Biological effects of conjugated linoleic acid: analysis, enrichment and mode of cytotoxicity in cancer cell lines.

Ph.D. thesis

By

Marianne O' Shea B.Sc. (Biochemistry)

School of Biotechnology,
Dublin City University,
Glasnevin, Dublin 9
and
Dairy Quality Department,
Dairy Products Research Centre,
Fermoy, Co.Cork.

Project Supervisors: Catherine Stanton M.Sc. Ph.D.
Rosaleen Devery Ph.D.

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Declaration

This thesis is submitted in fulfilment of the requirements for Doctor of Philosophy, by research and thesis. Except where otherwise acknowledged, this work was carried out by the author alone, on a full time basis between October 1996 and June 2000 at the School of Biotechnology, Dublin City University and the Dairy Products Research Centre, Moorepark, Fermoy, Co.Cork.

Signed: Marianne O' Shea Date: 18/9/2000

Marianne O' Shea

Student Number: 96970987

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AUTHOR: Marianne O' Shea

ABSTRACT

Conjugated linoleic acid (CLA) refers to a class of conjugated dienoic isomers of linoleic acid produced by ruminant animals. The predominant dietary sources of CLA (*cis*-9, *trans*-11) are ruminant fat products such as milk, beef and dairy products. CLA has been shown to be anti-carcinogenic, anti-atherosclerotic, and anti-catabolic. In this study methodology for analysis of *trans* fatty acids (TFA) was optimised using a combination of silver-ion chromatography and GLC, and applied to the analysis of CLA and TFA in a range of Irish dairy products. Having established levels of CLA in Irish dairy products, fractionation of anhydrous milk fat was investigated as a means of enriching the levels of this fatty acid within a food ingredient. Dry fractionation with a temperature range between 33-10 °C and a cooling rate of 0.54 °C/h, led to a soft fraction enriched in polyunsaturates and low in saturates with a CLA content that was 60.3 % elevated compared with the starting material. Its anticarcinogenic effect was demonstrated in mammary and colon cancer cell lines. A synthetic CLA mixture inhibited the growth of mammary MCF-7 and colon SW480 cancer cells in a dose and time dependent manner. Superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and thiobarbituric reactive substances (TBARS) were induced in both cell lines exposed to CLA (20ppm) over a period of 12 days. Similar effects were observed in MCF-7 cells treated with the pure *c*9, *t*11 CLA isomer and with bovine milk fat from animals fed pasture, rapeseed or soya. Growth suppression of these cells was independent of the source of CLA. Equimolar concentrations of linoleic acid (LA) promoted the growth of the MCF-7 cell line. CLA and LA exerted differential effects on protein farnesyl transferase (PFTase) activity, the rate limiting enzyme in farnesylation of *ras* oncoproteins in cancer cells, thus implicating modulation of PFTase activity in CLA-mediated growth suppression. Multiple biochemical mechanisms appear to be involved in the growth suppressive effects of CLA isomers *in vitro*.

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Abbreviations

AA	arachidonic acid
ACF	aberrant crypt foci
AMF	anhydrous milk fat
BCR	British certified reference
BHT	butylated hydroxy toluene
BMI	body mass index
CD	conjugated diene
c	*cis*
CHD	coronary heart disease
CFA	conjugated fatty acid
CLA	conjugated linoleic acid
COX	cyclooxygenase
CRM	certified reference material
DMEM	Dulbecco's Minimum Essential Medium
DAG	diacylglycerol
DPG	diphosphatidylglycerol
DPM	disintegrations per minute
DMBA	7,12-dimethyl-benz[a]anthracene
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
FAME	fatty acid methyl ester
FFA	free fatty acids
FFR	full fat rapseed
FFS	full fat soybeans
FID	flame ionisation detector
GAPs	GTPase activating proteins
GDP	guanidine-diphosphate
GLC	gas liquid chromatography
GLC-MS	gas liquid chromatography mass spectroscopy
GNRFs	guanine nucleotide releasing factors
GPx	glutathione peroxidase
GTP	guanidine-triphosphate
HDL	high density lipoprotein
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HETE	hydroxyeicosatetraenoic
HFCO	high fat dietary corn oil
HFFO	high fat fish oil
HPLC	high performance liquid chromatography
IGF	insulin-like growth factors
IGFBP	IGF binding proteins
IQ	2-amino-3-methyl-imidazo[4,5-f]-quinoline

LA	linoleic acid
LDL	low density lipoprotein
LPL	lipoprotein lipase
LPS	lipopolysaccharides
LOX	lipoxygenase
NADPH	β-nicotinamide adenine dinucleotide phosphate, reduced form
MDA	malondialdehyde
MEO	mammary epithelial cell organoids
MMTV	mouse mammary tumour virus
MNU	methylnitrosurea
mRNA	messenger ribonucleic acid
PBS	phosphate buffered saline
PC	phosphatidycholine
PGE_2	prostaglandin E_2
PI-PLC	phosphatidylinositol-specific phospholipase C
PKC	protein kinase C
PLC	phospholipase C
PLA_2	phospholipase A_2
PLPC	1-palmitoyl-2-linoleoyl phosphatidylcholine
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator response elements
PUFA	polyunsaturated fatty acid
R	chromatographic resolution
RF	response factor
SD	standard deviation
SOD	superoxide dismutase
TBARS	thiobarbituric acid-reactive substances
TCA	trichloroacetic acid
TFA	*trans* fatty acid
TLC	thin layer chromatography
TMG	tetramethylguanidine
TPA	12-O-tetradecanoylphorbal-13-acetate
t	*trans*
VLDLs	very low-density lipoproteins
WK	week

TABLE OF CONTENTS

CHAPTER 1

Literature Review

Milk is an important source of vital nutrients in the human diet. Nevertheless, many health-conscious consumers perceive milk and dairy products to contain excessive amounts of total fat, saturated fat and cholesterol. However, negative consumer perception of milk fat may soon be altered due to an explosion of research highlighting the potential therapeutic prowess of a certain class of conjugated diene isomers of linoleic acid (LA) known as conjugated linoleic acid (CLA) present in milk fat. Studies are showing that CLA may have potential in the prevention of a wide range of human medical conditions including cancer, vascular disease, diabetes, obesity and bone disorders. Extensive research is underway to determine mechanistic threads that tie these multifunctional health benefits together. This has led to efforts to naturally manipulate the CLA content of milk and animal tissues by dietary intervention. Such research may lead to the development of new high CLA-containing "functional foods" designed for cancer chemoprevention of the general population.

The aim of this chapter is to review the available literature on the role of dietary fats, in particular CLA, in health and provide a comprehensive background to the research work contained in this thesis.

1.1 Milk

Milk is defined in the Milk Ordinance and Code recommended by the United States Public Health Service (Webb and Johnson, 1974) as 'the lacteal secretion, practically free from colostrum, obtained by the complete milking of one or more healthy cows, which

contains not less than 8.25 % of milk-solids-not-fat (i.e. proteins, lactose, minerals, acids, enzymes and vitamins) and not less than 3.25 % of milk fat' (Table 1.1). The distinctive feature of milk as a food lies in the balanced content of essential nutrients which it contains. All species of mammals, from man to whales, produce milk for the purpose of feeding offspring. Perhaps as early as 6000-8000 BC, ancient man learned how to domesticate species of animals for the provision of milk. These included cows (genus Bos), buffaloes, sheep, goats and camels, all of which are still used in various parts of the world for the production of milk for human consumption (Mepham, 1987).

Table 1.1 Approximate composition of bovine milk.

Component	Average content (% w/w)
Water	87.3
Fat	3.9
Protein	3.2
Lactose	4.6
Ash	0.7

Adapted from Walstra and Jenness, 1984

1.1.1 Milk fat

Milk lipids provide a major source of energy and essential membrane structural components for the newborn in all mammalian species (Christie, 1994). The content and composition of lipids from milks of different species vary with such factors as diet, stage of lactation, number of lactations, breed and season (Hawke and Taylor, 1994). More than 400 fatty acids have been identified in milk fat, although it is generally accepted that 15 major and 12 minor fatty acids dominate the fatty acid spectrum (Mulder and Walstra, 1974). The major fatty acids found in milk are long chain ($C_{14:0}$ – myristic, $C_{16:0}$ - palmitic, $C_{18:0}$ – stearic, $C_{18:1}$ – oleic) and the minor fatty acids are short chain ($C_{4:0}$ – butyric, $C_{6:0}$ – caproic, $C_{8:0}$ – caprylic, $C_{10:0}$ – capric). Butyric acid is specific for milk fat of ruminant animals and is responsible for the rancid flavor produced when it is cleaved from glycerol by lipase action. Saturated fatty acids, such as $C_{14:0}$, $C_{16:0}$, and $C_{18:0}$ make up two thirds of milk fatty acids. $C_{18:1}$ is the most abundant unsaturated fatty acid in milk with one double bond. While the *cis* form of geometric isomer is the most commonly found in nature, approximately 5 % of all unsaturated bonds are in the *trans* position as a result of rumen hydrogenation.

1.1.1.1 Milk fat triglycerides

Milk fat is made up of 97 – 98 % triglycerides. These are accompanied by much smaller amounts of di- and monoglycerides, free cholesterol and cholesteryl esters, free fatty acids and phospholipids (Table 1.2). Triglycerides consist of a molecule of glycerol to which is esterified three fatty acids.

Table 1.2 Composition of lipids in milks from various species.

Lipid Class	Cow	Buffalo	Human	Pig
		Wt % of total lipids		
Triglycerides	97.50	98.60	98.20	96.80
Diglycerides	0.36	-	0.70	0.70
Monoglycerides	0.03	-	Trace	0.10
Cholesteryl esters	Trace	0.10	Trace	0.06
Cholesterol	0.31	0.3	0.25	0.60
Free fatty acids	0.03	0.5	0.40	0.20
Phospholipids	0.60	0.50	0.26	1.60

Adapted from Christie, 1994.

Synthesis of triglycerides occurs at the cytoplasmic surface of the smooth endoplasmic reticulum (SER) in mammary epithelial cells. The principal pathway of biosynthesis of triglycerides involves activation of fatty acids (obtained from the blood or synthesised by *de novo* synthesis) by formation of esters with coenzyme A (CoA), reaction of two molecules of such esters with *sn*-glycerol-3-phosphate (α-glycerol phosphate) to form phosphatidic acid, cleavage of phosphate by phosphohydrolase to form a 1, 2-diacyl *sn* - glycerol (1, 2-diglyceride), and finally, further acylation by another molecule of acyl CoA to form a triglyceride. Esterification of fatty acids on the glycerol molecule does not occur in a random order. Fatty acids of 4, 6 and 8 carbons are almost exclusively (95%) positioned on carbon 3 of the glycerol molecule, but occasionally do occur on carbon 1. Fatty acids of 10 carbons are primarily positioned on carbons 1 and 3 of the glycerol molecule in low molecular weight triglycerides (32 to 46 carbons in total) and on carbon 3 for higher molecular weight triglycerides. Fatty acids of 12 to 16 carbons are primarily positioned on carbon 2 of the glycerol molecule. Fatty acids with 18 carbons ($C_{18:0}$, $C_{18:1}$, and $C_{18:2}$) are primarily found on positions 1 and 3.

1.1.1.2 Minor components of milk fat

Phospholipids account for about 1 % of total lipid in milk fat; these are divided into the major classes of phosphatidylcholine (or lecithin), phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, sphingomyelin and plasmalogens (Sonntag, 1979). These phospholipids are located in the milk fat globule membrane (Christie, 1994). The phospholipids act as detergents and are capable of covering a large surface area so that their content may be of particular relevance during milk processing.

Cholesterol is the principal sterol present in milk fat, 10 –15 % of which is found esterified to fatty acids. LA has been reported to amount to 27 % in milk fat cholesteryl esters (Patton and McCarthy, 1963).

1.1.1.3 Origin of fatty acids in milk

Fatty acids in milk are derived from two sources, (i) *de novo* synthesis from acetate and β-hydroxybutyrate in the mammary gland and (ii) microbial activity in the rumen followed by uptake from circulating lymph and blood. The composition of the fatty acids from these two sources is very different e.g. endogenously produced fatty acids are of carbon length C_4-C_{16}, while a proportion of C_{16}, and all of the C_{18} fatty acids arise from blood (Hawke and Taylor, 1994). Synthesis of short and medium chain fatty acids occurs by *de novo* synthesis in the cytoplasm of the mammary epithelial cell. β-hydroxybutyrate provides approximately 8 % of the total fatty acids in milk, or 16 to 20 % of *de novo* synthesised fatty acids and seems to be the major source of carbons for short chain fatty acids. *De novo* synthesis also involves the provision of acetyl-CoA derived from acetate which acts as a primer for the reaction. Acetyl-CoA is a substrate for the synthesis of malonyl-CoA which is required for fatty acid elongation, and for provision of β-NADPH for the reductive steps of the pathway. The first committed step in the synthesis of fatty acids is the conversion of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase. The enzyme requires biotin, Mg/Mn as cofactors and its activity is proportional to the lipogenic capacity of the mammary gland. Elongation of the fatty acid requires the addition of more acetyl-CoA molecules which have been activated to malonyl-CoA. The reaction is catalyzed by fatty acid synthase. Approximately 30 % of $C_{16:0}$ is synthesised

de novo by the mammary gland (Bishop *et al.,* 1969). Most of the free fatty acids of plants in the diets of ruminants are long chain fatty acids and are usually highly unsaturated. The rumen microorganisms are capable of quickly saturating these unsaturated fatty acids which largely explains the high degree of saturation of the fat in milk of ruminants. Long chain fatty acids are absorbed from the small intestine into the lymph system where they become bound to proteins to form lipoproteins. These lipoproteins circulate into the blood stream and are absorbed by the mammary gland secretory cell. The uptake of very low density lipoproteins (VLDL) and chylomicron triglycerides by mammary tissue involves hydrolysis of the triglycerides by lipoprotein lipase (LPL). The lipase appears to be bound to the luminal surface of the endothelium of the blood capillaries and hydrolysis of triglycerides is regarded as a pre-requisite to the uptake of lipid by mammary epithelial cells. Estimates of the contribution of blood lipids to milk lipids in the cow vary from about 35 % to as high as 85 % (Annison *et al.,* 1967, Bishop *et al.,* 1969). Saturated long chain fatty acids absorbed from the intestine would cause milk fat to be solid if excessive amounts were incorporated. Melting point is lowered by activity of stearoyl Co A desaturase to convert $C_{18:0}$ (m.p. 70 °C) to $C_{18:1}$ (m.p. 5-7 °C). There is extensive desaturation of $C_{18:0}$ to $C_{18:1}$ on entry into the mammary gland by a desaturase located in the microsomes (Annison *et al.,* 1967). Thus, despite the high $C_{18:0}$ to $C_{18:1}$ ratios in triglycerides circulating in the blood of ruminants arising from extensive microbial biohydrogenation of dietary fat, desaturation of $C_{18:0}$ in mammary tissue results in the production of milk fat not markedly dissimilar in $C_{18:1}$ content to the milk fat of non ruminants. Figure 1.1 depicts an overview of lipid metabolism in dairy cows.

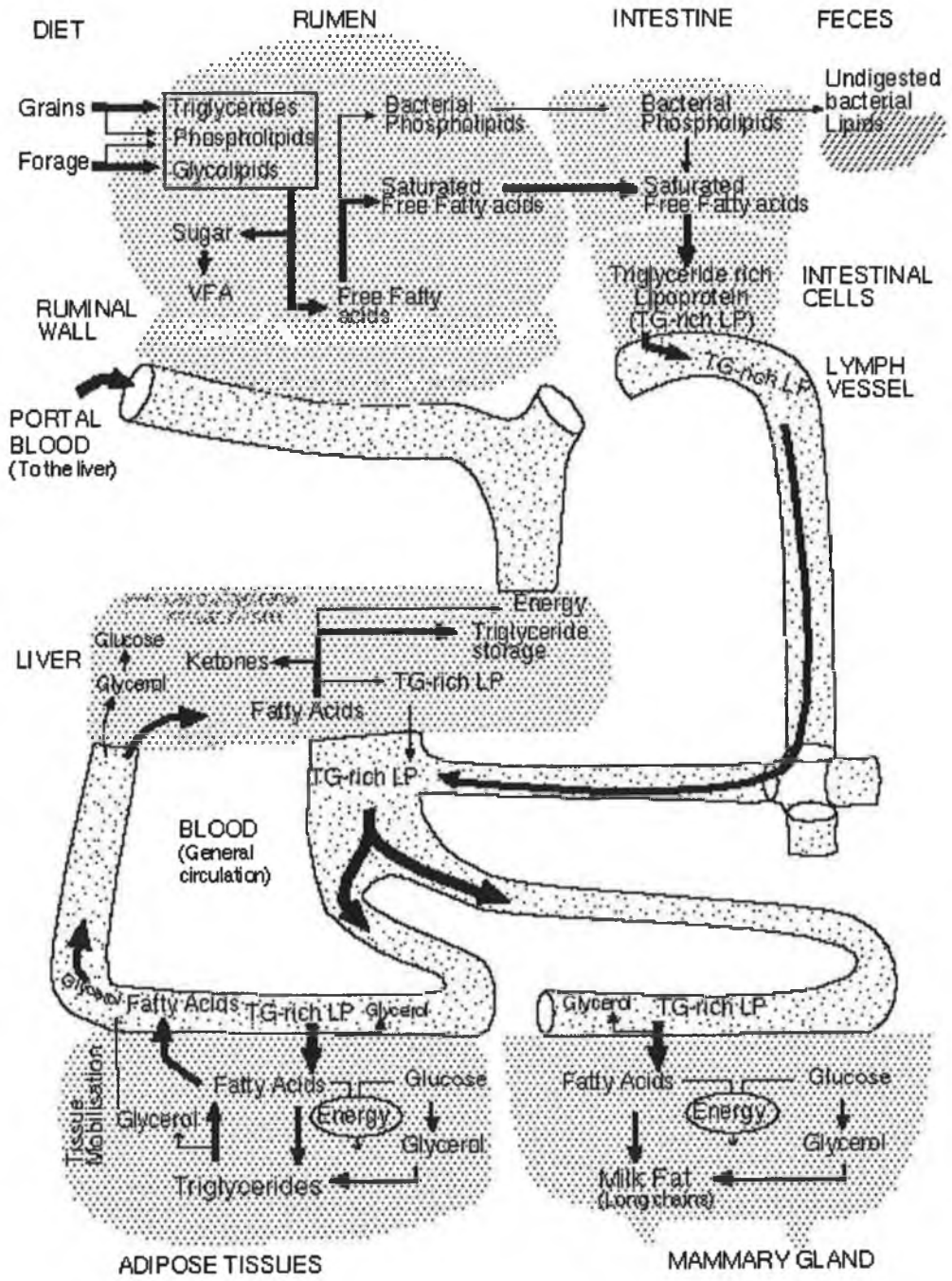


Figure 1.1 Overview of lipid metabolism in dairy cows. Babcock Institute 1997.

Fresh milk contains free fatty acids (FFA) which increase during storage of raw milk as a result of hydrolysis of the ester linkage by lipase enzymes secreted by psychotropic bacteria. Factors affecting free fatty acid levels in milk include animal nutrition, stage of lactation, storage temperature, time of milking and extent of foaming or agitation occurring during milking and milk assembly (Rajeh and Burgess, 1991).

1.2 Discovery of conjugated linoleic acid in milk

The presence of conjugated fatty acids in milk fat was first established by Booth *et al.* (1935) who reported that when cows were turned out to pasture after winter, the fatty acids of milk fat showed greatly increased absorption in the ultra-violet (UV) region at 230 nm. Although the nature of the fatty acid responsible for absorption at 230 nm was not known at this time, this was the first study to record the seasonal variation in conjugated fatty acid (CFA) content in milk fat. A survey published in 1963, repeatedly showed a 2-3 fold increase in conjugated diene content when cows were turned out to pasture (Riel *et al.,* 1963).

By 1939, it was known that absorption of fatty acids was the result of *two* conjugated double bonds and by 1949, it was known that conjugated unsaturation of milk fat fractions was associated with an 18-carbon chain length (Hilditch and Jasperson 1941, Mattsson, 1949). During the 1960's, lipid research was revolutionised by the application of thin layer chromatography (TLC), silver nitrate-TLC (Ag^{+}-TLC) and gas liquid chromatography (GLC). In 1977, Parodi isolated CFAs from milk fat after methylation

with boron trifluoride (BF_3) and the use of Ag^+-TLC. Using benzene and toluene as developing solvents CFA where shown to migrate between the *cis* (*c*) and *trans* (*t*)-monoenes and to possess characteristic infrared (IR) and UV absorption spectra. Partial hydrazine reduction followed by preparative Ag^+-TLC separation of resultant monoenes, reductive ozonolysis and GLC analysis of the products (aldehydes and aldehyde esters) showed that the stereochemistry of the major conjugated dienoic acids in milk fat was *c*9, *t*11-$C_{18:2}$ (Parodi, 1977) (Figure 1.2). Trace amounts of conjugated *trans/trans* dienoic acids (*t*8, *t*10-$C_{18:2}$ and *t*11, *t*13-$C_{18:2}$) were also observed in milk fat which were attributed to double bond migration during preparative GLC.

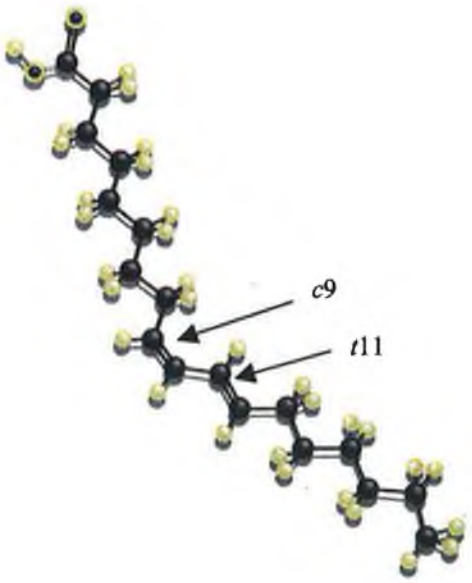


Figure 1.2 The structure of *c*9, *t*11 conjugated linoleic acid.

1.3 Origins of CLA in milk fat

CLA is an intermediate in the biohydrogenation of LA, and until recently it was generally accepted that CLA in ruminants originated from the incomplete biohydrogenation of LA by rumen bacteria (Fritsche and Steinhart, 1998). Complete biohydrogenation of LA in the rumen is a three step process, leading to the production of $C_{18:0}$ (Kepler *et al.,* 1966) (Figure 1.3). CLA is formed as the first intermediate of this pathway by the action of LA isomerase, an enzyme of the anaerobic rumen bacteria *Butyrivibrio fibrisolvens* (Kepler *et al.,* 1970) and is unusual in the respect that the reaction occurs in the middle of a hydrocarbon chain away from any activating functional groups and has an absolute substrate requirement for a *c*9, *c*12 diene structure and a free carboxyl group. The enzyme has been shown to exhibit maximum activity with the substrates LA and $C_{18:3}$ (Kepler and Tove, 1967). Lipid biohydrogenation in the rumen is affected by the type and amount of fatty acid substrate (Nobel *et al.,* 1974), the forage to grain ratio (Gerson *et al.,* 1985) and the nitrogen content of the diet fed to ruminants (Gerson *et al.,* 1983).

In accepting this explanation for the presence of CLA in ruminant lipids, it was assumed that the amount of CLA escaping rumen biohydrogenation and being absorbed was adequate to account for CLA levels in milk and body fat. However the absence of any reported measurements of the amount of CLA escape and absorption, together with *in vitro* studies showing rapid conversion of CLA, formed by LA isomerase, to *trans*

vaccenic acid ($t11$-$C_{18:1}$) has cast considerable doubt on the rumen being the sole source of CLA in tissues and milk fat.

Recent work by Griinari *et al.* (1997) showed that CLA could be produced endogenously from $t11$-$C_{18:1}$ in tissues by Δ9 desaturase. Figure 1.4 illustrates the two pathways of CLA biosynthesis which together may account for the high CLA concentrations observed in milk fat even when cows are fed diets that are low in LA e.g. pasture feeding or fish oil supplements. It is proposed that $t11$-$C_{18:1}$ accumulates in the rumen and that a portion escapes further biohydrogenation (Griinari and Baumann, 1999). Following absorption from the digestive tract, $t11$-$C_{18:1}$ is utilised by different tissues where a portion is desaturated to CLA and incorporated into tissue and milk lipids. This 'desaturase hypothesis' has been proposed to explain the relatively constant ratio of $t11$-$C_{18:1}$ and CLA in bovine milk fat across a range of diets. The presence of *t*7 *c*9 CLA and *c*9 *t*13 $C_{18:2}$ supports the role of an active Δ9 desaturase, an enzyme that introduces a *cis* double bond between carbons 9 and 10 (Ulberth and Henninger, 1994, Yurawecz *et al.,* 1998). Evidence suggests that Δ9 desaturation of $t11$-$C_{18:1}$ may be more relevant in the production of CLA than previously thought. Several studies have reported a strong correlation coefficient between $t11$-$C_{18:1}$ and CLA concentrations in milk fat (Jahreis *et al.,* 1997, Jiang *et al.,* 1996, Lawless *et al.,* 1998, Precht and Molkentin, 1997). A close linear relationship between milk fat $t11$-$C_{18:1}$ and CLA was observed suggesting a precursor-product association where $t11$-$C_{18:1}$ is the precursor and CLA is the product (Griinari *et al.,* 1997). A slope of 0.5 suggests approximately one third of circulating $t11$-$C_{18:1}$ was desaturated by Δ9 desaturase (Griinari and Baumann, 1999).

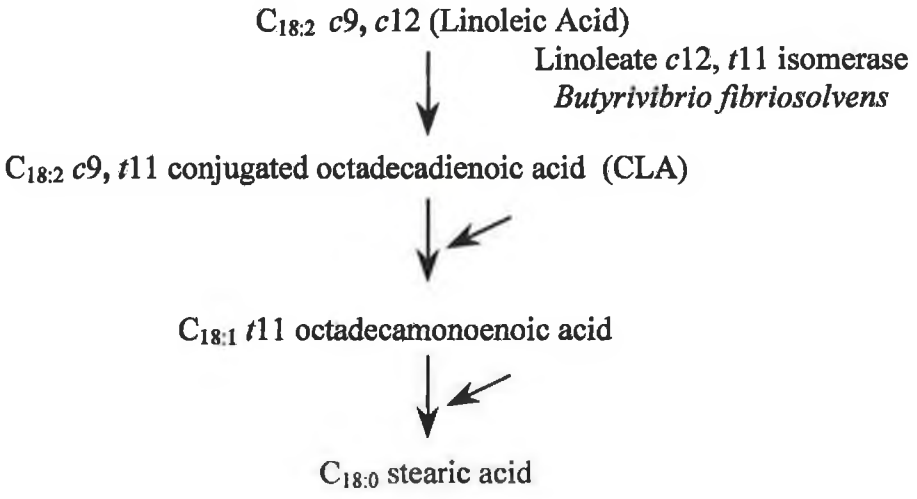


Figure 1.3. Biohydrogenation of LA in the rumen.

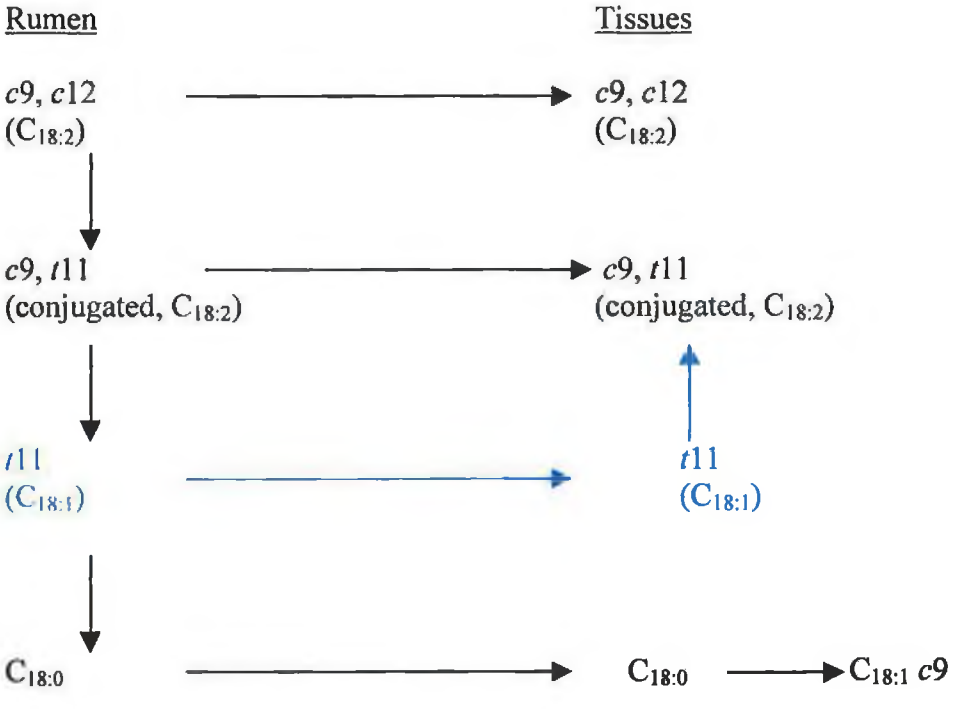


Figure 1.4 Pathways of conjugated linoleic acid (CLA) biosynthesis.

A recent study whereby *t*11-$C_{18:1}$ and CLA were fed in equal quantities to mice reported that 12 % of the *t*11-$C_{18:1}$ consumed during a 2-wk feeding period was recovered in the carcass as CLA (Santora *et al.*, 2000). Of the proportion of *t*11-$C_{18:1}$ in the tissues that was available for bioconversion, 48.8 % was desaturated. CLA was found in the carcass only when *t*11-$C_{18:1}$ or CLA was fed. CLA was found in both triglyceride and phospholipids when CLA was fed, but only in triglyceride when *t*11-$C_{18:1}$ was fed, suggesting that bioconversion occurred in the adipose tissue (Santora *et al.*, 2000).

1.4 CLA in the human diet.

Levels of total CLA in various foods range from as low as 0.2 mg/g fat in corn and peanut oil (Chin *et al.*, 1992) to as high as 17 mg/g in beef (Shantha *et al.*, 1995) and 30 mg/g in milk fat (Parodi, 1994), with *c*9, *t*11 being the predominant isomer in milk amounting to 90 % of the total CLA (Table 1.3).

Negligible amounts of *c*9, *t*11 CLA have been observed in seafood and vegetable oils. Estimates of CLA intakes for humans have been based on CLA databases in conjunction with 3 day or 5 week dietary records, national food intake surveys and data from biochemical analyses of food portions. Using the latter approach, in conjunction with 5 week dietary records, it was estimated that CLA intake by Finnish men and women ranged between ~ 40 and 310 mg /day (Salminen *et al.*, 1998). Such variation was attributed to marked differences in type of fat (dairy, *trans* and stearic acid) in the diet.

Herbel *et al.* (1998) reported that young men and women living in the United States consumed approximately 127 mg CLA/day. Another US study estimated that the dietary intake of CLA was lower in women (52 ± 44 mg/day) than in men (137 ± 84 mg/day) (Ritzenthaler *et al.,* 1998). Dietary intake of CLA in Germany was also estimated to be lower in women (350 mg CLA/day) than in men (430 mg CLA/day) (Fritsche and Steinhart, 1998). The differences between these studies can be accounted for by the fact that fat intake is higher in Germany than in the US (Aldolf *et al.,* 1994). The German study was not age-specific, while the US studies were limited to young men and women, suggesting that age may influence CLA intake in humans. Although extrapolations from animal studies to the human suggest that 3 g/day CLA is necessary to avail of the anticancer effects of CLA (Ip *et al.,* 1994) more information from human feeding trials is necessary before such a value can be verified.

Specific intervention studies have shown that increasing the CLA content of the diet increased the CLA content in human milk (Park *et al.,* 1999a), plasma (Huang *et al.,* 1994) and adipose tissue (Jiang *et al.,* 1999). Feeding a high dairy fat diet containing 291 ± 75 mg CLA /day led to a 1.6 fold increase (13.5 ± 0.1 μmol/ g fat) in CLA content of human milk (Park *et al.,* 1999a). Plasma CLA increased 19-27 % to 9.6 ± 1.1 μmol/L when men were fed Cheddar cheese containing 178.5 mg CLA each day for 4 weeks (Huang *et al.,* 1994).

Table 1.3 Total CLA content and % *c*9, *t*11 CLA in food products

Food	Total CLA (mg g^{-1} fat)	*c*9,*t*11CLA (% of total CLA)	Reference
Butter	9.4 -11.9	91	Shantha *et al.*, 1995
Non fat frozen dairy dessert	0.6	90	Chin *et al.*, 1992
Condensed milk	7	90	Chin *et al.*, 1992
Natural cheeses	0.6-7.1	17-90	Ha *et al.*, 1989, Chin *et al.*, 1992
Processed cheeses	3.2-8.9	17-90	Ha *et al.*, 1989, Chin *et al.*, 1992, Garcia-Lopez *et al.*, 1994
Cheddar cheese	5.1-5.4	82-88	Werner *et al.*, 1992
Ice cream	3.8-4.9	73-76	Fritsche *et al.*, 1998
Sour cream	7.5	78	Fritsche *et al.*, 1998
Yogurt	5.1-9.0	82	Fritsche *et al.*, 1998
T-bone (cooked)	4.7-9.9	65	Shantha *et al.*, 1994a
T-bone (raw)	4.4-6.6	59	Shantha *et al.*, 1994a
Round beef	2.9	79	Ip *et al.*, 1991
Milk fat	2-30	90	Parodi, 1994
Seafood	0.5	ND	Chin *et al.*, 1992
Vegetable oils	0.2	45	Chin *et al.*, 1992

ND= not detected

CLA in human serum has been shown to be derived in part from the diet and in part by conversion of dietary TFA (Salminen *et al.,* 1998). Serum levels of CLA varied between 0.17 - 0.43 %, when healthy subjects were fed a dairy fat diet for 5 weeks, followed by either a *trans* fatty acid (TFA) diet or a $C_{18:0}$ diet for 5 weeks (Salminen *et al.,* 1998). The proportions of CLA in the dairy fat, TFA and $C_{18:0}$ diets were 0.37, 0.04, and 0.10 % of total fatty acid methyl esters (FAME). The corresponding proportions in serum were 0.32, 0.43 and 0.17 % (Table 1.4). The difference in the CLA content of serum between subjects fed the dairy fat and $C_{18:0}$ diets was explained by different dietary intakes of CLA.

Table 1.4 Mean percentual fatty acid composition of total serum from healthy subjects fed a dairy fat diet followed by a *trans* fatty acid diet or a stearic acid diet.

Fatty acid	Dairy fat diet	*Trans* fatty acid diet (n=40)	Stearic acid diet (n=40)
$C_{16:0}$	23.74	20.31	21.42
$C_{18:0}$	7.74	6.68	10.30
$C_{18:1}$ *trans* fatty acid	0.59	4.47	0.49
$C_{18:1}$	23.39	24.39	23.11
$C_{18:2}$ LA	30.03	29.73	29.78
$C_{18:2}$ CLA	0.32	0.43	0.17

Adapted from Salminen *et al.* 1998.

1.5 Enrichment of CLA by dietary manipulation

There is a wide and natural variation in CLA content of raw milk. Such variation is a result of many different factors including diet (Dhiman *et al.*, 1996, 1997, 1999a,b, Jiang *et al.,* 1996, Kelly and Baumann, 1996, Lawless *et al.*, 1998, Murphy *et al.,* 1990, 1995, Parodi, 1977, Riel, 1963, Stanton *et al.,* 1997a, 1997b), age (Lal and Narayanan, 1984, Stanton *et al.,* 1997a) and breed (Lawless *et al.,* 1999).

It has been clearly demonstrated that animal diet has a major impact on the CLA content of milk fat. Several studies have shown that pasture feeding can increase milk fat CLA concentrations in lactating dairy cows (Dhiman *et al.*, 1996, Kelly and Baumann, 1998b, Lawless *et al.*, 1998, Stanton *et al.,* 1997a, 1997b). Cows receiving all of their daily feed as pasture produced milk fat higher in CLA content (22 mg/g fat) than cows receiving only one third (8 mg/g fat) or two thirds (14 mg/g fat) of their daily feed as pasture (Dhiman *et al.,* 1996). In an additional study, CLA concentrations in milk fat were doubled when cows consumed pasture only (10.9 mg/g fat) compared with a mixed diet (4.6 mg/g fat) (Kelly *et al.,* 1998b). This effect of pasture has been attributed to the provision of $C_{18:3}$ as a lipid substrate for the formation of *t*11-$C_{18:1}$ in the rumen and its subsequent desaturation to CLA in the mammary gland (Griinari and Bauman, 1999). Consistent with this hypothesis, milk fat CLA was reduced in a controlled feeding trial in which cows were fed a low grass diet over a 19 week period (Stanton *et al.,* 1997a).

Dietary supplementation of cows at pasture with full-fat rapeseeds (FFR) (Stanton *et al.,* 1997a) and soybeans (FFS) (Lawless *et al.,* 1998) resulted in substantial increases in

CLA content of milk over unsupplemented controls. A 27 % increase in CLA levels was obtained in milk fat following the FFS supplemented diet while a 60 % increase in CLA levels was obtained in milk fat following the FFR diet (Lawless *et al.,* 1998). Rapeseed oil is a relatively rich source of $C_{18:3}$ compared with soybean oil, and the amount of LA in rapeseeds is relatively low compared with its content in soybeans. This result is indicative of rumen biohydrogenation of $C_{18:3}$ in the FFR supplement and might have contributed to increased CLA concentrations observed in milk following this dietary treatment (Lawless *et al.,* 1998). The relatively lower increase in the CLA concentration following supplementation with FFS, which was rich in potential substrates for rumen biohydrogenation, suggests that these fatty acids were not readily accessible to the rumen microorganisms (Lawless *et al.,* 1998). Milk fat CLA was found to increase from 3.9 mg/g fat to 15.9 and 16.3 mg/g fat in cows fed a diet consisting of linseed oil at two concentrations (2.2 and 4.4 %) respectively, on a dry matter basis (Dhiman *et al.,* 1996). This increase in CLA concentration was attributed to the oils which were rich in $C_{18:3}$ polyunsaturated fatty acids (PUFA) and which were readily accessible to rumen microorganisms.

Comparisons between different types of plant oils suggest that those high in LA increased CLA concentration most effectively (Griinari and Bauman, 1999). Different dietary oil treatments (peanut oil, sunflower oil and linseed oil) have been shown to exert substantial variations in milk fat CLA concentrations (Kelly *et al.,* 1998a). Milk from cows fed sunflower oil (53 g/kg dry weight) for two weeks contained higher CLA content (24.4 mg/g fat), than milk from cows fed similar amounts of linseed oil (16.7 mg/g fat) or

peanut oil (13.3 mg/g fat) fed cows. Sunflower oil contains the most LA (69.4 g/100 g oil), compared with peanut (30.2 g/100g oil) and linseed oil (15.4 g/100g oil), therefore increasing the concentration of this precursor fatty acid led to the highest concentration of CLA in milk (Kelly *et al.,* 1998a).

The method of processing oilseed supplements has been shown to influence the degree and extent of biohydrogenation (Dhiman *et al.,* 1997, Murphy *et al.,* 1990, Lawless *et al.*, 1998) and therefore milk fat CLA content. A recent study showed that feeding dairy cows extruded forms of soybean and of cotton seed, whereby oil is readily available to ruminant microorganisms, resulted in higher levels of CLA in milk compared with cows fed a control diet containing 13.5 % soybean meal (Dhiman *et al.,* 1999a). Heat processing of soybeans produced a similar effect to extrusion (Chouinard *et al.,* 1998).

Other factors such as the presence of fish oil in the diet, the ratio of forage to concentrate in the diet and restricted feeding increase milk fat CLA by modifying the rumen population of bacteria involved in the biohydrogenation of unsaturated fatty acids. The mechanisms by which dietary supplementation with fish oil (Chouinard *et al.,* 1998) or fish meal (Dhiman *et al.,* 1996) increase CLA concentration are not known because biohydrogenation of the long chain polyunsaturated fatty acids in fish oils has not been shown to produce CLA or *t*11-$C_{18:1}$. However increased ruminal production of *t*11-$C_{18:1}$ has been reported (Chilliard *et al.,* 1999). It has been proposed that long chain PUFA in fish oil may inhibit complete ruminal biohydrogenation of $C_{18:2}$ fatty acids through

inhibition of the growth of bacteria that reduce *t*11-$C_{18:1}$ or through inhibition of their reductase enzymes (Griinari and Bauman, 1999).

A low forage to concentrate ratio in the diet increased CLA concentration significantly compared with a high forage to concentrate ratio (Chouinard *et al.,* 1998). A low forage to concentrate ratio reduces the fiber content of the diet and as a consequence reduces the ruminal pH (Romo, 1995). The increase in CLA in milk associated with a low ratio has been attributed to a low ruminal pH which may have favoured elevated production of *trans* octadecenoic acids in the rumen (Romo, 1995). Consistent with this hypothesis, addition of buffer to a low fiber diet increased the rumen pH and decreased production of *trans* octadecenoic acids (Kalsheur *et al.*, 1997).

As described above, a low grass intake (16 kg/cow/d) caused a significant reduction in the concentration of milk fat CLA over a 19 week period (Stanton *et al.,* 1997a). However, dietary restriction in another study led to an increase in the CLA concentration of the milk (Jiang *et al.,* 1996). This could be a result of diminished fat synthesis (fewer triglycerides increase the proportion of CLA). The different results obtained in these studies may be explained by the fact that feeding in the former study was restricted when the animals were lactating but feeding in the latter was restricted two weeks before calving. This may have more severely deprived the animals at a critical time with respect to energy requirements, resulting in mobilisation of body fat stores and direct transfer of tissue CLA to milk (Stanton *et al.,* 1997a).

Feeding patterns differ in certain countries due to variations in climate. Seasonal pasture feeding regimes exist in Ireland, New Zealand and Australia, whereas indoor feeding of forage and concentrates is more common in the US (Hawke and Taylor, 1994). The mean *c*9, *t*11 CLA content in Australian meats (Shantha *et al.,* 1995) such as lamb, beef, veal, pork and chicken was 2 to 3-fold higher than levels reported in equivalent US produce (Parodi, 1994). In Ireland a country with a seasonal milk production pattern, substantial variation in the CLA content of the milk supply has been observed (Lawless *et al.,* 2000, Stanton *et al.,* 1997b), much of the variation being attributed to seasonal influences on pasture supply and quality.

Lactation number (an index of age) is also a factor influencing CLA content of bovine milk fat. A positive correlation between milk fat CLA levels and lactation number has been reported (Lal and Narayanan, 1984, Stanton *et al.,* 1997a). Cows with seven or more lactations produced significantly more CLA in their milk than cows with one to six lactations (Lal and Narayanan, 1984) and cows with lactation numbers greater than four produced more CLA than cows with two to four lactation numbers (Stanton *et al.,* 1997a). The stage of lactation of the animals (i.e. the time elapsed after calving) which ranged from 12 to 193 days, had no effect on CLA levels in that study (Stanton *et al.,* 1997a).

A study investigating the effect of breed showed that Montbeliardes produced milk containing a significantly higher level of CLA than Dutch Holstein, Normandes and Irish Holstein cows (Lawless *et al.,* 1999). In a further study milk fat from Friesian herds exhibited a significantly higher CLA content than that from Jersey herds (MacGibbon and

Hill, 1998). These results suggest that animal breed has some influence over the CLA content of the milk.

1.6 Enrichment of CLA by non dietary means

Methods by which the CLA content of milk fat and oils might be enhanced is now the focus of many studies; such enrichment would be very advantageous in light of the many scientifically proven beneficial effects of this unique fatty acid (described in section 1.8). A few studies have investigated interesterification, hydrogenation, fractionation and microbial activity as means of enriching the CLA content of food (Garcia *et al.,* 1998, Jiang *et al.,* 1998, Jung and Ha, 1999, Kim and Liu, 1999, Lin *et al.,* 1999, 2000).

In one study Garcia *et al.* (1998) examined the possibility of increasing the CLA content of butter oil via a lipase mediated interesterification reaction. Microbial lipases from *Candida cylindracea*, *Pseudomonas sp.*, *Mucor miehei* and *Candida antartica* were screened for their ability to incorporate CLA into butter oil triglycerides by interesterification (acidolysis) whereby free CLA is incorporated into the glycerides of milk fat. CLA was produced by isomerisation of LA using the method of Chin *et al.* (1992) and added to butter oil with each lipase and subsequently the butter oil was analysed for CLA content. The lipase from *Candida antartica* increased the CLA content of the acylglycerides from 0.6 to 15 g/100 g fat. A solvent free approach was chosen for this study to minimise product bleaching, deodorisation and hydrolysis. However, the

observed reaction rates were slow in comparison with those in organic solvents (Oba and Witholt, 1994).

Microbial synthesis of CLA by food grade strains may also provide a means of enriching the CLA content of food products. The formation of CLA by certain rumen bacteria e.g. *Butyrivibrio fibrisolvens*, in the biohydrogenation of LA has been known for many years (Kepler and Tove, 1967). More recently, the intestinal flora of rats has been shown to be capable of converting free LA to *c*9, *t*11 CLA (Chin *et al.,* 1994). One hundred and eighty strains of common bacterial lung pathogens were studied to determine if they could produce a conjugated diene isomer of LA *in vitro* (Jack *et al.*, 1994). It was found that 12.8 % of the strains were capable of producing elevated levels of *c*9, *t*11 CLA from free LA. A study by Jiang *et al.* (1998) investigated the production of CLA by dairy starter cultures. Nineteen different strains of lactobacilli, lactococci, streptococci and propioni bacteria which are commonly used as dairy starter cultures were screened for their ability to produce CLA from LA *in vitro*. Three strains of propioni bacteria, including *Propionibacterium freudenreichii ssp. freudenreichii* and one strain of P. *freudenreichii ssp. sheramnii* were shown to convert free LA to extracellular CLA. The highest level of CLA formed in the media was 265 μg/ml by the strain *Propionibacterium freudenreichii ssp. freudenreichii.* This represented a 2-3-fold increase in CLA formation compared with the other strains examined. Of the different isomers, *c*9, *t*11 CLA represented more than 70 % of the total CLA formed. In a separate study, six lactic acid cultures including *Lactobacillus acidophilus* (CCRC14079), *L. delbrueckii subsp. Bulgaricus* (CCRC14009), *L. delbrueckii subsp. lactis* (CCRC14078),

Lactococcus lactis subsp. cremoris (CCRC12586), *Lactococcus. lactis subsp. lactis* (CCRC10791), and *Streptococcus salivarius subsp. thermophlius* (CCRC12257) were tested for their ability to convert free LA to extracellular CLA (Lin *et al.,* 1999). A sharp increase in CLA levels was observed following addition of LA to cultures of each of the six strains tested. Incubation of *L. acidophilus* in skim milk medium containing LA for 24 h was most effective in promoting CLA formation. The effects of additions of sucrose, lactose, fructose, and sodium chloride to these strains on CLA formation were also tested (Lin *et al.,* 2000). A significant decrease in *c*9, *t*11-CLA level was observed in all but one culture *Lactococcus. lactis subsp. cremoris* when sucrose, lactose, fructose, and sodium chloride were added. Inoculation of *Lactobacillus acidophilus* into sweeteners and sodium chloride-treated skim milk medium under aerobic conditions for 24 h incubation was most effective in promoting *c*9, *t*11-CLA formation (Lin *et al.,* 2000).

The effect of hydrogenation of soybean oil on CLA content has been examined. Two types of process exist: selective and nonselective hydrogenation. Selective hydrogenation involves the conversion of a diene to a monoene fatty acid and is more discriminating than the conversion of a monoene to a saturated fatty acid. By contrast, nonselective hydrogenation, being a random process does not specify desirable hydrogenation and isomerization reactions. Both are performed in the edible oils and fats industry with commercially available nonselective and selective catalysts. Most known selective hydrogenation catalysts contain one of the expensive metals rhodium, palladium or platinum. The fatty acid composition of oil is greatly dependent on the duration and

type of hydrogenation process. Selective hydrogenation of soybean oil was found to be more favorable for the production of CLA (98.27 mg total CLA/g oil) than nonselective hydrogenation (9.06 mg total CLA/g oil) (Jung and Ha, 1999).

