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Replacement of Dietary Fats – Effects on Serum Lipids and Plasma Fatty Acid Composition with Special Emphasis on the Metabolism of Essential Fatty Acids

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ACADEMIC DISSERTATION

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CONTENTS

ABSTRACT

The effects of fat substitution on plasma fatty acid composition, serum lipid levels and plasma lipid classes were investigated in two studies comprising a total of 100 and 48 subjects, respectively. The analytical methods included gas chromatography (GC) of total plasma and plasma phospholipid (PL) fatty acids, including octadecenoic *trans*-isomers. A high performance liquid chromatographic method using evaporative light-scattering detection (HPLC-ELSD) was applied for the quantitation of lipid classes.

Substitute fats included canola-type, ordinary or cold-pressed rapeseed oils containing *ca* 11% α-linolenic (α-LLA) and 23% linoleic (LA) acids, a test margarine, and olive and soybean oils. The average daily doses in the groups during the six-week substitutions, designed to replace butter or margarine on bread, ranged from 14 to 23 g (15-22% of total fat intake).

The results demonstrate a preference for n-3 polyunsaturated fatty acid (PUFA) metabolism from α-LLA to longer-chain n-3 PUFAs over LA and n-6 PUFA metabolism. This was most completely evident in plasma PL, when butter was replaced by rapeseed oil, as a simultaneous fall in saturated fatty acid (SaFA) and serum LDL cholesterol levels. The effect of monounsaturated oleic acid (n-9 MUFA), the main fatty acid in rapeseed oil (60%), remained neutral showing no increase in PL. The changes in PL followed the order of competition between the unsaturated fatty acid families: (n-3) > (n-6) > (n-9). The increase in n-3 PUFAs was predominant at three weeks, while that in n-6 PUFAs was highest at six weeks, without suppressing n-3 PUFAs. This delay is in line with higher desaturase selectivity for α-LLA conversion to long-chain n-3 PUFAs, and with their suppressive effect on n-6 PUFAs. The test margarine (3% α-LLA; 28% LA) lacked an n-3 PUFA effect, and an increase in PL LA (n-6) was seen already after the first three weeks.

Replacement of margarines by rapeseed oil first reduced both PL SaFAs and n-6 PUFAs, but simultaneously raised n-3 PUFAs and MUFAs. Oleic acid in dietary fat is thus a good counterpart with α-LLA. The amount of LA in the diet is in the key position during competition, since the rise in n-6 PUFAs at six weeks suppressed both n-3 PUFAs and MUFAs. Olive oil, instead of raising PUFAs, reduced LA levels in margarine users, which is desirable if the LA intake is high. However, due to the low α-LLA (<1%) and LA (<8%) contents, olive oil is a poor source of essential fatty acids for high SaFA diet.

Moderate amounts of α-LLA and LA, and a ratio of 1:2 in rapeseed oil, is close to the optimum for the progress of competitive mechanisms in both n-3 and n-6 PUFA metabolism, with a priority for n-3 PUFAs over n-6 PUFAs. Rapeseed oil should be a permanent constituent of daily food in western-type diets in order to replace excessive intake of SaFAs, to lower cholesterol levels, to moderate LA intake, and to ensure an essential fatty acid balance using one and the same vegetable oil. The preference of n-3 PUFAs and n-3 eicosanoids can be expected to provide protective effects against coronary heart disease in particular, as well as against many other diseases which may be caused by long-term imbalances of essential fatty acids in the diet.

LIST OF PUBLICATIONS

The dissertation is based on the following original publications, referred to by their Roman numerals (I-V), and a review (VI). Some unpublished data are also included.

I Seppänen-Laakso T, Laakso I, Hiltunen R.
Simultaneous analysis of bound and free fatty acids in human plasma by quantitative gas chromatography.
Acta Pharm Fenn 1990; 99: 109-117.

II Seppänen-Laakso T, Vanhanen H, Laakso I, Kohtamäki H, Viikari J.
Replacement of butter on bread by rapeseed oil and rapeseed oil-containing margarine. Effects on plasma fatty acid composition and serum lipids.
Br J Nutr 1992; 68: 639-654.

III Seppänen-Laakso T, Vanhanen H, Laakso I, Kohtamäki H, Viikari J.
Replacement of margarine on bread by rapeseed and olive oils. Effects on plasma fatty acid composition and serum lipids.
Ann Nutr Metab 1993; 37: 161-174.

IV Seppänen-Laakso T, Laakso I, Backman P, Vanhanen H, Viikari J.
Elaidic and *trans*-vaccenic acids in plasma phospholipids as indicators of dietary intake of 18:1 *trans*-fatty acids.
J Chromatogr B 1996; 687: 371-378.

V Seppänen-Laakso T, Laakso I, Vanhanen H, Kiviranta K, Lehtimäki T, Hiltunen R.
Major human plasma lipid classes determined by quantitative high-performance liquid chromatography, their variation and associations with phospholipid fatty acids.
J Chromatogr B 2001; 754: 437-445.

VI Seppänen-Laakso T, Laakso I, Hiltunen R.
Analysis of fatty acids by gas chromatography, and its relevance to research on health and nutrition.
Anal Chim Acta 2002; 465: 39-62.

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ABBREVIATIONS

AA	arachidonic acid (20:4n-6)
ACAT	acyl-coenzyme A-cholesterol acyltransferase
$AgNO_3$-TLC	silver nitrate thin-layer chromatography
α-LLA	alpha-linolenic acid (18:3n-3)
BF_3	bortrifluoride
BMI	body mass index (kg/m^2)
CAD	coronary artery disease
CE	cholesteryl ester
CETP	cholesteryl ester transfer protein
CHD	coronary heart disease
CLA	conjugated linoleic acid
CM	chylomicron
DHA	docosahexaenoic acid (22:6n-3)
DPA	docosapentaenoic acid (22:5n-3)
EFA	essential fatty acid
ELSD	evaporative light-scattering detector
EPA	eicosapentaenoic acid (20:5n-3)
FAME	fatty acid methyl ester
FC	free (unesterified) cholesterol
FFA	free fatty acid
FID	flame ionization detector
FTIR	Fourier transform infrared spectroscopy
GC-MS	gas chromatography-mass spectrometry
HDL	high-density lipoprotein
HGLA	homogamma-linolenic acid (20:3n-6)
HMG-CoA	β-hydroxy-β-methylglutaryl-coenzyme A
HPLC	high performance liquid chromatography
HUFA	highly unsaturated fatty acid (≥C20 with ≥3 double bonds)
IDL	intermediate density lipoprotein
IHD	ischaemic heart disease
LA	linoleic acid (18:2n-6)
LCAT	lecithin-cholesterol acyltransferase
LC-MS	liquid chromatography-mass spectrometry
LDL	low-density lipoprotein
LT	leukotriene
MI	myocardial infarction
MUFA	monounsaturated fatty acid
NaOMe	sodium methoxide
OA	oleic acid (18:1n-9)
PC	phosphatidyl choline
PG	prostaglandin
PL	phospholipid
PPP/PRP	platelet poor/rich plasma
P/S ratio	PUFA/SaFA ratio
PTV	programmed temperature vaporizer
PUFA	polyunsaturated fatty acid
RSD	relative standard deviation
SaFA	saturated fatty acid
SIM	single ion monitoring
sn	stereospecific numbering
SPE	solid phase extraction
TAG	triacylglycerol
TC	total cholesterol
TFA	*trans*-fatty acid
TLC	thin-layer chromatography
Tx	thromboxane
VLDL	very low-density lipoprotein

For fatty acids, see systematic names in the Appendix (p. 94).

1. INTRODUCTION

Fats in the food form a concentrated source of energy, and they carry fat-soluble vitamins and provide essential fatty acids (EFAs) for further modification in the body. Stored fats provide most of the energy needed to fuel muscular work and to protect against temperature extremes. In addition, the cellular plasma and organelle membranes within the human body contain 40-80% lipids (Goodman 1994; Sizer and Whitney 1997).

Differences in the effects of various types of fat led to the discovery of EFAs already more than 70 years ago. In animal studies, Burr and Burr found that unsaturated fat is essential, and subsequently demonstrated that linoleic and linolenic acids are essential for growth and reproduction (Holman 1992).

More detailed tissue fatty acid analyses became possible at the end of the 1950s along with the development of gas chromatography (GC) (Horning et al., 1964; Holman and Rahm 1966). The basic features of polyunsaturated fatty acid (PUFA) metabolism could be verified from animal tissues using GC (Mead 1968; Holman 1968), and the mechanisms were shown to involve strong mutual competition between n-6 and n-3 PUFAs derived from linoleic (LA, 18:2n-6) and α-linolenic (α-LLA, 18:3n-3) acids (Holman and Mohrhauer 1963; Mohrhauer and Holman 1963).

The two EFAs, LA and α-LLA, cannot be synthesized by the body, and they therefore have to be obtained from the diet. Edible plants are their original natural sources. Although most vegetable oils are rich in LA, only a few contain marked amounts of α-LLA (White 2000). EFAs are further converted to longer-chain n-6 and n-3 PUFAs, some of which, in turn, form their own series of eicosanoids (Sprecher 1981; Crawford 1983). Eicosanoids are found in very low quantities in human tissues, but they possess a number of diverse effects especially in the cardiovascular system and inflammatory processes (Moncada and Vane 1979; Hwang 2000). Imbalances of EFAs are additionally known to be related to many diseases (Chapkin 2000).

LA-rich oils have been considered to be the only sources of PUFAs, while n-3 PUFAs have been ignored even though data supporting their importance have been available for decades. Much of the α-LLA was hydrogenated away for technical reasons (Holman 1998). In contrast, hardening also produces *trans*-fatty acids, which in abundance are assumed to have adverse health effects (Kris-Etherton 1995; Craig-Schmidt and Holzer 2000).

In the early 1970s, middle-aged Finnish men had the highest mortality from coronary heart disease (CHD) in the world (Nikkari 1986; Vartiainen et al., 1994). At that time, dietary fat with high saturated fatty acid (SaFA) and low LA level resulted in a very low PUFA/SaFA ratio in the diet (Trygg 1991). In East Finland, a sharp decline in ischaemic heart disease (IHD) mortality occurred during 1972-92 in a programme to lower the main risk factors - high serum cholesterol (TC), high blood pressure and smoking - including a substantial change in dietary fat, *i.e.* from butter to vegetable fats. Almost half of the decline was related to the decrease in TC levels (Vartiainen et al., 1994). However, some reviews have pointed out that the link between serum cholesterol and CHD is not always clear (Gurr 1992; Bruckner 2000).

Increasing attention is being paid to the anti-inflammatory, antithrombotic and antiarrhythmic properties of long-chain n-3 PUFAs and cardiovascular disease (Leaf and Kang 1998; Lands 2003). A recent study shows that higher proportions of these PUFAs in tissues indicate association with lower CHD mortality rates (Lands 2003). Diet enriched with α-LLA has also been found beneficial in the secondary prevention of CHD (de Lorgeril et al., 1994).

This thesis is based on studies on the effects of fat replacement on serum lipids, plasma lipid classes and fatty acid composition in habitual butter and margarine users. The substitute fats included vegetable oils and a test margarine containing varying amounts of LA and α-LLA. This study focuses on analytical methods, fatty acid indicators during fat replacement, the metabolism of LA and α-LLA, and competitive interactions between n-6 and n-3 PUFAs at the plasma phospholipid (PL) fatty acid level. The importance of effective amounts of EFAs in the diet is specifically discussed.

2. REVIEW OF THE LITERATURE

2.1. Analysis of fatty acids and lipids

Lipids are major constituents in plants and animals and form usually the main structural components of tissues. They can be classified into simple, complex and derived lipids which are sparingly soluble or insoluble in water, but soluble in organic solvents. Their features are mainly determined by their constituent fatty acids, which differ in chain length, degree of unsaturation, configuration, double bond positions and other functionalities (Thiele 1979; Shahidi and Wanasundara 1998). Methodologies in lipid and fatty acid analysis have been dealt with in the reviews mentioned below.

Lipids are isolated by solvent extraction and separated and purified by thin-layer chromatography (TLC) or solid phase extraction (SPE) (Kuksis and Myher 1986; Myher and Kuksis 1995; Ruiz-Gutiérrez and Barron 1995; Touchstone 1995). Silver ion TLC ($AgNO_3$-TLC) is used to separate *cis*/*trans* positional isomers of fatty acids (Lie Ken Jie 1980) and fatty acids with a different degree of unsaturation (Dudley and Anderson 1975).

Gas chromatography (GC) is the main technique in fatty acid analysis owing to its sensitivity, speed, high resolution and reproducibility. The basic GC methodology have been widely described recently (Shantha and Napolitano 1992; Eder 1995; Gutnikov 1995; Ackman 2000a; Seppänen-Laakso et al., 2002). In addition, high-temperature GC and GC-mass spectrometry (GC-MS) are suitable for lipid profiling and for the determination of molecular species of acylglycerols (Kuksis and Myher 1986; Myher and Kuksis 1995).

High performance liquid chromatography (HPLC) is increasingly applied in fatty acid (Gutnikov 1995; Brondz 2002; Lima and Abdalla 2002) and lipid analyses (Christie 1987; Myher and Kuksis 1995; Ruiz-Gutiérrez and Barron 1995). Evaporative light-scattering detector (ELSD) and MS detection by LC-MS have significantly improved the quantitation and identification of lipid mixtures (Christie 1987; Myher and Kuksis 1995; Lima and Abdalla 2002).

2.1.1. Isolation and fractionation of lipids

Pretreatment of the sample starts by selecting solvents and modes of lipid extraction. The problem in total lipid extraction is twofold: some of the solvents are immiscible with water and some are poor solvents for polar lipids (mostly phospholipids; PL) (Ruiz-Gutiérrez and Barron 1995; Ackman 2000a). The traditional extraction by chloroform/methanol (2:1) including washing with a salt solution (Folch et al., 1957) is widely applied with modifications, e.g. one-step extraction using a ratio of 3:1 in excess of water (Chlouverakis and Hojnicki 1974). Another method still in use is that of Bligh and Dyer (1959), in which the ratio of solvents is changed to improve extraction from tissues with a higher water content.

Lipids in fatty oils and oilseeds that contain mainly triacylglycerols (TAGs) have been commonly extracted with non-polar solvents such as petroleum ether (Appelqvist 1968; Hiltunen et al., 1979), hexane (Shahidi and Wanasundara 1998), diethylether or chloroform (Ruiz-Gutiérrez and Barron 1995). Food products such as shortenings, fish fillets, baby foods and beef have been extracted with various chloroform/methanol mixtures (Slover and Lanza 1979; Lanza and Slover 1981; Sahasrabudhe and Smallbone 1983).

Human tissue lipids comprise complex mixtures containing a variety of lipid classes of different polarities, *i.e.* TAGs, free fatty acids (FFAs), sterols (e.g. cholesterol), sterol esters, PLs, gangliosides, ceramides and sphingolipids, as well as non-lipid substances (Goodman 1994; Shahidi and Wanasundara 1998). Plasma total lipid extraction is still based on chloroform/methanol (2:1) and (1:2), respectively (Folch et al., 1957; Bligh and Dyer 1959).

A wide range of PLs can be fractionated into subclasses on a silica gel column by increasing the methanol/chloroform ratio in the eluent from 1:6 to 9:1, for example (Thiele 1979). Lipid classes from plasma, serum and plasma lipoproteins have been isolated on silica gel G plates (Moilanen and Nikkari 1981; Skořepa et al., 1983; Kovács et al., 1988). SPE is also effective in separating lipid classes of different polarity (Touchstone 1995).

2.1.2. Derivatization of fatty acids

The preparation of methyl esters (FAMEs) is the most common procedure before analysing fatty acids by GC. These methods and their advantages and disadvantages have been dealt with in detail (Shantha and Napolitano 1992; Eder 1995; Gutnikov 1995).

Methods for esterifying both lipid-bound and free fatty acids, such as direct esterification (HCl or H_2SO_4/MeOH), saponification-esterification (KOH, HCl/ MeOH) or boron trifluoride (BF_3/MeOH) methods (Sheppard and Iverson 1975), are still widely used. Esterification by BF_3/MeOH has been applied for a variety of lipids (Bannon et al., 1982; Shantha and Napolitano 1992; Eder 1995). Derivatization with HCl/MeOH has been used for cholesteryl ester (CE), TAG and PL fractions and FFAs from human serum (Moilanen and Nikkari 1981; Nikkari et al., 1983a), for cerebrospinal and amniotic fluid, plasma or serum, erythrocytes, leukocytes and neuroblastoma tissue (Muskiet et al., 1983), and for plasma CE and TAGs (Hoving et al., 1988).

Derivatization by sodium methoxide (NaOMe), which does not esterify FFAs, is a rapid method for bound fatty acids. A 2-5 min reaction time appears to be effective for the conversion of seed oil TAGs to FAMEs (Appelqvist 1968). Transesterification of the TLC-isolated PL fraction at 40°C for 5 min was found to be optimum without the formation of FFAs (Seppänen-Laakso et al., 1990a). The method can be also used as the first step in removing fatty acids from *sn-2* and *sn-3* positions of alkylglyceryl ethers, after which free hydroxyl groups can be silylated (Seppänen-Laakso et al., 1990b). Fatty acids from CEs, TAGs and phosphatidyl choline (PC) from plasma and lipoprotein fractions have been derivatized by 0.5N NaOMe (Skořepa et al., 1983). Amide-bound fatty acids from sphingomyelin, in turn, are not hydrolyzed and derivatized by this procedure (Bittman and Verbicky 2000).

New applications of more specific derivatization techniques have been described in a number of reviews (Eder 1995; Hušek 1998; Ackman 2000a; Seppänen-Laakso et al., 2002).

2.1.3. Separation of *cis*/*trans*-isomeric fractions

Trans-octadecenoic (18:1) acid isomers form the most abundant group of *trans*-fatty acids in food fats and, together with *cis*-isomers, represent the most complicated samples in the analysis. Chromatographic separation of these isomeric groups is therefore necessary before the identification of individual isomers.

Fatty acids are first esterified to FAMEs, and the separation of *trans*- from *cis*-isomers is then carried out on $AgNO_3$-TLC plates (Lie Ken Jie 1980; Dobson et al., 1996; McDonald and Mossoba 1998). By using specific adducts, FAMEs can be fractionated according to the degree of unsaturation, and the *cis*- and *trans*-isomers then separated by $AgNO_3$-TLC (Ratnayake and Beare-Rogers 1990). Butyl or isopropyl esters can be also prepared instead of FAMEs before TLC isolation of the isomers (Ackman and Macpherson 1994; Chardigny et al., 1996).

Silver ion HPLC columns have been increasingly used for the separation of *trans*-fatty acids (Nikolova-Damyanova et al., 1992; Adlof 1994; Dobson et al., 1996). Reversed-phase HPLC is also applied for the isolation of the group of conjugated linoleic acid (CLA) isomers in FAME mixtures (Lavillonnière et al., 1998; Hurst et al., 2001).

In the determination of 18:1*trans*-isomeric profiles in a range of human tissues, the lipid classes have been first fractionated by TLC, and the *cis*- and *trans*-FAMEs then separated by $AgNO_3$-TLC (Ohlrogge et al., 1982). In human studies dealing with the incorporation of deuterium-labeled palmitic, oleic and linoleic acids (Emken et al., 1989a) or *trans*-isomers (Emken et al., 1989b), lipid classes have been separated by SPE or TLC, respectively, and then analysed by GC-MS.

2.1.4. Gas chromatography

The introduction of capillary columns marked the start of improved GC separation of fatty acid isomers (Ackman 1966; Ackman and Castell 1966). Later on, a number of liquid phases were applied in fatty acid analysis on glass capillary (Heckers et al., 1977; Hiltunen et al., 1979; Slover and Lanza 1979; Lanza and Slover 1981; Muskiet et al., 1983; Bohov et al., 1984) and fused silica columns (Gillan 1983; Arrendale et al., 1983; Kramer et al., 1985). Silica columns had better inertness, stability and resolution (Lipsky et al., 1980; Jennings 1980), and became highly applicable in practical work due to their flexibility and simplicity to connect to GC and MS (Kaiser and Klee 1986).

