POSITIONAL DISTRIBUTION OF FATTY ACIDS IN PLANT TRIACYLGLYCEROLS:
CONTRIBUTING FACTORS AND CHROMATOGRAPHIC/MASS SPECTROMETRIC ANALYSIS

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In memory of my beloved father,
who encouraged and supported me in my studies
CONTENTS

ABSTRACT ........................................................................................................................................ 6
LIST OF ABBREVIATIONS ............................................................................................................. 8
SYSTEMATIC NAMES, TRIVIAL NAMES AND ABBREVIATIONS OF FATTY ACIDS DISCUSSED IN THE TEXT ............................................................................................................ 10
LIST OF ORIGINAL PUBLICATIONS .................................................................................................. 11
1 INTRODUCTION ............................................................................................................................ 12
2 REVIEW OF THE LITERATURE ....................................................................................................... 14
  2.1 TRIACYLGLYCEROLS IN PLANTS .......................................................................................... 14
  2.2 INTRODUCTION TO TRIACYLGLYCEROL BIOSYNTHESIS IN PLANTS .................................................................................................................. 15
    2.2.1 BIOSYNTHESIS OF FATTY ACIDS IN PLANTS .............................................................. 17
    2.2.2 BIOSYNTHESIS OF STORAGE TRIACYLGLYCEROLS IN PLANTS ......................... 20
      2.2.2.1 General pathway of triacylglycerol biosynthesis .................................................. 20
      2.2.2.2 Modification of triacylglycerol fatty acids ............................................................. 24
        2.2.2.3 Selectivity and specificity of enzymes involved in triacylglycerol synthesis .......... 28
          2.2.2.3.1 sn-Glycerol-3-phosphate acyltransferase ...................................................... 30
          2.2.2.3.2 Lysophosphatidic acid acyltransferase ........................................................ 32
          2.2.2.3.3 Diacylglycerol acyltransferase ..................................................................... 38
          2.2.2.3.4 Cholinephosphotransferase ......................................................................... 43
          2.2.2.3.5 Lysophosphatidylcholine acyltransferase .................................................... 44
          2.2.2.3.6 Phospholipid:diacylglycerol acyltransferase .............................................. 45
          2.2.2.3.7 Desaturases and other enzymes modifying fatty acids ................................. 46
    2.2.3 ENVIRONMENTAL FACTORS AFFECTING LIPID METABOLISM .................................... 46
      2.3.1 GEOGRAPHICAL AND CLIMATIC FACTORS ............................................................ 47
      2.3.2 THE EFFECTS OF LIGHT ............................................................................................. 48
      2.3.3 THE EFFECTS OF TEMPERATURE ............................................................................... 49
  2.4 SUMMARY ................................................................................................................................. 53
3 AIMS OF THE STUDIES ................................................................................................................... 55
4 MATERIALS AND METHODS ........................................................................................................ 56
  4.1 ABBREVIATIONS AND NOMENCLATURE ........................................................................... 56
  4.2 MATERIALS .............................................................................................................................. 56
ABSTRACT

Storage triacylglycerols (TAGs) of plants are of great nutritional value for humans. Plant oils are also becoming increasingly important in petrochemical industry. Fatty acid (FA) distribution among the sn-1, -2, and -3 positions (sn, stereospecific numbering) of plant oil TAGs is non-random, and each sn-position has a characteristic FA pattern, which is also species-specific. The positional distribution of FAs in TAGs has been shown to affect the nutritional and technological properties of fats and oils and because of this, rapid and precise analytical methods are needed.

Sophisticated mass spectrometric (MS) methods combined with different high-performance liquid chromatographic (HPLC) methods were used in the practical work for the quantification of regioisomers of TAGs in various fats and oils. Regioisomer composition indicates the positional distribution of FAs between the sn-2 and sn-1/3 positions. The studies included development of both MS and HPLC methods as well as investigation of factors affecting the regioisomer compositions of plant TAGs.

The regioisomer compositions of storage TAGs was studied in the seed oils of currants and the pulp oils of sea buckthorn berries. The results show that in currant seed oils, α-linolenic acid has a stronger preference for the sn-1/3 positions than γ-linolenic acid and that linoleic acid has a stronger preference for the sn-2 position than linolenic acids. In sea buckthorn pulp oils, oleic acid is mainly located in the sn-2 position and vaccenic acid in the sn-1/3 positions. In addition, oleic acid has an overall higher affinity and vaccenic acid a lower affinity for the sn-2 position than palmitoleic acid. The regiospecific distribution of FAs of TAGs varied significantly within species, subspecies, and varieties of the berries. Furthermore, different weather factors including temperature and radiation were found to affect the regioisomerism of TAGs in berry oils.

The stereospecific distribution, i.e. the positional distribution of FAs between the sn-1, sn-2, and sn-3 positions, of FAs in the storage TAGs of plants is determined mainly by the selectivity and activity of acyltransferases sn-glycerol-3-phosphate acyltransferase, lysophosphatidic acid acyltransferase, and diacylglycerol acyltransferase on certain acyl donors and acceptors as well
as modification and hydrolysis of acyl chains located in the $sn$-2 position of phosphatidylcholine. The selectivity and activity of the enzymes vary between different plant species. The selectivity of the enzymes towards different substrates can generate distinctive TAG species and thus overgeneralization concerning the TAG stereoisomer compositions may not give accurate results. More studies with detailed stereoisomer analysis of particular plant TAG species, combined with genetic background and environmental factors, are needed.
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>A/A/B</td>
<td>triacylglycerol composed of fatty acids A and B</td>
</tr>
<tr>
<td>ABA</td>
<td>symmetric regioisomer composed of fatty acids A and B</td>
</tr>
<tr>
<td>ACCase</td>
<td>acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>ACN</td>
<td>acyl carbon number</td>
</tr>
<tr>
<td>ACP</td>
<td>acyl-carrier protein</td>
</tr>
<tr>
<td>Ag-HPLC</td>
<td>silver ion high-performance liquid chromatography</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>APCI</td>
<td>atmospheric pressure chemical ionization</td>
</tr>
<tr>
<td>CoA</td>
<td>coenzyme A</td>
</tr>
<tr>
<td>CDP</td>
<td>cytidylyl phosphate</td>
</tr>
<tr>
<td>CMP</td>
<td>cytidine monophosphate</td>
</tr>
<tr>
<td>CPT</td>
<td>cholinephosphotransferase</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DB</td>
<td>double bond, number of double bonds between carbon atoms</td>
</tr>
<tr>
<td>DES</td>
<td>desaturase</td>
</tr>
<tr>
<td>DGAT</td>
<td>diacylglycerol acyltransferase</td>
</tr>
<tr>
<td>DGTA</td>
<td>diacylglycerol transacylase</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
</tr>
<tr>
<td>FA</td>
<td>fatty acid</td>
</tr>
<tr>
<td>FAD</td>
<td>fatty acid desaturase</td>
</tr>
<tr>
<td>FAME</td>
<td>fatty acid methyl ester</td>
</tr>
<tr>
<td>FID</td>
<td>flame ionization detector</td>
</tr>
<tr>
<td>GC</td>
<td>gas chromatography</td>
</tr>
<tr>
<td>GPAT</td>
<td>$sn$-glycerol-3-phosphate acyltransferase</td>
</tr>
<tr>
<td>Gro-3-P</td>
<td>$sn$-glycerol 3-phosphate</td>
</tr>
<tr>
<td>GroP</td>
<td>$sn$-glycerol 3-phosphate with specified acyl chain(s)</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>HSD</td>
<td>honestly significant difference</td>
</tr>
<tr>
<td>KAS</td>
<td>3-ketoacyl-ACP synthase</td>
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</table>
KCS       3-ketoacyl-CoA synthase
LPAAT     lysophosphatidic acid acyltransferase
LPCAT     lysophosphatidylcholine acyltransferase
LSD       least significant difference
LysoPtdCho lysophosphatidylcholine
LysoPtdOH lysophosphatidic acid
MAG       monoacylglycerol
MGAT      monoacylglycerol acyltransferase
MS        mass spectrometry
MS/MS     tandem mass spectrometry
MW        molecular weight
NIAPCI    negative ion atmospheric pressure chemical ionization
NICI      negative ion chemical ionization
PAP       phosphatidate phosphatase
PDAT      phospholipid:diacylglycerol acyltransferase
PLA₂      phospholipase A₂
PtdCho    phosphatidylcholine
PtdOH     phosphatidic acid
PUFA      polyunsaturated fatty acid
RP        reversed phase
sn        stereospecific numbering
sn-AAB    triacylglycerol composed of fatty acids A and B with specified stereochemistry
ssp.      subspecies
TAG       triacylglycerol
UHPLC     ultra-high-performance liquid chromatography
UPLC®     ultra performance liquid chromatography®
VLCFA     very long-chain fatty acid
### SYSTEMATIC NAMES, TRIVIAL NAMES AND ABBREVIATIONS OF FATTY ACIDS DISCUSSED IN THE TEXT

<table>
<thead>
<tr>
<th>Systematic name</th>
<th>Trivial name used</th>
<th>Abbreviations used</th>
</tr>
</thead>
<tbody>
<tr>
<td>hexanoic acid</td>
<td>caproic acid</td>
<td>6:0</td>
</tr>
<tr>
<td>octanoic acid</td>
<td>caprylic acid</td>
<td>8:0</td>
</tr>
<tr>
<td>decanoic acid</td>
<td>capric acid</td>
<td>10:0</td>
</tr>
<tr>
<td>dodecanoic acid</td>
<td>lauric acid</td>
<td>12:0</td>
</tr>
<tr>
<td>tetradecanoic acid</td>
<td>myristic acid</td>
<td>14:0</td>
</tr>
<tr>
<td>hexadecanoic acid</td>
<td>palmitic acid</td>
<td>16:0</td>
</tr>
<tr>
<td>9-hexadecenoic acid</td>
<td>palmitoleic acid</td>
<td>16:1(n-7) Po</td>
</tr>
<tr>
<td>6-hexadecenoic acid</td>
<td></td>
<td>16:1(n-10)</td>
</tr>
<tr>
<td>octadecanoic acid</td>
<td>stearic acid</td>
<td>18:0</td>
</tr>
<tr>
<td>octadecenoic acid</td>
<td></td>
<td>18:1</td>
</tr>
<tr>
<td>11-octadecenoic acid</td>
<td>vaccenic acid</td>
<td>18:1(n-7) V</td>
</tr>
<tr>
<td>9-octadecenoic acid</td>
<td>oleic acid</td>
<td>18:1(n-9) O</td>
</tr>
<tr>
<td>9t-octadecenoic acid</td>
<td>elaidic acid</td>
<td>18:1(n-9)t</td>
</tr>
<tr>
<td>6-octadecenoic acid</td>
<td>petroselinic acid</td>
<td>18:1(n-12)</td>
</tr>
<tr>
<td>12-hydroxy-9-octadecenoic acid</td>
<td>ricinoleic acid</td>
<td></td>
</tr>
<tr>
<td>12,13-epoxy-9-octadecenoic acid</td>
<td>vernolic acid</td>
<td></td>
</tr>
<tr>
<td>9-octadecen-12-ynoic acid</td>
<td>crepenynic acid</td>
<td></td>
</tr>
<tr>
<td>9,12-octadecadienoic acid</td>
<td>linoleic acid</td>
<td>18:2(n-6) L</td>
</tr>
<tr>
<td>octadecatrienoic acid</td>
<td>linolenic acid</td>
<td>18:3</td>
</tr>
<tr>
<td>9,12,15-octadecatrienoic acid</td>
<td>α-linolenic acid</td>
<td>18:3(n-3) Ala</td>
</tr>
<tr>
<td>6,9,12-octadecatrienoic acid</td>
<td>γ-linolenic acid</td>
<td>18:3(n-6) Gla</td>
</tr>
<tr>
<td>9c,11t,13t-octadecatrienoic acid</td>
<td>eleostearic acid</td>
<td></td>
</tr>
<tr>
<td>11-eicosenoic acid</td>
<td>gondoic acid</td>
<td>20:1(n-9)</td>
</tr>
<tr>
<td>5,8,11,14-eicosatetraenoic acid</td>
<td>arachidonic acid</td>
<td>20:4(n-6)</td>
</tr>
<tr>
<td>5,8,11,14,17-eicosapentaenoic acid</td>
<td>eicosapentaenoic acid</td>
<td>20:5(n-3) EPA</td>
</tr>
<tr>
<td>13-docosenoic acid</td>
<td>erucic acid</td>
<td>22:1(n-9)</td>
</tr>
<tr>
<td>4,7,10,13,16,19-docosahexaenoic acid</td>
<td>docosahexaenoic acid</td>
<td>22:6(n-3) DHA</td>
</tr>
</tbody>
</table>

*a Abbreviations: c,cis; t, trans. The configurations of the double bonds are cis unless otherwise stated.*
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by the Roman numerals I–V:


1 INTRODUCTION

Storage triacylglycerols (TAGs) of plants are of great nutritional value for humans. Furthermore, some oil plants are of major value in the cosmetic industry, in the production of detergents, coatings, plastics, and specialty lubricants, and increasingly in the fuel industry, because of increasing demands for biodiesel production (1-4). Modification of plant oils for nutritional and other industrial purposes has expanded to the exploitation of transgenic plants.

![Figure 1](image1.png)

**Figure 1.** Structure of triacyl-sn-glycerol. R’, R”, and R’’’ denote different alkyl chains of the fatty acyl groups (sn, stereospecific numbering).

Fatty acid (FA) distribution in the sn-1, -2, and -3 positions (sn, stereospecific numbering) (Figure 1) of plant oil TAGs is typically non-random and each position has a characteristic FA pattern in particular natural fats and oils. The positional distribution of FAs in TAGs has been shown to affect, for instance, the nutritional quality and technological properties of fats and oils. The regioisomer compositions, i.e. the positional distribution of FAs between the sn-2 and sn-1/3 positions, of TAGs have been shown to affect infant nutrition, lipid absorption, lipid metabolism and atherogenesis in humans (5-7). The use of structurally modified fats and oils as a result of hydrogenation and transesterification (8) is common and the proportion of these ingredients has rapidly increased in our diet. Thus knowledge of the regioisomer composition of TAGs is important and there is a need for more rapid and precise analytical methods for their determination. These methods can also provide new possibilities for examining the biological processes taking place in the biosynthesis of TAGs.
Mass spectrometry (MS) combined with separation by high-performance liquid chromatography (HPLC) is a rapid and reliable method of quantification of the regioisomers of TAGs (9). The positional distribution of FAs of TAGs has been studied mainly with enzymatic hydrolysis using lipases or chemical hydrolysis using Grignard reagent, ethyl magnesium bromide, combined with chromatographic methods (10, 11). The methods usually include partial hydrolysis of TAGs to 1,2-, 2,3-, 1,3-diacyl-sn-glycerols (DAGs), 1-, 2-, 3-monoacyl-sn-glycerols (MAGs) and free fatty acids, derivatization of acylglycerols, isolation by thin-layer chromatography, separation by HPLC, and the analysis of FAs by gas chromatography (GC) (10, 11). These methods are time-consuming and laborious, especially, if results concerning the positional distribution of FAs of individual TAG species are needed. In addition, the hydrolysis and derivatization of TAGs may affect results due to the increased susceptibility to acyl migration (10, 11). However, reversed-phase (RP) and chiral-phase-HPLC methods, in which no hydrolysis or derivatization treatments are needed, have recently been presented for the determination of the regio- or stereoisomer composition of TAGs (10, 12). With the above mentioned methods the difference between FA compositions in the sn-1 and sn-3 positions, i.e. the stereoisomer composition, can be determined. MS only allows the distribution of FAs between the sn-2 and sn-1/3 positions of TAGs to be distinguished, but sample sizes can be small and data analyses and calculations are relatively easy to conduct. The separation of individual TAG species with certain FA compositions by HPLC enables determination of the regiospecific FA distribution in particular TAGs (9). This is not easily achieved by common methods of hydrolysis.

It was shown in publications II, III, and IV that many factors contribute to the regioisomer composition of TAGs of currant (Ribes nigrum L. and R. rubrum L., Grossulariaceae) varieties. In the currant seed oils studied, the regioisomer compositions of TAGs 54:7 (ACN:DB, acyl carbon number : number of double bonds between carbon atoms) containing one α-linolenoyl [18:3(n-3)] or γ-linolenoyl [18:3(n-6)] acyl chain were substantially different. The affinity of α-linolenic acid for the sn-2 position was lower than that of γ-linolenic acid. In TAG 50:3 of pulp oils of sea buckthorn (Hippophaë rhamnoides L., Elaeagnaceae) subspecies (ssp.) and varieties, vaccenic acid [18:1(n-7)] had lower affinity for the sn-2 position than oleic acid [18:1(n-9)]. These observations suggest that the enzymes involved in TAG synthesis distinguish between these FA isomers. The regioisomer compositions of the TAGs studied were relatively constant.
regardless of the origin of the currant or sea buckthorn berries. However, the regiospecific
distribution of FAs was shown to vary statistically significantly between different currant
varieties and between different sea buckthorn subspecies. Furthermore, statistically significant
differences were found when comparing the berries that were grown under different weather
conditions. In the review of the literature, the factors affecting the final stereospecific structure
of the synthesized plant TAGs are discussed, and the main emphasis is placed on the
regiospecific distribution of FAs in storage TAGs of seed oils and pulp oils.

2 REVIEW OF THE LITERATURE

2.1 TRIACYLGLYCEROLS IN PLANTS

Lipids are essential constituents of all plant cells and they are mainly found in cell membranes.
However, cells of storage organs produce lipids in more abundant amounts. TAGs constitute the
major form of storage lipids in fruits, seeds and the pollen grains of several plant species. They
provide carbon in a highly reduced form and serve as an important chemical energy reserve for
seed germination and plant development (13, 14).