Milk fat consists of a mixture of triglycerides that exhibit a broad and variable melting range, from approximately –40 °C to +40 °C. This allows the crystallising out of a series of triglycerides at temperatures below their melting points by controlled cooling of the melt. Urea crystallisation allows fractionation of fatty acids by the degree of unsaturation; saturated fatty acids form complexes with urea to a greater extent than polyunsaturated fatty acids and as a consequence these can be separated from each other. The process yields two fractions: a soft fraction which contains fats with low melting points and is therefore liquid and a hard fraction which contains fats with high melting points and therefore is solid. This approach was investigated for production of milk fat enriched in CLA, which resulted in a 250 % increase in the CLA content, to 12.7 mg/g fat (Kim and Liu, 1999). The CLA-enriched soft fraction was similar in saturated fatty acid content and higher in unsaturated fatty acids compared with the parent milk fat.

1.7 Factors influencing CLA content of food products

1.7.1 Beef

The initial data from Pariza's laboratory demonstrated that the CLA content of ground beef could be increased four-fold after heating as a result of free radical mediated oxidation of LA (Ha *et al.,* 1987). The proposed model for CLA formation by heating is

shown in Figure 1.5. When heated in the presence of moisture LA undergoes thermal oxidation, which involves abstraction of a hydrogen from the double bond system, resulting in a shift in the double bond structure to form a conjugated diene. The highly reactive radical formed may be converted to CLA via proteins functioning as H donors. Different cooking methods (frying, broiling, baking and microwaving) did not greatly influence CLA content of ground beef (Shantha *et al.,* 1994a).

Dietary manipulation of cattle prior to slaughter was shown to influence the CLA concentration of beef (French *et al.*, 2000). Decreasing the proportion of concentrate in the diet of steers, which effectively increased grass intake, led to a linear increase in the intra-muscular CLA concentration ($P < 0.001$) and the PUFA to saturated fat ratio ($P < 0.01$). Furthermore, a linear decrease in the concentration of intra-muscular saturated fatty acids ($P < .01$) and in the n-6 to n-3 PUFA ratio ($P< 0.001$) was observed as a result of increased pasture intake over concentrate.

1.7.2 Dairy products

The CLA concentration in raw milk fat is a major determinant of CLA concentration in dairy products. Levels of CLA in a range of dairy products such as low fat yoghurt, whole fat yoghurt, whole fat and regular ice cream, sour cream or cheese such as Mozzarella, Gouda, and Cheddar were the same as in the starting milk (Shanta *et al.,* 1995). This would suggest that the effect of processing was negligible. As cheese is generally the richest dietary source of CLA this product has received the most attention in relation to the effects of processing on CLA (Garcia-Lopez *et al.,* 1994, Jiang *et al.,* 1998, Lin *et al.,* 1995, Shantha *et al.,* 1994a, 1994b, Werner *et al.,* 1992).

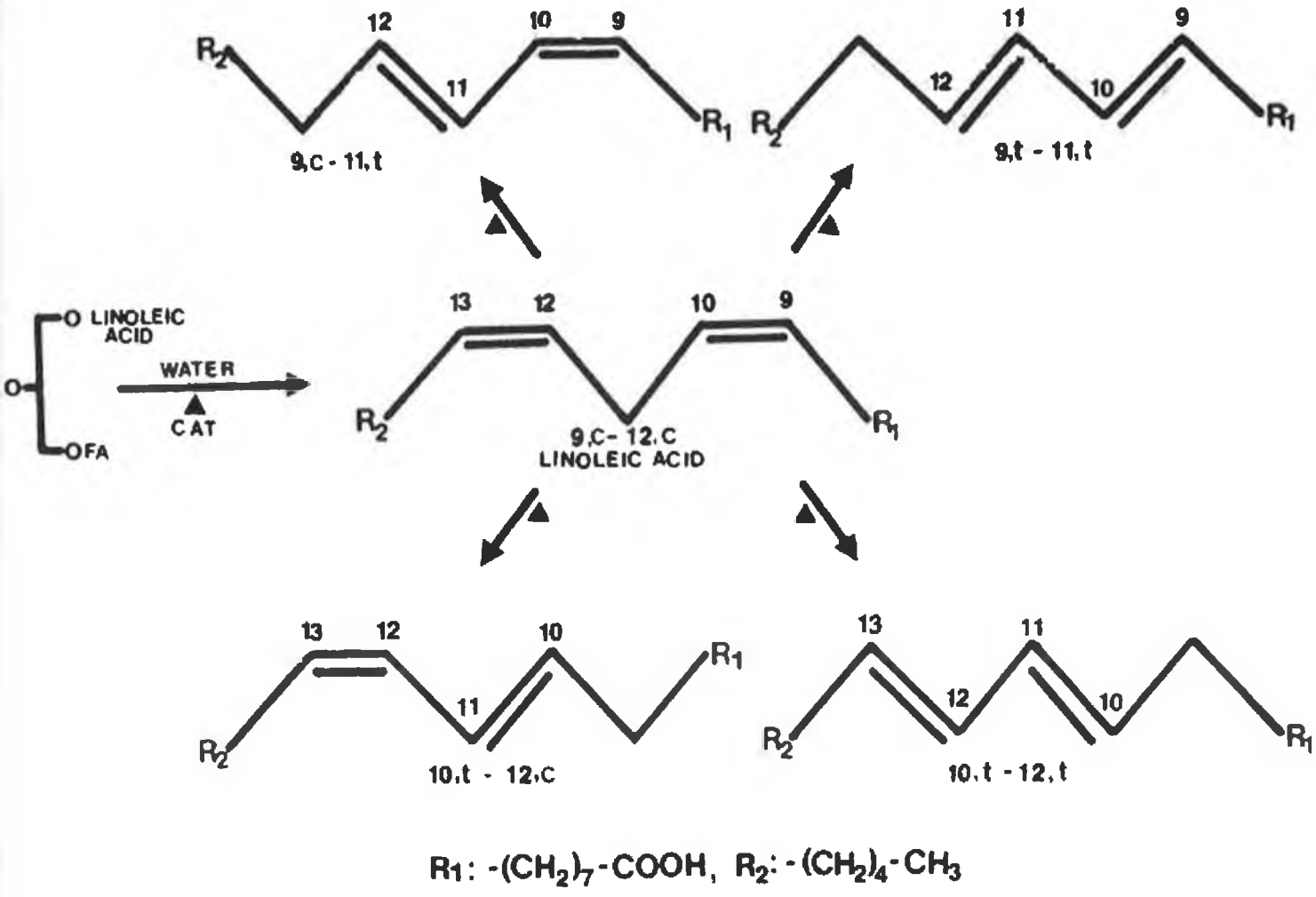


Figure 1.5 Model for formation of CLA from linoleic acid in ground beef during frying (Ha *et al.*, 1987)

High heat treatment of Cheddar cheese increased CLA content compared with low heat treatment. This was attributed to heat-induced formation of LA radicals which, in the presence of proteins functioning as H donors can be converted to CLA (Shantha *et al.,* 1992). Air is also important in the formation of CLA through the free radical mechanism. The CLA content of cheese processed under atmospheric conditions was significantly higher than that of cheese processed under nitrogen (Shantha *et al.,* 1992). Incorporation of air into butter also increased CLA content (Shantha *et al.,* 1995). While free radical oxidation mechanisms are proposed to be the major mechanism for CLA formation in cheese prior to aging, biohydrogenation of linoleic or linolenic acids to CLA is proposed to predominate during aging (Ha *et al.,* 1989). Addition of hydrogen donor supplements to the intermediates of free radical oxidation and biohydrogenation reactions may increase CLA by conversion of LA radicals to CLA, but may also decrease CLA by enhancing hydrogenation of CLA to stearic acid. Addition of supplements (BHA, tyrosine, lysine) yielded cheeses with lower CLA contents after 6 months compared with a control additive- free cheese (Lin *et al.,* 1998). Addition of supplements allowed the reduction of CLA to monoenoic or saturated fatty acids and thereby led to a decreased CLA content. Cheese that was milled at pH 5.5 and 5.9 had lower CLA than cheese milled at pH 5.7 (Lin *et al.*, 1998). Milling at pH 5.5 and 5.9 may retard the production of lactic acid, and the development of hydrogen donors therefore reducing enzymatic activities of the starter culture. High CLA cheeses had higher titratable acidity than low CLA cheeses; thus indicating that higher enzyme activity of cultures during aging may

lead to enhanced CLA formation via isomerisation and biohydrogenation (Lin *et al.*, 1998).

1.7.3 Stability of CLA during storage

While free radical mediated oxidation of linoleic acid has been shown to increase the CLA content of beef and cheese, CLA itself is not prone to oxidation during storage. Oxidative reactions measured during 7-day refrigerated storage of cooked beef did not alter CLA content suggesting that CLA was stable during storage (Shanta *et al.*, 1994a). Another study in which dairy products were examined during refrigerated storage also demonstrated that CLA is a stable food component (Shanta *et al.*, 1995).

1.8 Health benefits of CLA

The *c*9, *t*11 isomer of CLA acid is the most abundant isomer amounting to as much as 90 % of the total CLA content of dairy products (Chin *et al.*, 1992). A number of reports show an association between this isomer and protection against cancer (Belury *et al.*, 1996, 1997, Belury and Kempa-Steczko, 1997, Brodie *et al.*, 1999, Cunningham *et al.*, 1997, Durgham and Fernandes, 1997, Ha *et al.*, 1990, Ip *et al.*, 1991, 1994, 1995, 1996, 1997, 1999a, 1999b, Ip, 1997, Ip and Scimeca, 1997, Liu and Belury 1997, 1998, Liew *et al.*, 1995, Scimeca *et al.*, 1994, Schut *et al.*, 1997, Shultz *et al.*, 1992), atherosclerosis (Doyle, 1998, Lee *et al.*, 1994, Nicolosi *et al.*, 1997) and diabetes (Houseknecht *et al.*, 1998). The *t*10, *c*12 isomer of CLA has also been implicated in many biological activities, particularly in relation to alteration of body mass (Park and Pariza, 1998).

Additionally, CLA has been shown to regulate production of cytokines acting as growth factors to increase bone (Li and Watkins, 1998) and muscle mass (Miller *et al.*, 1994, Park *et al.*, 1997) while reducing subcutaneous fat (Dugan *et al.*, 1997).

The most studied bioactivity of CLA is its anticancer effect. The following sections describe the biological events involved in the development of cancer, the modulation of these events by specific dietary fat components and the particular studies that have been conducted to elucidate mechanism(s) of chemoprotection by CLA.

1.9 Development of cancer

Cancer is synonymous with malignant tumour; the Latin term 'cancer' is actually a literal translation of the Greek '*karkinos*' for crab, and refers to claws reaching out to grasp surrounding tissues (invasion). The key ingredient in order to produce a tumour is a cell type capable of dividing. The birth of a tumour cell requires at least 2 successive events. Firstly, an initiator must strike the DNA of a cell and introduce a "suitable" defect (i.e. a mutation); at this point the cell is potentially a cancer cell but is held in check by DNA repair enzymes. Secondly, a promoter must cause the cell to multiply and to generate a tumour, thus revealing the latent curse of the initiated cell. Initiation is conceived as a quick, almost instantaneous process; if it is repeated, the effect on the tissue is additive. Initiators can be chemical, physical or biological. 7,12 -dimethyl-benz[a]anthracene (DMBA), a polycyclic hydrocarbon was one of the first specific carcinogens isolated from tar in the 1930's and is still used today as an initiator in experimental animal models of carcinogenesis. It is metabolised stepwise by the enzymes of the endoplasmic reticulum such as P-450 cytochromes to yield the ultimate carcinogen, an electrophilic

species capable of covalent bonding with nucleophilic sites on DNA. Promotion is viewed as a slow process, its effect is reversible and non-additive. Many promoters cause cells to multiply and inhibit cell-cell communication. Promoters may be chemical (e.g. tetradecanoylphorbol-13-acetate (TPA)) or biological (e.g. estrogen). TPA activates protein kinase C (PKC), for which the physiological activators are diacylglycerols. In this way TPA inserts itself as a link in the transduction of signals from the cell surface to effectors in the cytoplasm. Once malignant tumours have started to grow, they acquire a genetic instability which favours such accidents as chromosomal breaks and translocations that result in the appearance of new clones (tumour progression). The overall result is clinically apparent; there is an increase in tumour growth rate, an increase in invasiveness and metastasis. The latter is the most lethal expression of malignancy and poses the most important concern for the treating physician by virtue that tumours have spread to other parts of the body usually via the blood stream or lymph. In addition, malignant tumours show a reduced sensitivity to drugs and hormones, decreased antigenicity and decreased radiosensitivity. Molecular genetic studies of cancer have shown that carcinogenesis is a continuous evolving process involving the accumulation of a series of genetic alterations, particularly in genes controlling cellular proliferation and the integrity of the genome (Ames *et al.,* 1995). Some tumours are driven by normal genes that are expressed inappropriately (dominant oncogenes). Other tumours are triggered by the loss of normal genes (suppressor oncogenes). To date, well over 60 dominant and suppressor oncogenes have been identified (Baserga, 1994). Because each gene is best defined by its product much effort is concentrated in finding out what proteins are coded by these oncogenes. Despite many loose ends a picture is emerging: gene products of oncogenes are either growth factors, growth factor receptors or links in an intracellular network through which external stimuli induce cell proliferation (Figure 1.6).

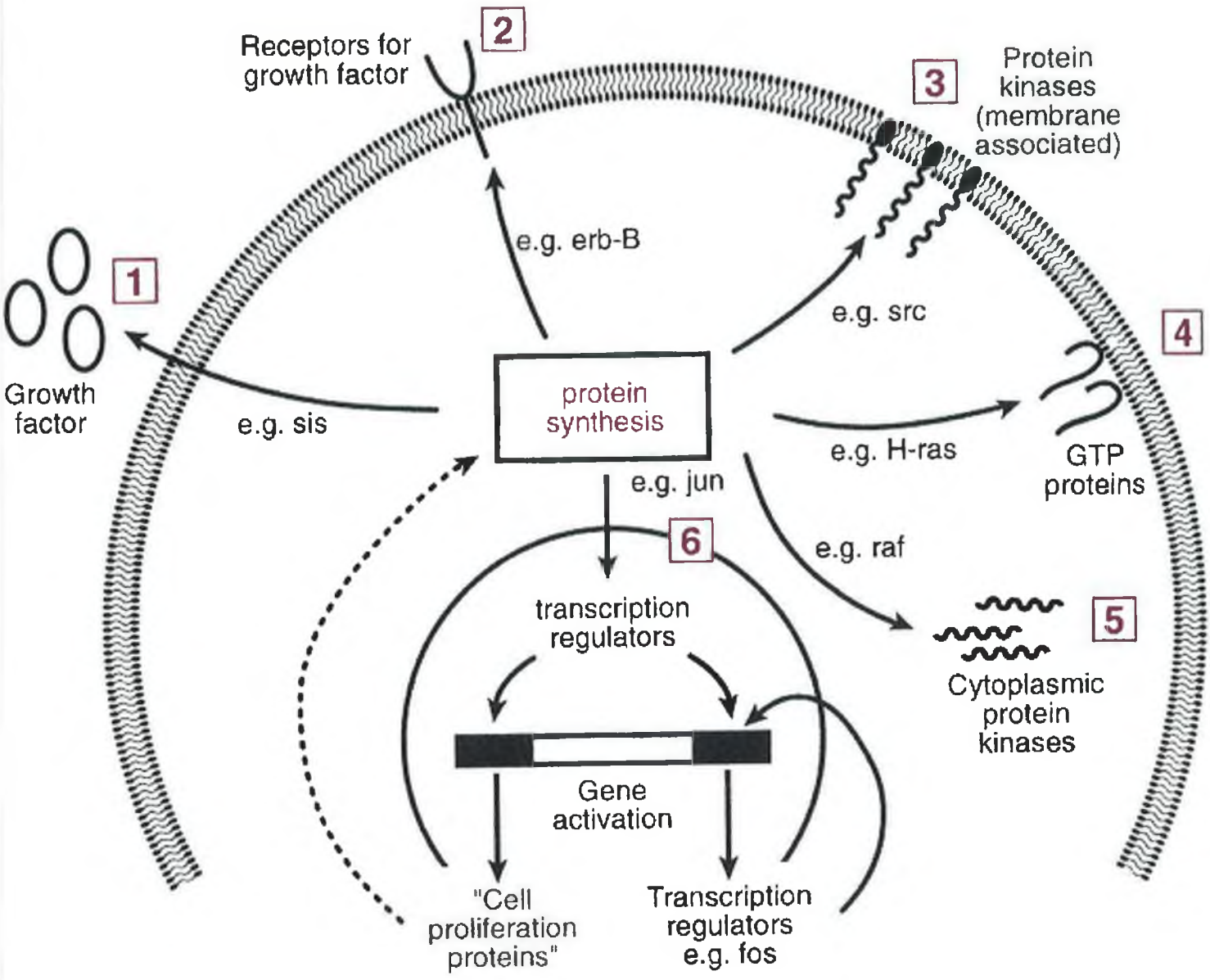


Figure 1.6 Diagram of a cell indicating the six major oncogene products: (1) secreted; (2), (3), (4) associated with the cell membrane; (5) active in the cytoplasm; (6) active on the nucleus. Adapted from Alberts *et al.* 1989.

One of the best understood dominant oncogenes is *ras; ras* genes code for 21-kilodalton proteins called p21, which are located in cell membranes, bind guanine nucleotides (GTP and GDP) and function as GTPases (Ames *et al.,* 1995). Ras acts positively to stimulate a cascade of kinase-driven phosphorylation events that culminate in the activation of nuclear transcription factors (Downward, 1990). Figure 1.7 shows that the active form of Ras has a molecule of GTP bound in the nucleotide binding site whereas the inactive form contains a GDP in that site. The intrinsic GTPase activity of Ras therefore converts the active Ras-GTP form into the inactive Ras-GDP moiety (Downward, 1990). Two types of proteins, GAPs (GTPase activating proteins) and GNRFs (guanine nucleotide releasing factors) control the activity of Ras such that GAPs are negative regulators and GNRFs are positive regulators of Ras (Milburn *et al.,* 1990). About 15 % of human solid tumours contain one or more *ras* genes with single-base alterations. Activated *ras* genes have also been found in premalignant and malignant lesions induced experimentally with carcinogenesis. The most prevalent dominant mutations in *ras* occur on codons 12, 13 and 61 and cause a defect in the Ras GTPase activity such that it is always in the active ras-GTP form (Milburn *et al.,* 1990). Under these circumstances, the cell receives a 'false' signal to activate growth-promoting genes, even in the absence of growth factors (Milburn *et al.,* 1990). Figure 1.8 depicts a proposed phosphorylation -signalling cascade showing how Ras connects the epidermal growth-factor membrane receptor to fos and jun through a sequential series of kinase-activated steps. Fos and jun are nuclear trancription regulators and when activated signal growth stimulation in the cell (Baserga, 1994). The role of Ras in signalling is known to involve localisation of Ras to the membrane which occurs via the farnesylation pathway (Marshall, 1996).

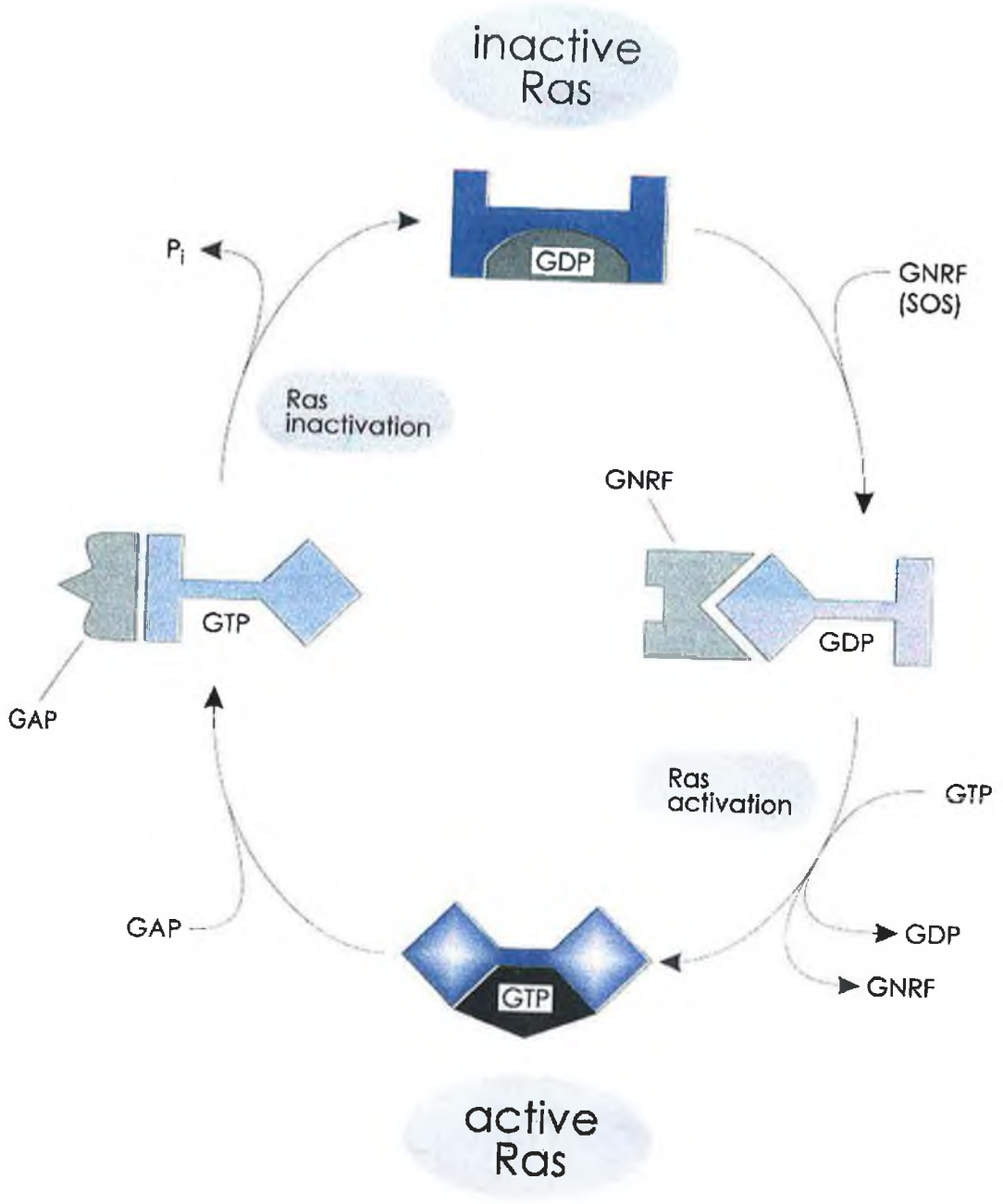


Figure 1.7 Ras activation by proteins that modulate Ras GTPase activity (Matthews *et al.*, 1997). SOS: Son of Sevenless, GAPs: GTPase activating proteins, GNRFs: guanine nucleotide releasing factors, GDP: guanine-diphosphate, GTP: guanine-triphosphate.

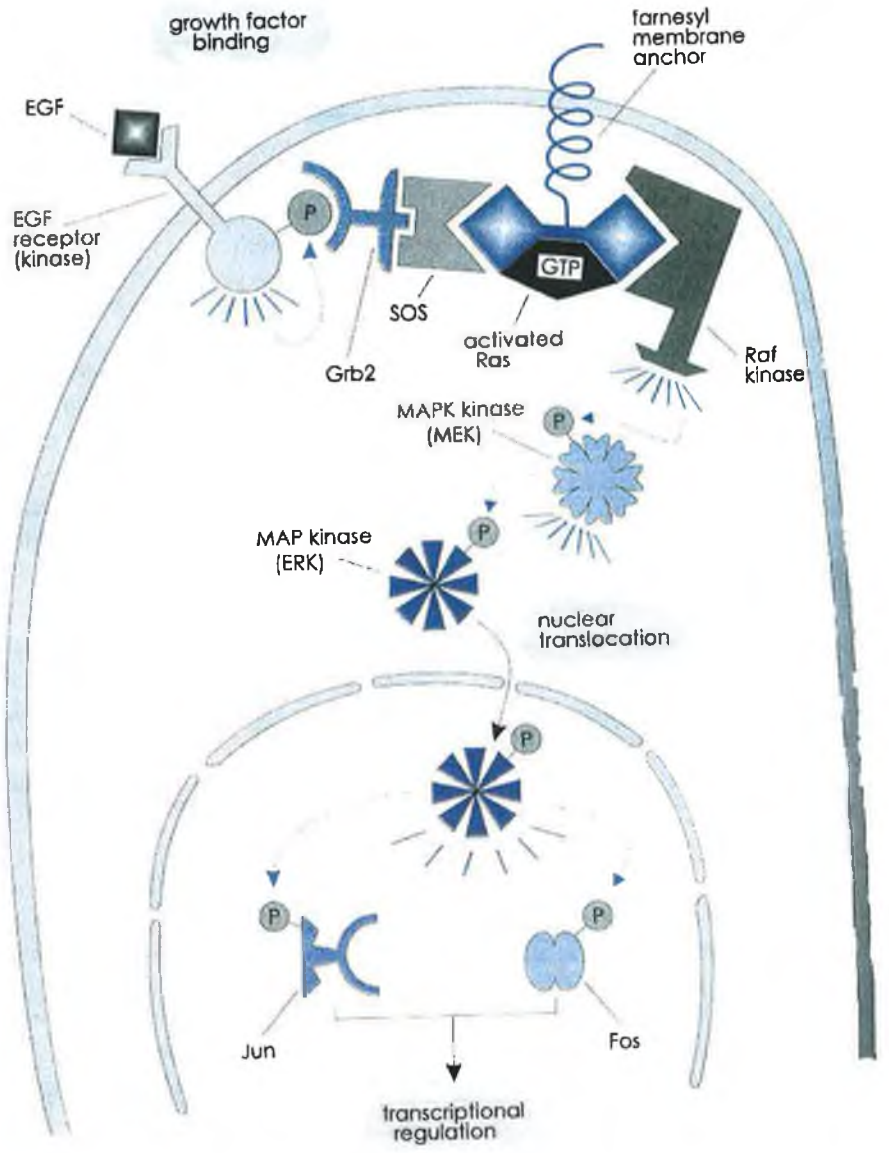


Figure 1.8 Proposed phosphorylation-signaling cascade from EGF receptor to *Fos* and *Jun* through a sequential series of kinase activation steps (Matthews *et al.*, 1997). MAPk: MAP kinase, MEK: MAP kinase kinase, SOS: son of sevenless, ERK: activated MAP kinases, Grb2-SOS adapter complex, EGF: epidermal growth factor, p: phosphate.

The farneslyation pathway involves the addition of a farnesyl moiety to the GTP-bound Ras oncoprotein enabling it to bind to the cell membrane (Singh *et al.,* 1997a). Several Ras inhibitors are being developed to target the enzyme protein farnesyl transferase responsible for Ras farnesylation and thereby disrupt the dominant activity of mutated Ras in cancer cells (Singh *et al.,* 1998).

1.9.1 Dietary Chemoprotective agents

Cancer chemoprotection is defined as the use of chemical agents or dietary components either to protect against the initiation of carcinogenesis or to retard the progression of neoplastic disease once it has begun. Compounds that reduce the rate at which mutations arise can be broadly classified into two groups (bioantimutagens and desmutagens), according to their mechanism of action. Bioantimutagens act mainly on DNA replication and repair processes, reducing the extent of DNA damage that has been induced by mutagens (Clark and Shankel, 1975). They also act as 'suppressing agents' by promoting expression of special features of the normal cell (i.e. differentiation), inhibiting oncogene activation, inhibiting proliferation of initiated cells and enhancing cell death via apoptosis (Ferguson, 1994). Desmutagens are considered blocking agents (i.e. they block mutagen activation) because they induce proteins involved in detoxification of carcinogens, inhibit the activation of carcinogens or prevent the uptake of carcinogens by the cell. Carcinogen activation may induce free radicals that initiate a series of reactions that have irreparable effects on lipid, protein, carbohydrate and DNA molecules. Lipids are prime

molecular targets for free radical mediated attacks and polyunsaturated fatty acids present in cell membranes are especially at risk. The hydrogens on carbons between two double bonds of a polyunsaturated fatty acid are easily abstracted by free radicals leading to a rearrangement of the double bonds. This rearrangement changes the shape of fatty acids and under aerobic conditions they are more likely to combine with oxygen to form a peroxyl radical. This peroxyl radical is capable of initiating the same process with another fatty acid, so propagating a chain reaction. Cells have two lines of defense, antioxidant vitamins (e.g. α-tocopherol, ascorbic acid and β-carotene) and antioxidant enzymes that scavenge free radicals induced by carcinogen activation and are important desmutagens. Antioxidant enzymes are the first line of defense eliminating superoxide radicals and hydrogen peroxides responsible for the production of dangerous hydroxyl radicals. Superoxide dismutase (SOD) eliminates superoxide radicals and occurs in several different compartments of the cell. The cytosolic enzyme is composed of two similar subunits, each one containing one equivalent of Cu^{2+} and Zn^{2+}, whereas the mitochondrial enzyme contains Mn^{2+} (Halliwell and Gutteridge, 1989). True to its important function SOD is very stable and one of the most active enzymes known. Catalase and glutathione peroxidase (GPx) eliminate hydrogen peroxide. Catalase acts specifically on hydrogen peroxide converting it to water and oxygen. GPx requires cosubstrates glutathione and NADPH and acts on all hydroperoxides. Antioxidants are an important component of the desmutagen artillery found in the diet (Table 1.5). The following section contains a synopsis of research showing that particular components of dietary fat can influence the development of cancer during promotion and metastasis.

Table 1.5 Selected dietary anticarcinogens and their postulated mechanism of action.

Compound	Bioantimutagen ←			Desmutagen →		
		Direct chemical inactivator	Enzyme modulator	Binding scavenger	Antioxidant	Tumour growth suppressor
Ascorbic acid	-	-	X	-	X	X
BHA	-	X	X	-	X	-
Calcium	-	-	-	-	-	X
Carotenoids	-	-	-	-	X	X
CLA	-	-	-	-	-	X
Coumarins	-	-	X	-	-	-
Dithiolthiones	-	-	X	-	-	-
Dietary fiber	-	-	-	-	-	-
Flavonoids	-	-	X	X	X	X
Folic acid	-	-	-	-	-	X
Genistein	-	-	X	-	X	X
Indoles	-	-	X	-	-	X
Isothiocyanates	-	-	-	-	X	-
Lignins	-	-	X	-	-	-
Omega 3 Fatty Acids	-	-	-	-	X	X
Organosulphur compounds	-	-	-	-	X	X
Phenolics	X	-	X	-	X	X
Phytates	-	-	X	-	X	-
Phytoestrogens	-	-	-	-	?	X
Porphyrins	-	-	?	-	X	X
Protease inhibitors	-	-	-	X	-	X
Retinoids	-	-	-	-	-	X
Saponins	-	-	-	-	-	X
Selenium	-	-	-	-	X	X
Terpenoids	-	-	X	-	-	-
Tocopherol	-	X	-	-	X	-
Vanillin	X	-	-	-	-	-

- represents no effect.
X represents an effect.
? represents conflicting results. Adapted from Ferguson, 1994.

1.9.2 Dietary fat and cancer

Evidence that dietary fat can exert both non-specific and specific effects on carcinogenesis has come from experimentation in animal cancer models and from epidemiological data on human populations. Non-specific effects relate to dietary fat as a whole and specific effects relate to particular components of dietary fat (Guthrie and Carroll, 1999). Non-specific effects of dietary fat on carcinogenesis are deleterious, being primarily related to its high energy density (38 kJ/g) relative to carbohydrates and proteins (17 kJ/g) and the low energy cost required for its metabolism and deposition into adipose tissue (Pariza and Boutwell, 1987). Accumulation of fat in adipose tissue over a long period of time is conducive to obesity. Obesity has been associated with an increased risk of cancer at a number of different sites, including endometrium, breast and kidney (Goodman, 1997). It could be postulated that the growth of a cancer requires energy, and an excess of energy from the diet might serve as a growth stimulant, whereas a deficit of energy from under-nutrition could help inhibit cancer growth (Guthrie and Carroll, 1999).

1.9.2.1 Monounsaturated fatty acids

$C_{18:1}$ is the most abundant dietary monounsaturated fatty acid and is a major constituent of many fats and oils, including olive oil, high-oleic safflower oil, canola oil and palm oil. The role of $C_{18:1}$ on mammary carcinogenesis is controversial and many conflicting results have been published. A carcinogenic effect was observed in rats fed $C_{18:1}$ - enriched safflower oil and treated with DMBA (Carroll and Khorr, 1975, Carroll, 1977). However, little effect on tumourigenesis was observed when methylnitrosourea (MNU)

treated animals were fed olive oil (a rich source of $C_{18:1}$) (Cohen *et al.,* 1986a). The seeming inconsistency may be related to the amount of LA in the olive (5 %) and safflower oils (17 %). In a further study when the LA content of the two oils were equal the differential effects of safflower and olive oil in DMBA treated rats were not evident (Laeskan *et al.,* 1990). This argues against any specific effect of $C_{18:1}$ on mammary carcinogenesis (Guthrie and Carroll, 1999).

1.9.2.2 Saturated fatty acids

Saturated fats seem to have little effect on cancer unless combined with small amounts of polyunsaturated fats (Carroll and Khor, 1971, Carroll *et al.,* 1981). $C_{16:0}$ is the most abundant dietary saturated fatty acid derived mostly from milk fat but diets contain smaller amounts of other saturated fatty acids of varying chain lengths. Little is known about the effects of individual saturated fatty acids on cancer, but a study on medium chain saturated fats C_{10}-C_{14} present in large amounts in coconut oil, showed little evidence that they had any promoting effect on mammary cancer (Cohen, 1986b).

1.9.2.3 Polyunsaturated fatty acids

Particular components of dietary fat have been shown to exert specific effects on the development of cancer in animals. Whereas n-6 (double bond positioned six carbons from the methyl terminal end of the fatty acid chain) fatty acids (e.g. LA) have been associated with enhancement of the promotional phase of experimental rat mammary carcinogenesis (Ip *et al.,* 1987), long chain n-3 (double bond positioned three carbons from the methyl terminal end of the fatty acid chain) fatty acids present in fish oils have

been associated with inhibition of this spectrum of the cancer process (Appel *et al.*, 1994). Dietary fish oil supplementation is associated with reduction of both mammary and colon tumourigenesis in rodent models (Rose and Connolly, 1993, Singh *et al.*, 1998). Docosohexaenoic acid (DHA) ($C_{22:6}$) is an n-3 fatty acid present in fish oil. DHA has been shown to reduce the onset of colon cancer by inhibiting the farnesylation pathway, responsible for membrane localisation of ras oncoproteins (Singh *et al.*, 1998). When mixtures of n-3 and n-6 PUFA were fed to rodents, it appeared that a relatively high proportion of n-3 fatty acids was required to overcome the promotional effect of the n-6 fatty acid, LA (Cave, 1991a, 1991b, 1997, Rose and Connoly, 1997).

LA undergoes desaturation and elongation to yield arachidonic acid ($C_{20:4}$), (AA) a substrate for the biosynthesis of eicosanoids. Eicosanoids are potent bioregulatory compounds involved in a wide variety of cell signalling processes. Upon stimulation by inflammatory stimuli or tumour promoter agents, AA is released from cell membranes by the action of phopholipase A_2 (PLA_2). Eicosanoids derived from AA which may modulate carcinogenesis include those formed as a result of cyclooxygenase (COX) pathways (prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$,), prostaglandin D_2 (PGD_2)) and of lipoxygenase pathways (hydroxyeicosatetraenoic acids (HETEs) and leukotrienes (LTs)). Eicosanoids have been linked to cancer by the findings that human tumours synthesise elevated levels of prostaglandins when compared with normal tissue (Karim, 1976). Much of the subsequent research has concentrated on the COX products of AA metabolism with particular emphasis on prostaglandin PGE_2, which has been shown to promote growth and metastasis in many experimental tumours (Fulton, 1998).

It is not yet clear how PGE_2 levels are regulated in tumour cells but it has been suggested that the overexpression of COX 2, one of the two isoforms of COX, leads to elevated PGE_2 production (Sano *et al.,* 1995). A simple pathway outlining eicosanoid synthesis is shown in Figure 1.9.

The release of AA from membrane phospholipids is the rate-limiting step for the production of eicosanoids. PLA_2 catalyses the hydrolysis of the *sn* 2 fatty acyl bond of membrane phospholipids to liberate AA and lysophospholipids. Though the major pathway of AA release is via PLA_2, AA can also be released from sequential cleavage of phospholipids by phospholipase C (PLC) to generate diacylglycerol (DAG) that is subsequently acted on by DAG lipase to liberate AA. Because PLA_2 preferentially hydrolyses phosphatidycholine (PC) (Hanel *et al.,* 1993), the pattern of AA incorporation into phospholipid classes may have an effect on the release of AA and so effect eicosanoid production.

The growth-enhancing effect of LA on mouse mammary epithelial cells was inhibited by indomethacin, a COX inhibitor, and was partially reproduced by the COX product PGE_2 (Rose and Connolly, 1997). However the growth response to PGE_2 was less than that to LA. For full stimulation, both PGE_2 and one of the three HETE acids formed from AA under the influence of the lipoxygenase activity were required. Thus the optimal growth response of mouse mammary epithelial cells to LA appears to represent a synergism between HETE and PGE_2, which occurs under the added influence of epidermal growth factor (EGF) (Rose and Connolly, 1997) (Figure 1.10).

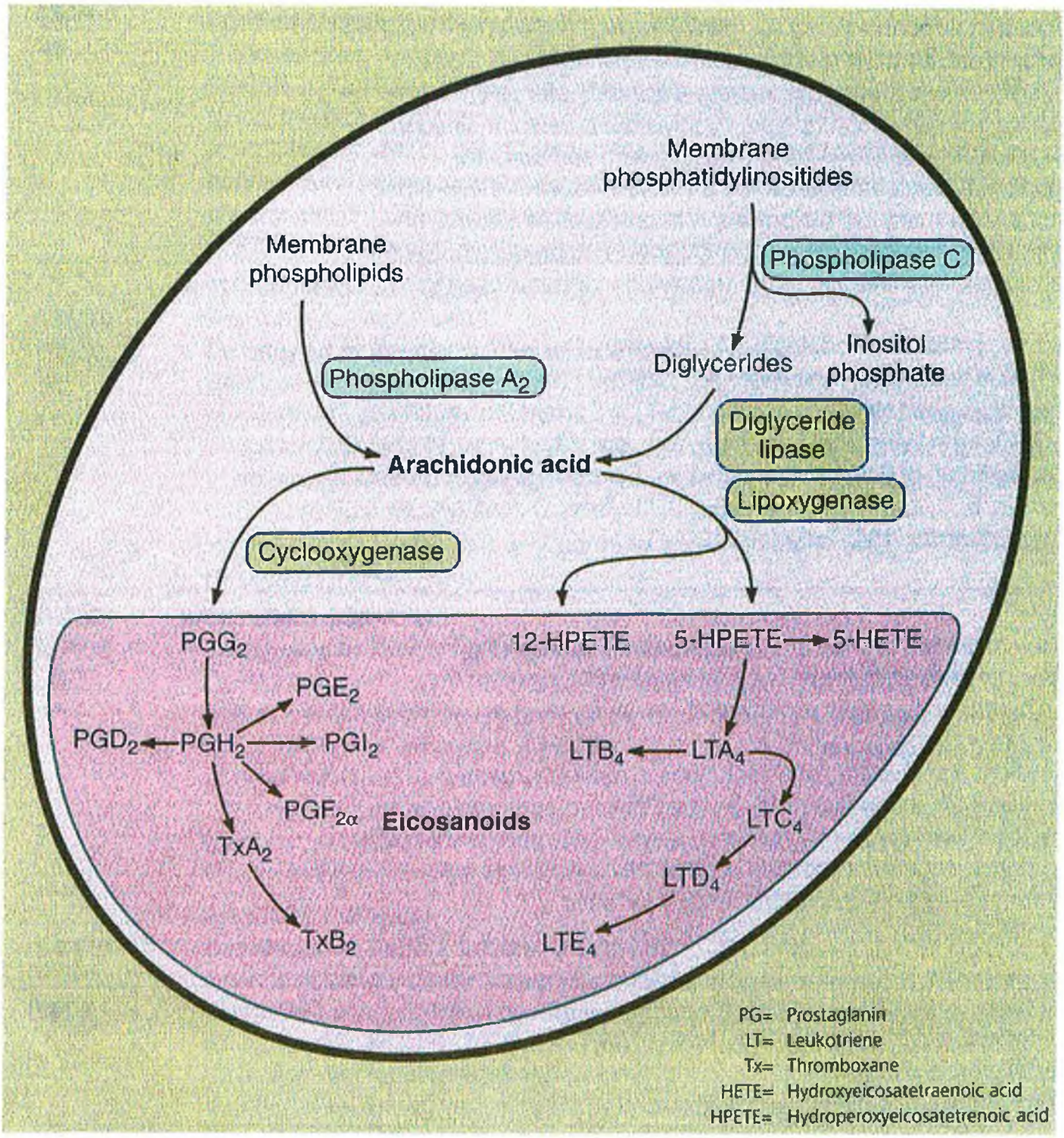


Figure 1.9 The eicosanoid pathway.

EGF when bound to its membrane bound receptor stimulates the proliferation of cells. Connolly and Rose, (1993) demonstrated a metastatic effect due to LA in the MDA-MB-435 human breast cancer cell line and provided evidence that local invasion of tumour cells through the extracellular matrix was associated with induction of HETE mediated expression of matrix metalloproteinases.

Similar effects of polyunsaturated fatty acids have been reported in the development of colon cancer. High fat dietary corn oil (HFCO) rich in n-6 fatty acids was shown to enhance COX-2 expression, while a high fat fish oil (HFFO) diet reduced COX-2 expression in colonic mucosa and in tumours (Singh *et al.,* 1997). These studies suggest that HFCO may promote colon tumourigenesis by up-regulating PGE_2 production and that moderation of PGE_2 production by HFFO may exert an antitumour effect in colon.

As in human breast cancer, mouse mammary tumours develop through a multi-step process (Medina, 1976). An intermediate pathologically defined stage is the formation of hyperplastic alveolar nodules, which appear with increasing frequency over time in mice expressing the mouse mammary tumour virus (MMTV). Telang *et al.* (1984, 1988) investigated the effects of fatty acids on the hyperplastic nodule-like mammary alveolar lesions that develop in explants cultured from the mammary tissue of mice expressing MMTV. They found that both LA and AA enhanced replicative DNA synthesis in these lesions, whereas both $C_{18:0}$ and the long chain $C_{20:5}$ n-3 fatty acids were inhibitory. These alterations in proliferation were associated with changes in c-ras^H p21 expression. The oncogene c-ras^H p21 signals an initiation event in carcinogenesis.

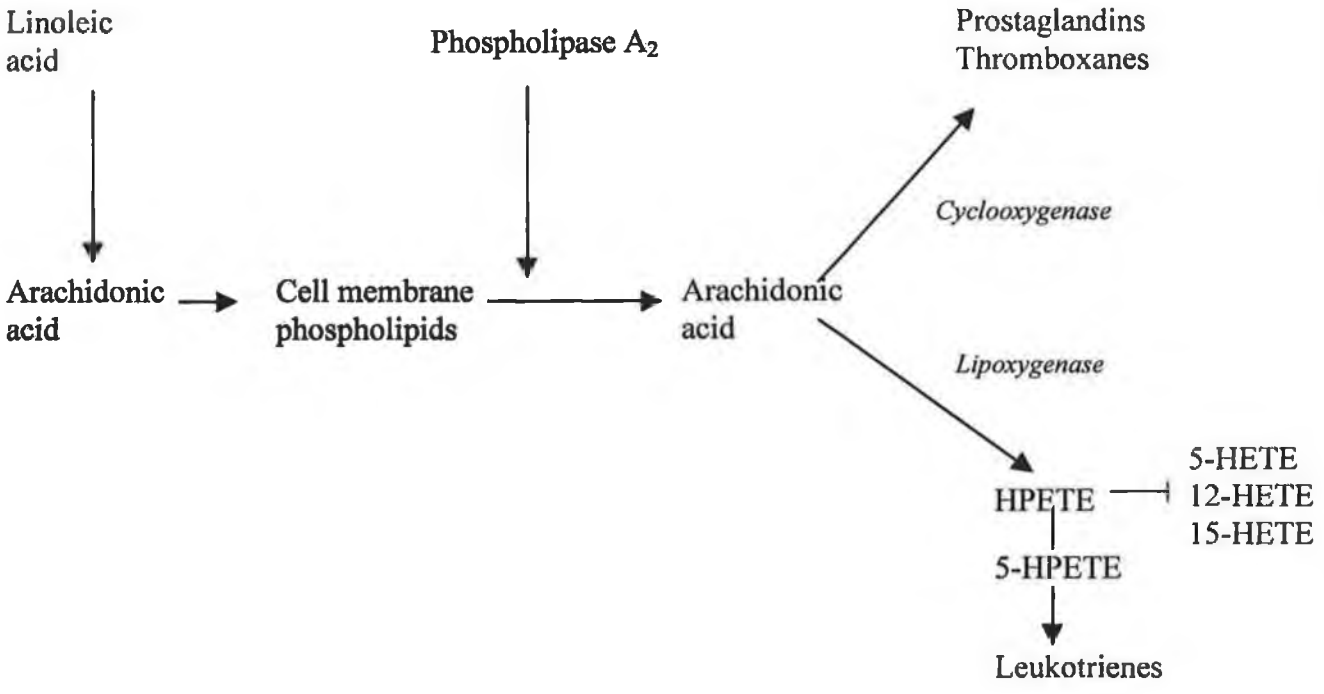


Figure 1.10 The biosynthesis of eicosanoids from linoleic acid. HPETE, hydroperoxy-5, 8, 10, 14-eicosatetraenoic acid (Rose and Connoly, 1997).

The demonstration that fatty acids affect events associated with cancer initiation, such as expression of MMTV and c-*ras*^{H} p21, in nontransformed tissue has important implications for breast cancer prevention. Growth modulatory effects of fatty acids have also been demonstrated in a number of *in vitro* studies involving human cells. A neoplastic, spontaneously immortalised, MCF-10A human mammary epithelial cell line and the same line expressing the c-Ha-*ras* protooncogene showed growth stimulatory responses to LA and inhibitory responses to n-3 fatty acids (Grammatikos *et al.*, 1994).

Despite the large number of experimental studies in animals showing that polyunsaturated fats enhance tumourigenesis more effectively than saturated fats (Connolly and Rose, 1993, Rose and Connolly, 1997), the involvement of dietary fatty acids in the etiology of human breast cancer remains controversial. Most available data on polyunsaturated fat and breast cancer risk was assembled by Howe *et al.* (1990) in their pooled analysis of 12 case control studies. The overall association of breast cancer with total dietary fat was due only to the saturated fat. In pooled prospective studies neither saturated nor monounsaturated fat intake was related to breast cancer (Hunter *et al.*, 1996). Case control studies of diet are based on contrasts between reports of recalled diets of people with and without cancer. A major problem with case control studies is their dependence on recall of past dietary habits, which may be inaccurate. This problem is not evident in cohort studies as subjects are asked to provide information on their present diet. In cohort studies a database of current diets is established; because this assessment of diet is made before diagnosis it is unbiased by the cancer experience. However these studies are limited because they only cover part of the life span. Due to

the presumed long latent period for some types of cancer, such as breast cancer, assessment of dietary fat intake during adolescence, when initiation may have occurred may be more important than assessment in later life (Hill, 1995).