New sampling methods, such as cold on-column injection (Schomburg et al., 1977; Grob and Grob 1978; Hiltunen et al., 1982) and programmed temperature vaporization (PTV) techniques (Poy et al., 1981; Schomburg et al., 1983), markedly improved the accuracy and precision of analyses by largely avoiding the problems due to the splitting system of the GC injector.

The present-day GC technique has been dealt with in reviews covering injection methods, optimization of GC parameters, the liquid phases of columns, isomeric fatty acid analyses, identification including GC-MS, quantification and applications in food and human tissue fatty acid analyses (Shantha and Napolitano 1992; Eder 1995; Gutnikov 1995; McDonald and Mossoba 1998; Ackman 2000a; Seppänen-Laakso et al., 2002).

Reproducibility. In capillary GC, normalized peak areas should be reproducible (<1%, RSD) (Yang et al., 1978) and, in practice, this is well achievable for main fatty acids, for example in the analysis of oilseeds and vegetable oils (Hiltunen et al., 1982; Caplan and Cronin 1983). For minor fatty acids (<0.5%), however, the values can be clearly higher (10-20%, RSD). The conventional split sampling mode, *i.e.* sample injection into a hot injector, is widely used in routine analysis, and the precision can be markedly improved by means of automatic sampling (Bannon et al., 1987).

The precision and accuracy attainable by the cold on-column technique are superior to those with conventional GC injection, and the analysis of minor fatty acids, in particular, becomes much more reliable (<5%, RSD) (Hiltunen et al., 1982; Badings and de Jong 1983; Geeraert et al., 1983; Eder 1995). The PTV injection modes, some of which are especially intended for dilute samples, are reproducible techniques (Eder 1995), and have proved to be especially suitable in the fatty acid analysis of plasma lipid fractions (Seppänen-Laakso et al., 2002).

Analysis and identification. Most of the separation problems in GC analysis are caused by fat mixtures containing 18:1*trans*-fatty acids, and therefore *trans*-isomers have to be separated from *cis*, as described in (2.1.3.). The *cis*- or *trans*-double bond positions have been traditionally determined by degradative methods such as ozonolysis, followed by identification of the derivatized products by GC or GC-MS (Ackman and Castell 1966; Ackman 1977; Thiele 1979; Sebedio et al., 1981; Fell and Schäfer 1991).

Trans-fatty acid analysis is useful for recognizing sources of fat in food products, as well as for profiling *trans*-isomeric patterns in tissues. For example, in the determination of *trans*-fatty acids in French fries, carried out by $AgNO_3$-TLC, ozonolysis and GC or GC-MS, marked contents of 18:1*trans*-isomers indicated the use of hydrogenated vegetable oils as the source of fat (Sebedio et al., 1994). Similarly, the 18:1*trans*-fatty acids of French infant formulae were characterized by vaccenic acid (18:1n-7*trans*) indicative of bovine milk, while di- and trienoic *trans*-PUFAs were similar to those in deodorized oils (Chardigny et al., 1996). The methods can be also applied in determining 18:1*trans*-distributions in human tissue PLs (Rocquelin et al., 1989).

Fatty acid methyl esters analysed on a specific stationary phase can be tentatively identified on the basis of standards, known composition of fats or, alternatively, by using the relative retention times of FAMEs *vs* reference FAMEs or equivalent chain length values. However, identification must be confirmed by on-line MS (Eder 1995).

The use of long capillary columns (30-100 m) and new phases markedly improved the separation (Jaeger et. al., 1975) and they have been frequently used in both food (Slover and Lanza 1979; Lanza and Slover 1981) and human tissue fatty acid analysis (Heckers et al., 1977; Bohov et al., 1984). For example, these columns enabled the determination of all four LA isomers (9*cis*,12*cis*-18:2, 9*trans*,12*cis*-18:2, 9*cis*,12*trans*-18:2, 9*trans*,12*trans*-18:2) in both foods (Lanza and Slover 1981) and human serum (Bohov et al., 1984; Krupčik and Bohov 1985).

Excellent resolution can be achieved on present-day long capillary columns in the direct analysis of a wide range of *cis*-isomeric fatty acids. Most of the menhaden oil fatty acids, for example, can be separated and identified by an isothermal GC run (Ackman 2000a). The resolution of the series of 18:1*trans* isomers in edible fats can be improved by lowering the GC oven temperature and by increasing the inlet pressure of the carrier gas. The modifications resulted in similar *trans*-fatty acid contents analysed either by $AgNO_3$-TLC/GC or by the direct GC method (Molkentin and Precht 1995).

Besides MS, other spectroscopic methods and specific fatty acid derivatives (Ratnayake and Beare-Rogers 1990) have been applied in the analysis of food fats. In the determination of *trans*-isomers in margarines the content of total *trans*-isomers has been quantified by infrared (IR) spectroscopy (Ratnayake et al., 1990). Identification using Fourier transform IR (FTIR) detector has been applied in the analysis of partially hydrogenated fats (Mossoba et al., 1990, 1993).

CLA isomers (e.g. 9*cis*,11*trans*-18:2) in cheese and milk chocolate can be identified as dimethyloxazoline derivatives (Hurst et al., 2001; Lavillonnière et al., 1998), and as FAMEs in a number of foods (Fritsche and Steinhart 1998), by using GC-MS and GC-FTIR. Both picolinyl esters and dimethyl-oxazoline derivatives have been applied in the identification of *trans*-fatty acids, including CLA isomers, by GC (Lavillonnière et al., 1998) or by MS (McDonald and Mossoba 1998; Hurst et al., 2001).

2.1.5. High performance liquid chromatography

The use of HPLC has become an important means of lipid analysis. The problems related to sensitivity in the detection and quantitation of non-chromogenic lipids by UV light have been largely overcome after developing ELSD (Christie 1987; Myher and Kuksis 1995). It is highly applicable for profiling and quantitative analysis of lipid classes of different polarities in tissue extracts (Christie 1985, 1986; Lutzke and Braughler 1990; Markello et al., 1991; Picchioni et al., 1996; Homan and Anderson 1998). Identification of lipids by LC-MS is useful for complex mixtures (Myher and Kuksis 1995).

Proper resolution of the analyses, usually carried out on silica columns, provides gradient elution from non-polar to polar solvents and careful re-equilibration of the chromatographic system. For quantitation it is necessary to calibrate lipid classes separately due to the high differences in responses (Christie 1985; Lutzke and Braughler 1990; Homan and Anderson 1998).

Plasma lipid classes. In humans, the cholesterol-containing lipid classes are dominant in the low-density lipoproteins (LDL), especially. CEs are carried in the core of lipoprotein particles, while free cholesterol (FC), PLs and apo-proteins are constituents of their outer monolayer (Eisenberg 1984; Voet and Voet 1995). A high proportion of PC, a subfraction of PLs, is typical for the high-density lipoprotein (HDL) fraction (Hemming and Hawthorne 1996). PC is rich in PUFAs and its *sn*-2 position is characterized by a high proportion of LA, which is utilized by the HDL-associated lecithin-cholesterol acyl transferase (LCAT) to esterify FC to CE (Eisenberg 1984; Jonas 1987; Voet and Voet 1995). A major part of the plasma cholesterol is esterified with fatty acids, mainly LA, and FC represents about 30% of the total cholesterol concentration (Vercaemst et al., 1989).

Analysis of lipid class composition by ELSD has been applied to several human tissue cell cultures and biopsy specimens (Markello et al., 1991), and animal tissue extracts (Homan and Anderson 1998).

2.2. Fatty acid composition of dietary fats

A variety of oils from plants (Hunter 1990; White 2000; Kris-Etherton et al., 2000) and fish (Hearn et al. 1987; Ratnayake et al., 1988; Kris-Etherton et al., 2000; Ackman 2000b) provide the most significant sources of EFAs and other PUFAs for the diet. In addition, vegetable oils have, almost without exception, the lowest SaFA contents. The fatty acid composition of major oils and fats are given below.

2.2.1. Vegetable oils

Vegetable oils are 100% fat and they have distinct fatty acid compositions, (Table 1) which can be roughly classified as high linoleic (LA>50%), oleic (OA>50%) and SaFA oils (about 50%). The principal sources of α-LLA are rapeseed (canola; 11%) and soybean oils (7%) (Hunter 1990). Other oils usually contain α-LLA below 1% and also have markedly higher LA/α-LLA ratios.

Table 1. Main fatty acid composition and vitamin E contents of common vegetable oils.

Fatty acids (%)	Sunflower	Corn	Palm	Olive 1)	Olive 2)	Soybean	Rapeseed 1)	Rapeseed 3)
Total SaFAs	12.6	14.5	51.4	17.1	13.3	15.4	6.8	5.6
16:0 Palmitic acid	6.8	12.2	45.1	13.7	9.4	11.0	3.9	3.3
18:0 Stearic acid	4.7	2.2	4.7	4.0	3.0	4.0	1.9	1.5
Total MUFAs	18.7	27.6	38.9	72.3	78.3	23.5	65.3	59.6
18:1 Oleic acid	18.6	27.5	38.8	71.1	77.4	23.4	64.1	57.0
Total PUFAs	68.7	57.9	9.7	10.6	8.4	61.0	27.9	34.8
Linoleic acid (n-6)	68.2	57.0	9.4	10.0	7.5	53.2	18.7	23.5
α-Linolenic acid (n-3)	0.5	0.9	0.3	0.6	0.8	7.8	9.2	11.2
LA / α-LLA ratio	136	63	31	17	9	7	2	2
Vitamin E (mg/100g) [4]	63.4	34.2	8.4	12.0		17.3	23.8	

[1] White 2000; zero-erucic acid (canola-type) rapeseed oil from rape (*Brassica napus* L.) and turnip rape cultivars (*B. campestris* L.).
[2] Seppänen-Laakso et al., 1993a.
[3] Canola-type rapeseed oil from *B. campestris* L. (Seppänen-Laakso et al., 1992).
[4] Anon. 2003a (Food Composition Database Fineli®, http://www.ktl.fi).

2.2.2. Fish and other seafoods

Comprehensive data on the fatty acid composition of fish species and oils therefrom, and on other seafoods, have been given by Hearn et al. (1987) and Ackman (2000b). Marine foods are rich in long-chain n-3 PUFAs, especially eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids (Table 2). Some fish oil concentrates can even have EPA and DHA contents of up to 20-35 and 25-50%, respectively (Ratnayake et al., 1988).

Table 2. Fatty acid composition and fat contents of common fish species.

Fatty acids (%)[1,2]	Herring	Mackerel	Pike	Sardines	Rainbow trout	Ocean trout	Tuna	Pink salmon
Total SaFAs	24.2	27.3	14.6	24.1	23.4	28.8	20.5	19.3
16:0	16.5	17.5	6.6	14.5	11.4	19.1	9.5	13.0
18:0	2.6	5.8	3.6	4.9	7.3	8.0	7.9	3.0
Total MUFAs	43.4	21.0	29.7	29.2	26.5	15.6	30.1	25.3
16:1	8.9	6.0	5.7	7.0	8.2	3.3	7.5	5.2
18:1	18.1	7.8	6.8	15.4	17.4	9.9	17.5	14.0
20:1	7.1	4.1	13.6	4.2	-	1.6	2.6	3.5
Total n-6 PUFAs	1.8	9.6	23.1	3.7	13.7	7.1	8.0	6.8
18:2n-6, LA	1.8	1.9	3.1	1.4	12.3	1.3	1.8	2.0
20:4n-6, AA	-	6.9	20.0	0.9	1.4	4.1	4.1	2.8
Total n-3 PUFAs	23.6	41.2	29.8	43.4	30.1	48.0	37.6	39.9
18:4n-3	2.9	2.4	4.6	3.8	2.1	-	1.2	4.2
20:5n-3, EPA	9.2	11.2	7.5	11.3	5.1	6.7	7.5	11.0
22:5n-3	1.8	4.1	2.5	2.5	2.6	2.0	2.5	3.1
22:6n-3, DHA	8.9	22.8	15.2	25.8	16.8	39.3	26.4	20.0
Fat (g/100g)[2]	12.0	20.6	0.9[1]	9.7	1.8	1.4	7.5	4.2[1]

[1] Hearn et al., 1987; [2] Ackman 2000b

Fish such as herring, mackerel, sardines, tuna, salmon and sturgeon are considered to be oily fishes (Ackman 2000b). The fat content of cod, on the other hand, is below 1% but contains 15% of EPA and 30% DHA (Hearn et al., 1987). In a number of more exotic seafoods like oysters, mussels, shrimps, scallops and lobsters the amount of fat ranges between 1-2.5%, but with marked proportions of EPA (7-20%) and DHA (6-18%) (Ackman 2000b).

2.2.3. Margarines and spreads

Margarines and spreads used on bread are major sources of dietary fats. They contain 28-80% of fat derived from vegetable oils and other vegetable fats, part of which can be hardened (Anon. 2003b). However, no exact composition can be given for majority of these fats due to their highly varying contents of SaFAs, MUFAs and PUFAs (Table 3). The composition (%) of fat was calculated from data in which the fatty acid contents (incl. *trans*) were given as g/100g product (Anon. 2003b). Rapeseed oil was an ingredient in most of the products, and the n-6/n-3 PUFA ratio was given in some cases.

Table 3. Fatty acid composition of margarines and spreads.

Fatty acids (%)	Common German margarines[1] 1994	1999		Finnish margarines and spreads 1988[2]	2003[3]	2003[4]
Total SaFAs	21.0	29.2	13.8-57.7	24.1	20.0-27.5	15.6-42.9
16:0	8.1	9.6		10.8		
18:0	11.3	16.8		7.8		
Total MUFAs	44.3	24.5	20.8-33.9	40.1	30.0-50.0	25.7-56.3
18:1n-9*cis*	23.1	19.3		31.1		
18:1*trans*	20.7	4.9	1.8-5.6	8.1	< 1.3-2.9	< 1.3-3.6
Total PUFAs	34.5	46.2	21.8-55.1	35.8	25.0-50.0	14.3-48.6
18:2n-6, LA	33.2	45.4		33.5	15.0-44.2	
18:3n-3, α-LLA	0.3	0.3		2.2	5.7-8.6	
LA (n-6)/α-LLA (n-3)	111	151		15	2.0-7.6	

1 German sunflower margarines (Precht and Molkentin 2000).
2 Mean of 5 common margarines (Seppänen-Laakso et al., 1993a).
3 Range of 7 products, n-3 and n-6 PUFAs reported, fat content 35-80% (Anon. 2003b, http://www.margariinitiedotus.fi).
4 Range of 16 products, fat content 28-80%, no data on n-3 and n-6 PUFA contents, 3 products contain stanolesters 8g/100g (Anon. 2003b).
3,4 Fatty acid composition calculated from Anon. 2003b and normalized to 100%.

An overall decreasing trend in *trans*-fatty acid contents in these fats is clear during the last decade (Table 3). New techniques, *i.e.* interesterification of liquid oils with SaFAs, is used as an alternative to hydrogenation in order to produce low-*trans* or *trans*-free margarines (deMan 2000). However, some fats can contain unexpectedly high proportions of SaFAs (>40%); this was even the case for two fats belonging to the low-fat spreads (28-40% fat).

2.2.4. Butter

The fatty acid composition of bovine milk (~3.5% fat) and butter (80% fat) (Table 4) are characterized by a high content of SaFAs (65-70%), about 20-30% of MUFAs (Jensen 2000), and a discernible profile of 18:1*trans*-isomers accounting for 2-6% of the total fatty acids (Ackman and Macpherson 1994; Kris-Etherton 1995; Craig-Schmidt and Holzer 2000). Oleic acid is the main MUFA, accompanied by *cis*-vaccenic acid and vaccenic acid (18:1n-7*trans*) as the most abundant *trans*-isomer (Ackman and Macpherson 1994). Butter/ vegetable oil (rapeseed oil) mixtures (fat content 40%) can contain 9% LA and 5% α-LLA (Table 4).

Table 4. Fatty acid composition of milk fat and butter.

Fatty acids (%)	Milk[1]	German milks[2]	Butter[3]	French butter[4]	Finnish butter [5)]	[6)]	Butter-vegetable oil[7]
Total SaFAs	64.9	63.5	62.3	69.9	69.7	67.5	44.4
10:0-14:0	18.1	17.6	17.0	19.6	19.7	18.1	10.8
16:0	29.9	28.6	26.2	33.3	36.0	26.1	17.5
18:0	9.7	9.5	10.8	9.0	13.8	11.1	8.1
Total MUFAs	32.4	25.2	26.2	20.6	28.1	26.7	37.9
16:1n-7	3.3	1.6	1.7	0.3	1.6	1.3	0.8
18:1 *cis*	26.5	23.6	24.1	19.2	24.3	23.1	35.5
18:1 *trans*				2.4	1.9	2.3	1.6
Total PUFAs	2.8	1.9	2.4	1.7	2.2	1.9	14.1
18:2n-6, LA	2.8	1.2	2.4	1.4	1.7	1.3	9.5
18:3n-3, α-LLA	0.7		0.3	0.5	0.4	0.6	4.6

[1] U.S. Bovine milk, in February, Jensen 2000; [2] Precht and Molkentin 1997.
[3] Iverson and Sheppard 1986; [4] French butters, in January, Wolff et al., 1995.
[5] Finnish butter, 4:0-8:0 fatty acids not included, Seppänen-Laakso et al., 1992.
[6] summer butter, Aro 1991; [7] butter-rapeseed oil mixture, fat content 40%, Aro 1991.

The cholesterol contents of milk and butter fat are 150 mg/L and about 2 g/kg, respectively (Sizer and Whitney 1997; Jensen 2000). In addition, dairy products contain a large number of other minor fatty acids, such as CLA-isomers, of which 9*cis*,11*trans*-18:2, especially, is considered to be bioactive (Lavillonnière et al., 1998; Fritsche and Steinhart 1998; Craig-Schmidt and Holzer 2000; Jensen 2000).

2.3. Essential fatty acid metabolism

2.3.1. Conversion of linoleic and α-linolenic acids up to eicosanoids

Dietary LA and α-LLA are the parent EFAs forming the two non-inter-convertible PUFA families. The same elongating and desaturating enzymes convert them to longer-chain n-6 and n-3 PUFAs (Sprecher 1981, 1992; Sprecher et al., 1995). Of these, homogamma-linolenic acid (HGLA), arachidonic acid (AA) and EPA act as the precursors of different eicosanoids (Fig. 1) (Sprecher 1981; Crawford 1983; Hwang 2000).

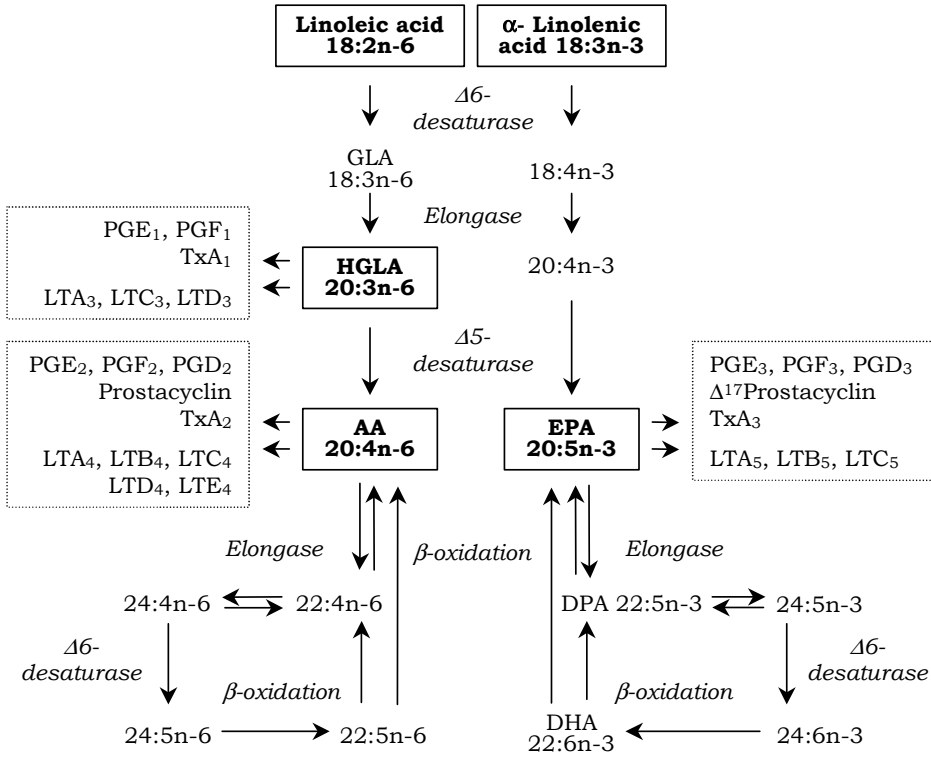

Fig. 1. Metabolic pathways of essential fatty acids. Eicosanoids are shown in dotted boxes.