Among the most important oil crops are oil palm (Genus Elaeis Jacq., Arecaceae), high- and
low-erucic acid rapeseed (Brassica napus L., Brassicaceae), sunflower (Helianthus annuus L.,
Asteraceae) and soybean (Glycine max (L.) Merr., Fabaceae) (3). The major lipids in all edible
plant oils are composed primarily of saturated FAs palmitic acid (16:0) and stearic acid (18:0)
as well as unsaturated FAs oleic acid [18:1(n-9)], linoleic acid [18:2(n-6)], and linolenic acid
[18:3(n-3)] (14-16). Some plant species also accumulate unusual FAs the structures of which
differ significantly from common FAs. Plants that accumulate these unusual FAs may also have
considerable industrial value. One example is castor bean (Ricinus communis L.,
Euphorbiaceae), which contains up to 90% of ricinoleic acid (12-hydroxy-9-octadecenic acid)
(17). The FA composition as well as the characteristic stereospecific distribution of FA in plant
oil TAGs appears to be under genetic control (18) and they are strongly dependent on species,
subspecies, variety and cultivar. In this way different plants have unique TAG profiles, FA
profiles and stereospecific distribution of FAs. One evident origin for the non-random
distribution of FAs of TAGs are the acylation steps in TAG synthesis. A general rule for the positional distribution of FAs of TAGs in plant oils is that the saturated FAs as well as FAs longer than C\textsubscript{18} occupy the \textit{sn}-1 and \textit{sn}-3 positions (18). In addition, the unsaturated FA content in the \textit{sn}-2 position is higher than that of \textit{sn}-1/3 positions. The amount of oil produced as well as the FA composition of a particular plant oil determines its usefulness in food and industrial applications and thus its commercial value. Nevertheless, the positional distribution of FAs in TAGs and the factors contributing to it are important regarding lipid metabolism in humans and the genetic engineering of plant lipids.

2.2 INTRODUCTION TO TRIACYLGLYCEROL BIOSYNTHESIS IN PLANTS

The biosynthesis of lipids in plant cells is a complex system consisting of many pathways. In addition, the steps of synthesis take place in different cellular organelles and the metabolites are transferred from one organelle to another for modification (14). Plant TAG synthesis can be divided into three phases. The first phase consists of the \textit{de novo} FA synthesis in plastids. The second phase includes the modification of FAs in the endoplasmic reticulum (ER) and the third phase the incorporation of FAs into TAGs which accumulate in oil bodies (14, 19). Current knowledge of lipid biosynthesis has expanded from a basic understanding of biochemical pathways to the structural information of individual enzymes and to identification of the corresponding genes. Understanding of synthesis and modification of FAs and the general pathways leading to the formation of TAGs is currently extensive. However, there is only little data concerning the control of the metabolic flux through these pathways as a whole (15, 19-22). Knowledge of regulatory mechanisms responsible for the substrate selectivity of the enzymes and of the interdependence of the individual biosynthetic steps are crucial for the understanding of the biosynthesis of plant TAGs (23). Generally, in maturing plant seeds and pulp the major factors controlling the FA compositions of the different \textit{sn}-positions in TAGs are the substrate selectivities of the acyltransferases involved in the glycerol-3-phosphate pathway, desaturation of the FAs in phosphatidylcholine (PtdCho), and the composition of the acyl-CoA (CoA, coenzyme A) pool, which is affected by the \textit{de novo} FA synthesis and modification of the FAs (14, 15, 24).
Although seeds are the most common tissue for storage TAGs in higher plants, pulp or mesocarp of some plant fruits also contain considerable amounts of oil. Oil palm (22–28% of oil) (25), avocado (*Persea americana* Mill., Lauraceae) (4–25% of oil) (25), coconut (*Cocos nucifera* L., Arecaceae) (65% of oil) (25), olive (*Olea europaea* L., Oleaceae) (12–30% of oil) (26), and sea buckthorn (1-4% of oil) (27) are sources of commercially important vegetable oils originating from the fruit mesocarp. The mesocarp and seeds have different functions in plants and thus also display differences in lipid biosynthesis. Storage TAGs in seeds nourish the developing embryo during the germination, whereas TAGs in pulp oil accumulate to attract animals. In addition, raw, green fruits may have photosynthetic activity, which in part also affects the metabolism of lipids (28). However, some developing seeds such as green seeds of rape and soybean also have the ability to use light for photosynthesis, which expresses and activates seed enzymes involved in FA and oil synthesis (29, 30).

Naturally, research on lipid biosynthesis in plants is mainly targeted on commercially important oil crops or plants of other industrial importance because of their special FA composition. *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae) is also used in several studies because it is considered as a model organism for plant biology. Studies specifically concerning TAG biosynthesis in seeds and pulp of berries, which were the main emphasis in our publications (II, III, IV), are scarce, but it can be assumed that the basic mechanisms are the same as in other plant seeds and mesocarp. Furthermore, most of the studies are concentrated on the factors affecting oil quantities and FA composition, and not directly on the factors contributing to the stereospecific distribution of FAs in TAGs. Studies that involve genetically engineered plants are included in this review, because they provide detailed information concerning the individual steps involved in TAG synthesis and because they are good examples of the complexity of lipid biosynthesis in plants.

Detailed knowledge concerning pathways of TAG biosynthesis is important for the successful application of genetic engineering to plant oil production. Genetic strategies aim to produce oilseed crops that would accumulate TAGs with high yields in seeds and fruits, and would have the desired stereospecific composition and/or FA composition (1, 2, 5). Several challenges are faced in production of transgenic oil plants (31). Accumulation of, for example, a particular FA in a transgenic plant may require not only the expression of the enzymes responsible for the
synthesis of the FAs in the target, but also co-expression of the enzymes responsible for the TAG synthesis (19, 32). The very high expression of lauric acid (12:0) in transgenic rapeseed, for example, does not necessarily result in accumulation of lauric acid in rapeseed TAGs in any substantial amounts, because enzymes exclude lauric acid from TAGs, especially from the sn-2 position of glycerol (33, 34). Again, in A. thaliana seeds, mutation of a single gene resulting in the reduced activity of one acyltransferase, namely diacylglycerol acyltransferase, which is involved in TAG assembly, altered the FA composition of the final TAGs substantially (35).

2.2.1 BIOSYNTHESIS OF FATTY ACIDS IN PLANTS

The de novo FA biosynthesis pathway is briefly discussed here because it affects the formation and composition of TAGs, and is an important step in TAG synthesis. FAs in plants, as in other organisms, are the main components of most lipids including storage TAGs and membrane phospholipids. Altogether, higher plants can synthesize and accumulate over 200 different FAs (14). The major lipids in edible plant oils are assembled using saturated and unsaturated C_{16} and C_{18} FAs (14-16). Unsaturated FAs contain one or more double bonds, which are predominantly of the cis configuration; hence all FAs discussed in this thesis are of the cis configuration unless otherwise stated. The number, position and configuration of the double bonds affect the physical and physiological properties of the FAs (14).

FAs very rarely exist in cells as free FA and are mainly esterified to glycerol (Figure 1). FAs can also be esterified to other molecules to form e.g. steryl esters, waxes, and carotenoid esters (14). Lipid biosynthesis is essentially different in plants than in animals and fungi because the FA synthesis of plants takes place in the plastids, whereas in the cells of animals and fungi, FA production occurs primarily in the cytosol. FA metabolism in bacteria resembles that of plants (14, 15, 36).

Approximately 30 enzymatic reactions are needed for the synthesis of 16-carbon and 18-carbon FAs in the stroma of plastids (14-16, 36). In the first step (Figure 2, reaction 1), acetyl-CoA is carboxylated by acetyl-CoA carboxylase (ACCase) (EC 6.4.1.2) to malonyl-CoA, and malonyl is further transferred from CoA to acyl-carrier protein (ACP) (Figure 2, reaction 2), an
essential protein cofactor. The growing acyl chain is attached to the ACP as a thioester. Malonyl-ACP is decarboxylated and a carbon-carbon bond is formed with acetyl-CoA to form four-carbon product, 3-ketobutyryl-ACP, by 3-ketoacyl-ACP synthase III (KAS III) (Figure 2, reaction 3). The removal of CO$_2$ drives the reaction forward. The next reaction sequence consists of three reactions: reduction of the 3-oxo group (Figure 2, reaction 4), dehydration (Figure 2, reaction 5), and reduction of the double bond (Figure 2, reaction 6). The cycle of these four reactions (Figure 2, reactions 3–6) catalyzed by FA synthase adds two carbons to the chain and continues until 16:0-ACP or 18:0-ACP are formed. The FA synthase complex refers to all enzyme activities in FA synthesis except ACCase. The condensation (Figure 2, reaction 3) is performed by KAS I isoenzyme when acyl chain lengths of six to 16 carbons are produced. The final condensation to 18:0-ACP is catalyzed by KAS II isoenzyme. The elongation of acyl chains usually terminates at 16:0 or 18:0 (14, 15, 36). The plant may regulate the ratio of formed long-chain FAs C$_{16}$ and C$_{18}$, or medium-chain FAs C$_{8}$–C$_{14}$, by controlling KAS activity and expression (36, 37).

All the carbon atoms existing in FAs derive from acetyl-CoA in the plastid (15). The substrates for FA synthesis are also used in other biosynthesis pathways. Acetyl-CoA, in addition to malonyl-CoA, is consumed by multiple reactions in the cell such as in the production of amino acids and flavonoids (14, 16). It appears that the plastid-localized ACCase involved in reaction 1 in Figure 2 is highly regulated and determines the overall rate of FA synthesis in plants (13, 14, 38, 39). Over expression of ACCase from Arabidopsis leads to increased synthesis of both FAs and TAGs in potato (Solanum tuberosum L., Solanaceae) tubers (40). The acetyl-CoA pool remains relatively constant, even if the rates of FA synthesis vary greatly (15, 16). In light, FA synthesis is relatively high, whereas in dark, synthesis is low due to the regulation of ACCase activity (16, 22).
The FA synthesis in plastids produces saturated FAs, mainly palmitic acid and stearic acid, but in most plants and plant tissues over 75 % of the FAs are unsaturated (15). De novo synthesized FAs are not thought to be desaturated in vivo as free FAs but are esterified either to ACP for the soluble plastid desaturases or to lipids for the integral membrane desaturases (20). The first double bond is formed in the acyl chain by the soluble plastid-localized stearoyl-ACP Δ⁹-desaturase (EC 1.14.99.6), which is the most important desaturase in plastids (14, 15, 36). Different isoforms of this desaturase can be found in a given species and even in a single tissue (41). A number of other desaturases that function on palmitoyl-ACP or stearoyl-ACP exist (13, 20, 41). These include, for example, palmitoyl-ACP Δ⁶-desaturase (42) and palmitoyl-ACP Δ⁴-
desaturase (43, 44), the latter of which is involved in the formation of FA petroselinic acid [18:1(n-12)]. These enzymes are all structurally related, but have different substrate specificities.

Acyl-ACP thioesterases (EC 3.1.2.14) hydrolyze the acyl moiety from ACP and this prevents the extension of acyl groups and targets them for export out of the plastids. The selectivity of thioesterases on certain FAs partly determines the FA composition of TAGs (36, 45). Presumably in most plants the thioesterases show high activity with oleoyl-ACP, lower activity with palmitoyl-ACP and even lower activity with stearoyl-ACP (16, 36). Thioesterases play an important role in plants such as coconut, which accumulate shorter chain FAs. The thioesterases of these plants are active with decanoyl-ACP and lauroyl-ACP, which results in the formation of decanoic acid (10:0) and lauric acid TAGs (14). Although FA synthesis of plants takes place in the plastids, the formation of glycerolipids mainly occurs in the ER. Thus, plants must have mechanisms to export FA from the plastids to other cell sites. It is not exactly known how the free FAs released from ACP are exported from plastids (15). It has been suggested to occur by simple diffusion across the membrane (15). On the outer membrane of the plastid, an acyl-CoA synthetase (EC 6.2.1.3) catalyzes the formation of acyl-CoA thioester, which is then available for glycerolipid formation (15, 46). The fatty acid export from plastids is shown to be dependent on activation by acyl-CoA synthetase, which indicates that acyl-CoA synthetase also has a role in FA transport through membranes (46).

2.2.2 BIOSYNTHESIS OF STORAGE TRIACYLGLYCEROLS IN PLANTS

2.2.2.1 General pathway of triacylglycerol biosynthesis

A scheme of TAG synthesis is shown in Figure 3. Synthesis of TAGs takes place in the ER of plant seed and mesocarp cells (14-16, 24). Palmitic acid, stearic acid, and oleic acid originating from plastids are used as CoA thioesters in the synthesis of TAGs in the ER via a pathway described by Kennedy (47). In the first step (Figure 3, reaction 1), lysophosphatidic acid (lysoPtdOH) is formed by acylation of sn-glycerol-3-phosphate by sn-glycerol-3-phosphate acyltransferase (GPAT) (EC 2.3.1.15). The second acylation (Figure 3, reaction 2) is activated
by lysophosphatidic acid acyltransferase (LPAAT) (EC 2.3.1.51) leading to the formation of phosphatidic acid (PtdOH). PtdOH is then dephosphorylated to \( sn \)-1,2-diacylglycerol by phosphatidate phosphatase (PAP) (Figure 3, reaction 3) (EC 3.1.3.4). Pearce and Slabas (48) suggested that metabolic channeling between the Kennedy pathway enzymes must exist, because PAP can dephosphorylate both lysoPtdOH and PtdOH. The activity of both GPAT and LPAAT is controlled so that premature dephosphorylation of lysoPtdOH does not occur in the Kennedy pathway. The TAG is formed in the final step, in which a third FA is incorporated to the \( sn \)-3 position of DAG. This final acylation (Figure 3, reaction 4) is the only enzymatic reaction unique to TAG synthesis and is catalyzed by diacylglycerol acyltransferase (DGAT) (EC 2.3.1.20). DAGs are situated at the branch point of the pathway between TAG and phospholipid formation (14, 15, 24).

The synthesis of TAG molecules can be divided into two parts: the de novo production of FAs in plastids and TAG assembly in the ER and oil bodies. The de novo FA synthesis has been suggested to be a more important factor than the Kennedy pathway in TAG accumulation in oil plants (22, 49). FA formation in plastids has been shown to exert a higher impact (60%) on TAG accumulation than TAG assembly in the ER (40%) in olive and oil palm (\textit{E. guineensis} Jacq.) callus cultures (50). In embryos of developing seeds of \textit{Cuphea lanceolata} P. Browne (Lythraceae), \textit{Ulmus carpinifolia} Gleditch (Ulmaceae), and \textit{Ulmus parvifolia} Jacq. (Ulmaceae) the amount of synthesized TAGs has also been shown to be limited by the amount of de novo produced FAs (51). However, Vigeolas \textit{et al.} (52) showed that the increased expression of \( sn \)-glycerol-3-phosphate dehydrogenase, which led to an increase in the level of \( sn \)-glycerol-3-phosphate in developing rape seeds, resulted in an increase in the lipid content of the seed. Nevertheless, it seems likely that manipulation of a single enzyme step does not substantially affect the yield of the desired product oil.

TAG synthesis is more complex than that described in the Kennedy pathway (Figure 3, reactions 1–4). In many oilseeds, most FA species are not immediately available for TAG biosynthesis but are channeled into PtdCho and to a lesser extent into phosphatidyl ethanolamine, where they can be desaturated or otherwise modified (20). PtdCho is the main substrate for the desaturation of oleic acid to linoleic acid and \( \alpha \)-linolenic acid. FAs from PtdCho may become available for TAG synthesis either via acyl exchange between PtdCho and
the acyl-CoA pool, which provides inputs of linoleic acid and α-linolenic acid back into the cellular acyl-CoA pool, or by acyl-CoA independent acyl transfer from PtdCho to DAG. These reactions are discussed in the next section. Although FA are mainly modified in PtdCho, in some oilseeds that contain very long-chain FAs (VLCFAs) having 20 carbon atoms or more, oleoyl-CoAs are elongated to CoA thioesters of, for example, gondoic acid [20:1(n-9)] and erucic acid [22:1(n-9)] (Figure 3, reaction 13) (14). These elongated FAs can also be used in the synthesis of TAG and DAG via the Kennedy pathway. The activities of the enzymes involved in TAG and PtdCho synthesis vary between different substrates and between different plant species and tissues. These differences are discussed in the contexts of FA modification and FA selectivity in the synthesis of TAGs.