1.9.2.4 Trans fatty acids

A comparison of the effects of *cis* and *trans* fatty acid diets on the growth and metastasis of mammary tumour cells in female BALB/c mice showed no differences in either the length of the latency period, tumour growth or the final tumour size at the local transplantation site (Erickson *et al.,* 1984). More viable tumour cells were found in the liver and spleen of mice fed the *cis* diet compared with those fed the *trans* fat diet. Thus, the *trans* fatty acid diet appeared to behave similarly to the *cis* fatty acid diet regarding growth of the primary mammary tumour. However, *trans* fatty acids might be less effective than *cis* fatty acids in promoting blood borne homing and survival of tumour cells at distant organ sites (Ip *et al.,* 1997).

A study in which DMBA-treated rats were fed either a diet enriched in *trans* fatty acids (35 % *trans* monoene and 22.5 % *cis* monoene), or a non-hydrogenated fat diet (containing 55 % *cis* monoene) also showed a lower tumour incidence in the *trans*-fatty acid fed group (Selenkas *et al.,* 1984). Using the same experimental model rats fed hydrogenated soybean oil had a lower incidence and yield of mammary tumours than those fed unhydrogenated soybean oil (Kritchevsky *et al.*, 1992). These studies suggest that a *trans* fatty acid diet may be less carcinogenic than a diet containing predominantly *cis* fatty acids.

Epidemiological data showing an association between high intake of *trans* fatty acids and an increased risk of breast cancer, suggests that further studies in this area are waranted (Kohlmeier and Mendez, 1997). *Trans* fatty acid levels in gluteal fat biopsy samples from 698 post menopausal incident cases of primary breast cancer and controls showed a positive association with breast cancer. Women containing the highest level of *trans* fatty acid stores had a breast cancer risk 40 % greater than women with low *trans* fatty acid stores (Kohlmeier and Mendez, 1997).

1.9.3 Milk fat and cancer

Most of the studies reviewing the effect of milk fat on carcinogenesis present problems associated with insufficient adjustment for other confounding dietary and nutrient variables such as total energy, fat, protein, fiber and vitamin A. It is difficult to clearly separate the effects of fat from those of calories, and of fat from dairy foods from other dietary fats. The presence of substantial correlations in caloric intake among dairy foods and meats complicates the assessment of individual nutrient or food effects (Jain, 1998).

Milk fat contains many anticarcinogenic components: butyric acid, ether lipids, sphingomyelin and CLA. About one third of all milk triglycerides contains one molecule of butyric acid C_4, a potent inhibitor of proliferation and inducer of differentiation and apoptosis in a wide range of neoplastic cell lines (Hague and Paraskeva 1995, Lupton, 1995, Mandal and Kumar 1996). At the molecular level, butyrate causes histone acetylation that may benefit DNA repair and suppress the expression of various

oncogenes (Parodi, 1996). Interest in butyric acid as a chemopreventive agent stems from its role in the colon where it is produced during bacterial fermentation of dietary fiber and starch. Although butyrate produced by colonic fermentation is considered important for colon cancer protection, an animal study suggests that dietary butyrate may also inhibit mammary tumourigenesis (Yianagi *et al.,* 1993). Milk fat contains small quantities (0.17 %) of ether lipids comprising of alkylglycerols, alkylglycerophospholipids and their derivatives are potent antineoplastic agents, which inhibit growth, show antimetastatic activity and induce differentiation and apoptosis in cancer cells (Berdel, 1991, Diomede *et al.*, 1993). These are present in neutral lipids and to a greater extent in the phospholipids of milk fat (Hallgren *et al.*, 1974).

In vitro studies have shown that sphingomyelin (0.1-1.0 % of milk fat), through its biologically active metabolites, ceramide and sphingosine, participates in three major antiproliferative pathways influencing carcinogenesis, namely, inhibition of cell growth (Hannun, 1994, Hannun and Linardic 1993) and induction of differentiation (Merrill *et al.,* 1993) and apoptosis (Jarvis *et al.,* 1996). Mice fed sphingomyelin after initiation with DMBA had fewer colon tumours and aberrant crypt foci (ACF) than control fed animals (Dillehay *et al.,* 1994). These results obtained with a limited supply of sphingomyelin, short feeding time (28 wk) and limited numbers of mice, offer exciting prospects for future research.

1.10 Chemopreventive action of CLA

1.10.1 in vivo studies

The tide of anticancer research involving CLA began when Ha *et al.* (1987) topically applied a synthetic mixture of CLA isomers (prepared using the method of the AOCS, 1973) to the dorsal area of mouse skin prior to initiation of cancer with the carcinogen, DMBA and its promotion with TPA. CLA inhibited the *in vivo* initiation of mouse epidermal tumours (Figure 1.11). Sixteen weeks after promotion, CLA-treated mice consistently exhibited fewer papillomas and a lower tumour incidence than control or LA-treated mice.

In the knowledge that CLA was present in a wide range of food products these researchers began to investigate how oral CLA treatment might act in other tumour models. Figure 1.12 demonstrates the design of an *in vivo* experiment showing that CLA reduced forestomach neoplasia induced by the carcinogen, benzo(a)pyrene in mice by up to 50 % (Ha *et al.,* 1990). CLA or LA was administered by gavage with olive oil twice weekly for 4 weeks. Control mice were treated with olive oil. All mice were administered 2.0 mg of benzo(a)pyrene once weekly for 4 weeks. CLA-treated mice (total dose 800 mg/mouse) developed half as many neoplasms/animal as mice in the control groups. Body weight and food intakes were not affected by CLA; therefore calorie restriction, known to reduce tumour risk (Ip *et al.*, 1985, Pariza, 1988) was not a factor in the reduction of forestomach neoplasia by CLA.

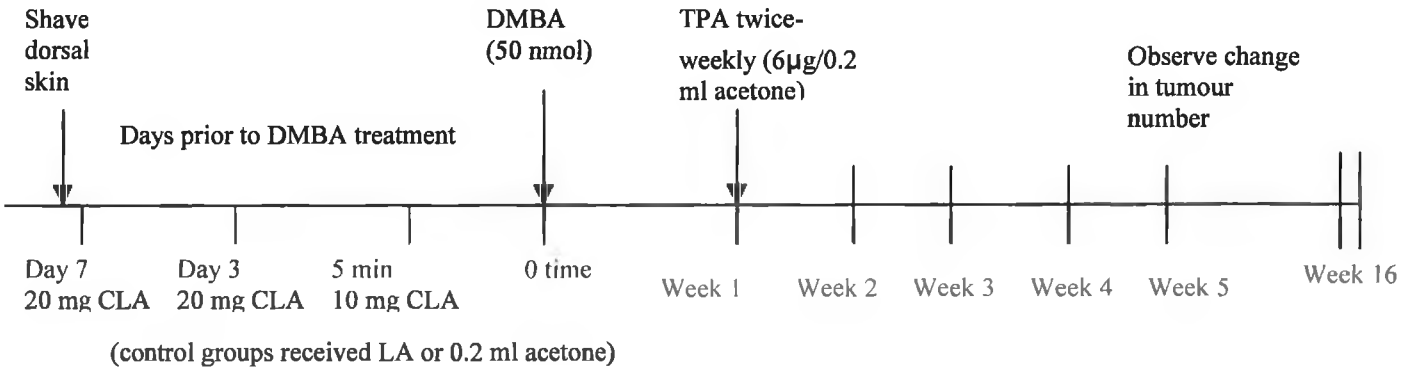


Figure 1.11 Design of 2-stage mouse skin cancer chemoprevention model (Ha *et al.*, 1987).

6 week old female CD-1 mice, 20 mice per treatment (topically applied CLA or LA) and 30 mice in the control group. DMBA : 7,12-dimethyl-benz[a]anthracene, TPA :12-O-tetradecanoylphorbal-13-acetate.

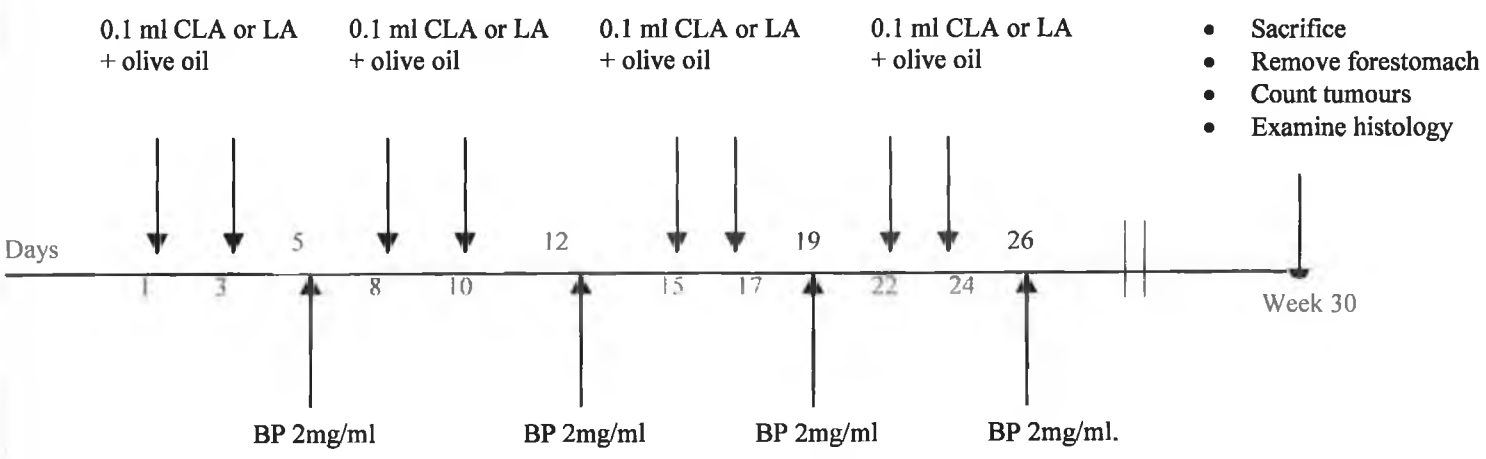


Figure 1.12 Design of mouse forestomach chemoprevention model (Ha *et al.*, 1990).

6-7 week old female ICR mice, 25 mice per treatment (0.1 ml CLA or LA + olive oil). BP: benzo(a)pyrene.

1.10.2 Mammary carcinogenesis.

Mammary cancer is usually detected when a primary tumour of malignant cells, which is clinically silent becomes a visible painless lump which the patient notices. Each breast has 15 to 20 sections called lobes, which have many smaller sections called lobules. The lobes and lobules are connected by thin tubes called ducts. The most common type of mammary cancer is found in the cells of the ducts and is called ductal cancer. Cancer that begins in the lobes or lobules is called lobular cancer. Lobular cancer is more often found in both breasts than other types of breast cancer.

The effects of CLA on the rat mammary gland are the most extensively studied of all effects on cells and tissues to date. In over ten separate studies Ip and coworkers (1999a, 1999b, 1997, 1997, 1997, 1996, 1995, 1994, 1991, 1985) have shown that dietary administration of CLA was an effective way of achieving cancer protection. CLA was effective at various doses, at various stages of carcinogenesis and for varying durations corresponding to particular stages of gland maturation, regardless of the level or type of fat in the diet. Morphological remodeling occurs during maturation of mammary gland in the rat. At birth and in the first week of postnatal life, the mammary gland is made up of a single primary duct, which branches into several secondary ducts, which end in club – shaped structures referred to as terminal epithelial buds. During the second and third weeks, additional sprouting of ducts occurs leading to a sharp increase in the number of terminal epithelial buds (Ip *et al.,* 1994). After reaching a peak at weaning (21 days of age for a rat) terminal epithelial buds begin to differentiate into alveolar buds and lobules.

After 40 days of age their population density has reached a level approximate to that of the mature gland (56 days).

Figure 1.13 shows the design of the rat mammary gland tumour model in which cancer was induced in adult female rats with a single dose of DMBA (10 mg). Feeding a basal diet supplemented with CLA (0.5, 1.0, 1.5 %) two weeks before DMBA treatment and for up to 24 weeks afterwards led to a dose responsive reduction in tumour number by 32 – 60 %. The maximum effect was observed at 1 % CLA (Ip *et al.,* 1991).

A subsequent study determined the sensitivity of dietary CLA treatment in rats treated with a lower dose of DMBA (5 mg) (Ip *et al.*, 1994). Feeding a lower dietary level of CLA (0.05 – 0.5 % of the diet) for a longer period of time (36 weeks) showed that as little as 0.1 % CLA in the diet was sufficient to cause a reduction in total tumour number (Ip *et al.*, 1994) (Figure 1.14). Total mammary tumour yield was reduced by 22, 36, 50 and 58 % in the 0.05, 0.1, 0.25 and 0.5 % CLA diets, respectively.

Studies in which timing of CLA feeding was carefully controlled have helped to delineate the role of CLA in modulating the kinetics of mammary gland development. Significant protection against tumour occurrence was offered (Table 1.6) when CLA feeding was initiated at weaning (i.e. 21 days) and limited only to 1 week after chemical carcinogen treatment (49 days) (Figure 1.15), such that it coincided with differentiation of terminal end buds and maturation of the rat mammary gland to the adult stage morphology. It is apparent from Table 1.6 that CLA exposure during mammary gland maturation caused a significant reduction in tumourigenesis in both DMBA (61 %) and MNU (65 %) treated animal models (Ip *et al.,* 1994).

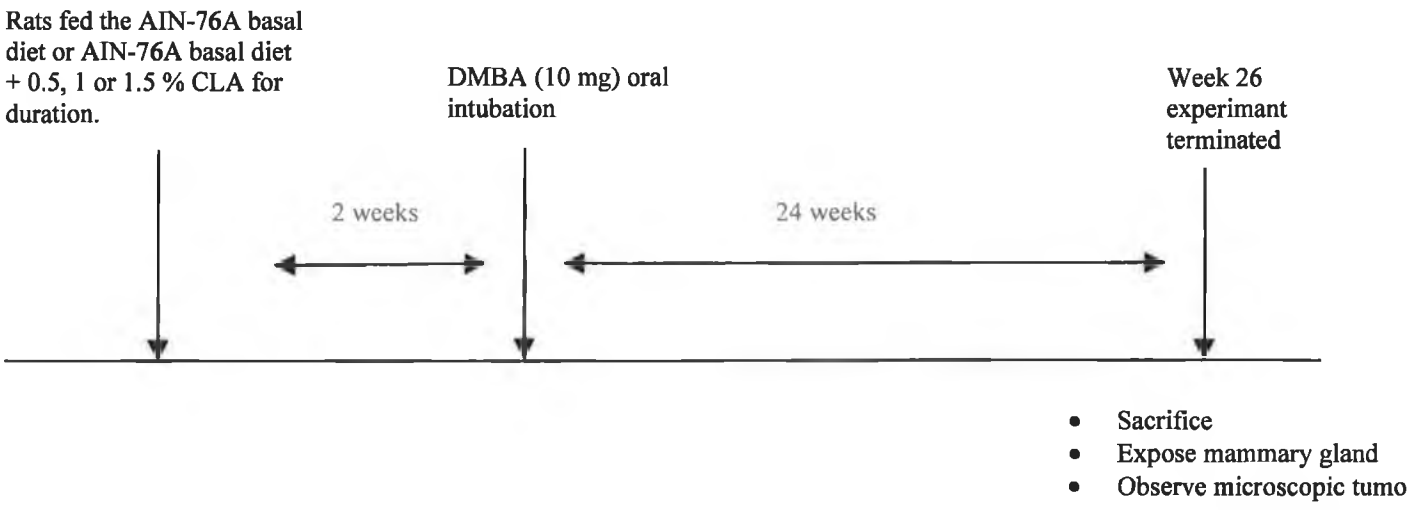


Figure 1.13 Design of rat mammary DMBA tumour model (Ip *et al.*, 1991).

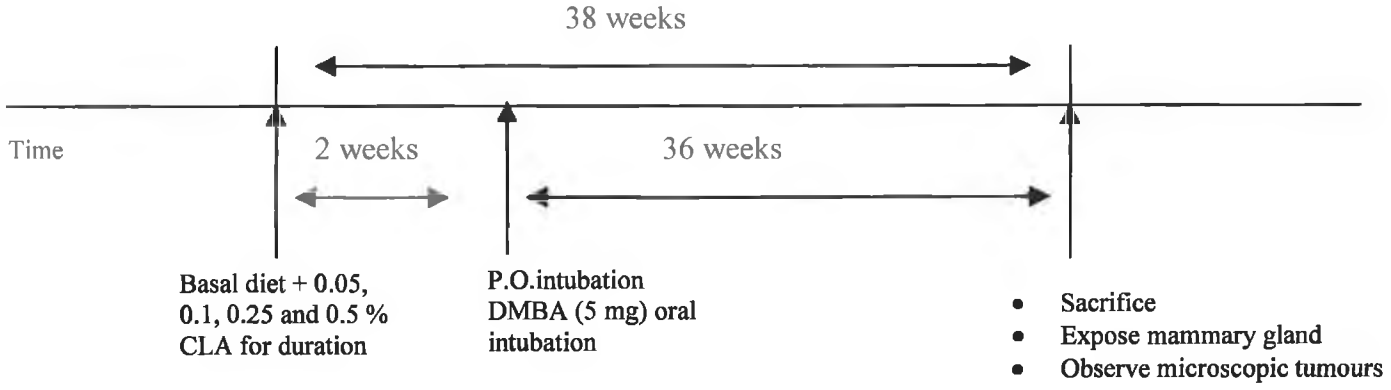


Figure 1.14 Design of rat mammary DMBA tumour model (Ip *et al.*, 1994).

Table 1.6 Mammary cancer prevention by short term feeding of CLA from weaning until 1 week post carcinogen treatment for a total period of 5 weeks.

Carcinogen	% dietary CLA supplementation	Tumour incidence	Total number of mammary tumours
DMBA (10 mg)	0	80	62
	1	52	38
MNU (6 mg)	0	88	76
	1	60	50

(Ip *et al.*, 1994).

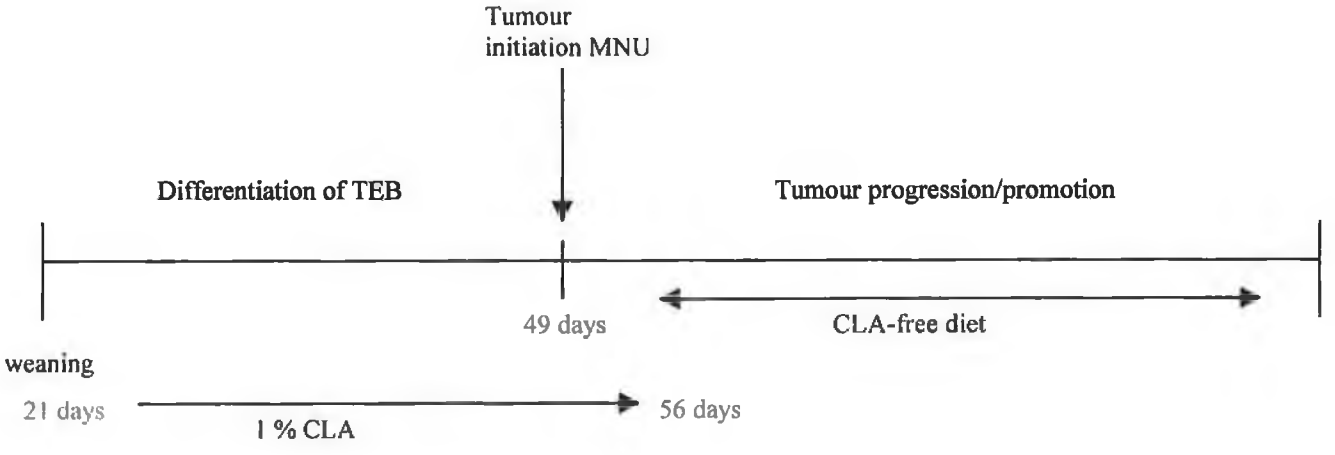


Figure 1.15 Design of rat mammary tumour model (Ip *et al.*, 1995).

When CLA was fed immediately after MNU treatment (i.e. in the post initiation phase only) for 1 or 2 months it was relatively ineffective in cancer protection. It was only when CLA was continued in the diet for 5 months that any significant inhibition occurred (Figure 1.16). This clearly showed that in the post initiation stage of mammary carcinogenesis a continuous supply of CLA was necessary for maximum tumour inhibition (Ip *et al.,* 1995).

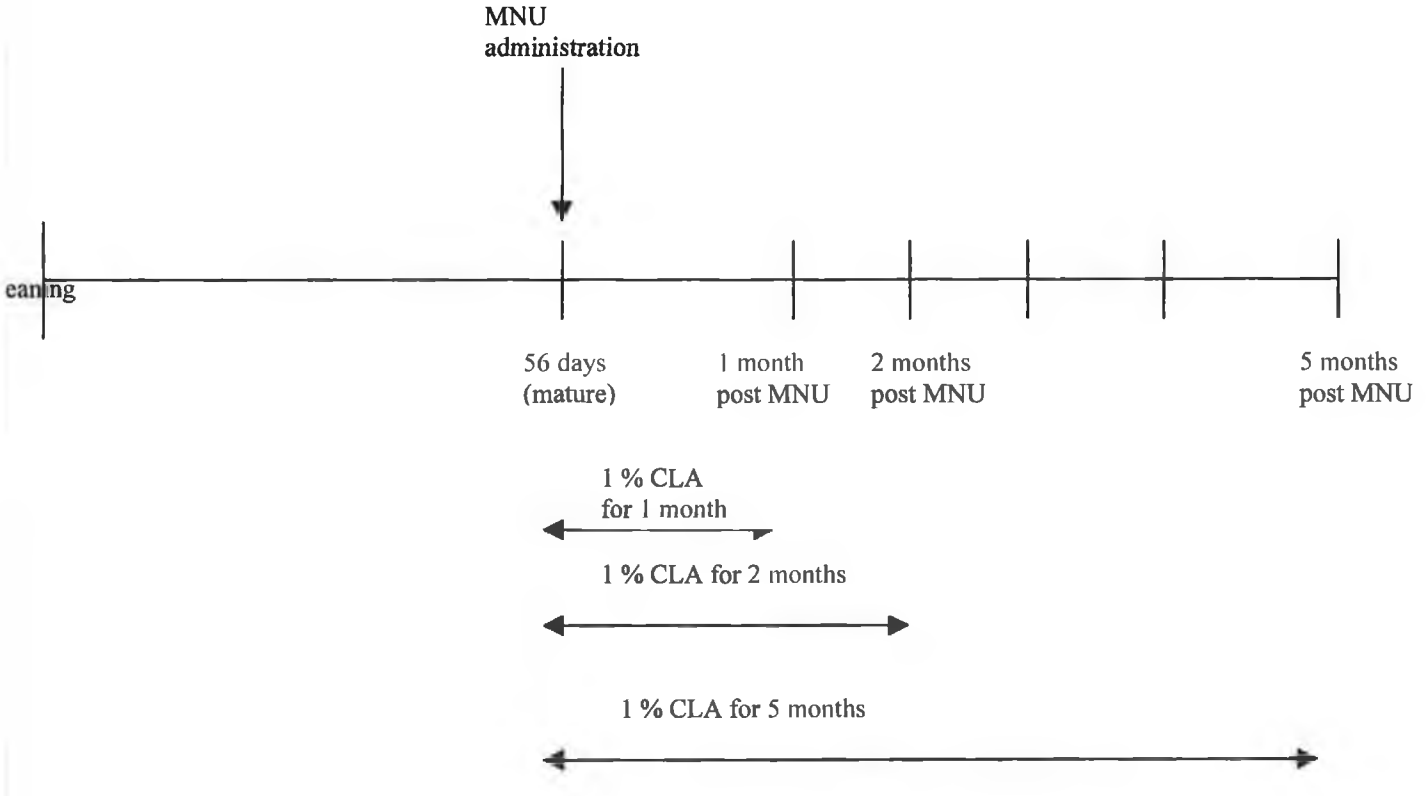


Figure 1.16 Design of mammary gland chemoprevention model (Ip *et al.,* 1995).

Subsequent work using the MNU-induced mammary gland tumour model showed that the tumour protective effect of the triglyceride form of CLA was similar to that of the free fatty acid and thus ruled out the probability of a non specific free fatty acid effect (Ip *et al.,* 1995).

The chemoprotective effect of dietary CLA present at 1 % in a basal diet containing 5 % corn oil (3 mg LA) was shown to be independent of the level or type of fat consumed (Ip *et al.,* 1996). Increasing the level of fat to 20 % by weight of the diet did not adversely affect the magnitude of tumour inhibition by 1 % CLA (Table 1.7). Switching to a predominantly saturated fat (lard) diet containing 1 % CLA did not affect the magnitude of tumour inhibition. Dose responses to CLA when the amount of corn oil was increased from 5 % to 20 % in the diet suggests that the effects of CLA were mediated via mechanisms separate and distinct from LA.

The hypothesis that CLA and LA were distinctive modulators of mammary carcinogenesis was tested by examining the dose response to CLA (at 0.5, 1.0, 1.5 and 2 %) in rats fed a 2 % or a 12 % LA diet (Ip and Scimeca, 1997). End points of investigation included measurement of tumour incidence and tumour number in addition to the incorporation of CLA, LA and AA in mammary glands.

As seen previously, the efficiency of tumour suppression by CLA was not affected by LA and no further protection was evident with CLA levels greater than 1 % of the diet.

Table 1.7 Mammary cancer prevention by CLA in rats fed different levels of fat.

Dietary fat level %	CLA %	Tumour incidence %	Total no. of tumours	Inhibition (%)
10	0	68.8	71	
10	1	40.6	31	56
13.3	0	81.3	74	
13.3	1	46.9	40	46
16.7	0	87.5	94	
16.7	1	59.4	46	51
20	0	90.6	98	
20	1	59.4	49	50

(Ip *et al.*, 1996).

GLC analysis of fatty acids in neutral and phospholipids of the mammary tissue indicated that the accumulation of CLA was dose dependent from 0.5 % to 2 % and that CLA concentration was 10 times higher in neutral lipids than in phospholipids regardless of the LA content of the diets (Table 1.8). CLA did not appear to displace either LA or AA in the mammary tissue phospholipids (Table 1.8) (Ip and Scimeca, 1997b).

A plausible explanation to address why no further protection was detected at CLA concentrations > 1 % while target tissue continued to accumulate CLA with dietary intakes > 1 % may be that CLA was converted to some active metabolite(s). A limited capacity of a biochemical pathway would explain maximisation of the protective effect at 1 % CLA, despite increasing accumulation of CLA in neutral lipids at dietary levels > 1 %. Studies by Banni *et al.* (1995) have provided evidence that desaturation and elongation of CLA may be one such pathway. Conjugated diene $C_{18:3}$ and $C_{20:3}$ were recovered in the liver of rats fed only 0.04 % CLA in the diet (Banni *et al.*, 1995).

CLA accumulated rapidly in neutral lipids following supplementation with CLA. When supplementation ceased there was a rapid disappearance of CLA from the neutral lipid with a return to basal levels (0.2 %) in 6 weeks. The rate of disappearance of neutral lipid CLA paralleled more closely the rate of occurrence of new tumours in the mammary gland. By contrast, the rate of change of phospholipid CLA during either exposure or withdrawal was slower. Maximum levels of phospholipid CLA (0.5 %) were not attained until 6 – 8 weeks. However a similar detectable amount of CLA (0.2 %) was present 6 weeks subsequent to removal of CLA from the diet (Figure 1.17). This suggests that a

continuous supply of CLA is necessary for maximum tumour inhibition in the post initiation phase of mammary carcinogenesis.

Table 1.8 Incorporation of CLA, LA and AA in neutral and phospholipids of mammary gland.

Dietary treatment		Neutral lipids			Phospholipids		
		% of total fatty acids			% of total fatty acids		
LA	**CLA**	CLA	LA	AA	CLA	LA	AA
2	**0**	0.2	14.8	0.4	0.1	12.6	23.2
	0.5	1.1	15.2	0.3	0.1	11.4	24.7
	1	2.8	14.5	0.5	0.3	13.1	21.3
	1.5	3.9	14.3	0.4	0.3	12.2	21.5
	2	5.2	13.9	0.3	0.4	11.8	22.8
12	**0**	0.2	45.1	0.6	0.1	13.0	24.6
	0.5	0.9	43.6	0.5	0.1	12.8	21.4
	1	2.7	46.5	0.7	0.3	13.5	23.9
	1.5	3.6	42.4	0.6	0.4	11.7	22.4
	2	5.0	42.7	0.5	0.4	12.4	23.3

Ip and Scimeca (1997).

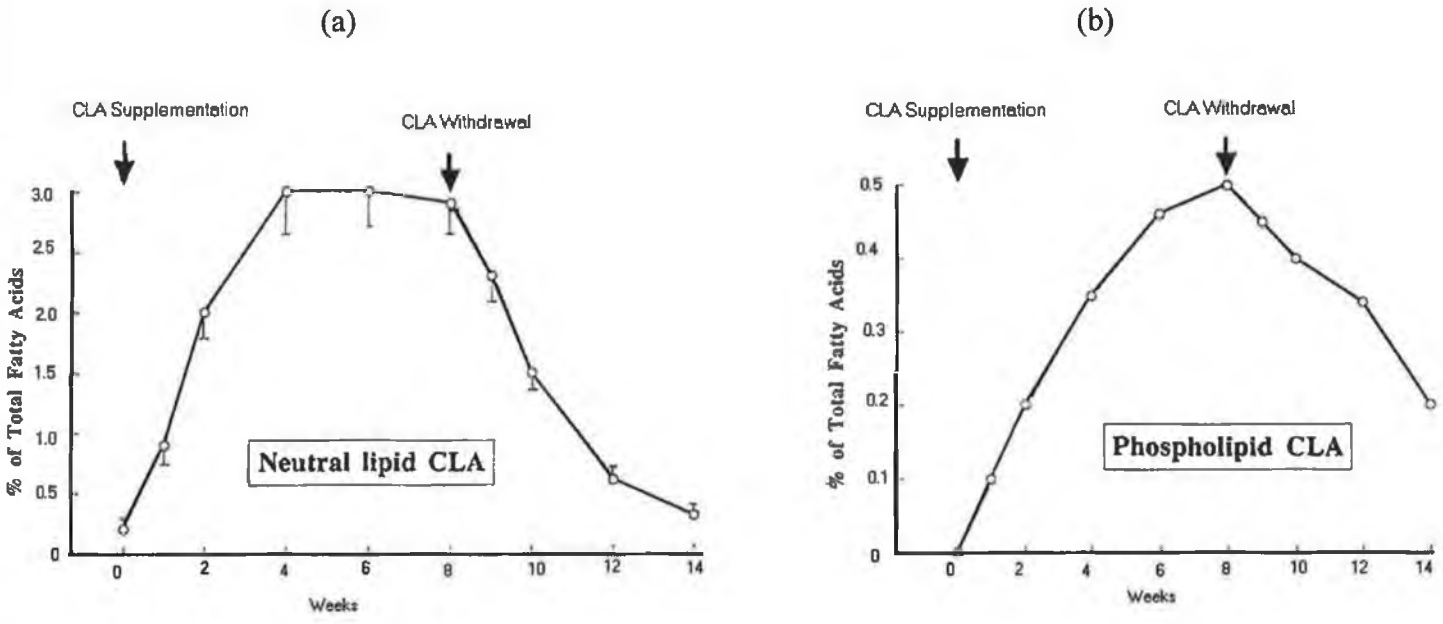


Figure 1.17 The kinetics of CLA retention in a) neutral lipids and b) phospholipids of mammary gland following CLA supplementation and withdrawal (Ip *et al.*, 1997).

Digitised image analysis of mammary gland mounts suggested that there was diminished epithelial branching as a result of CLA treatment (Thompson *et al.*, 1997). In particular, a 20 % reduction in the density of the ductal lobular tree was accompanied by a suppression of bromodeoxyuridine labeling (a thymidine analogue incorporated during DNA synthesis) in the terminal epithelial buds and lobuloalveolar buds. This change in morphology could account in part for the reduced susceptibility to chemical induction of carcinogenesis. Studies by Banni *et al.* (1999) related these morphological changes to particular biochemical profiles of CLA and LA metabolites. Specifically, there was a dose-dependent increase in desaturated and elongated products of CLA metabolism and a decrease in LA metabolites. Other biochemical changes were an increase in the vitamin A contents of mammary tissue and liver (Banni *et al.*, 1999). Retinoids related to vitamin A have become the focus of intense interest as potential anticancer agents. For example, it has been possible to obtain terminal differentiation of mouse teratocarcinoma cells *in vitro* and even *in vivo* by injecting a vitamin A analog directly into the tumour. In vitamin A treated experimental animals, inhibition has been obtained against cancers of the bladder, breast and skin (McCormick *et al.*, 1981).

Feeding butter fat (4 % CLA) to rats during the time of pubescent mammary gland development reduced mammary epithelial mass by 22 %, decreased the size of the terminal end bud population by 30 %, suppressed the proliferation of terminal end bud cells by 30 % and inhibited mammary tumour yield by 53 % (Ip *et al.*, 1999a). Furthermore, rats consuming CLA enriched butter fat consistently accumulated more

total CLA in the mammary gland and other tissues compared with those consuming free fatty acid CLA.

1.10.3 Skin carcinogenesis

Studies showing that CLA inhibited the promotion of tumours in skin in a manner that was independent of its anti-initiation activity have expanded knowledge of the role of dietary CLA in carcinogenesis (Belury *et al.,* 1996). Using the mouse skin multistage carcinogenesis model, female SENCAR mice were fed control diets during initiation and then fed diets containing various levels of CLA (0-1.5 % w/w) during skin tumour promotion with TPA (Figure 1.18). Twenty five weeks after TPA treatment was initiated, an inverse relationship was observed between tumour yield and level of dietary CLA; the average number of tumours per mouse was 39 % lower ($P < 0.05$) in mice receiving 1.5 % dietary CLA compared with mice fed the control diet (Belury *et al.,* 1996). There was a modest reduction in skin tumour incidence of approximately 15 % for mice fed the 1.5 % diet. It is apparent that the biological potency of CLA against mouse skin carcinogenesis (Belury *et al.,* 1996) was lower than had been observed in the mammary cancer model as described by Ip *et al.* (1994).

1.10.4 Colon carcinogenesis

Other evidence that CLA has anti cancer activity arose when CLA treatment reduced a number of early preneoplastic markers of carcinogenesis in rat colon (Liew *et al.*, 1995). Introduction of CLA by gavage prior to and during a 4 week exposure of rats to 2-amino-

3-methyl-imidazo[4,5-f]-quinoline (IQ) (Figure 1.19), a heterocyclic amine known to cause tumours at several sites, caused a 74 % decrease in the number of colonic aberrant crypt foci compared with control animals given IQ without any dietary treatment. In addition, CLA treatment was associated with a significant reduction in the number of IQ-DNA adducts formed in the colon as determined by ^{32}P –postlabeling analysis. These results were consistent with an earlier report (Zu and Schut, 1992) showing that CLA inhibited IQ-DNA adduct formation in liver, lungs, kidney and large intestine of CDF1 mice.

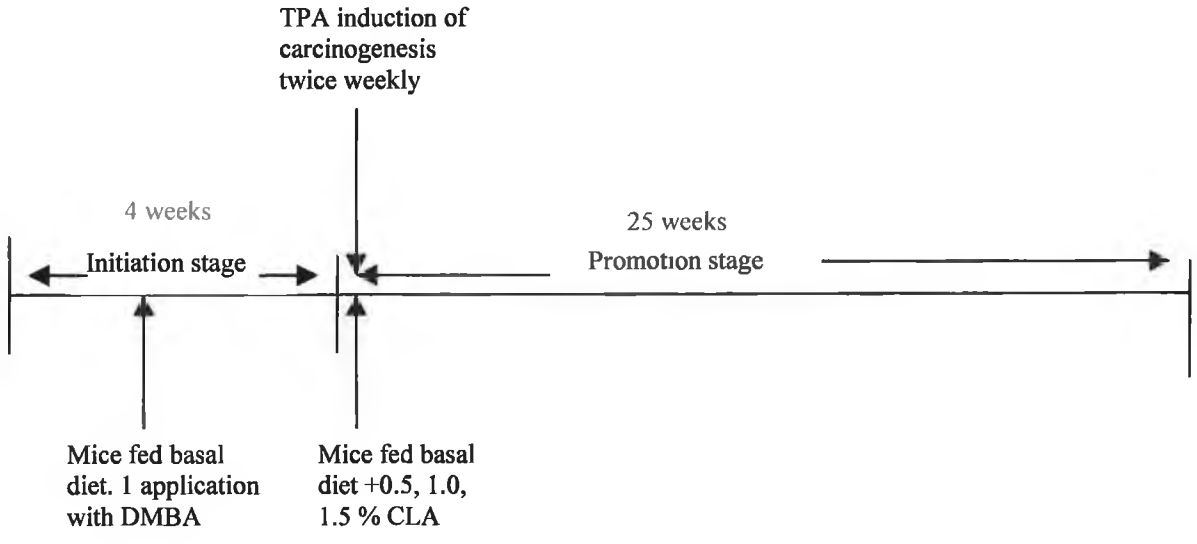


Figure 1.18 Design of 2-stage mouse skin chemoprevention model (Belury *et al.*, 1996).

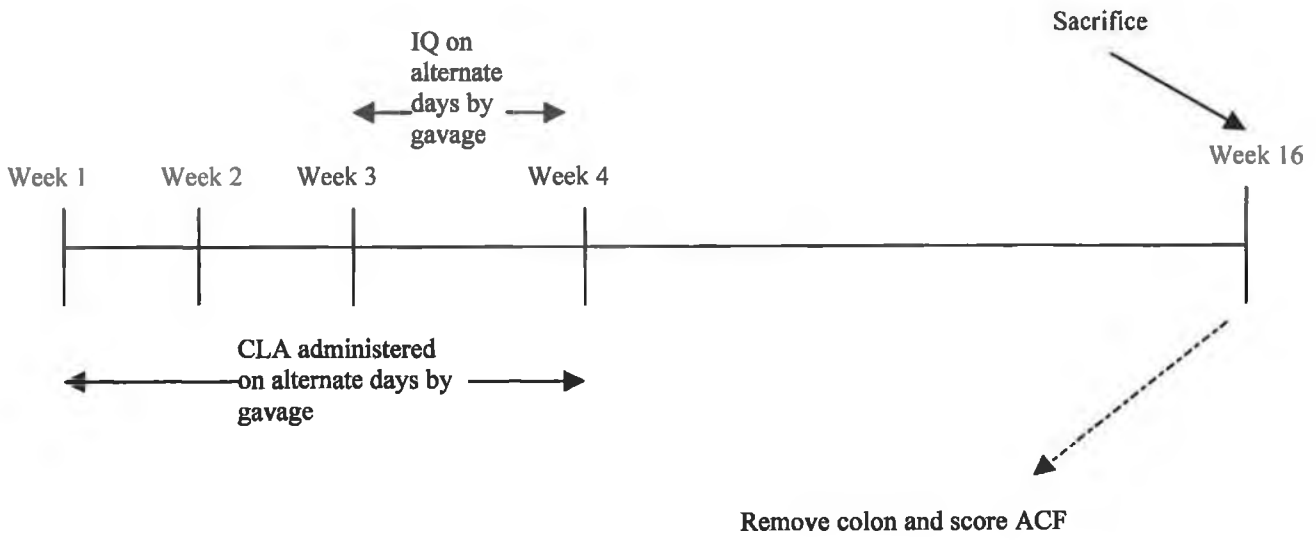


Figure 1.19 Design of rat colon tumour model (Liew *et al.*, 1995).

1.10.5 Prostate cancer

An inhibitory effect on growth of prostate tumours was reported by Cesano *et al.* (1998) in a study where DU145 human prostate cells were subcutaneously implanted into 1 % CLA-fed severe combined immunodeficient (SCID) mice. Control animals fed a non CLA diet showed tumours of ~1500 mm^3 by 12 weeks; CLA- fed mice showed significantly smaller sized tumours and reduced tumour growth.

1.10.6 Inhibition of progression (metastasis)

Mice fed 1 % CLA for two weeks prior to subcutaneous inoculation of 10^7 MDA-MB468 cells had 73 and 30 % less tumour growth at 9 and 14 weeks post inoculation, respectively (Visonneau *et al.*, 1997). Moreover, CLA completely abrogated the spread of breast cancer cells to the lungs, peripheral blood and bone marrow. These results indicate the ability of dietary CLA to block both local growth and systemic spread (metastasis) of human breast cancer (Visonneau *et al.*, 1997).

1.10.7 In vitro studies

The chemopreventive potential of CLA as seen *in vivo* has been verified in a number of *in vitro* studies wherein physiological concentrations of CLA (1.78-7.14 x 10^{-5} M) inhibited growth of various types of cultured cells especially tumour cell lines in a dose and time-dependent manner (Cunningham *et al.,* 1997, DesBordes and Lea, 1995, Durgam and Fernandes, 1997, Schonberg and Krokan, 1995, Shultz *et al.,* 1992a). A dose and time dependent decrease in cell proliferation was seen on human cancer cells

(M21-HPB, malignant melanoma; HT-29, colorectal; MCF-7, breast) incubated with CLA compared with control cultures. CLA also inhibited the growth of three human lung adenocarcinoma cell lines (A-427, SK-LU-1, A-549) in a dose and time dependent manner, but had no effect on a human glioblastoma cell line (A-172) (Schonberg and Krokan, 1995).

CLA was found to selectively inhibit proliferation of estrogen receptor positive MCF-7 cells compared with estrogen responsive negative MDA-MB-231 cells (Durgam and Fernandes, 1997). A higher percentage of the CLA treated MCF-7 cells remained in the G0/G1 phase (i.e. the resting and prereplication stage) compared to control cells or those treated with LA and thus did not progress to S (replication), G2 (post replication), and M (mitotic) stages of the cell cycle. CLA also inhibited c-*myc*, a transcriptional factor known to play a key role in biochemical pathways controlling cellular proliferation, in these cells. These authors concluded that CLA may inhibit MCF-7 cell growth by interfering with hormone regulated mitogenic pathway due to decreased expression of c-*myc*.

Both a mixture of CLA isomers and pure *c*9, *t*11 CLA inhibited the growth of rat mammary epithelial cell organoids (MEO) in primary culture (Ip *et al.,* 1999b), and this growth inhibition was mediated both by a reduction in DNA synthesis and stimulation of apoptosis. MEO number was reduced with increasing concentration of CLA (0 –128 μM). Table 1.9 demonstrates that the CLA mixture of isomers was (14 – 30 %) more potent than the individual *c*9, *t*11 CLA isomer. The effects of CLA did not appear to be

mediated by changes in epithelial PKC since neither total activity, expression nor localisation of PKC isozymes α, βII, δ, ε, η, or ξ were altered in the epithelium of CLA fed rats. In contrast adipocyte PKCs δ, ε, and η were specifically upregulated in CLA fed rats. PKC isozymes phosphorylate serine and threonine residues on a diverse group of proteins including growth factor receptor kinases associated with signal transduction. Taken together these observations demonstrate that CLA can act directly to inhibit growth and induce apoptosis of normal MEO and may thus prevent breast cancer by its ability to reduce mammary epithelial density and to inhibit the out growth of initiated MEO. The changes in mammary adipocyte PKC expression and lipid composition suggest that the adipose stroma may play an important *in vivo* role in mediating the ability of CLA to inhibit mammary carcinogenesis (Ip *et al.,* 1999b).

Table 1.9 Comparison of growth inhibitory effect of mixed isomers of CLA with pure *c*9, *t*11 CLA.

	MEO number at day 12 (x 10^{-5})	
Concentration (μM)	CLA isomers	*c*9, *t*11 CLA
0	21.0 ± 2.4	21.0 ± 2.4
16	17.5 ± 1.2	20.2 ± 0.2
32	15.5 ± 0.5	19.8 ± 2.1
64	11.8 ± 0.4	17.1 ± 1.4
128	5.9 ± 3.4	8.5 ± 0.7

(Ip *et al.,* 1999b).

1.11 Mechanistic studies

CLA has been associated with a variety of biological events that are involved in three broad stages of carcinogenesis, initiation, promotion and progression. A number of studies have investigated the mechanism of action of CLA. This is an evolving area of research that has identified to date a few pieces of the puzzle, although the picture is far from complete. In the following sections the various biological events will be treated separately as it is as yet impossible to present a unified mechanistic theory.

1.11.1 Oxidative effect

As described in section 1.9.1 antioxidant vitamins and enzymes that protect against damaging effects of free radicals are important aspects of chemoprevention. An investigation of the potential antioxidant activity of CLA arose following the discovery that CLA reduced iron thiocyanate-induced peroxide and thiobarbituric reactive substance (TBARS) formation *in vitro* (Ha *et al.,* 1990). TBARS is a marker of lipid peroxidation. This led to the initial assumption that CLA may prevent carcinogenesis by its ability to act as an antioxidant and scavenge free radicals generated from carcinogen exposure. Ip *et al.* (1991) reported that feeding CLA resulted in lower levels of malondialdehyde (MDA) in the mammary gland, an end product of lipid peroxidation. However, this activity did not correlate with dietary concentrations of CLA producing maximum suppression of tumourigenesis. CLA also failed to change the levels of 8-hydroxyguanosine, a marker of oxidatively damaged DNA in mammary tissue. Taken

together, the available evidence suggests that antioxidant mechanisms do not account for the inhibitory activity of CLA.

Many studies have since reinvestigated the antioxidant potential of CLA. Experiments employing synthetic 1-palmitoyl-2-linoleoyl phosphatidylcholine (PLPC) membrane vesicles and precise analytical methods have shown that CLA (1-50 μM / 0.28-14.08 ppm) did not have a clear protective effect under a variety of experimental oxidative stress conditions (Van den Berg *et al.*, 1995). Chen *et al.* (1997) showed that two forms of CLA, the free fatty acid form and its methyl ester form induced oxidation of heated canola oil in a dose dependent manner. Triglyceride bound CLA had no protective effect against oxidation in the same model (Chen *et al.*, 1997). Analysis of conjugated diene hydroperoxy fatty acids using HPLC with a diode-array detector showed that conjugated diene fatty acids were more susceptible to oxidation than their parent non-conjugated fatty acids (Banni *et al.*, 1998). Another study has demonstrated that CLA may be oxidised by singlet oxygen yielding furan fatty acids upon decomposition (Yurawecz *et al.*, 1995). A significant increase in lipid peroxidation as measured by MDA was observed after treatment of three lung adenocarcinoma cell lines (A-427, SK-LU-1, A-549) with CLA (Schonberg and Krokan, 1995). LA did not exert the prooxidant effect shown by CLA (Schonberg and Krokan, 1995). Hence oxidation of CLA may play an important role in influencing cancer cell death.

1.11.2 Anti-mutagenic mechanism

Inhibition of IQ activation was proposed as the mechanism of chemoprevention by CLA in colon tissue (Liew *et al.,* 1995). IQ must be activated to an aryl nitrenium ion by various enzymes which include cytochrome P4501A2, prostaglandin H synthase, acetyltransferase and sulfotransferase before it reacts with guanine residues of DNA forming an adduct at the C-8 position leading to tumour production. Liew *et al.* (1995) showed that hepatic microsomes from CLA-treated rats exhibited lower methoxyresorufin O-deethylase (MROD) activity, an enzyme indicative of cytochrome P4501A2. Furthermore, their demonstration that CLA was antimutagenic in the presence of ram seminal vesicle microsomes, a rich source of prostaglandin H synthase (enzyme responsible for the production of prostaglandin H) is additional evidence that CLA might also inhibit prostaglandin-mediated activation of heterocyclic amines in extrahepatic tissues.

1.11.3 Modulation of eicosanoid production

Given the well established association between n-6 PUFA, of which LA is a representative, and promotion of carcinogenesis together with the requirement of LA for the metabolic pathways of eicosanoid biosynthesis, an obvious avenue to investigate a possible mechanism for the anticarcinogenic effect of CLA is the determination of its effect on eicosanoid production. Tables 1.8 and 1.10 present data that shows incorporation of CLA into membrane phospholipids and neutral lipids in a number of tissues (Belury and Kempa-Steczko, 1997, Ip and Scimeca, 1997). It is therefore plausible that CLA through its incorporation into membrane phospholipids may modulate

the fatty acid composition of membranes and subsequently alter eicosanoid synthesis (Liu and Belury, 1998).