Once ingested, LA and α-LLA are metabolized mainly in liver microsomes. In the first step, a double bond is introduced by Δ6-desaturase to position 6 of the carbon chain. The chain elongation up to C20 and desaturation at position 5 produces HGLA and AA, respectively, which are the eicosanoid

precursors of the linoleate (n-6) family. Correspondingly, EPA formed by Δ5-desaturase acts as the eicosanoid precursor of the linolenate (n-3) family (Sprecher 1981; Crawford 1983). Thus, equal number of steps are required for the formation of AA and EPA from their dietary precursors.

The long-chain PUFAs, *i.e.* HGLA, AA and EPA, form their own series of prostaglandins (PG) and thromboxanes (Tx) via cyclo-oxygenase and leukotrienes (LT) via lipoxygenase pathway, respectively (Crawford 1983). Substrate availability is a limiting factor and therefore the precursor fatty acids have to be incorporated into tissue PLs and cleaved as FFAs by phospholipases before initiation of the synthesis (Hwang 2000). The kinds of eicosanoid synthesized vary with the type of tissue and when and where the cells are stimulated. Eicosanoids are not stored in cells but rapidly metabolized, and thus their effects are locally expressed.

Docosahexaenoic acid (DHA, 22:6n-3) is the most highly unsaturated fatty acyl constituent in all cell membranes possessing marked functions in retinal and neuronal tissues, especially (Stubbs 1992; Bazan et al., 1992). However, the metabolic pathway is not yet fully clear, since direct synthesis from EPA to DHA via docosapentaenoic acid (DPA, 22:5n-3) does not occur in microsomes due to the lack of Δ4-desaturase. A pathway, in which DPA is rather elongated to 24:5n-3 and then to 24:6n-3 by Δ6-desaturase finally forming DHA in peroxisomes by β-oxidation (Fig. 1), is assumed to be the primary mechanism (Sprecher 1992; Sprecher et al., 1995). This also includes a retroconversion from DHA to EPA, that occurs especially after EPA-free intake of DHA (von Schacky and Weber 1985; Conquer and Holub 1997; Nelson et al., 1997).

The metabolism of dietary LA and α-LLA utilizes the same enzyme systems, which indicates that continuous competition takes place between the two PUFA families. To take advantage of this competition is considered to be important for modulating eicosanoid biosynthesis through changes in the composition of dietary fatty acids (Crawford 1983; Holman 1986; Lands et al., 1992; Hwang 2000).

2.3.2. Competition between unsaturated fatty acid families

The traditional studies performed on animal tissues in the 1960s by GC confirmed the basic properties of EFA metabolism. The conversion of LA (18:2n-6) to AA (20:4n-6) was found to be inhibited by dietary α-LLA (18:3n-3) in rat tissue lipids (Mohrhauer and Holman 1963), and dietary LA suppressed the levels of the metabolites of α-LLA (Rahm and Holman 1964).

A hypothesis was then presented, according to which the metabolic pathway of chain lengthening and dehydrogenation obviously favours the substrates in the order α-LLA (n-3) > LA (n-6) > OA (n-9) (Holman and Mohrhauer 1963). Therefore, the pattern of PUFAs in tissue lipids is controlled by the concentrations of competing substrates in a common metabolic pathway.

Competition between n-6 and n-3 fatty acids, occurring at the level of desaturation and chain elongation, has been demonstrated in a variety of *in vivo* and *in vitro* experimental models. With the Δ6-desaturase enzyme, α-LLA is a better substrate than LA (Cook 1991). Simultaneous feeding of deuterated LA and α-LLA has shown that the conversion of α-LLA to n-3 PUFA metabolites (EPA and DHA) is much greater than that of LA to n-6 longer chain PUFAs in man (Emken et al., 1992).

Later on, it was confirmed that the mixture of longer-chain (C20-C22) PUFAs maintained in the PLs of human plasma is related to the dietary intake of LA and α-LLA by empirical hyperbolic equations in a manner very similar to the relationship reported for rats. In addition, competition between the n-3 and n-6 PUFAs can diminish the abundance of eicosanoid precursors in tissues which, in turn, can diminish the intensity of tissue responses that are mediated by n-6 eicosanoids (Lands et al., 1992; Lands 2000).

The essential PUFAs, LA and α-LLA, in contrast, are maintained in tissue TAGs in a linear relation to their average dietary supply (Lands 1995, 2000).

2.3.3. Polyunsaturated fatty acid metabolism modulated by diseases

Abnormal fatty acid patterns in human serum, especially those of n-6 PUFAs, have been long known in several diseases. PUFA alterations can be caused either by dietary insufficiency of LA or its abnormal metabolism (Holman and Johnson 1981). Disorders, like cystic fibrosis, Crohn's disease, Sjögren-Larsson syndrome and congenital liver disease, have reduced capabilities for desaturation or chain elongation. Alcoholism, cirrhosis, Reye's syndrome and chronic malnutrition are also accompanied by abnormal PUFA profiles in serum PLs (Holman 1986, 1998; Cook 1991).

Research during the last decades has largely increased our knowledge of the metabolism of EFAs and the importance of the balance between longer-chain n-6 and n-3 PUFAs. The effects of eicosanoids on immune responses in a number of diseases, in particular, has become the topic of widespread research (Klurfeld 1998; Boissonneault 2000). Major abnormalities in diabetes are hyperglycemia and dyslipidemia, showing altered fatty acid metabolism. The desaturases responsible for the synthesis of PUFAs are decreased, leading to more SaFAs and less PUFAs, especially AA, in tissue PLs and other lipids. In addition, membrane fluidity is altered (Bhathena 2000). Some eicosanoids such as leukotriene LTB_4 derived from AA (20:4n-6) are potent mediators of inflammation in diseases such as arthritis and psoriasis. In order to reduce inflammatory symptoms by altering the n-6/n-3 PUFA ratio, inhibition by fish oil n-3 fatty acids, in particular, suggests desirable effects (Cleland et al., 1992; Boissonneault 2000).

The effects of DHA include the improvement of visual dysfunctions of the retina caused originally by an inadequate intake of n-3 PUFAs (Hoffman 2000). In dietary α-LLA deficiency, neurological symptoms were ameliorated after α-LLA supply and an increase in DHA in serum PLs (Holman et al., 1982). There is further evidence that schizophrenic and depressive patients have lower n-6 and/or n-3 membrane PUFA levels in association with symptom severity. In these cases, balanced EFA diets with a high intake of fish and vegetables had especially beneficial effects (Yao and Reddy 2000).

2.4. Lipoprotein metabolism

TAGs, which constitute about 90% of the dietary fat, are the principal form of metabolic energy storage. Fat digestion and absorption take place mainly in the small intestine, where lipases release fatty acids enhanced by the emulsifying bile acids (Voet and Voet 1995). The lipolytic products, together with the bile salts, form micelles which are mainly taken up by passive diffusion by the microvilli of the enterocytes. Then they are re-esterified and packaged into chylomicrons (CMs), that are soluble in an aqueous medium. CMs consist of a non-polar core containing TAGs and a variable amount of CEs that is covered by a surface coat of PLs, FC and apolipoproteins.

Table 5. Composition of human plasma lipoproteins (Voet and Voet 1995).

	Lipid and protein constituents (g/L)					Major apolipoproteins present
	FC	CE	TAG	PL	Protein	
CM	1-3	3-5	84-89	7-9	1.5-2.5	A-I, A-II, B-48, C-1, C-II, C-III, E
VLDL	5-10	10-15	50-65	15-20	5-10	B-100, C-I, C-II, C-III, E
IDL	8	30	22	22	15-20	B-100, C-III, E
LDL	7-10	35-40	7-10	15-20	20-25	B-100
HDL	3-4	12	3-5	20-35	40-55	A-I, A-II, C-1, C-II, C-III, D, E

Densities (g/cm^3): chylomicrons (CM; <0.95), very low density lipoproteins (VLDL; <1.006), intermediate density lipoproteins (IDL; 1.006-1.019), low density lipoproteins (LDL; 1.019-1.063) and high density lipoproteins (HDL; 1.063-1.210).

In the following step, CMs deliver TAGs to muscle cells or adipose tissue, and absorbed cholesterol to the liver. When CMs come into contact with the capillary endothelium of blood vessels, the lipoprotein lipase activated by apolipoprotein C-II hydrolyzes the TAGs from the CMs. The FFAs then enter muscle cells or adipocytes, where they are again re-esterified to TAGs or oxidized (Mensink et al., 2000). After delivering TAGs, the core of CM shrinks, and some of the protein, PLs and FC are transferred to HDL particles. The cholesterol-enriched CM remnant then binds to the LDL-apoprotein B-receptor and other hepatic receptors, and is catabolized in the liver. Cholesterol is esterified by acyl-CoA-cholesterol acyltransferase (ACAT) and temporarily stored as CEs (Dietschy 1997).

Cholesterol synthesized endogenously in the liver is either converted to bile acids for use in the digestive process or esterified by ACAT to CEs which are secreted into the bloodstream as a part of very low density lipoproteins (VLDL) together with endogenously formed TAGs (Table 5). The lipoprotein lipase hydrolyzes TAGs from VLDL and intermediate density lipoproteins (IDL *i.e.* VLDL remnant), and finally LDLs are formed. The cholesterol supply in cells is maintained by regulating the rate of both LDL-receptor synthesis and cholesterol esterification by ACAT, as well as by regulating β-hydroxy-β-methylglutaryl-CoA (HMG-CoA) reductase activity, the rate-limiting step in the *de novo* synthesis of cholesterol (Voet and Voet 1995; Bruckner 2000).

Most of the cholesterol-rich LDL-particles are removed from the circulation through the hepatic LDL-receptor pathway recognizing apolipoprotein B-100 (Table 5), and a smaller part via the macrophage scavenger pathway. Cholesterol is transported out of tissues by reverse transport mediated by ATP-binding cassette transporter 1 (ABCA1), HDL, lecithin-cholesterol acyl-transferase (LCAT), CE transfer protein (CETP) and scavenger receptor B1 (SR-B1) in the liver (Attie et al., 2001). HDL clears cholesterol from the cells through transesterification of acyl groups from PC with the aid of LCAT. It is synthesized by the liver and secreted into the plasma, where HDL is the preferred substrate. LCAT is particularly activated by apolipoprotein-AI (Table 5), and its best fatty acyl substrate is LA (Jonas 1987). HDL can also deliver part of its CEs in exchange with TAGs, with the aid of CETP, to VLDL, IDL and LDL, which can then be taken up by the liver (Mensink et al., 2000).

The serum concentration of LDL cholesterol (LDL-C) depends on the rate of synthesis and removal of LDL particles from the circulation which, in turn, depends on the number of functioning LDL-receptors on the liver cell surface. In familial hypercholesterolemia, liver LDL receptors are diminished or eliminated due to a genetic defect, while in normal subjects ingestion of a high-cholesterol diet represses the rate of LDL-receptor production (Voet and Voet 1995). Recent meta-analyses suggest that a decrease or an increase of 100 mg dietary cholesterol/d is estimated to cause a corresponding change in the plasma cholesterol level of between 0.04-0.07 mmol/L (Howell et al.,

1997; Weggemans et al., 2001). The higher the serum LDL-C level, the more LDL-C will be cleared via the macrophage pathway. When too much LDL-C is taken up, foam cells loaded with cholesterol are formed (Bruckner 2000), which can then initiate development of atherosclerotic plaque (Goor 1987).

2.5. Effects of dietary fatty acids

The amount and type of dietary fat have profound effects on plasma lipids and lipoproteins, which may explain many of the effects that lipids have on the risk factors associated with several major diseases in affluent societies (Norum 1992). The Seven Countries Study, which started in the late 1950s and included two Finnish and eight other European populations, showed marked differences in SaFA and monounsaturated fatty acid (MUFA) intake. Further, a high intake of SaFAs was associated with high serum cholesterol levels and high mortality of CHD (Keys 1970). However, confusion still existed about why populations with low CHD could have very different sources of dietary fats. After finding a connection between a high MUFA intake and a low CHD in Mediterranean regions (Keys 1970), studies among Eskimos with even higher cholesterol levels reported that marine foods could also be related to low CHD (Bang et al., 1971). These diets contained much more PUFAs (n-3) than typical western-type diets (Bang et al., 1976).

2.5.1. Saturated fatty acids and associations with serum cholesterol levels

SaFAs form a significant part of human tissue lipids derived either from the diet or from endogenous synthesis in the liver. However, a higher intake of SaFAs causes elevated plasma cholesterol levels by reducing the activity of LDL receptors, and thus the receptor-mediated removal of cholesterol from circulation is diminished (Grundy 1987; Nelson 2000).

A high intake of SaFAs from animal fats such as butter, especially if it forms the main part of the dietary fat, not only leads to raised plasma cholesterol levels but also to reduced PUFAs in the diet. Probably the highest known SaFA intake and the lowest PUFA/SaFA (P/S) ratio (0.17) was recorded in

Finland in the early 1970s (Trygg 1991), as well as a high CHD mortality among men (Nikkari 1986). Population surveys in eastern Finland during 1972-1992 indicated that the observed decline in IHD mortality, which was higher than predicted, was 55% and 68% in men and women, respectively (Vartiainen et al., 1994). As much as 3/4 of the decline could be explained by changes in common risk factors, *i.e.* serum cholesterol, blood pressure and smoking, half of which was related to the decrease in serum cholesterol levels. During that period, substantial changes in the quality of dietary fat were made, *i.e.* a decrease in the use of butter and an increase in the use of margarines.

The exceptionally high SaFA intake, serum cholesterol levels and CHD rate in Finland (Keys 1970) have usually had no point of comparison in other countries. Sometimes it is well documented that serum cholesterol levels in a given population are closely correlated with an increased risk for atherogenesis (Bruckner 2000). In contrast, the factors influencing plasma cholesterol have been also considered of secondary rather than primary importance in CHD, and that the true mechanisms are still not clear (Gurr 1992). What still remain questions are why there is lower CHD mortality in some countries (e.g. France) despite substantial cholesterol and fat intakes, or why the number of CHD deaths can clearly be different at the same cholesterol levels (Bruckner 2000).

2.5.2. *Trans*-fatty acids

High amounts of *trans*-fatty acids (TFAs), derived from hardening by partial hydrogenation of vegetable oils, also belong to the adverse components of dietary fats. However, the use of interesterification as an alternative to hydrogenation (deMan 2000) has resulted in a clearly lower intake of TFAs, especially in Europe. The average total *trans*-fatty acid intake in Finland is about 1 g/d (Laatikainen et al., 2003), while the supply of 18:1*trans* from margarines and shortenings in the Danish diet has been the same (Ovesen et al., 1998). In Germany, vegetable and fat-reduced margarines have contained 0.3-4%, and diet margarines ≤0.5% of *trans*-isomers (Fritsche and

Steinhart 1997). However, 'hidden' fat can sometimes contain an abundance of TFAs, as found in fast-food frying fats (up to 20%) (Ovesen et al., 1998).

The high intake of TFAs in North American diets is mainly derived from bakery products, cookies, biscuits, snacks and deepfried food products, while the proportion of margarines accounts for about 1/4. Deepfried foods can supply as much as 10 g/d of TFAs. However, the *trans*-contents do not have to be labeled, but the products can be advertised as cholesterol-free, low in SaFAs and cooked in vegetable oil (Ascherio et al., 1999). Adverse effects caused by a high intake of TFAs (Craig-Schmidt and Holzer 2000) have led demands for labeling high TFA levels in food products (Ascherio et al., 1999).

A major concern is especially focused on the safety aspects of a high intake of TFAs in pregnant and lactating women and in newborn infants. There are indications that TFAs correlate inversely with birth weight, and that they can impair EFA metabolism and early growth (Koletzko 1992). The most adverse effect is that a high intake is easily reflected in breast milk, and that high TFA levels even occur in other baby foods (Holub 1999).

Studies on the effects of TFAs on serum lipid levels have shown that a total energy intake covering 11% of TFAs not only raises LDL-cholesterol but also lowers HDL-cholesterol levels (Mensink and Katan 1990). These effects have been also reported at lower energy levels of TFAs (3-8%) (Ascherio and Willett 1995).

In the 1990s, several epidemiological studies on U.S. populations indicated that a high intake of TFAs can contribute to a risk of myocardial infarction (MI) (Ascherio et al., 1994) and CHD (Willett et al., 1993; Ascherio et al., 1996; Hu et al., 1997). In contrast, in a study including eight European countries no associations were found between adipose tissue TFA levels and the risk of MI (Aro et al., 1995). Serum lipid levels were not determined but, instead, cigarette smoking was found to be the only significant risk factor for sudden cardiac death in this population.

2.5.3. Monounsaturated fatty acids

Among edible oils, the effects of MUFAs can be best monitored on the basis of the most typical monoenoic oil: olive oil which is composed of about 75% OA and 8% LA. A low incidence of CHD found among Mediterranean populations (in Corfu and Crete) already in the 1960s was considered to be related to the use of olive oil as the principal dietary fat (Keys 1970; Grundy 1987; Kris-Etherton 1999). A number of studies based on olive oil diets have focused on serum lipid levels. Comparisons can be best made when diets simulate both the MUFA and especially the PUFA contents of the oil.

The effects of an olive oil-rich diet (64% MUFAs, 9% PUFAs) were compared with those of a corn oil diet (36% MUFAs, 36% PUFAs) in two groups of subjects having serum TC levels of 6.2 and 7.2 mmol/L, respectively. The olive oil diet had no TC-lowering effect, while the corn oil diet reduced it by 8% ($p<0.01$) and 7% ($p<0.05$), respectively. After a corn oil diet, olive oil had an HDL-C raising effect (5%; $p<0.05$) (Sirtori et al., 1986). An olive oil-rich diet with a similar MUFA/PUFA ratio was consumed by subjects with TC levels of 5.9 mmol/L (Baggio et al., 1988). Dietary compliance was assessed by erythrocyte membrane fatty acid analysis, which showed a rise of 15% in OA without changes in PUFA levels. The fall in TC, LDL-C and TAGs were 9, 12 and 25%, respectively, but without any effect on HDL-C levels.

In a recent study on young men with clearly lower TC levels (4.7 mmol/L), the plasma TAGs, TC and VLDL, IDL and LDL cholesterol concentrations remained higher after an olive oil-rich diet (60% MUFAs and 9% PUFAs) compared to rapeseed oil and sunflower oil diets. The different squalene and phytosterol contents of the oils were assumed to be partly responsible for the differences in plasma lipid levels (Pedersen et al., 2000).

The effects of diets with about 60% of MUFAs but higher amounts of PUFAs (13%) have been studied in subjects with TC levels of 5 mmol/L. The use of MUFAs derived from both olive oil and rapeseed-oil based margarine resulted in a decrease in TC and an increase in HDL-C concentrations (Mensink and

Katan 1987). In a study performed on 63 healthy male subjects aged between 18-75 years, an olive oil diet had no effect on TC but raised both HDL-C and TAG levels (Jacotot et al., 1988).