In almost all plant species, oil consisting of TAGs is stored in mature seeds as lipid bodies, which consist of TAGs surrounded by a phospholipid monolayer (13). The phospholipid monolayer in the oil bodies of seeds also contains protein components, oleosins. Oleosins may play an important role in stabilizing the oil bodies in desiccation and quiescence as well as facilitating the rapid breakdown of oil bodies during germination (13, 53). Oil bodies in fruit tissues do not contain oleosin homologs, which implies that the mesocarp lipids are not important to the germination and growth of the seedling but are present in fruits in order to attract animals (13, 14). The size of oil bodies in seeds is smaller (0.2–2.5 μm) (53) than in mesocarp (approximately 20 μm) (13). The size of the oil bodies does not grow during seed development but, instead, the number of oil bodies increases as storage TAGs accumulate (13). However, in transgenic plants the increase in oil content is observed to induce the formation of larger oil bodies (54). The lack of oleosins in the oil bodies of fruit tissues enables the coalescence of the oil bodies to form just a few very large oil droplets. Although the morphology of oil bodies in seeds and fruits is quite different, it appears that the storage TAGs in both of these tissues are synthesized by identical biochemical pathways (13).
Figure 3. A simplified scheme of fatty acid (FA) modification and triacylglycerol (TAG) biosynthesis in developing seeds and mesocarp of plants (23, 24, 55, 56). In addition to desaturation, other FA modifications such as hydroxylation, epoxygenation and conjugation also take place in phosphatidylcholine (PtdCho). The Kennedy pathway is shown as broader arrows. Abbreviations: ACP, acyl carrier protein; CDP, cytidylyl phosphate; CMP, cytidine monophosphate; CoA, coenzyme A; CPT, cholinephosphotransferase; DAG, diacylglycerol; DES, desaturase; DGAT, diacylglycerol acyltransferase; DGTA, diacylglycerol transacylase; FAD, fatty acid desaturase; Gro-3-P, sn-glycerol-3-phosphate; GPAT, sn-glycerol-3-phosphate acyltransferase; KCS, 3-ketoacyl-CoA synthase (elongase); lysoPtdOH, lysophosphatidic acid; LPAAT, lysophosphatidic acid acyltransferase; LysoPtdCho, lysophosphatidylcholine; LPCAT, lysophosphatidylcholine acyltransferase; MAG, monoacylglycerol; MGAT, monoacylglycerol acyltransferase; PtdOH, phosphatidic acid; PAP, phosphatidate phosphatase; PDAT, phospholipid:diacylglycerol acyltransferase; PLA2, phospholipase A2.
2.2.2.2 Modification of triacylglycerol fatty acids

To date, enormous progress has taken place in the identification, cloning, and expression of several acyl group desaturases of various organisms (57). The FA desaturation steps involved in TAG assembly are displayed in Figure 3 (reactions 11, 12, and 14). Except for the soluble acyl-ACP desaturase family, into which the plastidial acyl-ACP desaturase belongs, all other FA desaturases in plants are membrane-localized, and desaturate acyl chains of glycerolipids (14). Polyunsaturated FAs (PUFAs) are formed in PtdCho and possibly in other phospholipids by ER-localized FA desaturase 2 (FAD2) (Figure 3, reaction 11) and FAD3 (Figure 3, reaction 12) that utilize PtdCho as substrate (14-16, 58, 59). FAD2 uses the 18:1(n-9) acyl chain as substrate and inserts a double bond to the $\Delta^{12}$ position to yield linoleic acid (36, 41, 60), whereas FAD3 inserts a double bond to the $\omega^{3}$ ($\Delta^{15}$) position of linoleic acid to yield $\alpha$-linolenic acid (14, 36, 41). These FAs are the most common PUFAs in plant oils. Formation of $\gamma$-linolenic acid in seeds of common borage (Borago officinalis L., Boraginaceae) (61, 62), currants (Genus Ribes), and evening primrose (Genus Oenothera, Onagraceae) involves a $\Delta^{6}$-desaturase active on linoleic acid located in PtdCho (14). The desaturases can either position the double bond by acting from the carboxyl-end of the acyl chain ($\Delta$-desaturase), from the methyl-end ($\omega$-desaturase), or by initiating unsaturation three carbons toward the methyl end from an existing double bond (20). $\Delta^{6}$-desaturase uses mainly linoleic acid in the sn-2 position of PtdCho (61, 62) whereas FAD2 and FAD3 also insert double bonds to FAs located in the sn-1 position (14, 16, 61).

The highly unsaturated or otherwise modified FAs in DAGs and, furthermore, in TAGs originate from PtdCho (63, 64). TAGs may be formed in acyl-CoA-independent reactions using DAGs and PtdCho as the acyl donors. PtdCho can donate its entire DAG moiety to TAG synthesis via the reversible synthesis of PtdCho from DAG and CDP-choline catalyzed by cholinephosphotransferase (CPT) (Figure 3, reaction 5) (EC 2.7.8.2) (16, 65-67). TAGs can also be synthesized through the acyl transfer from PtdCho to a DAG by phospholipid:diacylglycerol acyltransferase (PDAT) (EC 2.3.1.158) (Figure 3, reaction 8), which results in the formation of lysophosphatidylcholine (lysoPtdCho) (68-70).
In addition to direct transfer of acyl chains from PtdCho to DAGs, PtdCho can also donate the PUFAs into the acyl-CoA pool (14, 15, 24). The acyl moiety in the \(sn-2\) position of PtdCho may undergo acyl exchange with the existing acyl-CoA pool (71). This reversible acyl exchange reaction is catalyzed by acyl-CoA:1-acyl-sn-glycerol-3-phosphocholine acyltransferase or lysophosphatidylcholine acyltransferase (LPCAT) (EC 2.3.1.23) (Figure 3, reaction 10) (16, 72, 73). Acyl exchange provides inputs of linoleic acid and linolenic acid FAs as well as other modified FAs back into the cellular acyl-CoA pool. Consequently, the exchange reaction allows newly produced FAs exported from the plastids to enter PtdCho and the resulting desaturated or otherwise modified FAs as acyl-CoAs can be used again for the acylation of the TAGs via the Kennedy pathway or for the acylation of other lipids. The acyl-CoA pool in the seed and mesocarp tissue cells can thus be a complex collection of FA groups. The acyl group from the \(sn-2\) position of PtdCho can be cleaved off by phospholipase A\(_2\) (PLA\(_2\)) (EC 3.1.1.4) resulting in the formation of LysoPtdCho (Figure 3, reaction 6) and this reaction may be important in the removal of unusual FAs from PtdCho and other polar lipids, which are used as membrane lipids (14, 24, 74). The incorporation of unusual FAs in membrane phospholipids could interrupt the membrane functions (14). Plants also contain other phospholipases than PLA\(_2\) acting on PtdCho. The microsomal phospholipases of Cuphea procumbens Ortega, Ulmus glabra Huds., Euphorbia lagascae Spreng. (Euphorbiaceae), and castor bean selectively removed the unusual FAs characteristic to the plant species from phospholipids (74). Epoxidized and hydroxylated acyl groups are removed from both the \(sn-1\) and \(sn-2\) positions of PtdCho and from the \(sn-1\) position of lysoPtdCho. Hydrolysis of decanoyl groups, on the other hand, is highly dependent on the position of the FA in the phospholipid (74). In castor bean cotyledons, PLA\(_2\) released ricinoleic acid but not oleic acid from PtdCho (75). The same phenomenon was demonstrated in developing cotyledons of safflower (Carthamus tinctorius L., Asteraceae) and rapeseed (75).

Diacylglycerol transacylase (DGTA) (Figure 3, reaction 9) catalyzes the transfer of an acyl moiety between two DAG molecules to form a TAG and a MAG (24, 55, 70, 76). Stobart et al. (55) proposed that the reaction of DGTA, which yields MAGs and TAGs, is reversible. The activity of DGTA was approximately 25% of the activity of DGAT in developing safflower seeds. Thus the oleic acid in \textit{in situ} synthesized TAGs can enter PtdCho by DGTA and CPT for further desaturation to linoleic acid (55). In microsomal fractions from castor beans, again,
transfer of oleic acid from PtdCho to \textit{in situ} formed TAGs and ricinoleic acid in the opposite direction was detected (77). However, no evidence was found that TAGs in the oil bodies are rearranged (77). Nevertheless, Garcés \textit{et al.} (78) found that the TAGs in oil-bodies of sunflower were also modified by unsaturation but the exact mechanism was not known. Furthermore, lysoPtdCho may be formed from MAG (\textbf{Figure 3, reaction 7}), which indicates that it was catalyzed by CPT in an analogous fashion to the reversible reaction between DAG and PtdCho (55). It has also been suggested that MAG generated by the lysoPtdOH phosphatase, which has been observed in developing peanut (\textit{Arachis hypogaea} L.) cotyledons, may serve as a precursor for TAG biosynthesis using the interconversion between MAG, DAG, and TAG (79). LysoPtdOH phosphatase shows higher preference for 1-oleoyl-\textit{sn}-glycerol-3-phosphate (1-oleoyl GroP) than for lysoPtdOH containing saturated FAs (79). In addition, DAGs have been shown to be synthesized from MAGs and acyl-CoA in developing peanut cotyledons by soluble MAG acyltransferase (MGAT) (EC 2.3.1.22) (\textbf{Figure 3, reaction 15}) (56). Both 1-acyl-\textit{sn}-glycerol and 2-acyl-\textit{sn}-glycerol were substrates for MAG acyltransferase but the enzyme preferentially uses 1-acyl-\textit{sn}-glycerol (56). This enzyme preferred 1-oleoyl-\textit{sn}-glycerol over 1-palmitoyl-\textit{sn}-glycerol, and the acyl-CoA preference was as follows: stearoyl > palmitoyl > oleoyl (56). The above-mentioned scientific data suggest that several alternative synthesis mechanisms for TAGs exist in plants and that TAGs can not be considered as metabolically inert end products in plants.

In addition to desaturation, FAs can also be modified to unusual FAs, such as hydroxy, conjugated, or epoxy FAs (20, 36). Epoxygenases, hydroxylases, acetylenases, conjugases and \textit{trans} desaturases encoded by \textit{fad2} like genes also use PtdCho as a substrate (80). Some oilseeds also accumulate medium-chain FAs or VLCFAs. The formation of medium-chain FAs was discussed in the context of FA synthesis. According to several studies, there seems to be no evidence that phospholipids are involved in the formation of VLCFAs (64). VLCFAs are not usually detected in the phospholipids, but small amounts of VLCFAs have been found in PtdCho of developing seeds of cabbage (\textit{Brassica oleracea} L.) (81, 82). The elongase reactions have several features in common with FA synthase reactions in plastids and both are multisubunit complexes. But the elongases, such as 3-ketoacyl-CoA synthase (elongase KCS), localized in the cytosol (\textbf{reaction 13}), are membrane bound and do not use ACP (14, 36). In some plants, TAGs may contain large amounts of these unusual or modified FAs and some of
these FAs such as lauric acid, erucic acid, and ricinoleic acid are commercially important (14).

Unusual FAs are present almost exclusively in TAGs and are excluded from the membrane lipids, although some of the modified, unusual, FAs are synthesized in PtdCho, which is abundantly found in membranes. Unusual FAs may also be transferred in substantial amounts into PtdCho before they are incorporated into TAG, which suggests that PtdCho has a function in the biosynthesis of TAGs apart from its involvement in FA modification reactions. The uncommon FAs 16:1(n-10) in *Thunbergia alata* Bojer ex Sims (Acanthaceae) seeds and petroselinic acid in developing endosperm of coriander (*Coriandrum sativum* L., Apiaceae) and carrot (*Daucus carota* L., Apiaceae) were shown to be readily incorporated into PtdCho during TAG synthesis, despite the fact that they are not synthesized in PtdCho and are present only in small amounts in the membrane lipids (83, 84). Petroselinic acid was more abundant in the *sn*-1 and 16:1(n-10) in the *sn*-2 position of PtdCho.

Incorporation of unusual FAs into the membranes could alter the physical and chemical properties as well as the fluidity of the membrane (14). It seems that the mechanisms in plants for accumulating unusual FAs are not evolved only for the production of these FAs but also evolved to channel them from PtdCho to TAG (85, 86). One possible mechanism might be subcellular compartmentalization, in which membrane phospholipid and storage TAG assembly occurs in different cellular locations (14). Metabolically engineered soybean and *A. thaliana* produced as high as 20% of conjugated FAs, but failed to incorporate those FAs in substantial amounts from the *sn*-2 position of PtdCho to TAGs, which is in contrast to the plants that naturally accumulate those FAs (87). Although the incorporation of unusual FAs into membranes may cause problems in transgenic plants accumulating these FAs, an opposite challenge is the rapid transfer of common FAs to TAGs. In transgenic plants that are supposed to accumulate very long-chain PUFA into TAGs, common Δ⁶ desaturated FAs were rapidly channeled from PtdCho to TAGs resulting in a lack of Δ⁶ desaturated precursor FAs in the acyl-CoA pool, which limited the synthesis of elongated C₂₀ FAs (88).

In contrast to plants accumulating polyunsaturated FAs, the turnover of the FA groups and DAG moiety to PtdCho has been shown to be rather low in plants accumulating TAGs that contain mainly monounsaturated FAs and saturated FAs. In the early stages of developing
cocoa (Theobroma cacao L., Sterculiaceae) cotyledons, mainly DAGs containing palmitic acid and oleic acid were formed, whereas in the older tissues TAGs appeared with the concomitant accumulation of stearic acid (89). The accumulation of phospholipids was rather low in both maturation stages, which is in contrast with oil seeds that accumulate polyunsaturated oil. In addition, avocado mesocarp, which is particularly rich in oleic acid with only small quantities of PUFA, seems to have neither DAG to PtdCho interconversion nor acyl exchange between acyl-CoA and PtdCho, which suggests that avocado mesocarp lacks the enzymes responsible for the entry of oleic acid into PtdCho (73). In addition, the FA composition of TAGs can be more radically altered in seeds than in other tissues, which implies that non-seed tissues have efficient mechanisms to degrade unusual or excess FAs and in this way prevent their incorporation into membrane lipids (22).

2.2.2.3 Selectivity and specificity of enzymes involved in triacylglycerol synthesis

The non-random distribution of FAs in TAGs can be observed in various plant oils. The stereospecific position of an FA is dependent on its chain length, degree of unsaturation, and functional groups such as hydroxyl groups. For example, unsaturated FAs in olive oil are distributed in the following order of preference: \( sn-2 > sn-1 > sn-3 \) (90). This preference was stronger in polyunsaturated FAs. The saturated FAs were esterified into the \( sn-3 \) position as a function of chain length in the sequence: 20:0 > 18:0 > 16:0 (90). The effect of the chain length was similar for 16:1 and 18:1 although to a lesser extent. Thus the preferential order of esterification according to the chain length of FAs was \( sn-3 > sn-1 \) for saturated FAs and \( sn-2 > sn-1, sn-3 \) for monounsaturated FAs.

Mattson and Volpenheim (91) studied FA composition in the \( sn-2 \) position of TAGs from 28 plant species. They showed that if palmitic acid, stearic acid, and VLCFAs, that are preferentially esterified in the \( sn-1 \) and \( sn-3 \) position, are excluded, the positional distribution of oleic acid, linoleic acid, and linolenic acid are roughly random and thus predictable between the \( sn-2 \) and the primary positions. However, the analyses on which this observation is based were conducted from all of the TAG species simultaneously. This means that the stereospecific distribution of FAs in a particular TAG is probably different from this generalization.
The stereospecific distribution of FAs in TAGs can vary between different plant subspecies. Ozerinina et al. (92) and Vereshchagin et al. (93) showed that two different mechanisms of assembly of TAGs exist, resulting in two different stereospecific distribution patterns of FAs in the mesocarp of different sea buckthorn subspecies. In berries grown in Central Asia (ssp. *turkestanica*) and the Baltic area (ssp. *rhamnoides*), the sn-2 positions of all TAGs includes mainly monounsaturated FAs, and palmitic acid is almost exclusively esterified in the sn-1/3 positions (93). On the other hand, palmitic acid is located in significant proportions in the sn-2 position of berries grown in Caucasian regions (ssp. *caucasica*) (93).

The substrate preferences of the enzymes involved in TAG synthesis have been studied in the seeds and fruits of several plants. Specificity is studied using one single acyl-CoA species or other substrate at a time, whereas selectivity is measured using a mixture of acyl-CoA species or other substrates. The latter approach is considered more similar to the *in vivo* situation than the former approach. The order of specificity for various substrates can differ from that of selectivity. The problem in some of the *in vitro* studies is the use of only a limited selection of different acyl acceptors and acyl donors. The concentration of these substrates can also affect the selectivity and specificity of the enzyme (94). Nevertheless, these studies can offer valuable information about the corresponding *in vivo* reactions, because the substrate specificities usually reflect those present *in vivo* (66).

Selectivities of the enzymes involved in TAG synthesis are strongly dependent on the particular plant, and genetic strategies aim to combine these different selectivities. As an example, the LPAAT of the developing cabbage seeds (*Brassica oleracea*), but not rapeseed (*Brassica napus*), can incorporate erucic acid into the sn-2 position of 1-erucoyl GroP (81). The DGAT of rapeseed, again, can use 1,2-dierucoyl-sn-glycerol for the production of trierucoylglycerol, a desired feedstock for the industry, whereas DGAT of cabbage excludes erucic acid from the sn-3 position (81). Unusual FAs may not be readily used by the enzymes in TAG synthesis in oil plants that do not normally accumulate them. In transgenic rapeseed, even the very high expression of lauroyl-ACP thioesterase did not result in accumulation of lauric acid into the sn-2 position of TAGs, because of the selectivity of the sn-2 acyltransferases against lauric acid (33). The surplus lauric acid was directed to β-oxidation. Wiberg et al. (95) also showed that the LPAAT and DGAT of rapeseed did not have the same preference for lauric acid substrates.
as the enzymes of plants naturally accumulating lauric acid. The futile cycling of other unusual FAs through β-oxidation is also demonstrated in *Arabidopsis* (96). This indicates their limited integration into lipids.

Most enzyme specificity and selectivity studies are conducted with the assumption that only one enzyme is involved in a particular esterification reaction step. However, different isoforms and families of the acyltransferases such as GPAT, LPAAT, LPCAT and DGAT are found (19, 24, 97-100) and these can also be involved in the synthesis of lipids other than TAGs, such as suberins, cutins, and waxes (101, 102). Different members of the same enzyme family can be located in different intra-cellular locations and can have different functions and substrates (19, 103, 104). Different enzyme isoforms and families can have different substrate selectivities. The specificities, selectivities and other features of particular enzymes are now discussed in detail.

### 2.2.2.3.1 sn-Glycerol-3-phosphate acyltransferase

The first acylase enzyme, GPAT, acylates the *sn*-1 position of *sn*-glycerol-3-phosphate in the Kennedy pathway. Plants are found to express many GPATs and the GPAT enzyme family such as that in *A. thaliana* is relatively large including enzymes with different biosynthetic functions (104). The acyl-CoA specificity and selectivity of GPAT in various plants is summarized in Tables 1 and 2. In oil seeds that accumulate C\textsubscript{18} FAs, GPAT transfers both C\textsubscript{16} and C\textsubscript{18} FAs to the *sn*-1 position. In developing cocoa cotyledons, which are rich in oleic acid and linoleic acid, GPAT utilizes saturated FAs preferring palmitoyl-CoA over stearoyl-CoA. However, GPAT was found to exclude oleic acid from the *sn*-1 position (89). In the developing cotyledons of both sunflower (105) and safflower (105, 106), palmitic acid mainly entered in the *sn*-1 position of TAG by GPAT. In another study with developing safflower cotyledons, the saturated FAs palmitoyl-CoA and stearoyl-CoA were incorporated exclusively to the *sn*-1 position. In addition, oleic acid was present mainly in the *sn*-1 position (107).