Table 1.10 Fatty acid composition in neutral and phospholipids of mice as a result of a CLA diet (0-1.5 % CLA). Values are expressed as percentages of total fatty acids.

Fatty acids	**Neutral lipids**				**Phospholipids**			
	g/100g FAME				**g/100g FAME**			
% dietary CLA	0	0.05	1	1.5	0	0.05	1	1.5
$C_{18:1}$;9	42.85	44.28	44.50	47.63	22.03	20.48	20.09	21.95
$C_{18:1}$;11	4.82	4.56	4.45	4.96	3.86	3.44	3.62	3.26
$C_{18:2}$;9,12	10.63	9.55	8.20	5.97	11.94	11.32	10.64	8.59
$C_{18:2}$;9,11 CLA	0.06	0.47	0.67	0.65	0	0.13	0.28	0.38
$C_{18:2}$;10,12 CLA	0	0.17	0.35	0.38	0	0.14	0.20	0.10
$C_{20:4}$	4.29	4.58	3.02	2.87	20.15	22.08	21.72	19.14

(Belury and Van den Heuval, 1997)

A murine epidermal cell line (HEL 30) was used to show that CLA did influence the cellular content of AA. Pre-treatment of cells with CLA reduced by 50 % ($p < 0.05$) the AA content compared with pre-treatment with LA (Liu and Belury, 1997). Levels of cellular AA decreased as increasing amounts of CLA were added to cells. Additionally, CLA decreased both uptake of ^{14}C AA into cellular phosphatidycholine and the release of ^{14}C AA compared with LA. TPA-induced ^{14}C PGE_2 production was also significantly reduced in cultures pre-treated with CLA compared with equimolar concentrations of LA (Liu and Belury, 1997). CLA was incorporated into phospholipids and neutral lipids in a dose dependent fashion in an *in vivo* study in which mice were fed increasing amounts of CLA (0-1.5 %) in the diet (Kavanaugh *et al.,* 1999). PGE_2 synthesis was 50 % lower in mice fed 1.5 % CLA compared with mice fed a control diet (Kavanaugh *et al.,* 1999). These data suggest that the chemoprotective effect of CLA in skin carcinogenesis is mediated by a change in the composition of the lipid pool of the plasma membrane which alters the availability of AA for eicosanoid synthesis during tumour promotion. The involvement of the eicosanoid pathway in the growth modulatory influence of CLA and LA has also been demonstrated in human cancer cells (Cunningham *et al.,* 1997). LA stimulated growth and [^3H]-thymidine incorporation into normal human mammary cells (HMEC) and MCF-7 cells, while CLA was inhibitory. Incubation with LA and eicosanoid inhibitors (indomethacin; INDO or nordihydroguaiaretic acid; NDGA), resulted in growth inhibition in both cell lines. Incubation of normal HMEC with CLA and eicosanoid inhibitors stimulated growth. Incubation of MCF-7 cells with CLA and an inhibitor of the leukotriene pathway caused growth suppression suggesting that CLA

effects were mediated through inhibition of leukotriene synthesis (Cunningham *et al.*, 1997).

CLA was shown to be a substrate for liver microsomal Δ6 desaturase, an enzyme that catalyses conversion of LA to AA, in an *in vitro* model (Belury and Kempa-Steczko (1997). This suggests that CLA may compete with LA for this enzyme and thereby ultimately reduce levels of AA that would be incorporated into tissue phospholipids. Uptake of CLA into liver lipids was associated with an alteration in fatty acid composition: amounts of $C_{18:1}$ were increased at the expense of LA and AA. In support of a hypothesis that CLA may exert a general chemoprotective effect via a mechanism that modulates eicosanoid metabolism was the finding that the CLA-dose response effect on cancer protection in the mammary gland corresponded with dose-dependent increases in conjugated CLA metabolites in mammary tissue and dose-dependent decreases in LA metabolites (Banni *et al.*, 1999). A number of conjugated C_{20} metabolites of CLA have recently been identified in tissues from rats fed increasing amounts of Nu Chek CLA in the range 0.5 to 2 % (Banni *et al.*, 1995, 1999, Banni and Contini, 1996, Sebedio *et al.*, 1997). Conjugated metabolites included *c*8, *t*12, *c*14 $C_{20:3}$ and *c*5, *c*8, *t*12, *c*14 $C_{20:4}$ both of which undoubtedly arose from the *t*10, *c*12 CLA isomer and *c*5, *c*8, *c*11, *t*13 $C_{20:4}$ which arose from the *c*9, *t*11 isomer (Sebedio *et al.*, 1997).

1.11.4 Peroxisomal-proliferator actived receptor- mediated effects

It was hypothesised that the widespread chemoprotective actions of dietary CLA in extrahepatic tissues may be dependent upon its role in modulating lipid metabolism in

liver (Belury and Kempa-Steczko, 1997). Feeding 1.5 % CLA in the diet caused a decrease in body weight and an enhancement of lipid accumulation in livers of female SENCAR mice. Additional findings also suggest that CLA alters lipid homeostasis. Diets containing 1.0 % and 1.5 % CLA were associated with the induction of genes encoding acyl Co A oxidase, the first and rate limiting enzyme in peroxisomal β-oxidation, liver fatty acid binding protein and cytochrome P450IVA1, a lipid metabolising enzyme involved in hydroxylation of fatty acids (Belury and Van den Heuvel, 1997). Diets containing 0.5 %, 1.0 % and 1.5 % CLA were associated with approximately six to nine-fold increases in hepatic acyl Co A oxidase mRNA, compared with mRNA levels in livers from mice fed the control diet (0 % CLA). Quantification of acyl Co A oxidase protein by Western blot analysis revealed that the hepatic acyl Co A oxidase protein of mice fed 0.5 %, 1.0 % and 1.5 % CLA were 2.5 to 3.0 times the level of acyl Co A oxidase protein of mice fed the control diet (0 % CLA). All of these proteins and enzymes are regulated to some extent by a PPAR, an intracellular receptor that belongs to the steroid hormone receptor superfamily. Because several isomers of CLA are known to be high affinity ligands and activators of PPARα (Moya-Camarena *et al.,* 1999b) it is believed that CLA isomers (in the order of *c*9, *t*11 > *t*10, *c*12 > *t*9, *t*11) exert a modulatory influence on lipid metabolism and liver cell growth through activation of the PPARα receptor subtype predominantly expressed in liver. Once bound, the PPAR-CLA complex is expected to form a heterodimer with another nuclear hormone receptor, a 9-*cis*-retinoic acid receptor (RXR) and the PPAR-RXR heterodimer interacts with specific DNA response elements known as peroxisomal proliferator response elements (PPREs) located upstream of responsive genes (Moya-Camarena *et al.,* 1999b).

Such genes include those encoding lipid metabolising enzymes mentioned already and several growth regulatory genes (i.e. *c-myc*, *c-jun* and *COX 2*).

These PPAR-mediated effects of CLA on lipid metabolism and gene expression appear however to be species specific. Incremental levels of CLA were associated with enhanced hepatic ornithine decarboxylase activity, a marker of tumour promotion and cell proliferation in mice (Belury *et al.*, 1997). Although CLA also activated PPAR α and β subtypes in Sprague Dawley rats, it did not induce hepatic peroxisome proliferation (Moya-Camarena *et al.*, 1999a). In three strains of rats studied (Sprague Dawley, lean and obese Zucker diabetic and Fisher 344 rats) dietary CLA did not have a significant effect on the content of lipid in liver (Moya-Camarena *et al.*, 1999a). By contrast in two strains of mice (CD-1 and SENCAR) 1.5 % CLA increased the lipid content in liver by approximately 2-3 fold in both strains. Species-specific differences in absorption, metabolism, optimal dose as well as sex of the animal are potential factors that may affect the ability of a PPAR ligand such as CLA to exert biological responses (Moya-Camarena *et al.*, 1999a).

The alteration of lipid homeostasis, being much greater in mice than in rats is therefore not a unifying hypothesis to explain the ubiquitous widespread chemoprotective actions of dietary CLA in extrahepatic tissues in rodent models. Studies on the short and long term effects of consuming CLA on lipid metabolism in humans are warranted to determine if CLA decreases the risk of cancer in extrahepatic tissues at the expense of

increased risk for liver cancer. Table 1.11 is a summary of the reported effects to date of CLA on events associated with carcinogenesis.

1.12 Effect of CLA on atherosclerosis

Atherosclerosis, an underlying cause of myocardial infarction, stroke and other cardiovascular diseases is responsible for 50 % of all mortality in Europe, USA and Japan (Ross, 1993). The earliest recognisable lesion of atherosclerosis is the so-called 'fatty streak', an aggregation of lipid-rich macrophages, T lymphocytes and lipoproteins within the innermost layer of the artery wall, the intima. Much evidence has accumulated to suggest that oxidised low density lipoprotein (oxLDL) is a key component in endothelial cell injury (Ross, 1993). Once formed by the endothelium, oxLDL may directly injure the endothelium and play an initial role in the increased adherence and migration of monocytes and T lymphocytes into the subendothelial space. It can induce formation of adhesive cell glycoproteins, such as vascular cell adhesion molecules by the endothelium. During the subsequent progression of the lesion, macrophages are transformed into lipid-laden foam cells, presumably by uptake of ox LDL and smooth muscle cells migrate into the lesion to form a fibrous cap around the lipid rich core (Ross, 1993). By projecting into the arterial lumen these fibrous caps may impede the flow of blood. Studies showing that vitamin E and other lipophilic antioxidants e.g. probucol inhibited lipoprotein oxidation as well as the macrophage component of atherosclerotic lesions indicate that antiatherogenic effects of antioxidants may in part be mediated by interference with oxidation-dependent expression of adherence molecules, cytokines and chemotactic factors (Fruebis *et al.,* 1999, O'Brien *et al.,* 1991, Reaven *et al.,* 1993).

Table 1.11 CLA effect on events associated with carcinogenesis.

Event	Species/cell type	Experimental model	Route of administration	Major finding
Tumourigenesis	Mouse	DMBA skin tumours	Dermal	Inhibition of incidence and multiplicity
	Mouse	BP forestomach tumours	Gavage	Inhibition of incidence and multiplicity
	Rat	DMBA breast tumours	Diet	Dose response Inhibition of incidence and multiplicity
	Rat	AOM intestinal tumours	Diet	Inhibition w/short term feeding
	Rat	DMBA and MNU breast tumours	Diet	No effect
Promotion biomarker	Mouse	Forestomach ODC	Gavage	Inhibition of TPA-induced ODC activity
Mitogenesis	Rat	BrdU labelling of mammary gland	Diet	Inhibition of lobulo-alveolar proliferation
	M21-HPB HT-29 MCF-7	Cell growth curves	In vitro	Cytostatic and cytotoxic effects at micromolar concentrations
Mutagenesis	Salmonella	Ames assay	Gavage	CLA incorporated into S-9 inhibits IQ activation, but not BP or DMBA activation
Carcinogen activation & detoxification	Mouse	IQ-DNA adducts	Gavage	Inhibition in certain target and non target organs
	Rat	Mammary and liver phase II enzymes	Diet	No effect on liver and mammary GST
Signal Transduction	Mouse	Forestomach extracts	Gavage	PKC like activity refractory to activation
	3T3	Phospholipid	In vitro	Modulation of TPA-stimulated phospholipase C activity
Antioxidant capacity	Rat	Ex vivo TBARS	Diet	Inhibition in mammary gland but not in liver
Eicosanoid modulation	Mouse	TPA skin tumours	Diet	Decreased PGE_2 synthesis
Expression of genes for lipid metabolism	Mouse	Liver tissue	Diet	Activation of PPARα

Adapted from Scimeca *et al.*, 1994.

A small number of studies have demonstrated that CLA reduces aortic plaque formation in experimental animals (Lee *et al.*, 1994, Nicolosi *et al.,* 1997). Lee *et al.* (1994) reported that dietary CLA (0.5 g CLA/ rabbit/ day) fed in conjunction with an atherogenic diet (containing 0.1 % cholesterol for 22 weeks) reduced the severity of atherosclerotic lesions developing in the aorta of rabbits. Figure 1.20 shows that the ratio of LDL cholesterol to HDL (high density lipoprotein) cholesterol was significantly reduced in CLA fed rabbits compared with the control group (Lee *et al.*, 1994). The percentage of total aortic surface covered by fatty lesions was significantly lower in the CLA-fed group (43 ± 11 %) compared with the control group (55 ± 14 %). Other histological evidence of a less severe form of atherogenesis was the reduction in the plaque to wall volume ratio and a 30 % reduction in cholesterol deposition in the CLA-fed group.

Nicolosi *et al.* (1997) reported that dietary CLA (0.025-0.5 %) reduced fatty streak area in arteries of hamsters fed an atherogenic diet. Total plasma cholesterol, non-HDL cholesterol and triglyceride levels were significantly reduced in these animals with no effect on HDL cholesterol compared to controls. Plasma tocopherol /total cholesterol ratios were markedly higher in CLA-fed animals (48-86 %) compared with LA-fed animals (29 %) (Table 1.12) suggesting that CLA treatment caused a tocopherol sparing effect. However, the antiatherosclerotic effects of CLA in rabbits were not associated with a reduction in TBARS (Lee *et al.,* 1994).

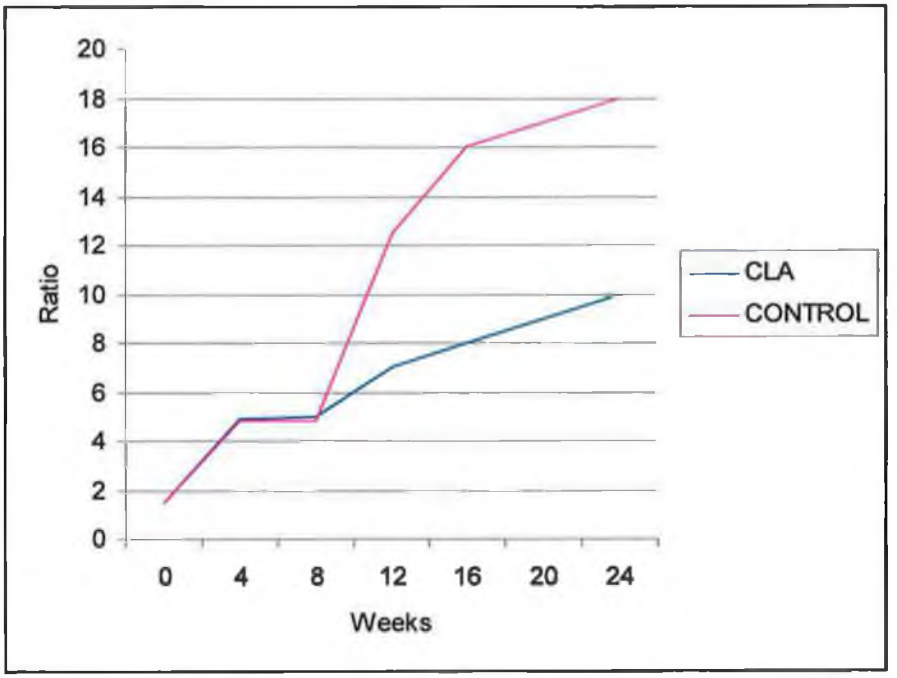


Figure 1.20 LDL cholesterol /HDL cholesterol ratio in rabbits fed experimental diet with or without 0.5 g CLA/ rabbit/ day (Lee *et al.*, 1994).

Table 1.12 Effect of dietary CLA and LA on plasma lipids, plasma tocopherol and aortic fatty streak area in hamsters.

	Control	Low CLA	Med CLA	High CLA	LA
Total Cholesterol (mmol/L)	17.85 ± 0.62	13.19 ± 2.56*	14.12 ± 1.06*	13.71 ± 1.56*	15.25 ± 1.09
Non HDL-C (mmol/L)	16.5 ± 0.6	12.08 ± 2.38*	12.73 ± 1.14*	12.39 ± 1.56*	13.91 ± 1.08
HDL-C (mmol/L)	1.35 ± 0.06	1.3 ± 0.11	1.4 ± 0.13	1.32 ± 0.08	1.34 ±0.16
Triglycerides (mmol/L)	12.41 ± 2.4	8.97 ± 2.35*	7.93 ± 2*	11.33 ± 1.79	8.93 ± 2.25*
α-tocopherol (mmol/L)	0.036 ± 0.001	0.0359 ± 0.004	0.04 ± 0.004	0.051 ± 0.005	0.0379 ± 0.004
Fatty streak area ($\mu m^2/mm^2$ x 100)	53 ± 14	43 ± 18	39 ± 11	37 ± 15	40 ± 18

(Nicolosi *et al.*, 1997)
*significantly different from control ($p < 0.05$)

Clearly, further studies are warranted to determine if CLA inhibits early aortic atherosclerosis via specific inhibition of LDL oxidation and/or via reduction of the macrophage component of the lesions. Although the evidence indicates that dietary CLA can modify plasma lipids and lipoprotein cholesterol levels, the mechanism for this action remains to be elucidated.

Recently a study has shown that short term feeding of the pure *c*9, *t*11 CLA isomer (Matreya CLA), thought to be the active component in CLA did not produce the same lipid lowering effects as a mixture of CLA isomers (Nu-Chek CLA) in hamsters fed an atherogenic diet (Gavino *et al.,* 2000). In addition, the CLA-fed group had significantly lower weight gain but greater food intake than either *c*9, *t*11 CLA- or LA- fed animals. The data suggested that CLA may disrupt assimilation and/or transport of fat as lipoprotein triglyceride and that *c*9, *t*11 CLA may need to act synergistically with another CLA isomer to exert its biological effects. A clue to the identity of which isomer may be responsible for lower weight gain has come from a study by Park *et al.* (1999c) in which it was reported that CLA-induced body composition changes in mice were associated with the *t*10, *c*12 CLA isomer and not *c*9, *t*11 CLA. This isomer has also been shown to suppress triglyceride and cholesteryl ester synthesis in a human liver cell line while also reducing apolipoprotein B (apo B) secretion (Yotsumoto *et al.,* 1999). The latter is essential for the accumulation and secretion of VLDL by liver which when secreted into blood are metabolised to LDL with apo B in LDL acting as a ligand for the LDL receptor (Brown and Goldstein, 1986). Clearly, more studies are needed to investigate the effects

of other individual CLA isomers on the level of apo B in blood as this is an independent risk factor for coronary heart disease and atherosclerosis (Huff and Burnett, 1997).

1.13 Effect of CLA on immunity

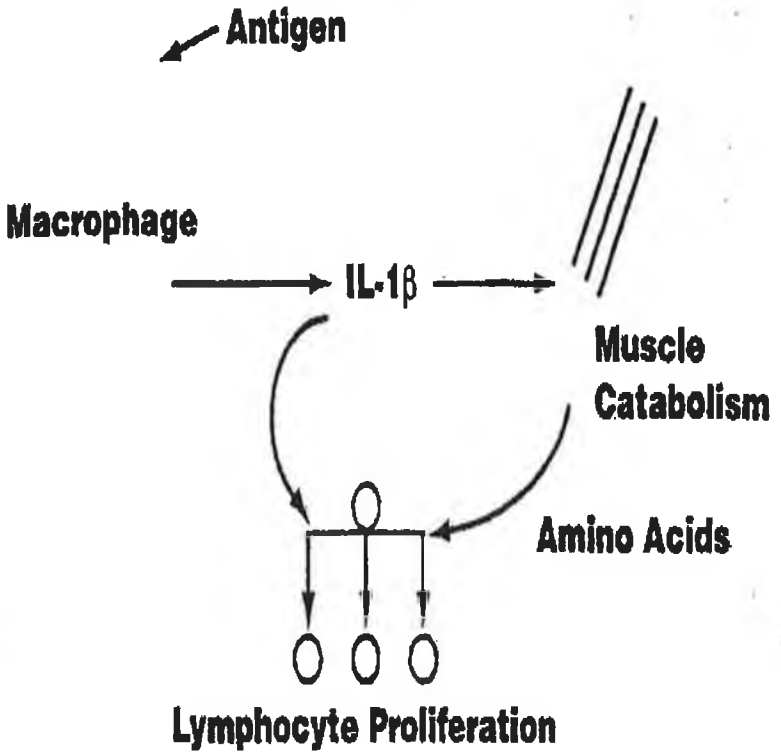
Considerable evidence exists that stimulation of the immune system either by vaccination or exposure to environmental lipopolysaccharides (LPS) partitions energy away from growth and causes catabolism of body protein stores (Klasing *et al.,* 1987). The resultant cachexia (immune-induced weight loss) is mediated by cytokines released from stimulated macrophages or other immune cells. Two cytokines of central importance in this process are interleukin 1 (IL-1) and tumour necrosis factor α (TNF). These cytokines induce antibody synthesis and an inflammatory response in immune cells and induce catabolism and changes in cell surface proteins in skeletal muscle cells. Figure 1.21a depicts the stimulatory effect of IL-1 released from antigen-stimulated macrophages on muscle catabolism and lymphocyte proliferation (Cook *et al.,* 1999).

An increase in PGE_2 production upon induction of skeletal muscle degradation by cytokines indicates a metabolic basis for immune-induced catabolism (Goldberg *et al.,* 1984). During cytokine mediated signalling events in skeletal muscle, AA is cleaved from phospholipids by the action of a phospholipase (Figure 1.21b). Upon the action of COX, AA can be converted to PGE_2 or other eicosanoids depending on the cell type and signal received. Since AA is a desaturated and elongated product of the dietary fatty acid, LA, dietary manipulation of membrane phospholipid composition and hence

eicosanoid synthesis may modulate cytokine-induced catabolism. Figure 1.21b shows a proposed mechanism for interplay of eicosanoids in immune function and muscle catabolism.

CLA was first shown to prevent immune-induced weight loss in chickens (Cook *et al.,* 1993). CLA-fed chickens had significantly less growth suppression when injected with LPS compared with control -fed chickens. Subsequent studies in mice and rats showing reduced cachexia confirmed that CLA was protective against immune-induced catabolism despite enhancement of a select immunological function (spleen lymphocyte blastogenesis). The antibody response in rats or chickens to bovine serum albumin and sheep red blood cells was not adversely affected by feeding CLA (Cook *et al.,* 1993). Other measures of immune function enhanced in CLA-fed animals include phagocytic activity of peritoneal macrophages, lymphocyte blastogenesis and proliferation in response to mitogens (Cook *et al.,* 1993, Michal *et al.,* 1992, Wong *et al.,* 1997). CLA feeding significantly raised the levels of immunoglobulins in serum such as IgA, IgM and IgG but decreased levels of IgE, particularly in rats fed 1 % CLA (Sugano *et al.,* 1998). Age-related depression of mitogen-induced splenocyte blastogenesis appeared to be slightly alleviated by feeding CLA (Hayek *et al.,* 1999). A study with the macrophage cell line RAW264.7 showing that CLA prevented LPS-induced increases in PGE_2 production is further evidence of the potential of CLA as a unique immunostimulatory and anticachectic nutrient. An elongated and desaturated product of *t*10, *c*12 CLA isomer (*c*5, *c*8 *t*12, *c*14 $C_{20:4}$) has been known to inhibit COX activity (Nugteren, 1970). This suggests that *t*10, *c*12 CLA could be the biologically relevant isomer capable of enhancing select immunological responses while protecting against adverse effects of immune cytokines (Cook *et al.,* 1999).

(a)



(b)

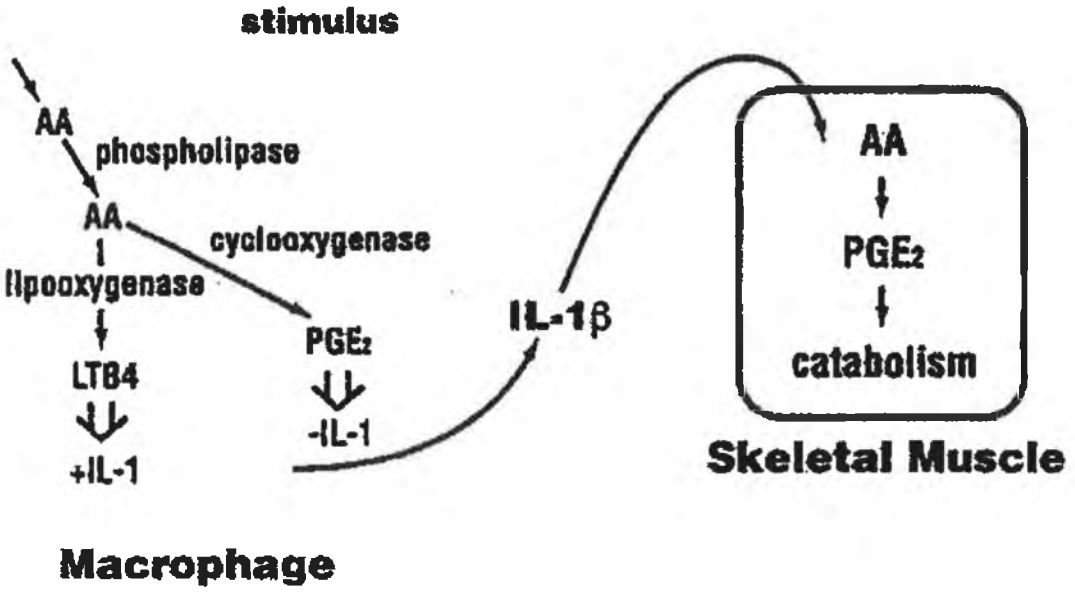


Figure 1.21 (a) IL-1 stimulates muscle catabolism, **(b)** PGE_2 induced muscle catabolism (Cook and Pariza, 1998).

1.14 Effect of CLA on growth

The growth performance of young rats was significantly improved (8.6 %) when the maternal diet was supplemented during gestation and lactation with 0.5 % CLA in the diet (Chin *et al.,* 1994). A 0.25 % dietary CLA dose was inefficient, as was a 0.5 % dose during lactation only. These observations suggest that a threshold level of CLA supplementation during foetal life is necessary for optimal growth preformance. After weaning, dietary supplementation with 0.25 % CLA (in the diet) allowed detection of a small but significant enhancement in body weight (5.6 g for male pups after 5 weeks and 7.1 g for female pups after 7 weeks) and better feed efficiency. Increasing the concentration of CLA in the supplemented diet to 0.5 % (of the diet) enhanced body weight gain and feed efficiency further.

Supplementation with 0.5 % CLA by weight of the diet for 4 to 8 weeks reduced body fat mass by 57-70 % in young mice, by 23 % in rats and by 22 % in chickens (Pariza *et al.*, 1996). Such an effect was not limited to young animals but was also noticed in adult mice fed for 4 weeks with a diet containing 20 % of lipids comprising 0.5 % CLA. The CLA group exhibited a 46 % lowering of the body fat mass and a 9 % increase in the lean body mass, leading to a decrease of body weight for a similar food intake to control group. Albright *et al.* (1996) reported that a continuous supply of CLA was required to maintain this effect. CLA withdrawal led to a 19 % decrease in whole body protein. Park *et al.* (1997) showed that feeding mice CLA (0.5 % by weight of the diet) caused a

60 % lower body fat and 14 % increase in lean body mass relative to control. This physiological effect was associated with an increase in carnitine palmitoyltransferase, a key regulatory enzyme controlling fatty acid β-oxidation in fat pad and skeketal muscle. Taken together these studies suggest that effects on body composition may be due to reduced fat deposition and increased lipolysis in adipocytes, possibly coupled with enhanced fatty acid oxidation in both muscle cells and adipocytes.

A study of the effects of high and low fat diets with and without CLA (1.2 and 1.0 % in high and low fat diets, respectively) on rodent metabolism showed that CLA significantly reduced energy intake, growth rate, adipose depot weight, and carcass lipid and protein content independent of diet composition (West *et al.*, 1998). A reduction of 43 to 88 % in adipose depot weight was observed, with the retroperitoneal (abdominal) depot being the most sensitive to CLA. CLA significantly increased metabolic rate and decreased night-time respiratory quotient (the molar ratio of CO_2 production to O_2 consumption). These findings demonstrate that CLA may reduce body fat by several mechanisms, including a reduced energy intake, increased metabolic rate and a shift in the nocturnal fuel mix (West *et al.*, 1998).

A recent study has attributed CLA-associated body compositional changes to the *t*10, *c*12 CLA isomer (Park *et al.,* 1999c). Feeding mice CLA preparations enriched in either *c*9, *t*11 or *t*10, *c*12 isomers of CLA led to reduced body fat and enhanced body water, body protein and body ash contents in *t*10, *c*12 CLA fed mice compared to controls. By contrast, the *c*9, *t*11 CLA isomer had no effect on these biochemical parameters.

1.15 Effect of CLA on bone metabolism

Long bones in children increase in length and diameter by a process called bone modeling (Watkins and Seifert, 1996). Bone modeling represents an adaptive process of generalised and continuous growth and reshaping of bone, governed by the activities of osteoblasts and osteoclasts until the adult bone structure is attained. Osteoblasts produce and mineralise bone matrix, whereas osteoclasts are responsible for bone matrix resorption (Baron, 1993). Osteoblasts have receptors for hormones (parathyroid hormone, Vitamin D and oestrogen), cytokines and growth factors which regulate differentiation, growth and metabolism of bone cells. Osteoclasts are associated with bone resorption via creation of a secondary lysosomal space for solubilisation of hydroxyapatite and digestion of matrix proteins. Abnormal bone modeling has implications in a number of adult pathologies such as osteoporosis or arthritis, which can be avoided if bone modeling is optimised early in life or if diets are supplemented with nutrients that reduce tissue concentrations of factors that undermine skeletal health (Watkins *et al.,* 1999). Recent investigations suggest that dietary lipids influence bone formation by modulating PGE_2 synthesis (Watkins *et al.,* 1996, 1997). Dietary lipids, in particular n-6 fatty acids which increase production of PGE_2 by bone, stimulated an increase in bone resorptive activity and thus reduced bone volume in chickens. By contrast, the rate of bone formation was significantly greater in chickens fed n-3 fatty acids. This increase in bone formation was associated with a 50 % decrease in AA and a 3.5 fold decrease in *ex vivo* PGE_2 production in tibia. This suggests that the role of PGE_2

on bone formation may be biphasic, stimulating bone formation at a low concentration but inhibiting it at higher concentrations.

PGE_2 has been shown to be a stimulator of IGF-1 (insulin like growth factor 1) production in osteoblast-enriched cell cultures (Baylink *et al.,* 1993). CLA like n-3 fatty acids has also been reported to lower PGE_2 production while n-6 fatty acids stimulated PGE_2 production and increased serum IGF levels in chickens and rats (Li *et al.,* 1999). Recent data suggests that inclusion of butter fat containing CLA in chicken diets can modulate PGE_2 and IGF-1 production to support higher bone formation (Watkins *et al.,* 1997).

Several reports have revealed that CLA may affect bone density (Cook *et al.,* 1997, Lee *et al.,* 1994, Watkins and Seifert, 1996). Cook *et al.* (1997) found that whole body ash content was higher in CLA-fed animals across a number of animal species suggesting that CLA enhanced bone mineralisation. Tibia from chickens fed CLA were ashed and found to have a higher dry, fat-free bone ash than control fed chickens (Cook *et al.,* 1997). This prompted a study to see if CLA would inhibit post menopausal bone loss. Adult ovariectomised rats, however, did not appear to be protected from bone loss due to oestrogen depletion. Another study showed that CLA prevented a skeletal disease known as valgus and varus leg deformity in chickens. Valgus and varus leg deformities have been related to a number of nutrient deficiencies, which have affected the poultry industry for some time (Cook *et al.,* 1997).

1.16 Effect of CLA on diabetes

Non insulin-dependent diabetes mellitus (NIDDM) is characterised by a resistance to insulin and a relative elevation of postprandial blood glucose levels (Belury and Van den Heuval, 1999). Currently available therapies for the treatment of NIDDM, e.g. exogenous insulin and sulphonylureas largely address the insulin secretary defects of this disease but do not improve the response of peripheral tissues such as adipose tissue and muscle to insulin. While the exact causes of insulin resistance are unknown, it has been speculated that insulin resistance may be mediated in part by an increase in free fatty acids (Belury and Van den Heuval, 1999). Thiazolidinedione analogs are a new class of drugs that act by improving insulin action, thereby lowering blood sugar levels in patients with diabetes. They block release of free fatty acids and reduce insulin resistance in adipose tissue via activation of the adipose enriched novel nuclear receptor PPARγ. Activation of PPARγ is a pivotal event in the adipocyte differentiation program, and is important in the transcriptional regulation of many genes that characterise the adipocyte, for example aP2 (Hollenberg *et al.,* 1997, Loftus and Lane, 1997, Torontoz *et al.,* 1994). aP2 is an adipocyte lipid-binding protein which plays a central role in facilitating the trafficking of fatty acids within adipocytes. Like thiazolidinediones, CLA has been shown to significantly increase the steady state level of aP2 mRNA in adipose tissue from fatty Zucker diabetic rats consistent with stimulation of adipocyte differentiation (Vanden Heuval *et al.,* 1994). This may be due to the ability of CLA to activate PPARγ, since increasing levels of CLA induced a dose-dependent transactivation of PPARγ in CV-1

cells cotransfected with PPARγ and a PPRE X 3-luciferase reporter construct. As CLA may share a common mechanism of action with thiazolidinediones via activation of PPARγ, a study on the effects of 1.5 % dietary CLA (by weight) on diabetic rats was undertaken. CLA normalised glucose intolerance and improved hyperinsulinema in the pre diabetic rat (Houseknecht *et al.*, 1998). The enhancing effects of CLA on glucose tolerance and glucose homeostasis indicate that dietary CLA may be an important therapy for the prevention and treatment of NIDDM (Houseknecht *et al.*, 1998).

1.17 Antithrombotic effect of CLA

The control of bleeding is a system of fine tuned checks and balances. In the formation of a thrombus (blood clot), platelets must undergo three processes; adhesion to exposed collagen in blood vessels, release of their contents and aggregation. Thrombin formed from the blood coagulation cascade interacts with its receptor on the platelet plasma membrane to initiate aggregation and release of the contents of the various types of platelet granules. Collagen-induced activation of a platelet PLA_2 by increased levels of cytosolic calcium results in liberation of AA from platelet phospholipids, leading to the formation of thromboxane A_2 (TxA_2), which in turn can further activate PLC, promoting platelet aggregation. Drugs (e.g. aspirin) that modify the behavior of platelets by inhibiting production of TxA_2 are important in management of angina, myocardial infarction and also in prevention of stroke and death in patients with transient cerebral ischemic attacks. Many studies have shown that C_{18} PUFAs inhibit human platelet aggregation caused by thrombin, collagen and the calcium ionophore A23187 (MacIntyre

et al., 1984). A recent study has shown that CLA also possesses antithrombotic properties (Truitt *et al.,* 1999). It was found that *c*9, *t*11 CLA, *t*10, *c*12 CLA and the CLA isomer mix inhibited AA- and collagen-induced platelet aggregation and that LA was a less potent inhibitor. The *c*9, *t*11 CLA and *t*10, *c*12 CLA and the mixture of isomers of CLA inhibited formation of TxA_2. No significant differences in the inhibitory potencies between the *c*9, *t*11- and the *t*10, *c*12-CLA were observed. The inhibitory effect of CLA on platelets was reversible and dependent on the time of addition of the aggregating agent.

1.18 Bacteriostatic effect of CLA

A bacteriostatic activity of CLA against *Listeria monocytogenes,* a human food-borne pathogen, that can grow even at refrigeration temperatures was demonstrated in a brain and heart infusion broth and in milk (Wang and Johnson, 1992). Although 5 to 10 fold less potent than lauric acid, monolaurin or LA, CLA as a potassium salt was bacteriocidal in brain and heart infusion broth at 50 to 200μg/ mL for up to six days. Potassium CLA was potent in inhibiting bacterial growth in whole and skim milk at 4 °C. At 25 °C, potassium CLA (300 μg/ml) prolonged the bacteriostatic lag-phase by 30h. This antibacterial activity of CLA in foodstuff requires further investigation with other microorganisms.

1.19 Analysis of conjugated linoleic acid isomers

The term CLA refers to *mixtures* of positional and geometric isomers of LA. Commercial production of CLA by the base catalysed isomerisation of LA yields a mixture of isomers (Mounts *et al.,* 1970, Sturve, 1983). The two double bonds in CLA are in positions 7 and 9, 8 and 10, 9 and 11, 10 and 12 or 11 and 13 along the carbon chain (Parodi, 1994, Yurawecz *et al.,* 1998). Each of the bonds can be in the *cis* (*c*) or *trans* (*t*) configuration. Pure samples of CLA have been prepared commercially by the dehydration of ricinoleic acid and by the microbial conversion of LA (Aldolf, 2000).

Accurate analysis of CLA mixtures is imperative as specific effects of CLA in nutritional studies may be attributed to specific isomers. The CLA found in dietary supplements and used in all, but one *in vivo* study to date has been shown to be composed of a complex mixture of positional (8, 10-, 9, 11-, 10, 12- and 11, 13) and geometric isomers (Nu Chek Prep CLA) (Ip *et al.,* 1999a). Analysis of this complex mixture by a single chromatographic method has proven difficult. GLC utilising 100 m capillary columns can separate (as fatty acid methyl esters (FAME)) the major *cis/trans* isomers but the *cis/cis* isomers overlap and the 8, 10 through 10, 12 *trans/trans* isomers remain unresolved. Separation of 12 positional and geometric isomers of CLA as their methyl esters is possible using Ag^+ HPLC with 0.1 % acetonitrile in hexane as the mobile phase, operated isocratically and using UV detection at 233nm. Figure 1.22 shows a separation using one Ag^+ HPLC column. The mixture is dominated by four *trans/trans, cis/trans*

and *cis/cis* positional isomers as follows: 8, 10-, 9, 11-, 10, 12- and 11, 13-$C_{18:2}$. Geometric pairs could not be separated with the use of only one column, however resolution was improved when two or more Ag^+ HPLC columns were used in series (Figure 1.23). The identities of the CLA isomers can be determined by gas chromatography- electron impact mass spectrometry (GC-EIMS) of their 4,4-dimethyloxazoline (DMOX) derivatives. This method was successfully used to separate and identify numerous minor new CLA isomers (*t*7, *c*9, *c*8, *t*10 and *c*7, *t*9) in milk, cheese, beef, human milk and adipose tissue (Rickert *et al.,* 1999, Sehat *et al.,* 1998, Yurawecz *et al.,* 1998).

GLC and Ag^+ HPLC are complementary techniques in the total analysis of CLA isomers. The analysis of CLA isomers by GLC and Ag^+ HPLC requires their conversion to derivatives that can be separated from other fatty acids by using GLC. The procedure for conversion of CLA to methyl ester derivatives (methylation) depends on the type and chemical composition of the material to be methylated. In early studies, acid catalysed methanolysis was used at high temperatures (100 °C) which resulted in excessive formation of *trans/trans* CLA isomers (Ha *et al.*, 1990, Ip *et al.*, 1991), however this isomerisation was greatly reduced by lowering the temperature and duration of methylation. A 4 % (v/v) HCL-catalysed methylation for 20 min at 60 °C resulted in only ~ 5 % *trans/trans* isomerised CLA product (Chin *et al.,* 1992). Under these conditions test samples consisting mainly of triglycerides (such as milk fat) were completely methylated but phospholipids were not (Kramer *et al.*, 1997).

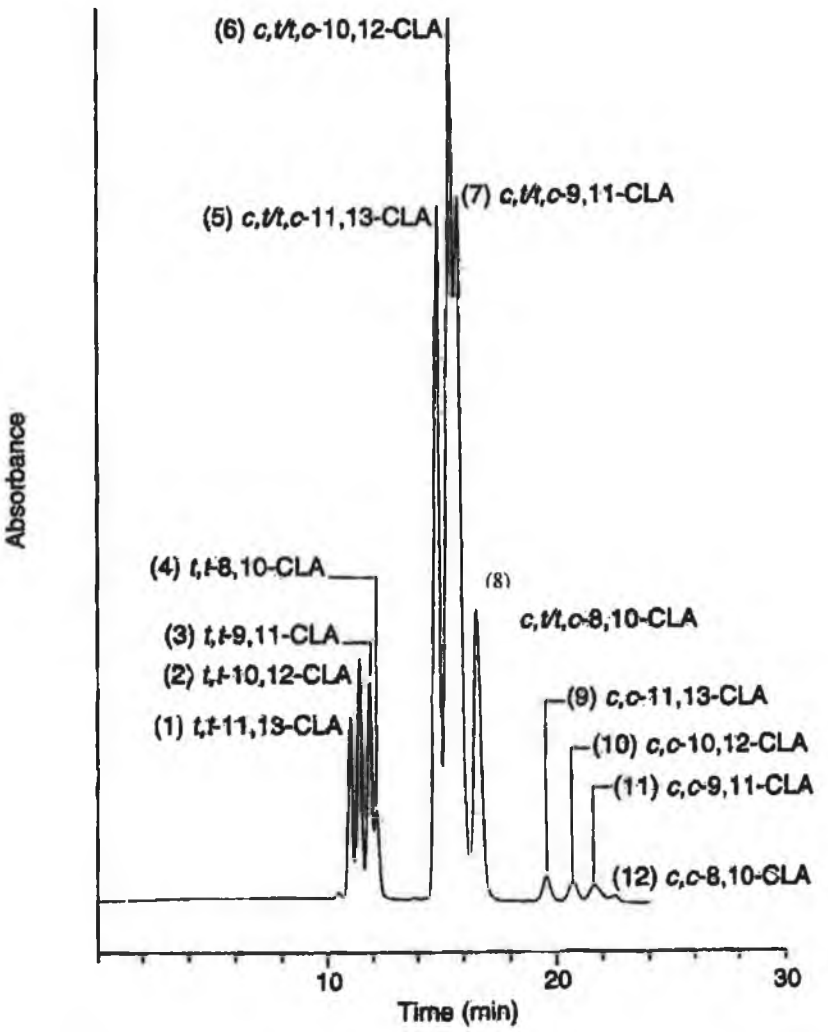


Figure 1.22 Ag^{+}HPLC separation of twelve CLA isomers using a single column (Sehat *et al.*, 1998).

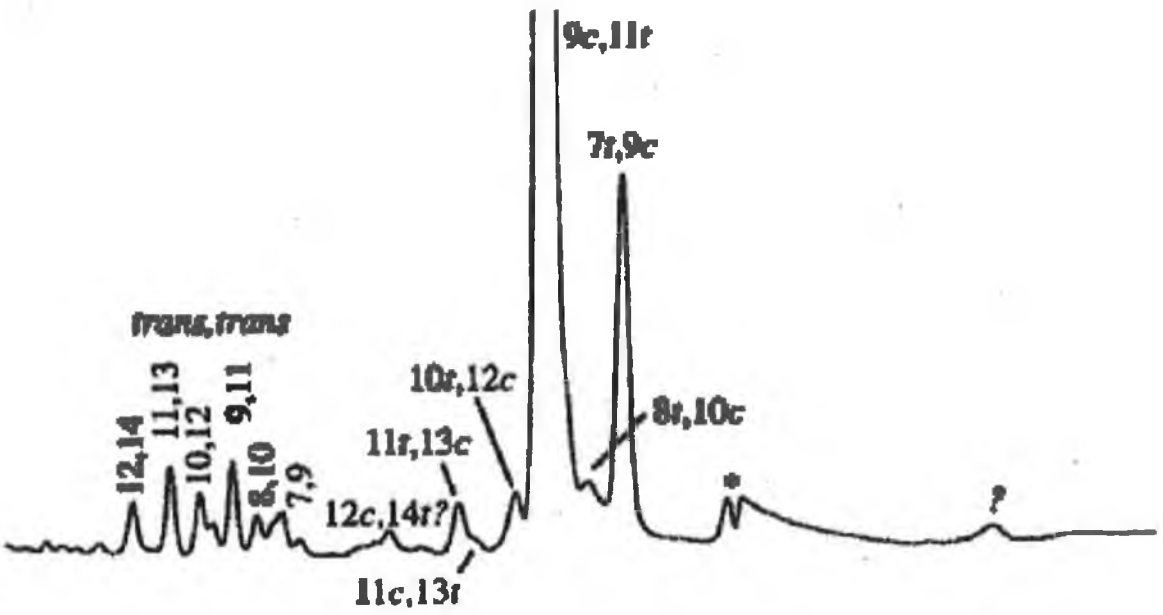


Figure 1.23 Separation of CLA geometric isomers by Ag^{+}HPLC columns used in series (Kramer *et al.,* 1999)

However this methylation procedure, while suitable for samples composed of triglycerides such as milk fat, poses a problem when determining the CLA content in animal tissues and biological samples, due to their high content of phospholipids. The use of tetramethylguanidine (TMG) as a base catalyst in the methylation of CLA-containing lipids has been extensively studied by Shantha *et al.* (1993) and appears to be the method of choice for biological samples as it does not isomerise conjugated double bonds (Yurawecz *et al.*, 1999). However free fatty acids are not methylated under these conditions. Therefore choice of methylation procedure depends largely on the lipid composition of the sample. GLC resolves pairs of geometric isomers (*c, t* from *t, c*) whereas Ag^+ HPLC separates only those with odd Δ (9,11- and 11,13-$C_{18:2}$) but not even (8,10- and 10,12-$C_{18:2}$) Δ values. Ag^+ HPLC resolves all of the *t, t* and *c, c* CLA isomers. In GLC the dominant *c*9, *t*11 peak in dairy products and meat from ruminants severely overlaps with a number of CLA isomers such as *t*7, *c*9 and *t*8, *c*10 whereas three Ag^+ HPLC columns in series clearly resolve these isomers. Both methods helped identify and quantitate the isomeric distribution of CLA isomers in tissues of pigs fed a commercial CLA mixture containing four positional CLA isomers (18.9 % *c*11, *t*13 $C_{18:2}$, 26.3 % *t*10, *c*12 $C_{18:2}$, 20.4 % *c*9, *t*11 $C_{18:2}$ and 16.1 % *t*8, *c*10 $C_{18:2}$) at 2 % of basal diet (Kramer *et al.*, 1998). Analysis of omental fat, liver triglycerides and heart triglycerides showed that the distribution of CLA isomers was similar to that of the diet. The liver phospholipids, particularly phosphatidylcholine and phosphatidylethanolamine selectively accumulated *c*9, *t*11-$C_{18:2}$, except diphosphatidylglycerol (DPG) which showed a preference to accumulate *c*11, *t*13-$C_{18:2}$. Heart phospholipids, particularly DPG also showed a preference for incorporating *c*11, *t*13-$C_{18:2}$. The remaining isomers were distributed

evenly across liver, heart, backfat and omental fat (Kramer *et al.*, 1998). The health benefits associated with the CLA content in milk may be countered by the metabolic effects of *trans* fatty acids, high levels of which are generally accepted as unhealthy. Therefore the accurate analysis of *trans* fatty acids is essential when analysing CLA levels in food products.

1.20 Trans fatty acid analysis

Previously, to separate methyl esters of the same carbon chain length, a packed or fused-silica capillary column coated with polar stationary phases such as SP-2340 was necessary. This was based on the assumption that $C_{18:1}$ *cis* and *trans* isomers are completely separable on the high polar column. However, because of the multiplicity of positional and geometric isomers present in partially hydrogenated vegetable oil (PHVO), these columns can give overlapping results for *trans* and *cis* monoenes of the same chain length (Ratnayake *et al.*, 1998). To quantify the fatty acids, the analyst must use his/her judgement as to where the point of division between each group lies (Christie, 1991). On capillary columns coated with cyano-alkyl-siloxane (highly substituted cyano-propyl phase that has been stabilised) stationary phases (e.g. SP 2560 or CP Sil 88), $C_{18:2}$ *trans* and $C_{18:3}$ *trans* fatty acids (TFA) are separated as distinct groups without any interference or overlaps and levels of these *trans* polyunsaturated fatty acids can be obtained directly by GLC analysis.

