result in loss of enzyme activity and denaturation (Ó Fágáin, 1995; Sellek and Chaudhuri, 1999). In contrast, enzymes are extremely thermostable in low water environments since bulk water does not contribute to stabilization or denaturation of the protein (Kvittingen, 1994; Persson *et al.*, 2002).

Stabilization of globular proteins in low water systems such as organic solvent depends on protein-solvent interactions (Sellek and Chaudhuri, 1999). A protein can maintain its proper conformation in organic solvent if a layer of bound or 'essential' water remains associated with the folded polypeptide (Zaks and Russel, 1988; Ó Fágáin, 1995; Halling, 2002). Literature also suggests that a lipase possessing a stronger binding force to water exerts a higher activity (Negishi *et al.*, 2003). Conversely, decreased stability may be seen in cases where there is easily removed or distorted bound water (Ó Fágáin, 2003). This tightly bound water is necessary to preserve the proper enzyme conformation that will allow the enzyme to retain its catalytic activity. Moreover, since unfolded polypeptides require many more water molecules for effective solvation, equilibrium in low water systems is shifted towards the folded form and makes unfolding of the polypeptide much less likely (Ó Fágáin, 1995). Further stabilization of enzymes in organic media occurs when protein-solvent interactions favour exclusion of solvent molecules from the enzyme hydration layer. According to Sellek and Chaudhuri (1999), this causes compaction of the enzyme due to an increase in surface energy and also results in added stability.

Enzymes in low water systems such as organic solvent have previously shown good selectivity and stability in reaction systems; however, enzymes show extremely low catalytic activities in these environments (i.e. several orders of magnitude) compared with native aqueous solutions (Gandhi *et al.*, 2000; Schmid *et al.*, 2001). This has been attributed in part to enzyme rigidity associated with low water systems which makes it difficult for the enzyme to undergo conformational changes, such as those required in the formation of enzyme-substrate complexes. Furthermore, this limited internal enzyme flexibility is itself a factor in enzyme stability and relates to the hydration state of the

protein molecule (Adlercreutz, 2000; Persson *et al.*, 2002). Another explanation for reduced lipase activity in organic solvent media relates to its increased ability to solubilize hydrophobic substrates. Typically, hydrophobic substrates bind readily with the hydrophobic active centers of lipase molecules due to energetic incentives to partition from the aqueous phase; however, because hydrophobic substrates are stabilized in organic media, the energetics of such reactions is less favourable. Overall, this results in a higher activation barrier for reactions in organic media and a slower reaction rate (Klibanov, 1997). Increasing quantities of enzyme are generally needed to compensate for the lowered enzymatic activities found in most organic solvent reaction systems (Sellek and Chaudhuri, 1999).

2.5.1. Advantages of Using Organic Solvent Media

Increased solubility of hydrophobic compounds in organic solvent media permits for greater interactions between substrates and enzymes as well as advantageous partitioning of substrates and products; specifically, this is because partitioning of products away from the enzyme can decrease the possibility of inhibition due to excess product around the biocatalyst (Sellek and Chaudhuri, 1999; Biselli *et al.*, 2002; Halling, 2002).

Organic solvent media is particularly suited for synthetic reactions as a distinct ability to alter the equilibrium position exists. In particular, esterification reactions proceeds more readily in non-aqueous media with improved yields obtained through removal of excess water produced (Sellek and Chaudhuri, 1999; Adlercreutz, 2000). Fine tuning of specificity was also noted as an important advantage when working in organic media since changes in solvent have been known to affect parameters such as enantioselectivity (Kvittingen, 1994; Persson *et al.*, 2002). Moreover, water-dependent side reactions may be reduced or prevented since unwanted side reactions which lead to irreversible enzyme inactivation like deamidation, peptide hydrolysis and cystine decomposition cannot occur to the same extent in the absence of water, required as a reactant (Zaks and Russell, 1988; Kvittingen, 1994; Secundo and Carrea, 2003). Increased stability, decreased microbial contamination and easier integration into already

established chemical steps are also among the virtues associated with biocatalysis in organic solvent media.

2.5.2. Selected Parameters Influencing Lipase Activity

The rate of enzyme biocatalysis, denoted by its activity value, is a very important factor when considering the feasibility of an enzymatic process. Factors which affect this rate include enzyme source, reaction type as well as reaction conditions employed (Gandhi *et al.*, 2000). Below, selected parameters found to influence lipase activity in organic solvent media are examined in greater detail.

2.5.2.1. Organic Solvent

Organic solvents are most often expressed in terms of their polarity, measured quantitatively as the logarithm of the partition coefficient (log P value) of solvent between 1-octanol and water (Laane *et al.*, 1987; Halling, 2002). The reason for this is that enzymatic activity in non-aqueous media has been found to correlate well with this parameter as opposed to others such as the Hildebrand solubility parameter and dielectric constants. According to guidelines reported by Laane *et al.* (1987), solvents with a log P value of less than two (< 2) are not favourable for enzymatic systems, solvents with a log P value between two and four ($2 \leqslant \log P \leqslant 4$) are typically unpredictable and solvents with a log P greater than four (> 4) can maintain active biocatalysts (Laane *et al.*, 1987; Kvittingen, 1994).

These predications are based on the ability of organic solvent to distort water-enzyme interactions essential for activity. Specifically, organic solvents that penetrate into the bound or 'essential' layer of water molecules surrounding an enzyme act to strip them away. Hydrophilic solvents ($\log P < 2$) generally possess the ability to strongly distort this layer while less hydrophilic solvents with their decreased ability to dissolve water, have less of an effect. Least distortion of water-enzyme interactions takes place in hydrophobic solvents where minimal interaction occurs due to the partitioning of water into the enzyme rather than the solvent itself. Consequently, it is in fact the concentration of water on the surface of the enzyme that determines the specific activity in a particular solvent system (Zaks and Russell, 1988; Wehtje and Adlercreutz, 1997). In view of the

countless studies' that have been performed examining the relationship of log P and enzymatic activity in a wide range of reaction systems (Gubicza, 1992; Hazarika *et al.*, 2002; Wu *et al.*, 2002), it is now widely accepted that hydrophobic solvents function best for biocatalysis in organic solvent media (Kvittingen, 1994; Klibanov, 1997).

Furthermore, Pogorevc *et al.* (2002) recently correlated the degree of enzyme deactivation of selected lipases upon exposure to organic solvent to their respective log P values; however, little deactivation by an organic solvent could not necessarily be correlated to catalytic activity in the medium.

According to Nakamura *et al.* (1995), in addition to its hydrophobicity, the basic structure of solvent also influences the activity and stereoselectivity of lipase-catalyzed reactions. In particular, enantioselectivity in a structurally linear solvent was found to be higher than for its corresponding branched chain solvent while cyclic solvents had decreased enantioselectivity based on the size of their ring structure. Based on these findings, the selectivity of a reaction can be controlled or even reversed by use of an appropriate solvent (Klibanov, 1997; Ottosson *et al.*, 2002) due to its influence on the rigidity of the enzyme. Kvittingen (1994) also noted the ability of solvents to act as substrates in lipase-catalyzed reactions.

2.5.2.2. Water Activity

Water content refers to the total amount of water present in a system (Pomeranz and Meloan, 1994) and is generally agreed on to be an extremely important parameter affecting enzymatic activity in organic solvent media (Halling, 1994; Wehtje *et al.*, 1997; Ma *et al.*, 2002); however, presenting enzymatic activity as a function of the water content of the whole reaction mixture is not very informative (Halling, 1992; Valivety *et al.*, 1992). This is so because a portion of the water present may be dissolved in the organic phase as individual molecules or small oligomers, in the more polar phase around the biocatalyst or even associated with other components present in the reaction media such as other proteins, impurities or support; without changing the composition of these phases, alterations in their relative volumes will affect the total water content, but not the microenvironment of the biocatalyst or its behaviour (Halling, 2002).

As a result, a more appropriate measure of the amount of water available in an enzymatic system is the thermodynamic water activity (a_w). The a_w is a better indicator of enzymatic activity because it does not change considerably with changes in solvent, support or other components which might be present in the enzyme preparation (Halling, 1992; Valivety *et al.*, 1992; Halling, 1994). Thermodynamic a_w is expressed as the partial pressure of a solution over the partial pressure of pure water and is measured through the vapour phase (Pomeranz and Meloan, 1994, Halling, 2002). At equilibrium, a_w values are the same for all phases, regardless of the critical water contents of individual phases. Additionally, this means that the water bound by an enzyme is likely a function of the a_w of the reaction system (Halling, 2002; Adlercreutz, 2000).

An abundance of research has shown the dependence of catalytic activity on a_w using a range of solvents with different critical total water contents (Hadzir *et al.*, 2001; Chowdary and Prapulla, 2002; Ma *et al.*, 2002; Cheng and Tsai, 2003). In general, an enzyme's optimum a_w reflects both the nature of the reaction environment as well as the structure of the enzyme. For instance, lipases from *Rhizomucor miehei* and *Candida rugosa* were found to catalyze the identical esterification reaction optimally at a_w values of 0.5 and > 0.9, respectively (Valivety *et al.*, 1992). Ma *et al.* (2002) further noted that most lipases are active down to $a_w \leqslant 0.1$. Still, too little water in the system results in decreased catalytic activity due to inadequate hydration of the protein molecules while too much water may cause agglomeration of particles and unwanted side reactions. Protein hydration is an extremely important phenomenon, with Zaks and Klibanov (1988) suggesting approximately 1000 molecules of water per enzyme molecule, corresponding roughly to a monolayer, for optimum catalytic activity.

2.5.2.3. Substrate Diffusion

Diffusion may be defined as random molecular motions taking place over small molecular distances which eventually lead to complete mixing. It can be a slow process, with rates of diffusion of gases being much faster than either liquids or solids. When diffusion occurs sequentially with other phenomena, the rate of diffusion has the potential to influence the overall rate of the process (Cussler, 2000*a*). Currently of interest is the

rate of diffusion of substrate from the bulk organic solvent to the active sites of immobilized enzyme present in the reaction system. Figure 6 illustrates a schematic relevant to substrate diffusion in monophasic organic solvent media.

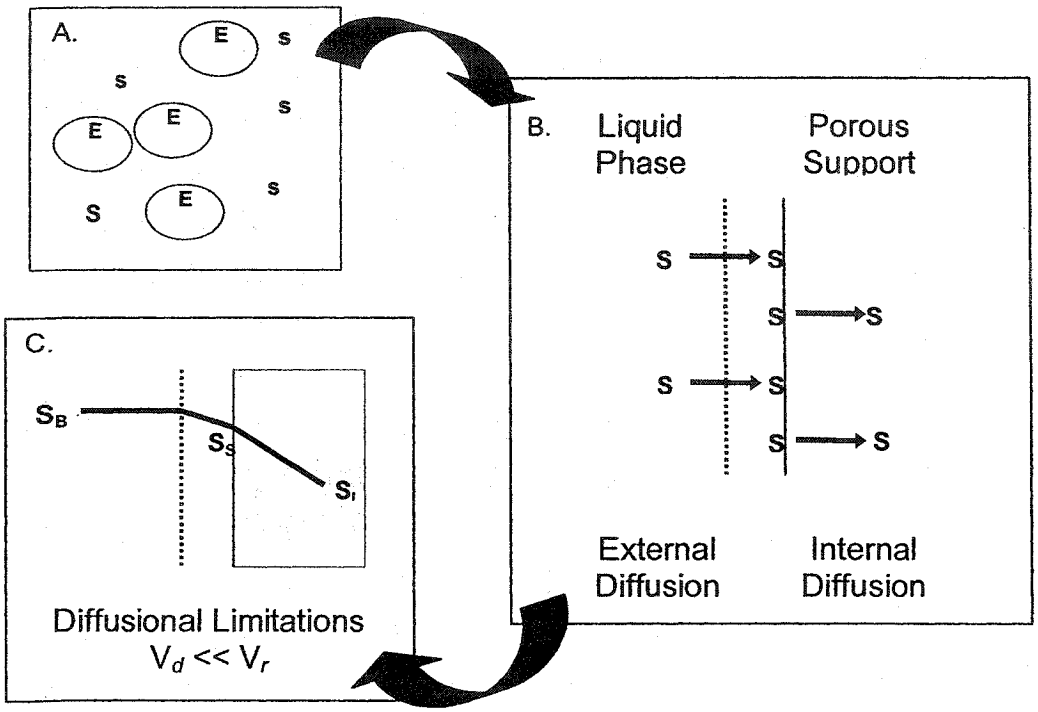


Figure 6. Diffusion of substrate in a heterogeneous system. (A) Heterogeneous system consisting of substrate and enzyme, (B) interface between solvent and immobilization matrix and (C) schematic of concentrations of substrate in a system limited by diffusion. Identification of species include immobilized enzyme (E), substrate (S) in addition to substrate concentrations in bulk solvent (S_B), at the surface (S_S) and interior (S_I) of the immobilization matrix. Adapted from Seagrave (1971) and Cussler (2000*a-d*).

In heterogeneous systems such as one containing an immobilized biocatalyst suspended in organic solvent, two types of substrate diffusion exist: external diffusion and internal diffusion. External diffusion refers to the diffusion of substrate across a boundary layer to the surface of the porous catalyst while internal diffusion refers to

diffusion of substrate from the surface of the biocatalyst to the active sites of the enzyme molecules (Almeida *et al.*, 1998; Salis *et al.*, 2003). Diffusional limitations occur when the velocity of diffusion (v_d) is much less than the velocity of the reaction (v_r). When this happens, diffusion is limiting the rate of the biocatalytic reaction since the actual concentration of substrate in the area of the biocatalyst is much lower than that observed in the bulk media. The result is generally a lowered enzymatic activity value (Seagrave, 1971; Cussler, 2000*a-d*).

The rate of diffusion can often be accelerated by agitation (Klibanov, 1997; Barros *et al.*, 1998; Shintre *et al.*, 2002). In cases such as these, diffusion still depends on random molecular motions. Agitation is not a molecular process; it is a macroscopic process that moves portions of the fluid over much larger distances. Following this macroscopic motion, diffusion mixes newly adjacent portions of the fluid (Cussler, 2000*a*). In enzymatic systems, one can check for the presence of external diffusional limitations by varying the agitation speed of the reaction. Specifically, increasing the agitation speed of a reaction where diffusional limitations exist will result in an increase in the initial enzymatic activity due to a decrease in the size of the boundary layer.

Internal diffusional limitations may be investigated through studying the effects of enzyme loading on catalytic activity. Almeida *et al.* (1998) reportedly investigated the effects of internal diffusion by sieving commercial immobilized enzyme into different size series; the dependence of reaction rates on particle size was indicative that internal diffusional limitations were present. Moreover, it should also be noted that type of immobilization support plays a role in diffusion, and ultimately the determination of catalytic activity (Adlercreutz, 1992).

2.6. Biosynthesis of Phenolic Lipids

Guyot *et al.* (1997) were among the first to study the biosynthesis of a range of phenolic acids with fatty alcohols to produce phenolic lipids. Particular substrates included cinnamic, caffeic, ferulic, dihydrocaffeic and 3, 4-dimethoxycinnamic acids as well as fatty alcohols varying between 2 – 18 carbon molecules in length. Based on these studies, the biosynthesis of phenolic lipids was deemed possible with highly variable

yields (3 – 98% bioconversion). Highest yields were obtained for the lipase-catalyzed esterifications of dihydrocaffeic acid with oleyl alcohol (up to 90% bioconversion after 8 days) as well as 3, 4-dihydroxyphenylacetic acid with octanol (98% bioconversion after 5 days) in a solvent-free reaction system in the presence of excess alcohol substrate. Somewhat less efficient, the esterification of cinnamic acid with oleyl alcohol resulted in a 56% bioconversion yield of phenolic lipid after a 7-day period under similar conditions. Not technically a phenolic compound itself, cinnamic acid reflects the basic skeleton of many phenolic compounds of interest and accounts for its importance and inclusion in numerous studies. Results from selected biosynthesis reactions found in the literature are detailed more fully in Table 2.

Certain interesting trends emerged from the work of Guyot *et al.* (1997) relating to optimal phenolic acid structure and fatty alcohol chain length. Regarding phenolic acid structure, electronic induction was found to modify the reactivity of the carboxylic function. This phenomenon resulted in close to zero bioconversion yield of phenolic lipid in the cases of caffeic (3, 4-dihydroxycinnamic acid) and ferulic (4-hydroxy-3-methoxycinnamic acid) acids with butanol under defined conditions. In contrast, cinnamic, dihydrocaffeic and 3, 4-dimethoxycinnamic acids resulted in yields of 97, 78 and 60%, respectively, after a 15-day reaction period. From this, one may conclude that para-hydroxylation had a negative effect on phenolic lipid biosynthesis. Moreover, the presence of a double bond on the side-chain conjugated with the ring structure (i.e. hydroxycinnamic acids) resulted in higher bioconversion yields than for those without (i.e. benzoic acids). Concerning isomers, Guyot *et al.* (1997) further concluded that meta isomers of hydroxycinnamic acid were favoured during biosynthesis followed by ortho and para isomers, which behaved similarly when esterified with octanol. Fatty alcohol chain length also influenced the degree of bioconversion to phenolic lipids. Overall, biosynthesis with ethanol was not successful; however, varying degrees of success were achieved using other fatty alcohol chain lengths. Highest bioconversion yields for cinnamic acid were reached with butanol and octanol whereas the biosynthesis of phenolic lipids employing ferulic acid could only be achieved using fatty alcohols with carbon chain lengths greater than or equal to eight.

Table 2. Lipase-catalyzed biosynthesis of selected model phenolic lipid compounds reported in the literature.

Phenolic Derivative	Lipid Derivative	Biocatalyst	Bioconversion Yield[a] (%)	Reaction Time[b] (day)	Reference
Cinnamic acid	Butanol	*CAL B*	85	5	Buisman *et al.* (1998)
Cinnamic acid	Butanol	Novozym 435	97	15	Guyot *et al.* (1997)
Cinnamic acid	Oleyl alcohol	Novozym 435	56	7	Guyot *et al.* (1997)
Caffeic acid	Octanol	Lipozyme	trace	12	Stamatis *et al.* (1999)
o-Coumaric acid	Octanol	Novozym 435	21	12	Stamatis *et al.* (1999)
m-Coumaric acid	Octanol	Novozym 435	38	12	Stamatis *et al.* (1999)
p-Coumaric acid	Octanol	Novozym 435	25	12	Stamatis *et al.* (1999)
Dihydrocaffeic acid	Oleyl alcohol	Novozym 435	90	8	Guyot *et al.* (1997)
Ferulic acid	Oleyl alcohol	Novozym 435	14	15	Guyot *et al.* (1997)

[a]Bioconversion yield was calculated based on the amount of phenolic substrate reacted compared with the initial quantity present in the reaction mixture, expressed as a percentage value.

[b]Reactions were incubated between 50 – 60°C with continuous stirring throughout the duration of the reaction period.

Further studies on cinnamic and benzoic acid derivatives with fatty alcohols from 4 – 12 carbons in length were performed by Buisman *et al.* (1998) with limited success; however, maximum bioconversion of phenolic lipid was improved compared with work performed by Guyot *et al.* (1997) for the biosynthesis of cinnamic acid with butanol (85% bioconversion after 5 days). On the whole, Buisman *et al.* (1998) essentially confirmed the work of Guyot *et al.* (1997), noting that electron donating substituents conjugated to the carboxylic group in cinnamic and benzoic acid derivatives intrinsically deactivated the electrophilic carbon center of that group for nucleophilic attack of the alcohol. Buisman *et al.* (1998) also investigated the esterification of polyphenols bearing primary hydroxyl groups at the aromatic ring with octanoic acid; excellent results were obtained using hydroxytyrosol (85% bioconversion after 15 hours), an antioxidant present naturally in olive oil as well as 3, 5-di-*t*-butyl-4-hydroxybenzylalcohol (98% bioconversion after 2.5 hours) which is a compound structurally similar to the synthetic antioxidant BHT.

Works by Stamatis *et al.* (1999 and 2001) were also in agreement, noting that both alcohol chain length and aromatic acid structure influenced the bioconversion of lipophilic antioxidant compounds. Moreover, maximum yields of 82 and 97% were obtained after a 12-day period for cinnamic and *p*-hydroxyphenylpropionic acids, respectively, when esterified with octanol (Stamatis *et al.*, 2001). Based on these and other results, *p*-hydroxylation was found to have no negative impact on the bioconversion of product when the side chain on the aromatic ring was saturated (Stamatis *et al.*, 1999 and 2001). Similar investigations were also carried out employing flavonoids such as rutin and naringin with a range of fatty acids from between 8 – 12 carbons in length whereby maximum bioconversion yields were found to range between 50 – 60% after a 9-day period (Kontogianni *et al.*, 2001).

In keeping with the generally low yields previously reported for the biosynthesis of esters of ferulic acid (Guyot *et al.*, 1997; Stamatis *et al.*, 1999), Compton *et al.* (2000) obtained maximum bioconversion yields following a 13-day period of 20 and 14%, for the esterification of ethanol and octanol, respectively, with ferulic acid. Transesterification of ethyl ferulate with triolein was also investigated by Compton *et al.*

(2000) to yield feruloyl monoolein and diolein species for possible use as active components in waterproof sunscreen formulations; a maximum bioconversion yield of 77% was achieved after a 6-day period.