Comparisons have been also made when MUFA diets contain PUFAs equivalent to 20% of total fat even (Mensink and Katan 1989). As dietary enrichment with olive oil increases LA in plasma CE by 4 %-units (n.s.) but not OA, the effect cannot be due to MUFAs. Instead, such a rise in CE can be obtained from a moderate LA content in rapeseed oil diet, for example (Valsta et al., 1995). In plasma PLs and CEs, olive oil brings about an increase in OA and a decrease in LA (Seppänen-Laakso et al., 1993a; Nydahl et al., 1994).

The effects of MUFAs, *i.e.* OA, are beneficial when replacing SaFAs (Grundy et al., 1988). After a SaFA diet or in subjects with hyperlipidemia, rapeseed oil-based diets have reduced serum cholesterol levels by 15-20% (McDonald et al., 1989; Valsta et al., 1992; Nydahl et al., 1994; Gustafsson et al., 1994). MUFA-rich oils such as olive, canola-type rapeseed or high oleic sunflower oils may not have similar plasma cholesterol-lowering effects (Truswell and Choudhury 1998), but they favourably affect on CHD risk factors related to thrombogenesis, *in vitro* susceptibility of LDL to oxidation compared with PUFAs, and insulin sensitivity (Kris-Etherton 1999).

2.5.4. N-6 and n-3 polyunsaturated fatty acids and eicosanoids

Since the discovery of the essentiality of linoleic and linolenic acids in 1930, more detailed research on their effects in humans remained in the background for decades simply because fatty acids were difficult to measure before the appearance of GC techniques. In contrast, cholesterol, a non-essential, abundant compound also synthesizable by the body, could be easily measured by colorimetry (Holman 1992). Much later on, the use of edible oils rich in LA increased to replace SaFAs and to lower cholesterol levels, as in the U.S. diet in the 1970s. Not until recently, however, has α-LLA been accepted as a significant member of the n-3 PUFA family

together with marine n-3 PUFAs (Holman 1998; Simopoulos 1999; Chapkin 2000).

N-6 PUFAs. LA-poor diets, in contrast, have indicated a marked imbalance in the PUFA/SaFA intake, and low LA levels in serum and adipose tissue lipids were frequently found in CHD patients (Nikkari 1986). Several studies on populations with different CHD mortality also concluded that lower LA levels rather than classical risk factors would explain the higher CHD rates (Logan et al., 1978; Wood et al., 1984, 1987; Riemersma et al., 1986). A five-year follow-up study on Finnish men showed that low PUFA contents in serum PLs is predictive for IHD (Miettinen et al., 1982). The subjects who had sustained fatal or non-fatal myocardial infarction not only had lower LA and longer-chain n-6 PUFA, but also lower n-3 PUFA levels.

It has become clear that LA, earlier considered to be the entire dietary PUFA, not only has cholesterol-lowering properties but also a regulatory role via n-6 PUFA and eicosanoid metabolism (Lands et al., 1992; Holman 1998; Hwang 2000). However, the different dietary PUFA composition in populations with a low CHD are difficult to put into the same category. In traditional Mediterranean diets, olive oil was low in LA and practically free of α-LLA, while marine food diets in Japan (Keys 1970) and among Eskimos (Dyerberg 1986) were rich in n-3 PUFAs.

Increased use of LA-rich (>50%) vegetable oils in some western diets, which means placing too much emphasis on n-6 PUFAs at the expense of n-3 PUFAs and thereby affecting eicosanoid balance, has been a matter of increasing concern (Budowski et al., 1984; Lands et al., 1992; Holman 1998; Lands 2000; Chapkin 2000). The eicosanoids possess diverse actions on the cardiovascular, reproductive, respiratory, renal, endocrine, skin, nervous and immune systems. An imbalance in eicosanoid synthesis is found in pathological conditions like thrombosis, inflammation, asthma, ulcers and kidney disease (Hwang 2000). LA (n-6) is metabolized to HGLA and AA. HGLA forms prostaglandin PGE_1 which inhibits platelet aggregation, while thromboxane TxA_2 and leukotriene LTB_4 derived from AA are potent

aggregatory and inflammatory agents, respectively (Crawford 1983; Drevon 1992; Hwang 2000). The metabolism of α-LLA (n-3) to EPA or more efficient direct intake of EPA leads to antiaggregatory prostacyclin PGI_3. Leukotriene LTB_5, in turn, is less aggregatory than its competitive analog LTB_4 (Dyerberg et al., 1978; Crawford 1983; Cleland 1992).

N-3 PUFAs. The low mortality rate of IHD among Eskimos has provided significant evidence of the benefits of n-3 PUFAs in the diet (Dyerberg et al., 1975; Dyerberg 1986; Leaf and Kang 1998). The traditional Eskimo diet was rich in protein and fat but poor in carbohydrates, and consisted mostly of the meat of whales, seals, sea birds and fish. Potatoes and other vegetables, and dairy products were eaten in very small amounts. In their plasma, typical features included low TAG levels. These findings were supposed to explain the low CHD and the absence of diabetes in the population (Bang et al., 1971). In particular, high EPA and low AA levels were suggested to be responsible for the antithrombotic state via the functions of prostacyclin involved in the platelet-vessel wall interaction (Dyerberg et al., 1978).

The majority of the studies on fish oils have shown an effective decrease in serum TAG levels (25-30%), and an increasing trend for LDL-C (5-10%) and for HDL-C (1-3%) (Harris 1997). Consistent effects of long chain n-3 PUFAs also include the inhibition of platelet aggregation, prolonged bleeding time (Siess et al., 1980; Goodnight et al, 1981; Lorenz et al., 1983; Ahmed and Holub 1984), and a reduction in AA-derived thromboxane TxA_2 formation (Siess et al., 1980; Lorenz et al., 1983; von Schacky et al., 1985). Desirable effects of n-3 PUFAs have been often reported, and diets containing fish have become an important means to maintain n-3 PUFA intake.

Moderate consumption of fish already as little as once or twice a week in the western-type diet has been shown to be inversely associated with twenty-year mortality from CHD (Kromhout et al., 1985). Evidence on cardio-protective effects has been obtained in animal studies, which showed that all major dietary n-3 PUFAs (α-LLA, EPA and DHA) significantly prevented fatal arrhythmias (Leaf and Kang 1998). Although LA and AA were also anti-

arrhythmic several cyclo-oxygenase metabolites derived from AA appeared to induce violent arrhythmias (Kang and Leaf 2000).

Dietary intake is the only route of entry for eicosanoid precursors. These long-chain n-6 and n-3 PUFAs are obtained from the metabolism of LA and α-LLA and also by direct intake. Thus, the n-6/n-3 PUFA balance can be affected much by food choices (Lands 2003). New evidence of the significant role of the n-6 PUFAs (HGLA and AA) is shown in Fig. 2, where the highest tissue proportions of long-chain n-6 PUFAs (*i.e.* the lowest n-3 PUFAs; EPA, DHA) out of total long-chain PUFAs are closely related with the highest CHD.

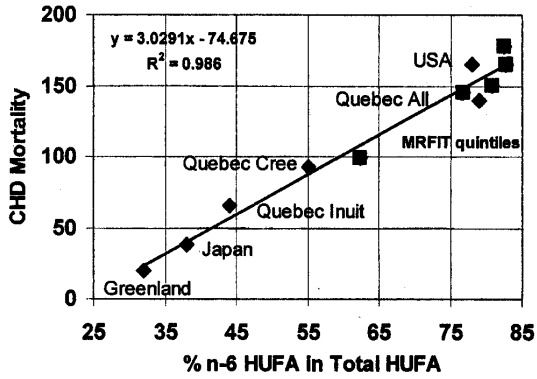

Fig. 2. The association between CHD mortality and the proportion of tissue long-chain n-6 PUFAs (HUFAs) from total long-chain PUFAs (HUFAs). (Printed with permission of AOCS from Lands 2003).

When seven populations are compared, the proportions of tissue n-6 PUFAs vary by as much as 30 to 80%. The results from the three latest studies on Canadian populations (Québecers, James Bay Cree and Inuit of Nunavik) with a 10-fold difference in the daily intake of fish (Dewailly et al., 2003), fitted closely to the relationship between populations with the lowest (Greenlanders and Japanese) and the highest CHD (U.S.) (Lands 2003).

An increase in long-chain n-3 PUFAs in tissues via metabolism of α-LLA cannot be, of course, compared with that derived from direct intake of EPA and DHA-containing fats (Li et al., 1999). However, a high intake of long-chain n-3 PUFAs was not predominant in the Mediterranean populations

having a low CHD. Their traditional diets contained abundantly plant foods (fruits, especially leafy vegetables like lettuce, spinach and purslane, breads, other forms of cereals, potatoes, legumes, nuts and seeds), olive oil as the principal source of fat, and dairy products (mainly cheese and yogurt), fish and poultry in low to moderate amounts (Keys 1995; Willett et al., 1995). Relatively high tissue contents of α-LLA were found in Cretan men especially, indicating that sources like green leafy vegetables can even supply α-LLA in sufficient amounts for effective n-3 PUFA metabolism, especially when it is not interferred by high intakes of SaFAs and LA (Simopoulos 1998).

Early evidence on the antithrombotic effects of α-LLA-containing diets has been obtained from long-term studies in farmers, where an increase in EPA and a decrease in AA level both in plasma lipids and platelet PLs led to a reduced platelet aggregation and prolonged clotting time of PRP. Beneficial functions were attributed to a higher intake of α-LLA from rapeseed oil (Renaud and Nordøy 1983; Renaud et al., 1986), which has also often been found in more recent studies (McDonald et al., 1989; Kwon et al., 1991). Linseed (flaxseed) oil is another marked source of α-LLA, which also has desirable platelet functions (Budowski et al., 1984; Mantzioris et al., 2000).

Long-chain n-3 PUFAs derived from α-LLA are especially enriched in plasma PL subclasses. An increase in EPA after a one-week rapeseed (canola) oil intake was already seen in the alkenylacyl ethanolamine fraction and choline phosphoglycerides of platelet PLs, showing concomitant reduction in long-chain n-6 PUFAs (Weaver et al., 1990; Chan et al., 1993). After replacing highly saturated fat, rapeseed oil increased both EPA and LA in the *sn*-2 position plasma PC fraction (Seppänen-Laakso et al., 1993b). An increase in long-chain n-3 PUFAs was similarly detected in the serum PL of the subjects with even higher cholesterol levels (Gustaffsson et al., 1994). The most consistent n-3 PUFA effect of linseed oil, containing about 16% LA and 53% α-LLA, is a rise in plasma PL EPA (Budowski et al., 1984; Dyerberg 1986; Cunnane et al., 1993; Mantzioris et al., 1994; Li et al., 1999). An enrichment of n-3 PUFAs has been also seen in PL subfractions during linseed oil supplementation (Sanders and Younger 1981; Chan et al., 1993).

An increasing number of studies on populations with western-type diets suggest an inverse association between α-LLA intake and CHD (Ascherio et al., 1996; Hu et al., 1999; Djoussé et al., 2001). When using plasma PL fatty acids as indicators, higher combined EPA and DHA levels were related to a lower risk of fatal IHD, while only a similar tendency was found for α-LLA (Lemaitre et al., 2003). In a 5-year study, higher EPA contents in CEs derived from fish intake were associated with a lower risk of CAD death, whereas higher α-LLA, EPA and DHA levels were related to a lower risk of all-cause mortality (Erkkilä et al., 2003). In a secondary prevention trial, a Mediterranean diet enriched with α-LLA resulted in a marked reduction in CHD mortality (de Lorgeril et al., 1994).

2.5.5. Associations between polyunsaturated fatty acids and lipid oxidation

The few adverse effects of PUFAs include enhancement of the susceptibility to oxidation of LDLs. A number of oxidation products formed especially from LA and AA have been detected in the oxidized LDL fraction (Esterbauer et al., 1987, 1997; Aviram and Vaya 2001). These n-6 PUFAs are abundant in LDL, while n-3 PUFAs occur in minor amounts only (Esterbauer et al., 1987). An increase in LDL oxidation has been observed, in particular after a high intake of LA (Reaven et al., 1991; Louheranta 1996).

The adverse effects of n-3 PUFAs after fish oil intake are thought to arise from high doses or too long supplementation, wheras low (<1 g) doses do not influence LDL oxidation (Higgins et al., 2001). A moderate supply (2.5 g/d) of n-3 PUFAs increases EPA and DHA in LDL and decreases n-6 PUFAs without enhancing the susceptibility to oxidation. By using n-3 PUFAs originally not abundant in LDL, it is thus possible to suppress the dominant effect of n-6 PUFAs in the LDL fraction (Bonanome et al., 1996). Studies on rapeseed oil containing moderate amounts of LA and α-LLA have also indicated no marked effects on lipid peroxidation (Corboy et al., 1993; Turpeinen et al., 1995; Södergren et al., 2001). The relatively high tocopherol content of the oil, which was reflected as an increase in the serum gamma-tocopherol level, was assumed to act as a potent antioxidant (Södergren et al., 2001).

3. AIMS OF THE STUDY

The main interest in the research on the health effects of dietary fats is focused on their response in the serum lipids and plasma fatty acid composition. In the present investigations, a gas chromatographic (GC) technique using a novel programmed temperature vaporizing (PTV) injection in fatty acid analysis and high-performance liquid chromatography (HPLC) in the determination of plasma lipid classes were applied. The effects of vegetable oils on serum lipids and the plasma fatty acid composition were studied in free-living, mildly hypercholesterolemic subjects during partial fat substitutions. The changes in PUFA levels in plasma PL derived from dietary EFAs were particularly examined. The specific aims of the studies were:

1. To optimize GC and other methods for total plasma and plasma PL fatty acid analyses including octadecenoic *trans*-fatty acid isomers, and to study their variation in subjects having habitual diets (I, IV).

2. To study the effects of rapeseed oil and margarine substitutions on serum lipid levels, and the plasma total and PL fatty acid composition, and their associations when replacing butter on bread (II, IV).

3. To study the effects of rapeseed oil and olive oil substitutions on serum lipid levels, and the plasma total and PL fatty acid composition, and their associations when replacing margarine on bread (III, IV).

4. To optimize an HPLC method using evaporative light-scattering (ELS) detection in quantitative analysis of plasma lipid classes, and to study their associations with serum lipid levels and the plasma PL fatty acid composition. In addition, the effects of ordinary and cold-pressed rapeseed oil and soybean oil substitutions were examined (V).

5. To review the methodology in food and human tissue fatty acid analysis, and to describe the responses in plasma during fat substitutions (VI).

4. EXPERIMENTAL

4.1. Subjects and substitutions

4.1.1. Selection and grouping of the subjects

Study 1 (I-IV). In a screening study, completed in the Turku area in January 1988, a total of 400 middle-aged men and women participated. The subjects answered a questionnaire about the consumption of fat, bread, fat on bread, milk products, eggs, fat-containing supplements and medical drugs. A number of subjects were excluded due to minor use of bread and fat on bread (<3 slices of bread/day, n = 38), drug therapy (n = 37), or the use of fat-containing supplements, such as cod-liver oil (n = 31).

The subjects with TC levels between 5.0 and 8.5 mmol/L, TAG less than 3.5 mmol/L and body mass index (BMI; kg/m^2) not more than 25, were accepted for the final study (n = 100). Subjects using butter on bread were divided alphabetically into rapeseed oil (n = 20; II) and margarine (n = 23; II, IV) groups, and subjects using common margarines (III, Table 2) (Table 7) into rapeseed oil (n = 23; III) and olive oil (n = 23; III, IV) groups. The control group (n = 11; II-IV) consisted of mixed fat users.

Table 6. The fat on bread before and during substitution, age, BMI, and serum TC and TAG levels of the study groups. Values are expressed as means (SEM).

	Butter users		Margarine users		Mixed fat users
Characteristics of the groups	Rapeseed oil, n=20	Test margarine n=23	Rapeseed oil, n=23	Olive oil n=23	Control n=11
Age (years)	45.7 (2.0)	44.8 (1.7)	45.5 (2.0)	44.6 (1.8)	41.7 (1.9)
Men / Women	10/10	11/12	12/11	13/10	5/6
BMI (kg/m^2)	25.7 (0.7)	25.4 (0.9)	26.9 (0.8)	24.7 (0.6)	24.6 (0.7)
TC (mmol/L)	6.32 (0.18)	6.15 (0.13)	6.07 (0.14)	6.28 (0.21)	6.06 (0.19)
TAG (mmol/L)	0.99 (0.09)	0.92 (0.09)	1.34 (0.12)	1.00 (0.13)	0.85 (0.10)

Study 2 (V). The subjects (n = 48) for another study (1996) were selected from the registry of the Helsinki University Hospital of Skin and Allergic Diseases and by an advertisement in a newspaper. The participants visited a medical doctor and filled in a diet questionnaire. The study was accepted by the Ethics Committee of the hospital. The subjects, who were mainly margarine users, and the majority used no fish in their habitual diets, formed cold-pressed rapeseed oil (n = 26), ordinary rapeseed oil (n = 16) and soybean oil (n = 6) groups.

4.1.2. Substitutions and substitute fats

Study 1 (I-IV). The substitution period lasted for 6 weeks, during which butter on bread was replaced by rapeseed oil or substitute test margarine prepared for the study (II). Similarly, margarine on bread was replaced by rapeseed oil or olive oil (III). No other changes were made in the diet. The subjects returned to using butter or margarine on bread during the post-experimental period (the following 6 weeks). The control group was asked to maintain its normal diet during the 12-week trial.

Rapeseed oil or olive oil was used on bread as a water-oil emulsion (fat content 65%). The test margarine contained rapeseed oil (24%), sunflower, coconut and partially hydrogenated soybean oils (fat content 80%). The fatty acid compositions, including 18:1*trans*-fatty acids, are shown in Table 7, as well as those of margarines and butter used before the substitutions.

In addition, the proportions of OA, LA and α-LLA in the *sn*-2 position of rapeseed oil TAGs were determined, and they accounted for 50.2, 33.0 and 16.5%, respectively. The corresponding values for olive oil were 85.8, 12.3 and 1.1% (Seppänen-Laakso et al., 1995a).

Study 2 (V). Rapeseed oils and soybean oil (Table 7) were given for 6 weeks in parallel design. They were used as a part of the habitual home-diets as salad dressings, for instance. Margarine, cheese or butter on bread were designed

to be replaced by a corresponding dose of cold-pressed or ordinary rapeseed oil or soybean oil.

Table 7. Fatty acid composition of the fats before and during substitutions.

Fatty acids (%)	Fat on bread before substitution		Substitute fat					
			Study 1			*Study 2*		
	Butter	Margarines[1]	Rape-seed oil	Olive oil	Test margarine	Cold-pressed rapeseed oil	Rape-seed oil	Soy-bean oil
Total SaFAs	69.7	24.1	5.7	13.9	20.6	4.6	5.6	14.8
10:0-14:0	19.7	4.8	0.1	0.1	4.5	0.1	0.1	0.1
16:0 Palmitic	36.0	10.8	3.3	10.6	8.0	2.6	3.3	10.2
18:0 Stearic	13.8	7.8	1.5	2.7	7.2	1.3	1.5	3.8
20:0-24:0	0.2	0.7	0.8	0.5	0.9	0.6	0.7	0.7
Total MUFAs	28.1	40.1	59.8	75.7	48.5	62.2	59.8	23.1
16:1 Palmitoleic	1.6	0.2	0.1	0.5	0.1	0.1	0.2	0.1
18:1 *cis* Oleic	24.3	31.1	57.2	74.6	31.1	61.1	57.2	22.7
18:1 *trans*	1.9	8.1	-	-	16.4	-	-	-
20:1 Eicosenoic	0.3	0.5	1.6	0.5	0.6	0.9	1.5	0.2
22:1 Erucic	-	0.2	0.9	0.1	0.3	0.1	0.9	0.1
n-6 PUFAs[2]	1.7	33.6	24.4	9.2	28.2	21.2	23.6	54.9
18:2 Linoleic acid	1.7	33.5	24.3	9.1	28.1	21.1	23.5	54.8
n-3 PUFAs								
18:3 α-LLA	0.5	2.2	10.1	1.2	2.7	12.0	11.3	7.2

[1] Mean of 5 common margarines; [2] incl. 0.1% 20:2 in vegetable fats.