The combination of GLC with Ag^+-TLC is a more effective method of separating TFA than GLC alone. Argentation chromatography can separate fatty acids according to the

configuration and number of their double bonds and also according to the position of the double bonds. A complex is formed between the silver ions and the double bonds by interaction of *pi* electrons of carbon-carbon double bonds, and separation is based on position and geometry of the bond (Ratnayake *et al.,* 1998). Because of steric hindrance, *trans* double bonds form weaker complexes compared with *cis* double bonds (Nickolova-Damyanova, 1992), thus Ag^{+}-TLC is an effective means of fractionation of either triglycerides or FAME.

Pre-coated plastic backed sheets with 0.2 mm layer of silica gel impregnated with 10 % (w/v) silver nitrate in acetonitrile allow equally good separation of TFA compared with the glass plates used previously (Ulberth and Henninger, 1992). The first step in Ag^{+}-TLC involves the preparation of the TLC plate in a solution of 10 % silver nitrate in acetonitrile, prior to application of FAME which are dissolved in non-polar organic solvents. Common TLC solvent systems include hexane:diethyl ether, toluene-hexane and hexane-cholorform in different proportions. The separated bonds are visualised under UV light at 254 nm by spraying the TLC plates with fat binding dyes such as 2', 7' –dichloroflourescein or rhodamin B, (Rao *et al.*, 1995). A TLC band corresponding to the *trans* fatty acids can then be scraped off and extracted with diethyl ether or any suitable solvent and analysed by GLC. A limitation of Ag^{+}-TLC is that complex mixtures of FAME containing a range of chain lengths and higher degrees of unsaturation, such as are encountered in fish oils, produce very complex chromatograms (Sebedio *et al.,* 1981). In partially hydrogenated fish oils, numerous mixed bands are expected due to separation according to chain length, and are superimposed on the

separation of geometric and positional isomers. The cross overlap occurs especially in FAME isomers containing more than three double bonds.

The same principal of separation by Ag-TLC can be applied as a HPLC technique in which silver ions are bound to an ion exchange support. However, the main disadvantage of this method is reproducibility as a result of rapid loss of resolution. This can occur after a short time of daily use (4 weeks) in contrast to capillary GC column which can be used daily for up to a year without loss of resolution (Ratnayake *et al.,* 1998). Ag^{+}-HPLC may be a good alternative to Ag^{+}-TLC for preliminary isomer fractionation before GLC analysis and was recommended by the American Oil Chemists' Society (AOCS, 1997) for TFA analysis. The combination of GLC with reverse phase HPLC is an additional means of analysing TFA (Christie, 1991). Reverse phase HPLC separates on the position as well as on the configuration of the bond. Epoxidation of the double bonds of TFA prior to GLC analysis is also beneficial for TFA analysis as *trans* epoxides elute clearly ahead of the corresponding *cis* derivatives, but it seems that conjugated isomers cannot be epoxidised quantitatively (Christie, 1991).

By selecting the proper operating parameters and a polar cyanopolysiloxane capillary column, detailed fatty acid composition including *trans* isomers and other isomeric fatty acids in partially hydrogenated vegetable oils can be obtained by GLC analysis alone. For applications requiring more precise *trans* fatty acid data and detailed information on individual isomers, it is necessary to couple GLC with either Ag-TLC or Ag-HPLC (Ratnayake *et al.,* 1998).

1.21 Aims

The aims of this study were to validate methodology for *trans* fatty acid analysis, and to investigate dry fractionation as a means of enriching milk fat CLA. The resulting milk fat fraction was then compared with a range of food products with respect to levels of CLA and *trans* fatty acid. The second stage of this work was to investigate specific mechanisms of action that may be responsible for the anticarcinogenic effect of synthetic CLA in human cancer cell lines and to compare these effects with those induced by milk fat triglyceride bound CLA.

1.22 Specific objectives

- To investigate dry fractionation as a means of enriching milk fat CLA, and to compare CLA levels as a result of dry fractionation with CLA and *trans* fatty acid contents of a range of food products. In parallel, to establish and validate Ag-TLC and GLC methodologies for the accurate detection and quantification of *trans* fatty acids in edible oils.

- To evaluate the relative growth effects of a synthetic mixture of CLA isomers, pure *c*9, *t*11- and *t*10, *c*12- CLA on human cancer cell lines by examination of viability, protein, RNA and DNA synthesis in the cells.

- To compare the cytotoxic effects of the synthetic isomers *c*9, *t*11 CLA, *t*10, *c*12 CLA and Nu-Chek CLA with CLA-enriched milk fat, consisting primarily of the *c*9, *t*11 isomer, present in triglyceride bound form on the growth of mammary cancer cells.

- To investigate if the growth suppressive effects of CLA are mediated by specific effects on lipid peroxidation, the enzymes of the antioxidant defence system and on farnesylation of Ras oncoprotein.

CHAPTER 2

Validation of methodology for *trans* fatty acid analysis

2.1 Introduction

As described in chapter 1 *trans* fatty acids (TFA) are formed during biohydrogenation of unsaturated fatty acids in the rumen and consequently are present in milk and meat of ruminant animals (Craig-Schmidt, 1998). Non-natural TFA are formed in industry during partial hydrogenation of vegetable and marine oils. There are highly unsaturated oils and as such are generally unsuitable for food fats because of their low melting points and ready susceptibility to oxidative deterioration. Industrially produced TFA differ in many respects from TFA formed in the ruminant animal during biohydrogenation; some of these differences are related to isomer distribution. The small amounts of TFA in butter and other ruminant fats are mostly *t*11-$C_{18:1}$, while TFA in vegetable oils, margarine and shortenings are mostly $C_{18:1}$ fatty acids with double bonds distributed in a Gaussian fashion around Δ 9, Δ 10, Δ 11 and Δ 12. Other differences relate to their amounts (1 – 2 % of total fat in dairy products such as butter, versus 35 – 50 % of total fat in edible oils) and their speed of metabolism (*t*11-$C_{18:1}$ is metabolised much faster than TFA in partially hydrogenated vegetable oil (PHVO)).

Epidemiological data has suggested an association between TFA consumption and the incidence of coronary heart disease (CHD) (Ascherio *et al.*, 1994). However, due to the nature of epidemiological studies it is difficult to assess the effects due to TFA because of the different amounts of TFA consumed and the different combinations of TFA with saturated and *cis* unsaturated fatty acids in foods. A survey of the literature carried out by Aro (1998) suggested that when consumed in large amounts, the effects of TFA on

CHD are expected to be comparable with those of saturated fatty acids in the diet. Ultimately the effects of dietary TFA depend on how they affect the balance between the hypercholesterolaemic saturated C_{12} to C_{16} fatty acids and the hypocholesterolaemic *cis* unsaturated fatty acids in food (Aro, 1998). Several experimental studies have now indicated that substitution of a *cis* fatty acid diet with a TFA diet resulted in increased total LDL-cholesterol concentrations and decreased HDL-cholesterol concentrations in blood. Changes in blood concentration of apolipoprotein B and apolipoprotein A-1 in response to TFA intake have been reported for the most part to mirror changes in LDL and HDL cholesterol, respectively. TFA intake has also been reported to increase blood lipoprotein (a) (Lp[a]) concentration, although this observation has not been consistent (Aro *et al.,* 1997, Almendingen *et al.,* 1995, Judd *et al.,* 1994, Mensink and Katan, 1990, Zock and Mensink, 1996).

In view of the possible harmful effects of dietary TFA, accurate analysis of these fatty acids is necessary to monitor their levels in foods. Owing to the complexity of hydrogenated fat mixtures, a simple definitive method for simultaneous determination of both total TFA content and fatty acid composition does not exist (Ratnayake *et al.,* 1998). GLC in combination with other techniques, particularly Ag^+-TLC, IR or mass spectrophotometry (MS) allows for a complete analysis of fatty acids and these are now the most commonly used methods for analysing TFA composition.

Current analytical tools are still, however, inadequate for complete analysis of TFA, mainly due to the unavailability of suitable reference materials particularly for minor,

unusual *cis* and *trans* isomers (Ratnayake and Beare-Rodgers, 1990). Certified reference materials (CRM) are defined as reference material whose properties are certified by a technically valid procedure accompanied by or traceable to other documentation which is issued by a certifying body (Laboratory of Government Chemists (LCG)). These reference materials are then used for the following four main purposes; calibration and verification of measurement processes under routine conditions, internal quality control and quality assurance schemes, verification of the correct application of standardised methods and development and validation of new methods of analysis.

2.2 Objectives

Due to the lack of a certified reference material and the wide variation in methodologies used in TFA analysis, this study was undertaken to validate combined Ag^+-TLC and GLC methodology for the accurate quantification of TFA in vegetable oils, provided by F. Ulberth (Department of Dairy Research & Bacteriology, Agricultural University, Gregor Mendel-Str. 33, A-1180 Vienna). This study was undertaken in parallel with 11 other analysing laboratories with a view to making a comparison of data obtained by a variety of analytical methodologies including GLC, using CP SIL88 and BPX70 columns and IR spectrometry, ultimately with a view to harmonisation of methodology for TFA analysis. Only the data obtained by analysis of a variety of TFA-containing samples, by Ag^+-TLC and GLC as performed in our laboratory are reported in this chapter. Results were compared with data obtained from the other laboratories. This permitted intercomparison of results and a critical assessment of methodology for TFA analysis, and where appropriate, alterations to the Ag^+-TLC and GLC methodology were made to improve accuracy.

2.3 Materials and Methods

2.3.1 Materials

Solvents: n-hexane, n-heptane and acetonitrile (HPLC grade) were purchased from Labscan (Stillorgan Industrial Park, Dublin). Free fatty acids, standard FAME and 0.25 mm silica gel plates were purchased from Sigma (Sigma Chemical Co., St. Louis, USA). CRM 162 soya-maize oil blend was purchased from the Laboratory of the Government Chemist (LCG, Queens Road, Teddington, Middelsex, UK). *Trans* polyene FAME standards were purchased from Supelco (Supelco Inc, Bellefonte, PA). Oil and FAME samples containing known levels of TFA where provided by F. Ulberth (Department of Dairy Research & Bacteriology, Agricultural University, Gregor Mendel-Str. 33, A-1180 Vienna). These included elaidinised linseed oil FAME, a mixture of FAME standards ($C_{14:0}$, $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$), 3 soya oil samples spiked with approximately 1 %, 5 % and 30 % methyl elaidate and trieladin, and 4 vegetable oil (soya, palm, linseed) samples containing varying levels of TFA (1, 1.5, 5 and 25 % TFA).

2.3.2 Sample preparation and methylation.

FAME standard mixtures of known composition were prepared freshly on each day of analysis. Standards were prepared gravimetrically, approximately 10 mg of each standard was weighed, pooled and dissolved in n-heptane to a concentration of 0.1 mg/ml each in the solvent for GLC analysis. Vegetable oils (soya, palm, linseed) of unknown composition were also analysed. On each working day, a fresh ampoule of each sample was melted by immersion in a water bath at 45-50 °C. When a clear oil was obtained, the

contents were mixed by repeated inversion for 30 sec, and the free fatty acids were converted to FAME using base-catalysed methanolysis as described previously (ISO, 1978). An aliquot (100 mg) of the triglyceride mix was dissolved in 5 ml of n-heptane and added to 0.4 ml of 2 M methanolic KOH. This was mixed for 1 min and allowed to stand for 5 min at room temperature. Sodium sulphate (0.5 g) was then added and the sample was mixed for 1 min. The sample was centrifuged (Sanyo MSC Mistral 2000R, Gallencamp PLC, Leicester, UK) at 280 x g for 5 min, and 1 µl of the supernatant was used for GLC analysis.

2.3.3 Silver ion thin layer chromatography (Ag^+-TLC)

Prior to GLC analysis, the FAME were separated using Ag^+-TLC, according to the method of Ulberth and Henninger (1992) described as follows. Initially, 0.25 mm silica gel plates (Sigma) were placed in a solvent tank with 10 % (w/v) $AgNO_3$ in acetonitrile (stationary phase) for approximately 2 h to impregnate the plates with $AgNO_3$. The FAME samples (100 µl) were applied to the plates manually using a pipette and allowed to develop in a mobile phase of hexane : diethyl ether (9:1 v/v) for approximately 3 h. The plates were allowed to dry at room temperature and were stained by spraying with 0.05 % (w/v) rhodamin B in ethanol. The *trans* and saturated fatty acid tracks, visible under UV light, were scraped from the plates individually and dissolved in diethyl ether by frequent agitating for 30 min. The resulting extracts were dried in a stream of N_2 and redissolved in hexane prior to GLC analysis. The calculation of the *trans*-monoene content was based on the concentration of $C_{18:0}$ in the pre-TLC sample and in the fractionated sample, using the following equation:

Trans monoene content =

$$\frac{\text{mass of the pre-TLC } C_{18:0}}{\text{total sample mass}} \times \frac{\text{peak area of } \textit{trans}\ C_{18:1} \text{ post-TLC}}{\text{peak area of } C_{18:0} \text{ post-TLC}} \qquad \textbf{Equation 1}$$

2.3.4 GLC analysis

Separation of FAMEs was performed using a Varian 3400 GLC (Varian, Walnut Creek, CA, USA) fitted with a flame ionisation detector (FID). Samples (1 μl) were injected onto the column using a splitless injector and a Varian 8035 auto-sampler (Varian). Separations were performed on a Chrompack CP Sil 88 column (60 mm x 0.25 mm i.d., 0.02 μm film thickness) (Chrompack, Middleburg, The Netherlands). The N_2 carrier gas and H_2 flow rates were 20 ml/min, and the airflow rate was 300 ml/min. The injector and detector temperatures were 140 °C and 250 °C, respectively. The column temperature was programmed from 140 °C with an initial delay of 8 min followed by an increase of 8.5 °C/min to a final temperature of 200 °C, which was held for 15 min. Fatty acids were identified by retention times with reference to standard FAME. Collected data were analysed on a Minichrom PC system (VG, Data Systems, Manchester, UK).

2.3.5 Accuracy assessment

The accuracy of the GLC system was evaluated by performing a series of tests to verify the levels of decomposition (deterioration of sample) and degree of irreversible adsorption (sample remaining on the column) of saturated and monounsaturated fatty acids that occurred during chromatography. Thus analysis of samples of known composition allowed determination of the degree of decomposition and irreversible

adsorption that occurred: composition of the sample following GLC was compared with known data relating to that sample. This involved preparation of three identical test mixtures as described in 2.3.2 composed of approximately equal quantities (10 mg) of $C_{14:0}$, $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ FAME standards and analysis was performed in triplicate by GLC. In addition, a mixture of FAME ($C_{14:0}$, $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$) in unknown quantities (provided by F. Ulberth), which was prepared as described in 2.3.2 was also analysed in triplicate.

Response factors

Response factors (RF), i.e. correction factors which compensate for the variable response of the flame ionisation detector (FID) to individual fatty acids were calculated relative to $C_{18:0}$ and were used to convert the peak areas to mass percentages, using the following equation :

$$RF = (m_i\ \%) / (A_i\ \%) = m_i \times \Sigma A_i / \Sigma m_i \times A_I \qquad \textbf{Equation 2}$$

where m_i is the mass of the individual fatty acid,

Σm_i is the sum of the masses of all fatty acids,

A_i is the individual area of the fatty acid and

ΣA_i is the sum of total areas.

Individual RFs were standardised with respect to the RF of $C_{18:0}$. To maintain specified limits of accuracy, it was established that these response factors should not deviate by more than 0.05 from 1.0.

2.3.6 Validation of methylation procedure

The effect of derivative formation on glyceride bound unsaturated fatty acids was assessed as follows. Three triglyceride samples of known composition ($C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$) (provided by F. Ulberth) were methylated prior to GLC analysis as described in 2.3.2. A further FAME sample (provided by F. Ulberth) with identical fatty acid compositions ($C_{16:0}$, $C_{18:0}$, $C_{18:2}$ and $C_{18:3}$) to the triglyceride samples above was also analysed in triplicate as described in 2.3.4. It was established that the average FAME composition (area %) of the triglyceride sample should not differ by more than 0.2 % (absolute) from those of the FAME mix.

2.3.7 Separation power

The chromatographic parameters leading to an effective separation of *cis/trans* fatty acids were optimised using specific mixtures of *cis* and *trans* fatty acids that are difficult to separate with conventional methods. Sample mixtures of equal quantities of 13-*trans* $C_{18:1}$, 9-*cis* $C_{18:1}$, $C_{20:0}$, all *cis* $C_{18:3}$ and 11-*cis* $C_{20:1}$ (Sigma) were prepared for analysis by GLC as described in 2.3.2. The parameters of the GLC program were modified (i.e. ramping temperature (rate of temperature increase of the column and injector), detector temperature, flow rate, flow rate temperature and injector temperature) until chromatographic resolution (R) (baseline resolution) between components was ^{R}s 1.

Chromatographic resolution was calculated using the following equation;

$$R = 2d / (w_1 + w_2) \qquad \textbf{Equation 3}$$

where w_1 and w_2 are the widths, (mm) of the peaks for $C_{18:0}$ and $C_{18:1}$, respectively, measured between the points of intersection of the tangents at the inflexion points of the curve with the baseline,

d is the distance (mm), between the respective peak maxima for methyl $C_{18:0}$ and methyl $C_{18:1}$.

To verify that these adjustments to the GLC programs were accurate for separation of *cis* and *trans* isomers, a vegetable oil containing *trans* was analysed to achieve similar resolution. A sample of nitrous acid isomerised linseed oil (provided by F. Ulberth) was analysed by GLC and the R value between 13-*trans* $C_{18:1}$, and 9-*cis* $C_{18:1}$ components was determined. Equal quantities of methyl esters of *trans*-9 and *cis*-9 $C_{18:1}$, (Sigma) were also prepared as described in 2.3.2 and separated by Ag^+-TLC as described in 2.3.3. The efficiency of separation by TLC was validated by analysis of the samples post-TLC by GLC.

2.3.8 Proficiency testing

The precision of the methodology for TFA analysis was established by verifying the repeatability and reproducibility of the Ag^+-TLC and GLC methodologies. Repeatability was assessed by examining the difference between two single results found on an identical test material using the same method of analysis within a short time frame. Reproducibility was assessed by examining the difference between two operators working in different laboratories on an identical test material. Three oil samples and three FAME samples (provided by F. Ulberth) were analysed by GLC, both before and after separation by Ag^+-TLC as described in section 2.3.2. The oil samples consisted of

fatty acids from soya oil in fatty acid methyl ester and triglyceride form. They had been spiked with approximately 1 %, 5 % and 30 % methyl elaidate and trielaidin. The 3 oil samples in triglyceride form were methylated prior to analysis by GLC and the 3 FAME samples were analysed directly by GLC. GLC analysis prior to TLC gave the composition of the samples and in particular the $C_{18:0}$ content which was necessary to calculate the TFA content of the post-TLC fraction as described in section 2.3.3. All 6 samples were then separated by Ag^+-TLC to enable separation of the *trans* isomers as described in 2.3.3, and analysed again post TLC by GLC to quantify the TFA content.

2.3.9 Intercomparison testing

Four test materials (provided by F. Ulberth) containing different TFA levels (1, 1.5, 5 and 25 % w/w TFA) were analysed using the combined Ag^+-TLC and GLC methods for quantification of TFA content and the results were compared with those from other laboratories involved in similar analyses, which allowed the intercomparison of results and critical assessment of the methodology employed. The sample containing 1 % TFA was a physically refined soya oil, the sample containing 1.5 % TFA was a physically refined rapeseed oil, the sample containing 5 % TFA was an oil as used for manufacturing margarine (primarily palm oil) and the oil containing approximately 25 % TFA was a partially hydrogenated sunflower oil, all of which had previously undergone homogenicity and stability testing based on BCR guidelines (ISO Guide 30, 1981). The oils were methylated and analysed by GLC both pre- and post-TLC to determine the TFA content of the oils.

A set of performance criteria, described below were established and had to be successfully achieved daily, prior to analysis of samples. Initially the performance of the GLC system was examined by injecting a solution containing equal amounts of the critical pairs *trans*-13/*cis*-9 $C_{18:1}$ and *cis*-11 $C_{20:1}$/all-*cis* $C_{18:3}$ to achieve a resolution (R) of critical pairs > 1.0. A solution containing elaidinised linseed oil FAME (provided by F. Ulberth) was analysed to determine retention times of *trans*-containing polyunsaturated fatty acids (single analysis) to achieve separation of the geometrical isomers of $C_{18:2}$ into 4 components (tt, ct, tc, cc) and $C_{18:3}$ into 6 components (ttt, ctt+tct, ttc+cct, ctc, tcc, ccc). These *trans* polyenes were identified using *trans* polyene FAME standards. A FAME solution obtained by methylation of CRM 162 (LCG) was analysed to determine accuracy of quantification. The average concentration of individual FAME (y) in CRM 162 following analysis must be within the range outlined in BCR guidelines (Table 2.1) for sufficient accuracy of the GLC system. Composition of the sample must be within the following range for the GLC system to be accurate,

$$(\text{certified value} - 2\ \text{SD}) < y < (\text{certified value} + 2\ \text{SD})$$

where y is the individual fatty acid following analysis by GLC,

the certified value is the concentration of that fatty acid present according to the BCR guidelines.

SD is the standard deviation (Table 2.1).

Table 2.1 BCR guidelines for quantification of CRM 162.

(soya/maize oil blend)

FAME	Certified mean	Standard deviation	Range
	% total lipids		% total lipids
$C_{16:0}$	10.65	0.279	10.09 - 11.21
$C_{18:0}$	2.87	0.123	2.63 - 3.11
$C_{18:1}$	24.14	0.392	23.36 - 24.92
$C_{18:2}$	56.66	0.760	55.14 - 58.18
$C_{18:3}$	4.68	0.309	4.06 - 5.30

2.4 Results

2.4.1 Assessment of accuracy of GLC methodology for FAME analysis

Mixtures of FAME of known concentrations were analysed by GLC to determine if decomposition or irreversible adsorption onto the GLC column occurred which would lead to inaccurate results. Three identical test mixtures were prepared, as described in section 2.3.2, composed of approximately equal quantities (10 mg) of $C_{14:0}$, $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ FAME standards and analysis was performed in triplicate by GLC. The performance criteria for this analysis recommended that response factors (relative to $C_{18:0}$) should not differ by more than 0.05 from 1.0 as described in section 2.3.5. The FAME mixture contained between 22 and 28 % of the fatty acids $C_{14:0}$, $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ (Table 2.2) which, when analysed by GLC, produced peak areas corresponding to the concentration of the individual FAME in the samples. The % peak areas for individual FAME (Table 2.2) corresponded closely to the known % mass of the fatty acids suggesting that decomposition and irreversible adsorption did not occur. This conclusion was supported by calculation of the relative RF values as described in section 2.3.5. The RF values for each of the individual fatty acids were between 0.95 and 1.05 (Table 2.2) and therefore fulfilled the required performance criteria, which required that this value should not deviate from unity by $\pm$ 0.05.

The FAME sample of unknown composition was also analysed by GLC, and the composition by area is reported in Table 2.3.

Table 2.2 GLC compositional analysis of a FAME standard mixture ($C_{14:0}$, $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$)*.

FAME	Mass (g)	Mass (%)	Area (counts)	Area (%)	RF	Relative RF
$C_{14:0}$	0.010 ± 0.003	26.54 ± 3.79	7035694 ± 136567	25.32 ± 4.04	1.05 ± 0.02	1.05 ± 0.02
$C_{16:0}$	0.009 ± 0.001	22.87 ± 2.74	6669243 ± 899979	24.03 ± 2.68	0.95 ± 0.01	0.95 ± 0.00
$C_{18:0}$	0.011 ± 0.002	28.24 ± 0.64	7827426 ± 490155	28.23 ± 0.57	1.00 ± 0.00	1.00 ± 0.00
$C_{18:1}(t9)$	0.009 ± 0.002	22.35 ± 0.80	6224554 ± 578919	22.43 ± 1.13	1.00 ± 0.02	1.00 ± 0.03

*(Data represent the mean ± SD of triplicate analyses)

Table 2.3 Quantification of FAME in an unknown mixture (provided by F. Ulberth)*.

FAME	Area (counts)	Area (%)
$C_{14:0}$	4781198 ± 139428	22.76 ± 1.02
$C_{16:0}$	5646724 ± 247213	26.89 ± 2.71
$C_{18:0}$	5207316 ± 401251	24.80 ± 1.50
$C_{18:1}(t9)$	5365559 ± 99802	25.55 ± 0.13

*(Data represent the mean ± SD of triplicate analyses)

As the % mass of the individual fatty acids in this sample was unknown, RF values could not be calculated. The composition by area of the FAME sample was similar to that of the previous standard mixture suggesting that the RF values may also be similar.

2.4.2 Validation of methylation procedure

Three triglyceride samples of known composition ($C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$) were methylated and analysed by GLC using the procedures outlined in sections 2.3.2 and 2.3.4 and the results presented in Table 2.4. A further preformed FAME sample with identical fatty acid composition ($C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$) to the triglyceride samples above was also analysed by GLC and the results presented in Table 2.5. The performance criteria for this analysis recommended that the average FAME composition (area %) of the triglyceride samples (Table 2.4) should not differ by more than 0.2 % (absolute) from those of the preformed FAME mix (Table 2.5). Tables 2.4 and 2.5 report the composition by weight of the individual fatty acids in each sample and the composition by area as determined by GLC. Using these data, the RF and relative RF values were calculated (as described in section 2.3.5) for the triglycerides methylated in the laboratory and the pre-formed FAME. It was clear that the acceptance criteria were not fulfilled in this section, as the difference between the % area values obtained for $C_{16:0}$, for preformed FAME (Table 2.5) and the samples methylated in the laboratory (Table 2.4) was greater than 0.2 %. However, criteria were fulfilled for the other fatty acids analysed $C_{18:0}$, $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$ as values obtained for the % area for preformed FAME (Table 2.5) and the samples methylated in the laboratory did not differ by more than 0.2 % (Table 2.4).

Table 2.4 GLC compositional analysis of a triglyceride sample ($C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$) (provided by F. Ulberth)*.

FAME	Area (counts)	Area (%)	RF	Relative RF
$C_{16:0}$	3627146 ± 103980	21.26 ± 0.06	0.94 ± 0.00	0.95 ± 0.01
$C_{18:0}$	3354706 ± 48524	19.67 ± 0.23	1.02 ± 0.01	1.03 ± 0.02
$C_{18:1}$	3439466 ± 124501	20.16 ± 0.23	0.99 ± 0.01	1.00 ± 0.00
$C_{18:2}$	3365179 ± 112612	19.72 ± 0.17	1.01 ± 0.01	1.02 ± 0.01
$C_{18:3}$	3272151 ± 57554	19.18 ± 0.17	1.04 ± 0.01	1.05 ± 0.02

*(Data represent the mean ± SD of triplicate analyses)

Table 2.5 GLC compositional analysis of a preformed FAME standard sample ($C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$) (provided by F. Ulberth)*.

FAME	Area (counts)	Area (%)	RF	Relative RF
$C_{16:0}$	5375159 ± 230471	20.93 ± 0.02	0.96 ± 0.01	0.97 ± 0.01
$C_{18:0}$	5034423 ± 211087	19.60 ± 0.16	1.02 ± 0.02	1.04 ± 0.00
$C_{18:1}$	5229824 ± 143775	20.37 ± 0.41	0.99 ± 0.01	1.00 ± 0.02
$C_{18:2}$	5099931 ± 213151	19.86 ± 0.22	1.00 ± 0.00	1.03 ± 0.01
$C_{18:3}$	4940485 ± 514832	19.24 ± 0.11	1.04 ± 0.00	1.06 ± 0.01

*(Data represent the mean ± SD of triplicate analyses)

2.4.3 Separation power

The chromatographic parameters leading to an effective separation of *cis/trans* fatty acids were optimised. Sample mixtures of equal quantities of 13-*trans* $C_{18:1}$, 9-*cis* $C_{18:1}$, $C_{20:0}$, all *cis* $C_{18:3}$ and 11-*cis* $C_{20:1}$ (Sigma) were analysed by GLC and the results presented in Table 2.6. The composition by weight of these isomers ranged from 18.43 % to 21.61 %. The retention times and peak base width of the individual FAME in the samples were obtained from the chromatograph (Figure 2.1) and are reported in Table 2.6. These values were required for calculation of chromatographic resolution (R) between the pairs 13-*trans* $C_{18:1}$/9-*cis* $C_{18:1}$ and all *cis* $C_{18:3}$/11-*cis* $C_{20:1}$ as described in section 2.3.7. The predefined criteria for this separation was a chromatographic resolution between the pairs of at least 1.00. The chromatographic resolution was acceptable, being 2.26 for 13-*trans* $C_{18:1}$/9-*cis* $C_{18:1}$ and 4.70 for all *cis* $C_{18:3}$/11-*cis* $C_{20:1}$.

A sample of nitrous acid isomerised linseed oil was analysed by GLC and the resolution of the pairs 13-*trans* $C_{18:1}$/9-*cis* $C_{18:1}$ within the oil was calculated from the chromatograph (Figure 2.2). This value (2.14) being > 1.00, demonstrated that efficient separation was achieved for these pairs in the presence of other fatty acids in the oil sample. The efficiency of separation by Ag^+-TLC was examined by separation of equal quantities of methyl esters of *trans*-9 and *cis*-9 $C_{18:1}$ (Sigma) by Ag^+-TLC and subsequent analysis of the *cis* and *trans* fractions by GLC. The *cis* and *trans* isomers were efficiently separated as seen from the chromatograph (Figure 2.3 a, b), as there was no cross contamination in either fraction (i.e. no *cis* peak in the *trans* fraction or *vice versa*).

Table 2.6 Data from chromatograph (Figure 2.1) representing GLC analysis of a FAME sample (13-*trans* $C_{18:1}$, 9-*cis* $C_{18:1}$, $C_{20:0}$, all *cis* $C_{18:3}$ and 11-*cis* $C_{20:1}$) which is used to calculate the separation power.*

FAME	Mass (g)	Mass (%)	Retention time (min)	Peak base width (min)
13-*trans* $C_{18:1}$	0.009 ± 0.01	18.43 ± 2.12	19.48 ± 0.002	0.17 ± 0.00
9-*cis* $C_{18:1}$	0.001 ± 0.01	19.99 ± 1.02	19.70 ± 0.001	0.14 ± 0.01
$C_{20:0}$	0.010 ± 0.06	19.68 ± 0.021	22.00 ± 0.002	0.19 ± 0.02
all-*cis* $C_{18:3}$	0.011 ± 0.02	21.61 ± 1.79	22.69 ± 0.001	0.19 ± 0.01
11-*cis* $C_{20:1}$	0.010 ± 0.11	20.25 ± 1.22	23.20 ± 0.010	0.19 ± 0.00

*(Data represent the mean ± SD of triplicate analyses)

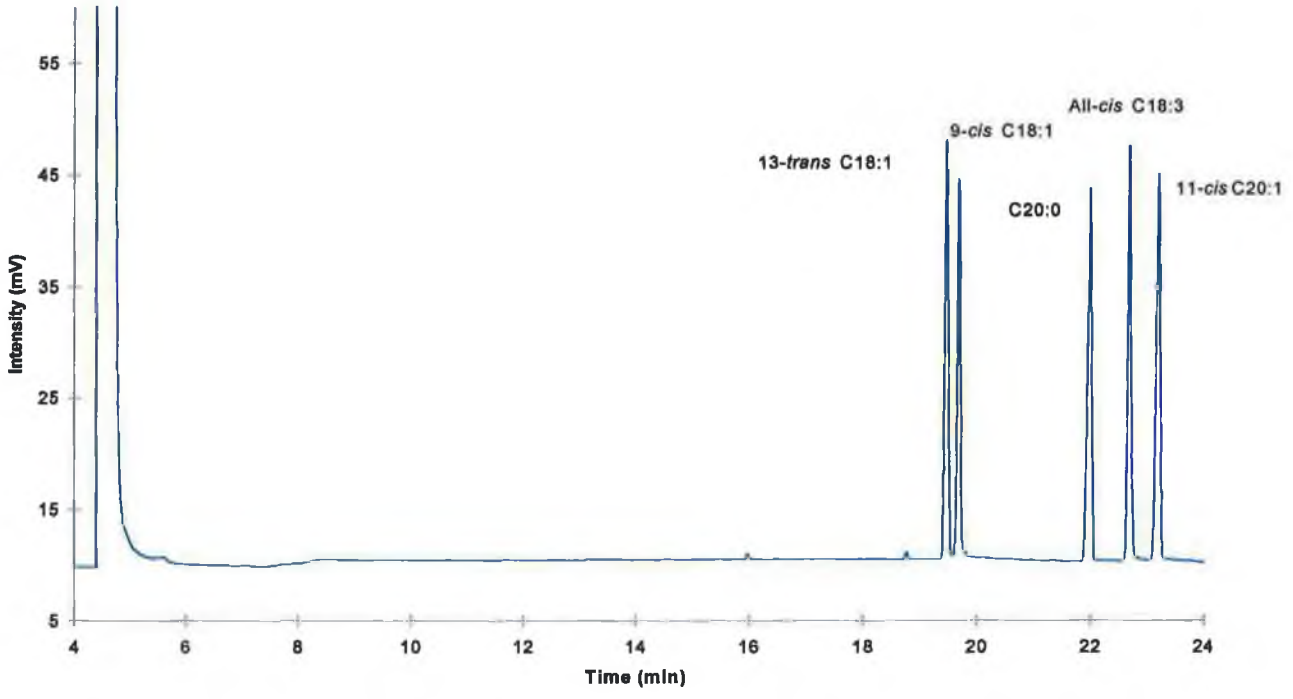


Figure 2.1 Separation of critical pairs 13-*trans*/9-*cis* $C_{18:1}$, all *cis* $C_{18:3}$/11-*cis* $C_{20:1}$ Resolution of 13-*trans* $C_{18:1}$/9-*cis* $C_{18:1}$ = 2.26. Resolution of all-*cis* $C_{18:3}$ /11-*cis* $C_{20:1}$ = 4.70.

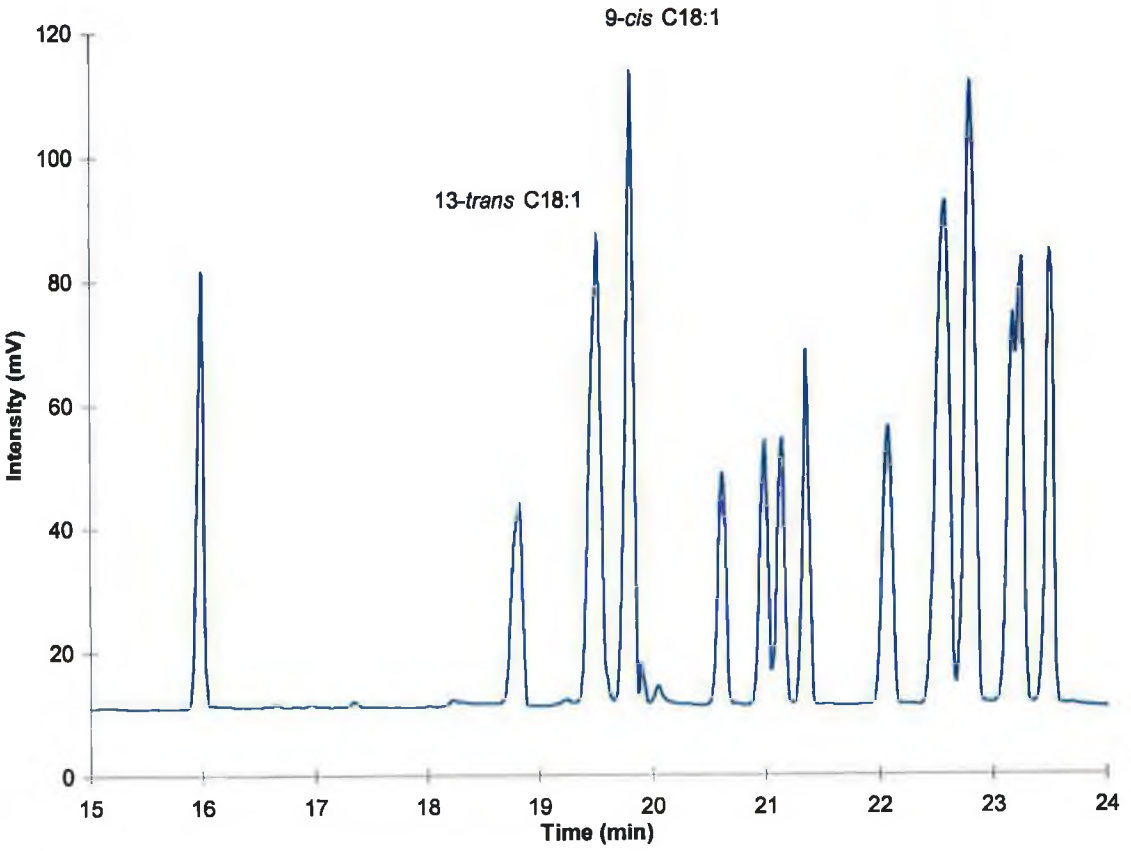
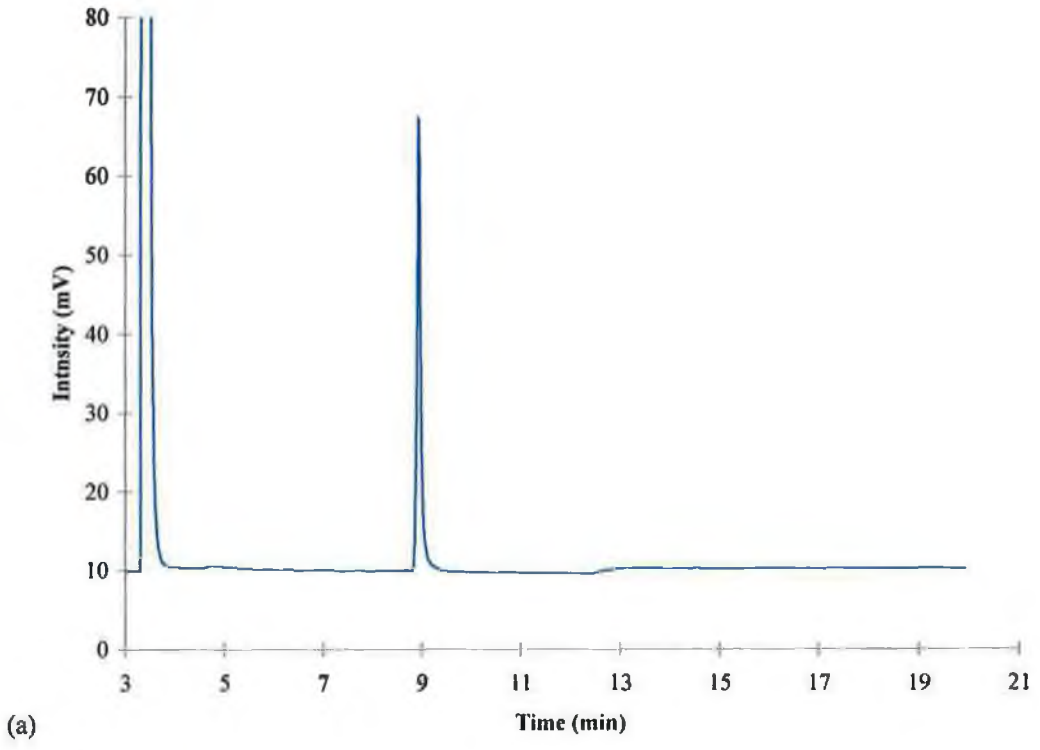
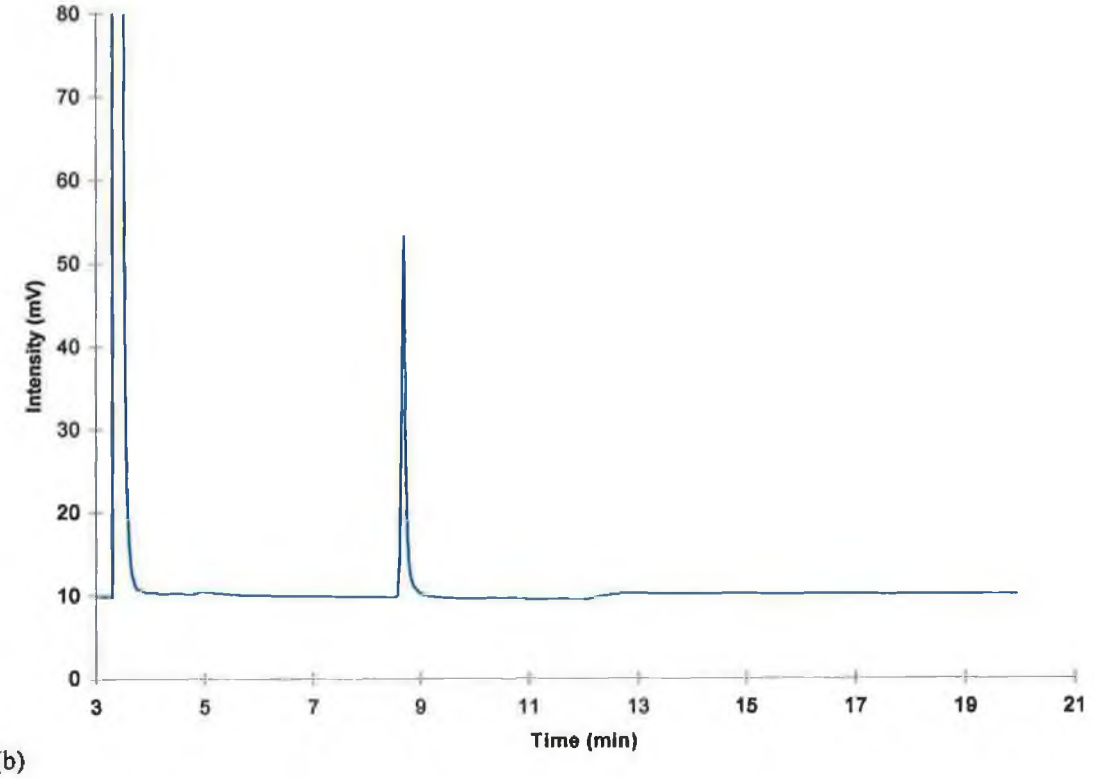


Figure 2.2 Separation of isomerised linseed oil by GLC. Resolution of 13-*trans* $C_{18:1}$/9-*cis* $C_{18:1}$ = 2.14.



(a)



(b)

Figure 2.3 Separation Power of Ag^+-TLC. GLC analysis following Ag^+-TLC (a) *cis*-9 $C_{18:1}$ and (b) *trans*-9 $C_{18:1}$.

2.4.4 Proficiency testing

The precision of the methodology for TFA analysis was established by verifying the repeatability and reproducibility of the Ag^+-TLC and GLC methods. Three oil samples spiked with trielaidin and three FAME samples spiked with methyl elaidate containing variable TFA contents were analysed by GLC, both prior to and following separation by Ag^+-TLC and the results presented in Tables 2.7 – 2.12. The criteria established to assess precision of the methodology was a recovery rate of TFA in excess of 90 % following separation by Ag^+-TLC; repeatability and reproducibility were assessed by fulfilling the precision criteria for triplicate analysis of samples on three separate days. The fatty acid composition of the soya oil sample spiked with approximately 1 % methyl elaidate as measured by GLC, pre- and post-Ag^+-TLC are presented in Table 2.7. Using these data the quantity of TFA present in the oil was calculated relative to $C_{18:0}$ (using equation 1 in section 2.3.3) and was found to be 1.16 % and 1.1 % for pre and post Ag^+-TLC analysis, respectively. This represented a 95 % recovery of TFA, (post Ag^+-TLC compared with pre Ag^+-TLC) and therefore met the performance criteria for precision analysis of this sample.

The fatty acid composition of the soya oil sample spiked with approximately 5 % methyl elaidate as measured by GLC, pre- and post-Ag^+-TLC are presented in Table 2.8. Using these data the quantity of TFA present in the oil was calculated relative to $C_{18:0}$ (using equation 1 in section 2.3.3) and was found to be 4.91 % and 4.65 % for pre- and post-Ag^+-TLC analyses, respectively. This also represented a 95 % recovery of TFA and therefore met the performance criteria for precision analysis of this sample. Figure 2.4 represents a chromatogram of the sample (a) pre TLC and (b) post TLC.