Lastly, according to Petel (2003), transesterification of catechin with tricaprylin in a monophasic hexane system resulted in bioconversion yields of 48 and 37% using Lipozyme after 4 days and Novozym 435 after 8 days, respectively. Further investigations using tricaprylin with catechol resulted in 13 and 34% bioconversion yields for Lipozyme and Novozym 435 lipase, respectively, after an 8-day reaction period. The lipase, nature of the phenolic compound as well as length of the incubation period were found to affect the final bioconversion yields.

According to studies by Kikuzaki *et al.* (2002), both the radical scavenging abilities of antioxidants as well as their affinities with lipid substrates influence overall effectiveness in food systems. Multiple model systems were employed to test the antioxidant and radical scavenging activities of a wide range of compounds, including ferulic acid and its related esters. While results were variable according to the physical system tested and chain length of ester employed, ferulic acid and its related compounds were found to be effective agents against oxidative reactions. Other investigations done on the antioxidant activities associated with phenolic lipid compounds include the monitoring of antioxidant performance of both hydroxytyrosol and 3, 5-di-*t*-butyl-4-hydroxybenzylalcohol octanoates in refined sunflower oil using an oil stability index apparatus (Buisman *et al.*, 1998). While results were promising, compared with both pure 3, 5-di-*t*-butyl-4-hydroxybenzylalcohol and synthetic BHT, these esters displayed lower antioxidant activities, later attributed to the loss of primary hydroxy groups following addition of the fatty acid moieties. Slightly apart, additional lipophilic structures demonstrating antioxidant properties included ascorbic acid fatty acid esters such as ascorbyl palmitate (Stamatis *et al.*, 1999).

2.7. Characterization of Phenolic Lipids

Phenolic lipid products have been characterized using a wide range of techniques and instrumentation, including thin-layer chromatography (TLC, Guyot *et al.*, 1997;

Buisman *et al.*, 1998), high-performance liquid chromatography (HPLC, Guyot *et al.*, 1997; Stamatis *et al.*, 1999; Compton *et al.*, 2000), gas-liquid chromatography (GLC, Buisman *et al.*, 1998), nuclear magnetic resonance spectroscopy (NMR, Buisman *et al.*, 1998; Stamatis *et al.*, 1999 and 2001) and more recently, liquid chromatography-mass spectrometry (LC-MS, Compton *et al.*, 2000; Kontogianni *et al.*, 2001).

TLC was used more often for initial qualitative analyses of biomolecules; it made use of a wide range of mixtures of chloroform, methanol, acetic acid and water to separate phenolic lipids, including esters of cinnamic acid and derivatives (Guyot *et al.*, 1997; Buisman *et al.*, 1998; Stamatis *et al.*, 1999 and 2001) as well as flavonoid fatty esters (Kontogianni *et al.*, 2001). Visualization of TLC plates was previously accomplished by either UV absorbance (Stamatis *et al.*, 1999) or using selected chromogenic reagents (Guyot *et al.*, 1997; Buisman *et al.*, 1998; Stamatis *et al.*, 1999 and 2001; Kontogianni *et al.*, 2001).

HPLC was often used over other instrumentation for the separation and quantification of substrates and phenolic lipid products following biosynthesis reactions. Methodologies varied depending on the analytes; however, in all cases, HPLC was carried out using reversed-phase chromatography employing C8 (Compton *et al.*, 2000) or C18 (Guyot *et al.*, 1997; Stamatis *et al.*, 1999 and 2001; Kontogianni *et al.*, 2001; Petel, 2003) columns in conjunction with a range of organic solvents and either UV or evaporative light scattering detection (ELSD). Selection of the UV wavelength was based primarily on the absorbing properties of the compounds of interest. Preferred over other modes of detection, the use of UV (205 – 300 nm, Guyot *et al.*, 1997; Lin *et al.*, 1997; Stamatis *et al.*, 1999 and 2001; Kontogianni *et al.*, 2001) was only feasible in the absence of strongly absorbing solvents such as chloroform and acetone. In contrast, ELSD detection was most appropriate for larger molecules, including the phenolic lipid transesterification products of ethyl ferulate with triolein (Lin *et al.*, 1997; Compton *et al.*, 2000; Andrikopoulos, 2002). GLC analysis was also employed for the quantification of smaller, more volatile phenolic lipids, including fatty alcohol esters of cinnamic acid (Buisman *et al.*, 1998).

Conclusive structural analysis of phenolic lipid end products was accomplished by infrared (IR) spectroscopy, NMR as well as LC-MS analysis. Specifically, the formation of ester bonds of cinnamic acid and its derivatives were monitored over the course of the biosynthesis reactions by IR spectroscopy (Guyot *et al.*, 1997); similarly, ester bond formation was also monitored by fourier transform infrared spectroscopy (FTIR, Tarahomjoo and Alemzadeh, 2003) throughout the biosynthesis of selected sugar fatty acid esters. Moreover, characteristic shifts in ^{13}C-NMR (Stamatis *et al.*, 1999, Kontogianni *et al.*, 2001) and ^{1}H-NMR (Buisman *et al.*, 1998) spectroscopy allowed for the structural elucidation of several phenolic lipid compounds in addition to carbohydrate esters of fatty alcohols (Villeneuve *et al.*, 2002). Recent research on phenolic lipids has also made use of atmospheric pressure chemical ionization-mass spectroscopy (APCI-MS) as well as electrospray ionization (ESI) techniques in conjunction with liquid chromatography (Compton *et al.*, 2000; Kontogianni *et al.*, 2001). Due to its ability to produce intact ions from large and complex species in solution, ESI-MS is considered a powerful technique for the characterization of biomolecules and among the most versatile ionization techniques in existence today (Giusti *et al.*, 1999). ESI-MS has also been used in the structural characterization of lipids (Andrikopoulos, 2002) and a wide range of phenolic compounds (Es-Safi *et al.*, 2000; Stobiecki *et al.*, 1999; Pérez-Magariño *et al.*, 1999).

3. MATERIALS AND METHODS

3.1. Materials

Commercially immobilized lipase from *Candida antarctica* (Novozym 435, with activity of 10,000 Propyl Laurate Units, PLU, per gram) was obtained from Novozymes A/S (Bagsværd, Denmark). *Trans*-cinnamic acid (>99%) and triolein (99%) substrates as well as oleic acid standard (99%) were purchased from Sigma Chemical Co. (St. Louis, MO), while oleyl alcohol substrate, diolein and monoolein standards (>99%) were purchased from Nu-Chek Prep Inc. (Elysian, MN). HPLC grade methyl ethyl ketone (2-butanone) and ACS grade glacial acetic acid were obtained from A.C.P. (Montreal, Qc). All other solvents were of HPLC grade or higher and were purchased, along with ACS grade salts used for pre-equilibration of water activity, from Fisher Scientific (Fair Lawn, NJ).

3.2. Methods

3.2.1. Lipase-Catalyzed Esterification of trans-Cinnamic Acid with Oleyl Alcohol

3.2.1.1. Preparation of Substrate Solutions

Prior to each enzymatic reaction, a stock solution of *t*-cinnamic acid (26.7 mM) was freshly prepared in 2-butanone while that of oleyl alcohol (40 mM) was prepared in hexane.

3.2.1.2. Esterification Reaction

The esterification reaction of cinnamic acid with oleyl alcohol was carried out according to a modification of the procedures described by Stamatis *et al.* (1999), Buisman *et al.* (1998) and Kermasha *et al.* (1995). Defined quantities of *t*-cinnamic acid and oleyl alcohol stock solutions were diluted with appropriate amounts of hexane in 50-mL Erlenmeyer flasks to yield final concentrations of 4 mM for each substrate in a 10 mL mixture of hexane/2-butanone (85:15, v/v).

Enzymatic reactions were initiated by the addition of 20 mg of Novozym 435 lipase (200 PLU). The Erlenmeyer flasks were incubated under vacuum at 55°C with continuous shaking at 150 rpm in an orbital incubator shaker (New Brunswick Scientific

Co., Inc., Edison, NJ). Control reactions without lipase were carried out in tandem with reactions under identical conditions. In addition, control reactions containing immobilized enzyme with only *t*-cinnamic acid or oleyl alcohol were also incubated to monitor for potential side-reactions.

Individual enzymatic reactions were halted at selected time intervals by the removal of immobilized lipase by decantation from each reaction flask. A quantity of 1 mL of reaction media was dried down under vacuum using an Automatic Environmental Speed Vac system (Savant Instruments Inc., Holbrook, NY) at 20°C. Dried samples were then flushed with nitrogen and stored at -80°C until time of analysis. Both enzymatic reactions and controls were run in duplicate.

3.2.1.3. Analysis of Reaction Mixtures

Sample mixtures were re-solubilized in appropriate amounts of methanol (0.25 – 2.0 mL) and subject to HPLC analysis for quantification of substrates and end product, according to a modification of the procedures described by Guyot *et al.* (1997) and Stamatis *et al.* (1999).

HPLC analysis was carried out with a Zorbax SB-C18 reversed-phase column (5 μm, 250 x 4.6 mm, Agilent, Canada) using Beckman HPLC system (Model 126, Beckman Instruments Inc., San Ramon, CA). This system was equipped with an autosampler (model 507) and an ultraviolet diode-array (UV-DAD) detector (model 168) with computerized data handling and integration analysis (System Gold software, version 5.0, Beckman). Injection volume was 20 μL of sample. The isocratic mobile phase, consisting of methanol and deionized water (95:5, v/v), was maintained at a flow rate of 1 mL/min over 40 min. Detection was carried out at 205 and 275 nm, with additional scanning from 190 – 400 nm at 1-sec intervals.

Calibration curves were constructed from a wide range of concentrations of cinnamic acid and oleyl alcohol substrates; however, since no commercial standard was readily available for the phenolic lipid end product, it was necessary to recover it by preparative HPLC analysis for the construction of a standard curve.

3.2.1.4. Determination of Lipase Activity and Percent Bioconversion

Initial enzymatic activities and bioconversion yields of the esterification reaction were calculated on the basis of the concentrations of substrates and end product, determined by HPLC analysis.

The initial enzymatic activities were calculated from the slopes (m) of the regression of data points obtained when plotting the concentration of product as a function of the amount of biocatalyst (Eq. 1) or the reaction time (Eq. 2):

$$v = \frac{(m)\,(V_R)}{(E)} \qquad \text{[Eq. 1]}$$

$$v = \frac{m}{t_R} \qquad \text{[Eq. 2]}$$

where, initial enzymatic activities (v) were calculated from the slopes (m), total reaction volume (V_R), mass of immobilized biocatalyst (E) and total reaction time (t_R). Biocatalyst concentrations ranged from 0.5 – 4 g/L per reaction flask while total reaction time was ⩽16 days. The unit of initial enzymatic activity was defined as nmol of product produced per g of immobilized lipase per min.

The percent bioconversion of the lipase-catalyzed reaction was defined as the concentration of the product (mM) produced, divided by the concentration of product (mM) assuming a complete conversion, multiplied by 100.

3.2.2. Optimization of Lipase Activity in Organic Solvent Media

3.2.2.1. Determination of Optimum Solvent Mixture

A wide range of solvent mixtures containing 2-butanone (85:15, v/v) were evaluated for the effect of co-solvent on the lipase-catalyzed esterification reaction, including iso-octane (log P = 4.5), heptane (log P = 4.0), hexane (log P = 3.0), octanone (log P = 2.4) and 2-butanone (log P = 0.29). The selection of these co-solvents was based on their diverse hydrophobicities (Laane *et al.*, 1987) and their miscibility with 2-butanone. Enzymatic reactions were analyzed following removal of reaction flasks at regular time intervals over the course of a 16-day reaction period.

3.2.2.2. Effect of Initial Water Activity

The effect of initial water activity (a_w) of the reaction system was investigated according to the method described by Valivety *et al.* (1992). The lipase and the most appropriate organic phase (iso-octane/2-butanone, 85:15, v/v) were pre-equilibrated separately to the required a_w. The equilibration was carried out at 4°C for the solid enzyme and at room temperature for the iso-octane and 2-butanone solvents, in sealed containers using saturated salts with characteristic water activity values, including NaOH ($a_w = 0.05$), LiCl ($a_w = 0.11$), $K(C_2H_3O_2)$ ($a_w = 0.23$), $MgCl_2$ ($a_w = 0.33$), $Mg(NO_3)_2$ ($a_w = 0.53$) and NaCl ($a_w = 0.75$). The minimum equilibration period was 72 h.

3.2.2.3. Effect of Agitation Speed

The effect of agitation speed on the initial lipase activity was investigated by varying the agitation speed from 0 to 200 rpm, using a range of lipase concentrations (1 – 4 g/L). Enzymatic reactions were carried out for a period of 2 days under the optimized conditions of solvent mixture (iso-octane/2-butanone, 85:15, v/v) and initial a_w (0.05).

3.2.2.4. Effect of Reaction Temperature

The optimum reaction temperature was investigated by varying the incubation temperature from 35 to 65°C, using a range of lipase concentrations (0.5 – 4 g/L). Enzymatic reactions were carried out for a period of 2 days under the optimized conditions of solvent mixture (iso-octane/2-butanone, 85:15, v/v), initial a_w (0.05) and agitation speed (150 rpm).

The energy of activation (E_a) was calculated according to the Arrhenius equation:

$$V = A_i e^{-(Ea/RT)} \qquad \text{[Eq. 3]}$$

where, the rate constant at a specific temperature (V), a pre-exponential constant (A_i), the universal gas constant ($R = 8.314$ mol · k/J) and the absolute temperature (T) were used to calculate the energy of activation (E_a) of the reaction system.

3.2.2.5. Effect of Cinnamic Acid to Oleyl Alcohol Substrate Ratio

The effect of cinnamic acid to oleyl alcohol ratios were investigated by increasing the initial concentration of oleyl alcohol in the reaction system (2 to 24 mM) at a defined cinnamic acid concentration (4 mM). The ratios of cinnamic acid to oleyl alcohol, therefore, ranged from 1.0:0.5 to 1.0:6.0. Enzymatic reactions were carried out for a 16-day reaction period, using optimized assay conditions (iso-octane/butanone, 85:15, v/v; a_w = 0.05; 150 rpm; 55°C) and were analyzed following removal of reaction flasks at regular time intervals.

3.2.2.6. Kinetic Parameters

The initial enzymatic activity of the esterification reaction was measured at substrate concentrations from 1 to 4 mM cinnamic acid and 0.25 to 4 mM oleyl alcohol, using the optimized assay conditions (iso-octane/butanone, 85:15, v/v; a_w = 0.05; 150 rpm; 55°C). Enzymatic reactions were monitored at time intervals over the course of a 3-day reaction period.

3.2.3. Characterization of the Model Phenolic Lipid Product

3.2.3.1. HPLC Separation of Reaction Components

HPLC analysis was carried out according to a modification of the procedures described by Guyot *et al.* (1997) and Stamatis *et al.* (1999), as described previously.

3.2.3.2. Spectrophotometric Scanning of Substrates and End Product

Stock solutions of cinnamic acid and oleyl alcohol substrates and the phenolic lipid end product were prepared in methanol (2 mg/mL). A wide range of methanol diluted solutions (1/2 to 1/125) were scanned spectrophotometrically from 200 to 800 nm, using Beckman spectrophotometer (Model 650, Beckman Instruments, Inc., Fullerton, CA).

3.2.3.3. Electrospray Ionization Mass Spectroscopy Analysis of End Product

Mass spectrometric analysis in the positive ion mode was performed on a triple quadrupole mass spectrophotometer (SCIEX API III Biomolecular Mass Analyzer, Thornhill, ON, Canada). The collected HPLC fraction was reconstituted in 0.5 mM

ammonium sulfate in methanol. The resulting solution was introduced into the electrospray ion-source using a syringe pump (Harvard Apparatus Model 22, South Natick, MA) at a flow rate of 1.5 µL/min. The ionspray voltage was set at 5.5 kV, and the orifice potential at 50 V.

3.2.4. Lipase-Catalyzed Transesterification of t-Cinnamic Acid with Triolein

3.2.4.1. Transesterification Reaction of Cinnamic Acid with Triolein

Using the optimized assay conditions for the esterification of cinnamic acid with oleyl alcohol (iso-octane/2-butanone, 85:15, v/v; a_w = 0.05; 150 rpm; 55°C), the lipase-catalyzed transesterification of cinnamic acid with triolein was also investigated. The reaction mixture was composed of 4 mM cinnamic acid and 4 mM triolein in a 10 mL mixture of iso-octane/2-butanone (85:15, v/v). Enzymatic reactions were initiated by the addition of 20 mg of solid lipase per reaction flask. Transesterification reactions were carried out in duplicate, and monitored alongside control trials containing no enzyme, at specific time intervals over the course of a 21-day reaction period.

Relative hydrolysis was calculated on the basis of the concentrations of oleic acid (divided by 3), monoolein and diolein (mM) at a given time divided by the initial concentration of triolein present in the control in mM, multiplied by 100. Relative transesterification yield was calculated on the basis of the concentration of esterified cinnamic acid (mM) at a given time divided by the initial concentration of cinnamic acid present in the control in mM, multiplied by 100.

3.2.4.2. HPLC Separation of Reaction Components

Sample mixtures were re-solubilized in appropriate amounts of 2-propanol (0.25 to 3 mL) and subject to HPLC analysis, according to a modification of the procedures described by Compton *et al.* (2000) and Lin *et al.* (1997).

The HPLC analysis was performed as described previously, with the exception that the detection was carried out using UV detection (235 nm) as well as an evaporative light scattering detector (drift tube 82°C, nitrogen flow 40 mm, ELSDII, Varex Corporation, Burtonsville, MD). In addition, a binary gradient elution system composed

of solvent A (190 mL methanol, 10 mL 2-propanol, 1.5 mL deionized water and 0.5 mL glacial acetic acid) and solvent B (2-propanol) was used. The elution sequence was 10 min of solvent A, followed by a 10 min linear gradient to 100% solvent B and a 15 min period of 100% solvent B; the total run time was 35 min. Calibration curves were constructed using cinnamic acid, triolein, diolein, monoolein and oleic acid standards.

3.2.4.3. Electrospray Ionization Mass Spectroscopy Analysis of End Products

Mass spectrometric analysis was carried out according to the procedure described previously, using mass spectrometric analysis in the positive ion mode performed on a triple quadrupole mass spectrophotometer (SCIEX API III Biomolecular Mass Analyzer). For MS/MS analysis, argon was used as the collision gas at a collision gas thickness (CGT) of 1.5×10^{14}.

4. RESULTS AND DISCUSSION

4.1. Lipase-Catalyzed Esterification of *t*-Cinnamic Acid with Oleyl Alcohol

A model enzymatic system for the biosynthesis of phenolic lipids was investigated, using cinnamic acid and oleyl alcohol as substrates. In order to optimize both the initial enzymatic activity as well as the overall bioconversion yield, the effects of selected parameters, including the nature of the solvent mixture, initial a_w, agitation speed, temperature and acid to alcohol ratio were investigated.

4.1.1. Determination of Optimum Solvent Mixture

A range of co-solvents, including methanol, ethyl acetate, chloroform, 1,4-dioxane and 2-butanone, were initially investigated for their capacity to solubilize substrates of interest, their miscibility with other organic solvents and their non-interference with the enzymatic system. The experimental results (not shown) indicated that 2-butanone was the most appropriate solvent. As such, a binary solvent mixture was established using selected co-solvents and 2-butanone at a ratio of 85:15 (v/v) for the biosynthesis of phenolic lipids. Already used with lipids intended for nutritional purposes, an additional advantage of using 2-butanone was its low solvent toxicity (Yan *et al.*, 2002).

Selected co-solvents with a wide range of hydrophobicities, denoted by their log P values, were evaluated in a monophasic reaction medium as a mixture containing the defined co-solvent and 15% of 2-butanone. The use of 15% of 2-butanone was the minimum amount necessary to ensure adequate solubilization of the more polar substrate, cinnamic acid, initially present at 4 mM.

On the whole, control trials carried out in tandem containing all reaction components without enzyme or individual substrates with enzyme, showed no chemical side reactions or significant changes in the concentrations of either substrate throughout the course of the reaction period. Lipase-catalyzed esterification reactions in the octanone/2-butanone (85:15, v/v) mixture and 2-butanone resulted (Table 3) in low initial

Table 3. Effects of selected solvent mixtures on the initial enzyme activity and percent bioconversion after a 16-day lipase-catalyzed esterification of cinnamic acid and oleyl alcohol substrates.

Solvent[a]	*Log P* Value[b]	Initial Enzyme Activity (nmol product/g enzyme/min)[c]	Bioconversion (%)[d]
Iso-octane	4.50	155.87 (±8.73)[e]	91.14
Heptane	4.00	124.97 (±4.49)[e]	80.67
Hexane	3.50	128.65 (±4.86)[e]	98.43
Octanone	2.40	3.92 (±0.42)[e]	1.58
Butanone	0.29	1.00 (±0.32)[e]	0.51

[a]Monophasic system consisting of a co-solvent and butanone (85:15, v/v).

[b]*Log P* value was defined as the partition co-efficient of the individual co-solvent between water and 1-octanol.

[c]Initial enzyme activity was defined as nmol cinnamic acid ester/gram enzyme/min.

[d]Percent bioconversion based on the conversion of substrate into product after a 16-day bioconversion reaction (55°C, agitation speed 150 rpm), was calculated as the concentration of the product divided by the maximum theoretical concentration, multiplied by 100.