4.1.3. Dietary control

Study 1 (II-IV). All the subjects kept 3-day dietary diaries before and towards the end of the substitution period, and the data obtained were analysed for nutrients using a food table-based Nutrica® programme. In addition, the consumption of oil emulsions and margarine was controlled twice (at 3 and 6 weeks). Body weight was recorded at blood sample drawings, *i.e.* before substitution and at 3, 6 and 12 weeks.

Study 2 (V). The 6 weeks' use of substitute oils was controlled by 3-day dietary diaries, and the consumption was also recorded when the subjects

returned the unused oil at the end of the study. The 3-day dietary diaries were made before and during substitution (at 3 weeks). The data were analysed by Nutrica® programme. Compliance to the substitutions was also followed by total plasma (II, III) and plasma PL fatty acid analyses (II-V).

4.1.4. Blood sampling

Fasted blood samples were taken two weeks before the substitutions. During *Study 1* (I-IV), fasted blood samples were taken at the baseline, after 3 and 6 weeks' substitution, and at the end of the post-experimental period (at 12 weeks) at Vagus Medical Laboratory, Turku, Finland. Platelet-rich (PRP) and platelet-poor EDTA plasma (PPP) samples were prepared by centrifugation at 180 G (8 min) and 2000 G (20 min), respectively, and stored at -20°C.

In *Study 2* (V), fasted blood samples were taken before the substitutions (at the baseline), at 3 weeks (data not shown) and at the end of the study (6 weeks), in the Laboratory of Helsinki University Central Hospital.

4.2. Analytical methods

4.2.1. GC analyses of plasma fatty acids (I-V)

Lipid extraction. For GC analysis, all the plasma samples (0.5 ml) were extracted with chloroform/methanol (2:1) by a partially modified Folch et al. (1957) method. For quantitative analysis (I), internal standards for bound and free fatty acids (FFA), respectively, were added. The lower phase was separated and evaporated to dryness and dissolved in petroleum ether.

Isolation of phospholipids. For the analysis of PL fatty acids, the residues of the lipid extracts were re-dissolved in chloroform and applied on Silica gel $60F_{254}$ glass plates. The plates were developed with petroleum ether/diethyl ether/acetic acid 85:15:2 (Nelson 1967). The PL fractions were scraped off, extracted with chloroform/methanol, evaporated and dissolved in petroleum ether for esterification (I).

Transesterification. Derivatization was optimized for bound plasma fatty acids by modifying earlier methods (Appelqvist 1968; Hiltunen et al., 1979). Sodium methoxide (NaOMe) in dry methanol was added to the lipid extracts in petroleum ether, and methylated at 40°C for 5 min (Seppänen-Laakso et al., 1989b). No decomposition of FAMEs during methylation was found (I), and the method was used throughout the studies. In II-V, total fatty acids refer to bound fatty acids. After neutralization, petroleum ether was added and a 1 µl aliquot was used for GC analysis.

Gas chromatographic conditions. The analyses were carried out on a DANI 3865 GC using a PTV sampling technique with split mode (I). The GC was equipped with an NB-351 fused silica column (25 m, 0.32 mm I.D., film thickness 0.20 µm; Nordion, Helsinki, Finland). The oven was programmed from 100°C to 235°C at 10°C/min under the following conditions: carrier gas H_2 (2.5 ml/min), split ratio 50:1, flame ionization detector (FID) 250°C, PTV-injector 70°C → 250°C (rate ~50°C/s). The effect of the split ratio on the relative amounts of FAMEs was controlled with a reference mixture.

Identification of fatty acids (I,IV). Identification of FAMEs and major FFAs was based on the retention times of reference standards and on those presented in the literature (Slover and Lanza 1979; Bohov et al., 1984; Krupčik and Bohov 1985). The methodological variation including the steps described above were determined by repeated GC analyses.

Isolation of the 18:1trans isomers (IV). The double-bond distributions of 18:1 *trans*-fatty acids were determined by $AgNO_3$-TLC and ozonolysis. FAMEs from the test margarine, butter and pooled plasma PL samples were applied on $AgNO_3$-impregnated Kieselgel 60 plates (Merck, Darmstadt, Germany). After development with chloroform containing 0.75% ethanol and spraying with 2',7'-dichlorofluorescein (0.2% in ethanol), the 18:1*trans*-bands were scraped off and extracted with hexane/chloroform (1:1, v/v) (Ratnayake and Beare-Rogers 1990).

Identification of ozonolysis products by GC-MS (IV). The isolated bands were ozonised by bubbling the samples for 2 min in 1.2% ozone (4.8 mg/sample) developed by an ozonizator. The products were then methylated with diazomethane and analysed by GC-MS (HP 5890 GC) equipped with an NB-54 fused-silica capillary column (15 m, 0.20 mm I.D., film thickness 0.25 μm; Nordion, Helsinki, Finland) and interfaced with an HP 5970A MS detector operating at EI mode (70 eV). Pure elaidic acid (18:1n-9*trans*) was ozonized to confirm the elution order and spectra of the four major products: nonanal, nonanoic acid, nonanoic acid aldehyde and nonanoic diacid.

Analysis of plasma PL 18:1trans fatty acids (IV). Routine analyses were performed on an SP-2340 fused-silica capillary column (60 m; 0.25 mm I.D., film thickness 0.20 μm; Supelco, Gland, Switzerland). A DANI 3865 GC equipped with a PTV injector was used in solvent split sampling mode (split ratio 50:1) (Poy et al., 1981). After manual injection (PTV at 70°C) the split valve was open for 6 s, then closed (and PTV heated to 235°C) and opened again after 60 s. A two-step oven temperature programme from 70°C (at 7°C/min) to 150°C and then to 180°C (at 1°C/min) was used. Carrier gas (H_2) flow was 0.6 ml/min and the detector (FID) temperature 235°C.

Stereospecific analyses. Pancreatic lipase was used to hydrolyse *sn*-1 and *sn*-3 fatty acids from TAGs in vegetable oils (Seppänen-Laakso et al., 1995a). The incubation (37°C, 15 min) products were extracted with diethylether. Monoacylglycerols containing fatty acids in the *sn*-2 position were isolated by TLC (Myher and Kuksis 1979), transesterified and analysed by GC.

Plasma PC fractions from 20 subjects (II) were isolated by TLC (Kovács et al., 1988), and fatty acids from *sn*-2 position were cleaved with phospholipase A_2 (20°C, 24 h). After hydrolysis, transesterification produces FAMEs from *sn*-1, whereas FFAs originate from *sn*-2. Major FAMEs and FFAs were analysed by GC (Seppänen-Laakso et al., 1993b). Minor long-chain *sn*-2 PUFAs (C20-C22, as FFAs) were trimethylsilylated and analysed by GC-MS-SIM according to the method as described earlier (Seppänen-Laakso et al., 1990b).

4.2.2. HPLC analyses of plasma lipid classes *(Study 2;* V)

For quantitative determination of plasma lipid classes by HPLC, 200 µl aliquots of plasma were extracted by chloroform/methanol and centrifuged. The lower layer was separated and filtrated, and 7 µl aliquots were used for injection.

Plasma CE, FC, TAG and PC concentrations were determined by HPLC using ELS detection (Cunow DDL21, Cergy St. Christophe, France). Separation of the lipid classes was carried out on a Spherisorb™ S3W column (100 × 4.6 mm I.D., particle size 3 µm; Phase Separations Ltd., UK) using a gradient elution based on solvent mixtures described by Christie (1986). The flow rate was 2 ml/min, detector temperature 40°C and air flow 27 psi. The method was validated by determining the average repeatabilities of the injections, intra- and inter-day variations and extraction recoveries for each plasma lipid class.

4.2.3. Serum cholesterol and triacylglycerol measurements

Study 1 (I-IV). Serum lipid concentrations were determined at Vagus Medical Laboratory, Turku, Finland. TC and TAG levels were analysed enzymatically using CHOD-PAP (Boehringer) and Mercotest (Merck) kits, respectively. HDL-C was determined enzymatically after precipitation of VLDL-C and LDL-C with polyethylene glycol 6000 (Viikari 1976). LDL-C levels were calculated using Friedewald's formula: LDL-C = TC - HDL-C - TAG/2.2 (Friedewald et al., 1972).

Study 2 (V). Plasma TC and TAG levels were determined enzymatically in the Laboratory of Helsinki University Hospital. HDL-C was measured with the same technique after precipitation of LDL-C and VLDL-C with dextran sulphate-magnesium chloride reagent (Finley et al., 1978). Serum LDL-C was calculated according to the Friedewald's formula.

4.3. Statistical methods

The two-week variabilities for total plasma and PL fatty acids were calculated by the following formula: $[| X_{2Weeks} - X_{0Weeks} |/(X_{0Weeks} + X_{2Weeks})/2] \times 100\%$, where X_{2Weeks} is the sample taken 2 weeks before fat substitution, and X_{0Weeks} is the sample taken at the baseline (just before substitution).

Statistical differences in lipid and nutrient levels were analysed by the paired or unpaired t-test (within groups and between groups, respectively). Pearson's correlation coefficients and regression analysis were used in studying the relationships between plasma PL fatty acid compositions and serum cholesterol concentrations and plasma lipid classes. All the significance levels ($p \leq 0.05\%$) are given two-sided.

5. RESULTS

5.1. Reproducibility of the analytical methods

GC analysis of total plasma (PPP) fatty acids. The determinations included all the study subjects who were habitual butter (n = 43), margarine (n = 46) or mixed fat users (n = 11). The mean reproducibility of the method including lipid extraction, derivatization and GC run was 4.5% (RSD) for quantitative (I, Table 1) and 2.4% for relative amounts of 14 FAMEs in total plasma. The total amount of the bound fatty acids in PPP correlated significantly ($r = 0.87$, $p<0.001$, $n = 100$) with the serum TAG values determined enzymatically (I, Fig. 2a).

GC analysis of phospholipid (PL) fatty acids. The average reproducibility of the method consisting additionally of TLC isolation of PLs, was 3.9% (RSD) for 12 FAMEs. The proportion of the methodogical variation out of the total variation of the population, based on variances, was the highest for palmitic (16:0) and stearic (18:0) acids and DPA (22:5n-3) and DHA (22:6n-3) (I, Table 3). The repeatability for the two main PL *trans*-isomers, elaidic (18:1n-9*trans*) and vaccenic acids (18:1n-7*trans*), analysed directly by GC *i.e.* without preceding TLC isolation, was 3.4 and 2.7% (RSD), respectively (IV, Table 4).

Stereospecific analyses. The reproducibility of the determination of major *sn*-2 fatty acids from vegetable oil TAGs was 3.9% (RSD), on the average (Seppänen-Laakso et al., 1995a). In the analysis of nine *sn*-2 fatty acids from plasma PC, the repeatability of the method, including additionally TLC isolation of PC and phospholipase A_2 treatment, was 8.7% (RSD).

HPLC-ELSD analysis of plasma lipid classes (V). The average intra- and inter-day precisions of the assay, including high and low lipid concentrations, ranged between 1.9 - 4.5% (RSD) and 2.3 - 7.2%, respectively, for CE, FC, TAG and PC in pooled plasma. The relative recoveries of the lipids varied between 97-110%.

5.2. Variation of plasma fatty acids and serum lipids

5.2.1. Two-week variability of plasma fatty acids before fat substitutions

The variability of total plasma fatty acids within the two-week period ranged from ±2.4 to ±13.9% (n = 89); the lowest variabilities were found for SaFAs (16:0; 18:0). The corresponding levels for α-LLA and OA were ±10.1 and ±4.4%, respectively (Seppänen-Laakso et al., 1995b). The mean increase in α-LLA during rapeseed oil substitutions (II+III, n = 43) was 29% and that in OA during olive oil substitution (III) 13% higher than at the baseline. These fatty acids were most indicative of the use of these oils.

The two-week variability of palmitic (16:0) and stearic acid (18:0) and LA in plasma PL ranged between ±3.0 to ±4.1% (Seppänen-Laakso et al., 1995b) (Fig. 3). The proportions of methodological variations for 16:0 and 18:0 (indicated by black bars in the figure) were 29 and 21%, respectively.

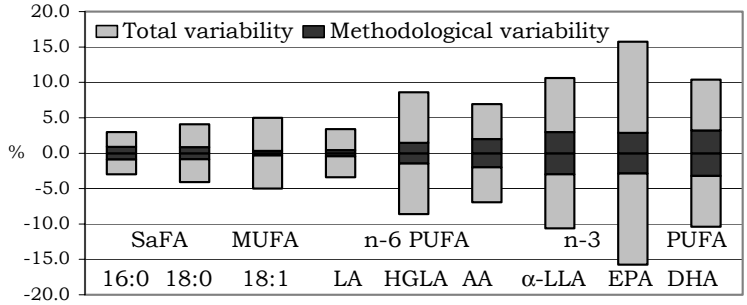

Fig. 3. Two-week variability of plasma PL fatty acids *vs* methodological variation.

The most fluctuating fatty acids in plasma PLs were n-3 PUFAs, especially eicosapentaenoic acid (±15%; EPA) (Fig. 3). After three weeks' rapeseed oil substitutions (II, III), the mean increase in PL EPA was 27% higher than at the baseline. The decrease in SaFAs (16:0 and 18:0) most clearly exceeded the two-week variability during replacement of butter, which was 6.3% lower than before substitution (II).

5.2.2. Variation of fatty acids and serum lipids at the baseline

The data in *Study 1* (Table 8) show that lipid concentrations were rather similar between the two groups, whereas a higher SaFA and lower LA and total n-6 PUFA level ($p<0.001$) in plasma PLs is typical for butter users. Thus, the higher P/S ratio (0.47; $p<0.001$) in the diet of margarine users is clearly reflected at plasma PL fatty acid levels.

Table 8. Summarized data of plasma PL fatty acids and serum lipids.

Plasma PL fatty acids (%)	Butter users *Study 1* (n=43, II)		Margarine users *Study 1* (n=46, III)		Margarine users *Study 2* (n=48, V)	
	Mean	RSD, %	Mean	RSD, %	Mean	RSD, %
Total SaFAs	45.9 [1]	3.9	44.6	2.3	44.3	3.7
Total MUFAs	14.8	10.0	13.9	9.8	14.4	13.7
Total n-6 PUFAs	31.6 [1]	7.2	33.4	5.9	35.1 [2]	6.9
18:2n-6, LA	21.2 [1]	10.7	22.8	9.9	22.7	13.4
20:3n-6, HGLA	2.8	21.8	2.9	21.1	3.2	21.8
20:4n-6, AA	7.6	18.2	7.6	14.3	9.2 [2]	17.2
Total n-3 PUFAs	7.8	20.3	8.1	19.3	6.2 [2]	22.9
18:3n-3, α-LLA	0.5	25.9	0.4	23.3	0.4	37.3
20:5n-3, EPA	1.5	36.0	1.4	40.2	1.3	37.0
22:5n-3, DPA	1.1	14.5	1.1	14.1	1.0	23.1
22:6n-3, DHA	4.7	23.4	5.2	20.1	3.5 [2]	30.4
n-6/n-3 PUFA ratio	4.3	23.2	4.3	25.2	6.0 [2]	25.3
Serum lipids (mmol/L)						
TC	6.2	11.2	6.2	13.7	4.9 [2]	19.3
LDL-C	4.3	16.6	4.2	21.0	3.0 [2]	29.0
HDL-C	1.5	28.0	1.4	23.8	1.5	25.0
TAG	0.9	44.3	1.2	53.0	1.0	50.7
Fat intake (E-%)	38.1	17.3	39.1	18.3	35.2	18.0
P/S ratio	0.33 [1]	40.9	0.47	40.3	0.43	45.0
Age	45.1		45.1		38.1	
Men/women	21/22		25/21		11/37	

Unpaired t-test: [1] $p<0.001$, butter *vs* margarine users; [2] $p<0.001$, between margarine users

Comparison between the two groups of margarine users show especially high arachidonic (AA) and low docosahexaenoic acid (DHA) levels ($p<0.001$) in *Study 2*. The fact that most of the subjects did not use fish in their habitual diet is reflected by the higher PL n-6/n-3 PUFA ratio ($p<0.001$). In addition, the group was younger, consisted mainly of women, and had a lower fat intake, and clearly lower serum cholesterol levels ($p<0.001$) (Table 8).

Quantitative variation of plasma lipid classes were examined in *Study 2* (V). PC (mean 3.27 g/L) was the most abundant lipid class, ranging from 1.73 to 5.60 g/L (27.3%, RSD). CEs were less variable (2.21 g/L; 18.9%, RSD) than FC (30.9%, RSD). TAGs had the highest relative variation (51.7%, RSD) and corresponded to the levels obtained by the enzymatic method.

5.3. Effects of fat substitutions on serum lipid levels and plasma fatty acid composition

5.3.1. Replacement of butter by rapeseed oil (II)

Intake of substitute oil. Based on the dietary data, the average consumption of rapeseed oil in the group (n = 20) was 18 g/day, accounting for 20% of the total fat intake (II, Table 3). The doses did not exceed 40 g/day (Seppänen-Laakso et al., 1989a). The P/S ratio of dietary fat increased ($p<0.01$) due to the fall in SaFAs ($p<0.01$) and rise in PUFAs ($p<0.01$) (II). The proportion of MUFAs ($p<0.05$) also increased. In the control group (n = 11) no changes were found in the nutrient intake.

Total plasma fatty acids. As a result of reduced use of butter the proportion of SaFAs decreased by 4%-units, reaching its lowest level at 6 weeks (II, Table 4) (Fig. 4a). α-LLA increased during the rapeseed oil diet ($p<0.001$) and decreased to the baseline at the end of the study (Fig. 4b).

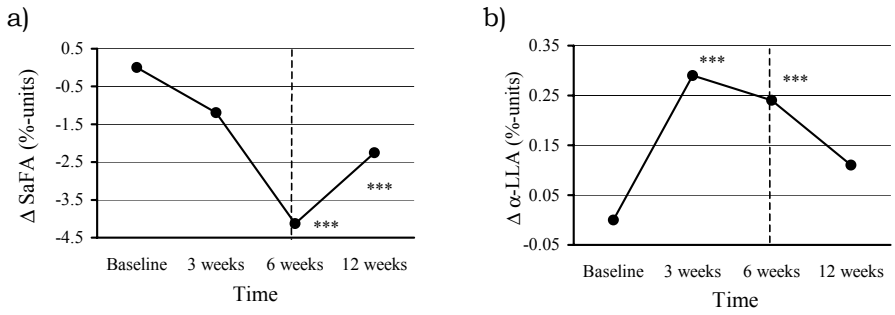

Fig. 4. Changes in plasma SaFA (a) and α-LLA (b) levels during fat replacement.

The mean intake of α-LLA was calculated to be 1.8 g/day, covering about 2.0% of total fat (II). The rise correlated with the amount of oil used ($r = 0.51$, $p<0.05$). A marked increase in LA occurred at 6 weeks (II, Table 4; 5 %-units, $p<0.001$), whereas that in OA remained minor. The changes in these fatty acids in the control group were non-significant.

Plasma PL fatty acids. The proportions of SaFAs, *i.e* those of palmitic and stearic acids, as well as the concentration of LDL-C, decreased significantly during the first 3 weeks of rapeseed oil substitution (II, $p<0.001$) (Fig. 5a-b). Both SaFA and cholesterol levels were restored at 6 weeks but remained below the baseline at the end of the study.

