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## ALPHA-LINOLENIC ACID Postprandial Lipid Metabolism and Enzymatic Interesterification of Triacylglycerols

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# ALPHA-LINOLENIC ACID

## Postprandial Lipid Metabolism and Enzymatic Interesterification of Triacylglycerols



**LUND**  
UNIVERSITY

Julia Svensson

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Lund University  
Doctoral Thesis  
May 2011

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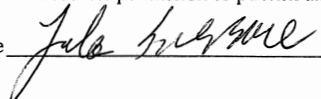
Academic thesis, which by due permission of the Faculty of Engineering at Lund University, will be publicly defended on Thursday, 26<sup>th</sup> of May, at 10.30 a.m. in lecture hall C, at the Center for Chemistry and Chemical Engineering, Sölvegatan 39, Lund, for the degree of Doctor of Philosophy in Engineering.

The Faculty opponent is professor Tom Sanders from the Nutritional Sciences Division, King's College, London, United Kingdom.

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Title and subtitle <b>Alpha-Linolenic Acid - Postprandial Lipid Metabolism and Enzymatic Interesterification of Triacylglycerols</b>		
<p><b>Abstract</b></p> <p>An important indicator of the metabolic capacity of humans is the ability to regulate plasma triacylglycerol levels and to clear triacylglycerol-rich lipoproteins (TRLs) from the circulation after a meal. This is crucial since most of the day is spent in the postprandial state. High concentrations and long circulation times of TRL remnants may be detrimental since these are considered to be highly atherogenic. Postprandial effects of alpha-linolenic acid (ALA) in men and women are poorly characterized. A new ALA-rich oil was produced from rapeseed and linseed oil by enzymatic interesterification. The postprandial effects of 3 meals containing 35 g of this new ALA-rich oil, olive oil, or butter were compared in two randomized crossover studies (26 men and 19 premenopausal women). Blood samples were drawn at regular intervals up to 7 h after the meals. We hypothesized that the postprandial lipid response might be attenuated by a preferential oxidation of ALA compared to other long chain dietary fatty acids.</p> <p>Premenopausal women showed lower postprandial lipemia and were less sensitive to variations in dietary fat than men. Butter resulted in lower postprandial lipemia than the oils in men, whereas no such difference was seen in the women. The ALA oil and olive oil meals induced similar plasma triacylglycerol concentrations. Women showed significantly lower NEFA responses after the olive oil and butter meals than men. The ALA-rich oil had significant effects on the different plasma lipid fractions and decreased the n-6:n-3 ratio in plasma several hours postprandially.</p> <p>ALA levels remained high in plasma triacylglycerols and NEFA even after 5-7 h. This late high concentration of ALA in NEFA is indicative of spill-over NEFA and/or preferential release of ALA by the adipose tissue into the circulation.</p> <p>In summary we did thus not find evidence that ALA has a beneficial effect on postprandial lipids by a selective partitioning to oxidation. This does not exclude the possibility that ALA over a longer time period may have health effects not only as precursor of longer chain n-3 fatty, primarily docosahexaenoic acid, but also because it is sorted out for oxidation.</p> <p>The enzymatic interesterification of triacylglycerols using immobilized <i>Thermomyces lanuginosus</i> lipase (Lipozyme TL IM) as catalyst has also been investigated. Three different reaction systems were studied: rapeseed oil + butter, rapeseed oil + linseed oil (ALA oil), and trilaurin + 1,3-palmitin-2-olein. The ALA oil (35% ALA) was the same as that used in the meal studies. All reactions were followed by reversed-phase HPLC and the triacylglycerol peaks were tentatively identified by calculating equivalent carbon numbers. The triacylglycerols in the rapeseed oil + butter mixture and products were also identified by HPLC-electrospray tandem mass spectrometry.</p> <p>In ideal sn-1,3-specific lipase-catalyzed interesterification, the fatty acid composition in the sn-2 position remains constant. In practice, however, slight changes are observed in the sn-2 position and, under certain conditions, a completely randomized fatty acid distribution can be obtained. Randomization is slower than interesterification. Uncontrolled hydrolysis should, however, be avoided as it lowers the TAG yield. Different triacylglycerol mixtures, i.e. products originating from 1,3-specific interesterification as well as totally or partially randomized products, can be produced by varying the reaction time.</p> <p>Enzymatic interesterification could be used as a method of designing dietary oils with new properties regarding fatty acid composition, susceptibility to oxidation and effects on blood lipids. The future of enzymatic processes relies on efficient, flexible, and easy-to-use systems that ensure high stability of the enzyme preparation and stable output of high-quality products at a reasonable cost.</p>		
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*To my Family*

Every day for us something new  
Open mind for a different view  
And nothing else matters

Forever trust in who we are,  
And nothing else matters  
(Metallica)



# Abstract

An important indicator of the metabolic capacity of humans is the ability to regulate plasma triacylglycerol levels and to clear triacylglycerol-rich lipoproteins (TRLs) from the circulation after a meal. This is crucial since most of the day is spent in the postprandial state. High concentrations and long circulation times of TRL remnants may be detrimental since these are considered to be highly atherogenic.

Postprandial effects of alpha-linolenic acid (ALA) in men and women are poorly characterized. A new ALA-rich oil was produced from rapeseed and linseed oil by enzymatic interesterification. The postprandial effects of 3 meals containing 35 g of this new ALA-rich oil, olive oil, or butter were compared in two randomized crossover studies (26 men and 19 premenopausal women). Blood samples were drawn at regular intervals up to 7 h after the meals. We hypothesized that the postprandial lipid response might be attenuated by a preferential oxidation of ALA compared to other long chain dietary fatty acids.

Premenopausal women showed lower postprandial lipemia and were less sensitive to variations in dietary fat than men. Butter resulted in lower postprandial lipemia than the oils in men, whereas no such difference was seen in the women. The ALA oil and olive oil meals induced similar plasma triacylglycerol concentrations. Women showed significantly lower NEFA responses after the olive oil and butter meals than men. The ALA-rich oil had significant effects on the different plasma lipid fractions and decreased the n-6:n-3 ratio in plasma several hours postprandially.

ALA levels remained high in plasma triacylglycerols and NEFA even after 5-7 h. This late high concentration of ALA in NEFA is indicative of spill-over NEFA and/or preferential release of ALA by the adipose tissue into the circulation.

In summary we did thus not find evidence that ALA has a beneficial effect on postprandial lipids by a selective partitioning to oxidation. This does not exclude the possibility that ALA over a longer time period may have health effects not only as precursor of longer chain n-3 fatty, primarily docosahexaenoic acid, but also because it is sorted out for oxidation.



The enzymatic interesterification of triacylglycerols using immobilized *Thermomyces lanuginosus* lipase (Lipozyme<sup>®</sup> TL IM) as catalyst has also been investigated. Three different reaction systems were studied: rapeseed oil + butter, rapeseed oil + linseed oil (ALA oil), and trilaurin + 1,3-palmitin-2-olein. The ALA oil (35% ALA) was the same as that used in the meal studies. All reactions were followed by reversed-phase HPLC and the triacylglycerol peaks were tentatively identified by calculating equivalent carbon numbers. The triacylglycerols in the rapeseed oil + butter mixture and products were also identified by HPLC-electrospray tandem mass spectrometry.

In ideal *sn*-1,3-specific lipase-catalyzed interesterification, the fatty acid composition in the *sn*-2 position remains constant. In practice, however, slight changes are observed in the *sn*-2 position and, under certain conditions, a completely randomized fatty acid distribution can be obtained. Randomization is slower than interesterification. Uncontrolled hydrolysis should, however, be avoided as it lowers the TAG yield. Different triacylglycerol mixtures, i.e. products originating from 1,3-specific interesterification as well as totally or partially randomized products, can be produced by varying the reaction time.

Enzymatic interesterification could be used as a method of designing dietary oils with new properties regarding fatty acid composition, susceptibility to oxidation and effects on blood lipids. The future of enzymatic processes relies on efficient, flexible, and easy-to-use systems that ensure high stability of the enzyme preparation and stable output of high-quality products at a reasonable cost.

**Keywords:** alpha-linolenic acid, triacylglycerol, postprandial lipemia, Lipozyme TL IM, interesterification

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# Populärvetenskaplig sammanfattning

Avhandlingen handlar om hur fett (lipider) i kosten bryts ner och transporteras i blodet för att slutligen antingen användas som energi, byggstenar i cellväggarna eller lagras i fettväven. I den här avhandlingen kan man också läsa om hur man kan modifiera fetter med hjälp av speciella proteiner: enzymer för att förbättra näringsinnehållet och dess fysikaliska egenskaper, som t.ex. att få rätt smälttemperatur och bredbarhet.

Enzymer finns överallt i levande celler och driver de flesta livsviktiga reaktioner i cellerna och är därför en förutsättning för liv. Jag har arbetat med en slags enzymer som kallas lipaser och dessa katalyserar reaktioner som innehåller fetter. Lipaser finns också hos människor och de är en del av det system som gör att vi kan bryta ner fett i magsäcken och tarmen. Andra lipaser hjälper till att lagra och bryta ner fett i fettceller. Lipaser kan också användas vid livsmedelstillverkning. Jag har tillverkat en ny olja (ALA-olja) av raps- och linfröolja med hjälp av lipaser.

Det finns olika sorters fetter, tex hårt fett som ister och smör och mjukt fett som vegetabiliska oljor. Fett består mestadels av triglycerider. Dessa består av tre fettsyror som är bundna till glycerol molekyl med esterbindningar. Det finns olika fettsyror och det är dessa som bestämmer fettets egenskaper. Det finns långa, korta, mättade, omättade, och fleromättade fettsyror. Jag har arbetat särskilt mycket med alfa-linolensyra (ALA) och den är en lång fettsyra med 18 kolatomer och 3 dubbelbindningar. ALA är en omega-3 fettsyra och är en av de två fettsyror vi inte kan tillverka i kroppen utan måste komma från maten. ALA finns i rapsolja, linfröolja, valnötter, bönor och gröna bladgrönsaker. Den används som energi och som byggsten i olika celler. ALA kan även förlängas och få fler dubbelbindningar och genom detta bilda EPA och DHA som är mycket viktiga för synen, nerverna och hjärnan, speciellt hos fostret och det nyfödda barnet. EPA och DHA, som finns i fet fisk och fiskolja, har och också visats ha sänkande effekt på blodfetter och vara inflammationshämmande vilket är fördelaktigt om man vill förebygga hjärt-kärlsjukdomar. ALAs effekter på hälsan har inte studerats tillräckligt, men det finns indikationer på att även ALA kan ha hälsobefrämjande egenskaper.

Förmågan att reglera triglyceridnivåerna och eliminera triglyceridrika lipoproteiner från blodomloppet är en viktig indikation på den metabola kapaciteten. Eftersom den största delen av dagen spenderas i det postprandiella tillståndet (efter måltid) är denna kapacitet avgörande. Ett ökat och förlängt postprandiellt lipidsvar är en riskfaktor i utvecklingen av ateroskleros och hjärt-kärlsjukdomar. Sammansättning på det dietära fettet påverkar triglyceridstegringen pga fördelning av olika fettsyror till oxidation och tillverkning av blodfetter och lipider i vävnader.

Vi har studerat hur blodfetterna påverkas hos män och kvinnor efter tre olika måltider. Dessa innehåll antingen 35 g smör, olivolja eller vår egentillverkade ALA-olja med hög ALA koncentration. Slutsatserna från studien på män är att smör resulterade i en lägre postprandial lipidstegring än de vegetabiliska oljorna. Skillnaderna var större än vad som var förväntat delvis pga av förekomsten av korta och medellånga fettsyror i smör. Hos kvinnor var det ingen skillnad mellan måltiderna. Dessutom hade de lägre blodfettstegring, vilket beror på att kvinnor innan klimakteriet har en effektivare postprandial fettmetabolism. I blodet speglar ALA-nivån direkt ett intag eftersom det finns så låga nivåer av kroppseget ALA. Efter intag av ALA-oljan förblev andelen ALA högre i både triglyceridfraktionen och i de fria fettsyrorna under hela mätperioden mellan ca 1 och 7 timmar. Detta tyder på att just ALA cirkulerar i olika fraktioner i blodet under lång tid efter en måltid och inte oxideras undan lika snabbt som vi trodde.

Raps och lin är väletablerade grödor i Sverige och dessa ALA-rika vegetabilier kan nu få ytterligare användningsområden inom livsmedelsindustrin. ALA-rika livsmedel har potential som ett hälsosamt, tillgängligt och säkert alternativ för dem som vill tillgodose sitt behov av EPA och DHA, samt förbättra sammansättningen av fettsyror i blodet.

# Papers

This thesis is based on the following papers, referred to in the text by their Roman numerals. The papers are attached at the end of the thesis. Reprints are published by kind permission of the publishers concerned.

- I** Identification of triacylglycerols in the enzymatic transesterification of rapeseed and butter oil, Julia Svensson and Patrick Adlercreutz  
European Journal of Lipid Science and Technology (2008) 110, p. 1007-1013
  
- II** Postprandial lipemic response to an alpha-linolenic acid-rich oil, butter, and olive oil, Julia Svensson, Anna Rosenquist, Patrick Adlercreutz, Åke Nilsson, and Lena Ohlsson  
European Journal of Lipid Science and Technology (2010) 112, p. 961-969
  
- III** Postprandial lipid responses to an alpha-linolenic acid-rich oil, butter, and olive oil in women: A randomized crossover trial, Julia Svensson, Anna Rosenquist, and Lena Ohlsson  
Nutrition Journal (re-submitted in revised-form March 28<sup>th</sup> 2011)
  
- IV** Effect of acyl migration in Lipozyme TL IM-catalyzed interesterification using a triacylglycerol model system, Julia Svensson and Patrick Adlercreutz  
European Journal of Lipid Science and Technology (re-submitted in revised-form April 1<sup>st</sup> 2011)

# My Contribution to the Papers

- I** I planned the study and wrote the manuscript together with Patrick Adlercreutz (PA). JS performed the experimental work.
- II** I and Lena Ohlsson (LO) were responsible for the performance of the study, the data collection, analysis of the results, and writing of the manuscript. Anna Rosenquist (AR) participated in the performance and the data collection. Åke Nilsson and PA were responsible for the design of the study, and participated in the analysis of the results and the writing of the manuscript.
- III** I and LO were responsible for the design of the study, its performance, data collection, analysis of the results, and the writing of the manuscript. AR participated in the performance, data collection, and analysis of the results, and commented on the manuscript.
- IV** I planned the study and wrote the manuscript together with PA. I performed the experimental work.

# Abbreviations

AA	arachidonic acid
ALA	alpha-linolenic acid
AUC	area under the curve
$a_w$	water activity = $\frac{\text{vapor pressure of water in sample}}{\text{vapor pressure of water above pure water}}$
CIE	chemical interesterification
CN	carbon number (number of carbon atoms in a fatty acid)
CVD	cardiovascular disease
DAG	diacylglycerol
DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
ECN	equivalent carbon number
EIE	enzymatic interesterification
ELSD	evaporative light scattering detector
EPA	eicosapentaenoic acid
ER	endoplasmic reticulum
FA	fatty acid
FAME	fatty acid methyl ester
FFA	free fatty acid (biotechnology, equivalent to NEFA)
FH	fully hydrogenated
GC	gas chromatography
HDL	high-density lipoprotein
HDL-C	high-density lipoprotein cholesterol
HPLC	high performance liquid chromatography



iAUC	incremental area under the curve
LDL	low-density lipoprotein
LDL-C	low-density lipoprotein cholesterol
Ln	linoleic acid
LPL	lipoprotein lipase
LCSFA	long-chain saturated fatty acid ( $\geq$ C 12:0)
MAG	monoacylglycerol
MCFA	medium-chain FA (C 8:0 to C 10:0)
MS	mass spectrometry
MUFA	monounsaturated fatty acid
NEFA	non-esterified fatty acid (metabolism, equivalent to FFA)
p-	plasma
PBR	packed-bed reactor
POP	1,3-palmitin-2-olein (C 16:0+C 18:1+C 16:0)
PUFA	polyunsaturated fatty acid
SCFA	short-chain fatty acid (C 2:0 to C 6:0)
SFA	saturated fatty acid
SFC	solid fat content
TAG	triacylglycerol
TLL	<i>Thermomyces lanuginosus</i> lipase
TRL	TAG-rich lipoprotein (VLDL, chylomicrons and remnants)
VLDL	Very Low Density Lipoprotein

# Introduction

This interdisciplinary research was carried out within the framework of the FUNCFOOD PhD program supported by the Functional Food Science Centre at Lund University, Sweden. The work was carried out partly at the Department of Biotechnology, Faculty of Engineering, and partly at the Department of Clinical Sciences, Laboratory of Gastroenterology and Nutrition, Faculty of Medicine. It consists of two parts: postprandial lipid metabolism, and enzymatic interesterification of triacylglycerols. Alpha-linolenic acid (ALA) is one of the important molecules in both projects.

During the past 30 years, overweight and obesity in men and women have increased in Sweden from 35.7 to 59% and from 17.7 to 31.8%, respectively (age group: 35-44, BMI>25) [1]. This is an alarming development since overweight and obesity are associated with hypertension, osteoarthritis, increased risk of developing type-2 diabetes, atherosclerosis, and cardiovascular disease (CVD) [2-3]. This increase in weight implies that the energy balance in today's society is not optimal. It also implies that our metabolic systems are at risk of becoming overloaded by the amount and frequency of food intake.