[e]Standard error was defined as the standard deviation divided by the square root of the number of samples and was calculated, using SigmaPlot statistical software, based on the concentration of product obtained as a function of time for duplicate samples.

enzymatic activities (< 4 nmol product/g enzyme/min) and bioconversion yields (< 2%), following a 16-day reaction period. In contrast, much higher initial enzymatic activities and bioconversion yields were obtained when hexane, heptane and iso-octane were used as co-solvents in a mixture with 2-butanone (85:15, v/v). For instance, when hexane (log P =3.5) was used as the co-solvent, the initial enzymatic activity and maximum bioconversion yield were 128.7 nmol product/g enzyme/min and 98.4%, respectively. A similar initial enzymatic activity of 125.0 nmol product/g enzyme/min and a moderately lower bioconversion yield of 80.7% were obtained with heptane (log P = 4.0) as co-solvent. The highest initial enzymatic activity of 155.9 nmol product/g enzyme/min was achieved with the most hydrophobic co-solvent, iso-octane (log P = 4.5), with a corresponding bioconversion yield of 91.1%.

The overall results (Table 3) show clearly that an increase in the hydrophobicity of the organic solvent mixture resulted in an increase in initial enzymatic activity. The initial velocity of the phenolic lipid biosynthesis reaction increased by a factor of 155.9 by increasing the hydrophobicity of the co-solvent in the mixture with 2-butanone, from a log P value of 0.29 to 4.5. The experimental results (Table 3) are in agreement with Laane *et al.* (1987) who also indicated that hydrophobic solvents (log P > 4) are more suitable media for biosynthesis reactions. Similar trends correlating hydrophobicity, using log P values, to enzymatic activity were also reported for a wide range of other lipase-catalyzed esterification and transesterification reactions (Gubicza, 1992; Bradoo *et al.*, 1999; Hadzir *et al.*, 2001; Hazarika *et al.*, 2002; Tarahomjoo and Alemzadeh, 2003; Priya and Chadha, 2003). Dordick (1989) indicated that the polarity of a solvent has a profound effect on the retention of enzyme-associated water and affects enzyme catalysis. More hydrophilic solvents, with lower log P values, have a tendency to strip the layer of water from the surface of the enzyme, distorting water-enzyme interactions essential for activity (Laane *et al.*, 1987; Kvittingen, 1994; Halling, 2002). Moreover, due to the existence of substrate-solvent interactions, the availability of substrate to the enzyme varies with the nature of organic solvent, and thus may affect the thermodynamic activity of the substrate, the overall activity of the enzyme-catalyzed reaction and the partition coefficients of substrates as well as products (Wehtje and Adlercreutz, 1997). The low

initial enzymatic activity values for the octanone/2-butanone (85:15, v/v) mixture and the 2-butanone media (Table 3) may therefore have been due to the low availability of oleyl alcohol substrate in increasingly hydrophilic media. Enzyme deactivation caused by an increase in polar solvent around the enzyme molecules could also account for these low initial enzymatic activities (Adlercreutz, 2000).

On the whole, initial enzymatic activity values for the esterification of cinnamic acid with oleyl alcohol (Table 3) are in the same range as others reported in literature. Initial enzymatic activity values from 58 to 320 nmol product/g enzyme/min were reported by Stamatis *et al.* (1999 and 2001) for the esterification of cinnamic acid with an excess of 1-octanol using selected lipases, with maximum activity obtained using Novozym 435 lipase from *C. antarctica.* Further substitution of cinnamic acid by its hydroxylated forms, such as coumaric and caffeic acids, or methoxylated derivatives, such as ferulic acid, yielded initial enzymatic activity values below 150 nmol product/g enzyme/min (Stamatis *et al.,* 1999).

4.1.2. Effect of Incubation Time

Figure 7 illustrates the production of phenolic lipid throughout a series of 16-day lipase-catalyzed reactions in a mixture of hexane or heptane or iso-octane with 2-butanone (85:15, v/v). The results show a steady increase of cinnamic acid ester product with bioconversion yields of 48.1, 45.7 and 56.4% after a 6-day period for reactions in hexane, heptane and iso-octane mixtures, respectively. Moreover, bioconversion yields were highest in the iso-octane/butanone mixture up to a period of 12 days. However, beyond the 12-day period, the bioconversion yield in the hexane/butanone mixture exceeded that of the iso-octane/butanone mixture by 7%.

The literature has shown a wide range of bioconversion yields ($\leqslant$ 98%) for the lipase-catalyzed esterification of cinnamic acid or its derivatives with fatty alcohols of varying chain lengths in both organic solvent and solvent-free systems; corresponding reaction periods also varied from several hours to in excess of 30 days (Guyot *et al.*, 1997). As such, bioconversion yields and associated reaction times obtained throughout

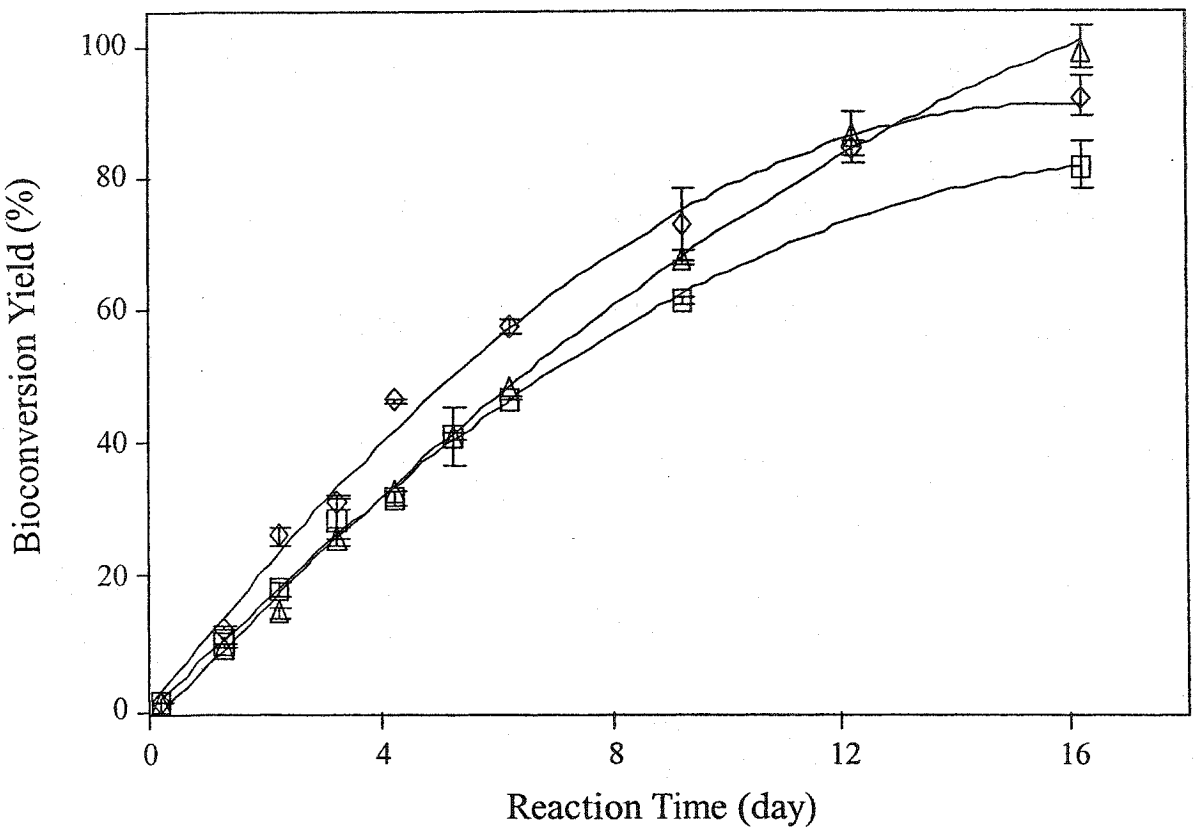


Figure 7. The effect of solvent mixture on the percentage bioconversion of cinnamic acid and oleyl alcohol esterification, using immobilized lipase from *Candida antarctica* monitored at 205 nm; iso-octane (◇), heptane (□) and hexane (Δ) solvent, respectively, present as a mixture of solvent and butanone (85:15, v/v).

this study (Table 3) are comparable to many of those reported in literature (Guyot *et al.*, 1997; Buisman *et al.*, 1998; Stamatis *et al.*, 1999 and 2001).

Guyot *et al.* (1997) reported on the esterification of cinnamic acid with an excess of oleyl alcohol using Novozym 435 lipase in a solvent-free system, noting a 56% bioconversion yield after 7 days. Similarly, Stamatis *et al.* (1999) obtained bioconversion yields from 38 to 57% after 12 days for the esterification of cinnamic acid with 1-octanol using Novozym 435 lipase in acetone, 2-methyl-2-propanol and 2-methyl-2-butanol solvent systems (Stamatis *et al.*, 1999). These bioconversion yields are significantly lower than those of 91.1, 80.7 and 98.4% obtained after 16 days of reaction in hexane, heptane and iso-octane mixtures, respectively (Fig. 7). Furthermore, large variations in bioconversion yields were reported by Buisman *et al.* (1998) for the Novozym 435 lipase-catalyzed esterification of cinnamic acid with *n*-butanol in a range of organic solvents; a maximum bioconversion yield of 85% was reported after a 5-day period in pentane, which is comparable to values obtained in the present study.

The overall results indicated that under the experimental conditions, iso-octane/2-butanone (85:15, v/v) was the most appropriate solvent mixture for the biosynthesis of phenolic lipids and consequently used as the reaction medium for further investigations throughout this study.

4.1.3. Effect of Initial Water Activity

The effects of initial a_w value on both the initial enzymatic activity of the reaction system and the bioconversion yield were investigated. The biosynthesis of the model phenolic lipid (Fig. 8) in an iso-octane/butanone (85:15, v/v) mixture was carried out at a wide range of a_w values (0.05 to 0.75); this was accomplished by pre-equilibration of solvent and enzyme separately through the vapor phase with saturated salts corresponding to known a_w values (Halling, 1994).

The results (Fig. 8, bar graph) show a large increase in the initial enzymatic activity of the lipase-catalyzed esterification reaction with a concomitant decrease in the initial a_w values. Likewise, the bioconversion yields obtained following a 16-day reaction

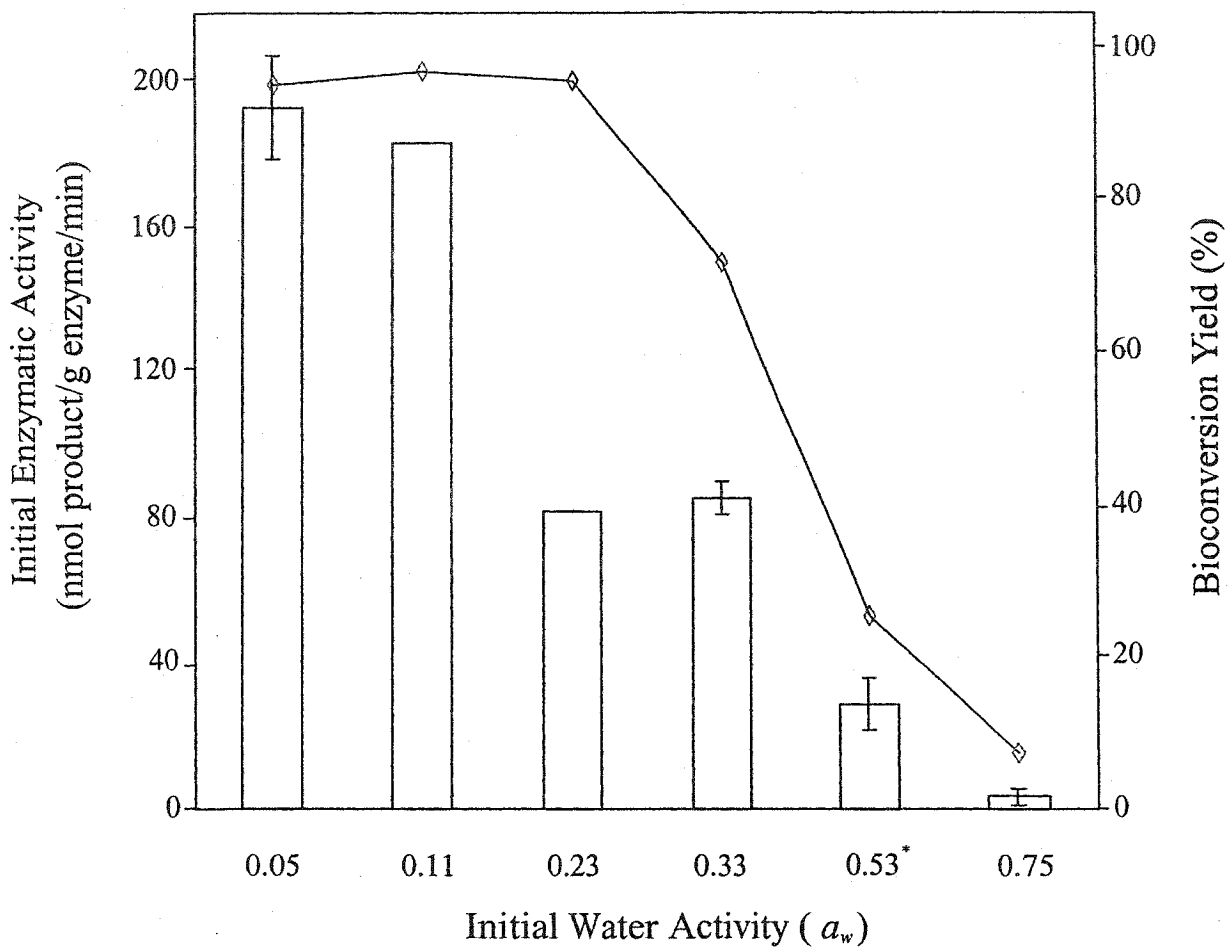


Figure 8. The effect of initial water activity on initial enzymatic activity and bioconversion yield following a 16-day lipase-catalyzed esterification employing cinnamic acid and oleyl alcohol as substrates in a mixture of iso-octane and butanone (85:15, v/v); enzymatic activity (▣) and 16-day bioconversion (◊). * The bioconversion value at 0.53 employed 20 mg immobilized lipase from *Candida antarctica* whereas the other values were obtained using 40 mg lipase per reaction.

period have a similar trend whereby increased yields were achieved by pre-equilibrating the reaction system to lower a_w values (Fig. 8, line graph). When the reaction system was pre-equilibrated to an initial a_w value of 0.75, both the initial enzymatic activity and the bioconversion yield after a 16-day reaction were extremely low, with values of 3.3 nmol product/g enzyme/min and 7.5%, respectively. Lowering the initial a_w of the reaction system to 0.23 increased the initial enzymatic activity and bioconversion yield considerably, resulting in values of 81.9 nmol product/g enzyme/min and 95.7%, respectively. Subsequent lowering of the initial a_w value to less then 0.23 maintained the same level of bioconversion yield; however, the highest initial enzymatic activity of 192.7 nmol product/g enzyme/min, with a corresponding bioconversion yield of 95.3%, was obtained at an initial a_w value of 0.05.

The increase in initial enzymatic activity and bioconversion yield for the esterification reaction at low initial a_w values (Fig. 8) may be due to the mass action effects of water; as a reactant in the system, excess water likely promoted hydrolysis while the equilibrium of the esterification reaction was more favorable as the water content in the system decreased (Klibanov, 1997; Kontogianni *et al.*, 2001; Halling, 2002; Secundo and Carrea, 2003). An excess of water in the reaction system has also been reported to cause agglomeration of biocatalyst particles and introduces the potential for water to act as a competitive inhibitor for substrates (Halling, 2002). In the present investigation, agglomeration was frequently observed during reactions at higher initial a_w values ($a_w \geq 0.53$).

The results (Fig. 8) hence show that the Novozym 435 lipase required minimal amounts of water to maintain its catalytic activity. Lipases have consistently been reported to require very little water to maintain their active conformation (Hadzir *et al.*, 2001; Ma *et al.*, 2002). Moreover, the amount of water needed in the present system for optimal catalytic activity was a function of the nature of the enzyme (Secundo and Carrea, 2003) as well as its environment (Halling and Valivety, 1992). Furthermore, the concentration of substrates has sometimes been reported to influence optimal initial a_w

values whereby a low a_w value was favored when lower substrate concentrations were present (Wehtje and Adlercreutz, 1997; Ma *et al.*, 2002; Salis *et al.*, 2003).

Kontogianni *et al.* (2001) also investigated the dependence of Novozym 435 lipase activity on the a_w of an enzymatic system and reported that the esterification of naringin with decanoic acid to produce a phenolic lipid compound was optimal in *tert*-butanol at an initial a_w value of 0.11. Similarly, Ma *et al.* (2002) reported that the transesterification of ethyl decanoate with hexanol by lipases from *Candida rugosa* and *Rhizopus oryzae* was optimal at low initial a_w values of 0.11 and 0.06, respectively. These results are comparable to those obtained in the present study (Fig. 8) whereby higher lipase activities and bioconversion yields were obtained at lower initial a_w values from 0.05 to 0.11. In contrast, Hadzir *et al.* (2001) reported the use of Novozym 435 as well as Lipozyme for the alcoholysis reaction of triolein with oleyl alcohol in hexane, with an optimal initial a_w of 0.32 for both lipases; however, Novozym 435 lipase demonstrated a much higher sensitivity to changes in a_w than Lipozyme.

The overall results indicated that the lowest initial a_w value of 0.05 was the most suitable one for further investigations, since it resulted in the highest initial enzymatic activity as well as a high bioconversion yield.

4.1.4. Effect of Agitation Speed

In heterogeneous catalysis, reaction kinetics may be affected by the mass transfer of substrates from the solvent through the boundary layer to the surface of the carrier, external diffusion, as well as inside the porous carrier to the active site of the enzyme, internal diffusion (Barros *et al.*, 1998). It is important to ensure that the substrate diffusion in heterogeneous systems does not limit the biosynthesis reaction. External and internal diffusional limitations are commonly studied by varying the agitation speed and concentration of enzyme, respectively. External diffusion may be excluded as a limiting factor in biosynthesis reactions when increased agitation does not result in an increase in initial enzymatic activity (Almeida *et al.*, 1998; Cussler, 2000; Shintre *et al.*, 2002; Salis *et al.*, 2003).

Figure 9 shows the effect of varying the agitation speed (0 to 200 rpm) as well as the concentration of the Novozym 435 lipase (1 to 4 g/L) on the relative initial enzymatic activity of the biosynthesis reaction.

Figure 9 shows an increase in relative enzymatic activity with increasing agitation speed at 2 and 3 g/L lipase concentrations. In the case of the 2 g/L concentration, initial enzymatic activity increased steadily from a value of 120.2 to 138.9 nmol product/g enzyme/min when the agitation speed was increased from 100 to 200 rpm. Likewise, initial enzymatic activity increased from 115.0 to 144.3 nmol product/g enzyme/min when the 3 g/L lipase concentration was raised from 0 to 150 rpm. The increase in initial enzymatic activity at constant lipase concentration may be due to a decrease in the boundary layer that separates the bulk solvent from the porous support and confirms the presence of external diffusional limitations (Barros *et al.*, 1998; Almeida *et al.*, 1998; Salis *et al.*, 2003).

Figure 9 also shows a decrease in relative initial enzymatic activity with an increase in lipase concentration at constant agitation speeds of 180 and 200 rpm. In particular, increasing the lipase concentration from 2 to 4 g/L resulted in a 9.0% decrease in relative initial enzymatic activity at a constant agitation speed of 180 rpm. Likewise, increasing the lipase concentration from 1 to 4 g/L resulted in a 15.1% decrease in relative initial enzymatic activity at 200 rpm. Similar trends were not observed at lower agitation speeds. These results may be due to the presence of protein-support interactions which alter the enzyme conformation at higher agitation speeds. Steric hindrance of the enzyme active site resulting from a more condense mixture, and excessive enzyme in the reaction system, may have also contributed to a decrease in initial enzymatic activity (Salleh, 1995; Hadzir *et al.*, 2001). Another possible explanation for the decrease in initial enzymatic activity is that lower agitation speeds may have resulted in enzyme aggregation and sharing of available water molecules; in this case, increased agitation speeds could have dispersed the enzyme and disrupted this water-sharing so that sufficient water was no longer present in the lipase vicinity to show suitable activity (Tarahomjoo and Alemzadeh, 2003).