Table 2.7 Determination of TFA content of FAME sample containing ca. 1 % methyl elaidate by GLC analysis pre and post Ag^{+}-TLC.*

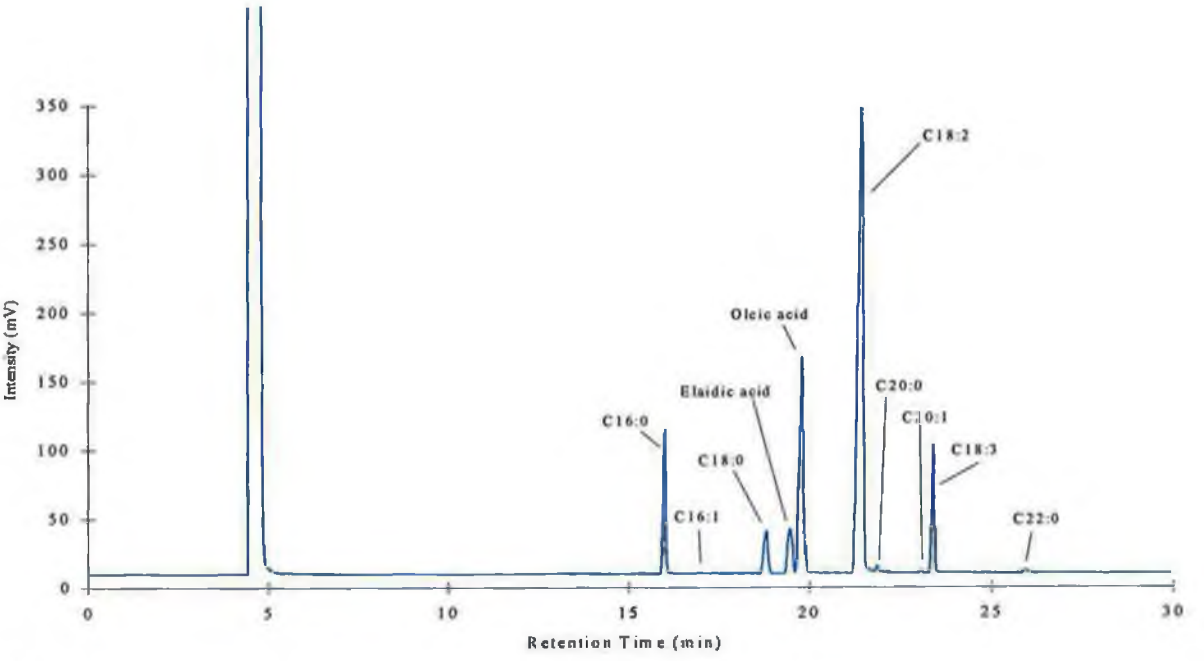
FAME	Area (counts) Pre-TLC	Area (%) Pre-TLC	Area (counts) Post-TLC	Area (%) Post-TLC
$C_{16:0}$	374006 ± 10231	8.88 ± 0.02	452334.6 ± 13554	62.48 ± 2.12
$C_{16:1}$	3755 ± 101	0.09 ± 0.01	0	0
$C_{18:0}$	192910.6 ± 11320	4.58 ± 0.15	219008.8 ± 20172	30.25 ±1.26
trans $C_{18:1}$(n-9)	48708.13 ± 11201	1.16 ± 0.13	52616.75 ± 5154	7.27 ± 3.04
cis $C_{18:1}$ (total isomers)	839057 ± 11246	19.92 ± 0.27	0	0
$C_{18:2}$ (total isomers)	2370369 ± 32278	56.28 ± 0.02	0	0
$C_{18:3}$ (total isomers)	339708.4 ± 39947	8.07 ± 0.84	0	0
$C_{20:0}$	18242.63 ± 12188	0.43 ± 0.01	0	0
$C_{20:1}$	6398.875 ± 62147	0.16 ± 0.01	0	0
$C_{22:0}$	18305.25 ± 45936	0.43 ± 0.02	0	0
Total	4211460 ± 362085	100	723960.1 ± 39481	100

*(Data represent the mean ± SD of triplicate analyses)

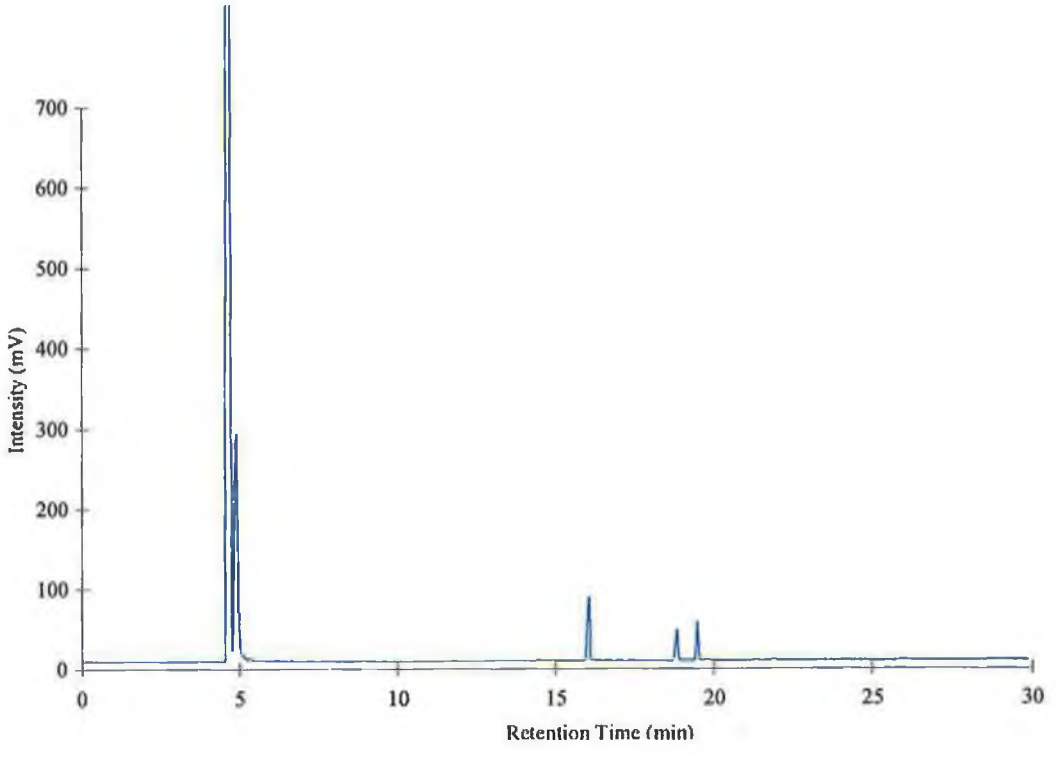
Table 2.8 Determination of TFA content of FAME sample containing ca. 5 % methyl elaidate by GLC analysis pre and post Ag^{+}-TLC.*

FAME	Area (counts) Pre-TLC	Area (%) Pre-TLC	Area (counts) Post-TLC	Area (%) Post-TLC
$C_{16:0}$	346013.4 ± 15039	8.51 ± 1.02	294717.5 ± 21947	47.61 ± 2.31
$C_{16:1}$	3322.75 ± 1021	0.08 ± 1.11	0	0
$C_{18:0}$	181357.7 ± 11592	4.46 ± 1.03	158730 ± 10711	25.64 ± 0.002
trans $C_{18:1}$(n-9)	199879.5 ± 12031	4.91 ± 1.54	165618.1 ± 2045	26.75 ± 1.07
cis $C_{18:1}$ (total isomers)	779103.8 ± 36251	19.15 ± 1.22	0	0
$C_{18:2}$ (total isomers)	2202521 ± 109921	54.15 ± 0.02	0	0
$C_{18:3}$ (total isomers)	315805.8 ± 11251	7.76 ± 1.25	0	0
$C_{20:0}$	16111 ± 1054	0.40 ± 0.01	0	0
$C_{20:1}$	6673 ± 2103	0.16 ± 0.05	0	0
$C_{22:0}$	16936.13 ± 1044	0.41 ± 0.00	0	0
Total	4067723 ± 331245	100	619065.6 ± 32579	100

*(Data represent the mean ± SD of triplicate analyses)



(a)



(b)

Figure 2.4 Chromatograms of FAME spiked with 5 % methyl elaidate (a) pre TLC and (b) post TLC.

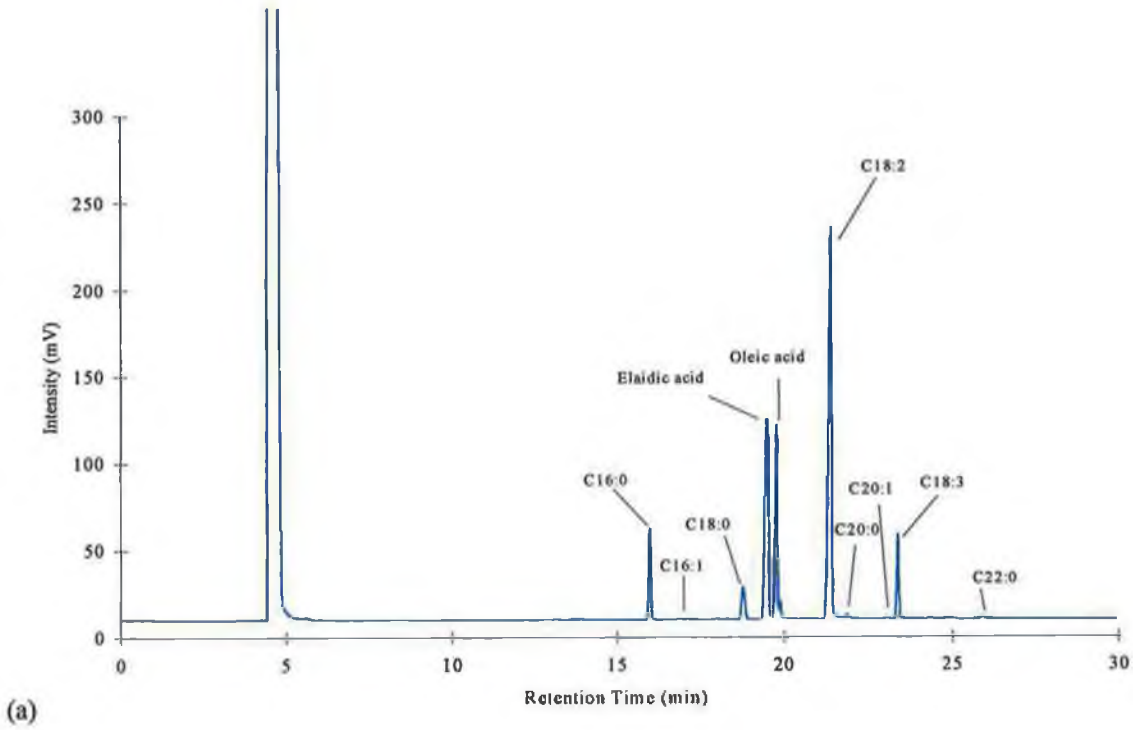
Similarly the soya oil sample spiked with approximately 25 % methyl elaidate was measured by GLC, pre- and post-Ag^+-TLC and the composition of the oil is presented in Table 2.9. The TFA present in the oil was found to be 23.4 % and 22.26 % for pre- and post-Ag^+-TLC analyses, respectively, again representing a 95 % recovery of TFA in the sample. Figures 2.5a and b show pre and post TLC chromatograms respectively, for the FAME sample spiked with 25 % elaidate.

In addition the analysis of soya oil spiked with 1 %, 5 % and 30 % trielaidin were analysed by GLC, pre and post-TLC and the results are presented in Tables 2.10-2.12. The purpose of this work was to compare the precision of analyses of the preformed FAME above with that of oil samples that were methylated in the laboratory prior to analysis using equation 1 in section 2.3.3. The fatty acid composition of the soya oil sample spiked with approximately 1 % trielaidin as measured by GLC, pre- and post-Ag^+-TLC is presented in Table 2.10. Using this data the quantity of TFA present in the oil was calculated relative to $C_{18:0}$ and was found to be 1.21 % and 1.13 % for pre- and post-Ag^+-TLC analysis, respectively. This represents a 93 % recovery rate of TFA and therefore fulfills the performance criteria. It is slightly lower than seen previously and may be a result of experimental error, arising from loss occurring during scraping of the *trans* band from the TLC plate.

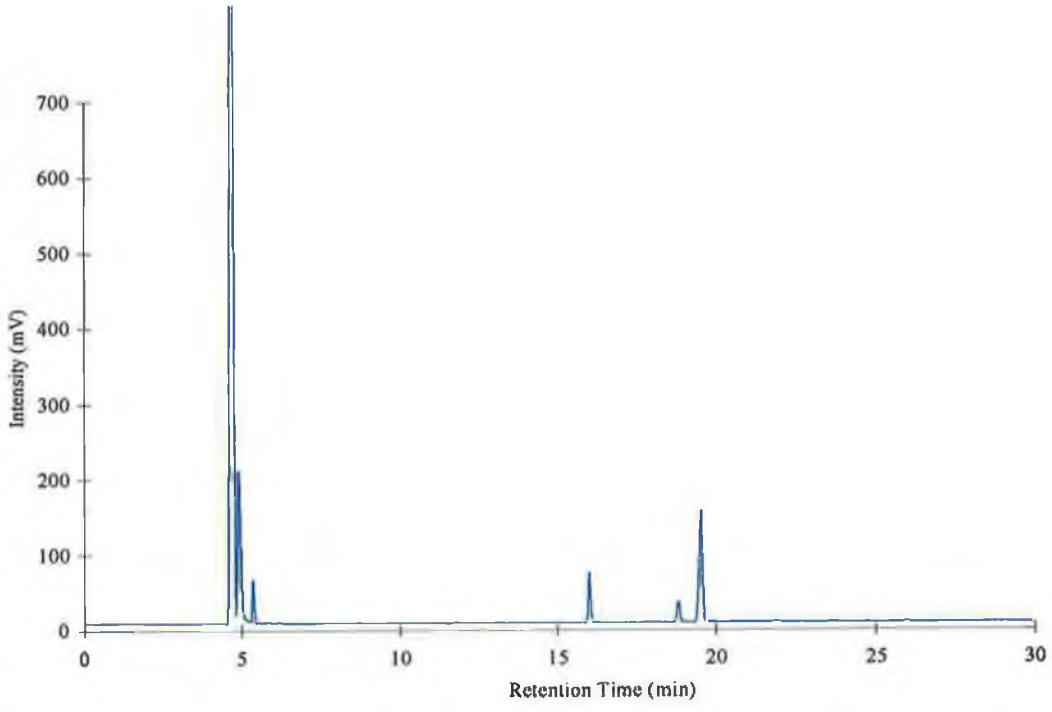
Table 2.9 Determination of TFA content of FAME sample containing ca. 25 % methyl elaidate by GLC analysis pre and post Ag^+-TLC.*

FAME	Area (counts) Pre-TLC	Area (%) Pre-TLC	Area (counts) Post-TLC	Area (%) Post-TLC
$C_{16:0}$	172898.3 ± 102214	7.08 ± 0.18	377310.8 ± 554691	21.44 ± 1.23
$C_{16:1}$	2080.75 ± 328	0.09 ± 0.01	0	0
$C_{18:0}$	91418.88 ± 5041	3.74 ± 0.21	195151.8 ± 30944	11.09 ± 5.07
trans $C_{18:1}$(n-9)	571724 ±4379	23.40 ± 0.71	1187132 ± 48715	67.47 ± 2.01
cis $C_{18:1}$ (total isomers)	381799.8 ± 10874	15.63 ± 0.58	0	0
$C_{18:2}$ (total isomers)	1051908 ± 45692	43.06 ± 2.35	0	0
$C_{18:3}$ (total isomers)	150007.6 ±4182	6.14 ± 1.08	0	0
$C_{20:0}$	8153.75 ± 1023	0.34 ± 1.11	0	0
$C_{20:1}$	4015.75 ± 546	0.16 ± 0.02	0	0
$C_{22:0}$	9001.25 ± 964	0.37 ± 0.01	0	0
Total	2443008 ± 51972	100	1759595 ± 129011	100

*(Data represent the mean ± SD of triplicate analyses)



(a)



(b)

Figure 2.5 Chromatograms of FAME spiked with 25 % methyl elaidate (a) pre TLC and (b) post TLC.

Table 2.10 Determination of TFA content of FAME sample containing ca. 1 % trielaidin by GLC analysis pre and post Ag^{+}-TLC.*

FAME	Area (counts) Pre-TLC	Area (%) Pre-TLC	Area (counts) Post-TLC	Area (%) Post-TLC
$C_{16:0}$	374133.3 ± 362014	9.03 ± 2.01	289966.8 ± 34561	67.71 ± 2.11
$C_{16:1}$	4326.75 ± 1023	0.10 ± 0.02	0	0
$C_{18:0}$	192658.6 ± 37251	4.65 ± 1.02	111247.5 ± 11021	25.98 ± 1.03
trans $C_{18:1}$(n-9)	50351.13 ± 41239	1.21 ± 0.01	27031.25 ± 1205	6.31 ± 0.02
cis $C_{18:1}$ (total isomers)	827941.5 ± 41236	19.98 ± 0.11	0	0
$C_{18:2}$ (total isomers)	2321006 ± 812054	55.99 ± 0.12	0	0
$C_{18:3}$ (total isomers)	329433.4 ± 354712	7.95 ± 0.23	0	0
$C_{20:0}$	19484.63 ± 102531	0.47 ± 0.17	0	0
$C_{20:1}$	6520.5 ± 1024	0.16 ± 0.01	0	0
$C_{22:0}$	18947.75 ± 11054	0.46 ± 0.12	0	0
Total	4144803 ± 542182	100	428245.5 ± 142897	100

*(Data represent the mean ± SD of triplicate analyses)

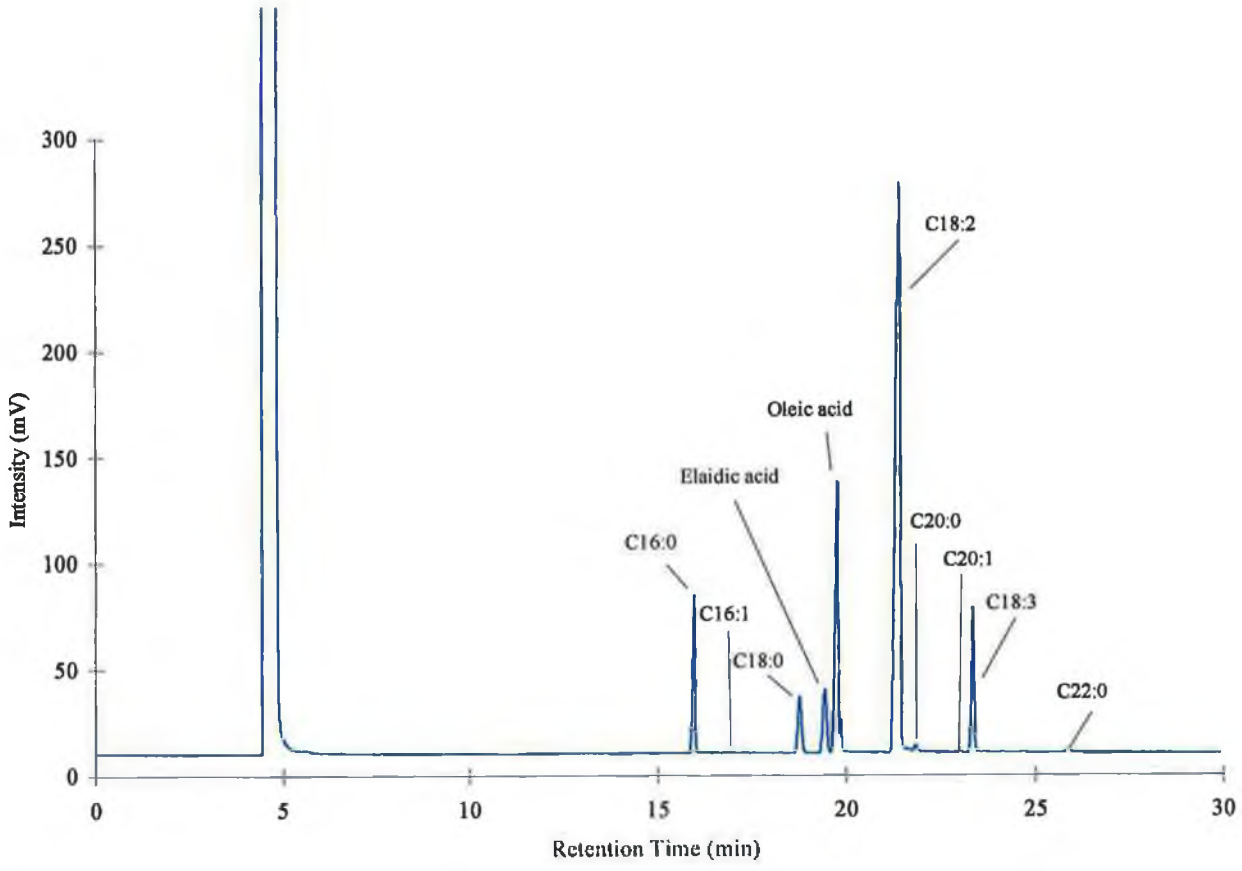
The fatty acid composition of the soya oil sample spiked with approximately 5 % trielaidin as measured by GLC, pre- and post-Ag^+-TLC is presented in Table 2.11. Using these data the quantity of TFA present in the oil was calculated relative to $C_{18:0}$ (using equation 1 in section 2.3.3) and was found to be 5.25 % and 5.12 % for pre- and post-Ag^+-TLC analysis, respectively. This represented a 97 % recovery of TFA and therefore fulfills the performance criteria. Figures 2.6a and b represent chromatograms of this sample pre and post-Ag^+-TLC.

Similarly the soya oil sample spiked with approximately 30 % trielaidin was measured by GLC, pre and post Ag^+-TLC and the composition of the oil is presented in Table 2.12. The TFA present in the oil was found to be 30.71 % and 27.34 % for pre- and post-Ag^+-TLC analysis, respectively, representing a 90 % recovery of TFA in the sample. Figures 2.7a and b represent the pre and post-Ag^+-TLC chromatograms for the FAME sample spiked with 30 % trielaidin. The criteria for precision analysis was fulfilled for all the samples within this section, and the repeatability and reproducibility of the method was verified by the low SD values for triplicate analysis on three separate days for each sample.

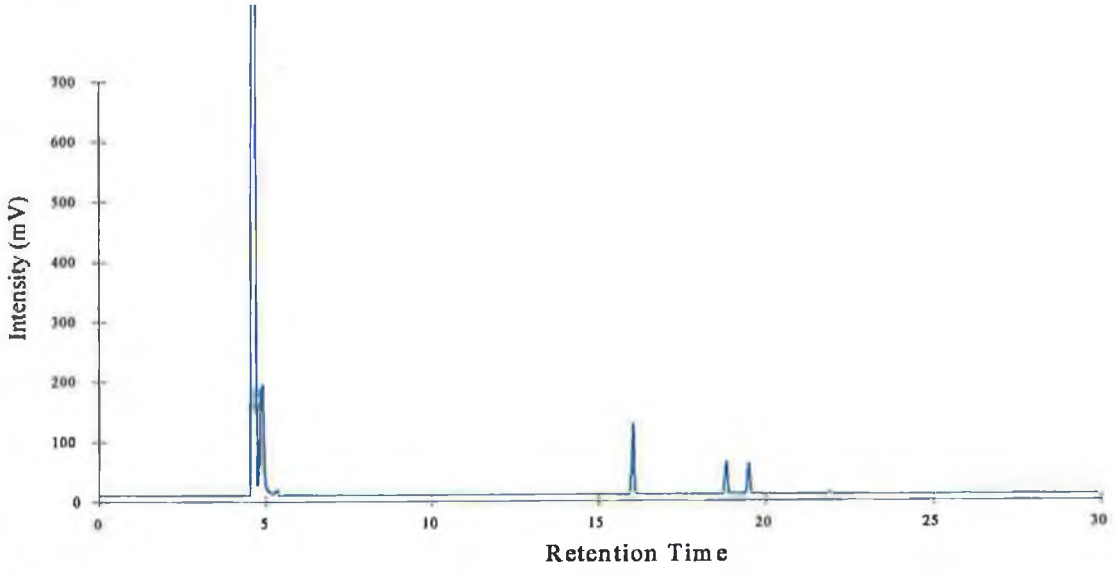
Table 2.11 Determination of TFA content of FAME sample containing ca. 5 % trielaidin by GLC analysis pre and post Ag^{+}-TLC.*

FAME	Area (counts) Pre-TLC	Area (%) Pre-TLC	Area (counts) Post-TLC	Area (%) Post-TLC
$C_{16:0}$	318282.4 ± 21301	8.59 ± 0.12	269178 ± 11203	47.96 ± 4.12
$C_{16:1}$	3650.5 ± 132	0.10 ± 0.01	0	0
$C_{18:0}$	164543.8 ± 1049	4.44 ± 0.04	135640.4 ± 52136	24.17 ± 3.70
trans $C_{18:1}$(n-9)	194350.8 ± 30124	5.25 ± 2.06	156446.1 ± 23882	27.87 ± 0.12
cis $C_{18:1}$ (total isomers)	707686.1 ± 4102	19.10 ± 1.20	0	0
$C_{18:2}$ (total isomers)	1990910 ± 52143	53.75 ± 1.39	0	0
$C_{18:3}$ (total isomers)	283267.3 ± 15764	7.65 ± 1.22	0	0
$C_{20:0}$	17903.63 ± 10244	0.48 ± 0.04	0	0
$C_{20:1}$	5969.375 ± 112	0.16 ± 0.01	0	0
$C_{22:0}$	17719.13 ± 1542	0.48 ± 0.01	0	0
Total	3704293 ± 50162	100	561264.5 ± 26314	100

*(Data represent the mean ± SD of triplicate analyses)



(a)



(b)

Figure 2.6 Chromatograms of FAME spiked with 5 % trielaidin (a) pre TLC and (b) post TLC.

Table 2.12 Determination of TFA content of FAME sample containing ca. 30 % trielaidin by GLC analysis pre and post Ag^{+}-TLC.*

FAME	Area (counts) Pre-TLC	Area (%) Pre-TLC	Area (counts) Post-TLC	Area (%) Post-TLC
$C_{16:0}$	113467.5 ± 1157	6.47 ± 0.75	245452.8 ± 30417	17.65 ± 1.47
$C_{16:1}$	1662.125 ± 102	0.09 ± 0.00	0	0
$C_{18:0}$	58299.5 ± 1064	3.32 ± 0.11	107902.5 ± 11012	7.76 ± 0.94
trans $C_{18:1}$(n-9)	538761.3 ± 21344	30.71 ± 0.02	1037611 ± 71121	74.60 ± 2.69
cis $C_{18:1}$ (total isomers)	247164.1 ± 26311	14.09 ± 1.22	0	0
$C_{18:2}$ (total isomers)	681634.9 ± 52413	38.86 ± 2.11	0	0
$C_{18:3}$ (total isomers)	97250.38 ± 1152	5.54 ± 0.31	0	0
$C_{20:0}$	6380.5 ± 1024	0.36 ± 0.02	0	0
$C_{20:1}$	2699 ± 374	0.15 ± 0.04	0	0
$C_{22:0}$	6804.75 ± 125	0.39 ± 0.03	0	0
Total	1754124 ± 10821	100	1390966 ± 21365	100

*(Data represent the mean ± SD of triplicate analyses)

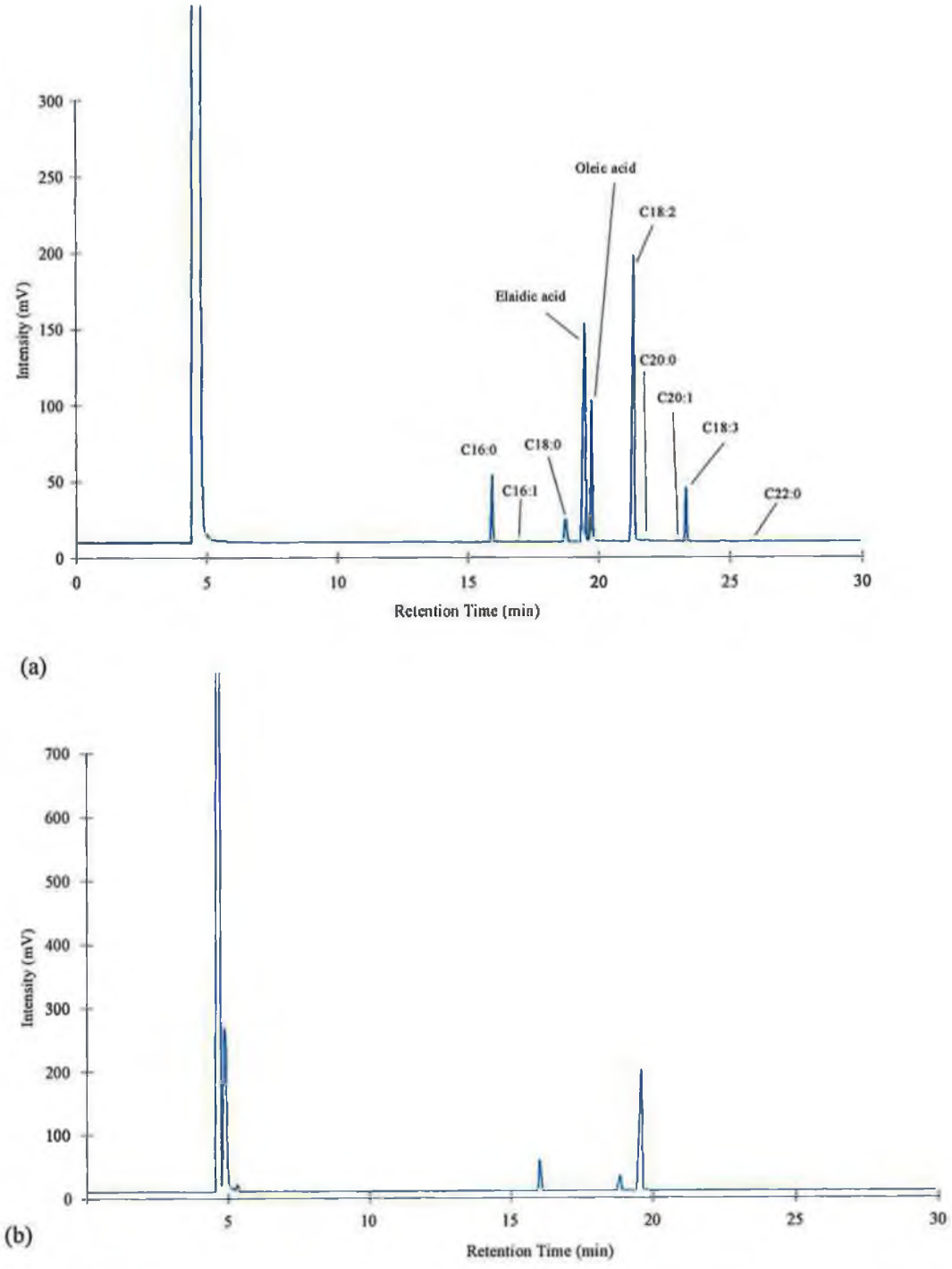


Figure 2.7 Chromatograms of FAME spiked with 30 % trielaidin (a) pre TLC and (b) post TLC.

2.4.5 Intercomparison testing

An intercomparison study was performed to analyse a number of samples using the methodology and conditions described above. Oils containing 1 % and 1.5 % TFA were analysed by GLC only, while oils containing 5 % and 25 % TFA were analysed pre-and post Ag^{+}-TLC by GLC. A series of performance criteria outlined in section 2.3.9 were met prior to analysis of each sample. Minor *trans* isomers of $C_{18:2}$ and $C_{18:3}$, in addition to $C_{18:1}$ were analysed as described in section 2.3.9 and the results for each oil, analysed in triplicate on two separate days, are summarised and presented as a series of tables (2.13-2.20).

The sample containing 1 % TFA, a physically refined soya oil, was analysed by GLC for TFA content and the results are presented in Table 2.13. The *trans* content of $C_{18:1}$ was found to be 0.11 % of total TFA, with *trans* $C_{18:2}$ content ranging between 0.56 and 0.62 % of total TFA which consisted mainly of the individual components *c*, *t* and *t*, *c* $C_{18:2}$ (Table 2.13). The *trans* $C_{18:3}$ was found to be 1 % of total TFA, which consisted mainly of *t*, *t*, *c* + *c*, *c*, *t* and *t*, *c*, *c* $C_{18:3}$ and the total TFA for this sample was found to be 1.7 % (Table 2.13). Certain performance criteria (as described in 2.3.9) were fulfilled prior to analysis of the sample, the average concentration of individual FAME in CRM 162 (Table 2.14) following analysis was within the range outlined in BCR guidelines (Table 2.1).

Table 2.13 TFA analysis of a physically refined soya oil containing approximately 1 % TFA.*

TFA	Day I	Day II
	Area (%)	Area (%)
trans $C_{18:1}$ (total isomers)	0.11 ± 0.033	0.10 ± 0.021
t,t $C_{18:2}$	0.01 ± 0.012	0.01 ± 0.001
*c*9, *t* 13 $C_{18:2}$	0.00	0.00
c, t $C_{18:2}$	0.30 ± 0.015	0.34 ± 0.019
t, c $C_{18:2}$	0.24 ± 0.002	0.26 ± 0.010
other $C_{18:2}$ isomers	0.01 ± 0.003	0.01 ± 0.001
t,t,t $C_{18:3}$	0.00	0.00
c,t,t + *t,c,t* $C_{18:3}$	0.04 ± 0.003	0.04 ± 0.007
t,t,c + *c,c,t* $C_{18:3}$	0.48 ± 0.005	0.49 ± 0.004
c,t,c $C_{18:3}$	0.00	0.00
t,c,c $C_{18:3}$	0.42 ± 0.001	0.42 ± 0.004
other $C_{18:3}$ isomers	0.04 ± 0.011	0.06 ± 0.002
trans $C_{18:1}$ (total isomers)	0.11 ± 0.033	0.10 ± 0.021
trans $C_{18:2}$ (total isomers)	0.56 ± 0.030	0.62 ± 0.029
trans $C_{18:3}$ (total isomers)	0.98 ± 0.018	1.01 ± 0.018
total *trans* isomers	1.65 ± 0.001	1.73 ± 0.002

*(Data represent the mean ± SD of triplicate analyses performed on separate days)

Table 2.14 Performance criteria for intercomparison study. Analysis of CRM 162 and *trans*-13/*cis*-9 $C_{18:1}$ and all-*cis* $C_{18:3}$/*cis*-11 $C_{20:1}$ for 1 % and 1.5 % TFA samples.*

CRM 162 (Individual FAME)	Day I % total fatty acids	Day II % total fatty acids	Mean
$C_{16:0}$	10.21 ± 0.12	10.05 ± 0.04	10.13 ± 0.113
$C_{18:0}$	2.95 ± 0.12	2.87 ± 0.14	2.91 ± 0.0569
$C_{18:1}$	24.9 ± 0.02	24.72 ± 0.11	24.81 ± 0.127
$C_{18:2}$	56.5 ± 0.17	56.61 ± 0.12	56.56 ± 0.077
$C_{18:3}$	4.17 ± 0.12	4.18 ± 0.17	4.18 ± 0.0056
RESOLUTION (R)		Retention time (min)	Peak base width (mm)
$C_{18:1}$ *trans* 13		18.93 ± 0.00	3.12 ± 0.01
$C_{18:1}$ *cis* 9		19.04 ± 0.01	3.20 ± 0.02
R			1.02 ± 0.01
all *cis* $C_{18:3}$		22.64 ± 0.00	5.30 ± 0.01
$C_{20:1}$ *cis* 11		22.34 ± 0.00	5.10 ± 0.00
R			1.42 ± 0.01

*(Data represent the mean ± SD of triplicate analyses performed on separate days)

An R value ranging from 1.02 to 1.42 (Table 2.14) between critical pairs *trans*-13/*cis*-9 $C_{18:1}$ and all-*cis* $C_{18:3}$ /*cis*-11 $C_{20:1}$ was achieved fulfilling the performance criteria.

The sample containing 1.5 % TFA, a physically refined rapeseed oil, was analysed by GLC for TFA content and the results are presented in Table 2.15. The *trans* content of $C_{18:1}$ was found to be 0.08-0.10 % of total TFA, with *trans* $C_{18:2}$ content ranging between 0.16 and 0.20 % of total TFA which consisted mainly of the individual components *c*, *t* and *t*, *c* $C_{18:2}$ (Table 2.15). The *trans* $C_{18:3}$ was found to be 0.64-0.67 % of total TFA, which consisted mainly of *t*, *t*, *c* + *c*, *c*, *t* and *t*, *c*, *c* $C_{18:3}$ and the total TFA for this sample was found to range from 0.88-0.97 % (Table 2.15). The performance criteria (as described in section 2.3.9) were fulfilled prior to analysis of the sample, as this sample was analysed on the same day as the sample spiked with 1 % TFA the performance criteria for both samples was the same (Table 2.14).

The sample containing 5 % TFA, an oil as used for manufacturing margarine (primarily palm oil), was analysed pre and post TLC by GLC in triplicate on two separate days (Table 2.16). The *trans* content pre-TLC of $C_{18:1}$ was found to be 4.2-4.3 % of total TFA, with *trans* $C_{18:2}$ content ranging between 2.41 and 2.68 % of total TFA which consisted mainly of the individual components *c*, *t* and *t*, *c* $C_{18:2}$ (Table 2.16).

Table 2.15 TFA analysis of a physically refined rapeseed oil containing approximately 1.5 % TFA.*

TFA	Day I	Day II
	Area (%)	Area (%)
trans $C_{18:1}$ (total isomers)	0.10 ± 0.002	0.08 ± 0.002
t,t $C_{18:2}$	0.04 ± 0.001	0.01 ± 0.001
*c*9, *t* 13 $C_{18:2}$	0.00	0.00
c, t $C_{18:2}$	0.08 ± 0.001	0.09 ± 0.003
t, c $C_{18:2}$	0.06 ± 0.000	0.05 ± 0.001
other $C_{18:2}$ isomers	0.02 ± 0.001	0.01 ± 0.000
t,t,t $C_{18:3}$	0.00	0.00
c,t,t + *t,c,t* $C_{18:3}$	0.01 ± 0.002	0.01 ± 0.004
t,t,c + *c,c,t* $C_{18:3}$	0.31 ± 0.032	0.31 ± 0.010
c,t,c $C_{18:3}$	0.00	0.00
t,c,c $C_{18:3}$	0.25 ± 0.012	0.25 ± 0.021
other $C_{18:3}$ isomers	0.10 ± 0.001	0.07 ± 0.002
trans $C_{18:1}$ (total isomers)	0.10 ± 0.000	0.08 ± 0.001
trans $C_{18:2}$ (total isomers)	0.20 ± 0.001	0.16 ± 0.031
trans $C_{18:3}$ (total isomers)	0.67 ± 0.011	0.64 ± 0.012
total *trans* isomers	0.97 ± 0.011	0.88 ± 0.021

*(Data represent the mean ± SD of triplicate analyses performed on separate days)

Table 2.16 TFA analysis of manufacturing margarine (primarily palm oil) containing 5 % TFA pre-TLC. *

TFA	Day I	Day II
	Area (%)	Area (%)
trans $C_{18:1}$ (total isomers)	4.32 ± 0.21	4.25 ± 1.05
t,t $C_{18:2}$	0.17 ± 0.01	0.13 ± 0.01
*c*9, *t* 13 $C_{18:2}$	0.00	0.00
c, t $C_{18:2}$	1.00 ± 0.01	0.90 ± 0.31
t, c $C_{18:2}$	0.97 ± 0.07	0.87 ± 0.24
other $C_{18:2}$ isomers	0.54 ± 0.03	0.51 ± 0.05
t,t,t $C_{18:3}$	0.00	0.00
c,t,t + *t,c,t* $C_{18:3}$	0.02 ± 0.00	0.02 ± 0.00
t,t,c + *c,c,t* $C_{18:3}$	0.11 ± 0.01	0.11 ± 0.01
c,t,c $C_{18:3}$	0.00	0.00
t,c,c $C_{18:3}$	0.08 ± 0.00	0.07 ± 0.00
other $C_{18:3}$ isomers	0.04 ± 0.00	0.00
trans $C_{18:1}$ (total isomers)	4.32 ± 0.21	4.25 ± 0.01
trans $C_{18:2}$ (total isomers)	2.68 ± 0.15	2.41 ± 0.04
trans $C_{18:3}$ (total isomers)	0.25 ± 0.01	0.20 ± 0.01
total *trans* isomers	7.25 ± 0.30	6.86 ± 1.02

*(Data represent the mean ± SD of triplicate analyses performed on separate days)

The *trans* $C_{18:3}$ content pre-TLC was found to range between 0.20 and 0.25 % of total TFA, which consisted mainly of *t*, *t*, *c* + *c*, *c*, *t* and *t*, *c*, *c* $C_{18:3}$ and the total TFA for this sample was found to be 6.8-7.2 % (Table 2.16). The TLC bands corresponding to $C_{18:0}$ and *trans* $C_{18:1}$ were analysed by GLC in triplicate on two separate days, and the data presented in Table 2.17. Using values from table 2.17, the *trans* monoene content found by Ag^+-TLC and GLC was calculated as described in section 2.3.3. The *trans* $C_{18:1}$ content post-TLC was 4.21-4.53 % (Table 2.17) which was similar to the pre-TLC *trans* $C_{18:1}$ content which was 4.2-4.3 % (Table 2.16). The performance criteria (as described in 2.3.9) were again fulfilled prior to analysis of the sample on each day (Table 2.18).

The sample containing 25 % TFA was a partially hydrogenated sunflower oil and was analysed pre- and post-Ag^+-TLC by GLC. The *trans* content pre-TLC of $C_{18:1}$ was found to be 11-12 % of total TFA, with *trans* $C_{18:2}$ content ranging between 5.54 and 6.22 % of total TFA which consisted mainly of the individual components *c*, *t* and *t*, *c* $C_{18:2}$ and other unidentified *trans* $C_{18:2}$ (Table 2.19). The *trans* $C_{18:3}$ pre-TLC was found to be 0.07 % of total TFA, which consisted of unidentified *trans* $C_{18:3}$ and was much lower than seen for the other oils analysed. The total TFA for this sample was found to range between 16.25 and 18.29 % (Table 2.19). The TLC bands corresponding to $C_{18:0}$ and *trans* $C_{18:1}$ from the hydrogenated sunflower oil were analysed by GLC in triplicate on two separate days, and are presented in Table 2.20. The % area of $C_{18:0}$ obtained prior to Ag^+-TLC analysis was found to be 9.28-10.36 % of TFA, and using these values (Table 2.20) the *trans* monoene content found by Ag^+-TLC and GLC was calculated. The *trans* $C_{18:1}$ post-TLC content was found to be 11.15-12.02 % (Table 2.20) which was similar to the pre-TLC *trans* $C_{18:1}$ content (Table 2.19). Certain performance criteria (as described in 2.3.9) were fulfilled prior to analysis of the sample on each day (Table 2.18).

Table 2.17 TFA analysis of manufacturing margarine (primarily palm oil) containing 5 % TFA post TLC. *

FAME	Day I	Day II
	Area (counts)	Area (counts)
$C_{18:0}$ (post-TLC)	271686 ± 10234	385478 ± 20915
trans $C_{18:1}$ (total isomers post-TLC)	193668 ± 10227	262691 ± 10477
$C_{18:0}$ by area (%) (pre-TLC)	6.36 ± 0.03	6.32 ± 0.04
% *trans* monoenes found by combined Ag^{+}-TLC and GLC	4.53 ± 0.21	4.21 ± 0.01

*(Data represent the mean ± SD of triplicate analyses performed on separate days)

Table 2.18 Performance criteria for intercomparison study. Analysis of CRM 162 and *trans*-13/*cis*-9 $C_{18:1}$ and all-*cis* $C_{18:3}$/*cis*-11 $C_{20:1}$ for 5 % and 30 % TFA samples. Data represent the mean and standard deviation of triplicate analysis carried out on two separate days.

CRM 162 (Individual FAME)	Day I % total fatty acids	Day II % total fatty acids	Mean
$C_{16:0}$	10.15 ± 0.01	10.17 ± 0.01	10.16 ± 0.01
$C_{18:0}$	2.91 ± 0.02	2.87 ± 0.02	2.89 ± 0.02
$C_{18:1}$	24.77 ± 0.12	24.79 ± 0.01	24.78 ± 0.01
$C_{18:2}$	56.64 ± 0.01	56.67 ± 0.01	56.66 ± 0.02
$C_{18:3}$	4.17 ± 0.02	4.23 ± 0.05	4.20 ± 0.04
RESOLUTION		Retention time (min)	Peak base width (mm)
$C_{18:1}$ *trans* 13		18.92 ± 0.01	3.10 ± 0.00
$C_{18:1}$ *cis* 9		19.07 ± 0.01	3.20 ± 0.01
R			1.11 ± 0.00
all *cis* $C_{18:3}$		22.64 ± 0.24	5.04 ± 0.04
$C_{20:1}$ *cis* 11		22.34 ± 0.12	5.01 ± 0.12
R			1.21 ± 0.01

Table 2.19 TFA analysis of a partially hydrogenated sunflower oil containing 25 % TFA Pre-TLC. *

TFA	Day I	Day II
	Area (%)	Area (%)
trans $C_{18:1}$ (total isomers)	10.64 ± 0.01	12.00 ± 0.44
t,t $C_{18:2}$	0.31 ± 0.01	0.34 ± 0.01
*c*9, *t* 13 $C_{18:2}$	0.00	0.00
c, t $C_{18:2}$	2.00 ± 0.01	2.24 ± 0.12
t, c $C_{18:2}$	1.95 ± 0.01	2.20 ± 0.01
other $C_{18:2}$ isomers	1.28 ± 0.02	1.44 ± 0.04
t,t,t $C_{18:3}$	0.00	0.00
c,t,t + *t,c,t* $C_{18:3}$	0.00	0.00
t,t,c + *c,c,t* $C_{18:3}$	0.00	0.00
c,t,c $C_{18:3}$	0.00	0.00
t,c,c $C_{18:3}$	0.00	0.00
other $C_{18:3}$ isomers	0.07 ± 0.01	0.07 ± 0.00
trans $C_{18:1}$ (total isomers)	10.64 ± 0.01	12.00 ± 0.44
trans $C_{18:2}$ (total isomers)	5.54 ± 0.11	6.22 ± 0.41
trans $C_{18:3}$ (total isomers)	0.07 ± 0.01	0.07 ± 0.00
total *trans* isomers	16.25 ± 0.12	18.29 ± 0.47

*(Data represent the mean ± SD of triplicate analyses performed on separate days)

Table 2.20 TFA analysis of a partially hydrogenated sunflower oil containing 25 % TFA post TLC. *

FAME	Day I	Day II
	Area (counts)	Area (counts)
$C_{18:0}$ (post-TLC)	515737 ± 2048	482259 ± 10332
trans $C_{18:1}$ (total isomers post-TLC)	619693 ± 61241	560827 ± 10662
$C_{18:0}$ by area (%) (pre-TLC)	9.28 ± 0.84	10.36 ± 0.33
% *trans* monoenes found by combined Ag^+-TLC and GLC	11.15 ± 1.69	12.02 ± 0.24

*(Data represent the mean ± SD of triplicate analyses performed on separate days)

2.5 Discussion

In view of the possible harmful effects of dietary TFA, accurate analysis of these fatty acids is necessary to monitor their levels in foods. Estimates of intakes (8-12 g/day) are approximations based on limited data and problematic analytical techniques (ASCN/AIN, 1996). Previous analytical tools were inadequate for complete analysis of TFA, mainly due to the unavailability of suitable reference material, particularly those of minor, unusual *cis* and *trans* isomers (Ratnayake and Beare-Rogers, 1990). Therefore a study to validate methodology was waranted.

This study validated combined Ag^{+}-TLC and GLC methodology for the quantification of TFA in vegetable oils. Initially the accuracy and separation power of the method was established and the effects of FAME preparation on the level of *trans* was investigated. Furthermore data from the analysis of common samples among different laboratories was intercompared which led to critical assessment and validation of the methodology for TFA analysis used in this study.

Using a test mixture composed of $C_{14:0}$, $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ (10 mg/ml) accuracy of the GLC was assessed by comparing the known mass with the % peak areas post GLC analysis. The % peak areas for individual FAME (Table 2.2) corresponded closely with the known % mass of the fatty acids, suggesting that decomposition and irreversible adsorption did not occur, which was supported by the relative RF values (relative to $C_{18:1}$), which did not differ by more than 0.05 from 1.0. Therefore the relevant performance criteria were met, whether self-prepared samples were analysed and the RF

values calculated within the laboratory or unknown test mixtures were analysed and the RF values compared with data from similar analysis in other laboratories (see Appendix 1).

The effect of derivative formation on glyceride bound unsaturated fatty acids was assessed by the methylation and analysis of three triglyceride samples of known composition ($C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$) and comparison of the data with those obtained from an identical preformed FAME sample. This acceptance criterion was not fulfilled, as the difference between the % area values obtained for $C_{16:0}$, for preformed FAME (Table 2.5) and the samples methylated in the laboratory (Table 2.4) was greater than 0.2 %. However, criteria were fulfilled for the other fatty acids analysed $C_{18:0}$, $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$ as the % area values for preformed FAME (Table 2.5) and the samples methylated in the laboratory did not differ by more than 0.2 % (Table 2.4). A similar result was seen by other laboratories when results were compiled and the 0.2 % target was missed (Appendix 2). The rRF (relative response factor) for pre-formed $C_{18:3}$ methyl ester, the fatty acid species most likely to autoxidise, was > 1.05 in every other lab. The same was true for glyceride bound $C_{18:3}$, regardless of type of derivative formation (methanolic KOH, sodium methoxide, BF_3). In order to find sources for the deviations observed within the analysis from all laboratories several critical parameters in the ester preparation step and the subsequent GLC process were tested (i.e. commercial sources of test mixtures, instrumentation (different capillary columns and different GLC equipment), injection technique, storage stability and type of derivative formation (HCL, BF_3, Na-methoxide as catalysts)). On examination neither equipment related factors such

as type of capillary column, GLC instrument, injection technique nor derivative formation procedures influenced the response factor of $C_{18:3}$ to a measurable extent. It has been observed previously that *trans* $C_{18:3}$ fatty acids co-elute with C_{20} fatty acids which may also be a possible source of error (Christie *et al.*, 1989).

Poor storage stability and / or purity of commercially available $C_{18:3}$ standards prepared from mono-acidic triglycerides may be a likely error source. Poor stability may be due to the fact that PUFAs are prone to autoxidise very rapidly (Christie *et al.*, 1989). Stability studies typically measure peroxide values (PV) and p-Anisidine values (PAV) (Christie *et al.*, 1989). Other potential error sources (pre-formed FAME) included the possibility of a weighing error, and oxidative deterioration of bound PUFAs. For triglyceride bound fatty acids it was found to be problematic to recover PUFAs quantitatively and stability problems were observed with glyceride bound PUFAs. A natural oil with a certified fatty acid profile such as CRM 162, proved to be the most effective means to demonstrate fitness for purpose of the GLC technique used for *trans* analysis in this case. It demonstrated that use of a proven methylation method in conjunction with a very long and highly polar capillary column is an effective process for the quantification of even highly unsaturated FAME.

The GLC program used was highly effective for the separation of critical pairs of geometrical and positional isomers of unsaturated fatty acids (e.g. *trans*-13 / *cis*-9 $C_{18:1}$ and all *cis* $C_{18:3}$/*cis*-11 $C_{20:1}$). The acceptance criterion was a chromatographic resolution of at least 1.00. The fractionation of *cis* and *trans* isomers of $C_{18:1}$ by Ag^{+}-TLC according

to the geometry of the double bond was also highly effective, as there was no cross contamination in either fraction (i.e. no *cis* peak in the *trans* fraction or vice versa) and recovered fractions were not contaminated with other FAME.