An important indicator of the metabolic capacity is the ability to regulate triacylglycerol levels and to clear triacylglycerol-rich lipoproteins from the circulation. This is crucial since most of the day is spent in the postprandial state [4-5]. In 1979, Zilversmit proposed that atherogenesis was a postprandial phenomenon [6] and since then substantial effort has been made to reveal the dietary, physiological, and genetic factors that influence the magnitude and duration of the postprandial lipemic response [6-8]. Indeed, an elevated, prolonged postprandial lipemic response is a risk factor for the development of CVD.

ALA and linoleic acid are the only true essential fatty acids for humans. ALA is the precursor of the n-3 fatty acid family, e.g. eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which play important roles in the development and function of the brain and vision, cardiovascular health and inflammatory response [9-10]. EPA and DHA have been thoroughly investigated in contrast to ALA, whose metabolism and biological effects have not yet been completely elucidated. Although the potential health benefits of ALA are still under discussion [11-14], it should be part of a healthy diet. Since ALA is easily accessible compared to EPA

and DHA, it provides an alternative means of increasing n-3 polyunsaturated fatty acid (PUFA) intake.

Rapeseed and linseed are well established crops in Sweden and their oils, especially linseed oil, have high concentrations of ALA. Rapeseed oil is used as edible oil in many food products, such as margarine, dressings, and baked goods. Linseed oil, on the other hand, has a very sharp taste and is therefore not very palatable.

Enzymatic interesterification can be used to produce tailor-made oils and fats. The ALA-rich oils can be mixed with other suitable oils and fats to obtain the nutritional and physical characteristics appropriate for use in the food industry. The desirable outcome is new products with a health-promoting fat composition.

Enzymatic interesterification has many advantages over the “classical” chemical method of modifying fats and oil. It produces fewer by-products, needs less water and equipment, and is easier to control; hence a wider range of products can be produced.

## Scope of this Thesis

The aims of the work presented in this thesis were:

- to examine the interesterification of triacylglycerols (TAGs) catalyzed by Lipozyme<sup>®</sup> TL IM and to produce ALA-rich oil,
- to improve the methods used to analyze TAGs, and
- to characterize the effects of ALA on postprandial plasma lipid metabolism in humans.

**Papers II and III** describe studies of the postprandial lipid responses in men and women after ingesting a meal containing ALA-rich oil. The results were compared with those after ingesting butter and olive oil. The fatty acid (FA) composition of the total lipids in plasma, TAGs and non-esterified FAs (NEFAs) was determined to investigate the effects of the dietary fats. Interesting aspects of the postprandial lipid response to butter were revealed (**Paper II**). The n-6:n-3 ratio and gender differences are discussed in **Paper III**. The design of the two postprandial studies is presented in the Methods chapter.

All four papers deal with aspects of the interesterification of TAGs catalyzed by the immobilized lipase *Thermomyces lanuginosus*, Lipozyme<sup>®</sup> TL IM (Novozymes, Denmark). The substrate and product distribution, the liberation of FAs, and the contingent randomization of the FA composition in the *sn*-2 position of TAGs during reactions were investigated. Different parameters affect the

outcome of interesterification and these are discussed in the chapter entitled Modification of Triacylglycerols using Enzymes. In **Papers I** and **II**, the reaction is denoted “transesterification”. After reviewing the literature and the reaction mechanisms, I felt that “interesterification” was a more suitable term. Some of the methods of analysis are presented in the Methods chapter.

**Paper I** describes a detailed study of the interesterification of rapeseed oil and butter. Reversed-phase HPLC was used together with either an evaporative light-scattering detector (ELSD) or electrospray tandem mass spectrometry (MS) to identify molecular species of particular interest. The degree of randomization in the *sn*-2 position of the TAGs was also estimated.

**Papers II** and **III** describe the production of a new ALA-rich oil from rapeseed and linseed oil by lipase-catalyzed interesterification in a 1 kg batch reactor, and its use in the meal studies. The HPLC-ELSD method described in **Paper I** was further developed (**Paper II**) to improve the separation of TAG species.

**Paper IV** deals with the randomization of the *sn*-2 position in TAGs during interesterification catalyzed by Lipozyme TL IM, in a solvent-free system, at different water activities and enzyme loads. Randomization can occur as a result of acyl migration or possibly a change in the regioselectivity of the enzyme. The performance of the reactions was evaluated by measuring the degrees of interesterification and hydrolysis.

Subjects outside the scope of this thesis include postprandial protein and carbohydrate metabolism and different genotypes and expressions of genes.

Concluding remarks and future considerations end this thesis.

# Triacylglycerols & Fatty Acids

A triacylglycerol consists of a glycerol moiety and three fatty acids, which are esterified to the hydroxyl groups of the glycerol at distinct stereo specific positions, denoted *sn*-1, 2, and 3 (Fig. 1). Fatty acids can either be saturated (SFAs) (i.e. having no double bonds), monounsaturated (MUFAs) (i.e. with one double bond), or polyunsaturated (PUFAs) (i.e. with more than one double bond). The double bonds can either have a *cis*- or *trans*- conformation, but *cis*- is the most common in nature. Fatty acids have different lengths, and are described by their carbon number (CN). Oleic acid has 18 carbon atoms and one double bond positioned at the ninth carbon from the methyl end, and is denoted 18:1 n-9. Various fatty acids are described in Table 1.

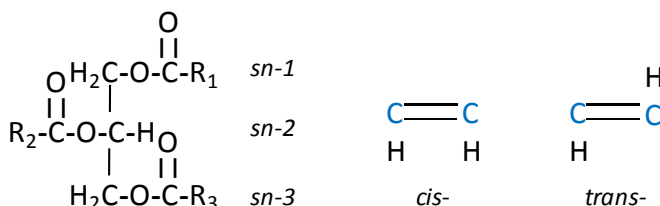


Figure 1. Structure of a triacylglycerol molecule with different fatty acids, R, positioned at stereo specific positions (*sn*). The numbering and *cis*-/*trans*- conformation of double bonds are also shown.

The positional distribution of fatty acids within the TAG molecule affects its physical properties (melting temperature and crystallization pattern), but may also influence its nutritional properties (absorption). Structured lipids, e.g. TAGs with MCFAs in *sn*-1 and 3 positions and a long chain PUFA in the *sn*-2 positions, have shown to have beneficial effects in patients suffering from malabsorption [15]. Triacylglycerols synthesized in plants often have MUFAs or PUFAs in the *sn*-2 position and SFAs in the *sn*-1 and 3 positions; whereas TAGs from animals often have SFAs in the *sn*-2 position. Human milk fat has predominately palmitic acid in the *sn*-2 position [16-17].

Solid fats, i.e. those with melting points above room temperature, can exist in three crystalline forms, resulting in different melting patterns. The  $\alpha$ -form is the least stable with the lowest melting point, while  $\beta$  is the most stable. The intermediate crystalline stability is denoted  $\beta'$ , and is the form preferred by margarine hard

stock producers because it gives the margarine a smooth mouth feel [18]. A TAG, with two palmitic acids (*sn*-1,3) and one oleic (*sn*-2) (POP), has a melting temperature of 26.5°C in the  $\alpha$ -form, and melting temperatures of 33.5 and 37.2°C, respectively, in the  $\beta'$  and  $\beta$  forms [16].

Table 1. Systematic names (and trivial names) of the most common fatty acids, together with their abbreviations, CN:DB ratio, molecular weight (Mw), and type.

Fatty acid	Abbr.	CN:DB	Mw (g/mol)	Type
Butanoic (butyric) acid		4:0	102.13	SCFA <sup>1</sup>
Hexanoic (caproic) acid		6:0	130.17	SCFA
Octanoic (caprylic) acid		8:0	158.23	MCFA
Decanoic (capric) acid		10:0	186.28	MCFA
Dodecanoic (lauric) acid	L	12:0	214.34	LCSFA <sup>1</sup>
Tetradecanoic (myristic) acid	M	14:0	242.39	LCSFA
Hexadecanoic (palmitic) acid	P	16:0	270.44	LCSFA
Octadecanoic (stearic) acid	S	18:0	298.47	LCSFA
Oleic acid (n-9)	O	18:1	296.47	MUFA
( <i>cis</i> -9,12) linoleic acid (n-6)	Ln	18:2	294.46	PUFA
( <i>cis</i> -9,12,15) $\alpha$ -linolenic acid (n-3)	ALA	18:3	292.44	PUFA
Eicosanoic (arachidic) acid	ARA	20:0	326.53	LCSFA
Arachidonic acid (n-6)	AA	20:4	304.47	PUFA
Eicosapentaenoic acid (n-3)	EPA	20:5	302.45	PUFA
Docosahexaenoic acid (n-3)	DHA	22:6	328.49	PUFA

<sup>1</sup> short-chain saturated fatty acid (SCFA), long-chain saturated fatty acid (LCSFA)

Dietary fat intake constitutes about 34% of our total energy intake (E%) (*Riksmaten* 1997-1998, Sweden) [19]; the recommended intake for adults being 25 to 35 E% [20]. The Swedish National Food Administration (NFA, *Livsmedelsverket*) in Sweden recommends that the intake of SFAs and *trans*-fatty acids should be less than 10 E%, MUFAs around 10-15 E%, and PUFAs 5-10 E% [20]. Triacylglycerols are the major component of dietary fat, while polar lipids and cholesterol/sterols are the minor ones. One gram of fat corresponds to 9 kcal, which can be compared to 1 gram of carbohydrates, which corresponds to 4 kcal,

hence the energy density of a meal will be increased considerably if carbohydrates are replaced by fat [21].

# The Digestion and Absorption of Lipids

The digestion of lipids starts in the mouth. Chewing grinds and mixes the food with saliva, which contains various enzymes ( $\alpha$ -amylases and proteases). In the stomach, secreted gastric lipase starts the hydrolysis of the fatty acid in the *sn*-3 position. Hydrolysis and peristalsis lead to the formation of increasingly smaller micelles, which facilitates the reaction. The stomach content is emptied into the small intestine as a result of neural and hormonal signals that arise in response to the nutrients [7, 21-22].

Lipid digestion takes place mainly in the upper part of the small intestine (the duodenum). The presence of food in the stomach as well as neural and hormonal signals stimulate acinar and duct cells in the pancreas, which subsequently secrete water, bicarbonate, and large amounts of inactive enzymes (zymogens) into the lumen of the duodenum. These zymogens are activated in the intestine and start to catalyze the breakdown of nutrients. At the same time, the gallbladder contracts and ejects bile salts into the duodenum through the common bile duct. The lipid products are emulsified by the bile salts and micelles are formed. A colipase binds to and activates pancreatic lipase, which starts region specific hydrolysis of the fatty acids in the *sn*-1 and 3 positions. The reactions result in free fatty acids (FFAs) and *sn*-2 MAGs, which are absorbed by the enterocytes [7, 21]. The lipids are also hydrolyzed by carboxylic ester hydrolase, which is a non-regio-specific enzyme that can act on several different substrates, such as TAGs, DAGs, MAGs, cholesteryl-, and retinyl esters [23].

It has been suggested that saturated LCFAs such as palmitic and stearic acid, together with calcium, form insoluble calcium soaps, which are poorly absorbed. However, in a recent review it was concluded that there is little evidence that calcium soap formation affects the digestibility and energy balance in adult humans [16].

It has been suggested that the absorption of *sn*-2 MAGs and fatty acids occurs through diffusion and facilitated transport. LCFAs pass through the cell membrane with the aid of the fatty acid transport protein system. These integral membrane transport proteins are also present in the heart, liver, adipose tissue, and other



fatty-acid-metabolizing tissues [24]. The fatty acids and the *sn*-2 MAGs are re-esterified to TAGs and the resulting lipid droplet enters the endoplasmic reticulum (ER), thus the fatty acid in the *sn*-2 position remains intact, which can have implications on the metabolism depending on the esterified fatty acid [16, 21]. The effect of different dietary fats on metabolism will be discussed later.

SCFAs and MCFAs pass through the enterocytes without being esterified. They are bound to albumin and transported as NEFAs via the hepatic portal vein into the circulation. NEFAs are used as an energy source by most tissues.

Inside the ER, the lipids will assemble with cholesterol, phospholipids, fat-soluble vitamins, and apolipoprotein B<sub>48</sub> (apoB<sub>48</sub>) to a nascent chylomicron. Chylomicrons are large lipoproteins with the main function of transporting dietary fat to the tissues. The nascent chylomicron moves to the Golgi apparatus where it matures and is subsequently released into the lymphatic system through exocytosis. In the circulation, it acquires surface components such as apoprotein C from high-density lipoprotein (HDL), together with free and esterified cholesterol and phospholipids, and becomes a mature chylomicron [17, 25].

Studies in humans after sequential meals have shown that chylomicrons secreted after the second meal contained TAGs from the first meal [26-27]. This observation led to the hypothesis that lipid droplets could be stored by the enterocytes for later use. Lipid droplets that emerge and then disappear in the cytosol have been seen in enterocytes in mice [28]. These lipid droplets may play a role in regulating p-TAG concentrations during chylomicron formation.

# Lipid Metabolism

Postprandial lipemia occurs after meals containing at least 20 g fat [29]. Thus, a daily intake of about 100 g fat elevates p-TAG levels several times each day. It is only after a night's sleep we experience a true fasting state. An augmented postprandial lipemic response is a sign of impaired lipid metabolism. Prolonged circulation of lipids in the blood is known to increase the risk of metabolic disorders [30].

TAGs are the main constituent of the adipose tissue, where they are stored until energy is needed. NEFAs are used as an energy source through  $\beta$ -oxidation by muscle fibers, heart, liver, and other tissues. Polar lipids, such as glycerophospholipids and sphingolipids, serve as structural components in membranes and are involved in cell signaling. Long-chain PUFAs are precursors of eicosanoids, which mediate a range of activities, such as blood clotting, regulation of vascular tonus, and pro- and anti-inflammatory responses [7, 21].

## The Lipoprotein Pathways

The core of the lipoprotein particle is made up of TAGs and cholesterol esters, while the outer part consists of phospholipids, proteins, and free cholesterol. Apo proteins are also found in lipoprotein particles, and their functions are to maintain the structure and solubility of the particle, and recognize and interact with other lipoproteins and receptors on cell surfaces. Moreover, they determine the activities of lipases such as lipoprotein lipase and hepatic lipase, receptors, and lipid transfer proteins.

### *The Exogenous Pathway*

Chylomicrons are part of the exogenous lipoprotein pathway and have a half-life in plasma of approximately 5-6 minutes [31]. Each particle contains one apoB<sub>48</sub> and several other apoproteins (A-I, A-II, C-I, C-II, C-III, and E). The chylomicrons undergo rapid lipolysis by the action of lipoprotein lipase (LPL) at the surface of the endothelial cells, and become chylomicron remnants. ApoC-II is a cofactor of LPL that enhances the interaction between substrate and enzyme. In

skeletal muscle, insulin decrease the activity of LPL, and fatty acids are consequently taken up by muscle cells in the post-absorptive- and fasting state. In contrast, LPL synthesis and secretion are activated by insulin in the adipose tissue. TAGs are then hydrolyzed and the fatty acids adsorbed. Moreover, LPL is sensitive to product (NEFA) inhibition. The resulting NEFAs and *sn*-2 MAGs are rapidly transported into the cells and used as energy in muscles, or re-esterified for storage in adipose tissue. NEFAs can, however, escape into the circulation, where they are denoted spill-over NEFAs [32].

Chylomicrons obtain ApoE from HDL, which is necessary for the clearance of the chylomicron remnants, which are taken up by the liver through the low-density lipoprotein (LDL) receptors that recognize ApoE, and the LDL-receptor related protein (LRP). Chylomicron remnants are considered to be atherogenic as they can take part directly in foam cell formation. It is thus of considerable importance that these remnants are efficiently cleared [33].

## *The Endogenous Pathway*

Very-low-density lipoproteins (VLDLs) contain apoB<sub>100</sub>, apoC-I, C-II, C-III, and E, and are produced in the liver. VLDLs distribute TAGs from the liver to the tissues, and they have a half-life of 1-3 hours. This pathway is always active, but during the postprandial state the hydrolysis of VLDLs is suppressed in favor of the hydrolysis of chylomicron-TAGs. NEFAs are transported continuously to the liver, hence the production of VLDLs never ceases. VLDLs carry most of the TAGs in the circulation, even during the postprandial state [21].

The secretion of VLDLs is closely regulated by insulin. In the fasting state hormone-sensitive lipase hydrolyzes TAGs in the adipose tissue. The NEFAs generated are major contributors to VLDL-TAGs. Other sources of fatty acids are the chylomicron remnants. TAGs synthesized *de novo*, in the liver, also contributes to VLDL-TAG [34], which is evident after high carbohydrate loads [35]. VLDL production is also controlled by the availability of hepatic cholesterol, which regulates the expression of LDL receptors [36]. After VLDLs are secreted from the liver they acquire apoC-II from HDL allowing them to be hydrolyzed by LPL. VLDL remnants, also called intermediate density lipoproteins, have two fates; they are either removed by the liver (LDL-receptors and cell surface heparin proteoglycans LRP) or most of the surface components are lost and they become LDLs [35-36].

LDL transports cholesterol to the tissues and regulates the synthesis of cholesterol in the cells. The apolipoprotein apoB<sub>100</sub> is recognized by the LDL receptor on the cell surface, and the LDL particle fuses with the cell. The cholesterol is used in cell membranes, for the synthesis of hormones, or stored.