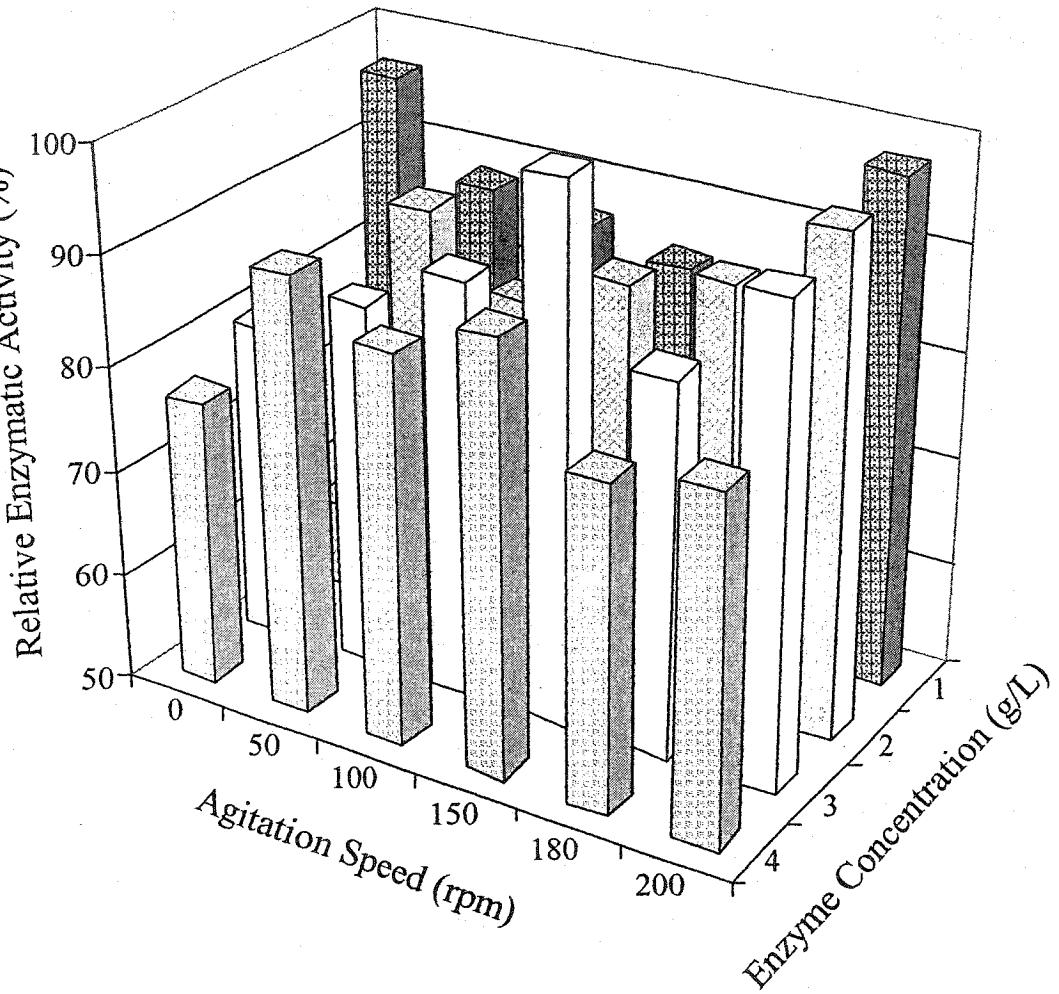


Figure 9. The effect of agitation speed and Novozym 435 lipase concentration on relative enzymatic activity following a series of 2 - day esterification reactions, using cinnamic acid and oleyl alcohol in a mixture of iso-octane and butanone (85:15, v/v) co-solvents.

On the whole, maximum initial enzymatic activity was achieved in the present study at an agitation speed of 150 rpm and a lipase concentration of 3 g/L. The same optimal conditions for Novozym 435 lipase activity were reported by Hadzir *et al.* (2001) for the transesterification of triolein with oleyl alcohol.

4.1.5. Effect of Temperature

The effect of temperature (35 to 65°C) on the initial enzymatic activity of the lipase-catalyzed esterification reaction was investigated. Variations in the initial enzymatic activity with changes in the temperature of the reaction system are shown in Figure 10A. The results show an increase in initial enzymatic activity from 17.4 nmol product/g enzyme/min at 35°C to a maximum of 189.8 nmol product/g enzyme/min at 55°C; this increase was followed by a sharp decrease in initial enzymatic activity to 11.5 nmol product/g enzyme/min at 65°C.

Previous research has shown that the optimal temperature for Novozym 435 lipase activity for the biosynthesis of phenolic lipids was in the range of 45 to 60°C (Guyot *et al.*, 1997; Buisman *et al.*, 1998; Stamatis *et al.*, 1999 and 2001; Compton *et al.*, 2000). The maximum temperature of 55°C (Fig. 10A) for the esterification of cinnamic acid with oleyl alcohol falls within the same temperature range; however, the drastic decrease in initial enzymatic activity (Fig. 10A) at higher temperatures could be attributed to enzyme deactivation.

The increase in initial enzymatic activity shown in Figure 10A follows an Arrhenius plot, shown in Figure 10B; based on the slope of this plot, the activation energy (E_a) of the reaction system, using Novozym 435 lipase as biocatalyst, was determined to be 43.6 kJ/mol.

E_a values reported in the literature for enzymatic reactions are between 20 and 60 kJ/mol (Biselli *et al.*, 2002); the E_a value calculated for the esterification of cinnamic acid with oleyl alcohol (Fig. 10B) falls within this range. Moreover, Almeida *et al.* (1998) reported E_a values from 17 to 63 kJ/mol for enzymatic reactions catalyzed by Novozym 435 lipase in a range of compressed gases. Comparable E_a values were also

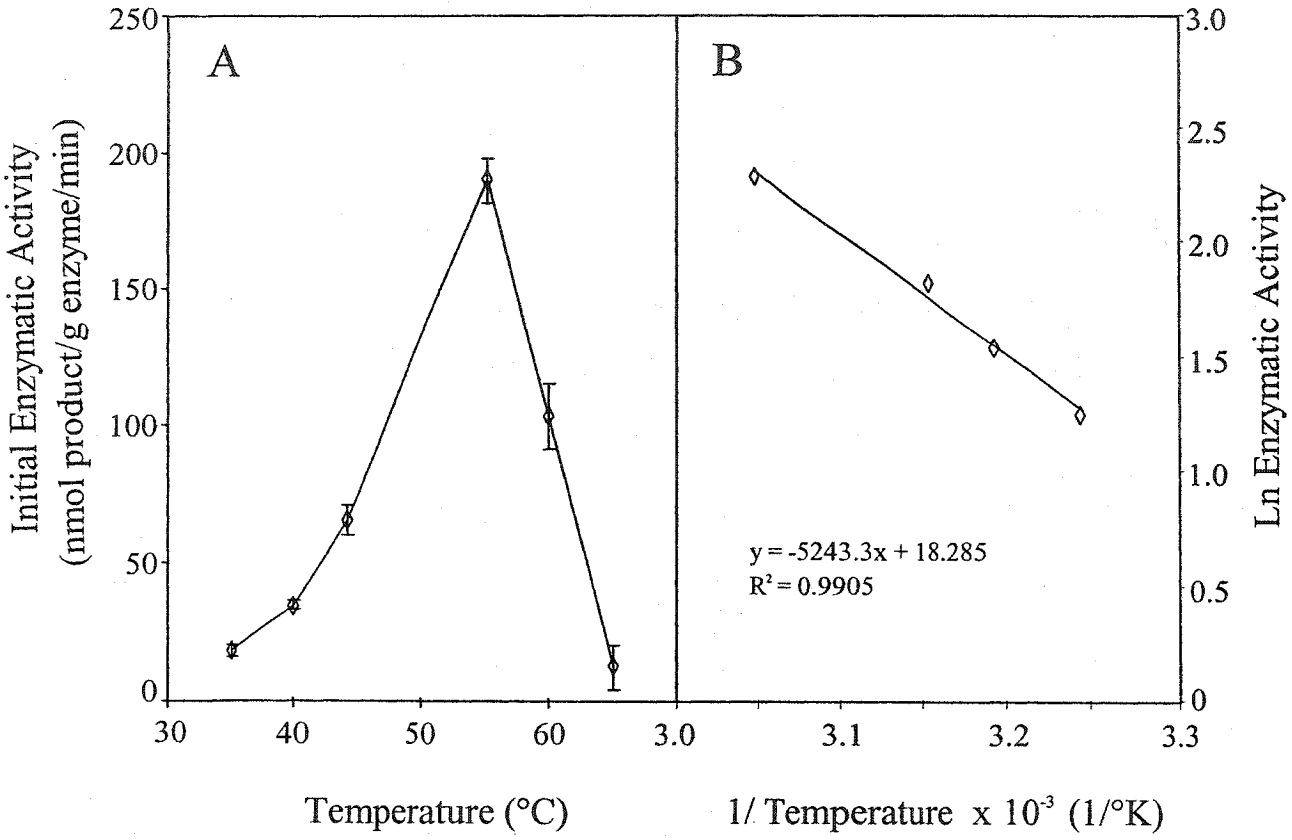


Figure 10. The effect of temperature on initial enzymatic activity (A) and the corresponding Arrhenius plot (B) for immobilized lipase from *Candida antarctica* (Novozym 435) following a series of 2-day esterification reactions using cinnamic acid and oleyl alcohol as substrates.

reported by Ottolina *et al.* (1994) and Habulin *et al.* (2001) for the transesterification of (*R*)-carvone by a lipoprotein lipase from *Pseudomonas sp.* (39.3 kJ/mol) and for the esterification of oleic acid with alcohols (C4 to C10) by a lipase obtained from *Rhizomucor miehei* (24 to 32 kJ/mol), respectively. Basheer *et al.* (1998) reported a somewhat lower E_a of 27.7 kJ/mol for the esterification of lauric acid and dodecyl alcohol catalyzed by a modified lipase from *Rhizopus japonicus*.

4.1.6. Effect of t-Cinnamic Acid and Oleyl Alcohol Ratios

In order to maximize the bioconversion yields of esterification and transesterification reactions, numerous studies have been reported on the optimization of the ratio of acid to alcohol substrates (Basheer *et al.*, 1998; Garcia *et al.*, 1999; Hadzir *et al.*, 2001; Paez *et al.*, 2003). The effect of molar ratio of substrates on the lipase-catalyzed esterification was investigated by maintaining the concentration of cinnamic acid at 4 mM and varying the oleyl alcohol concentration from 2 to 24 mM; in other words, cinnamic acid to oleyl alcohol ratios varied from 1.0:0.5 to 1.0:6.0. Figure 11 illustrates the results of these biosynthesis reactions, monitored at selected intervals over the course of a 16-day reaction period.

Increasing the molar ratio of cinnamic acid to oleyl alcohol resulted in increased bioconversion yields (Fig. 11). The lowest bioconversion yield was obtained at a cinnamic acid to oleyl alcohol ratio of 1.0:0.5 whereby relative bioconversion yields remained fairly stable (33.0 to 38.4%) beyond a 9-day period of reaction. Relative bioconversion yields of 70.6, 66.3, 86.0 and 90.5% were obtained for cinnamic acid to oleyl alcohol ratios of 1.0:1.0, 1.0:1.5, 1.0:2.0 and 1.0:4.0, respectively, after a period of 14 to 16 days. Maximum bioconversion yield was obtained after a 12-day incubation period using a cinnamic acid to oleyl alcohol ratio of 1.0:6.0. Table 4 shows a concomitant increase in initial enzymatic activity from 72.2 to 390.3 nmol product/g enzyme/min with increasing molar ratio of cinnamic acid to oleyl alcohol, from 1.0:0.5 to 1.0:6.0, respectively. The experimental results (Fig. 11, Table 4) suggest that the presence of an excess of oleyl alcohol in the reaction mixture may have increased interactions between the substrate and the enzyme, leading to an increase in the reaction rate. Furthermore, oleyl alcohol did not appear to cause any inhibition of the enzymatic

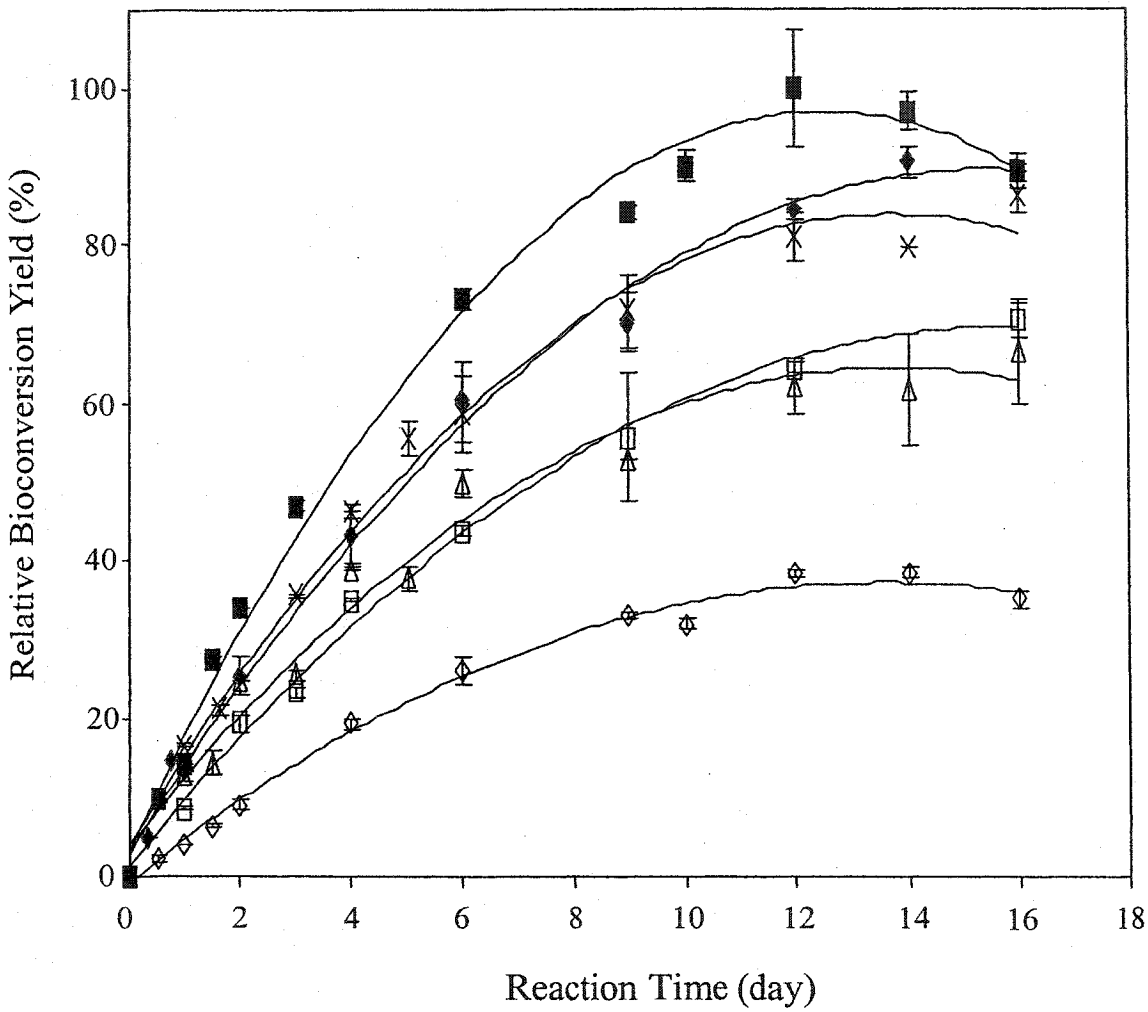


Figure 11. The effect of increasing oleyl alcohol concentration on the production of esterification product throughout a 16-day lipase-catalyzed reaction; cinnamic acid to oleyl alcohol ratio of 1:0.5 (◊), 1:1 (□), 1:1.5 (Δ), 1:2 (×), 1:4 (♦) and 1:6 (■).

Table 4. Effect of increasing the initial oleyl alcohol substrate concentration on the initial enzyme activity and the relative percent bioconversion, following lipase-catalyzed esterification with cinnamic acid in an iso-octane/2-butanone (85:15, v/v) solvent mixture.

Acid : Alcohol Ratio[a]	Initial Enzyme Activity (nmol product/g enzyme/min)[b]	Relative Bioconversion (%)[d]
1: 0.5	72.22 (±3.38)[c]	38.39
1: 1.0	185.73 (±11.60)[c]	70.55
1: 1.5	265.63 (±12.67)[c]	66.30
1: 2.0	240.24 (±12.53)[c]	86.04
1: 4.0	365.97 (±29.91)[c]	90.51
1: 6.0	390.28 (±9.06)[c]	100.00

[a]An initial cinnamic acid concentration of 4 mM was used while initial oleyl alcohol concentration varied from 2 to 24 mM, resulting in cinnamic acid to oleyl alcohol ratios ranging from 1:0.5 up to 1:6.0.

[b]Initial enzyme activity was defined as nmol cinnamic acid ester/gram enzyme/min.

[c]Standard error was defined as the standard deviation divided by the square root of the number of samples and was calculated, using SigmaPlot statistical software, based on the concentration of product obtained as a function of time for duplicate samples.

[d]Relative percent bioconversion was calculated based on the concentration of the ester product divided by the maximum theoretical concentration, multiplied by 100 and was expressed as a percentage of the maximum bioconversion obtained after 12 days using a 1:6.0 acid: alcohol ratio. Relative bioconversion values represent the maximum bioconversion yield possible after a period of between 12 and 16-days at 55°C and an agitation speed of 150 rpm.

reaction at concentrations of 2 to 24 mM.

The results (Table 4) also indicate that the maximum initial enzymatic activity of 390.3 nmol product/g enzyme/min, obtained at a cinnamic acid to oleyl alcohol ratio of 1.0:6.0, represents the maximum initial enzymatic activity possible using the optimized conditions of solvent type, a_w, agitation speed, reaction temperature and substrate ratio.

Although the experimental results (Fig. 11, Table 4) indicated no enzymatic inhibition, the literature did report enzyme inhibition at higher substrate ratios (Ramamurthi and McCurdy, 1994; Liu and Shaw, 1998). Tarahomjoo and Alemzadeh (2003) reported that increasing the substrate ratio of palmitic acid to glucose from 1:0.2 up to 1:1.0 resulted in a 21.5% increase in bioconversion yield while an additional increase to a molar ratio of 1:2.0 resulted in a 15.6% decrease in the yield. Ramamurthi and McCurdy (1994) indicated that the esterification of oleic acid with methanol by lipase from *C. antarctica* also followed the same pattern. Furthermore, Liu and Shaw (1998) showed that esterification yields of kojic acid monolaurate and kojic acid monooleate were maximal when the fatty acid to kojic acid ratio was 1:4. In contrast, Babali *et al.* (2001) reported that there was no inhibition of the enzymatic activity after a 2-day esterification, using lauric acid to menthol ratios of 1:0.5, 1:1.0 and 1:2.0, with corresponding bioconversion yields of 49, 93 and 98%, respectively.

4.1.7. Kinetic Parameters

Figure 12A indicates the effect of a range of oleyl alcohol concentrations (0 to 4 mM) on the initial enzymatic activity of the reaction system, using constant cinnamic acid concentrations of 1, 2 and 4 mM.

The experimental findings show (Fig. 12A) that initial enzymatic activity increased with increasing oleyl alcohol concentrations, for each defined quantity of cinnamic acid. At a cinnamic acid concentration of 4 mM, a linear increase in initial enzymatic activity as a function of oleyl alcohol concentration was observed, with no plateau obtained within the range of concentrations investigated. For lower cinnamic acid

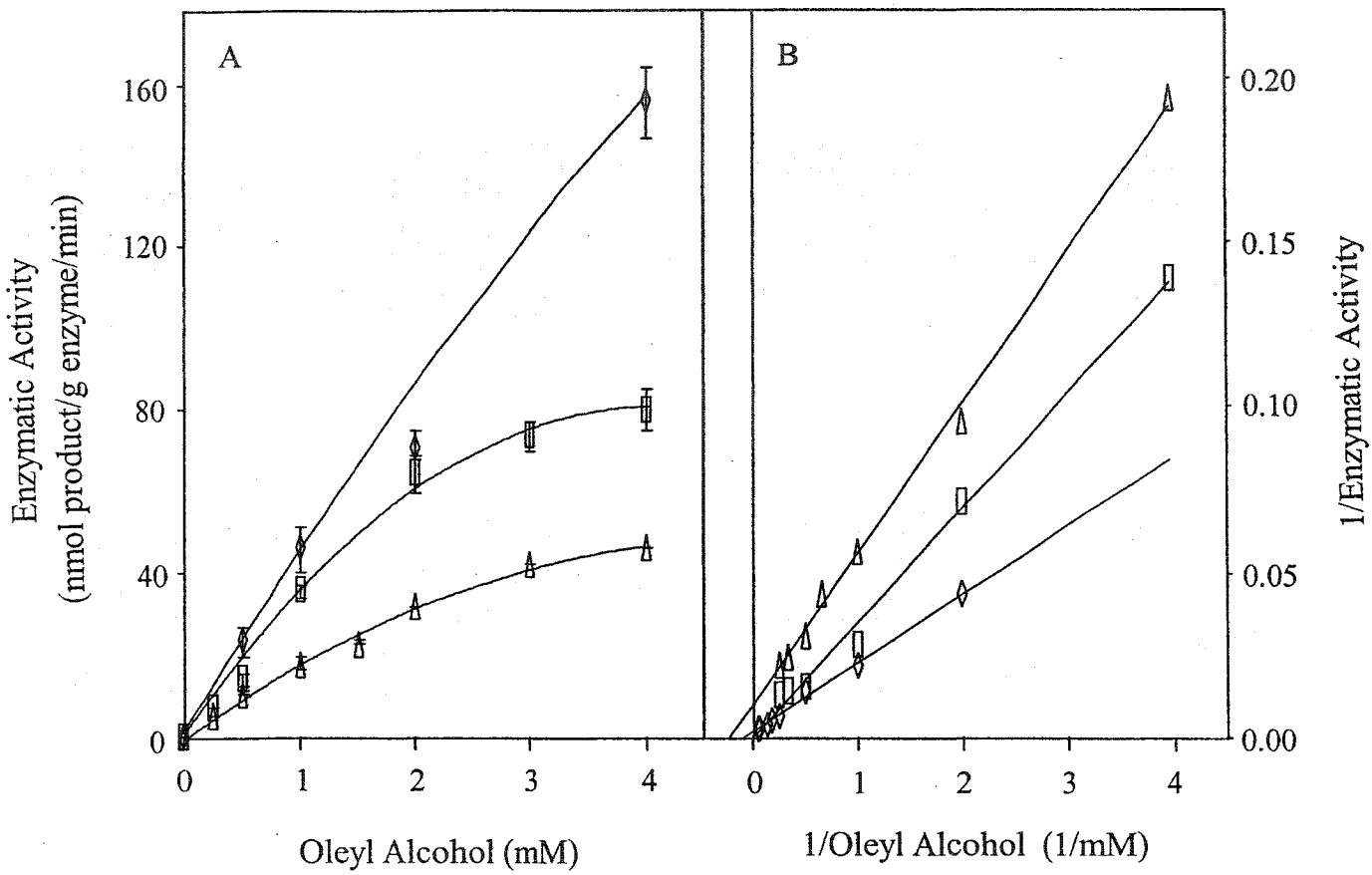


Figure 12. The effect of increased alcohol substrate concentration on initial enzymatic activity (A) and corresponding Lineweaver-Burke plots (B) for a series of bioconversion reactions employing a range of cinnamic acid substrate concentrations; 1 mM cinnamic acid (Δ), 2 mM cinnamic acid (□) and 4 mM cinnamic acid (◊), respectively.

concentrations of 1 and 2 mM, hyperbolic trends were observed from plots of initial enzymatic activity as a function of oleyl alcohol concentration.