Substrate specificities have also been studied in plants accumulating other FAs than the common C\textsubscript{16} and C\textsubscript{18} FAs. In developing rape seeds the acyl-CoA selectivity of GPAT seems to
be palmitoyl > erucoyl > oleoyl, but the FA composition of lysoPtdOH has been suggested to be determined mainly by the availability of different acyl-CoAs (108). The GPATs of developing seeds of both nasturtium (*Tropaeolum majus* L., Tropaeolaceae) and meadowfoam (*Limnanthes douglasii* R. Br., Limnanthaceae), which contain mainly unsaturated C$_{20}$ and C$_{22}$ FAs, have similar properties, which indicates that the FA composition in the *sn*-1 position of TAGs in both of these plant species is controlled by the acyl-CoA mixture available to the GPAT (109). *Cuphea* (Lythraceae) plants accumulate medium-chain FAs in their storage TAGs in seeds. *Cuphea wrightii* A. Gray GPAT has shown low activity with decanoyl-CoA, whereas it was efficiently utilized by *C. procumbens* GPAT (110). *C. wrightii*, again, showed overall higher activities towards saturated acyl-CoAs with chain lengths longer than C$_{10}$, but oleoyl-CoA was more preferred by *C. procumbens* GPAT. Stearoyl-CoA was used in negligible amounts by both plants. In the developing seeds of *C. lanceolata* the acyl-CoA specificities of GPAT were in the order decanoyl = lauryl > linoleoyl > myristoyl = oleoyl > palmitoyl (111). It seems that palmitoyl-CoA is the most favored substrate for GPAT except in plants accumulating medium-chain FAs. This is in accordance with the observations of Brockerhoff (18).

**Table 1.** Acyl-CoA selectivity of GPAT from various plant sources

<table>
<thead>
<tr>
<th>Plant species <em>a</em></th>
<th>16:0</th>
<th>18:0</th>
<th>18:1(n-9)</th>
<th>18:2(n-6)</th>
<th>18:3(n-3)</th>
<th>22:1(n-9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Safflower (106)</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Safflower (107)</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>4</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Rapeseed (108)</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Cocoa (89)</td>
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<td>2</td>
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</tbody>
</table>

For each sample, GPAT selectivity is ranked in order of activity: 1, highest; 5, lowest; N, negligible. *a* References are given in parentheses.
Table 2. Acyl-CoA specificity of GPAT from various plant sources

<table>
<thead>
<tr>
<th>Plant species</th>
<th>10:0</th>
<th>12:0</th>
<th>14:0</th>
<th>16:0</th>
<th>18:0</th>
<th>18:1(n-9)</th>
<th>18:2(n-6)</th>
<th>20:1(n-9)</th>
<th>22:1(n-9)</th>
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<tr>
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<td></td>
</tr>
<tr>
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<td>2</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Nasturtium (109)</td>
<td>5</td>
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<td>2</td>
<td>3</td>
<td>4</td>
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<td>1</td>
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<td>4</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><em>Cuphea procumbens</em> (110)</td>
<td>3</td>
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<td>2</td>
<td>4</td>
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<td></td>
</tr>
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<td><em>Cuphea wrightii</em> (110)</td>
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<td>N</td>
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<td></td>
<td></td>
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<tr>
<td><em>Cuphea lanceolata</em> (111)</td>
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<td></td>
</tr>
</tbody>
</table>

For each sample, GPAT specificity is ranked in order of activity: 1, highest; 5, lowest; N, negligible. *References are given in parentheses.

2.2.2.3.2 Lysophosphatidic acid acyltransferase

LPAAT is the second acylase enzyme in the Kennedy pathway. The specificities and selectivities of LPAATs from various plants have been more studied than those of GPATs probably because it is believed to have a more pronounced influence on the stereoisomer composition of TAGs. The acyl preferences of LPAAT from various plants are summarized in Tables 3 and 4. However, these tables are not exhaustive or completely accurate because many other factors than acyl-CoA species can influence the selectivity and specificity of LPAAT. These factors include lysoPtdOH acyl acceptors with different acyl moieties in the sn-1 position. Plants have various LPAAT enzymes that are expressed in different tissues and have different substrate specificities (100). In addition to LPAAT localized in the ER, soluble LPAAT was also recently recognized in *A. thaliana* (112).

LPAAT showed strict acyl-CoA specificity in maturing safflower seeds. The order of specificity with 1-oleoyl GroP was linoleic acid = oleic acid > palmitoleic acid > elaidic acid > vaccenic acid > stearic acid > palmitic acid (113). Petroselinoyl-CoA and erucoyl-CoA were ineffective as acyl donors. This observation suggests that the FA composition of sn-2 position in PtdOH synthesized in vivo in safflower depends primarily on both the acyl-CoA specificity/selectivity of the LPAAT and the composition of the acyl-CoA pool. The strict
specificity may also explain the absence of saturated FAs in the sn-2 position of safflower seed TAGs (113). In another study with safflower seeds, LPAAT incorporated α-linolenoyl into the sn-2 position (107). In developing cocoa cotyledons, which are rich in oleic acid and linolenic acid, LPAAT excluded unsaturated FAs (89). LPAAT from safflower used lysoPtdOH species in the following order of effectiveness (given as the sn-1 FA of lysoPtdOH): linoleic acid = oleic acid > palmitic acid (113).

Very long-chain C_{20} and C_{22} FAs are synthesized as their CoA esters, which implies their incorporation into TAG via the Kennedy pathway (64). These FAs do not usually exist in the sn-2 position, and Roughan and Slack (64) have suggested that LPAAT must exclude these in acylation. Substrate specificities of the LPAAT in different plant species accumulating erucic acid in TAGs have been shown to be quite different from each other. Erucic acid was practically excluded from the sn-2 position by LPAAT in the presence of oleoyl-CoA or palmitoyl-CoA in developing rape seeds (108) and theoretically the maximum amount of erucic acid in rapeseed that could be achieved without genetic manipulation is 66% (94). However, cabbage, also a member of Brassicaceae, has the ability to incorporate erucic acid in significant amounts into the sn-2 position of TAG by LPAAT (81). Compared with cabbage, the specificity of LPAAT to erucoyl-CoA was lower in nasturtium but significantly higher in meadowfoam (81). However Löhden and Frentzen (109) showed that the LPAAT of nasturtium, in contrast to that of meadowfoam, was inactive with erucoyl-CoA, which indicates that nasturtium has different mechanisms for the incorporation of very long-chain FAs into the sn-2 position, perhaps through acyl-exchange reactions. The incorporation of LPAAT from meadowfoam to rapeseed induced the accumulation of erucic acid into the sn-2 position in reasonable amounts and the formation of trierucoylglycerol in small amounts, while the FAs composition remained unchanged compared to non-transgenic rapeseed (114). This was also observed when the LPAAT of white meadowfoam (Limnanthes alba Hartw. ex Benth., Limnanthaceae) was expressed in rapeseed (115). This shows that the positional distribution of FAs of TAGs in one plant species can be altered by introducing acyltransferases with different substrate selectivity from another plant species. A recent success was the combination of non-transgenic rapeseed having low content of PUFA with transgenic high erucic acid rapeseed having the LPAAT gene from meadowfoam and an over-expressing elongase gene (116). This resulted in rapeseed lines that contained up to 72% erucic acid.
In LPAAT specificity studies, the maturing seeds of white meadowfoam, nasturtium, palm *Syagrus cocoides* Mart. (Arecaceae), castor bean, soybean, maize (*Zea mays* L., Poaceae), and rapeseed were able to use both 1-oleoyl GroP and 1-erucoyl GroP as acyl acceptors and oleoyl-CoA as an acyl donor, but only the enzyme from white meadowfoam could use erucoyl-CoA to produce 1,2-dierucoyl GroP (94). Furthermore, castor bean LPAAT had the ability to synthesize 1-oleoyl-2-erucoyl GroP. In white meadowfoam, enzyme activity was highest for 1,2-dioleoyl GroP production followed by 1-oleoyl-2-erucoyl GroP, 1,2-dierucoyl GroP and 1-erucoyl-2-oleoyl GroP, and the preference toward the oleoyl moiety in the substrates was slightly stronger. The white meadowfoam LPAAT is quite unique in its ability to produce 1,2-dierucoyl GroP. Overall, LPAAT preferred 1-oleoyl GroP over 1-erucoyl GroP. Oleoyl-CoA was preferred over erucoyl-CoA with 1-oleoyl GroP and erucoyl-CoA over oleoyl-CoA when 1-erucoyl GroP was the acyl acceptor (94). In selectivity studies, erucoyl-CoA was a less effective substrate for LPAAT, when both 1-oleoyl GroP and 1-erucoyl GroP were used, and also inhibited enzyme activity on oleoyl-CoA (94), which shows that it is not only the concentration of a particular FA in the acyl-CoA pool that affects acylation activity, but also the presence of other FAs that may be more preferred acyl donors or inhibit the acylation with certain FAs.

Sun *et al.* (117) suggested that the strong acyl specificity of LPAAT can be a blocking step in the synthesis of TAGs. *Butia capitata* palm (Becc., Arecaceae), maize, and rape seed microsomes had the ability to produce lysoPtdOH, PtdOH, DAG and TAG from oleoyl-CoA, whereas in the presence of erucoyl-CoA none of the plants produced these lipids (117). In the presence of lauroyl-CoA, only *B. capitata* palm was able to synthesize PtdOH, DAG and TAG. This lack of incorporation of lauroyl-CoA was also observed in castor bean, peanut, and soybean. In maize and rapeseed, kinetic studies suggested that lauric acid and erucic acid were incorporated into the sn-1 position by GPAT, but failed to enter PtdOH by LPAAT, and thus to subsequent lipid products. The strong acyl specificity of LPAAT could also be recognized in studies concerning other unusual FAs.

LPAAT in the immature endosperm of coconut had higher specificity for medium-chain FAs. The specificities were in the following order: lauroyl >> decanoyl ≈ myristoyl >> oleoyl > palmitoyl > octanoyl > stearoyl > hexanoyl (118, 119). A slight preference for 1-lauroyl GroP
over 1-oleoyl GroP was also observed (118). *C. wrightii* LPAAT showed low activity with decanoyl-CoA, whereas it was efficiently used by *C. procumbens* LPAAT with all lysoPtdOH species (110). The LPAAT of *C. wrightii* showed higher activity towards acyl-CoAs with acyl chains longer than C_{10}. In both plants, the utilization of acyl-CoAs with longer chain lengths was preferred with lysoPtdOH acyl acceptors that had longer acyl chains in the sn-1 position (110). In *C. lanceolata* (111), decanoyl-CoA was the most preferred substrate with all lysoPtdOH species, whereas oleoyl-CoA was utilized practically only with 1-oleoyl GroP. In transgenic rapeseeds capable of producing lauric acid, LPAAT excluded lauric acid from the sn-2 position of TAGs, which limited accumulation of lauric acid to TAGs (34).

Oo and Huang (120) found that in the endosperm of *S. cocoides* palm, the LPAAT specificity towards acyl-CoA is greatly influenced by the acyl acceptor lysoPtdOH and *vice versa*. LPAAT was equally active on oleoyl-CoA and lauroyl-CoA with 1-oleoyl GroP, whereas maize and rapeseed LPAAT were more active on oleoyl-CoA than lauroyl-CoA. With 1-lauroyl GroP, LPAAT in palm was three times more active on lauroyl-CoA than on oleoyl-CoA, while 1-lauroyl GroP was an inactive substrate in maize and rapeseed. Overall activity was, however, lower with 1-lauroyl GroP than with 1-oleoyl GroP in palm. In the selectivity tests, enzymes from all the three seeds had a stronger preference for the oleoyl moiety in both lysoPtdOH species and acyl-CoA, but only palm was able to use 1-lauroyl GroP. Enzymes from soybean and castor bean showed similar activity to those from rapeseed and maize. These results show that the FA in the sn-1 position of lysoPtdOH greatly influences the acyl preference in the sn-2 position in PtdOH formation (120).

Although it seems that the LPAAT of various plants are strict in their use of acyl moieties in substrates, Cao *et al.* (94) suggested that this enzyme was generally quite equal in its capability of using different lysoPtdOH in soybean, maize and castor bean. Brown *et al.* (121) also came to the conclusion that LPAAT might have less influence on the regiospecific distribution of FAs of TAGs in plant oils than is generally thought. They made extensive studies concerning the substrate selectivity of LPAAT in various plant species, including avocado, cocoa, meadowfoam, oil palm, rapeseed, shea tree (*Butyropermum parkii* (G.Don) Kotschy, Sapotaceae), paradise tree (*Simarouba glauca* DC., Simaroubaceae), and sunflower. In the majority of the species, they found no correlation between the final composition of sn-2 FAs in
oil or membrane lipids and the ability of LPAAT to use linoleic acid as a substrate. All LPAAT enzymes were capable of incorporating linoleoyl-CoA to lysoPtdOH when introduced together with oleoyl-CoA. The selectivity between these two acyl-CoA species was low, although several of the plants species do not normally accumulate linoleic acid in the sn-2 position in significant amounts. Palmitoyl-CoA was also an accepted substrate for all LPAATs, but oleoyl-CoA was generally more preferred when presented together. The LPAAT of oil palm was an exception, because oleic acid and palmitic acids were utilized at comparable levels. No selectivity was observed between 1-oleoyl GroP and 1-stearoyl GroP in oil palm. Generally, the amount of linoleic acid seemed to be more influenced by the activity of oleic acid desaturases using PtdCho, the availability of acyl-CoAs and the extent of post-synthesis modification of TAGs, than by the selectivity of LPAAT (121). When yeast LPAAT was expressed in *Arabidopsis* and rapeseed, the resulting transgenic plants exhibited elevated LPAAT activity and showed both a substantial increase in seed oil content and an increase in VLCFA proportions in TAGs (122). The amount of VLCFA in the sn-2 position of TAGs was particularly increased. This shows the importance of LPAAT on both total FA content and composition as well as on stereospecific FA distribution.

Generally, LPAAT seems to be specific for oleoyl-CoA and linoleoyl-CoA and does not use saturated substrates with the exception of plants accumulating medium chain saturated FAs. In addition, VLCFAs are generally excluded from the sn-2 position. The selectivity of LPAAT clearly influences the stereospecific distribution of FAs of TAGs, because it is generally known that unsaturated FAs are located in the sn-2 position of TAGs (18).

**Table 3. Acyl-CoA selectivity of LPAAT from various plant sources**

<table>
<thead>
<tr>
<th>Plant species</th>
<th>16:0</th>
<th>18:0</th>
<th>18:1(n-9)</th>
<th>18:2(n-6)</th>
<th>18:3(n-3)</th>
<th>22:1(n-9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Safflower (107)</td>
<td>N</td>
<td>N</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>N</td>
</tr>
<tr>
<td>Cocoa (89)</td>
<td>N</td>
<td>N</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For each sample, LPAAT selectivity is ranked in order of activity: 1, highest; 3, lowest; N, negligible. *References are given in parentheses.*

36
Table 4. Acyl-CoA specificity of LPAAT from various plant sources

<table>
<thead>
<tr>
<th>Plant species $^d$</th>
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<td>Nasturtium (109)</td>
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<td>Meadowfoam (109)</td>
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<td>Coconut (118)</td>
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<td>Coconut (119)</td>
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<td>Flax (123)</td>
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<td>C. procumbens $^d$ (110)</td>
<td>2</td>
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<tr>
<td>C. wrightii $^d$ (110)</td>
<td>6</td>
</tr>
<tr>
<td>C. lanceolata $^d$ (111)</td>
<td>1</td>
</tr>
</tbody>
</table>

For each sample, LPAAT specificity is ranked in order of activity: 1, highest; 6, lowest; N, negligible. $^d$ References are given in parentheses. $^b$ With 1-palmitoyl-ser-glycerol 3-phosphate (1-palmitoyl GroP) or 1-oleoyl GroP, respectively. $^c$ With 1-palmitoyl GroP and 1-oleoyl GroP or with 1-capryl GroP, 1-lauryl GroP, and 1-myristoyl GroP, respectively. $^d$ Genus: Cuphea.
2.2.2.3.3 Diacylglycerol acyltransferase