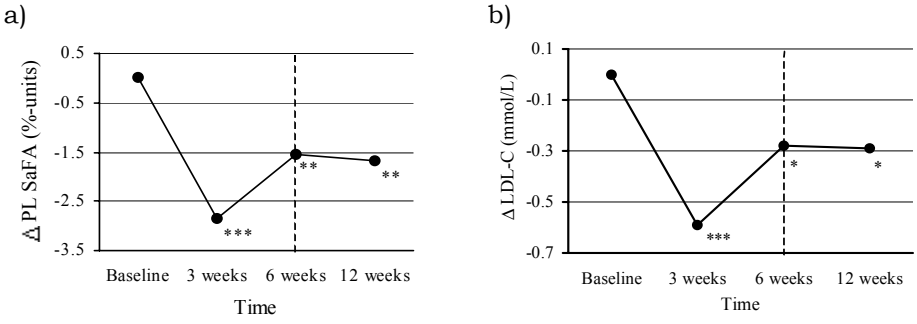

Fig. 5. Changes in plasma PL SaFA (a) and serum LDL-C (b) levels.

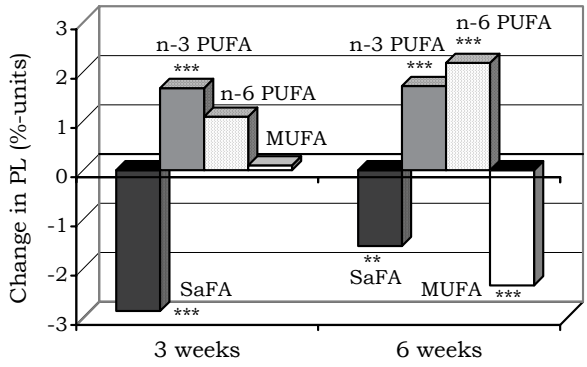

Fig. 6. Changes in PL fatty acids when replacing butter by rapeseed oil.

Rapeseed oil raised the levels of all the major n-3 PUFAs, *i.e.* α-LLA ($p<0.001$), EPA ($p<0.01$), DPA ($p<0.05$) and DHA ($p<0.001$), during the first 3 weeks. The levels of EPA, DPA and DHA remained significantly higher still at 6 weeks (II, Table 5) (Fig. 6). The rise in n-6 PUFAs ($p<0.001$) did not occur

until at 6 weeks, *i.e.* in LA (n.s.), HGLA ($p<0.05$) and AA ($p<0.001$), with a simultaneous fall in MUFAs ($p<0.001$).

Fatty acids in the plasma PC fraction were determined at the baseline and after 3 weeks' rapeseed oil substitution (n = 20). The *sn*-2 position mainly consisted of LA (46%) and OA (30%). Substitution caused an increase in LA and EPA ($p<0.05$) levels, with a simultaneous decrease in SaFAs and OA ($p<0.05$). In the *sn*-1 position, characterized by palmitic (41%), stearic (22%) and OA (19%), no changes appeared during substitution (Seppänen-Laakso et al., 1993b). Drastic differences in LA and SaFA levels in the *sn*-2 position existed between men and women (VI, Fig. 13). In men, the proportion of SaFAs (palmitic and stearic acids) was about 1.7-fold compared to that in women.

Serum lipids. During rapeseed oil substitution, the decrease in the serum TC concentration (range 5.4 - 8.4; mean 6.3 mmol/L) was 7.8% ($p<0.01$) at 3 weeks and 3.0% at 6 weeks (II, Table 6). The reductions in LDL-C were 13.4% ($p<0.001$), but only 6.4% ($p<0.05$) at 6 weeks due to strong restoration of the values (Fig. 5b). In subjects with initial TC levels higher than 6.0 mmol/L, the fall in LDL-C at 3 weeks was 16.3% ($p<0.01$) (II) (Seppänen-Laakso et al., 1989a). Fat replacement had no effect on HDL-C concentrations. In the control group the changes in cholesterol levels remained minor (n.s.). A rise in serum TAG levels was found during the experimental period in both the substitution and control groups ($p<0.05$).

5.3.2. Replacement of butter by margarine (II)

Substitute fat intake. When replacing butter by the test margarine the daily dose was 23 g, on the average, accounting for 22% of total fat (II, Table 3, n = 23). The nutrient data showed a rise in PUFAs ($p<0.05$) and in the P/S ratio ($p<0.01$), and a decrease in the amount of dietary cholesterol ($p<0.05$). The mean intake of LA derived from the test margarine was 6.5 g and that of 18:1*trans*-fatty acids 3.7 g/day.

Total plasma fatty acids. The decrease in SaFAs (3.6 %-units, p<0.001), as well as the increase in LA (4.7 %-units, p<0.001), was strongest at 6 weeks (II, Table 4) (Fig. 7a-b). During the post-experimental period, the LA level remained higher (p<0.01) and that of SaFAs lower (p<0.001) than at the baseline.

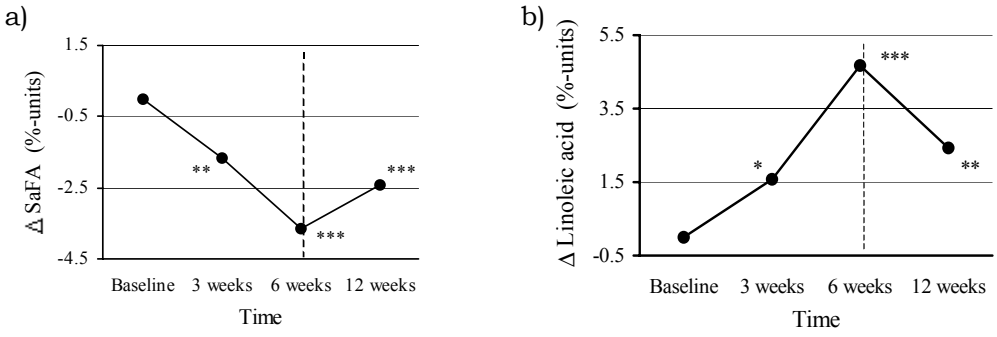

Fig. 7. Changes in total plasma SaFA (a) and LA (b) levels during replacement of butter by the test margarine.

Phospholipid fatty acids. Replacement of butter by the test margarine resulted in a decrease in the level of SaFAs (p<0.01) at 3 weeks (II, Table 5), but also in a partial restoration at 6 weeks. The proportion of n-6 PUFAs increased at 3 weeks (p<0.01) and remained elevated until the end of substitution. The changes in MUFAs and n-3 PUFAs appeared slight as in total plasma. PL 18:1*trans*-fatty acids are described in section 5.3.7.

Serum lipids. The serum TC (range 5.1–7.5; mean 6.1 mmol/L) and LDL-C levels fell by 6.3% (p<0.001) and 7.8% (p<0.01), respectively, during the first 3 weeks (II, Table 6). The values were restored at 6 weeks, but decreased again at 12 weeks. A temporary fall in HDL-C levels (p<0.05) occurred at 3 weeks.

5.3.3. Relationships between plasma PL acids and serum lipids (II)

At the baseline, PL α-LLA was the only fatty acid closely associated with the LDL-C levels in the combined group of habitual butter users (r = -0.40, p<0.01, d.f.= 41) (II). During rapeseed oil substitution at 3 weeks, the rise in

PL linoleic/stearic acid ratio was inversely related to the fall in TC ($r = -0.71$, $p = 0.01$; II, Fig. 2a) in subjects with TC baseline values higher than 6.0 mmol/L (n = 11). This was also the case between the rise in α-LLA and the fall in TC ($r = -0.62$, $p<0.05$). Between weeks 3-6, the increase in SaFAs correlated with that in TC ($r = 0.58$, $p<0.01$; d.f. = 18; II, Fig. 2c).

In the margarine group, the rise in PL linoleic/stearic acid ratio at 3 weeks similarly correlated with the fall in TC ($r = -0.59$, $p<0.01$, d.f. = 21; II, Fig. 2b). Between weeks 3-6, there was a close correlation between the rise in LDL-C and PL stearic acid ($r = 0.51$, $p = 0.01$; II, Fig. 2d).

5.3.4. Replacement of margarine by rapeseed oil (III)

Intake of substitute fat. In the group replacing margarine, the amount of rapeseed oil used was 17 g/day, accounting for 15% of the total fat, on the average (n = 23; III, Table 3). No changes were found in dietary cholesterol levels, whereas the P/S ratio increased during substitution ($p<0.05$) owing to the rise in PUFAs ($p<0.01$).

Total plasma fatty acids. The changes in plasma MUFAs appeared to be the best indicator of a reduced use of margarines (Fig. 8a) containing about 40% MUFAs (III, Table 2). The level of α-LLA increased during substitution ($p<0.01$; III, Table 4) (Fig. 8b), and decreased to the initial level at the end of the study. The rise was dose-dependent ($r = 0.79$, $p<0.001$).

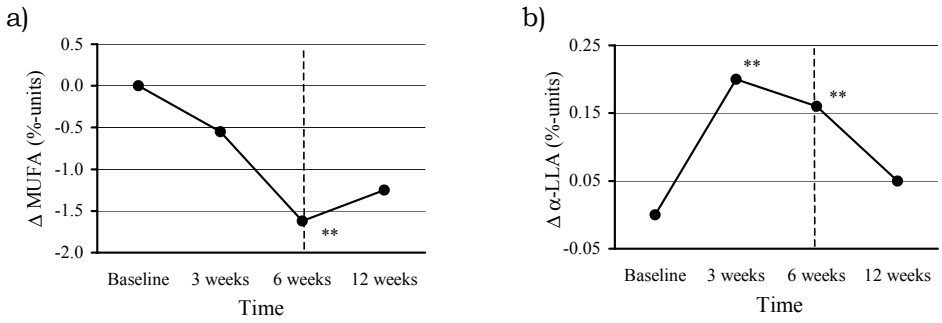

Fig. 8. Changes in total plasma MUFAs (a) and α-LLA (b) during replacement of margarine by rapeseed oil.

The average intake of α-LLA derived from rapeseed oil was calculated to be 1.6 g/day, which corresponded to 1.5% of total fat and 0.7% of the total energy intake. Compared to the baseline, the level of n-6 PUFAs remained higher ($p<0.001$) and that of SaFAs lower ($p<0.001$) at the end of the study.

Phospholipid fatty acids. Replacement of margarine by rapeseed oil caused a fall in palmitic acid ($p<0.001$), total SaFAs ($p<0.001$), AA ($p<0.01$) and total n-6 PUFAs ($p<0.01$) during the first 3 weeks (III, Table 5) (Fig. 9). At the same time, α-LLA ($p<0.001$), EPA ($p<0.01$), total n-3 PUFAs ($p<0.01$), OA and total MUFAs ($p<0.001$) increased, whereas no change occurred in the DHA levels.

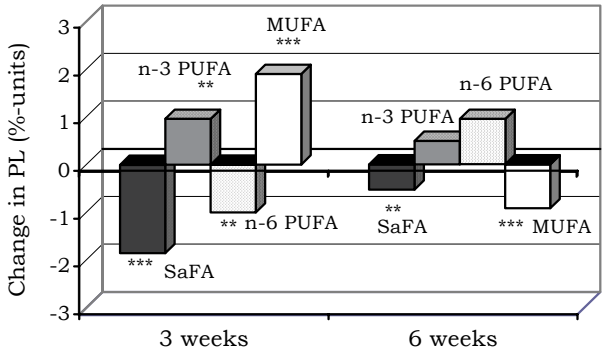

Fig. 9. Changes in PL fatty acid composition when replacing margarine by rapeseed oil.

The level of n-6 PUFAs (both LA and AA) markedly increased between weeks 3-6 (III, Table 5) (Fig. 9). Simultaneously, n-3 PUFAs remained lower than at 3 weeks, while the proportion of MUFAs ($p<0.001$) strongly decreased below the baseline.

Serum lipids. During rapeseed oil substitution, no change in TC levels (range 5.0–7.7; mean 6.1 mmol/L, n = 23) was found, while HDL-C temporarily increased by 6% (0.08 mmol/L, $p = 0.01$) at 3 weeks (III, Table 6). The HDL-C /TC ratio increased during the 6 weeks' diet period. In subjects with baseline TC levels higher than 6.0 mmol/L (n = 13), the fall in LDL-C was 10% ($p = 0.01$) at 3 and 11% ($p<0.01$) at 6 weeks.

5.3.5. Replacement of margarine by olive oil (III)

Substitute fat intake. In the group replacing margarine (n = 23), the amount of olive oil used was 19 g/day, accounting for 18% of the total fat (III, Table 3). A fall in the level of dietary PUFAs ($p<0.05$) and an increase in that of MUFAs ($p<0.01$) occurred. No changes were found in the P/S ratio or in dietary cholesterol.

Total plasma fatty acids. Olive oil substitution decreased the level of SaFAs ($p<0.001$) and raised that of OA during the 6 weeks' substitution ($p<0.001$; III, Table 4). The intake of oil correlated with the rise in OA at 3 weeks ($r = 0.41$, $p<0.05$).

Phospholipid fatty acids. Substitution had no effect on SaFAs, but decreased LA ($p<0.01$) and increased OA ($p<0.01$). The changes were the reverse for the whole study period (III, Table 5) (Fig. 10). A temporary rise in EPA at 3 weeks ($p<0.001$) and in AA levels at 6 weeks were found ($p<0.01$).

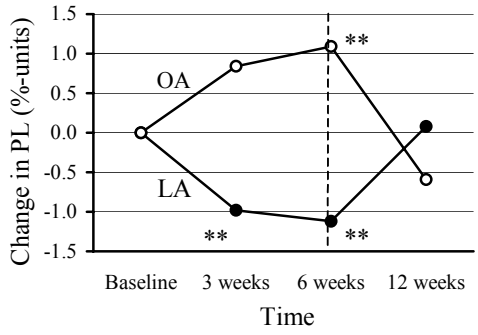

Fig. 10. Changes in PL linoleic and oleic acids during fat replacement.

Serum lipids. In the olive oil group (TC range 5.0-8.0; mean 6.3 mmol/L) the LDL-C level temporarily decreased at 3 weeks (7.5%, $p<0.01$), while that of HDL-C remained unchanged (III, Table 6). In subjects with TC baseline levels higher than 6.0 mmol/L (n = 13), the fall in TC was also significant at 3 weeks (7%, $p<0.01$). TC and LDL-C levels decreased ($p<0.01$) and were below the baseline during the post-experimental period.

5.3.6. Relationships between plasma PL fatty acids and serum lipids (III)

During replacement of margarine by rapeseed oil the increase in PL α-LLA at 3 weeks was inversely associated with the decrease in TC levels ($r = -0.42$, $p<0.05$; III) and with that of LDL-C ($r = -0.43$, $p<0.05$; III, Fig. 3a). The rise in α-LLA was also related to the rise (2.1 %-units) in the HDL-C/TC ratio ($r = 0.44$, $p<0.05$; III, Fig. 3b). During olive oil substitution the increase in OA at 3 weeks was associated with the decrease in TC ($r = -0.59$, $p<0.01$) and LDL-C levels ($r = -0.46$, $p<0.05$; III, Fig. 3c).

5.3.7. Changes in 18:1*trans* fatty acid levels in plasma phospholipids (IV)

At the baseline, the PL elaidic acid (18:1n-9*trans*) level was higher in margarine than in butter users ($p<0.01$; IV, Table 5), and the proportions of both elaidic and vaccenic acids (18:1n-7*trans*) were inversely associated with the HDL-C concentrations ($p<0.05$ and $p<0.01$, respectively).

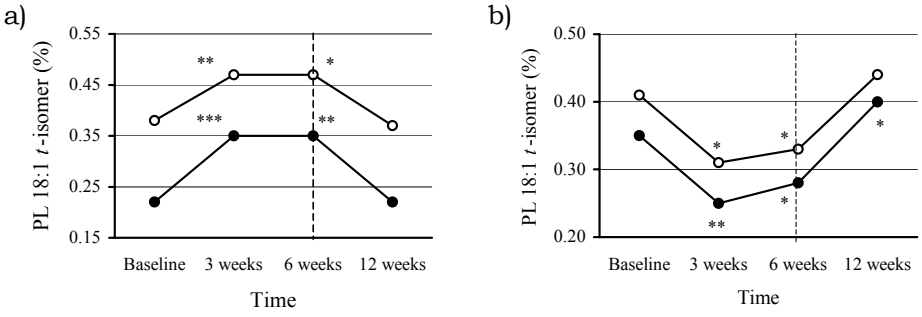

Fig. 11. Changes in PL elaidic (18:1n-9*trans*; filled dots) and vaccenic acid (18:1n-7*trans*; open dots) levels during replacement of butter bytest margarine (a) and margarine by olive oil (b).

During replacement of butter by the test margarine, the average intake of 18:1n-11 to n-7*trans*-isomers was 3.7 g/day. The use of margarines was best indicated by a rise of elaidic and vaccenic acids (IV, Table 5) (Fig. 11a). When margarines (8% 18:1*trans*-fatty acids, on average; IV, Table 2) were replaced by olive oil, the proportions of PL elaidic and vaccenic acids decreased during substitution (Fig. 11b).

5.4. Effects of fat substitutions on plasma lipid classes (V)

5.4.1. Effects of rapeseed and soybean oils

Substitute fat intake. In *Study 2* (V), the average amounts of cold-pressed (17 ml; n = 26) or conventional rapeseed oil (15 ml; n = 16) or soybean oil (16 ml; n = 6) accounted for 24% of daily fat intake during fat replacement.

Lipid levels. The average serum TC concentration of the subjects was 4.9 mmol/L (range 3.3 - 6.9 mmol/L; n = 48), but only slight changes (n.s.) were found in the groups during the 6 weeks' substitutions. The concentrations of the major plasma lipid classes, *i.e.* CE, FC, TAG and PC, were determined by HPLC before and after substitutions.

Soybean oil raised the CE (n.s.) and reduced the FC levels ($p<0.05$), resulting in a rise in the CE/FC ratio (from 3.6 to 5.1, $p<0.01$). In the groups using rapeseed oils the CE/FC ratio remained unchanged, whereas cold-pressed rapeseed oil more clearly increased the PC concentration ($p<0.01$) (V, Table 5). In addition, it reduced the non-lipidic compound fibrinogen ($p<0.01$).

5.4.2. Relationships between plasma lipid classes and PL fatty acids

In the whole group (n = 48), the PC concentrations at the baseline had a positive correlation with HDL-C ($r = 0.51$; $p<0.001$), plasma PL EPA (20:5n-3; $r = 0.41$, $p<0.01$) and MUFA ($r = 0.41$, $p<0.01$; V, Table 4). The increases in PC levels seemed to occur parallelly with those in n-3 PUFAs ($p<0.05$) during rapeseed oil substitutions (V, Table 5). PL LA, in contrast, had the highest inverse relationship with the PC concentration ($r = -0.48$; $p<0.001$; V, Table 4). Furthermore, LA was closely associated with FC ($r = -0.56$, $p<0.001$; V, Table 4) and it had, rather than serum lipids, an even closer relationship with the plasma esterified/free cholesterol ratio (CE/FC) ($r = 0.65$, $p<0.001$, Table 5).

6. DISCUSSION

The fatty acid composition of dietary fat is decisive in the prevention of a variety of adverse or even serious health effects. The diet has to contain more linoleic (LA, 18:2n-6) than α-linolenic acid (α-LLA, 18:3n-3), and both of them in sufficient amounts because they cannot be synthesized by the body. Continuous interactions take place in their metabolism to longer-chain n-6 and n-3 PUFAs because they compete for the same desaturating and elongating enzymes. Thus, the effects depend on the ratio between these two dietary fatty acids (Holman 1986; Lands et al., 1992; Chapkin 2000).

Major vegetable oils containing marked amounts of PUFAs also have the lowest levels of SaFAs. Although they are effective in replacing SaFAs, the majority of these edible oils have a high LA content (>50%) (Table 1) and thus the effects are due to n-6 PUFAs only. Among the MUFA oils, olive oil is rich in oleic (71-77%) but low in LA (7-10%). Canola-quality rapeseed oil is another high-MUFA oil, but contains moderate amounts of PUFAs in a more balanced ratio (LA, 18-23% and α-LLA, 9-11%).

The desirable effects of the α-LLA of edible oils and long-chain n-3 PUFAs of marine foods, demonstrated already decades ago, are now receiving ever more increasing attention in the prevention of cardiovascular disease (de Lorgeril et al., 1994; Leaf and Kang 1998; Simopoulos 1998; Lands 2003).