Reverse cholesterol transport is mediated by HDLs (apoA-I, A-II, B<sub>48</sub>, C-I, C-II, C-III, D, and E). Pre- $\beta$ -HDL is synthesized in the intestine and liver, and then secreted into the circulation where it gathers surface material. The nascent HDL binds to cell surface receptors and removes cholesterol from the cell. Cholesterol is esterified by the action of lecithin-cholesterol acyl-transferase and becomes the major constituent of the hydrophobic core of mature HDL. The mature HDL particle can remove the cholesterol by two pathways: the direct pathway, in which HDLs circulate directly back to the liver, and the indirect route, in which HDLs transfer the cholesterol esters to VLDLs or LDLs by the action of the cholesterol ester transfer protein in exchange for TAGs [37]. Upon arrival at the liver, the cholesterol can be excreted or transported to other cells. The HDL-cholesterol esters can selectively be taken up by the liver through the scavenger receptor B1 without parallel apolipoprotein uptake. Impaired LDL and HDL metabolism increases the formation of plaque in the artery walls, which is the first stage in the development of atherosclerosis [7, 21, 35, 38].

### ***Non-Esterified Fatty Acids***

The NEFA concentration in the circulation varies throughout the day. When the insulin level rises after a meal, the NEFA concentration is suppressed, but increases rapidly as the insulin level decreases. NEFA concentration is influenced both by nutritional state and physical activity [39]. Spill-over NEFAs, during chylomicron hydrolysis, as well as SCFAs and MCFAs (if dairy fat is consumed) can contribute from the exogenous pathway, while mobilization of fatty acids from adipose tissue and from *de novo* lipogenesis might contribute to NEFAs from the endogenous pathway.

### ***Differences in Lipid Metabolism between Premenopausal Women and Men***

The lipid metabolism of men and premenopausal women differs in several respects, such as lower body fat accumulation and lower circulation of blood lipids in women, whereas men exhibit greater lipolytic sensitivity and increased fatty acid uptake in visceral adipose tissue and less in femoral and gluteal fat depots [40]. Magkos et al.[41] showed that women had an increased clearance rate of VLDL-TAGs and a decreased rate of VLDL-apoB<sub>100</sub> secretion than men. The greater VLDL-TAG to VLDL-apoB<sub>100</sub> ratio in women also suggested that they secrete fewer but TAG-rich VLDL particles than men [41]. Women also show higher concentrations of HDL-cholesterol (HDL-C) and apoA-I, and a higher rate of apoA-I secretion than in men of the same age [33].

It has been shown in both previous work [42-44] and in the present work (**Papers II and III**), that postprandial lipemia is lower in women than in men, which is probably due to the more efficient clearance of chylomicron remnants in women [41, 44]. Significantly lower NEFA responses have been reported in women than in men (**Papers II and III**) [44]. The lower response may stem from more efficient capture and re-esterification of fatty acids after the hydrolysis of TRL in the adipose tissue, and the resulting lower probability of spill-over NEFAs [32] in women. Moreover, women are less sensitive to variations in dietary fat [29, 42, 45] (Fig. 3 A and B, page 32).

Men have a greater tendency to store fat in visceral adipose tissue, and higher hormone sensitive lipase activity, which may lead to a higher flux of NEFAs to the liver and higher VLDL production [40], which could explain the higher p-TAG levels in men. It has been proposed that estrogen promotes rapid clearance of chylomicron remnants due its effect on the LDL receptor, and promotes the efficient trapping of fatty acids in the adipose tissue. Moreover, estrogen could increase the surface area of the endothelium through its vasodilatory properties, hence a larger area could express LPL [38]. However, in a recent study, no effect was seen on postprandial TAG response in response to changes in estrogen level during the menstrual cycle [46]. It has also been shown that women exhibit lower postprandial oxidative stress after fat intake than men [47], which could, in part, explain the lower prevalence of CVD in women. The protective effect against CVD in women disappears after the menopause.

### ***Impaired Lipid Metabolism***

Excess intake of macronutrients, obesity, and insulin resistance impair the metabolism of lipids. The ingestion of large amounts of fat, or calories in general over a long time will overload the metabolism, leading to elevated levels of circulating NEFAs, VLDLs and remnants [39]. A high level of VLDL remnants is considered a risk factor for atherosclerosis, and a precursor of LDL, which has the ability to adhere and attract monocytes to endothelial cells in the arteries, promotes inflammatory response and triggers foam cell formation [33].

Insulin resistance has profound effects on lipid metabolism, such as elevated TAG concentrations and an overall increase in highly atherogenic particles [48-49]. The LPL secretion/activity is decreased in the endothelium. The liver starts to increase the production and secretion of apoB<sub>100</sub> and TAG-rich VLDL particles. These TAG-rich VLDL particles acquire cholesterol from LDL and HDL particles, in exchange for TAG. The TAG-enriched LDL and HDL particles are preferentially hydrolyzed by hepatic lipase, and the result is highly atherogenic, dense LDL and HDL particles. Moreover, the clearance rate of remnants in the liver is reduced.

# Factors Affecting the Postprandial Lipemic Response

Several factors are known to affect postprandial lipemia, including elevated fasting TAG level, increasing age and weight, gender, lack of physical activity, habitual diet, meal composition, as well as genetic factors [4-5, 8, 50]. The effect of different types of dietary fatty acids will be discussed in the next chapter. Aging and obesity increase fasting p-TAG and LDL-C concentrations, due to delayed chylomicron remnant clearance, elevated VLDL apoB<sub>100</sub> secretion, and delayed LDL apoB<sub>100</sub> clearance [33]. This will subsequently lead to an increase in postprandial response. Subjects with BMI > 26.18 have shown to respond with a higher iAUC p-TAG than subjects with BMI < 26.18 in a study by Lozano et al. [51].

The amount of fat in a meal affects the postprandial lipemic response. Studies have shown that 0-15 g fat in a meal elicited no increase in p-TAG, whereas a dose-dependent response was seen in subjects consuming 30-50 g of fat, and high doses, >80 g, resulted in highly elevated responses [29] [52]. Lopez-Miranda et al. [8] concluded that habitual diets rich in SFAs are more likely to generate higher postprandial lipemic response than diets rich in MUFAs, n-6 PUFAs, or long-chain n-3 PUFAs.

Carbohydrates and protein also affect the p-TAG concentration. Fructose tends to prolong and increase the postprandial lipemic response, whereas soluble viscous fibers decrease the response [53]. Bortolotti et al. [54] found that adding protein to a single meal did not affect the postprandial lipemic response, but after four days on a high-protein diet, the consumption of a protein-rich meal increased the level of chylomicron-TAG significantly. It was suggested that this could be due to impaired clearance of chylomicron remnants [54].

The hypotriacylglycerolemic effect of physical exercise is suggested to be due to secretion of fewer but TAG-rich VLDL particles by the liver and increasing concentration and activity of LPL in muscle [50]. Others have concluded that postprandial lipemia is reduced after physical exercise mainly due to the decreased fasting levels [55-56]. Hurren et al. [57] showed that a 90-minute brisk walk decreased both total and incremental lipemic responses after meals containing high or moderate amounts of fat.

## *Conclusion*

Postprandial lipemia is influenced by several factors including ageing and genetics, however, other factors can be influenced, leading to a reduction in postprandial lipemic response. Increased physical activity and reduced consumption of refined sugars and fat are recommended for everyone as well as weight loss in overweight subjects, in order to decrease the postprandial lipemic response.

## The Effect of Dietary Fatty Acids on Postprandial Lipemia

Postprandial lipemia is affected by the type of fatty acid present in the meal ingested. The purpose of the studies described in **Papers II** and **III** was to compare the effect on postprandial lipemia of meals containing 35 g of a ALA-rich oil to those of meals containing butter and olive oil, in 45 healthy volunteers (26 men and 19 women). The significant differences between the responses of butter and olive oil are discussed here, while the results for the ALA-oil are presented in the next chapter.

Butter elicited the lowest postprandial TAG response, and a significant difference was seen between the response to butter and olive oil in men, but not in women (Fig. 3 A and B, page 32). Women showed a significantly lower NEFA response after ingesting olive oil and butter than the men. Other researchers have also seen the reduction in TAG response elicited by butter compared to olive oil [42, 58-59].

Perez-Martinez et al. [60] found that the consumption of olive oil led to the formation of larger and a greater number of total chylomicron and VLDL particles compared to butter. Larger chylomicron sizes after olive oil and sunflower oil compared to butter have also been observed by Mekki et al. [58]. The p-TAG response after ingesting olive oil was higher during the first 3 hours after the meal, but cleared faster than butter [60]. Faster clearance of p-TAG after olive oil was not seen in the present study on men (**Paper II**) or by Mekki et al. [58]. A more rapid removal of MUFA-rich lipoprotein particles compared to SFA-rich during the postprandial period was seen in men consuming olive oil or palm oil + cocoa butter [61].

Jackson et al.[27] have reported that olive produce more chylomicrons than palm oil, safflower, and safflower+fish oil. This difference was retained after the second meal. Safflower, rich in n-6 PUFA, induced larger chylomicrons which may have been more rapidly hydrolyzed [27]. The authors suggested that olive oil may

influence one or several steps in the formation or metabolism of chylomicrons [27].

The low response to butter has been linked to the content of SCFAs, MCFAs, the formation of Ca-soaps [62], gastric lipase activity [63], and lipid storage droplets in enterocytes [26, 28]. The physical characteristics of the fat, as well as the fat load, also influence the postprandial response [58, 64]. Fats containing a high degree of fat that is solid at 37°C (e.g. stearic-rich fats) have been shown to induce lower postprandial lipemic response [65].

In studies using high doses of fat (>80 g), a higher postprandial TAG response has been found with butter than with olive oil [66-68]. Thomsen et al. [66] reported that 100 g butter elicited significantly larger incremental areas under the curve (iAUC) in both chylomicron-rich and -poor TAG fractions than 80 g of olive oil, in ten healthy young men and women.

Lopez et al. [67-68] compared the responses of ≈85 g olive oil (MUFA-rich) and butter (SFA-rich) in fourteen normotriglyceridemic subjects [68] and 14 subjects with fasting hypertriglyceridemia [67]. The postprandial p-TAG and NEFA responses to butter were higher than those to olive oil in both groups. A blend of vegetable oil and fish oil, and high-palmitic sunflower oil were also tested on the normal subjects and the vegetable oil + fish oil blend induced the lowest p-TAG response. Postprandial insulin sensitivity was measured, and it was concluded that normotriglyceridemic subjects became less insulin-resistant postprandially with a higher MUFA-to-SFA ratio in the meal [68]. In the study on hypertriglyceridemic men [67], it was stated that MUFA was superior to SFA in reducing insulin intolerance.

The postprandial effects of a butter and fish oil blend (1.1 wt% EPA and DHA) produced by enzymatic interesterification, were compared with a commercial butter product, following a single meal consisting of 32 g fat, white bread, and jam. No difference was seen in the iAUC p-TAG response, but the oil blend induced lower p-TAG levels after 2 h, and the concentration of long-chain n-3 PUFAs tended to be higher in plasma between 2 and 6 h. Long-chain n-3 PUFAs (40 g of oil) have been shown to attenuate postprandial lipemic response compared to corn oil, which is rich in n-6 PUFAs [69].

### *Conclusions*

The results of studies on LCFAs are inconsistent. Meals containing MUFAs and n-6 PUFAs, e.g. olive oil, and safflower oil, have been shown to produce fewer, larger TRL particles. These traits could be favorable since LPL preferentially hydrolyses large particles, and the presence of fewer particles would reduce the risk of saturation of LPL, thus facilitating the clearance of TRL. Moreover, the consumption of long-chain n-3 PUFAs has been shown to reduce the amount of



TAG remnants in the circulation. High concentrations and long circulation times of these remnants may be detrimental since they are considered to be highly atherogenic. Prolonged circulation of remnants increases the risk of unfavorable interactions with macrophages. Thus, rapid clearance of remnants is important. Further investigations in healthy, slightly overweight subjects on the effects of different fats on the size and number of lipoprotein particles are warranted.

However, the *type* of dietary fat may not be the most important factor, but rather the *amount*, as illustrated by studies on the response to butter. Sanders proposed in his review [38], that it could be favorable to limit fat consumption to less than 30 g per meal in order not to stress the system. I agree with this as the plasma lipids should then be able to return to baseline levels between meals.

# Alpha-Linolenic Acid

Alpha-linolenic acid (ALA, 18:3 n-3) is one of two fatty acids essential for humans; the other being linoleic acid (Ln, 18:2 n-6) (Fig. 2). The n-3 and n-6 fatty acids are defined by a double bond beginning at the third and sixth carbon from the methyl-end, respectively. Mammals have the capacity of inserting a double bond at the ninth carbon, using a  $\Delta$ -9 desaturase, but no more proximal than that. Mammals do not have the sets of desaturases required to synthesize n-3 or n-6 fatty acids *de novo*, nor can n-3 be converted into n-6 or vice versa. Hence, both ALA and Ln must be present in the diet. Essential acid deficiency is extremely rare. It has been reported in human infants fed milk formula and patients receiving long-term total parenteral nutrition without adequate lipid supply; the symptoms being scaling of the skin, impaired growth, weakness, and blurring of vision [11, 21, 70].

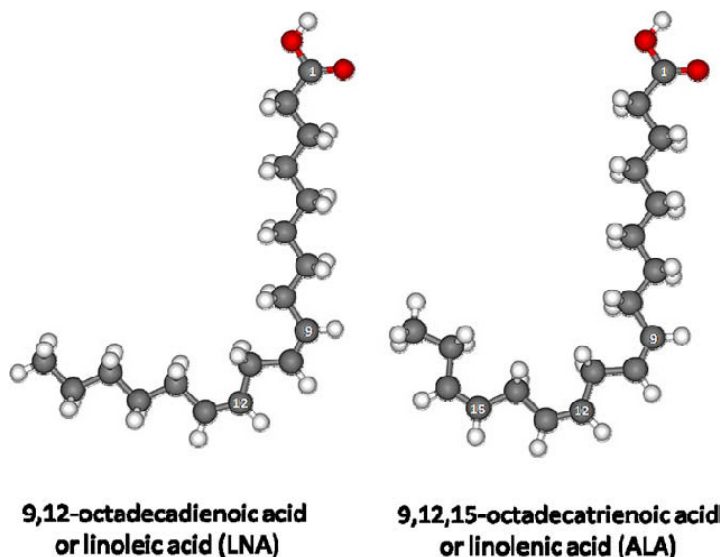


Figure 2. Model of the molecular structure of all-*cis*-9,12,15-octadecatrienoic acid (alpha-linolenic acid) and the all-*cis*-9,12-octadecadienoic (linoleic acid), adapted from Barceló-Coblijn and Murphy [11].

# Alpha-Linolenic Acid in Food

Statistics Sweden (*Statistiska centralbyrån*) and the Swedish NFA performed a survey on food intake and dietary habits in Sweden in 1997-98. It was found that the mean consumption of ALA (and EPA+DHA) per day was 1.2 (0.31) g in women and 1.6 (0.34) g in men [19]. The Swedish NFA recommends that one percent of energy intake should come from n-3 fatty acids, which corresponds to 2.5-3 g/day (approx. 1-2 tablespoons of rapeseed oil) or a portion of salmon [20]. Hence, there is a discrepancy between the actual intake and the recommended intake. Humans can acquire ALA by consuming rapeseed oil (also called canola), walnuts (*Juglans regia*), linseeds (*Linum usitatissimum* L), and linseed oil, legumes, green leafy legumes, and vegetables, (Table 2). The proportion of ALA in chloroplast membranes of leafy vegetables such as spinach is high, but the fat content is low, and they are therefore not a major source of dietary ALA. It is thought that ALA protects the plant membranes against low temperatures [70].

Table 2. Content of alpha-linolenic acid in various foods<sup>1,2</sup>.

<b>Food item</b>	<b>g<sub>ALA</sub>/100 g<sub>product</sub></b>	<b>Food item</b>	<b>g<sub>ALA</sub>/100 g<sub>product</sub></b>
<b>Linseeds raw</b>	16.7	<b>Rapeseed oil</b>	10.7
<b>Linseed oil</b>	53.3	<b>Wheat germ oil</b>	5.9
<b>Walnuts</b>	6.8	<b>Soybeans</b>	1.2
<b>Walnut oil</b>	10.4		

1) Data from Dietist XP, version 3.1, the Swedish NFA data base (20080306)

2) Data from Danish Food Composition Databank version 7.01, Technical University of Denmark, [www.foodcomp.dk](http://www.foodcomp.dk)

The bioavailability of the fatty acids from different food sources affects the metabolism. Berry et al. showed that there was a significant difference in postprandial p-TAG response and fatty acid composition in plasma, depending on whether whole almonds or almond oil and defatted almond meal were consumed. Whole almonds elicited a lower p-TAG response, while the fatty acid composition was less affected [71]. Hence, the intake of oils improves the bioavailability, increasing the concentration of any fatty acid in plasma, compared to the solid.

# The Effect of ALA on the Postprandial Lipemic Response

The effect of ALA on the postprandial lipid metabolism is not well known. Most studies have been conducted on healthy, young, men [72-76]. However, Lonzano et al. [51] included overweight men, and West et al. [77] investigated the effect on men and women suffering from type-2 diabetes. Garaiova et al. [78] and Lichtenstein et al. [79] also included both genders. To the best of the author's knowledge, the study presented in **Paper III** is the only one that focuses on the postprandial lipemic response of ALA in premenopausal women.