DGAT is involved in the terminal step of the Kennedy pathway (47) and catalyzes the only enzymatic reaction that is unique to the synthesis of TAGs. It has been suggested to have an essential role in controlling both the quantitative (35, 124-129) and qualitative formation of TAGs (19, 35, 125, 129-131). DGAT is assumed to have an important role in regulating the formation of TAGs because its substrates, DAGs, are located at the branch point between the biosynthesis of TAGs and phospholipids. Because of this, it is probably the most studied acyltransferase involved in the synthesis of TAGs. Many studies concerning the regulatory role of DGAT in TAG synthesis have been conducted. One suggestion is that the proposed rate-limiting role of DGAT in TAG synthesis may be important in the incorporation of unsaturated FAs from PtdCho to DAGs and thus to TAGs (24, 65, 124, 132). However, Fraser et al. (133) claimed that in the developing seeds of sunflower, DGAT may not have a major role in TAG formation, and that the other enzymes including DGTA may have important biosynthetic functions. Nevertheless, in mutants of A. thaliana (35) and tobacco (Nicotiana tabacum L., Solanaceae) (134), reduced DGAT activity correlated with a reduced TAG content, reduced seed oil content and induced delayed seed development. Reduced VLCFA synthesis with an accumulation of 18:3 in TAGs was also observed (35). Over-expression of DGAT cDNA in Arabidopsis also enhanced the oil content and average seed weight, which correlated with DGAT transcript levels (125). In addition, over-expression of DGAT1 from nasturtium increased the oil content in wild-type Arabidopsis and high-erucic rapeseed and canola (low-erucic acid rapeseed) (135). Lardizabal et al. (54) observed that DGAT2A from soil fungus (Umbelopsis ramanniana) increased the oil content of transgenic soybean with a concomitant increase of oil body size. Ramli et al. (136) proposed that the regulatory role of DGAT differs between different plant species. In the microsomal fractions of olive, the activity of DGAT was clearly a limiting factor in TAG formation, whereas in oil palm, accumulation of DAGs was not observed, which suggests that DGAT did not have a rate-limiting role. DGAT of oil palm has only minor control over carbon flux through the Kennedy pathway, whereas in olive DGAT exerts strong control in the Kennedy pathway (136). These studies show that DGAT activity may strongly affect the composition of FAs in TAGs and have an important role in regulating the quantity of seed TAGs.
Different DGAT isoforms and enzyme families have now been identified in various plants and organisms (24). DGAT1 and DGAT2 isoforms in seeds of the tung tree (*Vernicia fordii* Hemsl., Euphorbiaceae) had minor differences between the substrate specificity of both acyl-CoA and DAG species (19). The overall activity of DGAT2 was lower than that of DAGT1. Generally, the specificity towards a particular FA varied between different DAG species. DGAT1 and DGAT2 were shown to be enriched in different regions of the ER and to produce different types and proportions of TAGs when the enzymes were expressed individually in mutant yeast cells. DGAT2 showed a clear preference for the production of trieleostearoylgllycerol (eleostearic acid, cis-9,trans-11,trans-13-octadecatrienoic acid), the major TAG in tung tree oil and a desired lipid for industrial oil production. DGAT2 preferred the synthesis of TAGs containing PUFA, while DGAT1 showed preferential synthesis of TAGs containing monounsaturated FAs. Furthermore, DGAT1 was expressed at similar levels in leaves, flowers, and developing seeds of the tung tree, whereas DGAT2 was strongly induced in developing seeds during oil synthesis, functioning thus in the production of seed-specific TAGs containing eleostearic acid. These results suggest that DGAT1 and DGAT2 have different roles in plant cells and that the production of TAGs by these two enzymes occurs in distinct locations of the ER resulting in different TAG pools (19). DGAT1 may have a more general role in TAG metabolism in plant cells, whereas DGAT2 may be an important contributor in the processing and incorporation of unusual FAs into TAGs (19, 85). This may provide an effective mechanism for excluding the unusual FAs from membrane lipids (19). The DGAT of castor bean expressed in yeast cells showed the strongest preference for 1,2-diricinoleoyl-sn-glycerol as an acyl acceptor when oleoyl-CoA was used as the acyl donor, whereas 1,2-dioleoyl-sn-glycerol was the preferred acceptor for oleoyl-CoA compared to 1,2-dipalmitoleoyl-sn-glycerol (137). The DGAT2 of castor bean has been suggested to be more important in seed TAG synthesis than DGAT1 (99). DGAT1 has been proposed to function in the formation of a certain kind of TAG storage for FAs that are used during leaf senescence (138). In addition to DGAT1 and DGAT2, soluble (102) and ER located (139) enzymes having wax ester synthase activity and belonging to the DGAT enzyme family have been identified. However, further studies are needed to confirm the different functions of the various enzyme isoforms (85).

The acyl-CoA selectivity of DGAT depends on the composition and concentration of acyl-CoAs (24). As was the case concerning LPAAT, DGAT activity has also been shown to depend
on the FA composition of its acyl acceptor, the DAG molecule, and this selectivity varies between different plants (24). Acyl-CoA specificities and selectivities of microsomal DGAT from various plant sources are given in Tables 5 and 6. However, these tables are probably not entirely accurate because the specificity and selectivity towards a particular FA can be influenced by many factors and vary between different DAG species (19).

The DGAT of oil palm (E. guineensis) microsomes was active towards acyl-CoAs in the following order of specificity: oleic acid > palmitic acid > myristic acid > stearic acid. The DGAT specificities towards palmitic acid, stearic acid, and myristic acid were roughly the same in oil bodies (140). In developing cocoa cotyledons, DGAT also had almost equal selectivity for palmitic acid and stearic acid (89). Linoleoyl-CoA was most preferred in maize and soybean DGATs, whereas the DGATs of peanut and castor bean were most active on palmitoyl-CoA (141). DGAT1 from Echium pitardii Chev. Ex D. Bramwell (Boraginaceae), a plant that accumulates γ-linolenic acid, showed a preference for α-linolenic acid and γ-linolenic acid over linoleic acid in yeast cultures (142).

### Table 5. Acyl-CoA selectivity of DGAT from various plant sources

<table>
<thead>
<tr>
<th>Plant species a</th>
<th>Acyl-CoA</th>
<th>12:0</th>
<th>16:0</th>
<th>18:0</th>
<th>18:1(n-9)</th>
<th>18:2(n-6)</th>
<th>18:3(n-3)</th>
<th>22:1(n-9)</th>
</tr>
</thead>
<tbody>
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<td>N</td>
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</tr>
<tr>
<td>Safflower b</td>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td></td>
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<tr>
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<tr>
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</tr>
<tr>
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<td></td>
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</tr>
<tr>
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<td>1</td>
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<td>3</td>
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</tr>
<tr>
<td>Cocoa (89)</td>
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<td>Cuphea carthagenensis (150)</td>
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<td>2</td>
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<td>N</td>
<td></td>
</tr>
</tbody>
</table>

For each sample, DGAT selectivity is ranked in order of activity: 1, highest; 3, lowest; N, negligible. a References are given in parentheses. b From the review of Lung and Weselake (24). c With palmitoyl-CoA and stearoyl-CoA. d With palmitoyl-CoA and oleoyl-CoA.
<table>
<thead>
<tr>
<th>Plant species</th>
<th>Acyl-CoA</th>
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<th>14:0</th>
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<th>18:2(n-6)</th>
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<th>20:5(n-3)</th>
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For each sample, DGAT specificity is ranked in order of activity: 1, highest; 5, lowest; N, negligible. a References are given in parentheses. b From the review of Lung and Weselake (24). c Genus: Cuphea.

In mustard (Sinapis alba L., Brassicaceae) seeds the incorporation of erucic acid into TAGs, DAGs, and MAGs was found to be rapid, but appeared to be incorporated neither into lysoPtdOH, PtdOH, nor PtdCho (143). In honesty (Lunaria annua L., Brassicaceae) the long-chain monounsaturated FAs are also located in the sn-1 and sn-3 positions, and appeared to be incorporated into TAGs by DGAT and perhaps via an acyl exchange of the preformed TAG (144). However, Fehling et al. (82) found that lysoPtdOH, PtdOH, lysoPtdCho, and PtdCho were also intermediates in the incorporation of long-chain monounsaturated FAs to TAGs of honesty and mustard and the formation of TAGs via these intermediates was very rapid. Erucic acid was shown to be rapidly inserted into the sn-3 position of rapeseed by highly active DGAT, while oleic acid was incorporated into the sn-1 and sn-2 positions (145). Erucic acid
was also found in the sn-1 position, but the results did not support the theory that erucic acid would enter into this position by postsynthetic modification or transacylation of preformed TAGs. The authors did not have a clear explanation how erucic acid was incorporated into the sn-1 position (145). In addition, Taylor et al. (146) suggested that rapeseed DGAT was involved in the incorporation of erucic acid in the sn-3 position of TAG, while no incorporation of erucic acid in the sn-2 position was observed. In another study with developing rape seeds, erucoyl-CoA was also the preferred acyl donor for DGAT. The selectivity towards different acyl-CoAs was erucoyl > palmitoyl > oleoyl, and the sn-3 FA composition seemed to be primarily controlled either by the acyl-CoA pool composition or the DGAT specificity depending on the rapeseed variety (108). DGAT1 from nasturtium has been shown to be capable of synthesizing trierucylglycerol, and it can effectively use acyl-CoAs with VLCFAs (135). Based on these observations it could be suggested that DGAT has an important role in the incorporation of VLCFA into TAGs, at least in rapeseed.

DGAT may play an important role in excluding unusual FAs from the membrane phospholipids that consist of the common C\textsubscript{16} and C\textsubscript{18} FAs. DGAT exhibited 4-fold higher activity for didecanoylglycerol than for the DAG molecule species containing C\textsubscript{18} FAs in C. lanceolata, which is a plant that accumulates decanoic acid in its oil (147). Similarly in castor bean, which accumulates ricinoleic acid, diricinoleoylglycerol was the favored substrate for DGAT in TAG synthesis. In microsomal preparations of developing safflower seeds, the activities on dioleoylglycerol, dilinoleoylglycerol, and didecanoylglycerol were not substantially different but activity on dierucoylglycerol was the lowest. On the other hand, in the developing rape seeds, DGAT showed the lowest activity on DAGs containing erucic acid, but activities were low for all DAG species and the differences in the specificities were very small (147). Oleoyl-CoA was in all of the activity studies (147). Although 1,2-diacyl-sn-glycerol has been used in selectivity and specificity studies, both 1,2-diacyl-sn-glycerol and 2,3-diacyl-sn-glycerol have been shown to be substrates for DGAT in maturing safflower seeds (148). However, the 1,3-acyl-sn-glycerol did not serve as an acyl acceptor. DGAT from the plants Vernonia galamensis Less. (Asteraceae) and Stokesia laevis Greene (Asteraceae), which accumulate vernolic acid (12,13-epoxy-9-octadecenoic acid), preferred substrates containing vernolic acid, whereas DGAT from soybean preferred oleoyl-CoA and DAGs containing oleic acid (149).
In some studies such substrate selectivity and specificity shown by the preceding studies has not been found. In maturing safflower seeds, the DGAT did not exhibit significant specificity for saturated and unsaturated acyl-CoA species (148) nor strict selectivity for the acyl-CoA species consisting of palmitic acid, stearic acid and oleic acid (124), which suggests that the FA composition in the sn-3 position of safflower TAGs may depend on the composition of the acyl-CoA pool. As a conclusion it could be stated that both the composition of the acyl-CoA pool and the substrate selectivity contribute to the acylation of the sn-3 position and the formation of TAG stereoisomers.

2.2.2.3.4 Cholinephosphotransferase

CPT activates the reversible conversion of DAGs to PtdCho. Studies concerning the selectivity of CPT are scarce. Palmitic acid, stearic acid, and oleic acid are the major FAs that are channeled to PtdCho. In developing soybean seeds, the activity of CPT was highest with 1,2-dioleoyl-sn-glycerol. Enzyme activity was also increased by addition of the DAG species 18:0/18:1, 18:1/16:0, and 16:0/18:1, which is consistent with the general concept that PtdCho is synthesized in a more saturated form, followed by sequential desaturation of its oleic acid moieties (154). Triki et al. (155) also suggested that CPT in developing sunflower seeds preferred DAG species containing oleic acid in the transfer to PtdCho, but PtdCho species containing linoleic acid were preferred in the transfer to DAGs. No or only slight selectivity towards different DAG species has been reported for CPT in the safflower seeds (67).

Bafor et al. (75) suggested that DAGs containing ricinoleic acid were not totally excluded in the synthesis of PtdCho by CPT, because some ricinoleic acid was incorporated into PtdCho in the developing endosperm of castor bean. On the other hand, relatively poor DAG to PtdCho conversion was observed in developing seeds of C. lanceolata in the presence of decanoyl chains in DAGs, which suggested that CPT contributed to the exclusion of decanoic acid from PtdCho (111).
2.2.2.3.5 Lysophosphatidylcholine acyltransferase

Vogel and Browse (147) suggested that the selectivity of DGAT and CPT were not sufficient enough to explain the exclusion of unusual FAs from PtdCho and the lack of these FAs in membrane phospholipids. One enzyme transferring the modified FAs from PtdCho is LPCAT. It activates the exchange of acyl moieties between the \textit{sn}-2 position PtdCho and the acyl-CoA pool. In developing safflower cotyledons, the acyl exchange from the \textit{sn}-2 position was stimulated by the addition of free CoA to which the \textit{sn}-2 acyl chains from PtdCho were esterified (72). Oleic acid and linoleic acid in olive callus cultures were rapidly transferred to PtdCho but not to intermediates in the Kennedy pathway, which according to Hernández \textit{et al.} (156), was mainly due to the activity of LPCAT. The involvement of PDAT in transferring the unsaturated FAs to TAGs was also observed.

The selectivity of LPCAT towards acyl-CoA species in the developing seeds of various safflower varieties was as follows: oleic acid > linoleic acid > linolenic acid (72, 157). The selectivity toward stearoyl-CoA was negligible. In another study with safflower cotyledons, exogenous oleic acid was also rapidly transferred to the \textit{sn}-2 position of PtdCho and desaturated to linoleic acid, while exogenous supplied linoleic acid was more rapidly incorporated to DAGs than oleic acid (105). In contrast, sunflower cotyledons readily incorporated both oleic acid and linoleic acid into the \textit{sn}-2 position of PtdCho. Palmitic acid was strongly excluded from the \textit{sn}-2 position of PtdCho (105). Oleoyl-CoA and oleyl-glycero-3-phosphocholine were the most preferred acyl donor and acyl acceptor, respectively, in developing safflower seeds (158). The preference for linoleoyl-CoA and oleyl-CoA as well as 1-C\textsubscript{16}-\textit{sn}-glycero-3-phosphocholine, and 1-C\textsubscript{18}-\textit{sn}-glycero-3-phosphocholine was also observed by Ichihara \textit{et al.} (159). They showed that erucoyl-CoA, lauroyl-CoA, 1-octanoyl-\textit{sn}-glycero-3-phosphocholine, and 1-erucoyl-\textit{sn}-glycero-3-phosphocholine were not preferred as substrates.

LPCAT also seems to exclude unusual FAs from PtdCho. In the developing endosperm of castor bean, the incorporation of ricinoleic acid from the corresponding acyl-CoA to the \textit{sn}-2 position of PtdCho by LPCAT was negligible in the presence of competing oleyl-CoA substrate (75). In addition, LPCAT was completely inactive on erucoyl-CoA in developing rapeseeds (108). The selectivities and specificities of LPCAT seem to be quite straightforward.
LPCAT does not accept acyl-CoAs containing unusual FAs or saturated FAs as substrates but particularly prefers oleoyl-CoA and in some plants also linoleoyl-CoA.

2.2.2.3.6 Phospholipid:diacylglycerol acyltransferase

PDAT transfers an acyl chain from PtdCho into a DAG to form a TAG. It offers another mechanism for the transfer of unusual FAs from PtdCho and the exclusion of these FAs from membrane phospholipids, and it has been proposed to have an important role in transferring unusual FAs to TAGs at least in some oilseeds (69, 70). The involvement of PDAT in transferring unsaturated FAs to TAGs in olive callus cultures was also observed (156). PDAT from Arabidopsis root and leaves utilized widely different PtdCho species as acyl donors and accepted acyl groups ranging from C_{10} to C_{22} (70). The highest activities were observed for FAs containing several double bonds, epoxy, or hydroxy groups. These FAs included linolenic acid, ricinoleic acid, vernolic acid, and arachidonic acid [20:4(n-6)], which shows that this enzyme has selectivity towards unusual FAs. PDAT used FAs from both the sn-1 and sn-2 positions of PtdCho but had a 3-fold preference for the sn-2 position (70). In seeds of a knockout mutant of A. thaliana, PDAT activity did not play a major role in TAG synthesis (160), which suggests that PDAT may not have an important role in quantitative TAG formation.

The PDAT of Crepis palaestina Bornm. (Asteraceae), which accumulates vernolic acid in the oil, preferentially incorporated vernolic acid from PtdCho into TAGs, whereas the PDAT of castor bean preferred both ricinoleic acid and vernolic acid (69). In sunflower, most of the FAs were incorporated into DAGs regardless of the type of FA (oleic acid, ricinoleic acid or vernolic acid). It is clear that the FA specificity of the PDAT enzyme with respect to the FA that is transferred from PtdCho into TAG varies between different plant species (69). Banaś et al. (161) suggested that PDAT has a major role in removing ricinoleic acid and vernolic acid from phospholipids in castor bean and C. palaestina. However, vernolic acid, crepenynic acid (9-octadecen-12-ynoic acid) and decanoic acid were primarily removed from phospholipids by phospholipases in Euphorbia lagascae (accumulating vernolic acid) seeds, Crepis rubra L. (Asteraceae) (accumulating crepenynic acid) seeds, and elm (genus Ulmus, Ulmaceae) (accumulating decanoic acid) seeds, respectively (161). Results showed that the relative
contribution of PDAT in the removal of unusual FAs from phospholipids varies dramatically between oilseeds accumulating unusual FAs, even between species accumulating the same type of unusual FA to approximately the same level in the oil. Individual PDAT1 or PDAT2 homologues in *A. thaliana* were shown not to play a major role in the incorporation of hydroxy FAs into TAG (162).

2.2.2.3.7 Desaturases and other enzymes modifying fatty acids

The Δ⁶-desaturase used almost solely linoleic acid in the sn-2 position of PtdCho as substrate in maturing cotyledons of borage, which contains γ-linolenic acid, and showed only minor activity on linoleic acid in the sn-1 position (61). On the other hand, the Δ¹₂-desaturase utilized oleic acid in both the sn-1 and sn-2 positions. In developing castor bean, ricinoleic acid was formed almost exclusively from an oleoyl moiety located in the sn-2 position of PtdCho, whereas desaturation of an oleoyl moiety to a linoleoyl chain occurred in both the sn-1 and sn-2 positions (75). It seems likely that the modifying enzymes differ in their action between different FAs and sn-positions in PtdCho. The activity of the desaturation enzymes was different between the high-oleic acid and very-high-linoleic acid safflower seeds. The very-high-linoleic acid variety desaturated oleic acid in PtdCho rapidly, whereas only little desaturation of oleic acid was observed in the high-oleic variety (157).