In the present investigation, the effects derived from partial replacement of dietary fats on the plasma fatty acid composition, lipid class and serum lipid levels, and their associations, were examined in two studies consisting of 100 and 48 subjects, respectively. Substitute fats including ordinary (II-III, V) and cold-pressed (V) rapeseed oils, a test margarine (II, IV), olive oil (III-IV) and soybean oil (V), were used in amounts corresponding to the amount of fat on bread in the subjects' habitual diets. Fatty acids and lipids were in most cases measured at the baseline and after 3 and 6 weeks' substitutions. The methodology related to the GC of fatty acids and HPLC of lipid classes was optimized (I, IV, V).

6.1. Plasma fatty acid analysis by gas chromatography

Gas chromatography is an indispensable method in the fatty acid analysis of food fats and human tissues. Reproducible GC techniques and sample preparation procedures are required for the reliable determination of fatty acids over wide concentration ranges in plasma. The first steps usually include lipid extraction, isolation of lipid subclasses by TLC, derivatization of fatty acids to methyl esters and, if required, TLC separation of *cis/trans*-isomeric fractions. In the GC method, after selecting the carrier gas and an analytical column with sufficient resolution, optimization of the temperature programming, injection modes suitable also for dilute samples, gas flow rates and split conditions is necessary (Shantha and Napolitano 1992; Eder 1995; Ackman 2000a). Methodological points included in this study have also been treated in VI.

The total plasma and plasma PL fatty acid composition were analysed by GC using the PTV sampling technique with a mean reproducibility of 2.4 and 3.9% (RSD), respectively (I). Reliable analyses were needed for the main PL SaFAs (2%, RSD) even, because the methodological variation of palmitic acid, e.g. out of the total variation of the study subjects, accounted for as much as 20% in single measurements (I) and 30% within a two-week period (Fig. 3).

A low relative variation of palmitic acid in plasma PL (I, 5.2% RSD; n = 100) seems to be typical, since similar values have been found, for example, in populations of men (5.1%, n = 103; Nikkari et al., 1983a) and even children (4.1%, n = 162; Nikkari et al., 1983b). However, differences in PL SaFA levels caused especially by their long-term excessive intake, can be detected in lipids where they usually do not occur in abundance, as in plasma PC subfraction (Seppänen-Laakso et al., 1993b; VI, Fig. 13). The differences between groups at plasma PL n-3 and n-6 PUFA levels were usually very small, often 1-2 %-units only (Table 8). The changes in PUFAs but also in octadecenoic *trans*-isomeric levels, although remaining clearly below 1 %-unit during the fat substitutions (II-V), could be still reliably determined.

6.2. Fatty acids as indicators of dietary fat intake

The changes made in the quality of dietary fat are best identified in the plasma fatty acid composition, and fatty acid analyses are therefore necessary to ensure compliance with the fat substitution. However, the responses at the plasma level which are derived from dietary fat replacement can vary markedly depending on the age of the subjects, quality and quantity of fat, especially the amount of SaFAs in long-term habitual diets, fatty acid composition of the substitute fat, degree of fat replacement and the time period between the measurements on plasma lipids. It is therefore clear that the detailed responses can be very different (VI).

Saturated fatty acids. A high amount of SaFAs in the diet is the most adverse, and it is usually first evident as elevated serum cholesterol levels. When studying the effects of substitute fats, a clear-cut reduction in the plasma SaFA levels is required. However, decreasing trends are not always exactly the same in the different lipid fractions.

In the present study (II), the replacement of butter by canola oil indicated a 90% reduction of SaFAs on bread. The decrease in total plasma SaFAs (4 %-units) (II, Table 4) (Fig. 4a) was most prominent at the end of the 6-week period, thus definitely reflecting the reduced use of butter. A similar decrease in SaFAs was also observed when the test margarine was used (Fig. 7a). When margarine on bread was replaced, the olive oil substitution brought about a fall in total plasma SaFAs (III, Table 4).

The changes in SaFA levels were more complex in plasma PLs, but they also correlated with the cholesterol levels (II). When butter was replaced by rapeseed oil, the lowest levels of PL SaFAs (-3 %-units; II, Table 5) and serum LDL-C (-13%; II, Table 6) were found at 3 weeks, because SaFAs were restored by 1.3 %-units and LDL-C by 8.2%, respectively, during the following 3 weeks. The corresponding values in the margarine group were 0.8 %-units and 4.6%. In both groups, the decrease in cholesterol levels at 3 weeks was most closely related to the increase in the linoleic/stearic acid

ratio ($p \leq 0.01$), while the increase in cholesterol levels during restoration correlated with those in PL SaFAs, and especially in stearic acid ($p \leq 0.01$) (II, Fig. 2c-d).

The restoration of LDL-C observed in the habitual butter users (II), but not in the margarine users (III), is evidently related to cholesterol metabolism and LA. The increase of LA in total plasma of about 5 %-units (II, Table 4) represented the largest change derived from substitute fats found at the plasma level in these experiments. As shown by the strikingly parallel trends for PL SaFAs and LDL-C (Fig. 5a-b), the changes between weeks 3 and 6 can be considered as indicators of long-term accumulation of cholesterol (and SaFAs) in the body under a shortage of dietary LA, and their release from the tissues into the plasma during fat substitution (VI). A similar phenomenon is known in the opposite case too, *i.e.* when the preceeding diet is high in LA. Adipose tissue LA can be a continuous source of LA to tissue PLs when the intake of this fatty acid is restricted (Mantzioris et al., 1995).

Replacement of margarine by rapeseed oil, which also first reduced the PL SaFA levels (~2 %-units), led to a SaFA restoration at 6 weeks (1.3 %-units) (III, Table 5). This shows, despite the use of margarine on bread, that marked amounts of SaFAs have also been included in the diet of habitual margarine users. The fact that the decrease in PL stearic acid, instead of palmitic acid, most often correlated with that in TC when replacing butter by rapeseed oil (II), reflected the early features of Finnish high SaFA diets. In the adipose tissue of men, the stearic acid level especially has earlier been shown to be twice as high, and that of LA about half of that in Italian men (Riemersma et al., 1986).

The plasma PC fraction was the most indicative of an imbalance between LA and SaFAs (Seppänen-Laakso et al., 1993b; VI, Fig. 13). In the group of men, both stearic and palmitic acids were responsible for lower proportions of LA in the *sn*-2 position of PC. A sufficient LA level is important due to its relation to cholesterol metabolism, because LA in this position is the best substrate for HDL-associated LCAT to esterify cholesterol which, in turn, can

perform the reverse cholesterol transport from peripheral cells to the liver (Jonas 1987).

Trans-fatty acids. High amounts of 18:1*trans*-fatty acids also represent an undesirable component of food fat, the main sources being food products to which partially hydrogenated fats have been added. They represent about 0.3-3% of total fatty acids in human fat tissue (Ohlrogge et al., 1982; Fritsche et al., 1998). The profiles of 18:1*trans*-isomers have been determined in various lipid classes in several human tissues, most often in plasma (Ohlrogge et al., 1982; Emken et al., 1989a, 1989b) and adipose tissue (Hudgins et al., 1991; Fritsche et al., 1998).

Changes in the plasma PL 18:1*trans*-isomeric level were studied by replacing *trans*-containing margarines by vegetable oil, or by replacing butter by a *trans*-containing test margarine (IV). At the end of the 1980s, many of the margarines contained about 8% of 18:1*trans*-isomers (IV, Table 5). The two major isomers, elaidic and vaccenic acids, were excellent indicators in that they clearly indicated both decreased and increased use of *trans*-fatty acid-containing fats (Fig. 11). Despite the low levels of these isomers in plasma PL (<1%), they were inversely associated with HDL-C when the habitual diets contained hydrogenated fats.

One specific feature is that the *trans*-pattern of dietary fat is not reflected as such in plasma PLs (Ohlrogge et al., 1982; Emken et al., 1989b). The isomeric profiles demonstrate that the incorporation of elaidate (18:1n-9*trans*) derived from hydrogenated fats is particularly efficient compared to the other isomers (IV, Fig. 1). Based on the elaidate level, the isomeric profile is different from that derived from butterfat, and this can also be clearly distinguished by direct GC analysis of the plasma PL fraction (IV, Fig. 2).

Major unsaturated fatty acids. The fatty acid composition of adipose tissue, the principal site of the storage of body fat, provides suitable biomarkers of dietary fat quality during long periods of time (Katan et al., 1991; Marckman et al., 1995; Lands 1995). Dietary fat is mainly composed

of TAGs, and it is therefore the most variable lipid class in plasma, the concentrations varying even within a tenfold range (V). Thus, the TAG fraction as well as total plasma (I-III), in addition to giving the average fatty acid composition of lipids, can also be used in monitoring dietary fat intake.

The cholesteryl ester (CE) fraction is a suitable indicator for LA, since it is especially enriched in this lipid class. Early studies have compared CE LA levels in free-living populations of Finnish men (Nikkari et al., 1983a), in children using butter or margarine on bread (Nikkari et al., 1983b), and in North Karelians consuming sunflower oil diets (Nikkari 1986). CE α-LLA is a suitable indicator for rapeseed oil-based diets (Sarkkinen et al., 1994; Valsta et al., 1995), as well as LA for rapeseed oil and sunflower oil diets (Valsta et al., 1995). In a long-term intervention study on children, clearly higher CE LA but lower long-chain n-6 and n-3 PUFA levels were indicative of formula-fed infants compared to breastfed 7-month-old infants (Salo et al., 1999). After the intervention families had been counselled to replace part of saturated fat with more unsaturated fats, higher PL MUFA levels without any differences in PL 18:1*trans*-fatty acids were found in the intervention and control children at the age of 3 years (Salo et al., 2000).

Total plasma α-LLA was the best indicator of the course of the substitutions when butter or margarine were replaced by rapeseed oil (Fig. 4b, 8b) (II, III Fig. 2a). The daily doses of the oil, containing 1.8 and 1.6 g α-LLA, correlated with the rise in α-LLA. The dose of olive oil (19 g/day) also correlated with the increase in total plasma OA (III, Fig. 2b), while a rise in PL OA occurring at the expense of LA was indicative of the dietary change (VI, Fig. 7).

The control subjects (n = 11) who were asked to continue their habitual diets proved to be useful in monitoring seasonal changes in plasma lipid and fatty acid levels from February to May. However, no marked changes were found in the control group during the first 6-week period (II).

6.3. Competitive interactions caused by essential fatty acids in the diet

Competitive mechanisms are known to arrange the priority of unsaturated fatty acids. The enzyme-substrate affinities, which follow the order linolenic (n-3) > linoleic (n-6) > oleic acid (n-9), indicate that when linolenate is present in the substrate pool, its conversion to higher unsaturated fatty acids takes precedence over the metabolism of linoleate (Holman and Mohrhauer 1963; Mohrhauer and Holman 1963). An opposite effect has also been found, *i.e.* increasing the amounts of dietary linoleate suppresses the levels of linolenate metabolites (Rahm and Holman 1964).

The changes found in PL fatty acid levels after ordinary fat replacement (II, III) can be explained by the differences in the incorporation and desaturation of precursor α-LLA (18:3n-3) and LA (18:2n-6) and subsequent interactions between the three unsaturated (n-3, n-6, n-9) fatty acid families. This presupposes that substitute fat contains at least moderate amounts of both α-LLA (10%) and LA (24%) as was the case in this study in rapeseed oil. Since the composition of the substitute fat corresponded to that of the oil, the effects can be attributed to the oil itself.

Replacement of butter by rapeseed oil (II). This study shows, for the first time at the plasma PL level, a preference for α-LLA and its n-3 PUFA metabolites over LA and its n-6 metabolites during vegetable oil substitution. In addition, the subsequent increase in n-6 PUFAs did not affect the elevated n-3 PUFA levels (II, Table 5) (Fig. 6). Thus, only PUFAs increased when butter was replaced by rapeseed oil.

In plasma PL, substitution indicated an increase in all the major long-chain n-3 PUFAs during the first 3 weeks and, simultaneously, a clear reduction in PL SaFAs (II, Table 5) (Fig. 6). A marked rise in PL n-6 PUFAs was not seen until at 6 weeks, and the level of monoenoic OA (18:1n-9) fell even. Thus, the main compound in rapeseed oil (OA, 60%) could not limit the incorporation of PUFAs in plasma PL during substitution.

A time gap also existed between the changes, but this may not exactly indicate when PL n-3 and n-6 PUFAs are reaching their maximum levels. However, in these measurements, the rise in n-3 PUFAs (1.7 %-units) at 3 weeks was higher than that in n-6 PUFAs (1.1 %-units) (II, Table 5). Thus, it is clear that the conversion of α-LLA is faster than that of LA, and that an increase in long-chain n-3 PUFAs (EPA, DPA and DHA) can cause a delay by suppressing the conversion of LA to its longer-chain metabolites.

The metabolic preference for n-3 PUFAs is in accordance with *in vivo* studies using deuterated precursors (Emken et al., 1989a, 1992; Chapkin 2000). Although α-LLA is considerably less effectively incorporated into plasma PL, it has a clearly higher desaturase selectivity for conversion to long-chain n-3 PUFAs than LA has for conversion to n-6 PUFAs. Incorporation of LA, in turn, is much higher than that of α-LLA, as has been shown in the main lipid fraction of plasma PL, phosphatidylcholine (PC) (Emken et al., 1992).

During replacement of butter by rapeseed oil (II), moderate amounts of LA (24%) appeared to be appropriate (not too high), since no suppressive effect of n-6 PUFAs on n-3 PUFAs was found in plasma PL at the end of substitution (Fig 4). However, activated LA metabolism led to a considerable decrease in MUFAs (OA), showing that even a high OA level in dietary fat is not able to suppress the effect derived from moderate amounts of LA. The suggestion that OA is a neutral fatty acid in its relation to serum cholesterol (Keys 1957; Hegsted et al., 1965) seems to be the case during competitive processes when both EFAs become sufficiently available in high SaFA diets.

Replacement of butter by the test margarine (II). The effects were very different when the substitute fat contained 28% LA and 3% α-LLA acids. An increase in PL LA and AA levels already at 3 weeks showed no delay and response caused by n-3 PUFAs, but predominant n-6 PUFA effect for the substitution period (II, Table 5). This would indicate that dietary fat with a low α-LLA and moderate LA content, has no recognizable effect of n-3 PUFAs in plasma PL.

Replacement of margarine by rapeseed oil (III). The contents of SaFAs and LA in habitually used margarines were about 24 and 31%, respectively. Therefore, the change in fat on bread was not very drastic, and it was best reflected in plasma PL. During the first three weeks, when both SaFAs and LA decreased, there was not only an increase in α-LLA and EPA, but also in MUFAs (1.9 %-units) (III, Table 5). Figure 9 shows that the rise in n-3 PUFAs replaces the fall in n-6 PUFAs, and that MUFAs seem to compensate for SaFAs. Thus, reducing SaFAs and LA results in an increase in EPA and MUFAs especially, and apparently without interferring with each other. At least one study with subjects comparable to the present group reported very similar data as regards changes in PL SaFAs, MUFAs, n-6 PUFAs and EPA after a 3-week rapeseed oil-based diet (Gustafsson et al., 1994).

The dominant role of LA in the middle of the competitive ranking becomes evident when margarine replacement is continued (III, Table 5). The fact that a rise of 2 %-units in PL n-6 PUFAs did not occur until between weeks 3-6 means that LA from the rapeseed oil had started to be utilized (Fig. 9), but it had also a strong suppressive effect on n-3 PUFA metabolism and MUFA levels (-2.8 %-units). In fact, PL DHA (22:6n-3) in this group never increased as a result of α-LLA, which has earlier often been reported for this fatty acid. However, elevated levels in all the long-chain n-3 PUFAs during rapeseed oil substitution (II) show that this is not due to the weakness of α-LLA conversion, but rather the higher LA intake in the diet of habitual margarine users.

Effective α-LLA conversion to EPA, confirmed as an increase of ca. 25%, but not to DHA (III, Table 5), may also include retroconversion of DHA to EPA. Metabolism directed entirely to EPA can be seen as a protective mechanism to maximize the antithrombotic state when the thrombotic effects of linoleate /arachidonate/TxA_2 pathway are continuously active. As EPA has a number of unique pharmacological and biochemical actions, it is assumed that DHA, via retroconversion, supplies EPA on demand when the supply of α-LLA is inadequate (Nelson 2000).

Associations with plasma lipid classes (V). Linoleic acid is predominant also in its relationships with free cholesterol (FC) (r = -0.56; n = 48; V, Table 4) and PC concentrations (r = -0.48), and with the relation of CE to FC. At the baseline, PL LA rather than serum lipids showed a close link to cholesterol metabolism on the basis of its high correlation with the CE/FC ratio (r = 0.65, p<0.001). In a subgroup of subjects already having elevated levels of n-6 PUFAs in plasma PL (Table 8), the high LA content (55%) in soybean oil had a strong effect by further raising the n-6 PUFAs, as well as the CE/FC ratio (V, Table 5).

A high proportion of the high-density lipoprotein cholesterol (HDL-C) fraction in serum lipids is desirable due to its antiatherogenic properties (Miller and Miller 1975). In HDL, PC is a rich lipid fraction (Hemming and Hawthorne 1996), and plasma concentrations are closely related with the ratio of HDL-C to total cholesterol (V, Fig. 3). However, PC correlated positively not only with HDL-C (r = 0.51; V, Table 4), but also with PL MUFAs (r = 0.41) and n-3 PUFAs, especially EPA (r = 0.41). All the associations of LA were opposite to those of n-3 PUFAs. LA can easily suppress n-3 PUFAs and thereby reduce HDL-C, which has been reported after a high intake of LA (Vessby et al., 1980; Mattson and Grundy 1985). Despite a slight effect on serum lipids, the plasma PC levels were raised more clearly by cold-pressed than by ordinary rapeseed oil substitution (V, Table 5), while both oils showed similar trends by increasing EPA and decreasing the n-6/n-3 PUFA ratio in plasma PL.

The fat substitutions used in the present study indicate that the α-LLA and LA contents in rapeseed oil are high enough to give priority to proper n-3 and n-6 PUFA metabolism. LA already in moderate amounts gives a significant response when reducing a high-SaFA dietary fat. It is important that both EFAs can be obtained from the same vegetable oil, as this is the easiest way to fulfil the requirements of α-LLA and LA, and to maintain the balance of n-3 and n-6 PUFA metabolism.

It was also evident that the effectivity of α-LLA *vs* LA can be largely estimated on the basis of their relative amounts, *i.e.* percentages in a specific dietary

fat. When health claims for fatty acid compositions are presented, rapeseed oil should be the basis for comparison.

Rapeseed oil is, so far, the most balanced vegetable oil and a visible fat highly necessary for western-type diets that is capable of normalizing EFA metabolism. The basic functions of EFAs regulated by competitive mechanisms of the body have to be taken into account in long-term prevention and in the treatment of diseases that can be partly derived from imbalances in dietary fat composition.

6.4. Dietary fats and lowering the risk of CHD

N-3 PUFAs and populations with low CHD. The highly different sources and quality of fats in traditional diets in populations with a low rate of CHD have always appeared controversial. The Cretan diet mainly contained olive oil (Keys 1970), which is high in MUFAs but practically free of n-3 PUFAs (α-LLA), while the diet in Greenland Eskimos was based on seafoods (Bang et al., 1971) containing long-chain n-3 PUFAs. Thus, the fat in these diets had to include some basic properties that afforded protection against CHD. Such an effect could be due to the functions of n-3 PUFAs, combined with an abundance of MUFAs in the diet (Simopoulos 1998; Lands 2003).