## *Plasma Triacylglycerols and NEFA*

The postprandial lipemic response was investigated in 26 men and 19 women after three breakfasts containing: ALA-rich oil (11.5 g ALA, 31 E% of test fat), olive oil, or butter (**Papers II and III**). The meal was consumed after overnight fasting, and blood samples were withdrawn 0, 1, 3, 5, and 7 h after the meal. The test fat accounted for 35 g of the total amount of fat in the meals (47 g), which corresponds to 0.56 and 0.75 g fat/kg body weight in men and women, respectively, assuming an average body weight of 86 kg for men and 64 kg for women. The results showed that the p-TAG and p-NEFA response after the ALA-rich meal were lower than those to olive oil, but higher than those to butter, but neither of these differences was of statistical significance. Moreover, no significant differences in total p-TAG or total p-NEFA were seen between the genders after the ALA-rich meal (Fig. 3 A and B).

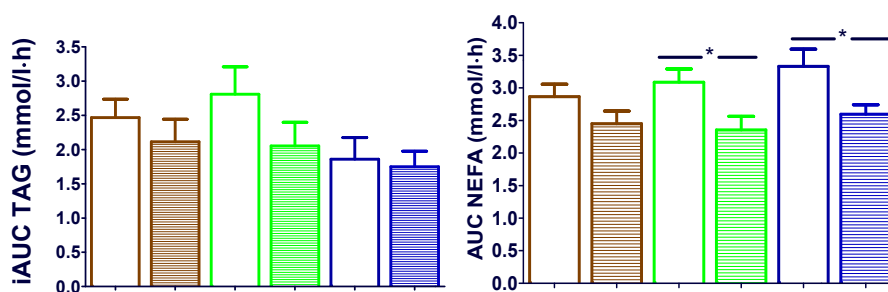


Figure 3. A) Postprandial change in plasma TAG iAUC (0-7 h, mmol/l·h, CI 95%) after ALA-rich oil (brown), olive oil (green), and butter (blue), in men (empty bars) and women (lined bars). \*Men: iAUC after olive oil significantly different from butter. B) Postprandial NEFA AUC (0-7 h, mmol/l·h, CI 95%). Women showed lower AUC NEFA after olive oil and butter than the men.

Lonzano et al. [51] investigated the postprandial p-TAG response in 10 normal and 11 overweight men after consuming a meal containing walnuts (4 E% ALA), butter, or olive oil (1 g fat/kg body weight). Before the tests, the subjects were required to adopt a baseline diet rich in saturated fats for four weeks. The different meals did not elicit any significant differences in p-TAG response or large TRL-TAG. However, the overweight subjects showed higher concentrations of small TRL-cholesterol and small TRL-TAG after the walnut and butter meals, than after the olive oil meal. Their overall conclusion was that overweight subjects exhibited a higher postprandial lipid response than normal subjects, and that the olive oil meal was favorable because of the higher concentration of large TRL particles. Non-significant differences in p-TAG and large TRL-TAG response were also seen in a study of 8 men by Bellido et al. [74]. Basically the same baseline diet, meals, and amounts of fat were used. After ingestion of the walnut meal the ALA content in large TRL-TAGs increased from 0.55 (0 h) to 3.91 (3 h).

Perez-Martinez et al. [60] and Fuentes et al. [75] studied the effects of meals containing walnuts, butter and olive oil (1 g fat/kg body weight), but before the trial the subjects (20 men in each) had followed three different diets with the corresponding fat composition for 4 weeks. The results from both these studies showed that the postprandial p-TAG concentration after the olive oil meal increased quickly and was significantly higher than those observed after the walnut and butter meals after 2 hours. After 6 hours, the p-TAG concentration was lower following the olive oil meal than the other two. The conclusion drawn from both studies was that the total p-TAG response was greater following a meal containing olive oil than the walnut - and butter-containing meals [60, 75].

Jiménez-Gómez et al. [73], who used a similar test protocol, did not see any difference in postprandial total p-TAG level. But the p-TAG response was higher 6 hours after the butter meal, than after the walnut and olive oil meals. After 9 hours, the p-TAG level was lower after olive oil than after the walnut meal. The amount of ALA in plasma fatty acids was increased from 0.41% (0 h) to 1.88% (3 h) after the walnut meal.

The effects of rapeseed, corn, and olive oils on fasting and postprandial plasma lipoproteins have been studied by Lichtenstein et al. [79]. The fifteen subjects (men and women, mean age 61 y) were overweight and had elevated LDL-cholesterol (LDL-C) levels, but were otherwise healthy. The subjects were first placed on a stabilization diet high in SFAs and MUFAs (fat 35.5 E% and carbohydrates 48.1 E%) before being given three different diets in which 66% of the fat content was given as rapeseed, corn or olive oil (fat 29.5 E% and carbohydrates 53.3 E%). A postprandial test was performed during the final day of each diet. All three oil diets induced significantly decreased fasting LDL-C concentrations compared to the stabilization diet; the olive oil diet having the least

LDL-lowering capacity. Fasting p-TAG and postprandial p-TAG concentrations were not significantly affected by the different diets, however, the oil diets tended to cause higher postprandial p-TAG concentrations. The authors suggested that this could be due to the higher carbohydrate load in the oil diets. Their overall conclusion was that significant reductions in LDL-C and apoB concentrations can be achieved when saturated fatty acids are replaced by mono- or polyunsaturated fatty acids [79].

Nielsen et al. [72] investigated the effects of diets rich in rapeseed oil, olive oil, or sunflower-seed oil on postprandial lipemia in eighteen young healthy men. The diets were consumed during three weeks and during the last day of each diet-period a postprandial test was made. The test meal was rich in rapeseed oil. The olive oil diet resulted in significantly higher postprandial chylomicron- and VLDL-TAG concentration than the rapeseed and sunflower diets. Fasting TAG concentrations were higher after the olive oil diet, which may have induced the higher postprandial TAG levels [72].

West et al. [77] investigated the postprandial lipid response in patients with type-2 diabetes (13 men, 5 women) after three MUFA-rich meals, which differed in the amount and type of n-3 fatty acids. The MUFA-rich meal was made up of high-oleic safflower oil and canola oil (MUFA 47 E%). In the other two meals the safflower oil was replaced by either canola oil (ALA meal 3.3 E% ALA) or sardine oil (EPA+DHA meal 6.9 E% EPA+DHA). The p-TAG response after the ALA meal was significantly improved compared to the MUFA meal, especially in the subjects with high fasting p-TAG levels.

Garaiova et al. [78] investigated the effect of pre-emulsification of PUFA-rich oil on the digestion, absorption, and postprandial p-TAG response and fatty acid composition in plasma. In this randomized cross-over study, 23 men and women received a meal containing either 30 ml of the pre-emulsified oil or normal oil. The main fatty acids in the oil were ALA (18.2 mol%), EPA (16.8 mol%), and DHA (11.0 mol%). The postprandial lipemia was greater after the pre-emulsified oil, but disappeared faster. The maximum p-TAG concentration was measured 3 h after ingestion of the pre-emulsified oil and 4½ hours after ingestion of the normal oil. The absorption of EPA, DHA, and ALA was found to be greatly enhanced by pre-emulsification. Interestingly, no difference was observed in the absorption of palmitic, stearic, oleic, and linoleic acid. The authors suggested that the low and delayed p-TAG response and low PUFA incorporation into p-TAG following intake of the normal oil was due to insufficient emulsification of the oil during digestion, and the subsequent decrease in hydrolysis by pancreatic lipase. This would lead to delayed and reduced incorporation of these fatty acids into chylomicrons. They hypothesized that the high EPA and DHA concentrations in the circulation following the pre-emulsified oil influenced lipoprotein lipase posi-

tively, thereby increasing the rate of fatty acid disappearance [78]. Since ALA followed the same pattern as EPA and DHA, it is possible that the proposed explanation also applies to ALA, although the effect is less pronounced.

The mechanism of the p-TAG-lowering effect of EPA and DHA is not fully understood. It is thought that long-chain n-3 PUFAs activate various hepatic transcription factors, resulting in more  $\beta$ -oxidizing enzymes, decreased hepatic-TAG production, and less VLDL production and secretion [80]. The high n-3 PUFA concentration in plasma after the pre-emulsified oil, discussed above [78], may have triggered these hepatic transcription factors, leading to a faster reduction of p-TAG.

### *Conclusions*

The conclusion that can be drawn from previous studies and those carried out as part of this work is that ALA- and MUFA-rich meals lead to similar p-TAG response. It is interesting to note that when significant differences regarding p-TAGs were found, they were in favor of the ALA meal. The number and size of the lipoprotein particles and their clearance rate are also of interest, especially the clearance rate of the remnants since these are considered to be among the most pro-atherogenic, together with small dense LDL particles [33, 38]. Unfortunately, the size, and number of the different lipoprotein particles were not measured in the present studies. In five of the studies discussed above whole walnuts were used in the test meals, and this may have affected the outcome of the studies due to the difference in bioavailability [71]. Moreover, three different test diets, which were consumed for four weeks prior to the postprandial tests, were used in three of the studies [60, 73, 75]. This is a very good approach to eliminating differences between the habitual diets of the subjects. However, two of the diets contained the same amount of fat (38 E%) but of different composition (high SFAs vs. high MUFAs), but the ALA diet was high in carbohydrates and low in fat (<30 E% fat). This may also have affected the results, since it is known that diets high in carbohydrates can increase lipid concentrations [81]. NEFA concentration was only measured in the present studies.

### *Alpha-Linoleic Acid in Plasma*

The fatty acid compositions in total p-lipids, p-TAGs and p-NEFAs were analyzed in men and women up to 7 hours after ingestion of the meal (**Papers II and III**). The concentration of ALA remained elevated in all three plasma pools several hours after the ALA meal in both men and women (Fig. 4). Endogenous sources are low (see Table 4, page 38), thus any increase in ALA originates from dietary sources. Women showed a greater increase in ALA in p-TAG and a lower increase in the NEFA pool than men. Both genders exhibited approximately the same

increase in ALA in total lipids (Table 3). Increases in ALA in total plasma fatty acids and p-TAG has also been reported by others [73-74, 78].

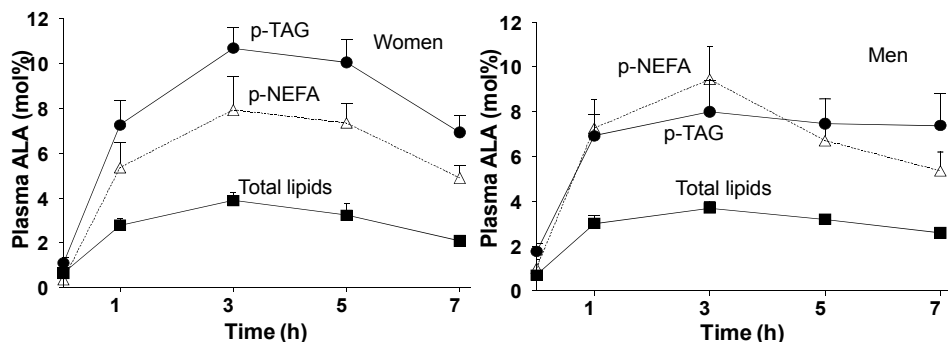


Figure 4. ALA content (mol%) in total p-lipids (-■-), p-TAG (-●-), and p-NEFA (--Δ--) in men (n=10 total lipids, n=8 TAG, n=6 NEFA) and women (n=10) after consumption of the ALA-rich oil (**Papers II and III**).

Table 3. Increase in ALA in different plasma pools, calculated as area under curve (AUC) mol%·h, in men and women (**Papers II and III**).

Plasma pool	Men	Women
ALA AUC (mol%·h)		
<b>Total lipids</b>	21	21
<b>TAG</b>	48	60
<b>NEFA</b>	49	42

### Conclusions

The conclusion drawn from these studies (**Papers II and III**) is that ALA is re-circulated to a great extent in p-TAGs and p-NEFAs. The high concentration and long circulation time of ALA in p-NEFAs is indicative of spill-over NEFAs in the dominant secretion phase and the preferential release of ALA by the adipose tissue into the circulation, as NEFAs, in the clearance phase. Other sources from which dietary ALA may recirculate are lipid droplets from the enterocytes, hepatic TAGs [32, 82], and phospholipids [83] in which ALA has been incorporated but sorted out and then released. No studies have been found in the literature in which circulating ALA has been measured during the postprandial period.



## Metabolic Fates of Alpha-Linolenic Acid

Much of the ALA ingested is subjected to mitochondrial  $\beta$ -oxidation [84]. Burdge et al. showed in their stable isotope studies that approximately 22% and 33% of the ingested ALA was subjected to  $\beta$ -oxidation, over 21 days, in women and men, respectively [85-86]. Carbon recycling into SFAs and MUFAs has been found to be higher in men than in women [13].

Another fate of ALA is to be incorporated into cell membranes and biliary components, particularly phosphatidylcholine [87]. PUFAs are incorporated into phospholipids, which are crucial for cell membrane structure and function, as well as second messenger formation. The long chain PUFA phospholipids regulate the fluidity of the membrane, which is important in ensuring that receptor functions and signaling pathways work efficiently.

Hodson et al. [88] presented an extensive review on fatty acids as biomarkers in plasma and adipose tissue. Some of the results are given in Table 4 and are compared with the results of the present investigations (**Papers II and III**). The present results correspond well with those of Hodson et al. The men and women in the present studies had slightly higher circulating ALA levels in p-TAGs and lower Ln concentration in total lipids than in the study by Hodson et al. [88]. ALA was present in all pools, but only in small amounts since the dietary intake is low.

ALA is also stored in adipose tissue. Burdge and Calder [13] estimated that women and men have ALA reserves corresponding to 70 and 53 days, respectively. This corresponds to an intake of roughly 1.5 g ALA per day. Moreover, Raclot [89] proposed in his review that fatty acids can be selectively mobilized from adipose tissue TAGs. He divided fatty acids into three groups: highly mobilized fatty acids (CN 16-20, double bonds 4-5), weakly mobilized (CN 20-24, double bonds 0-1), and moderately mobilized, including all others. Thus, ALA may be more easily mobilized than Ln. He concluded that the apparent selective storage of ALA in the adipose tissue was probably not only due to systematic mobilization, but also to fatty acid availability, enzyme selectivity, supply of NEFA, and differential oxidation of saturated and unsaturated fatty acids. Thus, if the supply of ALA, EPA, and DHA were to be insufficient, there would still be a way of rapidly mobilizing the amount needed.

Table 4. Fatty acid compositions in various parts of the blood<sup>1,2,3</sup> and adipose tissue<sup>1</sup>. Values adapted from Hodson<sup>1</sup> et al. [88] and from **papers II**<sup>2</sup> and **III**<sup>3</sup>. Numbers are presented as mean values. phospholipids (PL), cholesteryl ester (CE).

<b>FA composition in</b>	<b>16:0 (mol%)</b>	<b>18:1 (mol%)</b>	<b>18:2 (mol%)</b>	<b>18:3 (mol%)</b>	<b>20:5 (mol%)</b>	<b>22:6 (mol%)</b>
<b>Total p-lipids<sup>1</sup></b>	23	19.2	30.4	0.6	1.4	2.4
<b>Total p-lipids<sup>2</sup>♂</b>	19.1	16.1	21.5	0.7	-	-
<b>Total p-lipids<sup>3</sup>♀</b>	26.7	17.6	23.2	0.4	-	-
<b>p-TAG<sup>1</sup></b>	29.5	37.7	15.0	0.9	0.1	0.4
<b>p-TAG<sup>2</sup>♂</b>	25.4	43.3	16.0	1.4	-	-
<b>p-TAG<sup>3</sup>♀</b>	28.5	40.0	14.7	1.2	-	-
<b>p-NEFA<sup>1</sup></b>	28.3	32.7	13.5	0.9	0.1	0.4
<b>p-NEFA<sup>2</sup>♂</b>	27.6	34.5	11.8	1.1	-	-
<b>p-NEFA<sup>3</sup>♀</b>	24.6	30.7	9.0	0.7	-	-
<b>p-PL<sup>1</sup></b>	31.3	10.1	21.9	0.2	1.0	3.3
<b>p-CE<sup>1</sup></b>	13.6	19.3	52	0.6	0.7	0.4
<b>PL in platelets<sup>1</sup></b>	20.8	17.0	6.4	0.1	1.8	2.0
<b>Adipose tissue<sup>1</sup></b>	21.5	43.5	13.9	0.8	0	0.1

<sup>1)</sup> Baseline values average for men and women [88]. Tot FA: n=982, 9 studies; TAG: n=352, 5 studies; NEFA: n=348, 7 studies; PL: n=8119, 16 studies; CE: n=5902, 11 studies, PL in platelets: n=439, 7 studies, and adipose tissue: n=7354, 19 studies.

<sup>2)</sup> Baseline values: men (Paper II). Total: n=10, TAG: n=8, and NEFA: n=6. 20:5 and 22:6 were analyzed but the results were merged with other fatty acids.

<sup>3)</sup> Baseline values: women (Paper III). Total, TAG, and NEFA: n=10. 20:5 and 22:6 were analyzed but the results were merged with other fatty acids.