2.3 ENVIRONMENTAL FACTORS AFFECTING LIPID METABOLISM

A vide variety of environmental factors have been shown to affect the lipid metabolism in plants (28). Most of the studies related to the environmental effects on lipid metabolism in plants particularly concern adaptations and changes occurring in leaves and roots and more specifically in chloroplasts and in phospholipids i.e. at the membrane level. Thus the studies mostly cover the prokaryotic lipid synthesis pathways occurring in plastids. The environmental effects on storage TAGs and especially on the stereospecific distribution of FAs in storage TAGs, which is the emphasis of this review, have not been studied extensively. The effects of light and temperature are discussed in the following sections. The effects of temperature and
light are partly connected to each other. Temperature causes alterations to the cell membranes (28) and membrane fluidity affects the rate of photosynthesis (163). Soil constituents such as fertilizers, atmospheric constituents such as oxides of nitrogen, ozone and CO$_2$, xenobiotics such as herbicides, as well as physical damage and pest attacks can also affect lipid metabolism in plants (28). In addition, water deficit stress has been shown to induce lipid changes in plants mainly because it affects the cell membranes (164).

2.3.1 GEOGRAPHICAL AND CLIMATIC FACTORS

Plants grown in different places have been shown to have differences in oil yield, in FA composition, and in TAG stereoisomer composition probably due to the different environmental conditions, in particular temperature and light. Seeds of different soybean cultivars produced slightly more oil in the southern areas of the USA (Mississippi) than in the northern areas (Indiana), but the seeds were significantly smaller in size in the southern area (165). Thus in the south the oil content per seed was only 51 % of that in the north. Seeds produced in the south had lower proportions of myristic acid and linolenic acid and higher proportions of oleic acid than seeds from the north. The results indicated that a higher environmental temperature reduced the concentration of linoleic acid in soybean oil (165). Differences in oil content and FA composition were also observed in cloudberry (*Rubus chamaemorus* L., Rosaceae) and crowberry (*Empetrum nigrum* L., Empetraceae) grown in different locations in Finland (166). Cloudberry seeds had lower oil content and seeds of crowberries higher oil content in the south compared with the berries grown in the north. The seed oil of the crowberry from the north contained more TAG ACN (acyl carbon number) species of 52 and less those of 54 than berry oils from the south. The proportion of linoleic acid was the highest and the proportion of $\alpha$-linolenic acid the lowest in both cloudberrys and crowberries from the north (166). However, the berries were not cultivated but wild, so other variables than environmental factors may also have affected the results.

Different weather conditions in the three consecutive years caused significant differences in TAG profiles of hazelnuts grown in Portugal (167). The TAG profile of hazelnut (*Corylus avellana* L., Betulaceae) oil in one particular year was characterized by higher proportions of
dioleoylstearoylglycerol (O/O/S) and lower proportions of palmitoyllinoleoyloleoylglycerol (P/L/O) when compared with two other years. The difference was caused by high temperatures. The other two years had different proportions of L/O/O, P/L/L, and P/O/O, but the climatic effects causing these differences were not clear (167). A similar study with walnut (Juglans regia L., Juglandaceae) showed that oils originating from three different harvesting years also had different TAG profiles due to the different weather conditions. One year was characterized by high L/L/O and O/O/L proportions, whereas the other two years had different proportions of L/L/L and linolenoyllinoleoyloleoylglycerol (Ln/L/O) (168).

Vichi et al. (90) found that climatic and geographical conditions affected the positional distribution of the FAs of TAGs in olives grown in northeastern Italy. However, the results were obtained from different cultivars, which might have affected the results. Olive oils harvested in three consecutive years from different climatic areas of Umbria, Italy, characterized by either mild or more severe conditions, could be separated based on the different stereospecific compositions of TAGs (169). In contrast, the results of Damiani et al. (170) showed that the stereospecificity of the biosynthetic reactions involved in sunflower seed oil TAG formation are not affected by variety or growth conditions, which included different places of growth and irrigation. The relationship between changes in the FA composition of TAGs and the concomitant changes in the FA composition of each sn-position was taken into account. However, in the studies of Vichi et al. (90) and Damiani et al. (170), the overall stereospecific distribution of FAs in all TAGs was determined in general and the stereospecific compositions of individual TAG species were not quantified. Thus, the results do not show whether the stereoisomer composition of individual TAGs differed between varieties and different growth conditions.

2.3.2 THE EFFECTS OF LIGHT

Very few studies exist that concern solely the effects of light on plant lipid biosynthesis. The effect of light is mostly associated with the rate of photosynthesis. Light can stimulate the activities of certain enzymes such as acetyl-CoA carboxylase (28), which is a rate limiting factor in FA synthesis. In light, FA synthesis is relatively high whereas in dark synthesis is low.
due to the ACCase activity (22). In photosynthetic oil plant tissues such as the mesocarp of avocado and olive the photosynthetic metabolites for TAG synthesis originate both from the leaves as well as from the fruit tissue itself (171). However, light has been found to stimulate mainly the green epicarp of olive and not the yellow mesocarp (28). In callus cultures of developing olive fruit tissues, low light reduced oleic acid desaturation by affecting microsomal FAD2, which desaturates oleic acid to linoleic acid (156). Developing green oilseeds such as rapeseed and soybean also have an ability to utilize light for photosynthesis, which expresses and activates seed enzymes involved in FA and oil synthesis (29, 30, 172, 173).

2.3.3 THE EFFECTS OF TEMPERATURE

Alterations in temperature may cause several changes in membrane lipids. These include lipid remodeling, such as changes in the positional distribution of FAs, changes in cis/trans isomerism, changes in the chain lengths of FAs, changes in unsaturation, other modifications to acyl chains, changes in the lipid class proportions, and changes in the membrane lipid to protein ratio (28). The increase in cis-unsaturation at lower temperatures is a phenomenon which has been widely studied and is generally acknowledged (64). It plays an important role in determining the membrane fluidity at low temperatures and therefore in the survival of plants (174-176). The chill resistance and acclimatization to lower temperatures of higher plants is closely related with the levels of cis-unsaturated FAs of phospholipids in chloroplast membranes (174, 176, 177). Low temperatures have been shown to affect the transcription of plastidial FA desaturases (178). Specifically, the proportions of unsaturated FAs in the sn-1 position of phospholipids are thought to be particularly important in maintaining membrane functionality (177). Desaturation lowers the melting point of FAs and thus improves the membrane fluidity. For example, the desaturation of stearic acid to oleic acid lowers the melting point of the FA from 69 °C to 13.4 °C (14). The positions of the double bonds in the chain also affect the melting point of FAs and the lipid molecules containing them. For C_{18} FAs, melting points are lowest when the double bonds are near the center of the acyl group, C_{9} – C_{11} (14).

Many possible mechanisms are thought to cause this cold-induced desaturation. FA desaturase activity has been suggested to rise due to the increased supply of substrates such as oxygen,
increased expression of enzymes, and increased activation of the existing enzymes. The primary signal for gene transcription activation at cold temperatures may be a decrease in membrane fluidity (28). The effect of oxygen as a substrate has been proposed to be caused by its increased solubility in water at lower temperatures (28). However, changes in dissolved oxygen concentration affected neither the rate of oleoyl desaturation in PtdCho of safflower microsomes nor the relative rates of FA synthesis and desaturation in intact safflower cotyledons (179). Trémolières et al. (180) were of the opinion that it is evident that the synthesis of unsaturated FAs in plants is genetically controlled and that the physical factors such as oxygen concentration and membrane fluidity have only minor effects, and in this regard, the environmental changes can cause opposite changes in different plants and in different tissues. The amount of enzymes can be increased by the increased transcription, increased translation or decreased catabolism at cold temperatures. Cold-acclimation of plants is dependent not only on desaturase activity but also on the up-regulation and balance of various enzymes including those involved in FA and lipid synthesis (176, 181).

Sunflower and rapeseed display very different responses to variations in temperature and light intensity. The level of linoleic acid was increased and oleic acid decreased in maturing sunflower seeds as temperature and light intensity were lowered, but the total lipid content was not changed (180). However, under similar conditions, a decrease in lipid content and only a slight change in FA composition were observed in rapeseed. In rapeseed, both the total production of FAs as well as oleic acid were increased with increasing temperature and light intensity, which may result from the photosynthetic capacity of the seed and siliqua (180). As in rapeseed, the increase in temperature also increased the amounts of synthesized DAG and TAG in olive fruit and callus cultures (182) and reduced oleic acid desaturation and the content of PUFA (156). A different temperature effect was observed on plastidial lipids compared to microsomal lipids. Proportions of linoleic acid decreased in microsomal PtdCho, phosphatidylethanolamine, PtdOH, DAGs, and TAGs as the temperature rose, but the proportions remained essentially unchanged in plastidial monogalactosyldiacylglycerol and digalactosyldiacylglycerol. The amount of linolenic acid increased in phosphatidylethanolamine, PtdCho, and PtdOH, but decreased in monogalactosyldiacylglycerol and diacyldigalactosylglycerol (156). In another study with oil palm (*E. guineensis*) and olive callus cultures, an increase in temperature also caused an increase in TAG synthesis and a
decrease in PUFA synthesis (49, 136). At higher temperatures, both calli produced increased amounts of oleic acid, but in oil palm calli the amount of palmitic acid was also increased, which suggests that the relative activities of KAS I and KAS II were changed (49). High temperatures (30-35 °C) reduced both microsomal FAD2 and plastidial FAD7 desaturases in olive callus cultures (156).

In developing flaxseed (*Linum usitatissimum* L., Linaceae) and soybean cotyledons a change in temperature caused rapid and marked alterations in the proportions of unsaturated FAs in PtdCho and DAGs (183). The proportion of oleic acid increased when the temperature increased, whereas the proportions of linoleic acid and linolenic acid were lowered. Changes in the compositions of phosphatidylethanolamines and TAGs were small (183). The activity of stearoyl, linoleoyl and oleoyl desaturases decreased dramatically in cultured developing soybean seeds as a result of changes in growth temperature from 20 °C to 35 °C (184). Both linoleoyl and oleoyl desaturases had negligible activity at 35 °C compared to 20 °C. Plastidial stearoyl-ACP desaturase and CPT were most active in preparations at 20 °C. CPT activity was twofold lower at 25 °C, and threefold lower at 35 °C. Although the enzymes for FA desaturation and PtdCho synthesis were rapidly modulated in response to altered growth temperatures, the enzymes for FA synthesis and elongation were not (184). CPT activity was also rapidly decreased in maturing soybean seed cotyledons cultured at 35 °C compared to cotyledons at 20 °C, which may be due to either increased turnover or decreased synthesis of the enzyme at elevated temperatures (154). The oleoyl chains of TAGs in both the microsomal and oil-body fractions of developing sunflower seeds were unsaturated to linoleoyl chains when the seeds were transferred to low temperatures (78). The sn-2 FAs of TAGs were preferentially modified to linoleic acid but unsaturation also occurred in the sn-1 and sn-3 positions. The contents of lipids and TAGs remained unchanged, and no *de novo* synthesis of FA occurred (78). These observations could imply that the transfer of unsaturated FAs from PtdCho to DAGs by CPT and further to TAGs is increased at lower temperatures and decreased at higher temperatures. Temperature has also been shown to affect the acyl-CoA selectivity of DGAT (24). The incorporation of saturated FAs into glycerolipids was preferred at higher temperatures in cocoa microsomes (24). In *Cuphea carthagenensis* (Jacq.) J.F. Macbr. (Lythraceae) the increase in temperature induced higher activity of DGAT on lauroyl-CoA than oleoyl-CoA, whereas in maize, the selectivity of DGAT on oleoyl-CoA was enhanced (150). In rapeseed and
canola (*B. napus*) DGAT showed increased preference for erucic acid when the temperature was raised (150). The lipid biosynthesis rate increased with the increasing temperature in safflower cotyledons (179). An increase in temperatures also increased the proportions of stearic acid and oleic acid and decreased the proportions of linoleic acid in safflower cotyledons. This change in FA compositions was more rapid in PtdCho than in TAGs. The results suggested that the different temperature responses of FA synthesis and oleic acid desaturase could explain the sensitivity of the FA composition of an oil to temperature in oil seeds (179).

In maturing safflower seed preparations, with a rise in incubation temperature, LPAAT was observed to use more actively unsaturated acyl-CoA species than saturated for the acylation of the *sn*-2 position of lysoPtdOH in safflower seeds (113). In addition, safflower LPAAT preferentially utilized unsaturated the lysoPtdOH species. However, this acyl-acceptor specificity may not affect the FA composition of the *sn*-1 position, because acylation by LPAAT is much faster than the GPAT reaction and hence lysoPtdOH does not accumulate. This data would suggest that atmospheric temperature may affect the degree of unsaturation in the *sn*-2 position of PtdOH also *in vivo*. If the *sn*-1 FAs become more unsaturated, this would indicate that growth temperature would have to be a regulatory factor in GPAT specificity, because LPAAT specificity towards lysoPtdOH is probably not important regarding the degree of unsaturation of the *sn*-1 FAs (113). Temperature affected the specificity and selectivity of GPAT for different acyl-CoA species but it was considered to be a rather minor factor in the regulation of the FA composition in the *sn*-1 position (106). The specificity of GPAT on linoleoyl chains was decreased with increasing incubation temperature. In tomato (*Lycopersicon esculentum* Mill., Solanaceae) leaves, the overexpression of GPAT that exhibited selectivity to oleic acid over palmitic acid increased the proportions of *cis*-unsaturated FAs in the thylakoid membrane in chloroplasts, which was beneficial for the recovery of chill-induced photoinhibition and photosynthesis (185).
2.4 SUMMARY

Even though knowledge concerning lipid biosynthesis in plants has increased from a basic understanding of pathways to the structural determination of individual enzymes and the identification of the corresponding genes, the regulation and control of these pathways as a whole is not clear. The nonrandom distribution of FAs in TAGs is influenced by a number of factors, including the composition of the de novo synthesized acyl-CoA pool, the activity and selectivity of the acyltransferases on particular acyl donors and acceptors, the activity of desaturases and other enzymes involved in FA modification, acyl exchange between the sn-2 position and to a lesser extent the sn-1 position of PtdCho and available acyl-CoAs, particularly oleoyl-CoA, PDAT transacylase, and post-synthetic re-modeling of synthesized TAGs. The relative importance of these factors varies according to the plant species and varieties.

Nevertheless, the stereospecific distribution of FAs in TAGs is probably mainly determined by the selectivity and activity of acyltransferases on certain acyl donors and acceptors. Generally, with few exceptions, GPAT as well as DGAT can utilize both saturated and unsaturated acyl donors, although the preference is for saturated FAs. LPAAT is specific for oleoyl-CoA and linoleoyl-CoA and does not use saturated substrates with the exception of plants accumulating medium chain saturated FAs. VLCFAs are also generally excluded from the sn-2 position. Modification and hydrolysis of acyl chains located in the sn-2 position of PtdCho are also essential factors in the non-random distribution of FAs in plant TAGs. It seems that the post-synthetic remodeling of TAGs does not have a significant impact on the stereospecific distribution of FAs of TAGs, but this issue has been little studied.

Most of the studies are related to the membranes of non-storage tissues and particularly plastidial membrane functions at low temperatures. Synthesis and desaturation of FAs are well regulated by external factors such as temperature and light intensity at least in some plants. The changes in lipid synthesis caused by environmental factors are essentially caused by the genetically programmed activation of the enzymes or their biosynthesis. It is well known that low temperatures induce the increase of desaturated FAs, whereas an increase in temperature increases the amount of TAGs in many plants. However, studies concerning the environmental effects on the stereospecific distribution of FAs of TAGs are scarce. Specifically, long term
environmental effects on the composition of storage TAGs in plants have not been widely studied. The response to environmental changes may vary from one plant to another.

Previous studies have almost exclusively been conducted by analyzing the overall \( sn \)-specific distribution of FAs in the TAG pool, in which case the data concerning the stereospecific composition of particular TAG molecules with distinctive FA compositions is lacking. It has been clearly shown in this thesis that the selectivities of the enzymes toward substrates can generate distinctive TAG species and thus the generalizations concerning the stereospecific TAG composition may not result in accurate conclusions. More studies that combine a detailed stereoisomer analysis of plant TAGs with consideration of the genetic background and environmental factors are needed.
3 AIMS OF THE STUDIES

The aim of the research project in general was to develop and test different HPLC/MS methods for the analysis of the positional distribution of FAs of TAGs and apply these methods in the analysis of different nutritional fats and oils.

The first aim was to combine effective HPLC separations with MS methods in order to obtain detailed information concerning the regioisomer compositions of TAGs. The objective for the HPLC method development was successful separation of TAGs having the same molecular weight (MW) but different FA compositions. TAGs containing different double bond positional isomers of FAs such as vaccenic acid [18:1(n-7)] and oleic acid [18:1(n-9)] or α-linolenic acid [18:3(n-3)] and γ-linolenic acid [18:3(n-6)], which can not readily be differentiated by MS, were in particular focus.

The second aim was to test and develop different MS methods for the regioisomer analysis of TAGs. The objective for MS method development was to achieve a practical MS method with proper differentiation of regioisomers and small deviation between parallel analyses.

The third aim was to apply these methods for the analysis of different nutritional fats and oils. The project was expanded to the analysis of berry oils. Differences in the regiospecific distribution of FAs in TAGs containing FA double bond positional isomers, vaccenic and oleic acids or α- and γ-linolenic acids, were analyzed from a wide selection of currant seed oils and sea buckthorn pulp oils. In addition, the differences between different berry varieties and subspecies as well as the effect of different growth places and growth years having different climatic conditions were studied.
4 MATERIALS AND METHODS

4.1 ABBREVIATIONS AND NOMENCLATURE

A/A/B denotes a TAG containing two different FAs, A and B, in unknown \textit{sn}-positions. Regioisomers are written as ABA (symmetric regioisomer; B is in the \textit{sn}-2 position) and \textit{sn}-AAB + \textit{sn}-BAA (B is in the \textit{sn}-3 or \textit{sn}-1 position, respectively). In publications, \textit{sn}-ABA also denotes regioisomer ABA (I, II) and \textit{sn}-AAB denotes both \textit{sn}-AAB + \textit{sn}-BAA (I).