The food fat in the traditional diets of the Eskimos was unexpected rich in MUFAs (57%), contained half the LA and 2/3 of the SaFAs in the western-type Danish diets, while the contents of n-3 PUFAs *i.e.* EPA and DHA, often expected to be exceptionally high, were both about 2 %-units. However, they were 6-fold compared to those in Danish foods (Bang et al., 1976). The term 'traditional Mediterranean diet' has a specific meaning that reflected food patterns typical of Crete, much of the rest of Greece, and southern Italy in the early 1960s (Willett et al., 1995). Green leafy vegetables and wild plants at that time formed a significant source of n-3 PUFAs (α-LLA), which could also be seen as elevated serum α-LLA levels (Simopoulos 1998). The Cretan diet thus reflected how effective an n-3 PUFA metabolism, supported by a high MUFA intake, could be achieved on the basis of the food intake. The

conversion of α-LLA is expected to proceed normally when combined with low SaFA and rather low LA contents (Renaud et al., 1995; Simopoulos 1999).

The present study also showed that the conversion of α-LLA to EPA was best enhanced when both SaFAs and LA were reduced, *i.e.* by replacing margarine by rapeseed oil (Fig. 9). High amounts of OA in the oil caused a strong rise in PL MUFAs, but did not limit effective conversion of α-LLA to EPA (III). Olive oil substitution, instead, had a clear LA-reducing and MUFA-raising effect (Fig. 10). Olive oil may favour the conversion of α-LLA obtained from other dietary sources as in the early Mediterranean diets (Simopoulos 1998).

The fat in the traditional diet of the Eskimos was a natural part of marine foods, while the Mediterranean diet was characterized by vegetable-based foods with an oily visible fat, olive oil. These diets had apparent health benefits but only when followed strictly, and there have since been clear changes towards more western-style diets (Ferro-Luzzi et al., 1984; Dewailly et al., 2003). Plasma TC, blood pressure and obesity in Cretan men, for example, increased between 1960-90 (Kafatos et al., 1997), and similar TC levels exist even among Cretan, American and Dutch children (Truswell and Choudhury 1998). In elderly Greeks, a higher risk of death was reported among those whose diets deviated the most from the traditional ones (Trichopoulou et al., 1995).

Linoleic acid in the diet. LA is the primary EFA which is ubiquitous in the diet. Early animal studies showed that LA metabolism is predominant, as indicated also by its location in the middle of the competitive order: α-LLA (n-3) > LA (n-6) > OA (n-9) (Holman and Mohrhauer 1963). In the case of a complete lack of EFAs, reported once also in the human diet (Holman 1968), the ultimate attempt of the body is to synthesize PUFAs from OA. Similar selectivities of the metabolic systems of EFAs also appear in human tissue PL (Lands et al., 1992). On the basis of empirical equations, long-chain n-3 and n-6 PUFA levels in PL were shown to be in a competitive, hyperbolic relationship to the dietary supply of their precursors. Incorporation of α-LLA

and LA into tissue TAGs, in turn, follow a linear equation (Lands et al., 1992; Lands 1995, 2000).

During ordinary fat replacements, competitive interactions were also evident in the plasma PL fatty acid composition, and LA proved to be responsible for the most marked changes in the unsaturated fatty acid families (II, III, V). The rise in total plasma α-LLA best correlated with the daily dose of rapeseed oil, and clear effects derived from both α-LLA and LA metabolism were observed in plasma PL n-3 and n-6 PUFA levels (II, III).

The proportion of LA in the diet is highly critical. For example, a low intake and low tissue levels have been found to be characteristic in populations with high CHD (Riemersma et al., 1986), while an excessive intake can result in increased LDL oxidation (Reaven et al., 1991, 1993; Louheranta et al., 1996). These effects clearly reflect the highly variable LA contents in dietary fats. Early Finnish studies demonstrated regional differences even in serum LA levels (Nikkari et al., 1983a). There were drastic differences compared with Swedish populations, which showed 10 %-units higher serum LA levels than men living in eastern Finland (Nikkari 1986). There was also a clear difference in the use of margarines, (18 kg/person/year; 1970) *vs* that in Finland (7 kg/person/year; 1970) (Trygg 1991), suggesting a much longer history of sufficient amounts of LA in Swedish diets. For comparison, CHD mortality in men in Sweden in 1985 was only 60% of that in Finland (Trygg 1991). Since the 70s, the intake of LA in Finland has started to increase along with the use of margarines.

The fat replacements used in these studies showed that already moderate amounts of LA in dietary fat can be sufficient for marked responses at the plasma PL level (II, III). During rapeseed oil substitution, the intake of LA was apparently closest to the optimum, because the LA supply did not suppress the previously increased levels of PL n-3 PUFAs (II). In addition, moderate amounts of EFAs may not markedly affect lipid oxidation, as has been reported by studies on rapeseed oil (Corboy et al., 1993; Turpeinen et al., 1995; Södergren et al., 2001).

Dietary n-3/n-6 PUFA balance. In early Finnish studies, attention was already paid to the ratio of n-3 and n-6 PUFAs, and to the fact that the role of LA-rich fat in reducing lipid levels did not necessarily mean favourable platelet functions in the absence of an adequate supply of n-3 PUFAs (Salo et al., 1985). The amount of LA appeared to be significant when a high LA intake reduced n-3 PUFAs, especially EPA, by strong competitive effects (Nikkari 1986). In a study in Finnish men, a low intake of unsaturated fatty acids, which was reflected as low levels of both n-3 and n-6 PUFAs in serum PL, was found to be predictive for MI (Miettinen et al., 1982).

In addition to the imbalance between a high SaFA and low LA intake, the other imbalance has been between a low α-LLA and high LA intake, known for decades in some western-type diets, especially in the U.S. (Budowski et al., 1984; Lands et al., 1992; Holman 1998; Chapkin 2000). Ignoring α-LLA as an EFA, and emphasizing LA-rich vegetable oils as the only source of PUFAs and, if present, even removing the α-LLA, has been much criticized (Holman 1998). α-LLA-deficient diets have been considered to be responsible for the very low PL n-3 PUFA levels in U.S. adults and newborn infants.

A high n-6 PUFA intake increases n-6 eicosanoids; reducing their effects by dietary means can be achieved by increasing the intake of competing n-3 PUFAs (α-LLA, EPA, DHA) (Lands 2003). These, in turn, can be important in preventing cyclo-oxygenase from forming active n-6 eicosanoids in inflammation, thrombosis and arrhythmia. Choosing dietary fats to increase the n-3/n-6 PUFA balance can be an effective primary prevention strategy to reduce the risk of fatal CHD events (Lands 2003). The proportions of long-chain PUFAs have been shown to have a close relationship with CHD mortality rates (Fig. 2).

Vegetable oils are the most important sources of EFAs, and modification of oilseed fatty acid compositions by metabolic engineering would provide new possibilities for the development of more balanced α-LLA/LA ratios of dietary fats (Kris-Etherton et al., 2000). On the basis of the present study, moderate amounts of both EFAs, combined with a high OA content, should be

preferred (II, III, VI). α-LLA, especially, has to be present in effective amounts to give priority to n-3 PUFA metabolism, as was found with rapeseed oil.

Fish foods provide ever-more significant sources of n-3 PUFAs for western-type diets. Fish meals twice a week have also been shown to afford protection against CHD (Kromhout et al., 1985). A clearly reduced CAD mortality has been reported in patients who had suffered an MI, and then been advised to eat fatty fish (Burr et al., 1989) or EPA/DHA capsules (GISSI Prevenzione Trial, 1999). In a study in Finnish patients with established CAD, higher CE EPA contents derived from fish intake was related to a lower risk of CAD death, and higher α-LLA, EPA and DHA levels to a lower risk of all-cause mortality (Erkkilä et al., 2003). It was recently stated that fish is more beneficial than fish oil (Marckman 2003). In contrast, an oil supply is important for patients with a history of coronary events, and those with hypertriglyceridemia or rheumatoid arthritis.

During the last decade, α-LLA containing vegetable oils and fats have become widely available, and increasing evidence of the inverse relationship between CHD and α-LLA intake has been reported (de Lorgeril et al., 1994; Ascherio et al., 1996; Hu et al., 1999; Djousse et al., 2001; Lemaitre et al., 2003). However, no such relationships with the intake of α-LLA from *trans*-fatty acid-free foods have been found when the diet included LA-rich vegetable oils (Oomen et al., 2001).

A statement by international workshop has pointed out the importance of reducing n-6 PUFAs in order to reduce the adverse effects of excesses of AA and its eicosanoid products (Simopoulos et al., 1999). The presence of α-LLA in the diet can inhibit the conversion of high amounts of LA in western-type diets containing too much LA-rich vegetable oils (e.g. corn, safflower and soybean oils). An increase in α-LLA, together with EPA and DHA, are necessary to achieve a healthier diet. Adequate daily intakes of EFAs and other PUFAs for adults were recommended to be 2.2 g for α-LLA (n-3), 4.4-6.7 g for LA (n-6), 0.65 g for EPA+DHA (n-3) and 0.22 g (min) for both DHA and EPA (Simopoulos et al., 1999).

A high LA intake is not a major problem in the Finnish diet, but rather a too high intake of SaFAs. The recommended ratio for α-LLA and LA was 1:2, as it is in rapeseed oil, and a clear PUFA effect was obtained with a mean daily dose of 18 g (II). This required a clear reduction of SaFAs, leading first to an increase in all the major n-3 PUFAs in plasma PL (EPA, DPA and DHA), following an increase in n-6 PUFAs (HGLA and AA). In contrast, no significant effects of EFAs can be expected from olive oil due to their low contents (Table 1), which are even lower than those in the best margarines already at the end of the 50s (3% α-LLA; 12% LA) (Lampi et al., 1959).

Lowering SaFAs and cholesterol. Despite the decrease in the use of butter in the Finnish diet at end of the 80s, the positive trends have not continued, and the intake of SaFAs is still too high. A reduced use of fish has also been reported (Männistö et al., 2003). The consumption of cheese, especially, which is now the main source of hard fat in women's diets, has increased from 13 to 17 kg/person/year (Lahti-Koski and Kilkkinen 2001; Männistö et al., 2003). It is also important to note that, unless varying fat contents (28-80%), the proportion of SaFAs in spreads and margarines can range between 20-48% (Table 3) (Anon. 2003b). In addition, the use of vegetable oils (<5 kg/person/year) has increased by only 1 kg during the last decade (Lahti-Koski and Kilkkinen 2001).

Rapeseed oil has a clear plasma SaFA and cholesterol lowering effect (-13%, LDL-C) with a dose range of 10-40 g/day (II; Seppänen-Laakso et al., 1989a). Rapeseed oil (and the test margarine) also brought about cholesterol restoration from tissues in habitual butter users (Fig. 5b) (II). The fact that rapeseed oil did not cause a corresponding effect in habitual margarine users (III), shows the importance of LA for cholesterol transport when sufficient LA becomes available in habitual butter users. However, SaFAs were restored in all these groups. The fall in cholesterol depends on the SaFA content of the substitute *vs* replaced fat, and thus vegetable oils are the most effective. For example, the fat in spreads contains about 4-5-fold and that in butter 10-fold amounts of SaFAs compared to rapeseed oil (Tables 3-4).

The use of vegetable oils, green vegetables, berries etc. is highly important to obtain antioxidant protection from vitamin E. Comparison of European populations, for example, has shown that Finnish men had the lowest plasma vitamin E levels and South-European men highest ones (Gey et al., 1991). Similarly, the use of vegetable oils in Finland has been very low (2-3 kg/person/year; 1981-95) (Anon. 1996) compared to the traditional use in Italy (10-18 kg; 1963-65) (Ferro-Luzzi and Branca 1995).

A recent study in Finnish men with cholesterol levels of 6.6 mmol/L indicated that, besides many desirable effects, a change to a modified Mediterranean-type diet may not be very effective in reducing SaFAs. This was indicated by the relatively small decrease in LDL-C levels (-11%) (Jula et al., 2002). The impact of rapeseed oil when saturated fat mainly was replaced (II) was clear even in a small subgroup (mean TC 6.8 mmol/L; n = 11). An average fall in LDL-C of 16% was found already following partial fat replacement with doses below 40 g/day (Seppänen-Laakso et al., 1989a). The correlation in this group (II, Fig. 2a), which reflects the desired change in the dietary P/S ratio, also showed that an increase in LA is closely related to the decrease in TC, and that half of the reductions were between 10-25%. The fall in TC was 7.5-20% in the whole group, when butter was replaced by the test margarine with 28% of LA (n = 23) (II, Fig. 2b).

Rapeseed oil with relatively high tocopherol content (Södergren et al., 2001) could make a significant contribution when combined with drug therapy. A continuous supply of tocopherols would be beneficial, since simvastatin treatment, after a strong reduction in cholesterol levels (21%), also decreased the lipoprotein-bound α-tocopherol in plasma (Jula et al., 2002). Lovastatin, for example, is known to increase the desaturation of LA to AA, and thromboxane formation, in human hepatoma cells (Hrboticky et al., 1992). Therefore, vegetable oils with clear n-3 PUFA effects can be used to modulate the hepatic metabolism of n-3 and n-6 fatty acids. The conversion of α-LLA to EPA to suppress n-6 PUFAs, thus providing a more desirable n-3/n-6 PUFA ratio, is considered to have benefits for hypercholesterolemic patients (Hrboticky et al., 1996).

Besides the marked effects on plasma PUFA levels, the low SaFA content (6%) in rapeseed oil is especially effective when replacing visible saturated fat (II). It is also evident that most of the elevated cholesterol levels are still caused by a too high intake of SaFAs in the Finnish diet. However, instead of using drugs, such cholesterol levels should be simply reduced by changing the dietary fat, primarily to rapeseed oil. Reducing SaFAs (II) and also LA (III) indicated a preference for α-LLA conversion to EPA over the metabolism of LA, which may partly improve the antithrombotic effects (McDonald et al., 1989; Weaver et al., 1990).

The fatty acid composition of rapeseed oil provides significant health benefits in dietary fats. Rapeseed oil should be a daily constituent in the diet to maintain better balance between PUFAs and SaFAs, and also between n-6 and n-3 PUFAs, which can be expected to have important contributions in the long-term prevention of many diseases.

7. CONCLUSIONS

Gas chromatography (GC) using a programmed temperature vaporization (PTV) is a reproducible method for fatty acid analysis, and is especially suited for detailed follow-up of the changes in plasma fatty acid composition during dietary fat replacement. A high performance liquid chromatographic (HPLC) method with evaporative light-scattering (ELS) detection proved to be highly applicable in the analysis of plasma lipid classes for studying their variation and associations with serum lipids and plasma fatty acids.

Total plasma α-linolenic (α-LLA) and oleic acids (OA) were the best indicators of the use of rapeseed and olive oils, respectively, and also showed dose-dependent correlations. When replacing fat on bread for six weeks, it was possible to monitor the changes in saturated fatty acid (SaFA) levels, the metabolism of essential fatty acids (EFAs) to long-chain polyunsaturated fatty acids (PUFAs) and competitive interactions in the plasma PL fraction.

The decrease in LDL cholesterol levels was most prominent during the first three weeks when high saturated fat (butter) on bread was replaced by rapeseed oil or margarine (13 and 8%, respectively). The decrease in cholesterol levels most often correlated with an increase in the linoleic (LA)/stearic acid ratio in plasma PL, reflecting the desired change in the dietary PUFA/SaFA ratio. It was also indicative that SaFAs were enriched in the phosphatidylcholine (PC) fraction, and excessive palmitic and stearic acids were found in the *sn*-2 position.

The partial restoration of cholesterol levels, which occurred in habitual butter but not in margarine users at the end of the substitutions, was assumed to be due to the release of cholesterol from the tissues when sufficient LA became available in the diet. It was shown that plasma PL SaFA and stearic acid levels were also closely correlated with the rise in serum cholesterol levels. The results also suggested that most of the high cholesterol levels (mean 6.8 mmol/L) could be reduced (10-25%) by the use of rapeseed oil to replace visible high saturated fat.

An increase and a decrease in *trans*-fatty acid intake were clearly reflected at the levels of the most abundant octadecenoic *trans*-isomers in plasma PL. Thus, undesirable effects related to high amounts of *trans*-fatty acids can be avoided simply by avoiding food products containing hydrogenated fats.

Olive oil, which has no PUFA-raising effect, is basically a different vegetable oil compared to rapeseed oil. Olive oil clearly reduced PL LA levels in habitual margarine users, which can be desirable if their LA intake is high. However, n-3 PUFAs (α-LLA) still have to be obtained from other sources. In addition, some olive oils with very low LA contents (<8%) would be poor sources of LA in typical high SaFA Finnish diets, for example.

The results showed a balanced metabolism of both α-LLA (n-3) and LA (n-6) after replacing high saturated fat by moderate amounts of rapeseed oil containing 10% α-LLA and 24% LA. The changes in plasma PL fatty acid levels indicated a priority for desaturation of α-LLA to long-chain n-3 PUFAs and marked competitive effects over LA and n-6 PUFA metabolism. These interactions resulted in a rise in PL n-3 PUFAs, which was highest at 3 weeks, while in n-6 PUFAs this was not reached until after 6 weeks, *i.e.* there was a delay in the metabolism of LA.

Competitive interactions between fatty acids arise from the basic functions of the body and, since ancient times, they have ensured priority for EFAs from foods. These aspects have to be taken into account in preventing a number of adverse health effects derived from the highly variable fatty acid composition of dietary fats. For instance, the decreasing trends in SaFA in the Finnish diet have slowed down, reflecting that there is still a low consumption of edible oils. Oils should be the primary means of lowering elevated cholesterol levels, which are derived merely from incorrect fat and food choices, rather than the use of drugs with high public expense.

Rapeseed oil is widely recommended because of its moderate α-LLA and LA contents. Besides its cholesterol-lowering effects, it is so far the most promising edible oil as a balanced source of EFAs for western-type diets.

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APPENDIX

Trivial and systematic names for fatty acids in this study are listed below. The first double bond position in the carbon chain counting from the terminal methyl group is denoted by n-3, n-6, n-7, n-9 and n-12.

Trivial name	Systematic name	Shorthand designation
SaFAs		
capric acid	decanoic acid	10:0
lauric acid	dodecanoic acid	12:0
myristic acid	tetradecanoic acid	14:0
palmitic acid	hexadecanoic acid	16:0
margaric acid	heptadecanoic acid	17:0
stearic acid	octadecanoic acid	18:0
arachidic acid	eicosanoic acid	20:0
behenic acid	docosanoic acid	22:0
lignoceric acid	tetracosanoic acid	24:0
MUFAs		
palmitoleic acid	9-hexadecenoic acid	16:1n-7
oleic acid	9-octadecenoic acid	18:1n-9
cis-vaccenic acid (asclepic acid)	11-octadecenoic acid	18:1n-7
petroselinic acid	6-octadecenoic acid	18:1n-12
eicosenoic acid (gondoic acid)	11-eicosenoic acid	20:1n-9
erucic acid	13-docosenoic acid	22:1n-9
nervonic acid	15-tetracosenoic acid	24:1n-9
18:1*trans*-fatty acid isomers		
elaidic acid	9*trans*-octadecenoic acid	18:1n-9*trans*
vaccenic acid	11*trans*-octadecenoic acid	18:1n-7*trans*
petroselaidic acid	6*trans*-octadecenoic acid	18:1n-12*trans*
n-6 PUFAs		
linoleic acid	9,12-octadecadienoic acid	18:2n-6
γ-linolenic acid	6,9,12-octadecatrienoic acid	18:3n-6
homo-γ-linolenic acid	8,11,14-eicosatrienoic acid	20:3n-6
arachidonic acid	5,8,11,14-eicosatetraenoic acid	20:4n-6
n-3 PUFAs		
α-linolenic acid	9,12,15-octadecatrienoic acid	18:3n-3
stearidonic acid (moroctic acid)	6,9,12,15-octadecatetraenoic acid	18:4n-3
eicosapentaenoic acid (timnodonic acid)	5,8,11,14,17-eicosapentaenoic acid	20:5n-3
docosapentaenoic acid (clupanodonic acid)	7,10,13,16,19-docosapentaenoic acid	22:5n-3
docosahexaenoic acid (cervonic acid)	4,7,10,13,16,19-docosahexaenoic acid	22:6n-3

References: Lobb and Chow 2000; O'Keefe 1998.