ALA and Ln are the precursors of the long-chain PUFAs in the n-3 and n-6 families, respectively. The most well-known n-3 family members are EPA, docosapentaenoic acid (DPA, 22:5), and DHA. The corresponding n-6 member is arachidonic acid (AA, 20:4n-6). ALA and Ln compete for the same elongation and desaturation enzymes, and conversion takes place in the ER. The last step of conversion of 24:6 n-3 to 22:6 n-3 takes place in the peroxisomes (Fig. 5) [11, 13, 90].

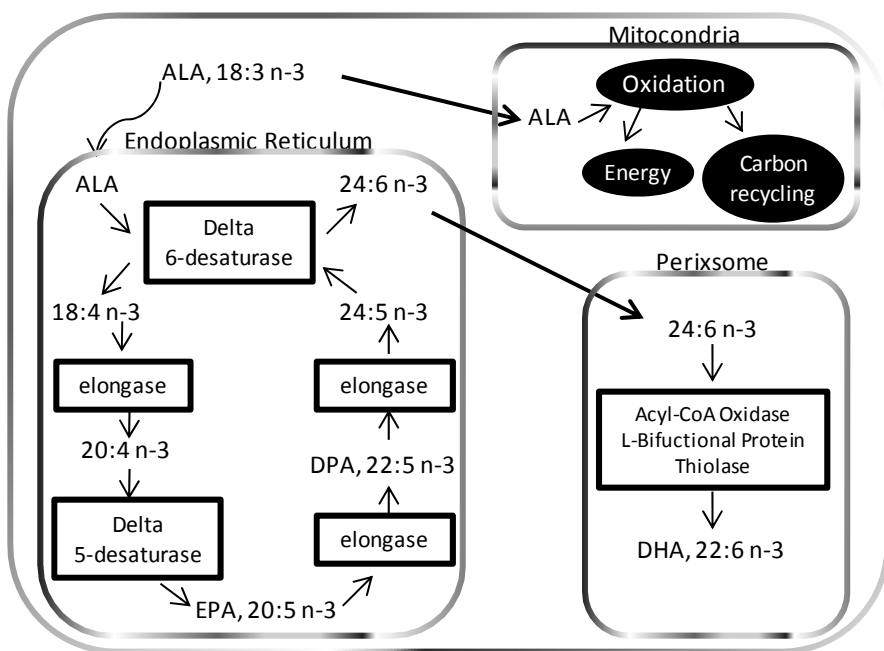


Figure 5. Overview of the elongation and desaturation pathway of ALA and Ln and the mitochondrial oxidation of ALA (adapted from Kitson et al. [91]).

The conversion rate of ALA and the capacity of the human body to sustain adequate amounts of long-chain PUFAs have been discussed intensively [11, 13, 70, 92]. Barceló-Cobijn and Murphy concluded that ALA is capable of maintaining DHA levels, especially in the liver and brain, provided that sufficient amounts of ALA (>1.2 g) are consumed [11]. Based on the amount of ALA estimated to be stored in adipose tissue (see above), there would be sufficient ALA for conversion to DHA to use in the brain for more than a month. Different tissues also have different capacity for converting ALA, for example, the heart does not possess the enzymes needed to elongate and desaturate ALA further than EPA. Conversion of ALA to long-chain PUFAs is positively correlated with increased dietary intake of ALA and negatively correlated with increased EPA, DHA, and Ln intake [11, 13, 93]. However, substrate competition between ALA and Ln, and potential feedback inhibition by EPA and DHA on the elongation-desaturation pathway may be too simplistic to explain the regulation of conversion, since it has been shown that regulation is also modulated at the genetic level [11].

Women of childbearing age exhibit a higher conversion of ALA than men of the same age, due to the action of estrogen [13, 91]. Circulating levels of DHA are also higher in women, especially during pregnancy. Results from the EPIC-

Norfolk study [94], show that women had higher n-3 PUFA levels in circulating plasma phospholipids, despite the fact that men had higher dietary intakes. It was also estimated that the conversion of ALA to EPA and DHA was greater in women and non-fish eaters than in men and fish-eaters, respectively. Geppert et al. [95] found that women had a higher percentage of DHA and lower ARA:EPA, and ARA:DHA ratios in platelet phospholipids than men, despite there being no difference in diets or n-6 and n-3 fatty acid intakes.

It has been argued that a low n-6:n-3 ratio is important in the diet, for example in order to relieve inflammatory states, since n-6 and n-3 fatty acids are precursors of eicosanoids, protectins, and resolvins [96-98]. The diets of today are often high in n-6 fatty acids, and n-6:n-3 ratios as high as 15-16.7 have been reported [98]. However, the estimate based on the results of a Swedish dietary study (*Riksmaten*) in 1998-99 was more modest, giving a ratio of 5 [19]. It has been suggested that humans evolved on a diet with an n-6:n-3 ratio of 1 [98]. Rheumatoid arthritis, inflammatory-bowel-disease [97, 99], atherosclerosis, and obesity are classified as inflammatory diseases [33], and it may thus be beneficial to decrease the n-6:n-3 ratio, for example, by increasing the ALA intake.

The concentrations of ALA and EPA have previously been measured in plasma cholesteryl esters, phospholipids, LDL, and red blood cells. The recently published results of the Alpha Omega trial in the Netherlands [100] showed that an intake of 1.9 g ALA per day significantly increased the content of ALA and EPA in plasma cholesteryl ester. Egert et al. [101] conducted a 6-week dietary intervention study in healthy men and women (mean age 25 y) using a blinded parallel design. The aim was to study the effects of increased ALA, EPA, and DHA consumption on plasma lipids and LDL fatty acid composition. Their results showed that an intake of 4.4 g ALA per day increased the ALA and EPA content, but decreased the AA content significantly in LDL, and as a result of this, the n-6:n-3 ratio in LDL was decreased. Lichtenstein et al. [79] found a threefold increase in ALA in plasma after the consumption of diets enriched in rapeseed oil, but no change in EPA or DHA was seen. Moreover, the n-6:n-3 ratio decreased from 33 at baseline, to 26 and 31 after the rapeseed and olive oil diets, and increased to 41 after the corn oil diet. The study by Lichtenstein thus showed that corn oil, which is high in Ln n-6, increased the n-6:n-3 ratio.

In a recent study [102], it was concluded that the consumption of food products from linseed-fed animals and linseed-enriched bread maintained the n-3 level in red blood cells in obese humans. Moreover, the ALA concentration was increased and the n-6:n-3 ratio decreased in the experimental group (low PUFA:SFA and low n-6:n-3 diet) compared to the control group (higher PUFA/SFA and high n-6:n-3 diet). The ingestion of 40 g ground flaxseed (8.8 g ALA) per day for a year increased the ALA, DPA, and total n-3 PUFA content in plasma, and lowered the

n-6:n-3 ratio significantly, compared with wheat germ placebo in postmenopausal healthy women [103]. Finnegan et al. [104] showed that supplementation of the diet with 4.5 g and 9.5 g ALA per day increased the ALA content in plasma phospholipids significantly. The EPA content also increased significantly, but only at the higher ALA dose.

### *Conclusions*

Alpha-linolenic acid has essential and diverse functions in the body. Apart from being used as an energy source, humans store enough ALA to sustain brain levels of DHA for more than a month. It has also been suggested that ALA can be selectively mobilized from the adipose tissue when there is a need for ALA elsewhere. Furthermore, ALA is stored and/or converted into long-chain PUFAs in women, rather than being subjected to  $\beta$ -oxidation, maybe because of the importance of ALA for fetal development.

However, circulating p-ALA is low in both men and women, mainly because of low intake, but also due to selective clearance and/or  $\beta$ -oxidation. The subjects in the present studies had higher fasting ALA concentrations in plasma than those in the study by Hodson et al. [88] (Table 4), and the recirculation of ALA in p-TAGs and p-NEFAs was high in the subjects investigated by the author. This could imply that the subjects in the present studies already had a good ALA status and fast clearance of ALA was not prioritized.

It is evident that ALA is converted into long-chain PUFAs, which may provide sufficient levels in healthy individuals. However, a substantial intake of DHA from marine sources seems to be needed to reduce the risk of CVD [12, 105]. And if I may take a more philosophical standpoint, was this perfectly regulated elongation – desaturation pathway really intended to prevent CVD? EPA and DHA are important for the growing fetus and the newborn, in cell-signaling and as precursors for the production of anti-inflammatory lipid mediators [11, 13, 70, 96, 106]. Is it not then possible that the elongation-desaturation pathway has the capacity to maintain lipid homeostasis by converting enough ALA when dietary long chain PUFA is scarce, and to assure health for the off-spring? This pathway was probably not evolved to prevent CVD, which partly results from suboptimal diets and insufficient physical exercise.

## Long-Term Effects of ALA Intake

A randomized controlled trial conducted by researchers at Uppsala University, Sweden (NORDIET) [107], showed that a Nordic diet (total n-3 PUFA 5-10 E%), which is rich in high-fiber plant foods, fruit, berries, legumes, rapeseed oil (ALA), nuts, fish, vegetables, and whole grain products, improved the lipid profile significantly in mildly hypercholesterolemic subjects. The concentration of LDL-C decreased by 21% and the LDL:HDL ratio decreased by 14% compared with controls, but no change in fasting p-TAG was observed. The subjects also showed a significant weight loss (-4%) compared to the controls, although the diet was prescribed *ad libitum*.

Egert et al. [101] showed that the intake of 4.4 g ALA per day for 6 weeks decreased the fasting p-TAG level significantly (-0.14 mmol/l), but not the VLDL cholesterol. This was in contrast to the EPA and DHA interventions in the same study, which caused a decrease in both parameters. Unfortunately, there was no control group in this study. The authors suggested that the lipid-lowering mechanism of ALA may differ from that of EPA and DHA. Finnegan et al. [104] observed no decrease in fasting p-TAG concentrations after supplementation of 4.5 or 9.5 g of ALA per day for 6 months, in moderately hyperlipidemic subjects (men and women, mean age 55). There is little evidence that ALA supplementation significantly affects blood lipids, LDL, HDL, or inflammatory markers (based on reviews covering 1983-2004 and 2008-2010, and the OPTILIP study [12-13, 108]).

It is also clear that ALA is not as potent as EPA and DHA in lowering the levels of risk markers for CVD [12, 105]. The multi-center, double-blind randomized Alpha-Omega trial [100] showed that dietary supplementation with EPA+DHA (0.40 g/day) and/or ALA (1.9 g/day) did not reduce the occurrence of major cardiovascular events in 4837 patients with a previous myocardial infarction, who were receiving good clinical care. However, a 27% reduction in cardiovascular events was observed in women following ALA supplementation ( $P=0.07$ ).

In contrast, a case-control study ( $n=1819$  subjects) in Costa Rica showed that a higher ALA consumption (assessed either in adipose tissue or using a food questionnaire) was associated with a lower risk of myocardial infarction [109]. Data from the multi-center NHLBI Family Heart Study in the USA ( $n>4500$  subjects) suggest that dietary ALA is associated with lower prevalent hypertension and lower systolic blood pressure in white subjects [110]. The NHLBI Family Heart Study also showed that dietary ALA intake was inversely related to plasma TAG concentration [111].

### *Conclusions*

Alpha-linolenic acid certainly has a place in a balanced diet, such as the NORDIET [107], although the evidence is weaker than for EPA and DHA regarding protection against CVD and improvement in blood lipids. A chronically low intake of ALA may have negative effects on health, and a cumulative negative health effect cannot be ruled out. Randomized controlled trials, investigating the role of ALA and its potential health-promoting effects in healthy, slightly overweight subjects in the age group 30 to 50 are warranted.

# Modification of Triacylglycerols using Enzymes

The first chapters of this thesis were devoted to the nutritional and metabolic aspects of fatty acids and TAGs, and we now turn to the chemical and biotechnical aspects. As described in previous chapters, lipases are an important part of the lipid metabolism. However, lipases (EC 3.1.1.3, members of the hydrolases) are also interesting as industrial biocatalysts because of their versatility and capacity to function in organic solvents [112]. In this chapter the enzyme preparation Lipozyme TL IM, its lipase from *Thermomyces lanuginosus* (TLL), and the most common reactions catalyzed by this lipase will be presented. Enzymatic interesterification (EIE) was the subject of investigation in **Papers I and IV**, and was used to produce the ALA-rich oil used in the meal studies presented in **Papers II and III**. The rest of the chapter is devoted to a discussion of the different factors concerning EIE.

## Industrial Processing of Fats and Oils

Margarine, baking fat, biscuit fillings and confectionary fats are produced in industrial processes affording them the desired characteristics in terms of melting point, crystallization pattern, taste, structure, and nutritional composition. Simply mixing fats and oils is often not sufficient to achieve the desired properties. Hence, other processes are needed, such as fractionation, hydrogenation, and interesterification.

Fractionation separates oils and fats into two or more components, depending on their solubility and melting point. The intention is to remove minor components, enrich certain TAGs, and increase the economic and nutritional value, and can be an alternative to hydrogenation [113-114]. Hydrogenation removes the double bonds in the fatty acids (totally or partially) in order to obtain fats with higher melting points. The reaction is carried out with a nickel catalyst at temperatures of 180-200 °C and a pressure of 3 bar. Partial hydrogenation produces *trans*-fatty acids [114]. Chemical interesterification (CIE) is catalyzed by sodium methoxide at 80-90°C for 30 to 60 min in a batch reactor. It completely randomizes the fatty



acids in the oil/fat, and products with different physical properties can be obtained depending on the starting materials [114].

The harsh reaction conditions mean that neither hydrogenation nor CIE is suitable for oils containing Ln (18:2), ALA (18:3), EPA (20:5) or DHA (22:6), because of their easily oxidizable double bonds [115]. This, together with health and legislation issues concerning *trans*-fatty acids [116], and the possibility of making new lipid products, makes the enzymatic approach of interest.

In industry, EIE is run continuously in packed-bed reactors (PBRs) coupled in series [117]. The processes of CIE and EIE are compared in Fig. 6. Both CIE and EIE start with pretreatment (washing, drying, and absorption of contaminants of the oil, depending on the source and purity. The stability and lifetime of the catalysts are enhanced if the oil is pure, i.e. low in oxidation products, FFAs, and residual acids [114, 117-119]. After EIE the FFAs are removed by deodorization. It is necessary in the case of butter to remove butyric acid [120]. More byproducts are produced during CIE, and the oil may be discolored. Major downstream processing is needed in order to obtain the final product. This requires chemicals, water, high temperatures, and more equipment [117].

EIE has several advantages [117], which include lower environmental impact and investment costs for equipment, less downstream processing, higher yields because of less byproduct formation, and a better working environment. Life-cycle assessment has shown that the major savings are in energy consumption and reduced loss of substrate per ton of final product. The process is also more flexible and faster than CIE since it is run continuously. The advantages stem from the fact that EIE is a simpler process (Fig. 6) and requires less handling of the catalyst [121]. The disadvantages have been the high cost and low stability of the enzymes. However, these have now been solved. It has already been shown that the products of EIE are at least as good as those obtained with CIE; however the future aim is to produce superior products. The EIE process was first introduced in Sweden by AAK Sweden in 2001, and ADM was the first company in the USA (2002) [117].

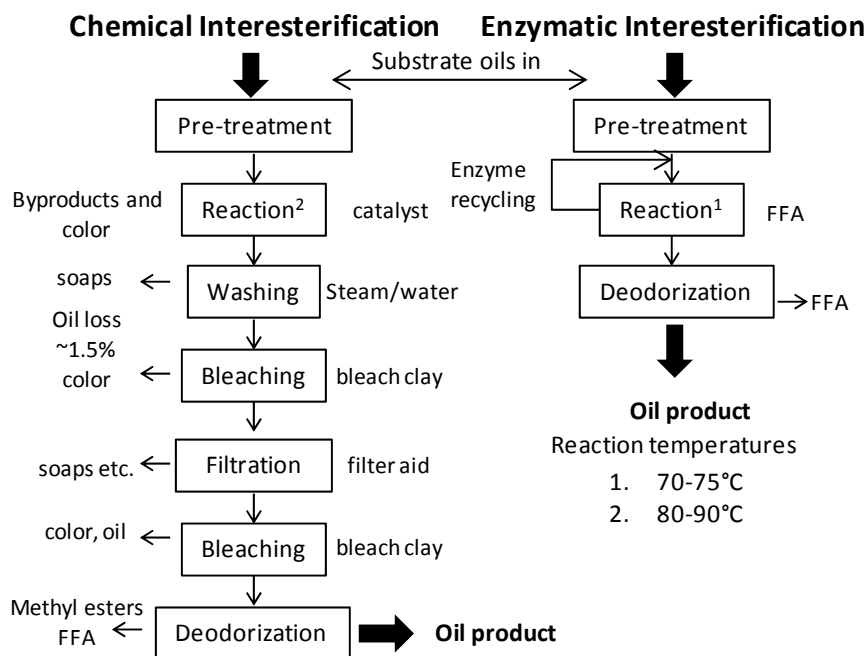


Figure 6. Schematic picture of CIE and EIE. The EIE requires fewer steps to reach the finished product. CIE produces more byproducts, such as DAGs, FFA, esters, and dialkylketones, whereas EIE only produces FFA. Picture adapted from Holm et al[117].