4.2 MATERIALS

Finnish rapeseed oil (I, V) and sunflower seed oil (I, V), as well as palm oil from Ghana (V) were purchased from local grocery stores. Lard was obtained from the Atria Group plc (Seinäjoki, Finland) and was purified by Raisio plc (Raisio, Finland). Black currant seed oil in study II was extracted from ground seeds by supercritical carbon dioxide (Aromtech Ltd., Tornio, Finland). For study III, black currant (\textit{Ribes nigrum} L.) varieties Ola, Melalahti, and Mortti, green currant (\textit{R. nigrum}) variety Vertti, red currant (\textit{Ribes rubrum} L.) variety Red Dutch, and white currant (\textit{R. rubrum}) variety White Dutch were collected from controlled cultivation sites in southwestern Finland (60° 23’ N, 22° 33’ E) and northern Finland (66° 35’ N, 26° 01’ E) in three consecutive years (2005–2007). In study II, sea buckthorn (\textit{Hippophaë rhamnoides} L.) varieties Tytti and Terhi of ssp. \textit{rhamnoides} were collected from controlled cultivation sites in Sammalmäki (60° 23’ N, 22° 09’ E) in southern Finland, Kittilä (68° 01’ N, 24° 38’ E) in northern Finland, and Quebec (46° 47’ N, 71° 23’ E) in Canada in 2007 and 2008. Wild berries of ssp. \textit{rhamnoides} were collected from Pyhämaa (60° 51’ N, 21° 11’ E) in southern Finland as well as Kemi (65° 37–40’ N, 24° 12–15’ E) and Taapajärvi (67° 07’ N, 24° 42’ E) in northern Finland in 2007 and 2008. The wild sea buckthorn bushes in Taapajärvi had been transferred from Pyhämaa. The wild berries of ssp. \textit{sinensis} were collected from three altitudes 2000 m, 2500 m, and 3000 m in the province of Sichuan (31° 01’ N, 106° 54’ E) in China in 2006, 2007, and 2008, and from Sammalmäki in 2007 and 2008. Because of the exceptional weather conditions and cold summer, the berries from Kittilä and Taapajärvi had to be picked slightly unripe. The seed oil of black currant variety Mortti and pulp oil of sea...
buckthorn variety Tytti were used also in study V.

FA methyl esters (FAMEs) were quantified and identified using FAME standard mixture (68D, Nu-Check Prep Inc, Elysian, MN, USA) and trinonadecanoylglycerol as an internal standard (I). The following pure TAG regioisomers were used as reference compounds: 1(3),2-dilinoleoyl-3(1)-α-linolenoyl-sn-glycerol (sn-AlaLL + sn-LLAla) (II, III), 1,3-dilinoleoyl-2-α-linolenoyl-sn-glycerol (LAlaL) (II, III), 1(3),2-dilinoleoyl-3(1)-γ-linolenoyl-sn-glycerol (sn-GlaLL + sn-LLGla) (II, III), 1,3-dilinoleoyl-2-γ-linolenoyl-sn-glycerol (LGlal) (II, III), 1(3),2-dilinoleoyl-3(1)-oleoyl-sn-glycerol (sn-LLO + sn-OLL) (I, V), 1,3-dilinoleoyl-2-oleoyl-sn-glycerol (LOL) (I, V), 1(3),2-dioleoyl-3(1)-linoleoyl-sn-glycerol (sn-LOO + sn-OOL) (I, V), 1,3-dioleoyl-2-linoleoyl-sn-glycerol (OLO) (I, V), 1(3),2-dipalmitoleoyl-3(1)-oleoyl-sn-glycerol (sn-PoPoO + sn-OPoPo) (IV, V), 1,3-dipalmitoleoyl-2-oleoyl-sn-glycerol (PoOPo) (IV, V), 1(3),2-dipalmitoleoyl-3(1)-vaccenoyl-sn-glycerol (sn-PoPoV + sn-VPoPo) (IV, V), 1,3-dipalmitoleoyl-2-vaccenoyl-sn-glycerol (PoVPo) (IV, V), 1(3),2-dioleoyl-3(1)-palmitoyl-sn-glycerol (sn-POO + sn-OOP) (I, V), 1,3-dioleoyl-2-palmitoyl-sn-glycerol (OPO) (I, V), 1(3),2-dipalmitoyl-3(1)-oleoyl-sn-glycerol (sn-PPO + sn-OPP) (I, V), 1,3-dipalmitoyl-2-oleoyl-sn-glycerol (POP) (I, V), and 1(3),2-dioleoyl-3(1)-decanoyl-sn-glycerol (sn-COO + sn-OOC), and 1,3-dioleoyl-2-decanoyl-sn-glycerol (POP). The reference TAGs were purchased from Sigma-Aldrich Co. (St.Louis, MO, USA) and Larodan Fine Chemicals (Malmö, Sweden). Purity of the reference TAGs was not verified before use.

Meteorological data from the harvesting years were obtained from the Finnish Meteorological Institute (III, IV) and from the National Climate Data and Information Archive of the Environment Canada (IV). The data covered temperature, radiation, and precipitation variables and were obtained by the weather stations closest to the harvesting sites. The harvesting dates varied between the years and between the berry samples, and this was taken into account when the weather variables were calculated. No daily weather data from Sichuan was available, but average weather variables were obtained from similar areas in western Sichuan covering the years 1963–1980 (IV).
4.3 SAMPLE PREPARATION

Berries were stored frozen at -18 °C until the seeds of currants (III) or pulp and peel of sea buckthorn berries (IV) were isolated. Seeds were removed from the berries mechanically and oil was extracted from the seeds (III) and the pulp (IV) using a modified Folch extraction procedure (186, 187). A sample of seeds or pulp was homogenized in methanol and chloroform (10 mL and 20 mL for 1 g of sample, respectively) and filtered. The procedure was repeated and the residue was extracted with another similar portion of methanol/chloroform. The combined filtrates were washed with 0.88% potassium chloride in water (one-fourth of the volume of the filtrate) and twice with methanol/water (1:1) (one-fourth of the volume of the filtrate) before evaporation of the solvent. The extracted oils were stored at -18 °C until analyzed. Oil was extracted once from each berry sample.

4.4 FATTY ACID ANALYSIS

FAMEs were prepared by sodium methoxide catalyzed transesterification (188) in duplicate and analyzed by GC (PerkinElmer Autosystem, Norwalk, CT, USA) using a DB-23 column (30 m × 0.32 mm i.d., 0.25 μm film thickness, Agilent Technologies, Inc, Santa Clara, CA, USA) and a flame ionization detector (FID) (I). FAMEs (0.33 mg/ml) were dissolved in hexane and injection volume was 1 μl. The temperatures of the injector and FID were 270 °C and 280 °C, respectively. The temperature program of the column oven was as follows: 1 min at 130 °C, raised at 6.5 °C/min to 170 °C, raised at 2.8 °C/min to 215 °C, 12 min at 215 °C, raised at 40 °C/min to 230 °C, and 3 min at 230 °C. Helium was used as the carrier gas. The FA compositions of rapeseed oil, sunflower seed oil, and lard were calculated from the averaged results obtained by a direct inlet ammonia negative ion chemical ionization (NICI) tandem mass spectrometric (MS/MS) method and compared with the GC-FID results in order to ensure the correctness of the MS/MS results (I).
4.5 LIQUID CHROMATOGRAPHY

4.5.1 ANALYTICAL CHROMATOGRAPHY

One RP-HPLC Discovery™ HS C18 column (I) and two Ascentis™ C18 columns (IV) (250 mm × 4.6 mm i.d., particle size 5 μm; Supelco Inc. Bellefonte, PA, USA) were used with an Hitachi L-6200 Intelligent Pump (Tokyo, Japan) (I) and Acquity Ultra Performance LC® (UPLC®) equipment (Waters Corp., Milford, MA, USA) (IV). The flow rates were 0.85 mL/min (I) and 1.0 mL/min (IV) of which approximately 0.3 mL was introduced to the MS. The gradients consisted of acetone in acetonitrile: 60 to 90% in 70 minutes (I) and 60 to 80% in 60 minutes (IV).

Acquity UPLC equipment and an Acquity BEH C18 column (100 mm × 2.1 mm i.d., particle size 1.7 μm, Waters Corp.) were used in studies I and V. The flow rate was 0.4 mL/min (I, V). The gradients consisted of acetone in acetonitrile (0 to 70% in 22 min) (I, V) (erratum in article I) and (A) methanol + 0.1% ammonium hydroxide and (B) isopropanol + 0.1% ammonium hydroxide (I) with the following program: A/B (100:0), isocratic to 1 min, linear to 3 min (98:2), isocratic to 6 min, linear to 10 min (97:3), isocratic to 18 min, linear to 35 min (60:40). Ammonium hydroxide was added in order to produce ammonium adduct ions by MS.

Silver ion HPLC (Ag-HPLC) columns were prepared according to Christie (189, 190) (II, III). The Ag-HPLC system consisted of either one (II) or two (III) silver ion bonded Nucleosil® 100-5 SA columns (250 mm × 4.6 mm i.d.; Macherey-Nagel GmbH & Co. KG, Düren, Germany) and Acquity UPLC equipment. The flow rate was 0.8 mL/min of which approximately 0.3 mL was introduced to the mass spectrometer. The gradient was modified from earlier methods (190, 191) and consisted of (A) acetone and (B) acetone/acetonitrile (4:1, v/v). The gradient program was as follows: A/B (100:0), isocratic to 5 min, linear to 30 min (70:30), linear to 40 min (47:53), linear to 41 (0:100), isocratic to 49 min.

In all HPLC runs, the reference TAGs were injected simultaneously onto the column in order to create similar chromatograms from the reference TAGs and sample oils (I, II, III, IV, V). The fragment ion (MS) or product ion (MS/MS) intensities were taken from the averaged mass
spectra, which were selected across the peak in the total ion chromatogram. The mass spectra were obtained from the segment of the peak where there was no overlapping of different TAG species. Mass spectra were extracted from the peaks in a similar manner from both the reference TAG chromatograms and the sample oil chromatograms in order to avoid possible fluctuations in product ion ratios across the peak, which was shown to happen in HPLC/APCI-MS analyses (192). However, in MS studies (I, II), it was impossible to completely follow this procedure, because of the overlapping TAGs that produced the same DAG ions.

4.5.2 FRACTIONATION OF BLACK CURRANT SEED OIL

The black currant seed oil was fractionated in study II because it was assumed that the abundance of different TAG species would complicate the silver ion HPLC analyses. Fractionation was conducted by a Discovery HS C18 guard column (20 mm × 4.0 mm, 5 μm particle size; Supelco Inc.), the Ascentis C18 column (250 mm × 4.6 mm i.d. particle size 5 μm; Supelco Inc.), the Hitachi L-6200 Intelligent Pump, and an evaporative light scattering detector (Sedex 75, S.E.D.E.R.E., Alfortville, France). The flow rate was 0.85 mL/min and the following linear gradient was used: acetone in acetonitrile increased from 40 to 90 % in 70 min. A total of eight fractions were collected and fraction 4, which contained TAGs 54:7, was used in the analyses.

4.6 MASS SPECTROMETRY

4.6.1 DIRECT INLET AMMONIA NEGATIVE ION CHEMICAL IONIZATION

The MW distribution as ACN:DB species of TAGs were determined in quadruplicate by the direct inlet ammonia NICI-MS method (193) using a triple quadrupole mass spectrometer (TSQ-700, Finnigan MAT, San Jose, CA, USA) (I). Parameters were set according to earlier optimizations (194). The regioisomer quantification was conducted in quadruplicate by the direct inlet ammonia NICI-MS/MS method using argon as the collision gas (195-197) (I). Spectra were scanned from \( m/z \) 750 to 1000 in NICI-MS and from \( m/z \) 200 to 1000 in NICI-
MS/MS with a scan time of 0.5 s. Both molecular weight distributions and regioisomer compositions of TAGs were calculated using the MSPECTRA program (197, 198) (Nutrifen, Naantali, Finland). The NICI-MS analyses were corrected for the natural occurrence of only the $^{13}\text{C}$ isotope.

### 4.6.2 METHODS BASED ON ELECTROSPRAY IONIZATION

Electrospray ionization (ESI) MS and MS/MS analyses were conducted using a Micromass® Quattro Premier™ tandem quadrupole mass spectrometer (Waters Corp.). ESI-MS and ESI-MS/MS methods had already been used in many earlier studies in the analysis of TAGs (199-205). The MS parameters were optimized for each study (I, II, III, IV). Nitrogen was used as the desolvation and cone gases and argon as the collision gas (I, II, III, IV). Ammonium adducts of TAGs ([M+NH$_4^+$]) were formed in the ionization source either by introducing ammonia water (I, III) to the post-column flow or ammonia gas (purity 5.0; Linde AG, Munich, Germany) to the nebulizer gas flow (nitrogen) (IV). The amount of ammonia water and the mass flow of ammonia gas were optimized to generate a maximal intensity for [M+NH$_4^+$] ions. Silver ion adducts ([M+107Ag]$^+$ and [M+109Ag]$^+$) were produced by introducing AgNO$_3$ to the post-column flow (II). Product ion spectra from $m/z$ 200 to 1000 (I), from $m/z$ 590 to 610 (III) or from $m/z$ 500 to 600 (IV) of the selected [M+NH$_4^+$]$^+$ precursor ions were collected in the positive ionization mode. For ESI-MS and ESI-MS/MS analyses in study II, mass spectra ($m/z$ 500-1000) were collected in the positive ionization mode. In the MS/MS analyses, both silver ion isotope adducts [M+107Ag]$^+$ and [M+109Ag]$^+$ were used as precursor ions (II).

### 4.6.3 METHODS BASED ON ATMOSPHERIC PRESSURE CHEMICAL IONIZATION

Atmospheric pressure chemical ionization (APCI) MS and MS/MS analyses were conducted using a TSQ-700 connected to HPLC (I). The suitability of APCI-MS for the analysis of TAGs had been shown in earlier studies (191, 192, 206, 207). Nitrogen was used as the sheath and auxiliary gas. Other APCI-MS and -MS/MS analyses were conducted using a Quattro Premier (I, II). Nitrogen was used as the desolvation and cone gas (I, II). Argon was used as the
collision gas in MS/MS analyses (I, II). The MS parameters were optimized for each study (I, II). Mass spectra in the ranges \( m/z \) 200-1100 (I) and \( m/z \) 500-1000 (II) were collected in the positive ionization mode. The results of APCI-MS analyses in study I were corrected for the natural occurrence of both the \(^{13}\text{C}\) isotope and the \(^{18}\text{O}\) isotope.

In study V, APCI was used in the negative ionization mode using ammonia (purity 5.0; Linde AG) as the nebulizer gas. Ammonia negative ion APCI (NIAPCI) MS/MS analyses were performed on a Quattro Premier and the MS parameters were optimized (V). Nitrogen was used as the desolvation and cone gases. Ammonia gas was used as the nebulizer gas and the flow was optimized to generate maximal intensity for negative [M-H]\(^{-}\) ions. Argon was used as the collision gas. The product ion spectra were collected in the range of \( m/z \) 380 to 510 depending on the precursor ion (V).

### 4.6.4 IONS EXAMINED

In the direct inlet ammonia NICI-MS analyses the ACN:DB species were determined from [M-H]\(^{-}\) ions, and in the NICI-MS/MS analyses the regioisomer compositions were calculated based on [RCOO]\(^{-}\) and [M-H-RCOOH-100]\(^{-}\) ions (I). [M-H-RCOOH-100]\(^{-}\) Ions were also used in ammonia NIAPCI-MS/MS analyses (V). In APCI-MS analyses the regioisomer compositions were calculated based on [M-RCOOH]\(^{+}\) ions (I, II). The same ions were examined in APCI-MS/MS analyses (I). [M+NH\(_{4}\)-RCOONH\(_{4}\)]\(^{+}\) Ions (I, III, IV) as well as [M+\(^{107}\text{Ag}\)-RCOOH]\(^{+}\) and [M+\(^{109}\text{Ag}\)-RCOOH]\(^{+}\) ions (II) were used in the regioisomer analyses conducted by ESI-MS/MS.

### 4.6.5 CALIBRATION CURVES

Analyses of reference TAGs were conducted in triplicate (I) and in quadruplicate (II, III, IV, V) as pure regioisomers and three regioisomer mixtures having the ratio \( \text{sn-ABA} / (\text{sn-AAB} + \text{sn-BAA}) \) of: 25/75, 50:50, and 75:25. The signal intensity ratios of the ions [M+NH\(_{4}\)-RCOONH\(_{4}\)]\(^{+}\) (I, III, IV), [M-H-RCOOH-100]\(^{-}\) (I, V), [M+\(^{107}\text{Ag}\)-RCOOH]\(^{+}\) and [M+\(^{109}\text{Ag}\)-RCOOH]\(^{+}\) (II) were calculated as 100 × \([\text{AB}]^{+} / ([\text{AB}]^{+} + [\text{AA}]^{+})\) (I, IV) or as 100 × \([\text{AB}]^{-} / ([\text{AB}]^{-} + [\text{AA}]^{-})\) in
studies (I, V) and as [AB]$^+$ / [AA]$^+$ in studies II and III. Ions [AB]$^+$ and [AA]$^+$ denote product or fragment ions of the TAG A/A/B from which either FA A or B is cleaved off. The ratios of ion intensities were plotted in the calibration curves on the y-axis and the corresponding proportions of the symmetric regioisomer ABA as 100 × ABA / (ABA + sn-AAB + sn-BAA) (%) on the x-axis. Calibration curves were plotted separately for each TAG regioisomer pair.

The natural occurrence of $^{13}$C and $^{18}$O isotopes was taken into account in the APCI-MS analyses in study I but not in study II, because the effect of the isotopes was considered to be similar in both reference TAGs and in the TAGs of sample oils. The overlapping of isotope mass peaks in mass spectra was taken into account by subtracting the calculated intensity of the M+2 isotope peak of the interfering DAG species from the mass peak of interest (I). The results from ammonia NICI-MS/MS analyses were calculated by MSPECTRA and the calibration curves obtained were not used in the calculations.

The intensity ratios of [M+107Ag-RCOOH]$^+$ and [M+109Ag-RCOOH]$^+$ ions from ESI-MS analyses were calculated differently because of the complexity of the spectra (II). The calculated intensity ratios were m/z 703.4/705.4, 705.4/707.4, and 703.4/707.4, where 703.4 is [M+107Ag-L]$^+$, 705.4 is [M+107Ag-Ln]$^+$ and [M+109Ag-L]$^+$, and 707.4 is [M+109Ag-Ln]$^+$ (Ln is linolenic acid).