Figure 12B shows the corresponding Lineweaver-Burke plots for the reaction system at 1, 2 and 4 mM cinnamic acid. The reciprocal plots yielded straight lines with correlation values, $r^2 \geq 0.994$. Based on these results, it was apparent that although this was clearly a multi-substrate reaction system (Palmer, 1995), the model for the phenolic lipid biosynthesis reaction seemed to follow Michaelis-Menten kinetics.

Kinetic parameters were calculated for each concentration of cinnamic acid, with K_m apparent values of 4.8, 3.2 and 7.0 mM and V_{max} values of 104.5, 141.6 and 485.1 nmol product/g enzyme/min, for 1, 2 and 4 mM of cinnamic acid, respectively. Increasing cinnamic acid concentration resulted in changes in K_m and V_{max} values (Fig. 12B) which suggest a mixed type inhibition (Price and Stevens, 1996; Copeland, 2000; Lee et *al.*, 2003). Mixed inhibition is a combination of competitive and uncompetitive inhibition where both the enzyme and the enzyme-substrate complex bind to the inhibitor (Cornish-Bowden, 1979; Copeland, 2000). In contrast, the lipase-catalyzed esterification of butyric acid with isoamyl alcohol was reported to follow Michaelis-Menten kinetics, with a Ping-Pong/Bi-Bi mechanism and competitive inhibition by both substrates, whereby the enzyme alone binds to the inhibitor (Hari Krishna and Karanth, 2001). The lipase-catalyzed esterification of butyl isobutyrate using isobutyric acid and *n*-butanol (Yadav and Lathi, 2003) as well as that of tetrahydrofurfuryl butyrate using butyric acid and tetrahydrofurfuryl alcohol (Yadav and Devi, 2004) have also been shown to follow a similar mechanism, with inhibition observed in the presence of excess alcohol substrate. Lastly, competitive inhibition by both substrates and products was reported for the lipase-catalyzed esterification of palmitic acid with isopropyl alcohol using an ordered bi-bi mechanism (Garcia *et al.*, 1999).

The highest cinnamic acid concentration of 4 mM, used throughout this study, was due to its limited solubility in the reaction system. Stamatis *et al.* (1999) also cited the solubility of cinnamic acid as a limiting factor during biocatalysis with 1-octanol, indicating that its solubility presented a particular problem limiting any extensive kinetic

study. In the present investigation, the biosynthesis reactions made use of cinnamic acid concentrations $\leqslant$ 4 mM, which is below the range used in most kinetic studies (Basheer *et al.*, 1998; Ramamurthi and McCurdy, 1994; Kontogianni *et al.*, 2001).

4.2. Characterization of Model Phenolic Lipid Product

4.2.1. HPLC Analysis of Substrates and End Product

The separation and quantification of substrates and end products, following the lipase-catalyzed esterification reaction of cinnamic acid with oleyl alcohol was carried out by HPLC analysis. The separation was accomplished using a reversed-phase (RP) C18 column with an isocratic methanol/water (95:5, v/v) mobile phase, whereby the more hydrophilic molecules were eluted prior to the hydrophobic ones. An UV detector was used at 205 and 275 nm to monitor the separation of mixtures, since oleyl alcohol was found to absorb at 205 nm while cinnamic acid absorbed more readily at 275 nm.

Characterization of phenolic lipid end products by HPLC analysis has been reported in literature (Guyot *et al.*, 1997; Stamatis *et al.*, 1999 and 2001; Compton *et al.*, 2000; Kontogianni *et al.*, 2001) using RP chromatography. Moreover, a methanol/water mobile phase was used to separate reaction components following the esterification of cinnamic acid with fatty alcohols of varying chain lengths (Guyot *et al.*, 1997; Stamatis *et al.*, 1999 and 2001). Overall, the mode of detection and specific wavelength employed were selected on the basis of the nature of the reaction components as well as the mobile phase (Thomson *et al.*, 1999; Andrikopoulos, 2002).

Figure 13A shows the HPLC elution profile of reaction components at 205 nm, obtained after a 3-day esterification reaction in an iso-octane/butanone (85:15, v/v) mixture. As the more hydrophilic substrate, cinnamic acid was eluted first (peak #1) with a retention time of 3.03 min while the more hydrophobic monounsaturated long chain oleyl alcohol substrate (peak #3) was eluted at 10.27 min. The main ester product of cinnamic acid and oleyl alcohol (peak #4) was eluted at a retention time of 32.97 min. A small side-reaction product (peak#2) was eluted at 3.83 min; this side-reaction product may be due to the reaction of cinnamic acid substrate with the traces of butanol present in the 2-butanone co-solvent used in the reaction system.

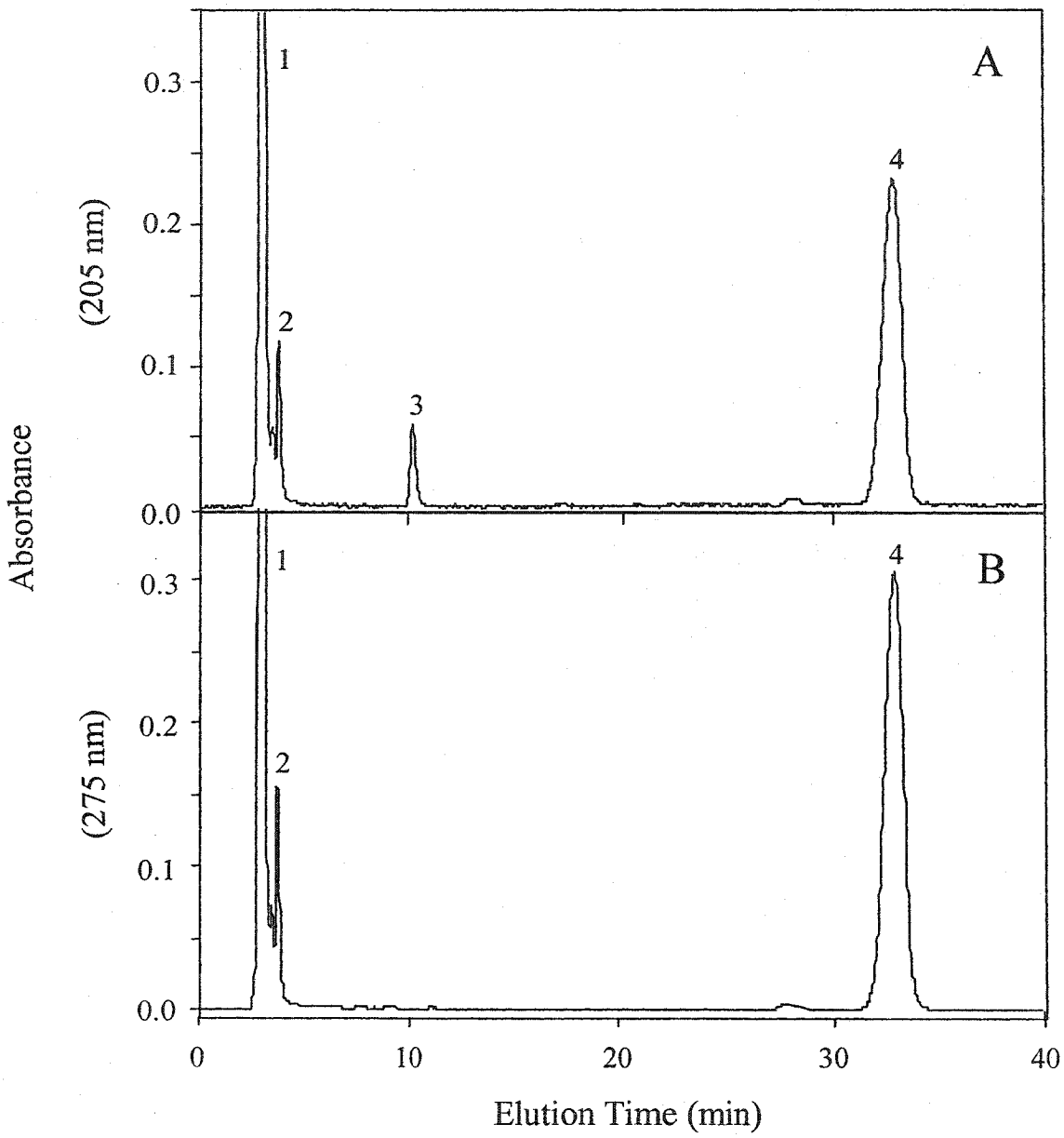


Figure 13. HPLC separation of substrates and products at 205 nm (A) and 275 nm (B) respectively, following a 3-day bioconversion reaction of cinnamic acid and oleyl alcohol in a mixture of iso-octane and butanone (85:15, v/v), using immobilized lipase from *Candida antarctica*; identification of peaks include cinnamic acid (peak #1), cinnamic acid side reaction (peak # 2), oleyl alcohol (peak # 3) and fatty alcohol ester product (peak # 4).

Figure 13B displays the elution profile of the reaction components at 275 nm. The results were similar to those obtained at 205 nm (Figure 13A); however, the oleyl alcohol substrate was not visible at this wavelength. Characterization of peaks was based on comparison of elution times and absorbance maxima with standards of cinnamic acid and oleyl alcohol; however, since no oleyl alcohol ester of cinnamic acid standard was readily available, it was subsequently separated and recovered by HPLC analysis for characterization by electrospray ionization mass spectroscopy (ESI-MS) analysis and construction of a standard curve.

4.2.2. Spectrophotometric Profiles of Substrates and End Product

The esterified end product was further characterized by a spectrophotometric scan; a comparison of its scan with those of cinnamic acid and oleyl alcohol is shown in Figure 14. Figure 14A displays the characteristic scanning of cinnamic acid (15.6 μg/mL) from 200 to 500 nm; the corresponding absorbance profile of cinnamic acid consisted of two main peaks, with a maximum absorbance at 275 nm. Figure 14B shows the absorbance profile of the oleyl alcohol substrate (1 mg/mL) within the same wavelength (λ) range (200 to 500 nm); the maximum absorbance was obtained at a λ of 205 nm. Figure 14C illustrates the characteristic scanning of the esterified end product (0.25 mg/mL) which has an absorbance profile somewhere between those of the cinnamic acid and oleyl alcohol. Similarities included a maximum absorbance identical to that of cinnamic acid (275 nm) as well as lesser peaks at 202 and 205 nm, which were consistent with the absorbance profile of oleyl alcohol. In order to monitor substrates and end products throughout the course of the HPLC analysis, the absorbance maxima of cinnamic acid and oleyl alcohol, determined as 275 and 205 nm, respectively, were used.

Although it is not typically used for lipids and their derivatives, spectrophotometric scanning has often been employed in the characterization of phenolic compounds (Revilla and Ryan, 2000; Chen *et al.*, 2001); flavonoids are among the phenolic compounds whose absorbance profiles are most frequently investigated (Mattila *et al.*, 2000). da Costa *et al.* (2000) indicated that the absorbance spectrum of a given anthocyanin compound may provide information on the nature of the aglycone, glycosylation pattern and possibly of acylation. Friedman and Jürgens (2000) used the

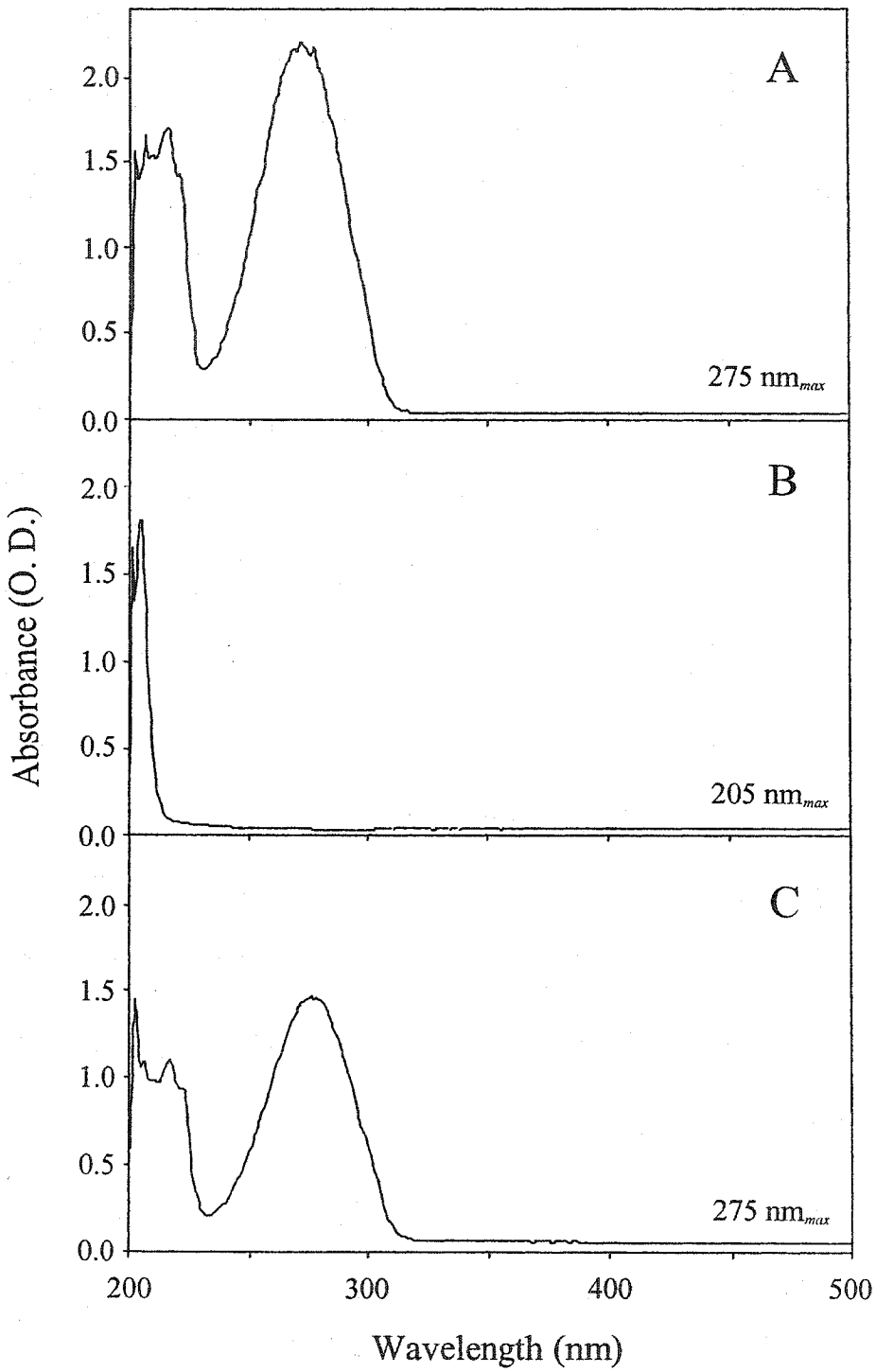


Figure 14. Absorbance profiles based on spectrophotometric wavelength scans of cinnamic acid (A) and oleyl alcohol (B) substrates as well as the fatty alcohol ester (C) produced through lipase-catalyzed biosynthesis in a co-solvent mixture of iso-octane and butanone (85:15, v/v).

changes in absorbance spectra of a wide range of phenolic compounds, including catechin, rutin and ferulic, chlorogenic, gallic and cinnamic acids as a means of determining their stability.

4.2.3. Structural Analysis of End Product by ESI-MS

Structural characterization of the HPLC purified phenolic lipid end product was proposed by ESI-MS analysis. Due to its ability to produce intact ions from large and complex species in solution, including thermally labile, non-volatile and polar compounds, ESI-MS is considered a powerful technique for the characterization of biomolecules and among the most versatile ionization techniques in existence today (Giusti *et al.*, 1999). Figure 15 shows the collision induced dissociation spectrum of the ammonated molecular ion of the oleyl alcohol ester of cinnamic acid following ESI-MS analysis.

The fragmentation pattern of the ester end product shows its relative intensity as a function of the mass over charge (m/z) ratio; peaks appearing at an m/z ratio of 399 and 416 represent the ionized $[M+H]^+$ and ammonated $[M+NH_4]^+$ end product following low energy bombardment (Fig. 15). Further increase in the bombardment resulted in the characteristic fragmentation pattern of cinnamic acid; however, the corresponding oleyl alcohol released during fragmentation of the ester molecules was too low to be likewise visible.

Figure 16 shows the mechanisms behind the two major cinnamic acid fragments, visible at an m/z ratio of 132 and 149 in Figure 15, respectively. Figure 16A indicates that an electron from the double bonded oxygen molecule of the ionized ester product formed a triple bond with the carbon atom, enabling the release of a molecule of oleyl alcohol and its corresponding cinnamic acid fragment. Figure 16B suggests that a bond was broken at the site of the ionized oxygen atom which resulted in the redistribution of charge within each fragment species. With regard to the cinnamic acid fragment, a double bond was formed at the site of the ionized oxygen atom and an alcohol group formed at the site of the previous double bonded oxygen atom. Smaller fragments were also observed at m/z ratios of 58, 70, 84, 98 and 112. Overall, ESI-MS results confirmed that

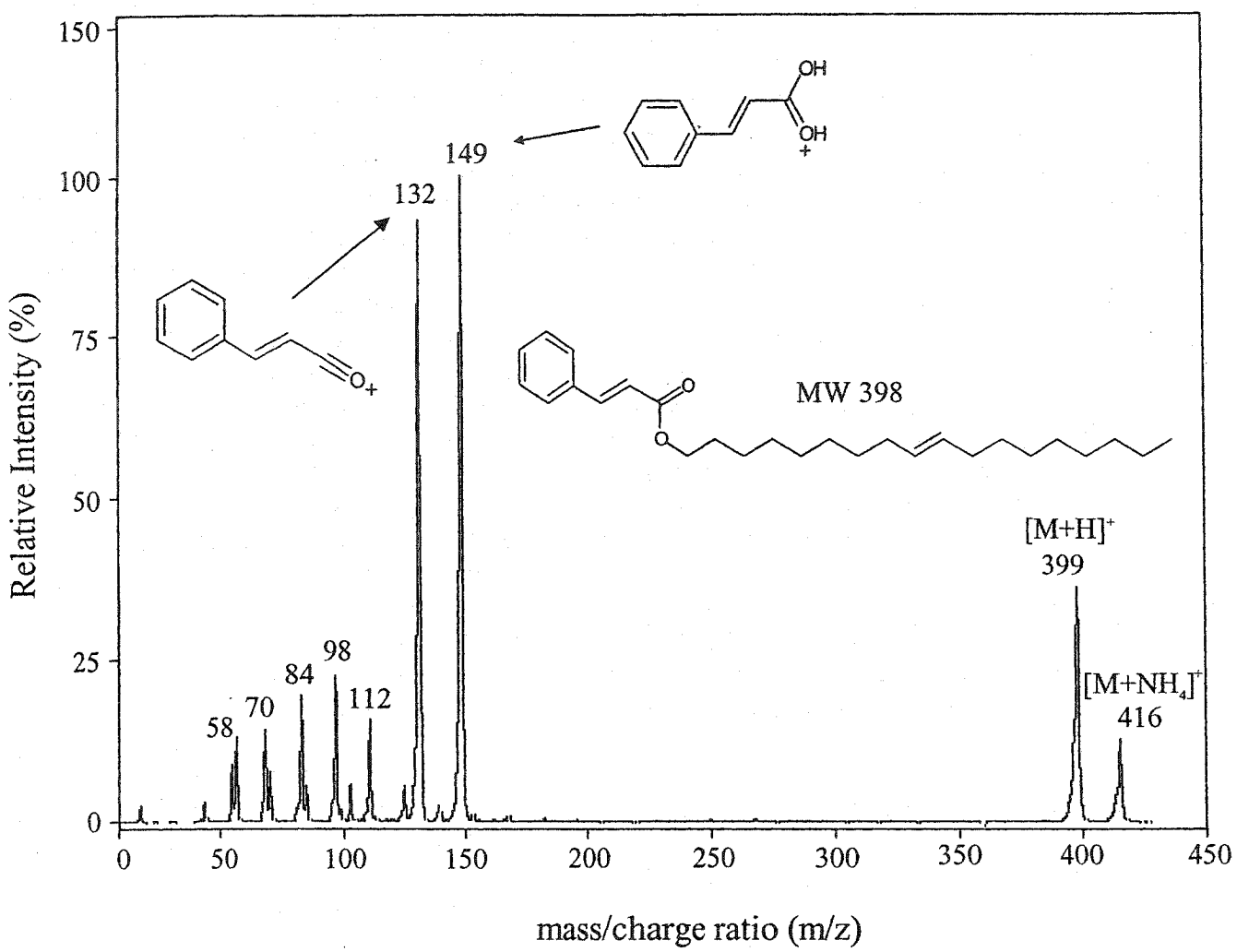


Figure 15. Collision induced dissociation spectrum of the ammonated molecular ion of cinnamic acid and oleyl alcohol ester following electrospray ionization mass spectroscopy. Relative intensity based on fragment with a mass/charge ratio of 149.