## Lipases

The *sn*-1,3-specific lipase from the fungus *Thermomyces lanuginosus* (formerly called *Humicola lanuginosa*) was used in all the studies described in this thesis. The commercial name is Lipolase<sup>TM</sup> (Novozymes A/S, Bagsvaerd, Denmark) and it is produced by a genetically modified *Aspergillus sp.* The main application is in detergents. Its molecular weight is 31700 g/mol and pH range 7-11. Its optimal working temperature is around 35-40°C [122]. However, in its immobilized form it has an operating range of 55 to 85°C [123]. It has a spherical shape with a mobile surface loop, which protects the active site. Upon contact with a substrate and/or placement in a hydrophobic environment, large conformational changes occur and the loop moves to reveal the catalytic triad (serine-histidine-aspartate). This interfacial activation is a special feature of lipases, but not all lipases have this characteristic (e.g. *Candida antarctica* lipase B), and the lid can be different in both appearance and size [124-125].

The catalytic mechanism of lipases is often referred to as the ping-pong bi-bi mechanism (Fig. 7). The substrate, also called the acyl donor (an ester or an acid) binds to the active site (acylation). The hydroxyl group of the serine attacks the carbonyl carbon of the ester and forms an ester linkage between the lipase and the acyl group of the substrate. During this stage, the histidine residue is used to transfer the proton from the hydroxyl group of the serine to the leaving group of the substrate. The histidine is now protonated and stabilized by the aspartic acid. Further stabilization is provided by the oxyanion hole, which interacts with the negatively charged oxygen through hydrogen bonding. Alcohol, or water, is then immediately released from the substrate. Hence, an acyl-enzyme is formed. The second stage is the deacylation step, and is the opposite of the first. The second substrate, the acyl acceptor, enters the active site, attacks the acyl-enzyme and the product is released [126-127].

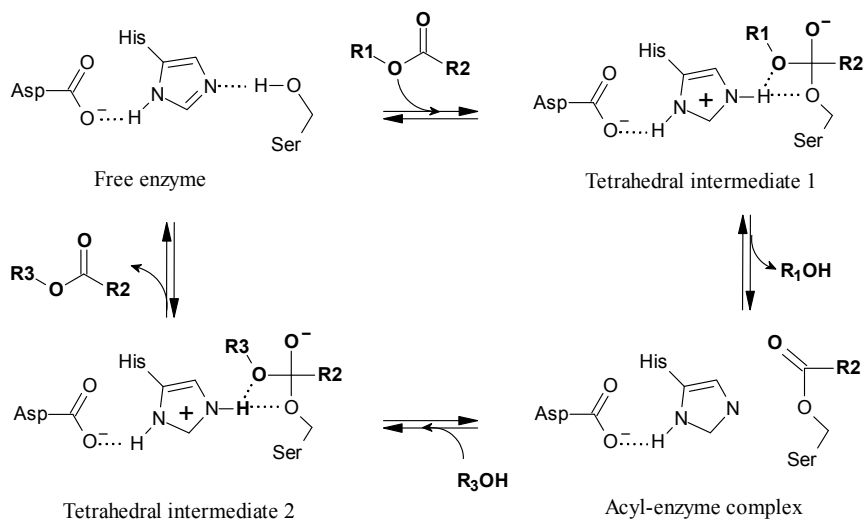


Figure 7. The catalytic mechanism of lipases (Adpated from Hagström [126]).

## Lipase Catalyzed Reactions

Various reactions catalyzed by lipases, such as acidolysis, interesterification, alcoholysis, transesterification, esterification, and hydrolysis, are described below. The catalytic mechanism described in Fig. 7 is applicable to all the reactions below.

### *Acidolysis*

Acidolysis is the replacement between a fatty acid ( $FA_1$ ) in a TAG and a free fatty acid ( $FFA_2$ ) [128-134]. This approach is often used to produce structured lipids, e.g. TAGs with two MCFAs in the *sn*-1 and 3 positions, and a LCFA in the *sn*-2 position. A TAG reacts with the lipase and forms an acyl-enzyme ( $E-FA_1$ ) and releases a di-acylglycerol (DAG). Another lipase molecule then forms an acyl-enzyme with  $FFA_2$  ( $E-FFA_2$ ), and finally the DAG deacylates  $E-FFA_2$ , forming a new TAG. The reaction is reversible and the yield is governed by the equilibrium constant. The substrate ratio ( $FFA:TAG$ ) is thus important, and ratios from 3 to 6 are often used [128-129, 131, 135-137]. Other important factors are the reaction temperature and time, enzyme load, water content and reactor system [138].

### *Enzymatic Interesterification and Side Reactions*

Intesterification, in this case EIE, is fatty acid replacement between esters. Examples of applications in which EIE has been used are the production of margarine and bulk fat, human milk fat substitute, and confectionary fats [76, 118, 120, 123, 139-145]. There are several reasons for using EIE: to obtain the desired physical properties, to avoid phase separation of the blend, to enable the use of less expensive fat, to minimize the susceptibility of PUFAs to oxidation, and to enhance the nutritional value. Interesterification can be used to produce new TAGs, not present in the substrate oils [139, 146]. The reaction scheme for an EIE reaction and possible side reactions (hydrolysis, acyl migration and potential imperfect regioselectivity of lipase) are depicted in Fig. 8. The products are formed when fatty acids are exchanged, mainly in the *sn*-1,3 positions, between TAGs. The intermediate TAG-enzyme complex releases a DAG and forms an acyl-enzyme intermediate containing a fatty acid. The acyl-enzyme then reacts with another DAG forming a new TAG. The reaction will eventually reach steady state.

Hydrolysis and acyl migration are side reactions that occur during EIE and acidolysis. When water is present in the system the hydrolytic activity of the lipase is improved, and the acyl-enzyme can then react with water forming a FFA

(hydrolysis product). A small degree of hydrolysis is important initially to produce DAGs, which facilitate the formation of new TAGs, but uncontrolled hydrolysis will decrease the TAG yield.

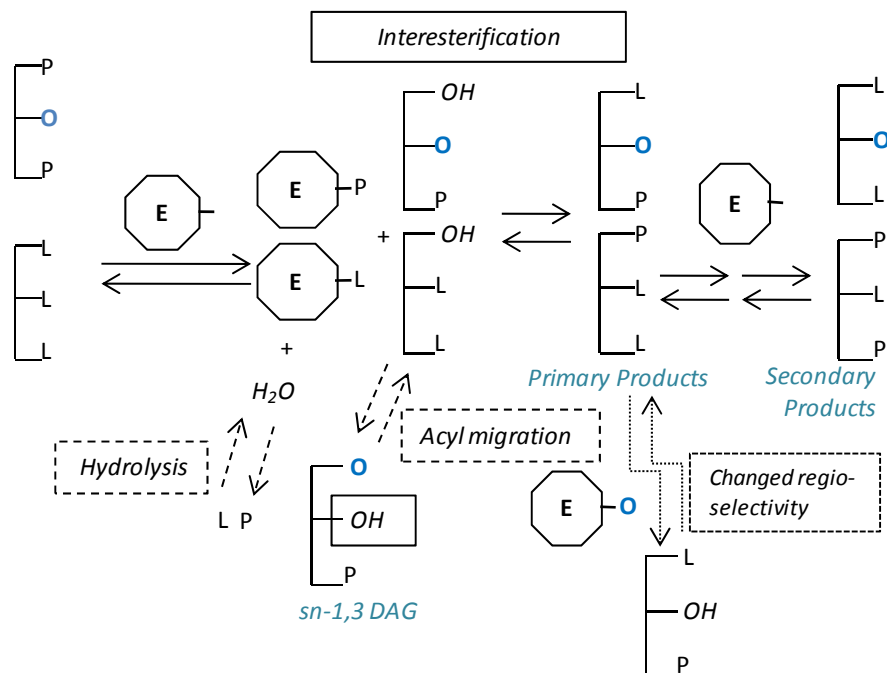


Figure 8. Schematic of the interesterification reaction between trilaurin and POP catalyzed by Lipozyme TL IM. The side reactions, hydrolysis and acyl migration, and the potential change in regioselectivity of the lipase are also shown. P, O, L, and OH denote palmitic, oleic, and lauric acid, and a hydroxyl group.

Acyl migration is a non-enzymatic reaction that takes place in the relatively unstable *sn*-1,2 (2,3)-DAGs [147]. If the aim is to obtain products originating from *sn*-1,3-specific interesterification, this intra-molecular transfer, of one fatty acid moiety from one hydroxyl group to an adjacent one, must be minimized. Acyl migration destroys the positional specificity of the TAG and lowers the yield. The resulting *sn*-1,3 DAGs can undergo hydrolysis leading to the formation of FFAs and MAGs. The mechanism is depicted in Fig. 9. Acyl migration is often a problem in selective lipid modification and different strategies can be used to suppress or enhance it. This will be discussed later in relation to EIE.

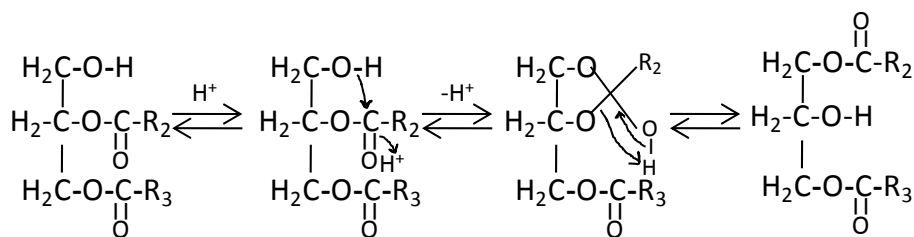


Figure 9. Mechanism for acid-catalyzed acyl migration [148]. The carbonyl carbon becomes more electrophilic in the presence of the proton. The hydroxyl oxygen in the *sn*-1(3) position attacks the carbonyl carbon, and an intermediate is formed. The intermediate hydroxyl oxygen attacks the carbon, the strained intermediate is opened, and the FA moiety moves to the *sn*-1(3) position. Acyl migration from *sn*-2 to *sn*-1 is favored because the primary hydroxyl oxygen is a better nucleophile than the secondary, and the resulting *sn*-1,3 DAG has a lower free energy than the *sn*-1,2(2,3) DAG [147].

## Alcoholysis and Transesterification

Alcoholysis can be used in the production of specialty oils and MAGs [149-151]. It is the reaction in which an ester and a free alcohol react to form a new ester. The ester and the lipase react, and the alcohol part is released. The acyl-enzyme containing the acid part reacts with the free alcohol and forms a new ester. When the ester substrate is a TAG, the reaction is often denoted transesterification, and is often used in biodiesel production [149, 152-154].

When reviewing the literature I have found that many, including myself, have had difficulties in knowing whether to use “interesterification” or “transesterification”. However, in my opinion, “interesterification” should be used when both substrates and products are esters. That is, when the fatty acid moiety is replaced but the resulting molecule (or part of the molecule) is still an ester. An example of this is EIE between two oils. “Transesterification” should be used when the ester bond is “transferred”, so that the substrate ester becomes an alcohol and the substrate alcohol becomes an ester. Biodiesel production is an example, where the lipase catalyzes the reaction between soybean oil and methanol, resulting in partial acylglycerols, glycerol, and fatty acid methyl esters.

## Esterification and Hydrolysis

Esterification [155] (reversed hydrolysis) starts with the formation of an acyl-enzyme of the fatty acid and the release of water. The alcohol reacts with the acyl enzyme and an ester is formed. Lipase-catalyzed hydrolysis is used to produce FFAs and partial acylglycerols from TAGs and water. Two examples are the use of pancreatic lipase to produce MAGs for *sn*-2 positional analysis [156], and the

hydrolysis of squid- and fish oil to evaluate the fatty acid selectivity of lipases [157].

## Properties of Lipozyme TL IM

The EIE described in the papers was catalyzed by Lipozyme TL IM, which contains TLL. It is immobilized on granulates of porous silica and is insoluble in oil [158]. Lipozyme TL IM is a food grade preparation and is GRAS (Generally Recognized As Safe), kosher, and halal certified [159]. Advantages of this preparation are that there is no need to add water to the reaction mixture (since the hydrophilic nature of the carrier ensures sufficient hydration of the enzyme [123]), it is suitable for PBRs [117-118, 142, 160-161], it is stable at the working temperatures of EIE, and is reasonably priced [117]. The preparation is currently used in industry to produce bulk fat for margarine production and other fat shortenings [117]. An extensive review of Lipozyme TL IM, its uses and prospects, has been presented by Fernandez-Lafuente [125].

The fatty acid specificity of TLL and Lipozyme TL IM has been investigated. Rønne et al. investigated the TAG/FA selectivity of Lipozyme TI IM in EIE reactions between two mono-TAGs in hexane. Tristearin was used as the reference, and a series of saturated (4:0, 8:0 to 20:0) and unsaturated (18:1 to 18:3) mono-TAGs were investigated. The results were evaluated by both the initial rate and the competitive factor,  $\alpha$ , which describe the whole reaction. The results indicated that Lipozyme TL IM was non-selective towards the fatty acids tested in this system [162].

Karabulut et al. carried out two extensive investigations concerning the fatty acid selectivity of lipases during acidolysis in hexane [128-129]. The reaction system consisted of triolein with individual saturated fatty acids (6:0-22:0) or a mixture of equimolar amounts of the fatty acids. The effect of reaction temperature, time, enzyme load, and substrate molar ratio on the percent saturated fatty acids incorporated into TAG was investigated. Lipozyme TL IM was found to have higher selectivity towards fatty acids 12:0 to 16:0 when tested individually, and towards 8:0 to 12:0 when tested in a mixture. In another system the incorporation of oleic acid into mono-TAG (6:0 to 18:0) was investigated. The highest incorporation was obtained in tricaproin and tristearin.

The fatty acid specificity of TLL towards EPA and DHA has been investigated by carrying out ethanolysis of squid oil. TLL was immobilized on MP1000 (macroporous polypropylene), which is very hydrophobic. The results were evaluated by calculating a competitive factor. TLL was found to have very low specificity towards DHA, but higher towards EPA [151]. Similar results regarding

low specificity towards PUFA have been obtained by Nascimento et al. [140] and Yang et al. [163]. It can thus be concluded that the specificity, if any, is highly dependent on the reaction system and the method of evaluation used. However, it appears that DHA is a poor substrate for Lipozyme TL IM. If the fatty acid selectivity in a system is important, then studies of that particular system are warranted.

## Factors Affecting the Outcome of Enzymatic Interesterification

In an ideal *sn*-1,3-specific lipase-catalyzed interesterification reaction, the fatty acid composition of the *sn*-2 position remains constant. In practice, small changes in the *sn*-2-position are observed and, under certain conditions, a completely randomized fatty acid distribution can be obtained. Interesterification, hydrolysis, and randomization occur in the reaction system, but at different times and rates. However, increased understanding of the reactions and the factors affecting them, such as the enzyme preparation, the presence of water, reaction time and temperature, substrate composition, and the type of reactor, can turn difficulties into opportunities. There is a potential to produce different TAG mixtures: i.e. products originating from *sn*-1,3-specific interesterification, as well as totally or partially randomized products.

### *Interesterification and Hydrolysis*

A typical EIE reaction starts with a rapid decrease in the main TAG species and an increase in primary products (one fatty acid exchanged, interesterification products), DAG, and FFA (hydrolysis products). Since the lipase removes and replaces one fatty acid at a time, the secondary products (two fatty acids replaced) are formed at a lower rate. A typical substrate/product distribution during EIE of rapeseed and linseed oil is shown in Fig. 10.



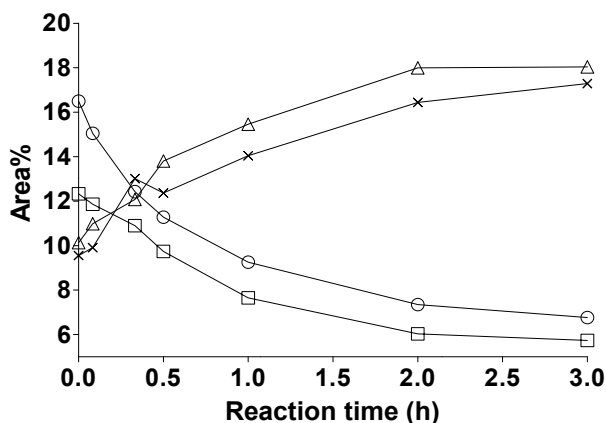


Figure 10. The reduction in substrates and increase in products, in area%, with reaction time during EIE. Rapeseed and linseed oil, Lipozyme TL IM 8 wt% (**Paper II**). Substrates: 18:1+18:1+18:1 (○-), 18:3+18:3+18:3 (□-). Products: 18:1+18:1+18:3 (Δ-), 18:3+18:3+18:1 (×-). Analyzed with HPLC-ELSD.

EIE and hydrolysis occur simultaneously until steady state is reached, but at different rates depending on the enzyme load, i.e. the amount of enzyme preparation and the water content in the reaction mixture, as well as in the preparation [119, 123, 164]. The enzyme load is an important factor, and steady state will be reached faster with a higher enzyme load. In batch experiments (small- and large-scale), the enzyme load is often around 5-10 wt% in order to achieve reasonable reaction times (2-6 h).

The results presented in **Paper IV** show that the reaction with water activity of 0.35 was the fastest, with regard to both substrate consumption and product formation, while a water activity above 0.64 led to slower reactions. The decrease in product formation at high water activity was mainly due to increased hydrolysis. The degree of interesterification and hydrolysis was dependent on the amount of water in the system, and the differences were visible already after 10 min. The increased byproduct formation seen with increased water content/activity and/or enzyme load has also been seen by others [119, 123, 141]. However, an initially low degree of hydrolysis produces DAGs, which can facilitate the formation of new TAGs. On the other hand, uncontrolled hydrolysis will lower the yield and result in poorer product quality, due to a high concentration of partial acylglycerols.