Precision of TFA methodology was demonstrated by the successful recovery of > 90 % of added methyl elaidate or trielaidin contained in soya oil FAME or soya oil using a combination of Ag^+-TLC and GLC. In the overall analysis involving all laboratories, the standard deviation (SD) for reproducibility varied between 2.18 % and 4.47 % for samples spiked with 23.09 g and 1.09 g methyl elaidate /100 g fatty acid methyl esters and between 3.28 % and 6.19 % for samples spiked with 30.21 g and 1.08 g trielaidin /100 g soya oil (Appendix 3). Therefore the methods employed i.e. Ag^+-TLC and GLC in this section produced accurate and precise data for TFA analysis and the method was deemed suitable for use in a certification exercise for oils of known quantities of TFA. Precision of the Ag^+-TLC and GLC was deemed comparable to data published in the certification report of CRM 162 regarding the low and medium TFA levels (ISO Guide 30, 1981), and somewhat poorer regarding high TFA levels. However the measuring principal used in these experiments was far more sophisticated compared with the simple GLC analysis of the fatty acid spectrum used in the certification exercise of CRM 162 (ISO Guide 30, 1981). Furthermore, RSD (R) (Residual Standard Deviation) figures were well within the expected range of reproducibility (RSD (R) between 10 % and 40 %) (F. Ulberth) (Appendix 4).

The intercomparison study involved the analysis of preliminary test materials (refined soya oil with approximately 1 %, 1.5 % 5 %, 25 % TFA) all of which had undergone homogenicity and stability tests based on BCR guidelines. Since the RSD(R) (Appendix 5) did not range between 10 and 40 % for the combined analysis from all groups the analysis was repeated. The problem areas with the first intercomparison were the quantification of *trans*-monoenes in the 1 % TFA sample and *trans*-polyenes in the 5 % and 30 % TFA samples (Appendix 4). The quantities of TFA were low and therefore underestimated. In order to correct this in the second intercomparison, the peak area reject value was decreased and all integrated peaks were reported. It has been shown previously that column overload or the presence of large amounts of *cis* isomers negatively affect the resolution of *trans* monoenes (Ratnayake and Beare-Rogers, 1990). In addition previous studies found many $C_{18:2}$ and $C_{18:3}$ *trans* polyenes were not correctly identified (Siguel, 1993) however in this study many peaks were found to overlap. This was corrected by dilution of the sample prior to GLC analysis and better resolution was obtained enabling identification of all *trans* polyenes present. This is in agreement with Ratnayake and Beare-Rogers (1990) who found that dilution and smaller sample load was found to be effective in improving resolution between *cis* and *trans* isomers. This is due to the presence of large quantities of saturated fatty acids leading to large peak areas, which co-elute with the minor *trans* polyene peaks. Therefore dilution to smaller quantities enables separation between these peaks as the peak area for each fatty acid is reduced. Wolff (1994) demonstrated that variation of the column temperature causes $C_{20:1}$ to elute in different positions before, after or with $C_{18:3}$ on CP Sil 88 column. There was poor resolution between $C_{20:1}$ and $C_{18:3}$ *tcc*, in this study; however following dilution

of the sample prior to GLC analysis, it was possible to separate these peaks, which was imperative as otherwise values for total *trans* $C_{18:3}$ would be greatly overestimated. Due to these modifications sufficient conformity of results was achieved, between the various laboratories. The RSD(R) for total TFA ranged between 10 and 40 % for the combined analysis from all laboratories; therefore the analysis was deemed successful and fulfilled the predefined criteria (Appendix 6).

The methodology used for TFA analysis in this study was validated and found to be useful for the accurate analysis of TFA in foods. In addition, the method involving Ag^{+}-TLC in combination with GLC may be used in the certification of reference materials containing TFA. These would then allow for the accurate quantification of TFA by all analysing laboratories given that such certified reference material for TFA are currently not available. In addition there is currently inadequate information on the TFA content of foods (ASCN/AIN, 1996). The fats and oils industry world wide is rapidly responding to pressure to give consumers a wide range of choices with respect to household table fats and spreads (Haumann, 1998). New food technologies, including improvements in the hydrogenation process (temperature, pressure, time, catalyst, methods, starting oils), interesterification and genetic engineering of oil seed plants to modify the fatty acid composition of oils have the potential to reduce the TFA content of foods (ASCN/AIN, 1996). There are movements in several countries to mandate the inclusion of the TFA content of margarine and other products on the food label (Katan *et al.,* 1994, 1995). This is a complex area and health claim issues have been raised, but because of inadequate data, modifications to labeling may be premature. Suggestions considered by

a Task Force (ASCN/AIN, 1996) included the addition or inclusion of *Trans* fatty acids with saturated fatty acids on labels. However, saturated fatty acids are currently defined without subtypes (ASCN/AIN, 1996). Including *trans* fatty acids with saturated fatty acids would then change this to a group. Secondly, *trans* fatty acids should be labeled as a separate class, as currently, *trans* fatty acids are not included on the nutrition label in the listing of unsaturated fatty acids. Some *trans* fatty acids may then be classified with *cis* monounsaturated fatty acids and some with polyunsaturated fatty acids. They are not mandated by law to be listed on the nutrition label. Some food manufacturers and processors have elected voluntarily to list unsaturated fatty acids (excluding *trans* fatty acids) on the nutrition label. However to reach a decision on the labeling issue, accurate data must be acquired, comparable with that for saturated fats concerning the intake of *trans* fatty acids, their biological effects and associated mechanisms of action, and their relation to disease (ASCN/AIN, 1996). Because of public health concerns regarding the amount of fat in the American diet, the Food and Drug Administration and the US Department of Agriculture have emphasized use of the recently reformed food label to inform consumers about the fat and fatty acid contents of foods (Scarbrough, 1997). Issues that must be addressed to further improve the communication effectiveness of the food label include health effects of n-3 and n-6 fatty acids; appropriate labeling of *trans* fatty acids, $C_{18:0}$, and other non-cholesterol-raising fatty acids; partially absorbed fats; and label claims, especially health claims, for specific fatty acids and fatty acids of biotechnologically altered foods (Scarbrough, 1997).

2.6 Summary

Trans fatty acids are formed during refining and catalytic hydrogenation of vegetable oils and occur naturally in ruminant fats. In view of current interest in their physiological effects, an accurate analysis of these fatty acid isomers in the human diet and in biological samples is of considerable importance. Since no single routine method exists for the accurate quantification of TFA, the analyst has to choose among different methods, taking into account what targets have to be met. In this study a combination of Ag^+-TLC and GLC was used for the analysis of test mixtures of soya oil FAME and soya oil samples spiked with 1, 5 and 30 % methyl elaidate and trielaidin and four samples of edible oils containing different levels of TFA (1, 1.5, 5, 30%). Results were compared with data obtained from other laboratories. A chromatographic resolution greater than 1.00 was achieved for separation of the critical pairs *trans*-13 / *cis*-9 $C_{18:1}$ and all *cis* $C_{18:3}$/*cis*-11. Recovery of TFA in all samples was in excess of 90 % following Ag^+-TLC. The methodology used for TFA analysis in this study was validated and found to be useful for the accurate analysis of TFA in foods. In addition, the method involving Ag^+-TLC in combination with GLC may be used in the certification of reference materials containing TFA. These would then allow for the accurate quantification of TFA by all analysing laboratories given that such certified reference material for TFA are currently not available.

CHAPTER 3

Enrichment of the conjugated linoleic acid content of bovine milk fat by dry fractionation*

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3.1 Introduction

Epidemiological studies have suggested a protective effect of dietary CLA against breast cancer in humans (Knekt *et al.,* 1996, Lavillonnière and Bougoux 1999). Relatively rich dietary sources of CLA include milk fat-containing foods, such as butter and cheese, whose CLA contents were reported as 9.4 and 8.4 mg/g fat, respectively (Fritsche and Steinhart, 1998).

One of the mechanisms by which CLA occurs in milk fat is by microbial biohydrogenation of dietary linoleic acid in the rumen (Kepler and Tove, 1967). The *c*9, *t*11 CLA isomer thus formed in the rumen is rapidly converted to *t*11-$C_{18:1}$ the main positional TFA isomer, which represents approximately 50 % of the total *trans* octadecanoic acids in milk fat (Kepler *et al.,* 1966). Milk fat CLA may also be produced endogenously from *t*11-$C_{18:1}$ in mammary tissue by Δ9 desaturase (Griinari and Bauman, 1999). Elaidic acid (*t*9-$C_{18:1}$) is a minor component of milk fat (Wolff *et al.,* 1998) but is the predominant *trans* isomer in partially hydrogenated vegetable fats. A number of studies have demonstrated that TFA intake, particularly those of vegetable oil origin, is associated with alteration of plasma lipid and lipoprotein concentrations, thereby implicating these as atherogenic fatty acids (Judd *et al.,* 1994, Mensink and Katan, 1990, Zock and Katan, 1992). Some epidemiological evidence also suggests an association between TFA intake and risk of coronary heart disease (CHD) (Ascherio *et al.*, 1994, Willett *et al.,* 1993). However, other studies that have estimated TFA intake, did not identify an association with increased risk of CHD (Aro *et al.,* 1995, Roberts *et al.*, 1995). Milk fat consists of a mixture of triglycerides that exhibit a broad and variable melting range, from approximately –40 °C to +40 °C.

This allows the crystallising out of a series of triglycerides at temperatures below their melting points by controlled cooling of the melt. Since the process does not use solvents, it is known as 'dry fractionation' and has resulted in the production of food grade ingredients for products such as spreadable butters, chocolate and bakery products (Kaylegian and Lindsay, 1995). The use of milk fat fractions was reported at approximately 50,000 tonnes per year, representing approximately 20 % of the butteroil market (Deffense, 1995). The slip-point of milk fat ranges between 31.5 and 32.5 °C (Keogh and Higgins, 1986). The fatty acid compositional changes observed in high melting fats (35 °C to 25 °C) include a decrease in C_4 to C_{10} fatty acids, a slight increase in C_{12} to C_{15} fatty acids and an increase in C_{16} to C_{18} saturated fatty acids (Dimick *et al.,* 1996). In the low melting fractions (25 °C to 10 °C), the main compositional changes include an increase in concentrations of C_4 to C_{10} and C_{12} to C_{15} fatty acids, and a decrease in C_{16} to C_{18} saturated fatty acids compared with the parent fat (Dimick *et al.,* 1996). The $C_{18:1}/C_{18:0}$ ratio is also generally higher for low melting fractions than for other fractions (Kaylegian and Lindsay, 1995). Increased utilisation of milk fat may thus be facilitated by dry fractionation into fractions markedly different from one another in composition and melting point. Few studies have examined the CLA content of milk fat fractions, although a recent study reported a 2.5-fold enrichment of milk fat in CLA following urea crystallisation (Kim and Liu, 1999).

3.2 Objectives

In this study, the effect of laboratory scale dry fractionation on CLA enrichment of milk fat fractions was investigated, the levels achieved were then compared with the CLA and TFA content of a range of food products.

3.3 Materials and methods

3.3.1 Milk fat fractionation

Anhydrous milk fat (Dairygold, Mitchelstown, Co. Cork) was fractionated into hard and soft fractions by the following method. Pre-melted milk fat was cooled from 60 °C to the initial fractionation temperature (33 °C) in a 500 ml beaker, placed in a Lauda programmable water bath (AGB Scientific, Dublin) capable of a variable cooling gradient. Programmed cooling at selected cooling rates (0.58, 0.74, 1.17 and 2.8 °C/h) was then initiated until the final fractionation temperatures (19, 15 and 10 °C) were reached. Agitation at 16 rpm was initiated immediately using a paddle stirrer which swept within 6 mm of the beaker wall and continued for 2 h at the final fractionation temperature. The fraction containing the crystallised fat (hard fraction) was then separated from the soft fraction by centrifugation (Sanyo MSC Mistral 2000R, Gallencamp PLC, Leicester, UK) at 2,500 g for 5 min at the final fractionation temperature.

3.3.2 Fat extraction from selected foods

Fat was extracted from a range of food products including spreads, butter, yoghurt and cheese and the CLA, TFA and total fatty acid composition analysed by GLC as described below. The probiotic cheese used, which harboured the strain *Enterococcus faecium* PR88 strain was manufactured as described previously (Gardiner *et al.*, 1999) and analysed following 7 months of ripening. Fat was extracted from the butter and spread samples according to the IDF method No. 593/E (IDF, 1992), from yoghurt by

the method by Falih *et al.* (1977) and from cheese by the method of McNeill and Connolly (1989).

3.3.3 Fatty acid analysis by GLC

For CLA analysis of fat samples, fatty acid methyl esters (FAME) were prepared using acid-catalysed methanolysis and analysed by GLC as previously described (Stanton *et al.*, 1997a). The FAME were separated on a Supelcowax-10 capillary GLC column (Supelco Inc, Bellefonte, PA) (60 m x 0.32 mm i.d., 0.25 μm film thickness), using a Varian 3500 GLC (Varian, Harbor City, CA, USA) which was fitted with a flame ionisation detector (FID). The *c*9, *t*11 isomer of CLA was identified by retention time with reference to a CLA standard mix (Nu-CheK Prep Inc., Elysian, MN, USA). Individual fatty acids (*t*11-$C_{18:1}$) were identified with reference to the retention times of FAME standards (Sigma Chemical Co., St. Louis, USA), and quantified as g/100g FAME, or with reference to the internal standard $C_{13:0}$, and expressed as mg/g fat.

For TFA (*t*11-$C_{18:1}$) analysis of fat extracted from foods, FAMEs were prepared using base-catalysed methanolysis and TFA were separated by silver ion thin layer chromatography (Ag^+-TLC), and subsequently analysed by GLC as previously described (Ulberth and Henninger, 1992). Separation of TFA by Ag^+-TLC is briefly described as follows. The FAMEs were applied to 0.25 mm silica gel plates impregnated with 10 % (w/v) $AgNO_3$ in acetonitrile (stationary phase) and placed in a mobile phase of hexane : diethyl ether (9 : 1 v/v) to develop for approximately 3 h. The plates were then allowed to dry at room temperature and were stained with 0.05 % (w/v) rhodamin B in ethanol. The *trans* track, visible under UV light, was eluted

from the silica in hexane prior to GLC analysis on a Chrompack CP Sil 88 column (60 mm x 0.25 mm i.d., 0.02 μm film thickness) (Chrompack, Middleburg, The Netherlands). The injector and detector temperatures were 140 and 250 °C, respectively and the N_2 carrier gas was 20 ml/min. The column temperature was programmed from 140 °C with an initial delay of 8 min, followed by an increase to 200 °C, at a rate of 8.5 °C/min and held for 15 min.

3.4 Results

3.4.1 Effects of dry fractionation on CLA content of the soft fraction

Using final fractionation temperatures of 19, 15 or 10 °C and variable cooling rates, increases in CLA content were obtained in the soft fraction over the parent fat (Table 3.1). Cooling rate had an influence on the CLA content of the resulting soft fraction, with increased cooling rate generally resulting in reduced CLA content (Table 3.1). The conditions for maximum CLA-enrichment were found to be a final fractionation temperature of 10 °C and a cooling rate of 0.58 °C/h, which resulted in an increase from 1.36 to 2.2 g/100g FAME (63 %) in the CLA content over the parent AMF. The yield of this CLA-enriched fraction was 30 % of the parent AMF (Table 3.1). Refractionation of this fraction using the same fractionation conditions resulted in a reduction in the CLA content of approximately 10 % (Table 3.1). The effect of agitation on CLA yield was also investigated, using the soft fraction generated at a final temperature of 19 °C and a cooling rate of 0.74 °C/h, which had a CLA content of 2.07 $\pm$ 0.01 g/100g FAME. Agitation had a negative impact on the CLA content of

this fraction, resulting in reductions of 8.3 and 12.0 %, after 1 and 2 h agitation, respectively.

3.4.2 Fatty acid composition

The fatty acid compositions of the parent AMF and the hard and soft fractions obtained following fractionation to a final temperature of 10 °C, at a cooling rate of 0.58 °C/h are given in Table 3.2. The concentration of C_{16} - C_{18} saturated fatty acids analysed was higher in the hard fraction and similar or slightly reduced in the soft fraction to the parent AMF. Conversely, the concentration of all unsaturated fatty acids analysed were higher in the soft fraction and similar or slightly reduced in the hard fraction, compared with the parent AMF. The concentrations of $C_{16:0}$ and $C_{18:0}$ acids were increased by 6.7 and 24.7 %, respectively, in the hard fraction. The concentrations of $C_{18:1}$ and CLA were increased by 25.6 and 63 %, respectively, compared with the parent AMF. C_{18} polyunsaturated fatty acids, in addition to *t*11-$C_{18:1}$ were also increased (by ~ 30 %) in the soft fraction, compared with the parent AMF. The concentration of C_{16} - C_{18} saturated fatty acids analysed was higher in the hard fraction and similar or slightly reduced in the soft fraction to the parent AMF.

Table 3.1 The effect of different temperature ranges, cooling rates and refractionation on CLA content of milk fat

Temperature range °C	Cooling Rate °C/h	CLA g/ 100g FAME [1]	Yield of soft fraction % (w/w) [1]
Parent AMF[2]		1.36 ± 0.02	
33 °C – 19 °C	2.80	1.91 ± 0.05	37.8 ± 1.20
33 °C – 19 °C	1.17	2.06 ± 0.12	40.1 ± 2.40
33 °C – 19 °C	0.74	2.07 ± 0.01	39.4 ± 1.60
33 °C – 19 °C	0.58	2.07 ± 0.21	36.5 ± 0.52
33 °C - 15 °C	0.74	2.10 ± 0.06	30.6 ± 0.11
33 °C - 15 °C	0.58	2.12 ± 0.10	31.2 ± 0.02
33 °C - 10 °C	0.74	2.14 ± 0.08	29.4 ± 1.21
33 °C - 10 °C	0.58	2.22 ± 0.01	30.0 ± 1.12
Refractionation			
33 °C - 10 °C	0.58	2.01 ± 0.10	26.4 ± 1.81

[1]Mean of triplicate analyses ± standard deviation

[2]AMF; anhydrous milk fat

Conversely, the concentration of all unsaturated fatty acids analysed were higher in the soft fraction and similar or slightly reduced in the hard fraction, compared with the parent AMF. The concentrations of $C_{16:0}$ and $C_{18:0}$ acids were increased by 6.7 and 24.7 %, respectively, in the hard fraction. The concentrations of $C_{18:1}$ and CLA were increased by 25.6 and 63 %, respectively, compared with the parent AMF. C_{18} polyunsaturated fatty acids, in addition to *t*11-$C_{18:1}$ were also increased (by ~ 30 %) in the soft fraction, compared with the parent AMF.

3.4.3 CLA and TFA content of selected food products

The CLA and TFA contents of a selection of butter, spreads, yoghurts and cheeses are shown in Table 3.3. Spreads containing hydrogenated vegetable fat only as the fat source contained negligible amounts of CLA (0.3 mg/g fat), but relatively high TFA content, compared with products containing only milk fat as the fat source. Dairy spreads, which contained a blend of milk fat and vegetable oils, contained substantially less CLA (0-3.3 mg/g fat) than butter (12.3–14.2 mg/g fat) (Table 3.3). Furthermore, the TFA content of butter was approximately 3-fold less than dairy spreads (Table 3.3). Of all the dairy products analysed, butter was found to be the richest source of CLA, while the yoghurt and cheese samples analysed, contained, on average, one and two-thirds, respectively, of the CLA content of butter. It should be noted that these samples were retail products, and as such were most likely manufactured from different milk, obtained at different stages throughout the season and most likely with varying milk fat CLA concentrations. The CLA content of goats milk yoghurt was found to be similar to that of cows' milk yoghurt. However goat cheese contained much less CLA than other cheese samples analysed (Table 3.3).

Table 3.2: Fatty acid composition of milk fat fractions obtained by dry fractionation[1]

Fatty acid	Starting AMF g /100g FAME [2]	Hard fraction g/100g FAME [2]	Soft fraction g /100g FAME [2]
$C_{12:0}$	4.66 ± 0.02	4.20 ± 0.01	4.00 ± 0.12
$C_{12:1}$	0.19 ± 0.01	0.18 ± 0.02	0.24 ± 0.01
$C_{14:0}$	15.68 ± 0.11	15.52 ± 1.02	12.2 ± 0.21
$C_{14:1}$	1.18 ± 0.15	0.94 ± 0.10	1.28 ± 0.16
$C_{15:0}$	0.83 ± 0.02	0.82 ± 0.12	0.98 ± 0.04
$C_{16:0}$	30.60 ± 0.12	32.65 ± 0.01	27.2 ± 0.21
$C_{16:1}$	1.89 ± 0.02	1.21 ± 0.12	2.27 ± 0.07
$C_{17:0}$	0.74 ± 0.01	0.81 ± 0.01	0.82 ± 0.01
$C_{18:0}$	14.00 ± 0.42	17.46 ± 1.51	12.83 ± 0.05
$C_{18:1}$	22.60 ± 0.51	19.68 ± 0.54	28.39 ± 1.62
$C_{18:1}$ vaccenic	4.05 ± 0.12	4.10 ± 0.11	5.18 ± 0.08
$C_{18:2}$	0.89 ± 0.01	0.71 ± 0.01	1.19 ± 0.12
$C_{18:2}$ CLA	1.36 ± 0.21	1.31 ± 0.01	2.22 ± 0.01
$C_{18:3}$	0.88 ± 0.01	0.41 ± 0.02	1.20 ± 0.10

[1]Fractionation conditions used to obtain the fractions were a temperature range of 33 to 10 °C and a cooling rate of 0.58 °C h^{-1}

[2] Mean of triplicate analyses ± standard deviation

Table 3.3 CLA and TFA content of selected food products.

Product	CLA mg/g fat *	TFA mg/g fat *
Dairy Spreads (n = 8) (vegetable oil: milk fat)		
1. 100 : 0	0	65.2 ± 11.21
2. 100 : 0	0.5 ± 0.01	102.4 ± 6.80
3. 99 : 1	≤ 0.01	112.2 ± 4.21
4. 99 : 1	0	125.2 ± 0.02
5. 70 : 30	2.5 ± 0.02	133.2 ± 0.57
6. 70 : 30	1.8 ± 0.04	134.7 ± 3.48
7. 70 : 30	3.3 ± 0.10	154.3 ± 2.05
8. 70 : 10	1.9 ± 0.01	155.7 ± 1.69
Butter (n = 4)		
1. Butter	12.6 ± 0.12	50.7 ± 0.02
2. Butter	13.4 ± 0.25	50.3 ± 0.51
3. Butter	12.3 ± 0.31	60.9 ± 0.11
4. Butter oil	14.2 ± 0.01	53.3 ± 0.12
Yoghurt (n=4)		
1. low fat milk + live cultures	4.2 ± 0.11	50.1 ± 2.40
2. low fat milk + live cultures	3.9 ± 0.05	39.5 ± 1.34
3. low fat milk + live cultures	3.3 ± 0.30	37.4 ± 0.02
4. goats' milk + live cultures	4.6 ± 0.17	34.8 ± 1.05
Cheese (n = 10)		
1. probiotic cheese (Cheddar)	9.3 ± 0.12	57.4 ± 1.02
2. Cheddar	9.2 ± 1.10	58.2 ±0.44
3. Cheddar	16.2 ± 0.51	45.6 ± 3.21
4. Cheddar	6.9 ± 0.24	36.3 ± 1.45
5. Emmental	6.6 ± 0.30	26.2 ± 0.20
6. Camembert	7.2 ± 0.01	22.9 ± 2.37
7. Processed Cheddar	8.3 ± 0.11	33.2 ± 1.11
8. Blue cheese	4.0 ± 0.11	32.7 ± 0.51
9. Gouda	2.3 ± 0.01	29.4 ± 0.12
10. Goat cheese	1.5 ± 0.04	11.5 ± 0.11

* Mean of triplicate analyses ± standard deviation

3.5 Discussion

This study demonstrates that fractionation of milk fat is a suitable means of enriching the CLA content over the starting AMF. In view of the protective effect against risk of mammary cancer development of CLA-enriched butter (Ip *et al.*, 1999a), milk fat fractionation may offer an alternative means by which a health-beneficial food can be produced. The maximum CLA enrichment obtained was 63 % over the parent fat, yielding a milk fat fraction containing 2.22 g CLA /100g FAME. A few approaches have already been investigated to increase the CLA content of milk fat, and in particular, animal feeding regimes, using dietary oil supplements have been effective at elevating milk fat CLA concentrations (Dhiman *et al.*, 1999a,b, Kelly *et al.*, 1998a, Lawless *et al.*, 1998, Stanton *et al.*, 1997a). An enrichment of milk fat CLA content to 41 mg/g total fatty acids (Ip *et al.*, 1999a) was achieved by feeding a selected herd of cows with supplements of sunflower oil, as described (Kelly *et al.*, 1998a). Milk fat CLA contents of 24.0 and 19.6 mg/g fat were achieved through supplementation of cows on pasture with full fat rapeseeds and full fat soybeans, respectively (Lawless *et al.*, 1998). Another approach for production of milk fat enriched in CLA involved urea crystallisation, which resulted in a 250 % increase in the CLA content, to 12.7 mg/g fat (Kim and Liu, 1999).

In addition to CLA, *t*11-$C_{18:1}$, the major *trans* fatty acid in milk fat, may also act as a beneficial fatty acid, since it is converted to the *c*9, *t*11-CLA isomer by the Δ9-desaturase enzyme (Griinari and Bauman, 1999). Following animal dietary supplementation, a strong positive correlation has been observed between milk fat CLA and *t*11-$C_{18:1}$concentrations (Jiang *et al.*, 1996, Lawless *et al.*, 1998). Following

milk fat fractionation in this study, the CLA-enriched milk fat fraction was increased in *t*11-$C_{18:1}$ content by 28 %, compared with the parent fat. The CLA-enriched soft fraction was similar in saturated fatty acid content and higher in unsaturated fatty acids compared with the parent milk fat. The compositional changes observed in the CLA-enriched fraction from this study are similar to those in the CLA-enriched milk fat fraction obtained by urea fractionation (Kim and Liu, 1999), which was higher in $C_{18:1}$ and lower in $C_{16:0}$ and $C_{18:0}$, compared with the parent fat.

As the final temperature of fractionation decreased from 19 to 10 °C, the yield of the soft fraction also decreased, from 38.5 to 30 %, respectively while the yield of the hard fraction increased. Previously, it has been shown that the final temperature had a significant effect on the yield of linoleic acid in the soft fraction (Keogh and Higgins, 1986), while our study showed that final temperature had a negligible effect on CLA yield. Furthermore, it was shown that slow stirring increased the yield of the soft fraction and produced cleaner fractions (Keogh and Higgins, 1986). In this study, agitation for 2 h of the partially crystallised fat post-fractionation further decreased the amount of CLA present in the soft fraction and increased the CLA content of the hard fraction. This may be due to the structure of the triglyceride containing the conjugated double bond, which has a less kinked structure than that of linoleic acid and therefore may have been incorporated more easily to a crystal structure containing $C_{16:0}$ and $C_{18:0}$. Crystal growth also occurs during agitation (Black, 1975) which possibly may entrap the triglycerides into the hard fraction. In previous studies using similar procedures for obtaining hard and soft fractions, the hard fractions have found applications in various products, including butter, confectionery items, chocolate and soups (Kaylegian and Lindsay, 1995).

The enrichment of 63.2 % in the CLA content represented approximately a 3-fold increase in CLA over a range of milk fat containing-food products analysed in this study. Previous studies (Chin *et al.,* 1992, Fritsche and Steinhart, 1998) have reported lower CLA contents of butter and cheese compared with the values reported in this study. The differences may be due to the higher CLA content of the milk fat from which the food products were manufactured, which in turn, may be due to differences in the feeding regimes of the dairy herds. Milk produced in Ireland is mostly from cows on pasture, and this is associated with relatively high milk fat CLA concentrations (Kelly *et al.* 1998b, Stanton *et al.,* 1997b). In addition, the milk may have been obtained at different stages throughout the season, which may also have influenced the CLA content of the milk fat, as was previously demonstrated (Stanton *et al.,* 1997b). Other factors shown to influence milk fat CLA concentrations include animal feeding regime and dietary supplements (Kelly *et al.,* 1998a, Lawless *et al.,* 1998). A large variation was observed in the CLA content of the cheeses (1.5–16.2 mg/g fat), which may be due to variations in the CLA content of the cheese milk, an effect of the different manufacturing processes, or the use of different cultures in the cheese. Studies have demonstrated that such parameters as temperature, protein quality, choice of starter culture and period of ageing can affect the CLA content of dairy and meat products (Fritsche and Steinhart, 1997, Garcia-Lopez *et al.,* 1994, Jiang *et al.,* 1997, Lin *et al.,* 1995, Shantha *et al.,* 1994 a,b), although no effect of cheese manufacture was observed by others (Dhiman *et al.*, 1999b, Werner *et al.*, 1992). Differences have also been observed in the CLA content of the milk of different species with the highest level reported in sheep's milk followed by cow's milk and then goat's milk (Jahreis *et al.,* 1999). This may account for the lower CLA

content of the goat cheese in this study, but yoghurt manufactured from goats' milk did not display lower CLA content that other samples. The TFA content of the products containing hydrogenated vegetable oils was higher than those containing milk fat, an observation made previously (Ackman and Mag, 1998).

The fractionation of milk fat for CLA-enrichment may provide an alternative method to approaches involving dietary oil supplements, which have led to elevated milk fat CLA and *t*11-$C_{18:1}$. Furthermore, milk fat fractionation had a positive impact on the *t*11-$C_{18:1}$ and polyunsaturated fatty acid content of the CLA enriched fraction. Consumption of CLA-enriched butter oil was associated with anticancer effects in rats, and enhanced accumulation of CLA in rat tissue resulted from the butter consumption, compared with CLA from synthetic sources (Ip *et al.*, 1999a). It is suspected that the *t*11-$C_{18:1}$ may act as the precursor of endogenous CLA synthesis. Thus, milk fat enriched in CLA and *t*11-$C_{18:1}$, by dry fractionation may offer a means to generate a food ingredient with potential benefits to human health.

3.6 Summary

This study investigated the effect of fat fractionation on the CLA (*c*9, *t*11-$C_{18:2}$) content of bovine milk fat. Anhydrous milk fat was fractionated into hard and soft fractions using controlled cooling and agitation. Fractionation of milk fat pre-melted at 60 °C using a temperature programme of 33 °C to 10 °C and a cooling rate of 0.58 °C/h yielded a soft fraction containing 63.2 % more CLA (2.22 g/100 g FAME), which was also enriched in polyunsaturated fatty acids and *trans* vaccenic acid (*t*11-$C_{18:1}$) compared with the parent fat. Agitation following fractionation was found to

have a negative effect on the CLA content of the soft fraction. Refractionation of the soft fraction did not increase the yield of CLA. The CLA and TFA content of 26 selected food products ranging in milk fat content from 0 to 100 % is reported. CLA concentrations ranged from 0 to 16.2 mg/g fat and were generally lower than the TFA content which ranged from 0 to 155.7 mg/g fat. Spreads containing vegetable oils contained higher TFA and lower CLA contents than milk fat-containing products. This study highlights that a milk fat fraction enriched in CLA may be achieved by dry fractionation.

CHAPTER 4

Antioxidant enzyme defence responses of human MCF-7 and SW480 cancer cells to conjugated linoleic acid *

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4.1 INTRODUCTION

A study of the relationship between conjugated polyunsaturated fatty acid-induced cytotoxicity and lipid peroxidation has revealed the significance of the conjugated double bond system in enhancing superoxide radical generation in cancer cells (Cornelius *et al.*, 1991). The ability of antioxidants to prevent cytotoxicity caused by parinaric acid, a polyunsaturated fatty acid with 4 conjugated double bonds, is evidence that lipid peroxidation significantly contributes to cytotoxicity. Whether CLA mediates a cancer cell - specific prooxidant effect similar to parinaric acid remains to be determined. Critical factors influencing *in vitro* oxidation of CLA include temperature, presence of metal ions, extent of aeration, hydration, concentration and degree of esterification (Chen *et al.*, 1997, Van den Berg *et al.*, 1995, Yurawecz *et al.*, 1995). The oxidative susceptibility of CLA was higher than that of LA when tested in a membrane model system composed of 1-palmitoyl -2-linoleoyl phosphatidyl choline under different metal-dependent and independent oxidising conditions (Van den Berg *et al.*, 1995). Such prooxidant inferences may need to be interpreted cautiously in view of other studies showing that CLA when oxidised produces furan fatty acids which may themselves have antioxidant properties (Yurawecz *et al.*, 1995). Although oxidising conditions have been implicated in a wide variety of degenerative processes including cancer, the influence of the oxidative susceptibility of CLA in cancer cells has yet to be elucidated. Antioxidant activity, as measured by the amount of TBARS in mammary tissue was reported by Ip *et al.* (1991) in the CLA-fed DMBA cancer inducing rat model. The antioxidant activity

was independent of dietary CLA concentration above 0.25 % and did not correlate with the concentration of CLA causing maximal tumour inhibition.

In another study (Ip *et al.,* 1996), supplementation of rats fed a 20 % corn oil diet with 1 % CLA for 2 months resulted in lower levels of mammary tissue MDA compared to control rats not receiving CLA, however the levels of 8-hydroxydeoxyguanosine (a marker of oxidatively damaged DNA) were unchanged between the two groups. It is apparent from these studies that the ability of CLA to inhibit peroxidation of neutral lipids in mammary tissue may not be effective at protecting cell DNA against damage by reactive oxygen species and that its antioxidant efficacy may not be important in cancer protection.

CLA is incorporated into rat mammary and mouse forestomach tumour phospholipids as well as neutral lipids (Ha *et al.,* 1990, Ip *et al.*, 1991), and therefore the potential of CLA to modulate cell membrane-mediated events and alter fat metabolism makes defining its role on cellular oxidative tone of great importance. In view of findings that genes for a number of proteins affecting cell proliferation and various antioxidants are induced in response to oxidative stress (Janssen *et al.,* 1993), a study of the effects of CLA on the antioxidant defence system of cancer cells may provide information on the oxidative stress status of cancer cells when exposed to CLA.

4.2 Objectives

To investigate the effects of varying dose and time responses of a mixture of conjugated linoleic acid isomers on the growth of MCF-7 breast cancer cells and SW480 colon cancer cells and to explore the effect of CLA on protein, DNA and RNA synthesis. To investigate if the growth suppressive effects of CLA are mediated by specific effects on lipid peroxidation, by examining the effect of CLA on the antioxidant defence enzymes catalase, SOD and GPx.

4.3 Materials and methods

4.3.1 Cell culture

Human breast (MCF-7) and colon (SW480) cancer cell lines were obtained from the American Type Culture Collection, (Rockville, MD, USA). Culture media and supplements were purchased from GIBCOBRL (Paisley, Scotland). The MCF-7 cells were maintained in Dulbecco's Minimum Essential Medium supplemented with 5 % v/v fetal bovine serum, 0.2 mM L-glutamine, 1 mM HEPES, and 1 unit/ml penicillin and streptomycin. The SW480 cells were maintained in the same medium as for MCF-7 cells containing, in addition, 10 mM sodium pyruvate. Cells were grown in Falcon T-25 cm^2 flasks and maintained at 37 °C in a humidified atmosphere. The pH of the media was maintained at 7.2-7.4 by a required flow of 95 % air and 5 % CO_2. Using these conditions the effects of CLA (Nu-Chek Prep, Inc., Elysian, MN, USA) on the growth of

colon and mammary cancer cells were determined. Initially 1 x 10^6 cells were plated in flasks and cultured for 24 hours allowing the cells to attach to the substratum. The medium was then replaced with medium alone or medium supplemented with various concentrations of CLA, 0 (control) 5, 10 and 20 ppm CLA, dissolved in ethanol. Control flasks were supplemented with ethanol to a final concentration of 0.1 % (v/v) as in experimental flasks. Following incubation, cells were harvested using phosphate buffered saline (PBS) containing 0.25 % (w/v) trypsin and counted on days 4, 8 and 12. Following each cell count, the cells were again incubated in fresh medium containing the appropriate concentration of CLA. Cell viability was determined using the Trypan blue exclusion (0.1 % w/v) method. Days 4, 8 and 12 corresponded to the times when the control flasks were at 20 %, 60 %, and 100 % confluency, respectively.

4.3.2 Radiolabelled precursor incorporation into protein, RNA and DNA

The MCF-7 and SW480 cells (1 x 10^6) were cultured in appropriate medium for 24 hours prior to beginning the experiment. The medium was then replaced with medium alone or medium supplemented with 20 ppm CLA. The SW480 cells were incubated with CLA at 37 °C for 2 days and the MCF-7 cells were incubated with CLA for 6 days, when both cultures were pulsed for 6 h with L-[4,5-^{3}H]leucine (163 Ci/mmol, 5μCi/flask), [6-^{3}H]uridine (17.8 Ci/mmol, 5μCi/flask), or [5-^{3}H]thymidine (163 Ci/mmol, 5μCi/flask) as described by Shultz *et al.* (1992a). Following incubation with the radioactive precursors, the medium was removed and cells harvested and centrifuged at 1000 x g for 5 min. Cells were then washed three times in cold PBS and the supernatant discarded. One ml of cold 10 % (w/v) trichloroacetic acid (TCA) was added to the cell pellet and the

mixture vortexed for 1 min. The tubes were maintained on ice for 15 min and centrifuged at 750 x g for 10 min. The pellet was washed three times in TCA (10 % w/v) and resuspended in 100 µl of Protosol tissue solubilizer (Amarsham Pharmacia Biotech, Little Chalfont, Buckinhamshire, UK) and digested for 60 min at 30 °C. The digested material was transferred to liquid scintillation vials with 3 ml methanol followed by 12 ml of scintillation fluid and counted in a Beckman LS 6500 scintillation counter. Counting efficiency of tritium [^{3}H] was 63 %. The amount of radioactivity in the TCA-insoluble material was a measure of the incorporation into protein, RNA and DNA.

4.3.3 Lipid peroxidation analysis

The TBARS test as described previously (Esterbaur and Cheeseman, 1990) was used to measure MDA. Cells were seeded as described earlier and after incubation for 24 hours the medium was replaced with medium alone or medium supplemented with varying concentrations of CLA as described above. At days 4, 8 and 12, cells were harvested and disrupted by the addition of 2 ml of 10 % (w/v) TCA to precipitate the protein. The precipitate was pelleted by centrifugation and an aliquot of the supernatant was reacted with an equal volume of 0.67 % (w/v) TBA in a boiling waterbath for 20 mins. After cooling, absorbance was measured at 535 nm and the concentration of MDA was calculated based on an ε value of 153,000. All solutions were freshly prepared on the day of assay.

4.3.4 Antioxidant enzyme assays

SOD and GPx activity were determined using Ransod and Ransel kits (Randox Laboratories Ltd., Armagh, Northern Ireland), respectively. The units of GPx activity are defined as nmol NADPH oxidised/min at pH 7.4 and 30 °C. 1 unit of SOD activity is that quantity of enzyme that inhibits by 50 % the conversion of xanthine by xanthine oxidase to a formazon dye. Catalase activity was determined as described (Aebi, 1984) and expressed in milli Bergmeyer units which are defined as nmol [H_2O_2] decomposed min^{-1} ml^{-1} at pH 7 and 25 °C.

4.3.5 Statistical analysis

The experimental design for GPx, TBARS, SOD, catalase and growth curve was a split-plot with three replicates. The main plot effects were CLA, cell lines, CLA x cell lines and replicates, the sub-plot effects were days, CLA x days, cell lines x days and CLA x cell lines x days. In the isotope studies, there was only a single time point and the design was a randomised block with effects CLA, cell lines, CLA x cell lines and replicates. The data was analysed using GENSTAT and the least significant difference was used as a guide to determine specific differences within the effects.

4.4 RESULTS :

4.4.1 Cell growth experiments

The effect of incubation with CLA (5-30 ppm) on the viability of MCF-7 and SW480 cancer cell lines is shown in Figure. 4.1. Though SW480 cells displayed more vigorous

growth than MCF-7 cells, there was a significant decrease ($p < 0.001$) in cell numbers for both cell lines when supplemented with varying concentrations of CLA over a period of 12 days, with a statistically greater ($p < 0.001$) cytotoxic influence on MCF-7 cells than on SW480 cells. Maximal growth suppression occurred in both cell lines following supplementation with 15-30 ppm CLA for 8 to 12 days.

4.4.2 Radiolabelled precursor incorporation studies.

The effects of CLA on radiolabeled leucine, uridine and thymidine incorporation into protein, RNA and DNA respectively is shown in Table 4.1. Both MCF-7 and SW480 cell lines incorporated 91-93 % less ^{3}H leucine, 56-91 % less ^{3}H uridine and 73-91 % less ^{3}H thymidine when supplemented with 7.14×10^{-5} M (20 ppm) CLA than did unsupplemented control cells. Though the inhibitory effect of CLA on uridine and thymidine incorporation was more pronounced in MCF-7 cells (8.6 % and 9.0 % respectively, relative to controls) than in SW480 cells (43.1 % and 27.3 % respectively, relative to controls) inhibition of leucine incorporation was similar in both cell lines.

4.4.3 Lipid peroxidation analysis.

Levels of intracellular MDA measured by the TBARS assay increased significantly ($p < 0.001$) in a dose and time-dependent manner in both cell lines, the effect being greater in the MCF-7 cells (Figure 4.2a) than in SW480 cells (Figure 4.2b). Following 4 days of supplementation of MCF-7 cells with CLA (30 ppm), TBARS increased six-fold compared with controls (0.03 nmoles MDA/10^{3} cells).

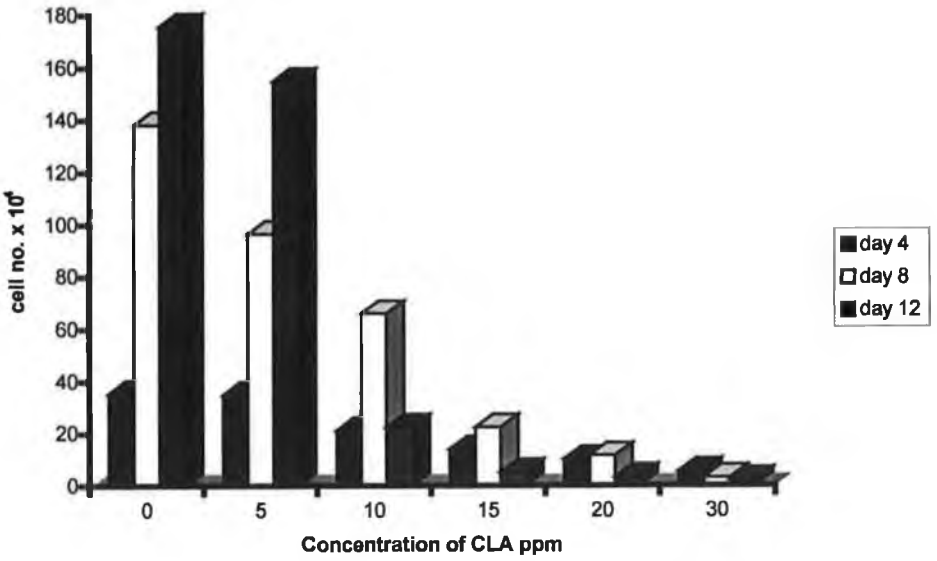

Figure 4.1 (a)

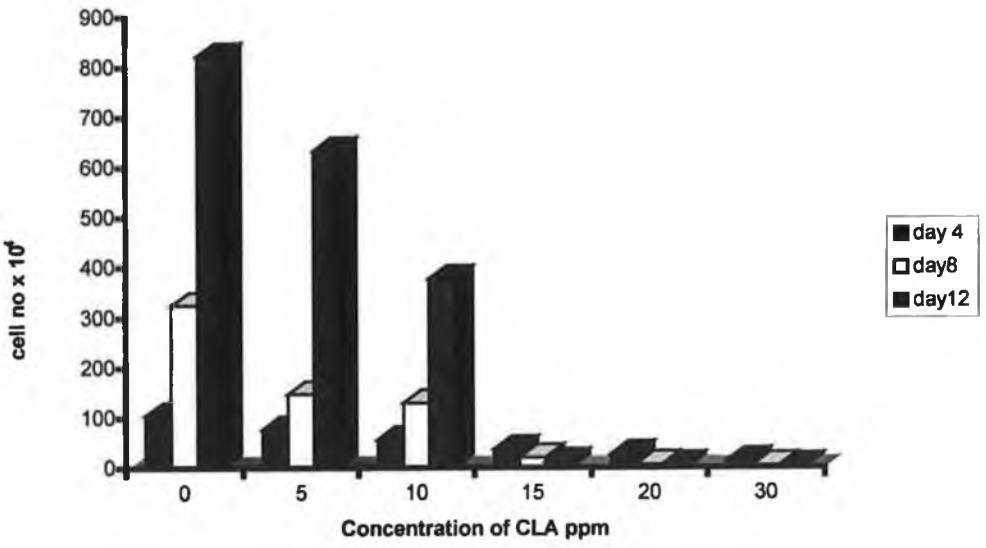

Figure 4.1 (b)

Figure 4.1 Growth curve of MCF-7 cells (a) and SW480 cells (b) over a 12 day incubation with varying levels of CLA. The SEM for these results was 226.98.

Table 4.1 The effect of CLA on uptake of tritium radiolabelled uridine and thymidine into MCF-7 cell lines

Precursor	Medium supplement level of CLA (M)	MCF-7		SW480	
		TCA-insoluble fraction μCi/flask	% incorporation relative to control	TCA-insoluble fraction μCi/flask	% incorporation relative to control
Leucine	Control	2.70	6.6%	1.54	8.4%
	7.14×10^{-5}	0.18		0.13	
Uridine	Control	1.97	8.6%	1.23	43.1 %
	7.14×10^{-5}	0.17		0.53	
Thymidine	Control	1.44	9.0%	1.98	27.3%
	7.14×10^{-5}	0.13		0.54	

Increasing the time period for supplementation to 8 and 12 days increased MDA levels to 0.1 and 0.105 nmoles MDA/10^3 cells, respectively. However, when cells were supplemented with lower concentrations of CLA (10-20 ppm), the onset of lipid peroxidation was delayed to between 8 and 12 days (Figure 4.2a). Supplementation of SW480 cells with 30 ppm CLA resulted in a significant increase in MDA on day 4 (0.012 nmoles MDA/10^3 cells), day 8 (0.062 nmoles MDA/10^3 cells) and day 12 (0.1 nmoles MDA/10^3 cells) (Figure 4.2b). Lipid peroxidation was induced only when these cells were supplemented with $\geq$15 ppm CLA for 8 days or more.

4.4.4 Superoxide dismutase activity.

SOD activity increased in both cell lines with the greatest effect being seen in MCF-7 cells following supplementation with 20 ppm CLA on all days ($p < 0.001$). Activity increased 3-4 fold above control levels on day 4 with 20 ppm CLA (0.018 mU/10^3 cells) but increased approximately 35-fold above control levels by day 12 (0.05 mU/10^3 cells) (Figure 4.3a). Levels of SOD activity in SW480 cells were similar to those of control cells on days 4 and 8 at all concentrations of CLA (Figure 4.3b). A significant increase ($p < 0.001$) in SOD activity in these cells was observed following 12 days of incubation with CLA (20 ppm), resulting in similar activity to that obtained in the MCF-7 cell line following 12 days of incubation with 20 ppm CLA (Figure 4.3).