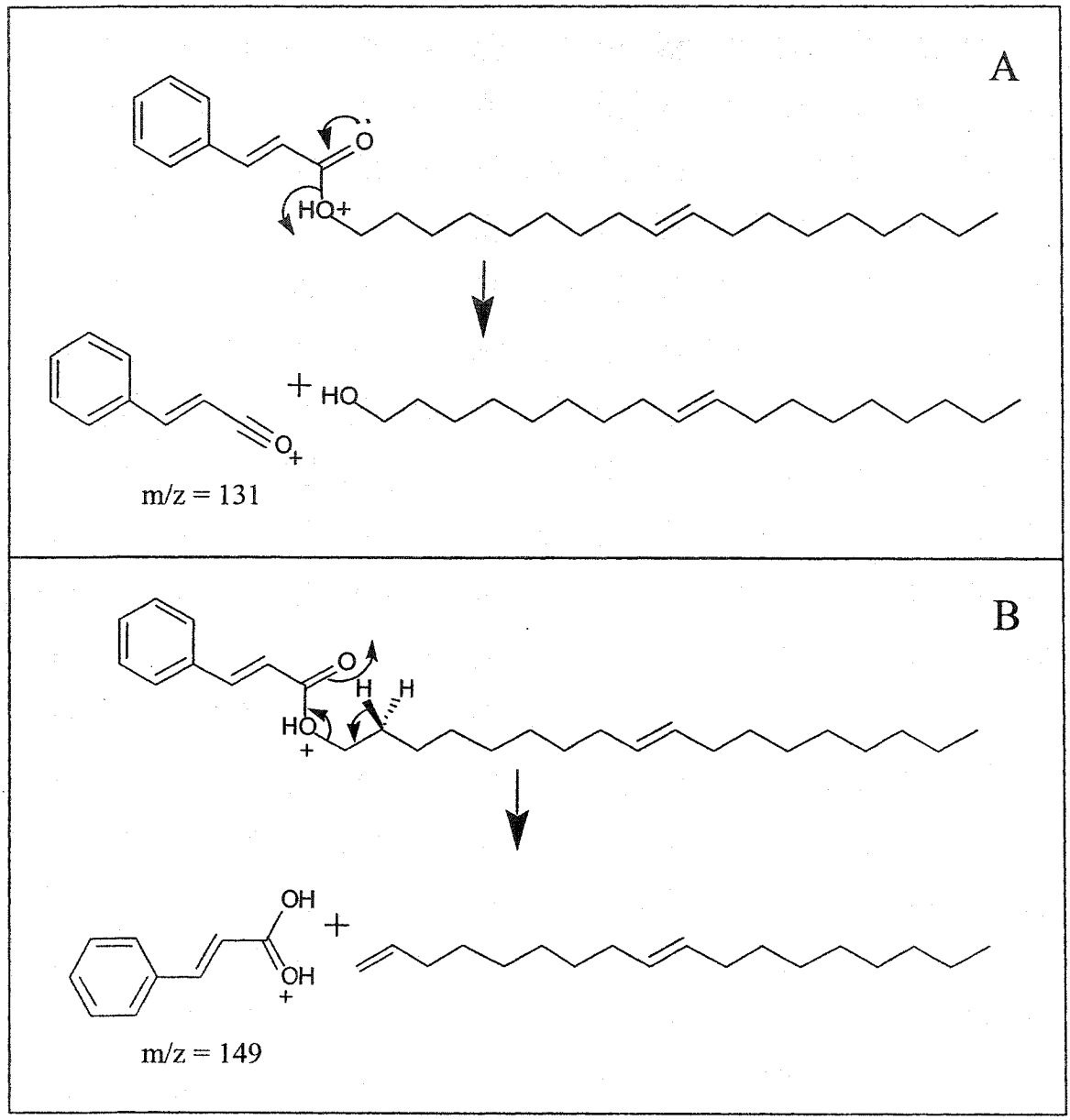


Figure 16. Schematic for the fragmentation of cinnamic acid and oleyl alcohol ester using electrospray ionization mass spectroscopy; fragment visible at a ratio m/z = 131 (A) and a ratio m/z = 149 (B).

the end product obtained by lipase-catalyzed biosynthesis was the ester of cinnamic acid and oleyl alcohol. Similarly, ESI-MS and ACPI-MS have also been reported in literature for the structural analysis of flavonoid esters (Kontogianni *et al.*, 2001) and feruloyl-substituted acylglycerols (Compton *et al.*, 2000).

4.3. Lipase-Catalyzed Transesterification of *t*-Cinnamic Acid with Triolein

4.3.1. Hydrolysis Profile of Triolein during Transesterification

The model phenolic lipid reaction system, using oleyl alcohol as substrate, was applied to its corresponding triacylglycerol, triolein, using the optimized reaction conditions defined throughout the present study, including solvent mixture (iso-octane/2-butanone, 85:15, v/v), initial a_w (0.05), agitation speed (150 rpm) and temperature (55°C).

Transesterification reactions proceed through intermediate stages involving hydrolysis as well as synthesis (Soumanou *et al.*, 1999), with the degree of hydrolysis influencing the nature of the final products. Figure 17 displays the concentrations of the hydrolysis products of triolein, namely diolein, monoolein and oleic acid, formed during lipase-catalyzed transesterification with cinnamic acid over the course of a 21-day reaction period. The triolein concentration decreased drastically from an initial concentration of 3.7 to 0.2 mM after a 2-day reaction period where it remained relatively stable; in the mean time, concentrations of diolein, monoolein and oleic acid increased in the reaction system to 1.3, 1.1 and 4.0 mM, respectively, with slight decreases in diolein and monoolein thereafter. Control reactions carried out in tandem with the enzymatic trials showed no chemical side reactions or significant changes in the concentrations of either substrate. Overall, these results (Fig. 17) indicate that triolein was almost completely hydrolyzed to its corresponding glycerides during the first 2 days of the biosynthesis reaction.

Decreased diolein and monoolein concentrations during the latter part of the reaction might suggest their transesterification with cinnamic acid. Relative percent transesterification, calculated from the esterified cinnamic acid, increased to a maximum of 10.5% after a 21-day period; these findings are in agreement with the decrease in total hydrolysis products of triolein (Table 5).

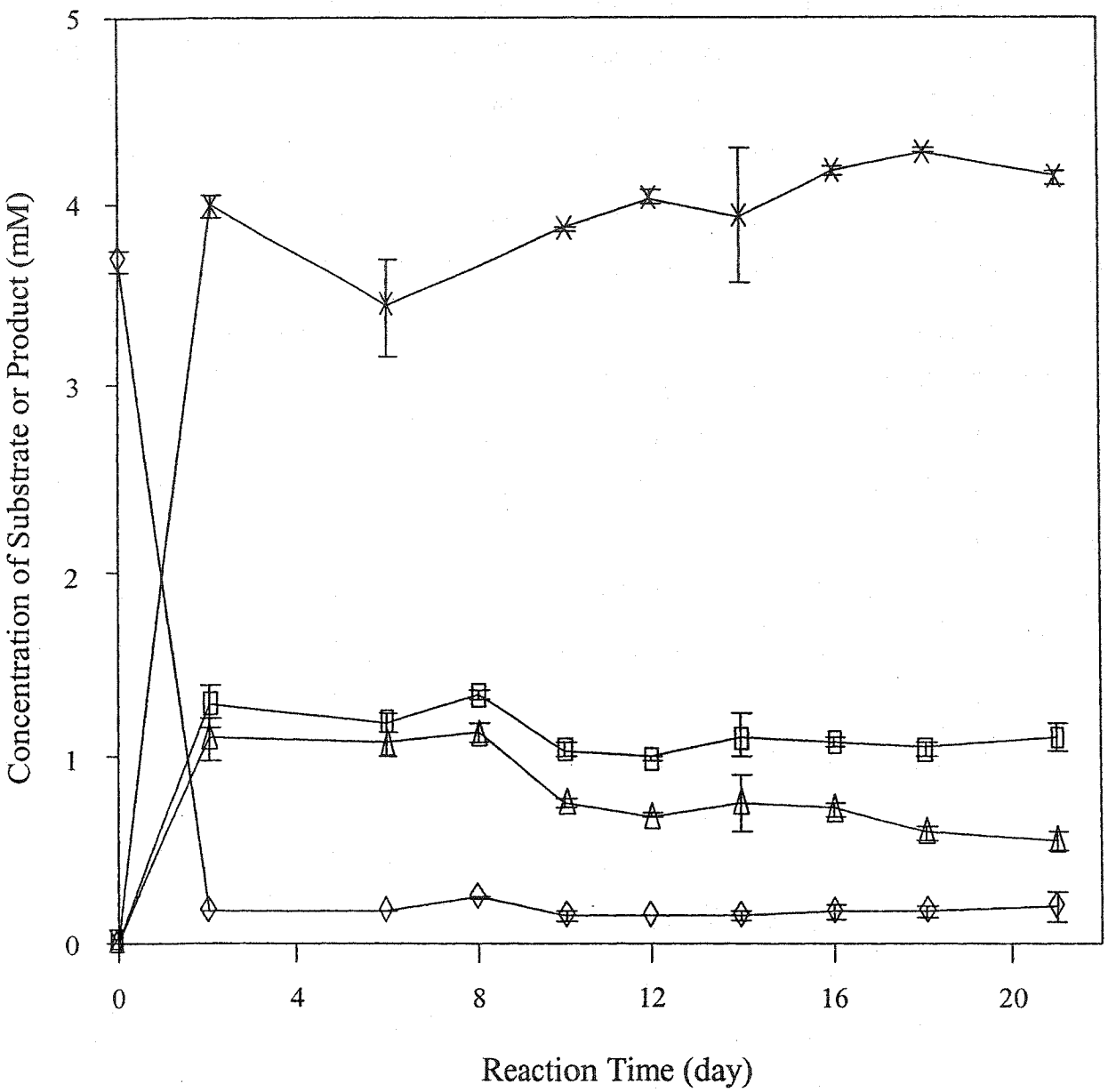


Figure 17. Monitoring of the hydrolysis products of triolein during transesterification with cinnamic acid by an immobilized lipase from *Candida antarctica* in a co-solvent mixture (85:15, v/v) of iso-octane and butanone; concentration of triolein (◊), 1-3-diolein (□), monoolein (Δ) and oleic acid (X) over the course of the reaction, determined using HPLC-ELSD.

Table 5. Lipase-catalyzed transesterification of triolein with cinnamic acid in an iso-octane and butanone mixture (85:15, v/v); Comparison of the extent of hydrolysis, phenolic lipid production and relative transesterification over a 21-day reaction period

Reaction Time	Triolein Hydrolysis (%)[a]			Total Hydrolysis	Phenolic Lipid Products[d]				Relative Transesterification
(day)	DG[f]	MG[f]	FA[g]	(%)[c]	1	2	3	4	(%)[e]
0	0.0 (0.00)[b]	0.00 (0.00)[b]	0.00 (0.00)[b]	0.00	0.00 (0.00)[b]	0.00 (0.00)[b]	0.00 (0.00)[b]	0.00 (0.00)[b]	0.00 (0.00)[b]
2	34.53 (8.54)[b]	29.82 (10.19)[b]	35.88 (1.37)[b]	100.0	152.48 (0.00)[b]	4.30 (0.00)[b]	0.00 (0.00)[b]	25.83 (0.00)[b]	0.00 (0.00)[b]
6	32.10 (3.99)[b]	29.25 (5.55)[b]	30.92 (7.54)[b]	92.27	188.07 (0.54)[b]	6.24 (4.77)[b]	13.42 (0.00)[b]	54.66 (19.91)[b]	2.36 (3.63)[b]
8	36.41 (2.43)[b]	30.99 (4.69)[b]	25.66 (2.61)[b]	93.05	199.56 (2.37)[b]	7.02 (0.88)[b]	27.23 (2.22)[b]	71.66 (2.84)[b]	3.39 (0.34)[b]
10	29.69 (3.48)[b]	21.62 (4.21)[b]	36.47 (0.25)[b]	87.78	204.85 (1.29)[b]	7.00 (1.65)[b]	26.29 (3.51)[b]	77.32 (0.73)[b]	2.96 (3.07)[b]
12	26.65 (1.34)[b]	18.44 (2.26)[b]	35.65 (0.98)[b]	80.74	200.97 (3.88)[b]	7.30 (8.56)[b]	31.11 (12.65)[b]	81.60 (22.69)[b]	5.73 (1.71)[b]
14	30.39 (10.66)[b]	20.64 (20.37)[b]	35.78 (9.39)[b]	86.81	204.63 (0.94)[b]	7.95 (1.40)[b]	36.20 (2.16)[b]	85.93 (5.37)[b]	2.65 (3.72)[b]
16	29.54 (2.46)[b]	19.66 (4.51)[b]	37.51 (0.64)[b]	86.70	204.91 (2.05)[b]	8.01 (2.48)[b]	29.54 (4.29)[b]	76.97 (5.54)[b]	4.63 (3.41)[b]
18	29.12 (4.16)[b]	16.55 (5.67)[b]	39.48 (0.38)[b]	85.15	207.61 (3.03)[b]	9.73 (7.50)[b]	37.87 (7.22)[b]	87.75 (8.64)[b]	4.91 (1.47)[b]
21	32.95 (7.01)[b]	16.76 (8.78)[b]	40.75 (0.91)[b]	90.46	215.91 (3.33)[b]	11.94 (3.61)[b]	45.66 (6.18)[b]	97.65 (9.75)[b]	10.52 (2.02)[b]

[a]Hydrolysis products include diolein (DG), monoolein (MG) and oleic acid (FA), monitored by HPLC with evaporative light-scattering detector (ELSD).

[b]Relative percent standard deviation, calculated as the standard deviation of duplicate samples divided by the mean, multiplied by 100.

[c]Total hydrolysis of triolein was calculated as the sum of the hydrolysis products, diolein, monoolein and oleic acid.

[d]Phenolic lipid compounds were determined by the appearance of peaks following HPLC-UV analysis at 235 nm.

[e]Relative transesterification was calculated based on cinnamic acid consumed divided by that present in the control, multiplied by 100.

[f]Percent hydrolysis was calculated as the concentration of hydrolysis product divided by the concentration of triolein at time *t*, multiplied by 100.

[g]Percent hydrolysis was calculated as the concentration of oleic acid divided by 3, divided by the concentration of triolein at time *t*, multiplied by 100.

Two major and two minor end products of the transesterification reaction of cinnamic acid with triolein were quantified by HPLC and were found to increase rapidly over the first 6 days of the reaction period, and more slowly thereafter (Table 5).

Compton *et al.* (2000) reported that the transesterification reaction of ethyl ferulate with triolein reached its equilibrium after 6 days, with a bioconversion yield of 77%. Similarly, the low bioconversion yield obtained for the transesterification of cinnamic acid with triolein (Table 5) could potentially be improved with the use of an ester of cinnamic acid rather than cinnamic acid.

4.3.2. HPLC Analysis of Substrates and End Products

HPLC analysis of substrates and end products of the reaction system was performed using a reversed-phase column with gradient elution and both UV (235 nm) and ELSD detectors. Figure 18 shows a typical HPLC chromatogram of substrates and products of the transesterification reaction of cinnamic acid and triolein after a 16-day incubation period.

Figure 18A shows the control reaction (containing no enzyme) after a 16-day reaction period monitored at 235 nm and by ELSD. The more hydrophilic compound, cinnamic acid (peak #1), eluted first with a retention time of 3.09 min, while the more hydrophobic compound, triolein (peak #9), was eluted with a retention time of 25.86 min.

The detection by ELSD (Fig. 18C) revealed peaks of both substrates, the hydrolysis products of triolein, and end products. Cinnamic acid (peak #1) was eluted first while the hydrolysis products of triolein, namely monoolein (peak #2), oleic acid (peak #3) and diolein (peak #7) were eluted at 4.81, 5.61 and 18.37 min, respectively. Three potential phenolic lipid compounds were eluted at 6.18, 9.47 and 20.89 min, respectively and correspond to product 1 (peak #4), product 3 (peak #6) and product 4 (peak #8) detailed in Table 5.

The UV detection at 235 nm (Fig. 18B) revealed the presence of cinnamic acid (peak #1) followed by four potential phenolic lipid products, three of which were also detected by ELSD (Fig. 18C). Peak #8, designated as product 2 was eluted after 7.21 min.

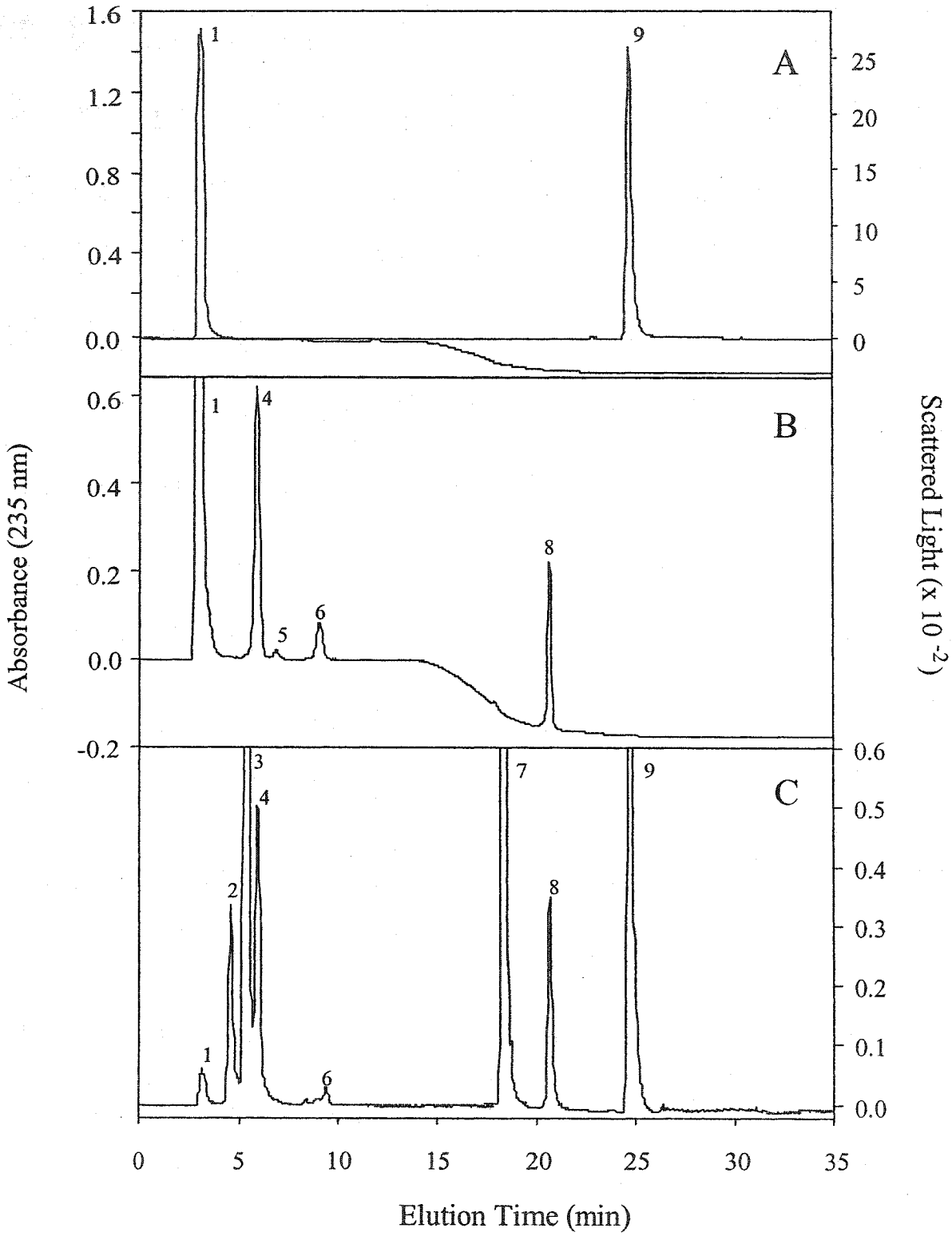


Figure 18. RP-HPLC analysis of substrates and products of transesterification monitored by UV detection at 235 nm and evaporative light scattering detector (ELSD); control (A), sample separation following a 16-day incubation period monitored at 235 nm (B) and by ELSD (C). Peak identification as followed; cinnamic acid (peak # 1), monoolein (peak # 2), oleic acid (peak # 3), product 1 (peak # 4), product 2 (peak # 5), product 3 (peak # 6), diolein (peak # 7), product 4 (peak # 8) and triolein (peak # 9), respectively.