High FFA concentration is associated with a risk of bad taste and smell, and increased susceptibility to oxidation [119-120]. The amount of FFAs in butter must be below 0.6% [120]. The oil/fat substrate is often dry, but Lipozyme TL IM contains a considerable amount of water (3.8% in the batch from 2010 and 5.8% in the batch from 2008). Although water is important in retaining enzyme activity,

much of the water in Lipozyme TL IM can be removed without lowering the activity [123]. The water content in the preparation can thus be decreased by running a short pre-reaction in order to dehydrate it. After the pre-reaction, the enzyme preparation is rinsed with the substrate oil and can then be used in the main reaction. Only short reaction times are needed since hydrolysis is initially very rapid. This approach was used by Zhang [119] and in the present work (**Papers II and III**), where rapeseed and linseed oil were interesterified at 60°C.

The effect of the pre-reaction to decrease the FFA content in reaction mixtures is depicted in Fig. 11A and B. Rapeseed and linseed oil were used in all the reactions, which were run at 60°C. Fig. 11A shows the results from a small-scale pilot study (3 g, with shaking), and Fig. 11B the results from the 1-kg stirred batch reactor (impeller stirring, nitrogen flow, **Paper II**). The FFA concentration increased sharply during the first 30 minutes of the reaction with fresh Lipozyme TL IM, i.e. the 4-h reaction, and then reached a high, stable level. The pre-reaction was stopped after 30 minutes and the products were removed. New substrate was added and the reaction was allowed to continue for 3 h. The liberation of fatty acids was much slower and reached a lower concentration (approx. half). Towards the end of the large-scale reactions the FFAs decreased (Fig. 6B). This was probably due to the bubbling of nitrogen gas through the reactor, which dried the oil. The decrease in water content shifted the equilibrium towards esterification instead of hydrolysis [138]. The pre-reaction approach can also be used in PBRs. Oil is pumped through the bed and the product is collected when the FFA concentration is low and stable [18, 118, 161].

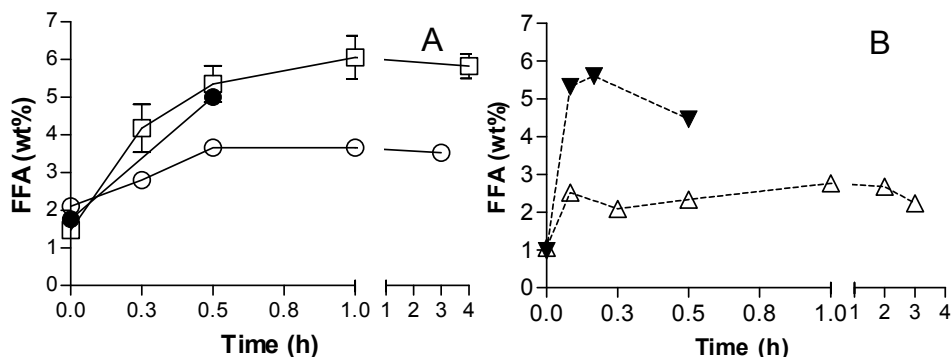


Figure 11. The effect of the pre-reaction to decrease the FFA content in reaction mixtures. A) Small-scale test with 3 g substrate: (□) 4-h reaction: 8 wt% of fresh Lipozyme TL IM. (●) Pre-reaction: 8 wt% of fresh Lipozyme TI IM. (○) Main reaction: The products from the pre-reaction were removed, and more oil (3 g) was added. B) 1-kg scale: (▼) Pre-reaction: ~445 g of oil, 17 wt% of fresh Lipozyme TL IM. (△) Main reaction: the products from the pre-reaction were removed, and ~995 g of substrate was added. Enzyme load: 8 wt%.

## Randomization in the *sn*-2 Position

Although the lipase from *Thermomyces lanuginosus* is *sn*-1,3-specific, changes occur in the *sn*-2 position during EIE. Immobilization procedures and the reaction conditions might change the specificity to a certain extent [165-166], but the most probable reason for the changes in FA composition in this system, is acyl migration. This was studied (**Papers I, II and IV**) and randomization of the *sn*-2 position was observed in all three reaction systems. In the final study (**Paper IV**) it was concluded that EIE was faster than randomization.

The total FA composition and the *sn*-2 composition were analyzed in the substrate and product (**Paper II**). The degree of randomization (**Eq. 2, Paper I**) was estimated, and the results are given in Table 5. The pre-reaction product exhibited a lower degree of randomization, despite the fact that the reaction was run with more enzyme (17 vs. 8 wt%). A high degree of hydrolysis results in a higher amount of DAG, which can undergo acyl migration. However, in this system, the reaction time was more important in causing randomization than the enzyme load or water content. The importance of optimizing the reaction time in order to control the randomization in the *sn*-2 position was demonstrated in **Paper IV**, and also by Zhang [119], Lee et al. [145], and Oh et al. [132].

Table 5. Randomization of FA in 1-kg batch reactor. Pre-reaction: enzyme load 17 wt%, 30 min. Main reaction: 8 wt%, 3 h. For further reaction conditions see Fig. 11.

FA	Randomization (%)	
	Pre-reaction	Main reaction
C 16:0	19	42
C 18:1	24	37
C 18:2	13	32
C 18:3	29	37

Factors that can enhance acyl migration are the support of the enzyme, in this case silica gel [147, 154], the use of hexane as solvent [131, 146, 149], and increased reaction temperatures [136, 145, 147]. A PBR rather than a batch reactor should be used if acyl migration is to be minimized, since the reaction times are much shorter [118, 120, 160-161]. It has previously been shown that the degree of randomization in a reaction between corn oil and tristearin could be lowered by adding molecular sieves [141]. This reduction was probably due to lower DAG formation resulting from the decreased water content.

## *Substrate*

The substrates used, and the ratio between them are of major importance in obtaining the optimal product. The purity of the oil/fat/FA mixture (i.e. no byproducts such as acids, partial acylglycerols, FFAs, or other impurities) is of crucial importance both for the stability of the lipase and for the reaction. Impure substrates will give impure products, with an increased need for downstream processing. The ratio of the ingoing substrates and the fatty acid composition dictate the resulting TAG product and its physical properties, such as solid fat content [161, 167]. Substrate mixtures containing high amounts of stearic and palmitic acids will result in harder fats than mixtures containing more MUFAs and PUFAs. Many different oils, fats and TAG mixtures have been used in EIE trials, e.g. palm stearin + coconut oil, rapeseed oil + butter, fully hydrogenated soybean oil (FHSO) + olive oil, sesame oil + FHSO, FH canola + olive oil, fish oil + MCT oil, sunflower, palm kernel, and rapeseed oil [118-120, 123, 161, 167-168]. In the present studies, rapeseed oil + butter, rapeseed oil + linseed oil and trilaurin + 1,3-palmitin-2-olein mixtures were used. The only limits on the mixtures of oils are the imagination and patents.

The rapeseed oil + linseed oil mixtures were used to produce the ALA-rich oil used in the meal studies (**Papers II and III**). The ALA oil had almost equal amounts of oleic acid and ALA, a combination seldom occurring in natural oils. The reason for using EIE instead of just blending the oils was to obtain a more homogeneous product, and to distribute the ALA more evenly between the TAGs. The risk of undesirable oxidation can increase if more than one PUFA is present in lipid molecules [169]. Also, since the two studies were conducted one year apart, it was important to assure that the products were the same. The correct fatty acid and TAG compositions were achieved by changing the ratio of the substrates linseed oil/rapeseed oil from 2:1 (**Paper II**) to 1.3:1 (**Paper III**). Just mixing the oils would have resulted in dissimilar TAG compositions, which may have affected the postprandial response.

## *Temperature*

Efficient interesterification relies on the mass transfer to and from the lipase. The reaction temperature must be above the melting point of the substrates, in order to ensure completely liquefied substrates with acceptable viscosity and a homogeneous reaction mixture. Temperatures lower than the melting point will decrease the reaction rate as a result of higher viscosity, and the resulting product will be different since all the TAGs will not have been available to the lipase. Moreover, solidification of the reaction mixture in the reactor must be avoided. Kim et al.

suggested that the decrease in reaction rate was due to the formation of TAG crystals on the enzyme preparation [170].

The reaction rate usually increases with increasing temperature. However, the risk of thermal deactivation of the lipase must be considered. High temperatures increase the risk of unfolding of the enzyme and hence a loss of activity. Lipozyme TL IM can retain 100% activity for 7 days when incubated at 70°C. All fat mixtures likely to be used in margarine production have melting points below this. It is possible to perform EIE at 80°C for short periods of time, but the rate of inactivation increases [117]. Zhang et al. showed, in an early study, that 100% interesterification could be achieved in 6 hours with 10 wt% Lipozyme TL IM at temperatures ranging from 55 to 80°C [123]. In a later study, using the same substrates (palm stearin and coconut oil) the optimal temperature was concluded to be 70°C. Higher temperatures did not provide any benefits with regard to viscosity or enzyme stability [119]. The optimal temperature for the EIE of olive oil and FH palm oil (melting point 63°C) was shown to be 75°C by Criado et al. [146]. In a previous trial using rapeseed oil/butter in a PBR, the degree of interesterification did not change significantly over the temperature interval of 60-90°C [142]. However, the same residence time was used in all experiments, and the maximum degree of interesterification was achieved already at 60°C; and it was therefore not possible to ascertain whether the reactions at higher temperatures reached equilibrium more quickly.

EIE trials with olive oil and FH canola oil and 1 wt% Lipozyme TL IM have been performed with stepwise lowering of the temperature [170]. The aim was to increase the working life of Lipozyme TL IM. Equilibrium was reached after 24 hours by carrying out the reaction at 70°C, or by initiating the reaction for 4 hours at 70°C and then lowering the temperature to 60°C and maintaining it for 20 hours. A stability test was performed and the enzyme preparation subjected to the lower temperature regime had a remaining activity of 90% after 168 hours, compared to 70% for the other. It was thus concluded that the stability of the preparation could be enhanced and the energy requirements of the process reduced when the lower temperature regime was used. However, a reaction requiring 24 hours to reach equilibrium is not feasible in commercial applications, despite the decrease in energy requirements. An increase in enzyme load together with a lower temperature regime may be the preferable option. On the other hand, the study described above shows that it is possible to save energy by lowering the temperature, while maintaining the reaction time and product composition.

## ***Solvents***

Organic solvents such as n-hexane, chloroform, and diethyl ether have been used in EIE and acidolysis to dissolve lipids, lower the viscosity of the reaction mixture, and make it possible to use lower reaction temperatures. However, this often leads to more problems than in solvent-free approaches. The use of organic solvents in food processing is tightly regulated by legislation, and of the solvents mentioned above only n-hexane can be used under certain circumstances [171]. Organic solvents should be avoided as they are toxic, inflammable, and a threat to the environment. The use of solvents increases the cost of the process, due to the cost of the solvent and the need for more extensive downstream processing. The use of solvents in EIE for bulk fat production is unnecessary, since Lipozyme TL IM is stable above the melting temperatures of the substrates used. Nevertheless, organic solvents have been investigated in some reactions. Criado reported that lower conversion was achieved when EIE was carried out with chloroform or hexane, than in a solvent-free system [146]. The incorporation of FAs into TAGs in acidolysis reactions is, on the other hand, positively influenced by the inclusion of the appropriate solvent [131, 172].

Solvents can also enhance acyl migration which, in most processes, is undesirable. Hexane has been shown to increase acyl migration in various studies [131, 146, 149]. The non-polar nature of hexane has been suggested to facilitate the partitioning of FFAs and the relatively polar partial acylglycerols into the microaqueous phase of the carrier. The hydrophilic environment and the acids could then promote acid-catalyzed acyl migration in the partial acylglycerols. The use of diethyl ether, which is more polar than hexane, resulted in less acyl migration [149]. FFAs and partial acylglycerols are more soluble in diethyl ether, thus the risk of high concentrations in the carrier is reduced. Solvents can be used in analytical methods and in the lab when conducting small-scale experiments, but they should be avoided in commercial processes.

## ***Stirring and Orbital Shaking***

Sufficient mixing to ensure good mass transfer in the reaction system is crucial for an efficient reaction. This was demonstrated in a study by Criado et al., who compared mixing with orbital shaking and magnetic stirring. The time required to reach equilibrium with orbital shaking was 8 hours, and with magnetic stirring only 1 hour [146]. Many investigators use orbital shakers when carrying out small-scale reactions. However, it is important to be aware of the risk of the mass transfer problems that can arise when using preparations in which the lipases are located in the pores of the carrier together with highly viscous substrates. Impeller stirring is a better choice since a magnetic stirrer can mechanically damage the

preparation. Process technology and the engineering of reactors are complex scientific areas. The future of enzymatic processes will rely on efficient, flexible, and easy-to-use systems that ensure high stability of the enzyme preparation and stable output of high-quality products [117, 126, 138].

# Methods

The chapter is divided into four parts: the design of meal studies, HPLC analysis of lipid profiles, analysis of fatty acid composition, and *sn*-2 positional analysis.

## Design of Meal Studies

Two meal studies were carried out: one on men (**Paper II**) and the other on women (**Paper III**) following the CONSORT guidelines [173], as far as they were applicable for a meal study. A single-blinded, randomized, crossover study design was used (see **Papers II** and **III** for details). Each subject consumed three breakfasts containing 35 g of oil or 42 g of butter. The subjects were randomized into three groups, beginning with ALA oil, olive oil or butter. The composition of the meals was: fat 53 E% fat, carbohydrate 35 E%, and protein 12 E%. The total amount of energy was 3290 KJ, which is approximately 33% and 38% of the habitual daily intake for men and women, respectively. The amount of fat (g) per kg body weight (mean  $\pm$  SD) was  $0.75 \pm 0.12$  for women and  $0.56 \pm 0.08$  for men. This was deemed a sufficient amount of fat to obtain an increase in blood lipids for all subjects. All subjects were given the same amount of fat although they differed in body weight. Another approach is to give subjects individual amounts of fat. Before using that approach, careful consideration should be taken regarding which should be the decisive parameter, e.g. body weight, or fat mass [4-5], both of which could affect the results.

The cholesterol content in each kind of meal was not adjusted. The butter meal contained 154 mg and the oil meals 47 mg of cholesterol. Cholesterol content has been found to have a minor effect on the postprandial TAG response [4-5], but this was not observed in the present studies. However, if amounts above 200 mg of cholesterol are administered in one of the test meals, should be adjusted in the other meals to eliminate bias.

The composition of the test meal is very important for the outcome of the study, but equally important is the status of the subjects upon entering the study. Standardization of the nutritional intake and physical activity the day before the trial is important because both affect the postprandial lipid response [4-5, 50]. Low-fat meals and a normal amount of physical activity (no strenuous exercise)



are recommended. Alcohol consumption also affects the response and should be avoided the days before the test. If resources allow, serving the subjects a standardized low-fat evening meal before the test day is the optimal solution.

The habitual diet can affect the postprandial lipid response [8], and subjects should be asked to fill in food intake diaries three to four days before the test. Several biochemical parameters were investigated. In future studies, quantitative analysis of the distribution, size, and amounts of the TRL particles, apoB-48, apoB-100, and inflammation markers should be included, to more accurately evaluate the atherogenic risk. It would also be interesting to analyze gut hormones related to hunger and satiety hormones such as PYY and GLP-1, which are influenced by the macronutrient content in meals and inhibit bowel movement (ileal break).

## HPLC Analysis of Lipid Profiles

The EIE reaction is often monitored by the solid fat content (SFC) in the fat, using nuclear magnetic resonance. The SFC is measured at different temperatures, which are related to the rheological behavior of fats at packaging, and during storage and utilization [118-119, 140, 161, 174]. If the SFC of margarine is too high, it will have a coarse, sandy structure. This method is very convenient when the desired physical properties are known. However, it does not reveal the type of lipid molecular species produced, i.e. TAGs, DAGs, MAGs, or FFAs. The HPLC methods presented here (**Papers I, II, and IV**) are useful tools in identifying molecular species in the oil during the reaction.

The lipids were separated on a reversed-phase C<sub>18</sub> column (250 mm x 4.6 mm, 5 µm particle size) with different proportions of acetonitrile (ACN, solvent A) and iso-propanol:hexane (2:1 vol:vol) (solvent B) as mobile phase [175]. The detector used was an ELSD (**Papers I, II, and III**) or a Corona<sup>®</sup>Plus CAD<sup>®</sup> detector (**Paper IV**). The complete lipid profile of TAGs, DAGs, MAGs and FFAs can be analyzed in one hour using a method that starts at 90% solvent A and ends at 30% solvent A after 47 minutes at flow rate of 1ml/min (section 2.4.1 in **Paper IV**). If a particular group of species is especially important, the gradient can be changed slightly to enhance their separation. The sample solvent and column temperature are also important factors in achieving optimum separation (Fig 12A and B; Fig. 13A and B). In Fig. 12A, the rapeseed oil was dissolved in dichloromethane and in Fig. 12B, 40 vol% solvent A and 60% solvent B. When using the mobile phase as sample solvent, baseline separation was achieved and the chromatogram was much cleaner. Dichloromethane is a stronger solvent than the mobile phase and TAGs are easily dissolved. However, the difference in solvent strength causes zone broadening, and thus poorer separation. Some lipids cannot be satisfactorily

dissolved in a mixture of solvent A+B, in which case only solvent B is used together with gentle heating. The importance of column temperature during separation is shown in Fig. 13A and B, and it is especially evident for FFAs and MAGs, which elute early. A higher temperature induces faster elution, but poorer separation.