The relative proportions of regioisomer ABA (100 × ABA / (ABA + sn-AAB + sn-BAA), %) of the analyzed sample oil TAGs were calculated based on the corresponding ratios of the formed [AB]$^+$ and [AA]$^+$ ion intensities and calibration curves, as was first done by Jakab et al. (208). The sample oil analyses were conducted in triplicate (I) or in quadruplicate (II, III, IV, V).

### 4.7 Statistical Analysis

SPSS 14.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for data analysis (I, II, III, IV). Normal distribution of the data was tested by the Shapiro–Wilk test, and the homogeneity of variances was tested with the Levene test. One-way analysis of variance (ANOVA) and Tukey’s honestly significant difference (HSD) test were used when comparing the slopes and
R² values of the calibration curves (I). Statistical differences between the regioisomer compositions were analyzed by one-way ANOVA or the Brown–Forsythe test, depending on the homogeneity of variances. Tukey’s HSD-test and Tamhane’s test were used as post hoc tests depending on the homogeneity of variances (I, II, III, IV). Fisher’s least significant difference (LSD) test (III, IV), the independent-sample t-test (III, IV), and one-sample t-test (IV) were also used when considered to be appropriate. When the data were not normally distributed, the Kruskal-Wallis test and the Mann-Whitney test were employed (I, III, IV). The required bonferroni corrections were applied. Differences of P < 0.05 were considered significant.

5 RESULTS AND DISCUSSION

5.1 FATTY ACID COMPOSITIONS

When comparing the FA composition obtained by GC-FID to the calculated FA composition from the ammonia NICI-MS/MS results, the results were consistent, and proved that the TAG regioisomer results obtained with direct inlet-ammonia NICI-MS/MS were reliable (I).

5.2 LIQUID CHROMATOGRAPHIC SEPARATION

The acetone/acetonitrile gradient (I, IV, V) and the methanol/isopropanol (I) gradient used with RP-HPLC (IV) and ultra-HPLC (UHPLC) (I, V) columns separated TAGs that had the same MW but different FA compositions. Po/Po/V and Po/Po/O were also successfully separated using an acetone/acetonitrile gradient and two RP-HPLC columns (IV). The separation was better than that obtained by the UHPLC column by which only partial separation was achieved (V). Ala/L/L and Gla/L/L) were separated efficiently enough by one Ag-HPLC column (II) but the separation was better with two columns (III). However, the separation achieved by UHPLC was almost as good with a much shorter elution time (V).
5.3 MASS SPECTROMETRIC METHODS

5.3.1 CALIBRATION CURVES AND CLEAVAGE PATTERNS OF TRIACYLGLYCEROLS

The regioisomers were readily differentiated by APCI-MS (I, II), ammonia NICI-MS/MS (I), ammonia NIAPCI-MS/MS (V), and ESI-MS/MS using both ammonium adduct formation (I, III, IV) and silver ion adduct formation (II). The calibration curves showed linear dependence between ion intensity ratios and regioisomer composition. If the selection of the best MS method in study I would have been conducted only based on the calibration curves, the choice would have been APCI-MS, because it seemed to differentiate the regioisomers more efficiently (I). No statistical differences were found between the $R^2$ values of different methods (I).

The cleavage of TAGs was shown to be different using different MS methods and on different MS instruments (I, II, III, V), which suggests that analyses of reference TAGs are needed in order to achieve accurate regioisomer composition results of TAGs in sample oils and fats. The cleavage patterns of Ala/L/L and Gla/L/L were different and the cleavage of Gla was more efficient than Ala using positive ESI-MS/MS and APCI-MS methods (II, III) but less efficient than Ala by ammonia NIAPCI-MS/MS (V). The calibration curves of different reference TAGs obtained by the ammonia NIAPCI-MS/MS method (V) differed considerably between each other. Thus the hypothesis that the use of only one calibration curve with this method would be justified was disproved (V).

5.3.2 APPLICABILITY OF THE METHODS

(U)HPLC/APCI-MS (I, II) was an otherwise excellent method but was found to be partially flawed. Although the HPLC gradient was efficient enough to separate TAGs having the same MW, some co-elution of TAGs inevitably occurred. This can affect the results obtained by APCI-MS if the co-eluting TAGs have the same DAG fragments. The co-eluting TAGs having different MW could be separated by (U)HPLC/MS/MS (I, II, III, IV, V). ESI-MS/MS of $[\text{M}+^{107}\text{Ag}]^+$ and $[\text{M}+^{109}\text{Ag}]^+$ ions was found not to be convenient, because the existence of two
different silver ion isotopes made the calculations complex (II). The major flaw of the ammonia NIAPCI-MS/MS method was the decrease of $[M-H]$ ion intensity with the increasing UHPLC flow rate (V). ESI-MS/MS of $[M+\text{NH}_4]^+$ ions (I, III, IV) was found to be the easiest and the most reliable method for quantifying the regioisomer composition, because it does not have the above mentioned disadvantages. Furthermore, ammonium adduct formation by ammonia gas was a convenient way to analyze the regioisomer composition of TAGs (IV).

The APCI-MS/MS method was shown not to be applicable for the quantification of TAG regioisomers (I). In addition, ESI-MS of $[M+\text{Ag}]^+$ was tested, but because of the complexity in the interpretation of mass spectra and the considerable deviation of the results, this method was not considered to be reliable for regioisomer quantification (II).

5.4 PROPORTIONS OF TRIACYLGLYCEROL SPECIES IN SEA BUCKTHORN PULP OIL

Proportions of Po/Po/V and Po/Po/O were different between sea buckthorn berries of different origin (IV). In the ssp. *rhamnoides* variety Tytti, Po/Po/V was more abundant than in the Terhi variety. The proportions of Po/Po/V and Po/Po/O were different in wild ssp. *rhamnoides* berries grown in Pyhämää and Taapajärvi. The sea buckthorn bushes in Taapajärvi were transferred from Pyhämää, and thus it can be concluded that latitudes with different weather conditions affect the profiles of these two TAGs in sea buckthorn pulp oil. The phenomenon may have also been partly explained by the half-ripe berries in Taapajärvi. In the wild ssp. *rhamnoides* berries, the proportion of Po/Po/O was much lower than the proportion of Po/Po/V.

5.5 REGIOISOMER COMPOSITIONS OF TRIACYLGLYCEROLS IN DIFFERENT FATS AND OILS

The proportions of the ABA isomer calculated from the averaged results of all four trials in study I for L/L/O, L/O/O, and P/O/O in rapeseed oil were 7.7, 57.9, and 4.5%, respectively, and in sunflower seed oil 12.2, 34.0, and 1.4%, respectively. The proportions of the ABA isomer of
P/O/O and P/P/O in lard were 95.3 and 4.9%, respectively (I). The proportions of the ABA isomer in study V for L/L/O, L/O/O, and P/O/O in rapeseed oil were 0.0, 68.4, and 5.7%, respectively, and in sunflower seed oil 7.4, 39.3, and 9.3%, respectively. The proportions of the ABA isomer of P/O/O and P/P/O in palm oil were 5.4 and 85.8%, respectively (V). The regioisomer compositions of various TAGs in sunflower seed oil and rapeseed oil (I, V) as well as in lard (I) and palm oil (V) were in accordance with several earlier studies (192, 209-213) although some studies (192, 208, 209) were not in full agreement with the present results. Possible reason for the different results might be the different origin of the oil samples. In addition, reference regioisomers were not used for regioisomer quantification in some of the earlier studies (192, 209).

The regioisomer compositions of Ala/L/L and Gla/L/L in black currant seed oil were different (II). The LAlaL proportion among Ala/L/L was 9.8% whereas that of LGlaL among Gla/L/L was 34.9%, which is near a random distribution (33.3%) (II). In different currant samples (III), the proportion of LAlaL ranged between 10.2 and 15.3% and that of LGlaL between 24.2 and 36.6%. These observations were in accordance with the results of Lawson and Bronwyn (214), who found that 36.8% of Gla was located in the $sn$-2 position of all TAGs, while the same proportion in Ala was 20.6%. The results of Kallio et al. (215) also showed that linolenic acid favored the $sn$-1/3 positions. The proportion of the regioisomer PoVPo among the Po/Po/V varied in different sea buckthorn pulp oil samples between 7.9 and 24.1%, whereas the proportion of PoOPo among the Po/Po/O was 43.3–61.2% (IV). The regioisomer compositions of Po/Po/V and Po/Po/O in the pulp of different sea buckthorn samples (IV) as well as Ala/L/L and Gla/L/L in the seeds of different currant samples (III) showed quite constant distributions between the two regioisomers ABA and $sn$-AAB + $sn$-BAA, which shows the well-controlled system of TAG synthesis in both of these oils.

The results show that $\alpha$-linolenic acid has a stronger preference for the primary $sn$-1/3 positions than $\gamma$-linolenic acid in currant seed oils and that linoleic acid is more strongly located in the secondary $sn$-2 position than $\alpha$-linolenic acid and $\gamma$-linolenic acid (III). Oleic acid has a preference for the $sn$-2 position whereas vaccenic acid has a preference for the $sn$-1/3 positions in the pulp oil of sea buckthorn berries (IV). In addition, oleic acid had overall higher affinity and vaccenic acid lower affinity for the $sn$-2 position than palmitoleic acid (IV). These
observations, however, apply only to TAGs Ala/L/L and Gla/L/L in currant seed oils as well as to TAGs Po/Po/V and Po/Po/O in sea buckthorn pulp oils.

The regioisomer composition results of the seed oil of Mortti (black currant) and the pulp oil of Tytti (sea buckthorn) obtained by ammonia UHPLC-NIAPCI-MS/MS (V) were not exactly the same as the results obtained by ammoniated TAGs by ESI-MS/MS (III, IV). However, this was expected, because the methods used were different.

5.6 REGIOISOMER COMPOSITIONS OF TRIACYLGLYCEROLS IN DIFFERENT BERRY SPECIES, SUBSPECIES, AND VARIETIES

In the *R. rubrum* species, the proportion of the symmetric regioisomer LAlaL among Ala/L/L was higher (14.1%) than in the *R. nigrum* species (12.1%) (III). Differences were also found between some of the currant varieties in both Ala/L/L and Gla/L/L (III). Kallio *et al.* (216) have also reported differences in the regioisomer composition between currant species *R. spicatum* and *R. alpinum*. However, in that study, the different currant species were grown in different geographical locations, which may have partly affected the regioisomer proportions.

Varieties Tytti and Terhi of sea buckthorn ssp. *rhamnoides* were different regarding the regioisomer composition of both Po/Po/V and Po/Po/O (IV). The berries of cultivated ssp. *rhamnoides* contained a higher proportion of PoVPo (19.8%) than berries of wild ssp. *sinensis* (15.1%) and ssp. *rhamnoides* (16.4%) regarding Po/Po/V, whereas the only difference in PoOPo was found between wild ssp. *rhamnoides* (55.5%) and wild ssp. *sinensis* (52.1%) (IV). It has been shown earlier that the regioisomer composition of Po/Po/Od (Od, octadecenoic acid) can vary between different sea buckthorn subspecies (92, 217) and it has also been demonstrated that different climatypes, i.e. subspecies, of sea buckthorn have different pathways of TAG biosynthesis (92, 93).
5.7 THE EFFECTS OF ENVIRONMENTAL VARIABLES

Generally in currants, the proportion of LAlaL was lower in plants grown in northern Finland (12.1%) than in southern Finland (13.5%), where temperature and radiation sums were higher (III). In *R. rubrum* varieties, the proportion of LGlaL was significantly higher in 2005 and 2007 (30.7–32.0%) than in 2006 (24.2-25.4%) when temperature and radiation sums were higher (III). However, these two observations may not be compared as such, because the conditions were different. The difference in the south vs. north values of LAlaL is due to the overall different growing conditions between south and north, whereas in the case of LGlaL in *R. rubrum*, the difference is only due to the year to year weather changes. The difference in the responses of *R. nigrum* and *R. rubrum* to environmental factors may be explained by the differences in the lipid metabolism. Some other differences in Ala/L/L and Gla/L/L regioisomerism between the years and growing places were also found within some currant varieties (III), but no clear tendency or dependence on weather variables was found.

Northern locations with overall lower temperatures, lower temperature sums, and lower radiation sums seemed to promote the accumulation of the regioisomers *sn*-PoPoV + *sn*-VPoPo and *sn*-PoPoO + *sn*-OPoPo in the berries of wild ssp. *rhamnoides* and *sn*-PoPoO + *sn*-OPoPo in varieties Tytti and Terhi of ssp. *rhamnoides* (IV). The berries in northern Finland had to be picked when they were slightly unripe, which could also affect the results. However, the unripeness of the berries was due to the weather conditions in the north. Higher temperatures in the same growth place promoted the formation of regioisomer PoOPo in the variety of Terhi grown in Quebec, in wild ssp. *rhamnoides* grown in Pyhämäa, and in wild ssp. *sinensis* grown in Sammalmäki (IV). In contrast, the proportion of the regioisomer PoVPo in Po/Po/V in wild ssp. *sinensis* grown in Sammalmäki was lower when temperatures were higher.

5.8 SUMMARY

In the present study, different (U)HPLC/MS/(MS) methods were developed and their suitability tested in order to find a practical and reliable method for the analysis of the positional distribution of FAs of TAGs. With only a small number of exceptions, all the (U)HPLC and MS
methods tested were suitable for the analysis of regioisomer compositions of TAGs. The present study showed that because the cleavage of TAG regioisomers differed between different MS methods, between different instruments, and between different TAG species, the use of proper reference TAGs is important in order to achieve accurate results. In addition, the cleavage pattern varies between different TAG species. As was seen in the current study with Ala/L/L and Gla/L/L, even the double bond positions in FAs can have a tremendous impact on the cleavage pattern. The (U)HPLC methods were efficient enough to separate TAG species having the same MW but different FA composition. The separation of Ala/L/L and Gla/L/L as well as Po/Po/V and Po/Po/O, which contain FA isomers differing only in the position of double bonds, was achieved. (U)HPLC/ESI-MS/MS combined with ammonium adduct formation was probably the most practical method for the quantification of regioisomers of TAGs because MS/MS separates the TAGs having different MW and ESI-MS/MS of ammonium adducts was shown to differentiate the regioisomers readily with a good linear fit of the calibration curves.

(U)HPLC/ESI-MS/MS of ammonium adducts was used in the analysis of the regioisomer compositions of TAGs in the seed oils of currants and the pulp oils of sea buckthorn berries and the primary interest was in TAGs containing either α- or γ-linolenic acid or either vaccenic or oleic acid, respectively. The results show that in currant seed oils, α-linolenic acid has a strong preference for the primary sn-1/3 positions and linoleic acid for the secondary sn-2 position whereas the distribution of γ-linolenic acid is nearly random. In the pulp oil of sea buckthorn berries, oleic acid has a preference for the sn-2 position and vaccenic acid for the sn-1/3 positions. Oleic acid had a higher affinity and vaccenic acid a lower affinity for the sn-2 position than palmitoleic acid. Another interesting finding was that the regioisomer composition of TAGs Ala/L/L and Gla/L/L in the seed oil of currants as well as Po/Po/V and Po/Po/O in the pulp oil of sea buckthorn berries showed quite constant distributions regardless of place of growth, harvest year, species, subspecies, or variety. However, some statistically significant differences were found within different currant species, different subspecies of sea buckthorn, and different varieties of both berries. In addition, temperature and radiation variables in different harvesting years and different growth places were shown to affect the regioisomer compositions of TAGs in currant seed oils and in sea buckthorn pulp oils.
6 CONCLUSIONS

The MS methods applied effectively differentiated the TAG regioisomers. Accurate results concerning the regioisomer composition of TAGs were achieved by good chromatographic separation, which enabled the examination of TAG species having the same MW but different FA composition. Based on the results obtained, the method of choice was (U)HPLC/ESI-MS/MS of ammoniated precursor ions, which has been used widely in the analysis of TAG. The method discriminates between the FAs located in the primary \( sn-1/3 \) positions and in the secondary \( sn-2 \) position of TAGs.

It was shown that Ala/L/L and Gla/L/L in the seed oil of currants and Po/Po/V and Po/Po/O in the pulp oil of sea buckthorn berries have distinctive and different regioisomer compositions, which suggests that the enzymes involved in the synthesis of TAGs in the berry tissues studied discriminate between the isomers of FAs differing in the positions of double bonds. The regioisomer compositions of these TAGs showed quite constant distribution regardless of place of growth, harvest year, species, subspecies, or variety. This shows that the TAG biosynthesis in berries is a well-regulated system. However, it was shown that species, subspecies, variety, and weather conditions have statistically significant effects on the regioisomer composition of TAGs. The stereospecific distribution of FAs in storage TAGs of plants is determined by the selectivity and activity of acyltransferases on certain acyl donors and acceptors. GPAT and DGAT preferably utilize saturated FAs, whereas LPAAT is specific for unsaturated FAs. Modification and hydrolysis of acyl chains located in the \( sn-2 \) position of PtdCho also affects the non-random distribution of FAs in TAGs. The selectivity and activity of the enzymes were shown to vary between different plant species.

The current literature is lacking in studies concerning the stereospecific composition of particular TAG molecules with distinctive FA composition. The selectivity of the enzymes towards different substrates can generate distinctive TAG species and thus the generalizations concerning the TAG stereospecific composition may not give accurate results. More studies with detailed stereoisomer analysis of plant TAGs combined with the genetic background and environmental factors are needed. The present work shows that rapid (U)HPLC/MS/MS methods can be exploited in studies where detailed structural compositional analyses of TAGs.
are needed. However, the present methods could be further ameliorated by developing a method which can also separate FAs located in the $sn$-1 and $sn$-3 positions.
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Heidi Leskinen
REFERENCES


APPENDIX: ORIGINAL PUBLICATIONS


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