Triolein and its hydrolysis products were not detected (Fig. 18B), which may be due to their weak absorption properties within the UV range. Based on the elution profiles and peak areas, it was concluded that a minimum of two major and two minor end products were produced by the lipase-catalyzed transesterification reaction of cinnamic acid and triolein.

Similarly, Compton *et al.* (2000) also reported the production of four new end products in addition to phenolic and lipid substrates, and hydrolysis products of triolein, as demonstrated by HPLC analysis coupled with UV and ELSD detectors; the separation was achieved on a RP-C8 column using an isocratic elution of acetone/acetonitrile (40:60, v/v). As in the present study, ELSD detection was used primarily to monitor the hydrolysis products of triolein. Moreover, although retention times were not comparable between studies, the order of elution in the present work and that reported by Compton *et al.* (2000) was the same.

4.3.3. Structural Analysis of End Products by ESI-MS

Figure 19 shows the collision induced dissociation spectrum of the ammonated molecular ions of a reaction mixture after a 16-day lipase-catalyzed transesterification of cinnamic acid and triolein following ESI-MS analysis.

Comparison of the spectrum of the reaction mixture (Fig. 19) with the characteristic fragmentation patterns of cinnamic acid, triolein and corresponding hydrolysis products confirmed their presence as well as two major end products (Fig. 20). These major phenolic lipid products were cinnamyl diolein, with a peak appearing at an m/z ratio of 769 representing the ammonated $[M+NH_4]^+$ end product as well as cinnamyl monoolein, with peaks appearing at m/z ratios of 504 and 487 representing the ionized $[M+H]^+$ and ammonated $[M+NH_4]^+$ end product following bombardment. Structures were further confirmed by MS-MS analysis.

The overall findings suggest that these two structures, cinnamyl diolein and cinnamyl monoolein, corresponded to product 4 (peak #8) and 1 (peak #2) (Fig. 18), respectively, due to their elution times relative to the hydrolysis products, diolein and

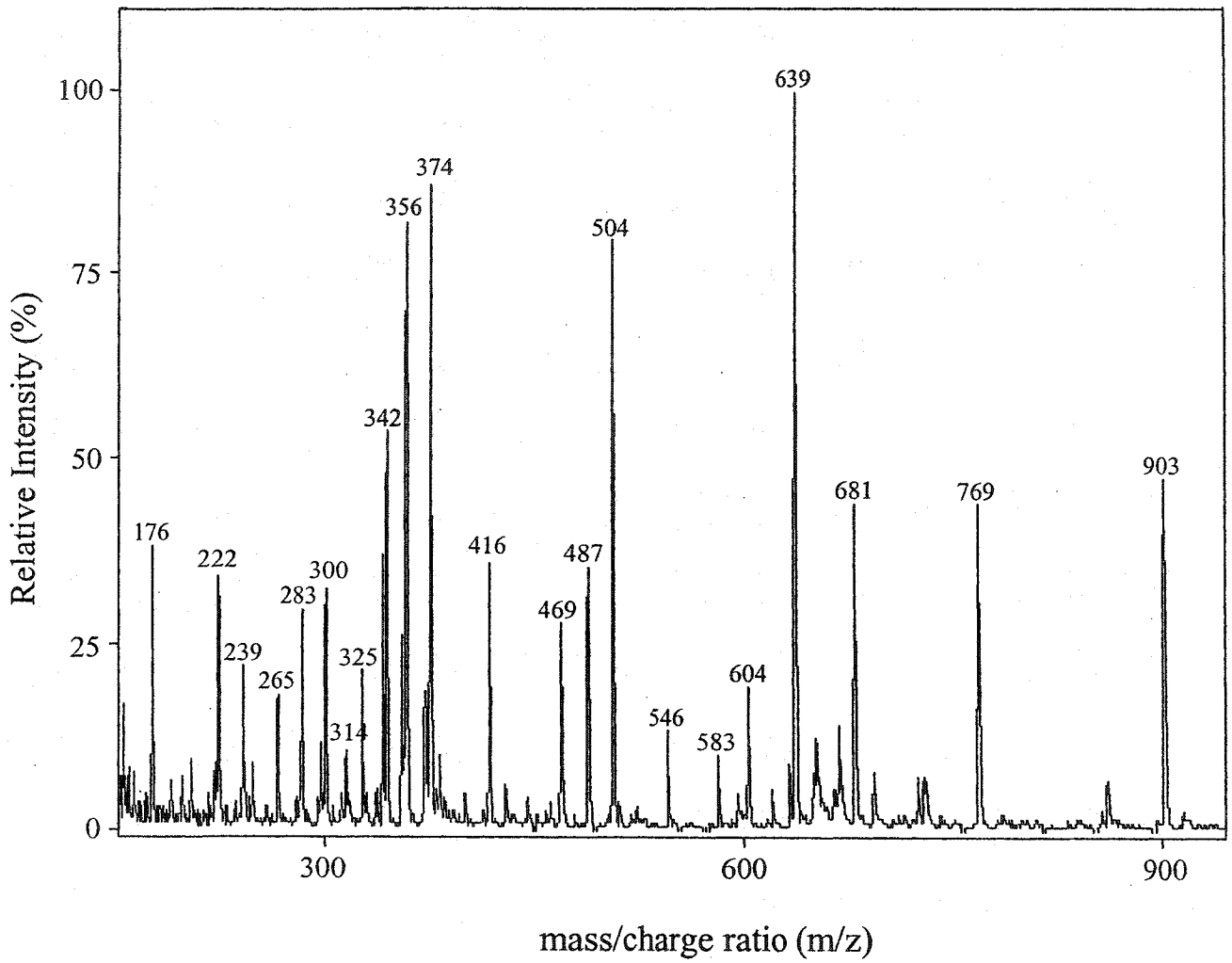


Figure 19. Collision induced dissociation spectrum of the ammonated molecular ions of a reaction mixture after a 16-day lipase-catalyzed transesterification reaction of cinnamic acid and triolein following electrospray ionization mass spectroscopy. Relative intensity based on fragment with a mass/charge ratio of 639.

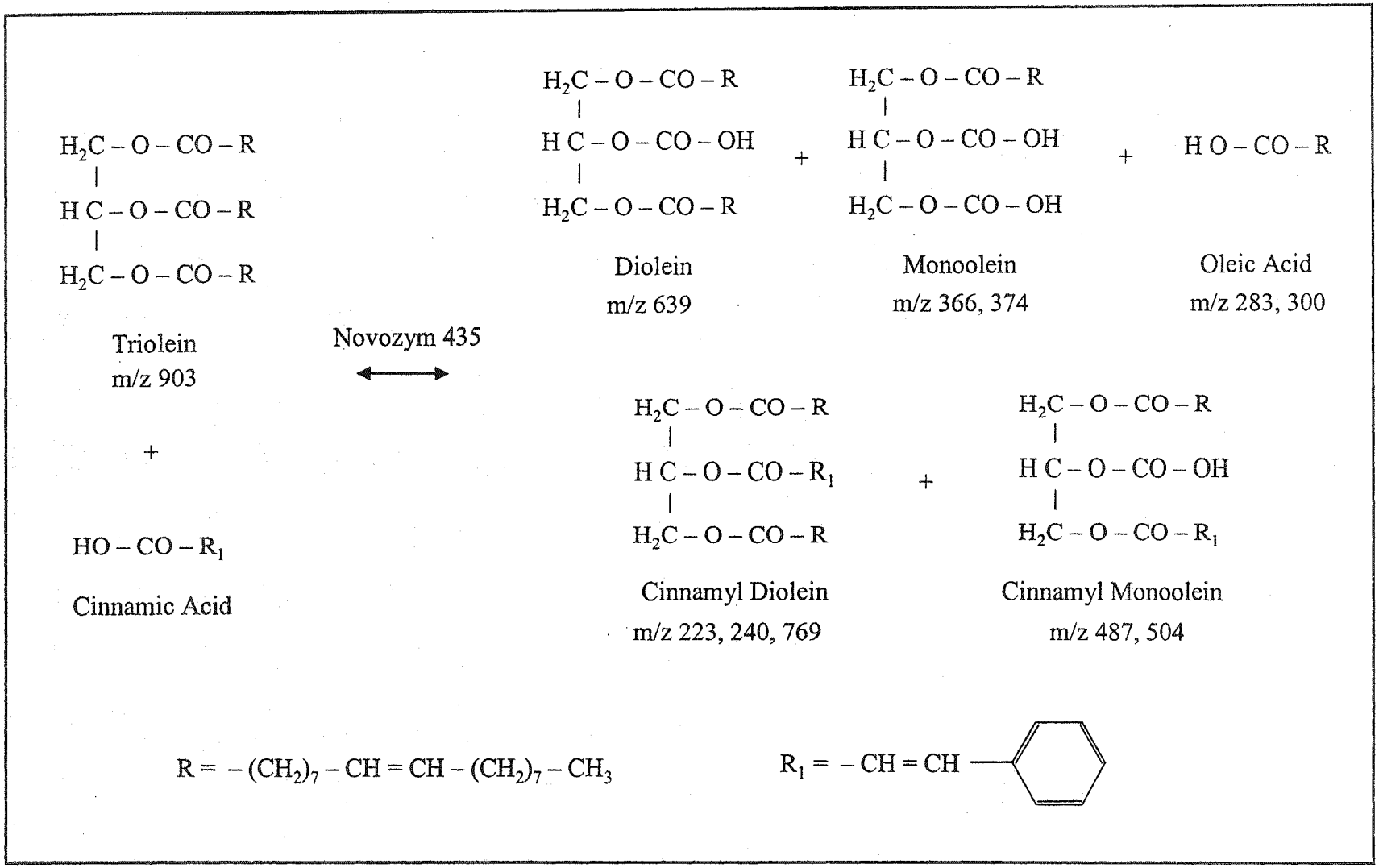


Figure 20. Transesterification of cinnamic acid and triolein to produce hydrolysis products of triolein and phenolic lipid products.

monoolein. Similarly, phenolic lipid products were also characterized by Compton *et al.* (2000) and Kontogianni *et al.* (2001) using ACPI-MS and ESI-MS techniques. In particular, Compton *et al.* (2000) reported major ion peaks for feruloyl monoolein and feruloyl diolein corresponding to $[M + H - H_2O]^+$ and $[M]^+$, at m/z ratios of 515 and 796, respectively.

5. CONCLUSION

A model enzymatic system in organic solvent media, using commercial immobilized lipase (Novozym 435) from *C. antarctica,* for the biosynthesis of phenolic lipids was established with *t*-cinnamic acid and oleyl alcohol as substrates.

The experimental results showed that the hydrophobic solvents provided a more appropriate environment for catalytic activity, while lower initial a_w values promoted phenolic lipid synthesis and limited unwanted side reactions. The results also revealed the existence of diffusional limitations, whereby an increase in agitation speed could only partially overcome these limitations. The temperature was also optimized and the E_a of the biosynthesis reaction, determined by an Arrhenius plot, was in the same range as others reported previously in literature. The increase in fatty alcohol concentration resulted in an increase in the initial enzymatic activity and the bioconversion yield, without any enzyme inhibition in the range investigated. The physico-chemical and structural analyses by HPLC, spectrophotometric scanning and ESI-MS confirmed the nature of the phenolic lipid end product.

Application of the optimized conditions of the model system, with triolein as substrate, resulted in the production of two major and two minor end products; the structural analysis confirmed that the two major phenolic lipid end products are cinnamyl monoolein and cinnamyl diolein.

In conclusion, the optimization of a model enzymatic system and its subsequent application for the production of phenolic lipids was demonstrated. As such, the development of the model system has laid the groundwork for future investigations of the biosynthesis of phenolic lipid ester compounds, using a wide range of triacylglycerols and phenolic substrates.

6. REFERENCES

Adlercreutz, P. (1992). On the importance of the support material for enzymatic synthesis in organic media. Support effects at controlled water activity. In *Biocatalysis in Non-Conventional Media,* Tramper, J., Vermüe, M.H., Beeftink, H.H. and von Stockar, U. (eds.), Elsevier Science Publishers B.V., New York, N.Y., pp. 55-61.

Adlercreutz, P. (2000). Biocatalysis in non-conventional media. In *Applied Biocatalysis*, 2nd Edition, Straathof, A.J.J. and Adlercreutz, P. (eds.), Harwood Academic Publishers, Amsterdam, Netherlands, pp. 295-316.

Almeida, M.C., Ruivo, R., Maia, C., Freire, L., de Sampaio, T.C. and Barreiros, S. (1998). Novozym 435 activity in compressed gases. Water activity and temperature effects. *Enzyme Microb. Technol.* **22**, 494-499.

Andlauer, W., Stumpf, C. and Fürst, P. (2000). Influence of the acetification process on phenolic compounds. *J. Agric. Food Chem.* **48**, 3533-3536.

Andrikopoulos, N.K. (2002). Chromatographic and spectroscopic methods in the analysis of triacylglycerol species and regiospecific isomers of oils and fats. *Crit. Rev. Food Sci. Nutr.* **42**, 473-505.

Auerbach, M.H., Klemann, L.P. and Heydinger, J.A. (2001). Reduced-energy lipids. In *Structured and Modified Lipids,* Gunstone, F.D. (ed.), Marcel Dekker, Inc., New York, N.Y., pp. 485-510.

Ayorinde, F.O., Eribo, B.E., Balan, K.V., Johnson, J.H.Jr. and Wan, L.W. (1999). Determination of major triacylglycerol components of polyunsaturated specialty oils using matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom* **13**, 937-942.

Babali, B., Aksoy, H.A., Tüter, M. and Ustun, G. (2001). Enzymatic esterification of (-)-menthol with fatty acids in solvent by a commercial lipase from *Candida rugosa*. *J. Am. Oil Chem. Soc.* **78**, 53-56.

Baldé, A.M., Claeys, M., Pieters, L.A., Wray, V. and Vlietinck, A.J. (1991). Ferulic acid esters from stem bark of *Pavetta owariensis*. *Phytochem.* **30**, 1024-1026.

Barros, R.J., Wehtje, E. and Adlercreutz, P. (1998). Mass transfer studies on immobilized α-chymotrypsin biocatalysts prepared by deposition for use in organic medium. *Biotechnol. Bioengineer.* **59**, 364-373.

Basheer, S., Cogan, U. and Nakajima, M. (1998). Esterification kinetics of long-chain fatty acids and fatty alcohols with a surfactant-coated lipase in n-hexane. *J. Am. Oil Chem. Soc.* **75**, 1785-1790.

Bibus, D. M. (2001). *Omega 3: Implications in human health and disease.* Simopoulos, A.P. (ed.), Power-Pak C.E., New York, N.Y., pp. 1-24.

Biselli, M., Kragl, U. and Wandrey, C. (2002). Reaction engineering for enzyme-catalyzed biotransformations. In *Enzyme Catalysis in Organic Synthesis: A Comprehensive Handbook, Volume I,* Drauz, K. and Waldmann, H. (eds.), Wiley-VCH Verlag GmbH, Weinheim, Germany, pp. 185-258.

Botía, J.M., Ortuño, A., Benavente-García, O., Báidez, A.G., Frías, J., Marcos, D. and Del Río, J.A. (2001). Modulation of the biosynthesis of some phenolic compounds in *Olea europaea* L. fruits: Their influence on olive oil quality. *J. Agric. Food Chem.* **49**, 355-358.

Bradoo, S., Saxena, R.K. and Gupta, R. (1999). High yields of ascorbyl palmitate by thermostable lipase-mediated esterification. *J. Am. Oil Chem. Soc.* **76**, 1291-1295.

Brenes, M., García, A., García, P., Rios, J.J. and Garrido, A. (1999). Phenolic compounds in spanish olive oils. *J. Agric. Food Chem.* **47**, 3535-3540.

Brockman, H.L. (1984). General features of lipolysis. In *Lipases*. Borgström, B. and Brockman, H.L. (eds.), Elsevier Science Publishers, Inc., New York, N.Y., pp. 1-46.

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 esterifications of functionalized phenols for the synthesis of lipophilic antioxidants. *Biotechnol. Lett.* **20**, 131-136.

Calder, P.C. (2001). More good news about fish oil. *Nutrition* **17**, 158-160.

Chen, H., Zuo, Y. and Deng, Y. (2001). Separation and determination of flavonoids and other phenolic compounds in cranberry juice by high-performance liquid chromatography. *J. Chromatogr. A* **913**, 387-395.

Cheng, Y.-C. and Tsai, S.-W. (2003). Effects of water activity and alcohol concentration on the kinetic resolution of lipase-catalyzed acyl transfer in organic solvents. *Enzyme Microb. Technol.* **32**, 362-368.

Chowdary, G.V. and Prapulla, S.G. (2002). The influence of water activity on the lipase catalyzed synthesis of butyl butyrate by transesterification. *Process Biochem.* **38**, 393-397.

Clough, P.M. (2001). Specialty vegetable oils containing γ-linolenic acid and stearidonic acid. In *Structured and Modified Lipids,* Gunstone, F.D. (ed.), Marcel Dekker, Inc., New York, N.Y., pp. 75-117.

Compton, D.L., Laszlo, J.A. and Berhow, M.A. (2000). Lipase-catalyzed synthesis of ferulate esters. *J. Am. Oil Chem. Soc.* **77**, 513-519.

Copeland, R.A. (2000). Enzyme reactions with multiple substrates. In *Enzymes; A Practical Introduction to Structure, Mechanism, and Data Analysis.* 2nd edition, Copeland, R.A. (ed.), Wiley-VCH, Inc., New York, N.Y., pp. 350-366.

Cornish-Bowden, A. (1979). Two-substrate reactions. In *Fundamentals of Enzyme Kinetics.* Cornish-Bowden, A. (ed.), Butterworth & Co. Ltd., Boston, MA., pp. 99-129.

Cui, H., He, C. and Zhao, G. (1999). Determination of polyphenols by high-performance liquid chromatography with inhibited chemiluminescence detection. *J. Chromatogr. A* **855**, 171-179.

Cuppett, S., Schnepf, M. and Hall, C. III. (1997). Natural antioxidants – Are they a reality? In *Natural Antioxidants: Chemistry, Health Effects & Applications*, Shahidi, F. (ed.), AOCS Press, Champaign, IL, pp. 12-24.

Cussler, E.L. (2000*a*). Models for diffusion. In *Diffusion Mass Transfer in Fluid Systems.* 2nd edition, Cussler, E.L. (ed.), Cambridge University Press, New York, N.Y., pp. 1-12.

Cussler, E.L. (2000*b*). Diffusion in dilute solutions. In *Diffusion Mass Transfer in Fluid Systems.* 2nd edition, Cussler, E.L. (ed.), Cambridge University Press, New York, N.Y., pp. 13-49.

Cussler, E.L. (2000*c*). Diffusion of interacting species. In *Diffusion Mass Transfer in Fluid Systems.* 2nd edition, Cussler, E.L. (ed.), Cambridge University Press, New York, N.Y., pp. 163-183.

Cussler, E.L. (2000*d*). General questions and heterogeneous chemical reactions. In *Diffusion Mass Transfer in Fluid Systems.* 2nd edition, Cussler, E.L. (ed.), Cambridge University Press, New York, N.Y., pp. 371-389.

da Costa, C., Horton, D. and Margolis, S. A. (2000). Analysis of anthocyanins in foods by liquid chromatography, liquid chromatography-mass spectrometry and capillary electrophoresis. *J. Chromatogr. A* **881,** 403-410.

de Deckere, E.A.M., Korver, O., Verschuren, P.M. and Katan, M.B. (1998). Health aspects of fish and n-3 polyunsaturated fatty acids from plant and marine origin. *Eur. J. Clin. Nutr.* **52**, 749-753.

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

Es-Safi, N.-E., Cheynier, V. and Moutounet, M. (2000). Study of the reactions between (+)-catechin and furfural derivatives in the presence or absence of anthocyanins and their implication in food color change. *J. Agric. Food Chem.* **48**, 5946-5954.

Escarpa, A. and González, M.C. (2000). Optimization strategy and validation of one chromatographic method as approach to determine the phenolic compounds from different sources. *J. Chromatogr. A* **897**, 161-170.

Fernandes, G. and Venkatraman, J.T. (1993). Role of omega-3 fatty acids in health and disease. *Nutr. Res.* **13**, Suppl. 1, pp. S19-S45.

Friedman, M. and Jürgens, H.S. (2000). Effect of pH on the stability of plant phenolic compounds. *J. Agric. Food Chem.* **48**, 2101-2110.

Gandhi, N.N. (1997). Applications of lipase. *J. Am. Oil Chem. Soc.* **74**, 621-635.

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

Garcia, T., Sanchez, N., Martinez, M. and Aracil, J. (1999). Enzymatic synthesis of fatty esters. Part I. Kinetic approach. *Enzyme Microb. Technol.* **25**, 584-590.

Gubicza, L. (1992). Effect of the solvent on enzyme enantioselectivity. In *Biocatalysis in Non-Conventional Media,* Tramper, J., Vermüe, M.H., Beeftink, H.H. and von Stockar, U. (eds.), Elsevier Science Publishers B.V., New York, N.Y., pp. 497-503.

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

Gunstone, F.D. (2001*a*). Minor oils, specialty oils, and superrefined oils. In *Structured and Modified Lipids,* Gunstone, F.D. (ed.), Marcel Dekker, Inc., New York, N.Y., pp. 185-208.