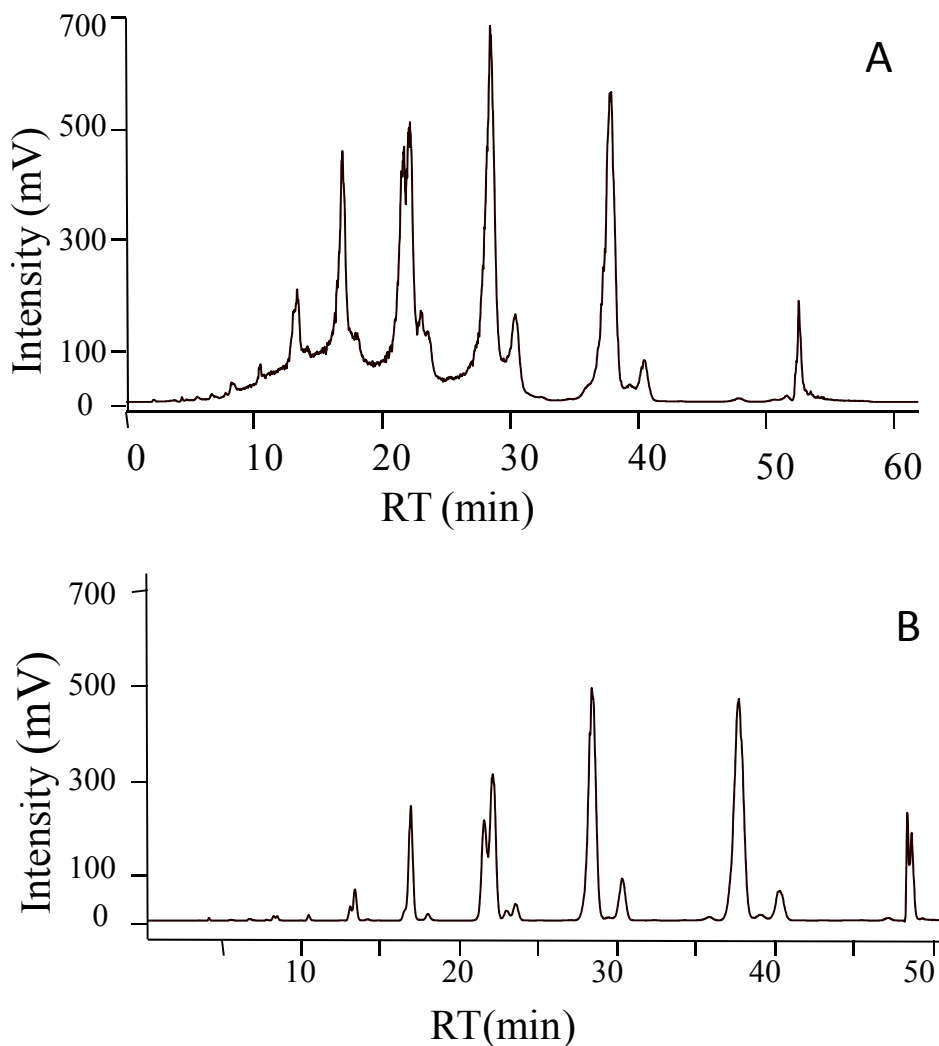


Figure 12. Analysis of lipid profiles using HPLC. A) Rapeseed oil dissolved in dichloromethane (method described in Paper I); B) Rapeseed oil dissolved in 40 vol% solvent A and 60 vol% solvent B (described in Paper II).

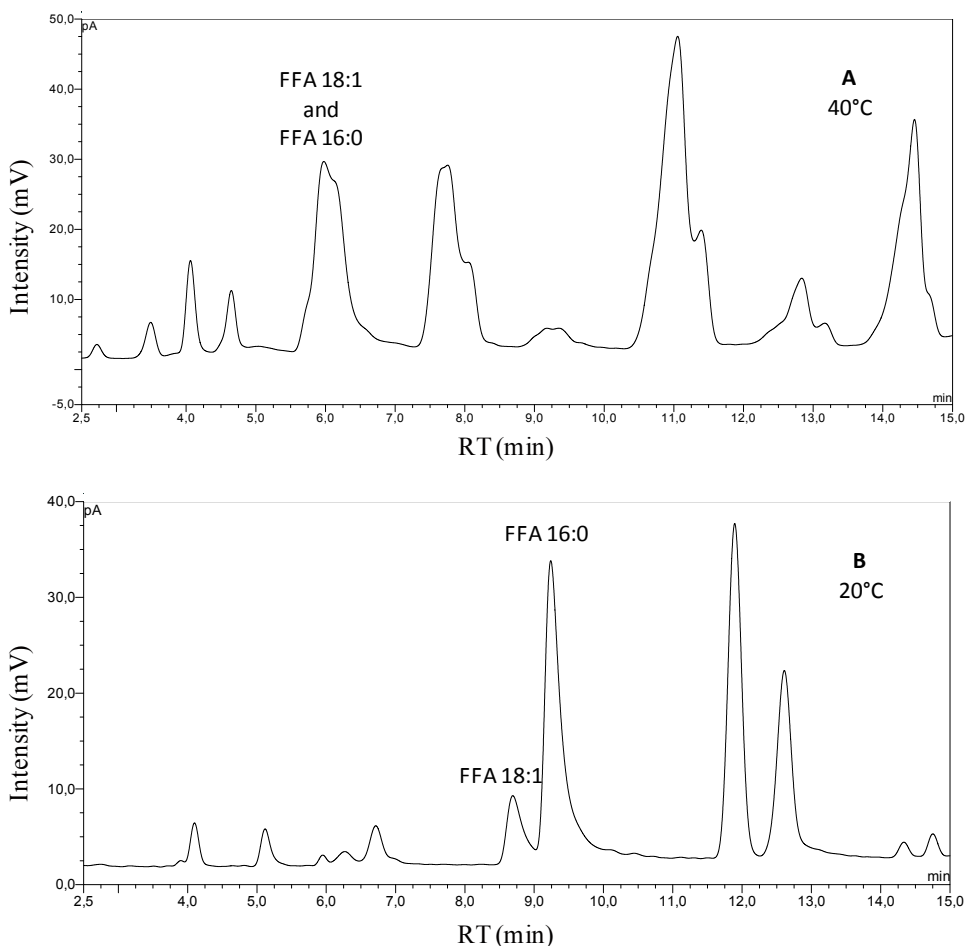


Figure 13. Analysis of FFAs using HPLC. A) Reaction mixture from the EIE of a mixture of trilaurin and POP (Paper IV). Separation carried out at 40°C; B) Same mixture as in A, but separation carried out at 20°C.

The order of elution of acylglycerols in reversed phase systems is dependent on the chain length and degree of unsaturation of the fatty acids. This allows tentative identification of TAGs using the concept of equivalent carbon number (ECN) (**Paper I**). The ECN can be defined for individual fatty acids and TAGs and is correlated with the elution time of the TAG [176]. The ECN of a TAG is the sum of the ECN of its fatty acids. For saturated fatty acids, ECN equal to the number of carbon atoms in the molecules. However, when confirmation of the molecular species is required, HPLC coupled with mass spectrometry (MS) is a better option.

## Mass Spectrometry

HPLC coupled with mass spectroscopy, HPLC-ESI-MS/MS, is useful in studying the formation and breakdown of specific TAG species and when the molecular weight is required, especially in oils in which complete separation of TAG species is difficult [139, 177]. This method was used together with ECN (**Paper I**). Interesterification of rapeseed oil and butter was performed, and the resulting products were identified. TAGs containing ALA and SCFAs and/or MCFAs were found. These were impossible to identify using ECN alone, due to poor separation. An improvement would be to use the HPLC methods presented in **Papers II** and **IV** together with MS. Another option is to fractionate the sample before analysis and only analyze the fraction containing the target molecule.

## Analysis of Fatty Acid Composition

The basic composition of the lipids studied is immensely important, since the properties of the lipid are governed by its fatty acid composition. Three different methods were used in this work to analyze the fatty acid composition in oils (**Papers I and IV**), and in human plasma (**Papers II and III**). The fatty acids are converted into fatty acid methyl esters (FAMES) by basic or acid methylation, and are then analyzed using gas chromatography (GC). The methods are modified versions of those described by Christie [178], and Lyberg and Adlercreutz [157], and can easily be scaled up or down depending on the requirements. Basic methylation converts esterified fatty acids, i.e. TAGs, DAGs, MAGs, and phospholipids into FAMES. The acid method also converts FFAs.

*Basic methylation* was carried out in two different ways. *The ordinary method* [76, 178] involved dissolving the lipid sample in dry cyclohexane and mixing it with 0.5 mM sodium methoxide in dry methanol, followed by incubation at 50°C. The reaction was stopped by adding water saturated with NaCl. The FAME-containing cyclohexane phase was removed and analyzed with GC. The *special method* for fats containing SCFAs [139, 178] was used because SCFAs will evaporate and/or dissolve in the water phase if water and high temperatures are used. The reaction was carried out using the same reagents and solvents but at 20°C, and was stopped by adding concentrated acetic acid and crushed CaCl<sub>2</sub>.

The two methods were compared by analyzing butter, rapeseed oil and linseed oil. Both methods gave the same total fatty acid composition for the two vegetable oils, but the results for butter differed: 4.0 mol% butyric acid and 2.6 mol%

hexanoic acid with *the ordinary method* and 11.8 mol% butyric acid and 4.7 mol% hexanoic acid with *the special method*. Therefore, when it is not known whether SCFAs are present in a sample, the *special method* should be used.

*Acid methylation* [76, 157, 178] was used for analyzing lipids including free fatty acids. Dry cyclohexane (0.7 ml) and 1.5 % sulfuric acid in dry methanol (1 ml) was added to the lipid sample (approx 5-10 mg) and mixed carefully, and then incubated at 50°C for 2 hours. The reaction was stopped, the FAMES extracted, and the solution neutralized by adding 3 ml of an aqueous, saturated solution of NaCl and 3 ml of a 2% aqueous solution of NaHCO<sub>3</sub>. The test-tube was vortexed and centrifuged, and the upper cyclohexane layer transferred to a vial and analyzed with GC. It is very important to neutralize the reaction solution before GC analysis, as any remaining acidity in the cyclohexane can destroy the stationary phase in the GC column. This is also the case for the special method described above.

## ***sn*-2 Positional Analysis**

Randomization of TAGs during reactions was investigated by analyzing the FA composition in the *sn*-2 position. The protocol includes four steps: (i) preparation of MAGs, (ii) separation of lipid classes by thin-layer chromatography, (iii) conversion of MAGs to FAME, and (iv) GC analysis. This analysis was employed in the studies presented in **Papers I, II and IV**. However, only the first step will be discussed here, since this is associated with the most difficulties.

### ***Preparation of Monoacylglycerols***

*sn*-2 MAGs can be prepared by both chemical and enzymatic routes [149, 179]. One of the difficulties in preparing *sn*-2 MAGs is avoiding acyl migration. Another is dissolving the lipid sample to ensure reproducible and representative results. Two different enzymes were used in this work: lipase from the fungus *Rhizopus arrhizus* (**Papers I and II**) and pancreatic lipase (**Paper IV**). In the first study (**Paper I**), the *sn*-2 MAGs were produced by mixing the lipid sample with tert-butyl methyl ether and the *sn*-1,3-specific lipase from *Rhizopus arrhizus* [144, 150] (20 g/l in a phosphate buffer, 50 mM, pH 7) and incubation at 37°C for 6 hours [139]. In the second study (**Paper II**), the lipase concentration was increased to 40 g/l and the reaction time decreased to 1 hour, in order to minimize the risk of acyl migration [76].

In the final study (**Paper IV**), a modified version of the methods by Luddy et al. [156] and Christie [178] using pancreatic lipase, was used. The lipid sample was mixed with TRIS buffer (0.5 M, pH 8), CaCl<sub>2</sub> (2.2%), 3.5 mM bile salt solution,

and 50  $\mu$ l cyclohexane. The mixture was equilibrated for 2 minutes 40°C and then sonicated. Pancreatic lipase (10 mg) was added and incubated for 4 minutes at 40°C.

Both methods have their advantages and disadvantages. The *Rhizopus arrhizus* method is suitable for lipid samples that are difficult to dissolve, since the reaction is carried out in an organic solvent. However, optimization of the extraction step is needed. The pancreatic lipase method is quick and widely used. However, difficulties in dissolving TAG samples with high melting points cause poor reproducibility in the results. This problem was also encountered by Lopez-Hernandez et al. when analyzing sesame oil and fully hydrogenated soybean oil [161]. It is thus clear that the solubility of the lipid sample must be considered when choosing the method of producing *sn*-2 MAGs, as both lipases produce *sn*-2 MAG and the acyl migration during reactions is minimized.

# General Conclusions and Future Considerations

## *Postprandial Lipemia and Alpha-Linolenic Acid*

Prolonged circulation of lipids in the blood increases the risk of metabolic disorders. Postprandial lipemia is influenced by several factors including ageing and genetics, however, other factors can be influenced, leading to a reduction in postprandial lipemic response. Increased physical activity and reduced consumption of refined sugars and fat are recommended for everyone. This would allow lipid levels to return to baseline levels between meals in order to not stress the metabolic system.

Various dietary fatty acids can affect TAG concentrations during the postprandial period. MUFAs and n-6 PUFAs have been shown to induce the production of fewer, larger TRL particles. This could be favorable since lipoprotein lipase preferentially hydrolyses large particles, reducing the risk of saturation of the enzyme. High concentrations and long circulation times of remnants may be detrimental since they are considered to be highly atherogenic.

Premenopausal women show lower postprandial lipemia and are less sensitive to variations in dietary fat than men. The intake of butter resulted in lower postprandial lipemia than the oils in men (**Paper II**), whereas no such difference was seen in the women (**Paper III**). The ALA oil and olive oil meals induced similar plasma TAG concentrations. Women showed a significantly lower NEFA response after olive oil and butter than men.

ALA circulated to a great extent in plasma TAGs and NEFAs. The high concentration and long circulation time of ALA in NEFAs is indicative of spill-over NEFAs in the dominant secretion phase, and preferential release of ALA by the adipose tissue into the circulation, as NEFAs, in the clearance phase. It could also imply that the subjects in these studies already had a good ALA status and fast clearance of ALA was, therefore, not prioritized.

In summary, we did thus not find evidence that ALA has a beneficial effect on postprandial lipids by a selective partitioning to oxidation. ALA certainly has an important role in a balanced diet, although the evidence is weaker than for EPA

and DHA regarding its cardiovascular protective effect and reduction in blood lipid levels. A chronically low intake of ALA may affect health negatively, and a cumulative effect cannot be excluded. Future studies should include randomized controlled trials investigate the role of ALA and its potential health-promoting effects in healthy, slightly overweight subjects in the age range 30 to 50 years. Postprandial and long-term meal studies on the effect of ALA on the size and number of lipoprotein particles and their clearance rate are warranted. The effects of ALA on inflammation are also an interesting area, which could be further explored.

### *Enzymatic interesterification*

Enzymatic interesterification can be used to produce tailor-made lipid products, which can be incorporated into various foods. I have investigated the interesterification of triacylglycerol catalyzed by Lipozyme TL IM, and produced a new alpha-linolenic-rich oil (ALA-oil) (**Paper II**). From this work, it was concluded that interesterification is faster than randomization in the *sn*-2 position, and that uncontrolled hydrolysis should be avoided because it lowers the yield (**Paper IV**). Product quality and enzyme stability are dependent on the quality of the substrates, which should be high. Moreover, the reaction temperature must be above the melting point of the substrates in order to ensure a homogeneous reaction mixture with acceptable viscosity. Good mass transfer is also required to obtain efficient interesterification, and this is dependent on adequate mixing in a batch reactor, or the flow properties in a packed-bed reactor.

Products originating from 1,3-specific interesterification can be produced by minimizing the acyl migration and using short reaction times. Completely or partially randomized products can be obtained by promoting acyl migration and using longer reaction times. Thus a range of products can be formed. The future of enzymatic processes relies on efficient, flexible, and easy-to-use systems that ensure high stability of the enzyme preparation and stable output of high-quality products at a reasonable cost.

### *Analysis Methods*

Reliable methods of analysis are of crucial importance. Triacylglycerols require special consideration as they are difficult to dissolve, and oils and fats contain many different triacylglycerol species that can be difficult to dissolve. A reaction mixture containing free fatty acids, mono-, di-, and triacylglycerols can be separated in one hour using HPLC. An evaporative light scattering detector or a Corona<sup>®</sup>Plus CAD<sup>®</sup> detector can be used. The mobile phase contains acetonitrile and isopropanol/n-hexane (2:1) and depending on the molecular species to be separated, different gradients or isocratic modes can be applied. Samples are dissolved in the mobile phase and can be tentatively identified using the



methodology of equivalent carbon numbers. Further identification can be achieved with mass spectrometry (**Paper I**).

Positional analysis of fatty acids in triacylglycerols is laborious, especially the preparation of *sn*-2 monoacylglycerols. Both the *Rhizopus arrhizus* lipase method and the pancreatic lipase method produce *sn*-2 monoacylglycerols and the acyl migration is also minimized. However, when working with triacylglycerols that have high melting points, it is recommended that the *Rhizopus arrhizus* lipase method be used since the reaction is run in *tert*-butyl methyl ether.

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