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

Gurr, M.I. (1992). Fat in food. In *Role of Fats in Food and Nutrition*, 2nd edition. Elsevier Science Publishing Co. Inc., New York, N.Y., pp. 21-38.

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

Habulin, M., Krmelj, V. and Knez, Ž. (1996). Synthesis of oleic acid esters catalyzed by immobilized lipase. *J. Agric. Food Chem.* **44**, 338-342.

Hadzir, N.M., Basri, M., Rahman, M.B.A., Razak, C.N.A., Rahman, R.N.Z.A. and Salleh, A.B. (2001). Enzymatic alcoholysis of triolein to produce wax ester. *J. Chem. Technol. Biotechnol.* **76**, 511-515.

Halling, P.J. (1992). Salt hydrates for water activity control with biocatalysts in organic media. *Biotechnol. Tech.* **6**, 271-276.

Halling, P.J. (1994). Thermodynamic predictions for biocatalysis in non-conventional media: Theory, tests, and recommendations for experimental design and analysis. *Enzyme Microb. Technol.* **16**, 178-203.

Halling, P.J. (2002). Enzymatic conversions in organic and other low-water media. In *Enzyme Catalysis in Organic Synthesis: A Comprehensive Handbook, Volume I,* 2nd Edition, Drauz, K. and Waldmann, H. (eds.), Wiley-VCH Verlag GmbH, Weinheim, Germany, pp. 259-286.

Halling, P.J. and Valivety, R.H. (1992). Physical-chemical nature of low water systems for biocatalysis: especially phase behaviour, water activity and pH. In *Biocatalysis in Non-Conventional Media,* Tramper, J., Vermüe, M.H., Beeftink, H.H. and von Stockar, U. (eds.), Elsevier Science Publishers B. V., New York, N.Y., pp. 13-21.

Haraldsson, G.G., Halldorsson, A. and Kulås, E. (2000). Chemoenzymatic synthesis of structured triacylglycerols containing eicosapentaenoic and docosahexaenoic acids. *J. Am. Oil Chem. Soc.* **11**, 1139-1145.

Haraldsson, G.G. and Hjaltason, B. (2001). Fish oils as sources of important polyunsaturated fatty acids. In *Structured and Modified Lipids,* Gunstone, F.D. (ed.), Marcel Dekker, Inc., New York, N.Y., pp. 313-350.

Hari Krishna, S. (2002). Developments and trends in enzyme catalysis in nonconventional media. *Biotechnol. Adv.* **20**, 239-267.

Hari Krishna, S. and Karanth, N.G. (2001). Lipase-catalyzed synthesis of isoamyl butyrate A kinetic study. *Biochim. Biophys. Acta* **1547**, 262-267.

Hazarika, S., Goswami, P., Dutta, N.N. and Hazarika, A.K. (2002). Ethyl oleate synthesis by porcine pancreatic lipase in organic solvents. *Chem. Engineer. J.* **85**, 61-68.

Ho, C.-T. (1992). Phenolic compounds in food, an overview. In *Phenolic Compounds in Food and Their Effects on Health, I Analysis, Occurrence & Chemistry,* Ho, C.-T., Lee, C.Y. and Huang, M.-T. (eds.), American Chemical Society Symposium 506, Washington, D. C., pp. 2-7.

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

Hooper, L. (2001). Dietetic guidelines: diet in secondary prevention of cardiovascular disease. *J. Hum. Nutr. Dietet.* **14**, 297-305.

Häkkinen, S. and Auriola, S. (1998). High-performance liquid chromatography with electrospray ionization mass spectroscopy and diode array ultraviolet detection in the identification of flavonol aglycones and glycosides in berries. *J. Chromatogr. A* **829**, 91-100.

Høy, C.-E. and Xu, X. (2001). Structured triacylglycerols. In *Structured and Modified Lipids,* Gunstone, F.D. (ed.), Marcel Dekker, Inc., New York, N.Y., pp. 209-240.

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.

Iwasaki, Y. and Yamane, T. (2000). Enzymatic synthesis of structured lipids. *J. Mol. Catal. B: Enz.* **10**, 129-140.

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

Jensen, R.G., Dejong, F.A. and Clark, R.M. (1983). Determination of lipase specificity. *Lipids* **18**, 239-252.

Jones, P.J.H. and Kubow, S. (1999). Lipids, sterols, and their metabolites. In *Modern Nutrition in Health and Disease.* 9th Edition, Shils, M. E., Olson, J.A. and Shike, M. (eds.), Williams & Wilkins, Baltimore, MD, pp. 67-94.

Kanikkannan, N. and Singh, M. (2002). Skin permeation enhancement effect and skin irritation of saturated fatty alcohols. *Int. J. Pharm.* **248**, 219-228.

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

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.

Kikuzaki, H., Hisamoto, M., Hirose, K., Akiyama, K. and Taniguchi, H. (2002). Antioxidant properties of ferulic acid and its related compounds. *J. Agric. Food Chem.* **50**, 2161-2168.

Kim, I.-H., Yoon, C.-S., Cho, S.-H., Lee, K.-W., Chung, S.-H. and Tae, B.-S. (2001). Lipase-catalyzed incorporation of conjugated linoleic acid into tricaprylin. *J. Am. Oil Chem. Soc.* **78**, 547-551.

Klibanov, A.M. (1997). Why are enzymes less active in organic solvents than in water? *Trends Biotechnol.* **15**, 97-101.

Kontogianni, A., Skouridou, V., Sereti, V., Stamatis, H. and Kolisis, F.N. (2001). Regioselective acylation of flavonoids catalyzed by lipase in low toxicity media. *Eur. J. Lipid Sci. Technol.* **103**, 655-660.

Krog, N.J. (1997). Food emulsifiers and their chemical and physical properties. In *Food Emulsions,* 3rd Edition, Friberg, S.E. and Larsson, K. (eds.), Marcel Dekker, Inc., New York, N.Y., pp. 141-160.

Kvittingen, L. (1994). Some aspects of biocatalysis in organic solvents. *Tetrahedron* **50**, 8253-8274.

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

Lee, M.H., Lin, Y. S., Hsu, F.L. and Hou, W.C. (2003). The mucilage of yam (*Dioscorea batatas*) tuber exhibited angiotensin converting enzyme inhibitory activities. *Bot. Bull. Acad. Sin.* **44**, 267-273.

Lehninger, A.L., Nelson, D.L. and Cox, M.M. (1993). Water: Its effect on dissolved biomolecules. In *Principles of Biochemistry,* 2nd edition, Worth Publishers, Inc., New York, N.Y., pp. 81-105.

Liebert, M.A. (1985). Final report on the safety assessment of stearyl alcohol, oleyl alcohol, and octyl dodecanol. *J. Am. Coll. Toxicol.* **4**, 1-29.

Lin, C.F. (1994). Flavor chemistry of fish oil. In *Lipids in Food Flavors*, Ho, C.-T. and Hartman, T.G. (eds.), American Chemical Society, Washington, D.C., pp. 208-232.

Lin, J.-T., Woodruff, C.L. and McKeon, T.A. (1997). Non-aqueous reversed-phase high-performance liquid chromatography of synthetic triacylglycerols and diacylglycerols. *J. Chromatogr. A* **782**, 41-48.

Liu, K.-J. and Shaw, J.-F. (1998). Lipase-catalyzed synthesis of kojic acid esters in organic solvents. *J. Am. Oil Chem. Soc.* **75**, 1507-1513.

Llorach, R., Tomas-Barberan, F.A., Vallejo, F., Garcia-Viguera, C. and Ferreres, F. (2002). Valorization of by-products of the handling and commercialization of vegetables. In *Natural Products in the New Millennium: Prospects and Industrial Application*, Rauter, A.P, Palmer, F.B., Justino, J., Araújo, M.E. and dos Santos, S.P. (eds.), Kluwer Academic Publishers, Boston, MA., pp. 337-343.

Lortie, R. (1997). Enzyme-catalyzed esterification. *Biotechnol. Adv.* **15**, 1-15.

Ma, L., Persson, M. and Adlercreutz, P. (2002). Water activity dependence of lipase catalysis in organic media explains successful transesterification reactions. *Enzyme Microb. Technol.* **31**, 1024-1029.

Martinelle, M. and Hult, K. (1994). Kinetics of triglyceride lipases. In *Lipases Their Structure, Biochemistry and Application,* Woolley, P. and Petersen, S.B. (eds.), Cambridge University Press, New York, N.Y., 159-180.

Mattila, P., Astola, J. and Kumpulainen, J. (2000). Determination of flavonoids in plant material by HPLC with diode-array and electro-array detections. *J. Agric. Food Chem.* **48**, 5834-5841.

Mauri, P.L., Iemoli, L., Gardana, C., Riso, P., Simonetti, P., Porrini, M. and Pietta, P.G. (1999). Liquid chromatography/electrospray ionization mass spectrometric characterization of flavonol glycosides in tomato extracts and human plasma. *Rapid Commun. Mass Spectrom* **13**, 924-931.

Moreno, J.J. and Mitjavila, M.T. (2003). The degree of unsaturation of dietary fatty acids and the development of atherosclerosis (Review). *J. Nutr. Biochem.* **14**, 182-195.

Nakamura, K., Kinoshita, M. and Atsuyoshi, O. (1995). Structure of solvent affects enantioselectivity of lipase-catalyzed transesterification. *Tetrahedron* **51**, 8799-8804.

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

Negishi, S., Shirasawa, S., Arai, Y., Suzuki, J. and Mukataka, S. (2003). Activation of powdered lipase by cluster water and the use of lipase powders for commercial esterification of food oils. *Enzyme Microb. Technol.* **32**, 66-70.

Opara, E.C. and Hubbard, V.S. (1993). Essential fatty acids (EFA): Role in pancreatic hormone release and concomitant metabolic effect. *J. Nutr. Biochem.* **4**, 498-509.

Opstvedt, J., Urdahl, N. and Pettersen, J. (1990). Fish oils – An old fat source with new possibilities. In *World Conference Proceedings Edible Fats and Oils Proceeding: Basic Principles and Modern Practices,* Erickson, D.R. (ed.), American Oil Chemists' Society, Champaign, IL, pp. 250-259.

Osório, N.M., Ferreira-Dias, S., Gusmão, J.H. and da Fonseca, M.M.R. (2001). Response surface modelling of the production of ω-3 polyunsaturated fatty acids-enriched fats by a commercial immobilized lipase. *J. Mol. Catal. B: Enz.* **11**, 677-686.

Ottolina, G., Gianinetti, F., Riva, S. and Carrea, G. (1994). Effects of enantiomeric solvents on the activity, thermostability and activation energy of lipoprotein lipase. *Catalysis Today* **22**, 511-516.

Ottosson, J., Fransson, L., King, J.W. and Hult, K. (2002). Size as a parameter for solvent effects on *Candida antarctica* lipase B enantioselectivity. *Biochim. Biophys. Acta* **1594**, 325-334.

Ó Fágáin, C. (1995). Understanding and increasing protein stability. *Biochim. Biophys. Acta* **1252**, 1-14.

Ó Fágáin, C. (2003). Enzyme stabilization – recent experimental progress. *Enzyme Microb. Technol.* **33**, 137-149.

Paez, B.C., Medina, A.R., Rubio, F.C., Cerdán, L.E. and Grima, E.M. (2003). Kinetics of lipase-catalysed interesterification of triolein and caprylic acid to produce structured lipids. *J. Chem. Technol. Biotechnol.* **78**, 461-470.

Palmer, T. (1995). Kinetics of multi-substrate enzyme-catalysed reactions. In *Understanding Enzymes,* 4th Edition. Prentice-Hall/Ellis Horwood, Hertfordshire, Great Britain, pp. 155-174.

Perona, J.S. and Ruiz-Gutiérrez, V. (1999). Characterization of the triacylglycerol molecular species of fish oil by reversed-phase high performance liquid chromatography. *J. Liquid Chromatogr. Rel. Technol.* **22**, 1699-1714.

Pérez-Magariño, S., Revilla, I., González-SanJosé, M.L. and Beltrán, S. (1999). Various applications of liquid chromatography-mass spectrometry to the analysis of phenolic compounds. *J. Chromatogr. A* **847**, 75-81.

Persson, M., Costes, D., Wehtje, E. and Adlercreutz, P. (2002). Effect of solvent, water activity and temperature on lipase and hydroxynitrile lyase enantioselectivity. *Enzyme Microb. Technol.* **30**, 916-923.

Petel, T. (2003). In *Biogeneration of Lipophenols by Lipases Using Selected Substrate Models*. M.Sc. Thesis, McGill University, Montreal.

Pogorevc, M., Stecher, H. and Faber, K. (2002). A caveat for the use of log P values for the assessment of the biocompatibility of organic solvents. *Biotechnol. Lett.* **24**, 857-860.

Pomeranz, Y. and Meloan, C.E. (1994). Lipids. In *Food Analysis: Theory and Practice,* 3rd edition, Chapman & Hall, New York, N.Y., pp. 678-724.

Price, N.C. and Stevens, L. (1996). An introduction to enzyme kinetics. In *Fundamentals of Enzymology,* 2nd edition, Price, N.C. and Stevens, L. (eds.), Oxford University Press, New York, N.Y., pp. 136-180.

Priya, K. and Chadha, A. (2003). Synthesis of hydrocinnamic esters by *Pseudomonas cepacia* lipase. *Enzyme Microb. Technol.* **32**, 485-490.

Quinlan, P. and Moore, S. (1993). Modification of triglycerides by lipases: process technology and its application to the production of nutritionally improved fats. *Inform* **4**, 580-585.

Ramamurthi, S. and McCurdy, A.R. (1994). Lipase-catalyzed esterification of oleic acid and methanol in hexane – A kinetic study. *J. Am. Oil Chem. Soc.* **71**, 927-930.

Ratledge, C. (2001). Microorganisms as sources of polyunsaturated fatty acids. In *Structured and Modified Lipids,* Gunstone, F.D. (ed.), Marcel Dekker, Inc., New York, N.Y., pp. 351-400.

Revilla, E. and Ryan, J.-M. (2000). Analysis of several phenolic compounds with potential antioxidant properties in grape extracts and wines by high-performance liquid chromatography – photodiode array detection without sample preparation. *J. Chromatogr. A* **881**, 461-469.

Rodríguez-Delgado, M.A., Malovaná, S., Pérez, J.P., Borges, T. and Montelongo, F.J.G. (2001). Separation of phenolic compounds by high-performance liquid chromatography with absorbance and fluorimetric detection. *J. Chromatogr. A* **912**, 249-257.

Salis, A., Svensson, I., Monduzzi, M., Solinas, V. and Adlercreutz, P. (2003). The atypical lipase B from *Candida antarctica* is better adapted for organic media than the typical lipase from *Thermomyces lanuginosa. Biochim. Biophys. Acta* **1646**, 145-151.

Salleh, A.B., Razak, C.N.A., Ampon, K., Yunus, W.M.Z. and Basri, M. (1995). Effect of some physical parameters on esterification reaction using lipase from *Rhizopus oryzae*. *Pertanika J. Sci. Technol.* **3**, 233-239.

Sargent, J.R. (1997). Fish oils and human diet. *Brit. J. Nutr.* **78**, Suppl. 1, S5-S13.

Schieber, A., Keller, P. and Carle, R. (2001). Determination of phenolic acids and flavonoids of apple and pear by high-performance liquid chromatography. *J. Chromatogr. A* **910**, 265-273.

Schmid, A., Dordick, J.S., Hauer, B., Kiener, A., Wubbolts, M. and Witholt, B. (2001). Industrial biocatalysis today and tomorrow. *Nature* **409**, 258-268.

Schmitt-Rozieres, M., Deyris, V. and Comeau, L.-C. (2000). Enrichment of polyunsaturated fatty acids from sardine cannery effluents by enzymatic selective esterification. *J. Am. Oil Chem. Soc.* **77**, 329-332.

Schuchardt, U., Sercheli, R. and Vargas, R.M. (1998). Transesterification of vegetable oils: A review. *J. Braz. Chem. Soc.* **9**, 199-210.

Seagrave, R.C. (1971). Fundamentals of mass transfer. In *Biomedical Applications of Heat and Mass Transfer,* The Iowa State University Press, Ames, Iowa, pp.114-170.

Secundo, F. and Carrea, G. (2003). Optimization of hydrolase efficiency in organic solvents. *Chem. Eur. J.* **9**, 3194-3199.

Sellek, G.A. and Chaudhuri, J.B. (1999). Biocatalysis in organic media using enzymes from extremophiles. *Enzyme Microb. Technol.* **25**, 471-482.

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

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

Shintre, M.S., Ghadge, R.S. and Sawant, S.B. (2002). Lipase catalyzed synthesis of benzyl esters of fatty acids. *Biochem. Engineer. J.* **12**, 131-141.

Smith, R.E., Finley, J.W. and Leveille, G.A. (1994). Overview of salatrim, a family of low-calorie fats. *J. Agric. Food Chem.* **42**, 432-434.

Soumanou, M.M., Bornscheuer, U.T., Schmid, U. and Schmid, R.D. (1999) Crucial role of support and water activity on the lipase-catalyzed synthesis of structured triglycerides. *Biocatal. Biotransform.* **16**, 443-459.

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*, Stansby, M.E. (ed.), An AVI book, New York, N.Y., pp. 6-39.

Stobiecki, M., Malosse, C., Kerhoas, L., Wojlaszek, P. and Einhorn, J. (1999). Detection of isoflavonoids and their glycosides by liquid chromatography/electrospray ionization mass spectrometry in root extracts of lupin (*Lupinus albus*). *Phytochem. Anal.* **10**, 198-207.

Tarahomjoo, S. and Alemzadeh, I. (2003). Surfactant production by an enzymatic method. *Enzyme Microb. Technol.* **33**, 33-37.

Tawata, S., Taira, S., Kobamoto, N., Zhu, J., Ishihara, M. and Toyama, S. (1996). Synthesis and antifungal activity of cinnamic acid esters. *Biosci. Biotechnol. Biochem.* **60**, 909-911.

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

Valivety, R.H., Halling, P.J. and Macrae, A.R. (1992). Effect of water activity on rate of lipase catalysed esterification. In *Biocatalysis in Non-Conventional Media,* Tramper, J., Vermüe, M.H., Beeftink, H.H. and von Stockar, U. (eds.), Elsevier Science Publishers B.V., New York, N.Y., pp. 549-555.

Villeneuve, P., Hills, G., Bachain, P., Pina, M., Caro, Y., Baréa, B., Guyot, B., Grüning, B. and Graille, J. (2002). Lipase-catalyzed synthesis of quinate and glucuronate fatty esters. *Eur. J. Lipid Sci. Technol.* **104**, 394-401.

Wang, J., Kalt, W. and Sporns, P. (2000). Comparison between HPLC and MALDI-TOF MS analysis of anthocyanins in highbush blueberries. *J. Agric. Food Chem.* **48**, 3330-3335.

Wehtje, E. and Adlercreutz, P. (1997). Water activity and substrate concentration effects on lipase activity. *Biotechnol. Bioengineer.* **55**, 798-806.

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

Whelan, J. (1997). Polyunsaturated fatty acids: Signaling agents for intestinal cancer? *Nutrition Today* **32**, 213-218.

Whitaker, J.R. (1996). Enzymes. In *Food Chemistry,* 3rd edition, Fennema, O.R. (ed.), Marcel Dekker, Inc., New York, N.Y., pp. 431-526.

Williams, C.M. and Roche, H.M. (2001). Achieving optimal fatty acid intake through manufactured foods. In *Structured and Modified Lipids,* Gunstone, F.D. (ed.), Marcel Dekker, Inc., New York, N.Y., pp. 465-484.

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.

Wu, J.-C., Song, B.-D., Xing, A.-H., Hayashi, Y., Talukder, M.M.R. and Wang, S.-C. (2002). Esterification reactions catalyzed by surfactant-coated *Candida rugosa* lipase in organic solvents. *Process Biochem.* **37**, 1229-1233.

Wu, X.Y., Jääskeläinen, S. and Linko, Y.-Y. (1996). An investigation of crude lipases for hydrolysis, esterification, and transesterification. *Enzyme Microb. Technol.* **19**, 226-231.

Yadav, G.D. and Devi, K.M. (2004). Immobilized lipase-catalysed esterification and transesterification reactions in non-aqueous media for the synthesis of tetrahydrofurfuryl butyrate: comparison and kinetic modeling. *Chem. Engineer. Sci.* **59**, 373-383.

Yadav, G.D. and Lathi, P.S. (2003). Kinetics and mechanism of synthesis of butyl isobutyrate over immobilized lipases. *Biochem. Engineer. J.* **16**, 245-252.

Yan, Y., Bornscheuer, U.T. and Schmid, R.D. (2002). Efficient water removal in lipase-catalyzed esterifications using a low-boiling-point azeotrope. *Biotechnol. Bioengineer.* **78**, 31-34.

Yankah, V.V. and Akoh, C.C. (2001). Zero energy fat-like substances: Olestra. In *Structured and Modified Lipids,* Gunstone, F.D. (ed.), Marcel Dekker, Inc., New York, N.Y., pp. 511-536.

Zaks, A. and Klibanov, A.M. (1988). The effect of water on enzyme action in organic media. *J. Biol. Chem.* **263**, 8017-8021.

Zaks, A. and Russell, A.J. (1988). Enzymes in organic solvents: properties and applications. *J. Biotechnol.* **8**, 259-270.