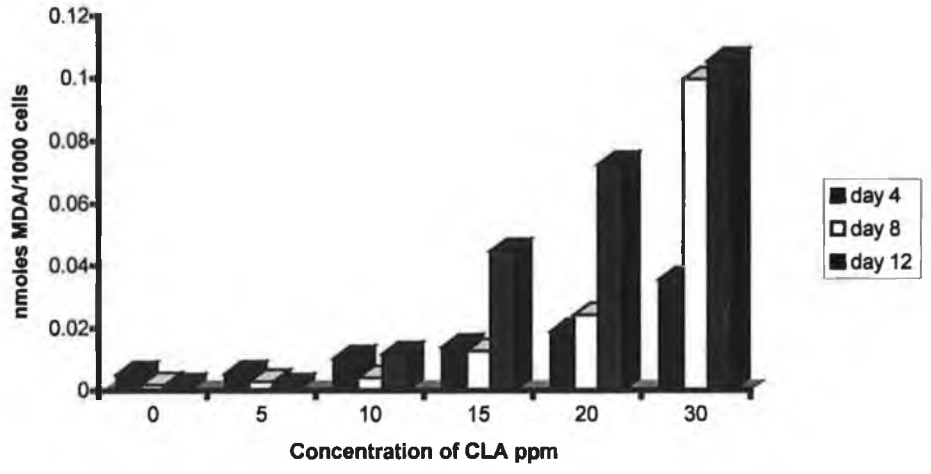

Figure 4.2 (a)

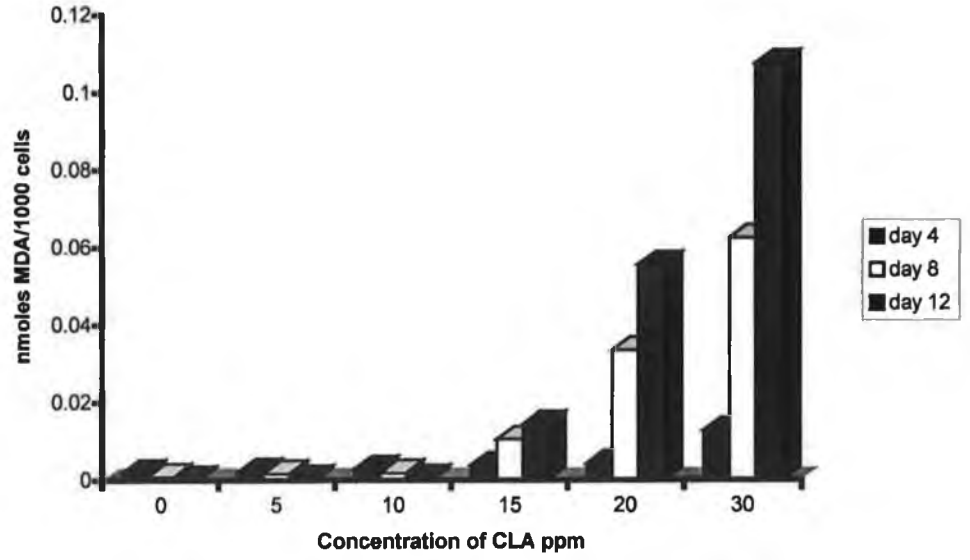

Figure 4.2 (b)

Figure 4.2 Lipid peroxidation activity in MCF-7 cells (a) and SW480 cells (b) as measured by TBARS. The SEM for these results was 0.0086.

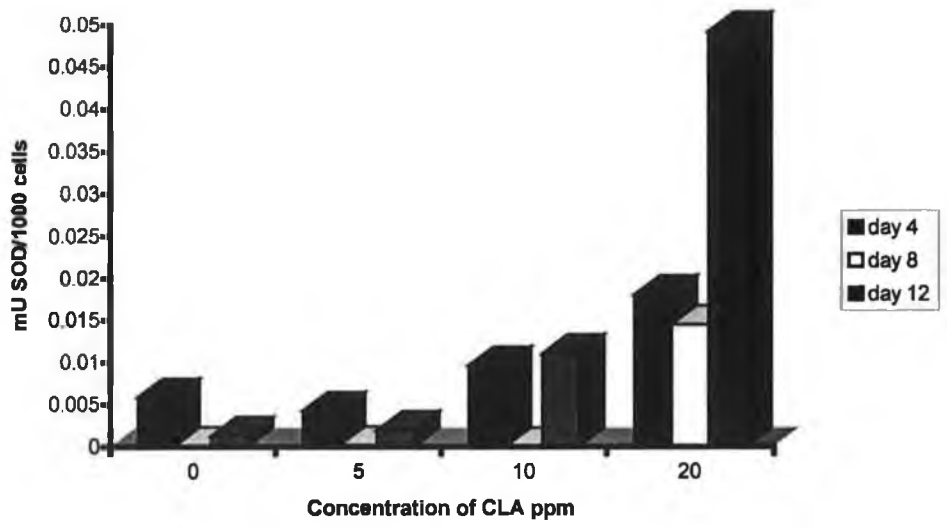

Figure 4.3 (a)

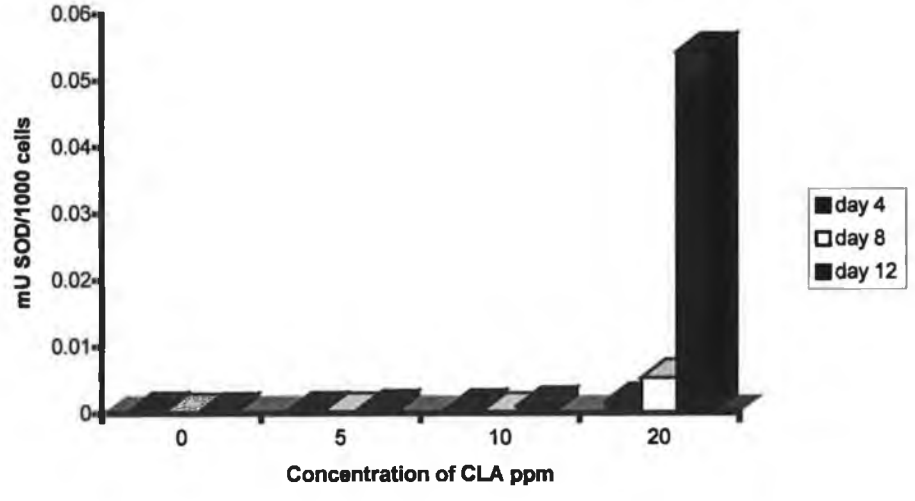

Figure 4.3 (b)

Figure 4.3 Superoxide Dismutase activity in MCF-7 cells (a) and SW480 cells (b). The SEM for these results was 0.0056.

4.4.5 Catalase activity.

In MCF-7 and SW480 cells, catalase activity was not significantly stimulated by incubation with CLA, at concentrations of 0 - 10 ppm for up to 12 days (Figure 4.4). Supplementation of MCF-7 cells with 20 ppm CLA induced catalase activity 8-fold over controls by day 4 (0.017 mBergmeyer units/10^3 cells) and 19-fold on day 8 (0.01 mBergmeyer units/10^3 cells) (Figure 4.4a). The highest level of catalase activity (0.04 mBergmeyer units/10^3cells) was observed following 12 days supplementation with 20 ppm CLA. By contrast, catalase was maximally expressed in SW480 cells on day 8 (0.042 mBergmeyer units/10 cells) when cells were supplemented with 20 ppm CLA (Figure 4.4b). Following 12 days of incubation of SW480 cells with CLA (20 ppm), catalase activity again declined but remained higher than control cells.

4.4.6 Glutathione peroxidase activity.

Supplementation of MCF-7 cells with CLA (5-20 ppm) for 4 days had minimal effect on GPx activity (Figure 4.5a). However, incubation for 8 days with 20 ppm CLA resulted in a 15-fold increase in GPx activity while lower concentrations of CLA had little effect. Highest GPx activity (2.2mU GPx/10^3 cells) was observed following incubation of MCF-7 cells for 12 days with 20 ppm CLA (Figure 4.5a). In the SW480 cells GPx activity was only expressed following 8 days of incubation with CLA at concentrations of 10 and 20 ppm. Following 8 days of incubation of SW480 cells with 20 ppm CLA, a 60-fold increase in GPx activity over control cells was obtained (Figure 4.5b) at 20 ppm CLA. The effect on GPx activity on incubation of MCF-7 cells with CLA (20 ppm) was more marked than in SW480 cells, where a 2.07 - fold higher response was obtained following 8 days of incubation (Figure 4.5).

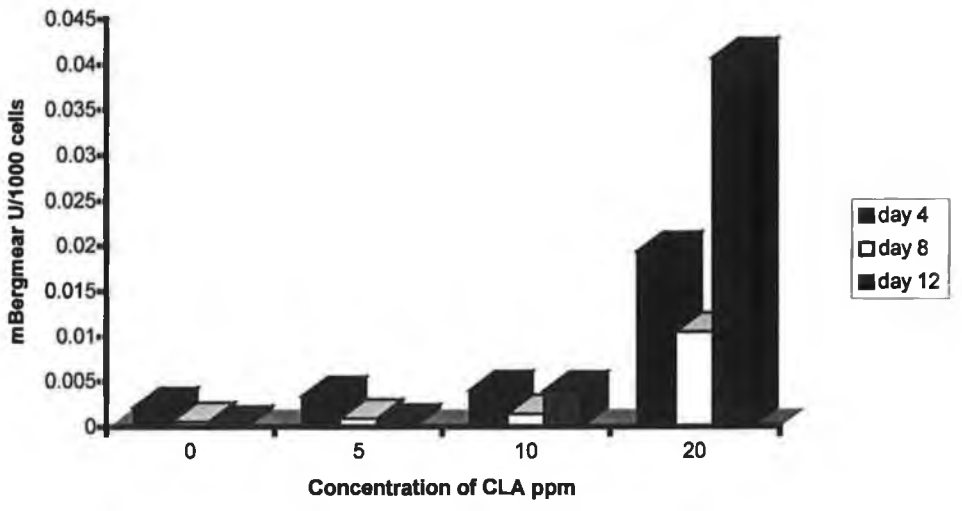

Figure 4.4 (a)

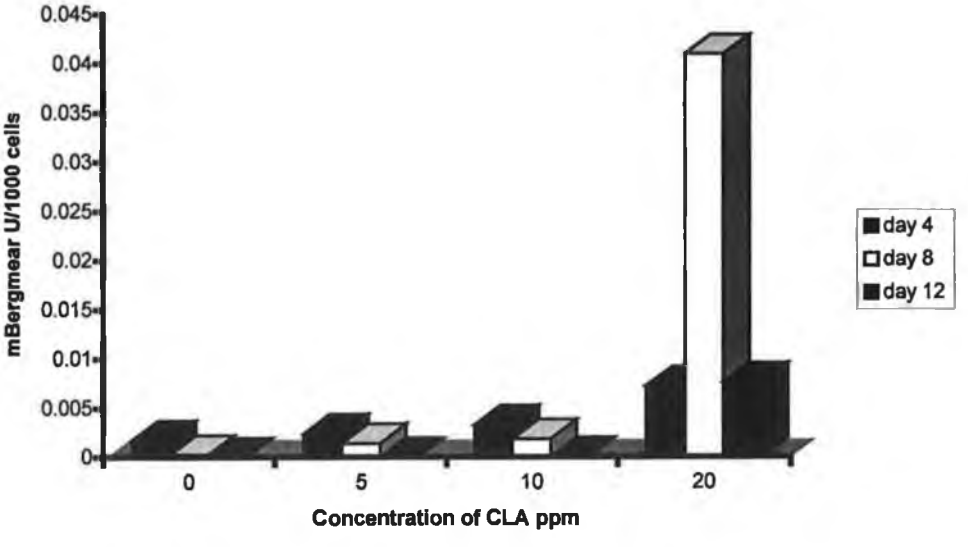

Figure 4.4 (b)

Figure 4.4 Catalase activity in MCF-7 cells (a) and SW480 cells (b). The SEM for these results was 0.0022.

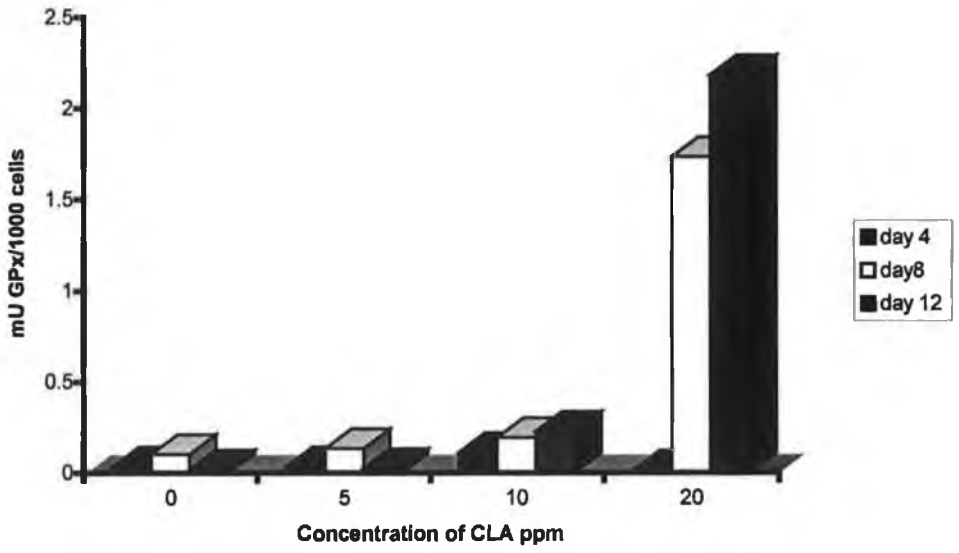

Figure 4.5 (a)

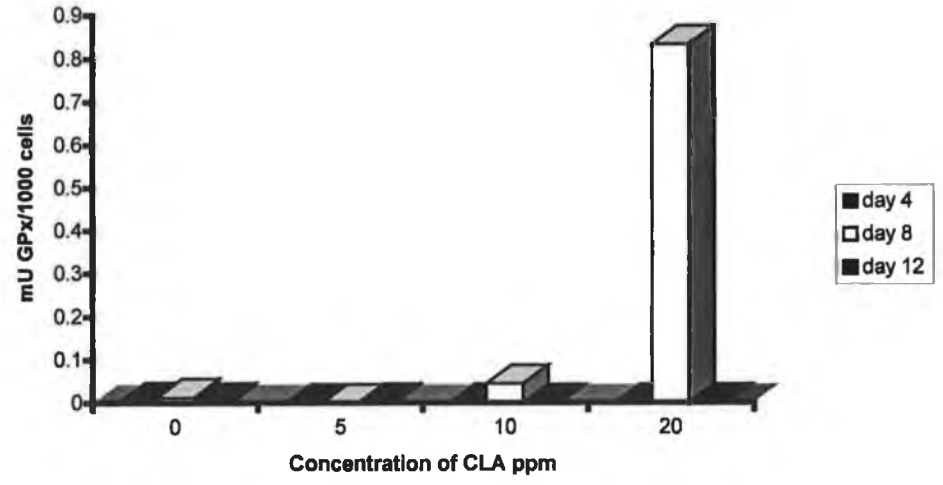

Figure 4.5 (b)

Figure 4.5. Glutathione Peroxidase activity in MCF-7 cells (a) and SW480 cells (b). The SEM for these results was 0.104156.

4.5 Discussion

The biochemical mechanism by which CLA exerts its activity has yet to be elucidated, despite existing evidence for a number of possible mechanisms (Belury and Kempa-Stecko, 1997, Belury *et al.,* 1997, De Voney *et al.,* 1997, Wong *et al.,* 1997) e.g. altered eicosanoid metabolism (Cunningham *et al.,* 1997, Liu and Belury, 1998), conversion to potent cytotoxic lipid peroxidation products in human tumour cell lines (Schonberg and Krokan, 1995) and inhibition of protein and nucleic acid biosynthesis (Shultz *et al.,* 1992a). In this study we have observed a cytotoxic effect of CLA, and have provided evidence for inhibition of protein and nucleic acid biosynthesis by CLA and increased peroxidation leading to activation of the cellular antioxidant defence enzymes.

A dose and time dependent inhibitory effect of CLA on human MCF-7 cells is well documented (Schultz *et al.,* 1992a). The results of this study confirm and extend previous findings of Schonberg and Krokan (1995) indicating that the inhibitory effect of CLA on the growth of human tumour cells was accompanied by increased lipid peroxidation. We found that CLA exerted a growth inhibitory effect in a dose- and time-dependent manner. In this investigation, inhibition of the growth of mammary and colon cancer cells *in vitro* following supplementation with CLA was characterised by inhibition of protein and nucleic acid biosynthesis as seen previously by Shultz *et al.* (1992a) and induction of lipid peroxidation and cellular antioxidant enzyme defences. In view of findings that lipid peroxides and free radicals can induce enzyme, protein, and DNA damage (Roubal and Tappel, 1996, Reiss and Tappel, 1973) it is probable that suppression of growth of both cell lines could have been mediated at least in part by an

increased susceptibility of CLA to undergo lipid peroxidation. Cellular prooxidant states promote lipid peroxidation, and is a mechanism by which polyunsaturated fatty acids may exert their inhibitory effects on cancer cell proliferation. It has been proposed that a conjugated double bond system may allow for more efficient trapping of electrons resulting in the likelihood of superoxide anion generation (Belury and Kempa-Steczo, 1997). Thus, addition of CLA to cancer cells may stimulate the initiation of its own oxidation by increasing the amount of $O_2^{\cdot -}$ radicals and by increasing the substrate concentration available for lipid peroxidation.

The occurrence of lipid peroxidation in both cell lines exposed to CLA is indicative of an overloading of the endogenous defence system and the generation of severe cellular oxidative stress. SOD represents the first line of antioxidant defence dismuting superoxide radicals to hydrogen peroxide and water. In this study, SOD was increased in both cell lines exposed to CLA indicating the presence of $O_2^{\cdot -}$ species, the major initiator of the oxygen radical cascade that feeds into the lipid peroxidation chain reaction. As hydrogen peroxide, the end product of SOD activity, is also causative of oxidative stress, a second enzyme either catalase or GPx is required to remove the hydrogen peroxide formed. Lipid peroxidation in the MCF-7 cells suggest that CLA at a concentration of 20 ppm may act as a prooxidant generating potential reactive oxygen species as a result of its conjugated diene double bond structure. The onset of lipid peroxidation after day 8 supplementation of the more vigorously growing SW480 cells is also suggestive of the presence of reactive species. The absence of SOD activity in SW480 cells following supplementation for 4 and 8 days suggests a possible role for non-enzymatic $O_2^{\cdot -}$

scavenging molecules. Since lipid peroxidation was maximally induced by day 8 it is apparent that as with MCF-7 cells, adding CLA to SW480 cells rendered them susceptible to lipid peroxidation. Activation of all enzymes in the antioxidant defence system supports the onset of an oxidatively stressed cellular milieu when cells are supplemented with CLA. We conclude that the antioxidant defence enzymes failed to protect cancer cells from the cytotoxic effects of lipid peroxidation products.

4.6 Summary

The relationship between antioxidant-enzyme defence responses and cellular growth suppression in human MCF-7 and SW480 cancer cells, exposed to CLA in culture was studied. MCF-7 and SW480 cells (1 x 10^6/flask) were cultured in appropriate medium for 4, 8 and 12 days with varying levels of CLA (0-30 ppm). A dose-dependent decrease in cell numbers and increase in lipid peroxidation, as determined by TBARS was observed in both cell lines following incubation with CLA. Exposure of both cell lines to 20 ppm CLA for 2 - 6 days produced a reduction (91-93 %) in ^{3}H leucine incorporation into protein while ^{3}H uridine and ^{3}H thymidine incorporation into RNA and DNA were reduced by 56-91 % and 73-91 %, respectively, compared with untreated control cells. The activities of SOD, catalase and GPx were induced in both cell lines exposed to CLA (20 ppm) over a period of 12 days, although to a greater extent in MCF-7 cells than in SW480 cells. The data indicate that CLA-induced cytotoxicity against MCF-7 and SW480 cancer cell lines is related to the extent of lipid peroxidation of CLA treated cells and affirm that the CLA-induced antioxidant enzymes failed to protect these cells from cytotoxic lipid peroxidation products.

CHAPTER 5

Milk fat conjugated linoleic acid (CLA) inhibits growth of human mammary MCF-7 cancer cells.*

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5.1 Introduction

CLA is a potent anticarcinogen in animal models (Ip *et al.,* 1994), and has been shown to inhibit skin papillomas, mammary tumours and colon aberrant crypt foci (Belury *et al.,* 1996, Ip *et al.,* 1996, Liew *et al.,* 1998). An epidemiological study showing an inverse association between risk of breast cancer and intake of dairy products provides indirect evidence that dietary CLA is a biologically active anticarcinogenic fatty acid (Knekt *et al.,* 1996). Furthermore, a study by Lavillonnière and Bougoux (1999) showing that CLA content was higher in adipose tissue from benign breast tumours than from malignant breast tumours, suggests a protective effect of dietary CLA against breast cancer in humans. In addition, feeding CLA-enriched butterfat to rats during the time of pubescent mammary gland development, led to alterations in mammary gland morphogenesis and a reduction in mammary cancer risk (Ip *et al.,* 1999a).

Potent anticarcinogenic effects of CLA have until recently been attributed to a synthetic mixture of CLA at levels as low as 1 % in the diet containing similar amounts of *c*9, *t*11 (29.5 %) and *t*10, *c*12 CLA isomers (29 %) (Ha *et al.,* 1990, Ip *et al.,* 1991, Pariza and Hargraves, 1985, Schut *et al.,* 1997). The *c*9, *t*11 CLA isomer was preferentially incorporated into phospholipids of rat mammary tumour extracts (Ip *et al.,* 1991) and studies have shown that this isomer inhibited the growth of normal rat mammary epithelial cell organoids (Ip *et al.,* 1999b). CLA and its metabolites have also been shown to accumulate in mammary tissue in a dose-dependent manner, while LA metabolites decreased ($C_{18:3}$, $C_{20:3}$ and $C_{20:4}$) (Banni *et al.,* 1999). The *c*9, *t*11 form is the

predominant (92 %) CLA isomer found in milk fat triglyceride form (Chin *et al.,* 1992). CLA has been identified in cholesteryl esters, triglycerides, phospholipids and free fatty acid extracts of human serum (Cawood *et al.,* 1983, Iversen *et al.,* 1984). Studies have shown that increasing dietary CLA led to increases in the CLA content of plasma (Britton *et al.,* 1992, Huang *et al.,* 1994, Salminen *et al.,* 1998), adipose tissue (Jiang *et al.,* 1999) and milk fat in humans (Park *et al.,* 1999a). The concentration range of CLA (0-20 ppm) used in cell culture studies (Cunningham *et al.,* 1997, Durgam and Fernandes, 1997, Shultz *et al.,* 1992a,b) spans the concentration of CLA reported in human plasma (Herbel *et al.,* 1998, Huang *et al.,* 1994, Iversen *et al.,* 1985).

5.2 Objectives

A number of studies have shown that CLA content of milk may be increased by modifying the dietary regime of the dairy cow (Dhiman *et al.,* 1999a, Lawless *et al.,* 1998, Kelly *et al.,* 1998a). We have previously shown that the mammary MCF-7 cancer cell line is susceptible to the cytotoxic effects of synthetic CLA (Chapter 4) and in this chapter, similar parameters were examined using CLA-enriched milk fat. The present chapter reports on the effects of incubation of CLA-enriched milk fat, obtained from cows on pasture, supplemented with full fat soybeans (FFS) or full fat rapeseeds (FFR), on the growth and antioxidant enzyme defence responses of MCF-7 cancer cells. These effects were compared with those of a control milk fat, not enriched in CLA, and with a CLA-enriched milk fat fraction obtained by dry fractionation using a temperature programme of 33 ^{o}C to 10 ^{o}C and a cooling rate of 0.58 ^{o}C h^{-1} (Chapter 3). The uptake of CLA by the MCF-7 cells from milk fat and synthetic CLA isomers was also examined.

5.3 Materials and methods

5.3.1 Culture of MCF-7 cells.

The human breast cancer cell line (MCF-7) was cultured exactly as outlined in chapter 4.

5.3.2 Preparation of milk fat samples.

The milk fat used in this study was obtained following supplementation of lactating dairy cows on pasture with full fat rapeseeds (FFR) and full fat soybeans (FFS) for 33 and 34 days, respectively. The level of supplementation and trial conditions were exactly as previously described (Lawless *et al.,* 1998). Composite milk samples for each herd, consisting of the morning and evening milk of each individual cow (based on the morning and evening milk yields) were taken on day 32 for animals fed pasture only (control), day 33 for FFS-fed animals and day 34 for FFR-fed animals. The fat was extracted from whole milk by centrifugation, as described previously (Murphy *et al.,* 1990) and stored at – 20 °C prior to GLC analysis. In addition, a soft milk fat fraction was obtained by dry fractionation of milk fat using a temperature range of 33 to 10 °C and a cooling rate of 0.58 °C/h (Chapter 3).

5.3.3 Treatment of MCF-7 cells.

Initially, 1 x 10^6 cells were seeded in flasks and cultured for 24 h allowing the cells to attach to the substratum. The medium was then replaced with medium containing ethanol to a final concentration of 0.1 % (v/v) (control) or medium supplemented with CLA

isomers, LA or various concentrations of milk fat dissolved in ethanol and incubated for 8 days prior to harvest. Three synthetic sources of CLA were used in this study, including a mixture of CLA isomers obtained from Nu-Chek Prep (Inc., Elysian, MN), containing 29.5 % *c*9, *t*11 and 29 % *t*10, *c*12 CLA isomers (in addition to minor components of other isomers (Sehat *et al.,* 1998)), and pure *c*9, *t*11-CLA and *t*10, *c*12 CLA isomers (Matreya, Pleasant Gap, PA). Cells were harvested in the presence of phosphate buffered saline (PBS) containing 0.25 % (w/v) trypsin and viability assessed using the trypan blue exclusion assay prior to analysis of lipid peroxidation and antioxidant enzyme defence activities.

5.3.4 Lipid peroxidation and antioxidant enzyme assays.

Cells obtained from incubation with varying concentrations of CLA, LA and milk fat were monitored for lipid peroxidation using the TBARS test, as described previously (Chapter 4). SOD and GPx activities were determined using Ransod and Ransel kits (Randox Laboratories Ltd., Armagh, Northern Ireland), respectively. The units of GPx activity are defined as nmol NADPH oxidised / min at pH 7.4 and 30 °C. A unit of SOD activity is described by the quantity of enzyme that inhibits by 50 % xanthine oxidase mediated conversion of xanthine to a formazon dye. Catalase activity was determined as described previously (Chapter 4) and expressed in Bergmeyer units, nmol [H_2O_2] decomposed min^{-1} ml^{-1} at pH 7 and 25 °C.

5.3.5 Uptake of ^{14}C-CLA

The lipids of MCF-7 cells, incubated with ^{14}C-CLA were separated into neutral lipid, phospholipid and monoglyceride fractions and analysed for uptake of radiolabelled CLA (Hamilton and Comai, 1988). Media containing 0.025, 0.05, or 0.1 μCi ^{14}C-CLA (American Radiolabeled Chemical Inc., St. Louis, MO, specific activity 55 mCi/mmol) was added to confluent MCF-7 cells for 24 h prior to harvest. Cells were harvested and total lipids extracted from the cell pellets obtained from each flask (n=3 per treatment) using the method of Folch *et al.* (1957) and resuspended in 1 ml of chloroform. Cellular lipids were fractionated into neutral lipids, monoglycerides and phospholipids using the procedure described by Hamilton and Comai (1988). An aliquot (200 μl) of each fraction was counted by liquid scintillation (LS6500 Beckmann) to determine the levels of ^{14}C-CLA present in each lipid fraction.

5.3.6 Uptake of CLA into MCF-7 cell membranes

The ability of MCF-7 cells to incorporate CLA from different sources was examined. Cells were seeded in T-75 cm^2 flasks at a density of 5 x 10^6 per flask and grown to 90 % confluency. Cells were then incubated for 24 hours with medium containing ethanol to a final concentration of 0.1 % (v/v) (control), medium supplemented with 20 ppm *c*9, *t*11 CLA (Matreya), or with medium supplemented with control milk fat or FFR milk fat dissolved in ethanol, each of which were included to yield a final *c*9, *t*11 CLA concentration of 20 ppm. Subsequently, total cellular lipids were extracted from the cells using the method of Folch *et al.* (1957), resuspended in 1 ml of chloroform and stored at – 20 °C prior to GLC analysis.

supplemented with full fat rapeseed, respectively. The fatty acid composition of the milk fat samples was variable between treatments, the most abundant fatty acid $C_{16:0}$, varied in content from 21.05 ± 0.08 to 27.2 ± 0.11 g/100 g FAME for the FFR and soft fraction milk fat samples, respectively. The $C_{18:1}$ content ranged between 20.28 ± 0.187 to 28.39 ± 1.62 g/100 g FAME for the control milk fat and soft fraction, respectively. The ratio of LA/CLA varied between samples from 0.74 ± 0.001 (control milk fat) to 2.34 ± 0.004 (FFS); the LA ($C_{18:2}$) content of the FFS milk fat was at least 2.5-fold higher than the other milk fat samples. *t*11-$C_{18:1}$ content also varied between samples with the control milk fat containing 3.14 ± 0.173 g/100 g FAME and the soft fraction containing the highest level at 5.18 ± 0.082 g/100 g FAME (Table 5.1).

5.4.2 The effects on cell viability of CLA enriched milk fat.

The effects of incubation of a mixture of CLA isomers (Nu-Chek Prep), LA, *c*9, *t*11 CLA, *t*10, *c*12 CLA (Matreya) and milk fat on the growth of MCF-7 cells during 8 days were determined. The milk fat content of the medium varied between 0.8 to 1.2 mg/ml to yield a final CLA concentration of 20 ppm. All milk fat samples significantly lowered ($p < 0.05$) cell numbers to approximately 7-9 % of untreated control cells, following 8 days of incubation (Figure 5.1a). However there was no significant difference between the final cell numbers (8.3-11.7 x 10^4 cells) for all milk fat treatments. Subsequently MCF-7 cells (1 x 10^6 cells/flask) were incubated for 8 days in the presence of 1 mg /ml milk fat to yield CLA concentrations from 16.9 to 22.6 ppm. This permitted an examination of the effect of increasing the milk fat CLA concentration, while the milk fat content was kept constant.

Table 5.1. Fatty acid composition of the milk fat samples.

	Milk Fatty Acids (g/ 100 g FAME)			
Fatty Acid	Control milk fat [1] (n=2)	FFS milk fat [1] (n=2)	FFR milk fat [1] (n=2)	Soft fraction milk [2] (n=3)
$C_{12:0}$	4.15 ± 0.004	3.19 ± 0.003*	2.74 ± 0.009*	4.00 ± 0.120
$C_{12:1}$	0.10 ± 0.001	0.07 ± 0.001*	0.07 ± 0.003*	0.24 ± 0.010
$C_{14:0}$	12.00 ± 0.005	9.58 ± 0.001*	9.53 ± 0.061*	12.20 ± 0.212
$C_{14:1}$	1.15 ± 0.003	0.89 ± 0.002*	1.03 ± 0.006*	1.28 ± 0.016
$C_{15:0}$	1.43 ± 0.015	1.08 ± 0.009	1.22 ± 0.022*	0.98 ± 0.042
$C_{16:0}$	25.93 ± 0.018	21.23 ± 0.023*	21.05 ± 0.079*	27.2 ± 0.210
$C_{16:1}$	1.70 ± 0.007	1.51 ± 0.003*	1.82 ± 0.008*	2.27 ± 0.071
$C_{17:0}$	0.62 ± 0.005	0.53 ± 0.001*	0.52 ± 0.003*	0.82 ± 0.011
$C_{18:0}$	10.20 ± 0.010	12.42 ± 0.016*	11.07 ± 0.037*	12.83 ± 0.050
$C_{18:1}$	20.28 ± 0.187	23.37 ± 0.605	26.92 ± 0.579*	28.39 ± 1.621
$C_{18:1}$ vaccenic	3.14 ± 0.173	4.41 ± 0.604	4.64 ± 0.465	5.18 ± 0.082
$C_{18:2}$	1.25 ± 0.045	4.28 ± 0.020*	1.69 ± 0.353	1.19 ± 0.120
$C_{18:3}$	0.80 ± 0.006	1.07 ± 0.001*	0.71 ± 0.007	1.20 ± 0.100
$C_{18:2}$ CLA	1.69 ± 0.004	1.83 ± 0.005*	2.26 ± 0.005*	2.22 ± 0.010

[1]Samples obtained through manipulation of the dietary regime of dairy cows (Lawless *et al.*, 1998). *Denotes values that are significantly different to the control milk fat ($p < 0.05$). [2]Sample obtained by dry fractionation of milk fat (Chapter 3).

The cell numbers obtained following incubation with all milk fat samples were significantly ($p < 0.05$) less than the untreated control cells following 8 days of incubation and a dose-dependent decrease in cell number was observed with increasing milk fat CLA content (Figure 5.1b). Maximal growth inhibition of 91 % occurred at the highest CLA concentration.

Synthetic mixtures were prepared, containing CLA (20 ppm) and LA at concentrations similar to those found in the milk fat samples (15, 47 and 16 ppm LA, for control (sample 1), FFS (sample 2) and FFR (sample 3), respectively). These were incubated with MCF-7 cells for 8 days and the effect on cell viability was assessed (Figure 5.2a). A significant ($p < 0.05$) inhibitory effect on cell numbers was obtained following incubation with all of the CLA/LA mixtures, compared with untreated control cells. Samples 1, 2 and 3 representing the CLA/LA mixtures were as effective in growth suppression as the milk fat treatments resulting in 89 %, 97 % and 92 % decrease in cell numbers, respectively, compared with control cells. There were no significant differences in cell numbers between the synthetic CLA/LA mixtures (Figure 5.2a).

Two individual isomers of CLA (*c*9, *t*11 CLA and *t*10, *c*12 CLA, Matreya) and a mixture of CLA isomers (Nu-Chek Prep) were incubated with MCF-7 cells (1×10^6 cells) at a concentration of 20 ppm for 8 days. In addition, the effects on cell growth of LA alone (20 ppm) and a mixture containing 20 ppm CLA (Nu-Chek Prep) and 20 ppm LA were compared with untreated controls (Figure 5.2b).

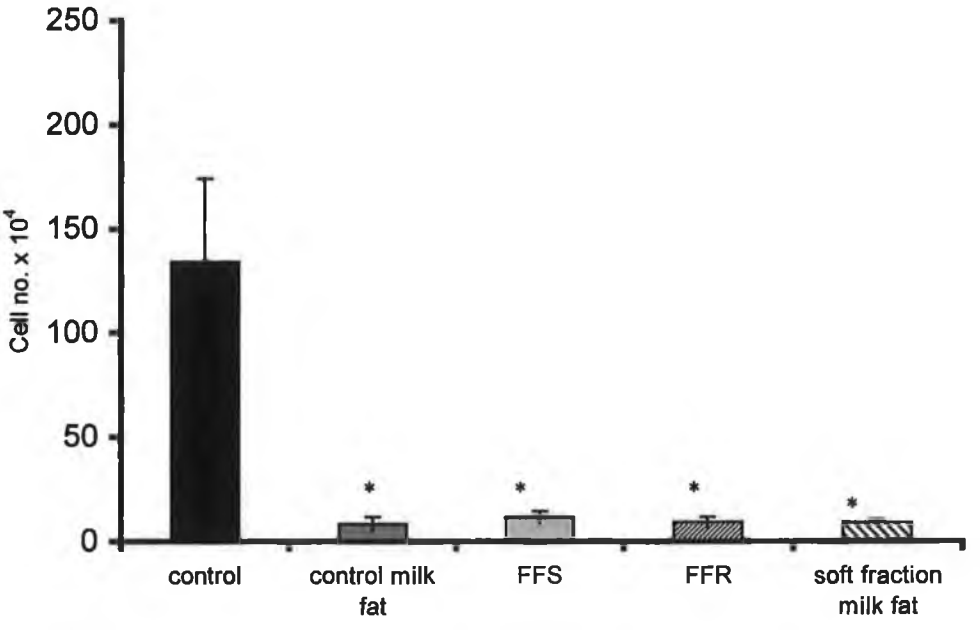

Figure 5.1 (a)

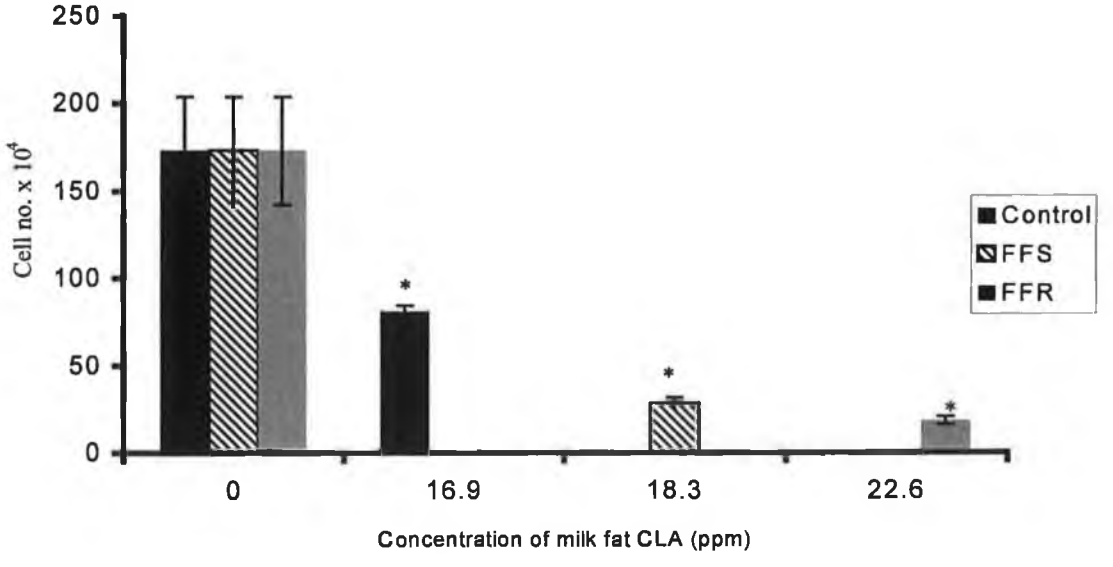

Figure 5.1 (b)

Figure 5.1. Growth curve of MCF-7 cells incubated with milk fat samples each containing 20 ppm CLA for 8 days (a) and milk fat samples containing varying concentrations of CLA (16.9-22.6 ppm) over 8 days (b). * Denotes results which are significantly ($p < 0.05$) reduced compared with controls.

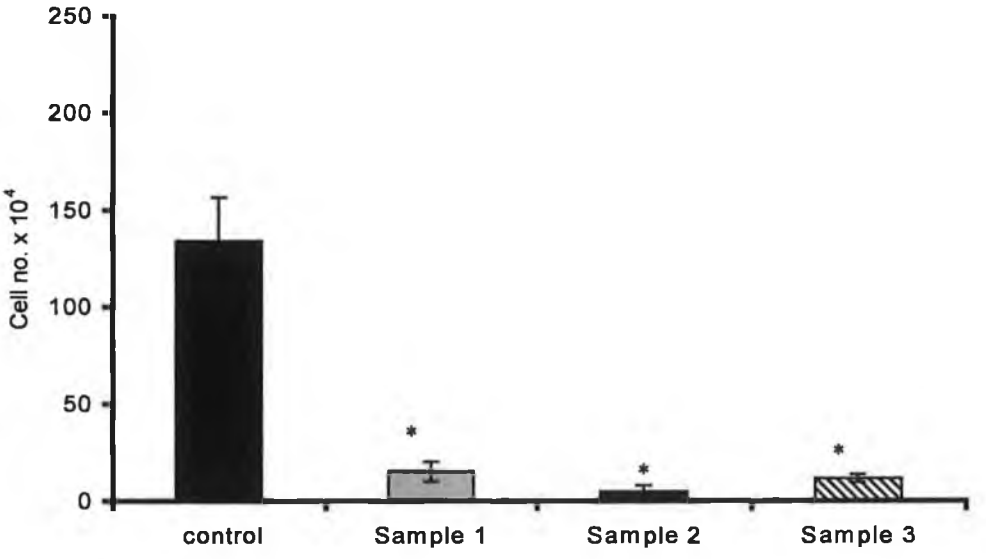

Figure 5.2 (a)

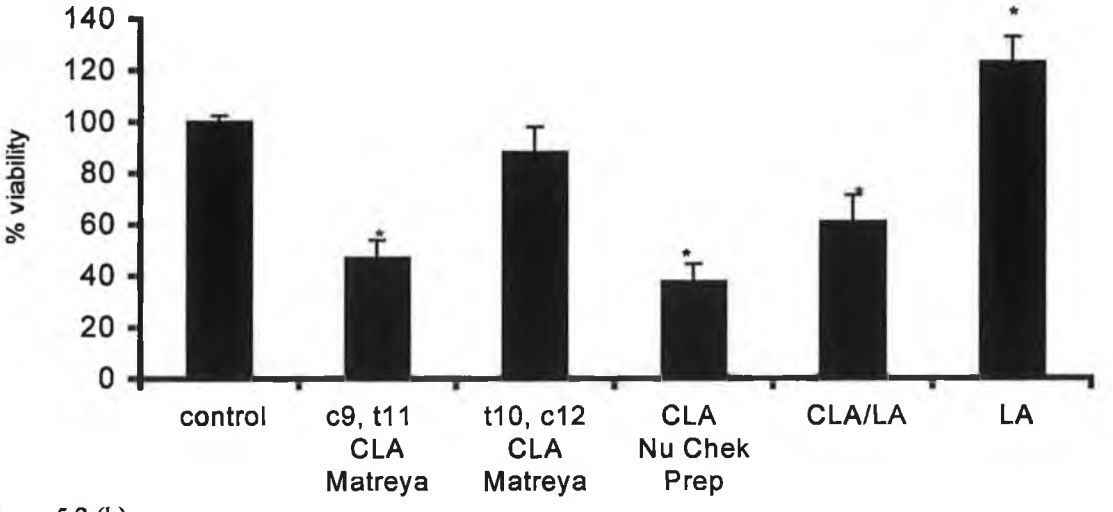

Figure 5.2 (b)

Figure 5.2. Growth curve of MCF-7 cells incubated with a synthetic mix of LA and CLA in similar concentrations to that found in the milk fat samples for 8 days (a) and 20 ppm *c*9, *t*11 CLA, *t*10, *c*12 CLA, Nu-Chek CLA and LA for 8 days (b). * Denotes results which are significantly ($p < 0.05$) different compared with controls.

Cell numbers obtained following incubation in the presence of the pure *c*9, *t*11 CLA isomer and the Nu-Chek Prep mixture were significantly ($p < 0.01$) reduced compared with untreated controls and the degree of inhibition of growth was not significantly different between the two CLA preparations (Figure 5.2b). It should be noted that the *c*9, *t*11 CLA concentration in the medium containing the Nu-Chek Prep mixture was only 6 ppm. Furthermore, there was also a reduction ($p < 0.03$) in cell numbers following incubation in the presence of the mixture containing 20 ppm CLA (Nu-Chek Prep) and 20 ppm LA (CLA/LA) compared with untreated controls (Figure 5.2b). This was not significantly different to the effect seen with CLA (Nu-Chek Prep) alone, suggesting that the presence of LA in combination with CLA did not have a significant effect on the growth suppressive potency of CLA on MCF-7 cells. However, the *t*10, *c*12 CLA isomer (20 ppm) had no effect on the growth of MCF-7 cells following incubation for 8 days compared with untreated controls. Finally, incubation of MCF-7 cells with LA alone (20 ppm) for 8 days resulted in an increase ($p < 0.05$) in cell numbers compared with untreated controls (Figure 5.2b).

5.4.3 Lipid peroxidation analysis.

LA (20 ppm) had a negligible effect on lipid peroxidation in MCF-7 cells (Figure 5.3a). However, when the cells were incubated with the three milk fat samples (0.8 – 1.2 mg/ml) MDA levels were significantly ($p < 0.01$) increased ($\geq$ 9-fold) compared with untreated controls (Figure 5.3a). No significant differences were obtained between the MDA levels from cells incubated with the different milk fat samples. When cells were incubated in the presence of 1 mg /ml milk fat to yield CLA concentrations from 16.9 to

22.6 ppm, a 2.7 fold increase in MDA levels was observed (Figure 5.3b). An increase in MDA levels was obtained following incubation for 8 days with mixtures containing 20 ppm CLA and LA at concentrations corresponding to those found in the milk fat samples (Figure 5.3c). There was no significant difference in MDA levels from cells treated with these synthetic CLA/LA samples (Figure 5.3c). There was a 2-3 fold increase ($p < 0.05$) in MDA levels when cells were incubated with the *c*9, *t*11 CLA isomer, *t*10, *c*12 CLA isomer and the Nu-Chek Prep CLA mixture of isomers at 20 ppm for 8 days (Figure 5.3d). Incubation with 20 ppm LA for 8 days reduced MDA levels compared with CLA/LA treated cells (Figure 5.3d).

5.4.4 Superoxide dismutase activity.

SOD activity increased to 0.006 ± 0.002 mU SOD/10^3 cells in MCF-7 cells following supplementation with 20 ppm LA on day 8 (Figure 5.4a). The MCF-7 cells incubated with 1 mg /ml of each milk fat sample (control, FFS and FFR) to yield CLA concentrations from 16.9 to 22.6 ppm for 8 days showed an increase in SOD activity compared with control cells. SOD activity in cells was proportional to CLA concentration and was 6-fold higher ($p < 0.05$) in cells treated with 22.6 ppm CLA (0.06 ± 0.01 mU SOD/10^3 cells) compared with untreated controls (Figure 5.4b).

5.4.5 Catalase activity.

Catalase activity was increased ($p < 0.05$) upon incubation with 20 ppm LA for 8 days (0.005 ± 0.002 mBergmeyer units/10^3 cells ($n = 3$)) (Figure 5.5a). Catalase activity was increased in MCF-7 cells incubated in the presence of milk fat samples (1 mg /ml) containing between 16.9 to 22.6 ppm CLA for 8 days. A 12-fold increase in catalase activity was observed in cells incubated with FFR milk fat after 8 days (0.13 ± 0.01 mBergmeyer units/10^3 cells) compared with untreated controls (0.011 ± 0.001 mBergmeyer units/10^3 cells ($n = 3$). An increase in catalase activity was also observed in MCF-7 cells incubated with the FFS milk fat containing 18.3 ppm CLA for 8 days, this was 17-fold higher (0.08 ± 0.02 mBergmeyer units/10^3 cells ($n = 3$)) (Figure 5.5b) than that observed in cells treated with 20 ppm LA (0.005 ± 0.002 mBergmeyer units/10^3 cells ($n=3$)) (Figure 5.5a).

5.4.6 Glutathione peroxidase activity.

GPx activity was 0.08 ± 0.06 mU GPx/10^3 in MCF-7 cells treated with 20 ppm LA for 8 days (Figure 5.6a). An increase in GPx activity was obtained when the cells were incubated with each milk fat sample (1 mg /ml) to yield CLA concentrations from 16.9 to 22.6 ppm for 8 days (Figure 5.6b). Maximum activity (0.62 ± 0.15 mU GPx/10^3) was observed with the FFR milk fat sample (Figure 5.6b). This represented an approximate 3-fold increase in GPx activity over similar cells incubated with the same concentration of LA.

5.4.7 Uptake of ^{14}C-CLA.

^{14}C-CLA was preferentially incorporated into the phospholipid fraction in a dose-dependent manner over a 24-hour incubation period in MCF-7 cells (Figure 5.7a). Incubation with 0.025 μCi ^{14}C-CLA resulted in 40 % of radiolabelled CLA being incorporated in the neutral lipids, 7.5 % in the monoglycerides and 52 % in the phospholipids. When the dose was increased to 0.1 μCi ^{14}C-CLA, 6.5 % of radiolabelled CLA was found in the neutral lipids with 93.5 % in the phospholipid fraction.

5.4.8 Uptake of milk fat-CLA.

There was a 4-fold higher uptake (1.57 g/100 g FAME) of CLA in the lipids extracted from cells treated with milk fat compared with untreated controls (Table 5.2). Uptake of CLA from cells treated with the pure *c*9, *t*11 CLA isomer was negligible (0.28 g/100 g FAME) and similar to untreated controls (0.26 g/100 g FAME). $C_{14:0}$ $C_{16:1}$ and *t*11-$C_{18:1}$ fatty acids were notably increased in the lipids obtained from milk fat treated cells. In addition, $C_{18:0}$ and LA contents in lipids from the FFR milk fat-treated cells were increased compared with untreated controls.

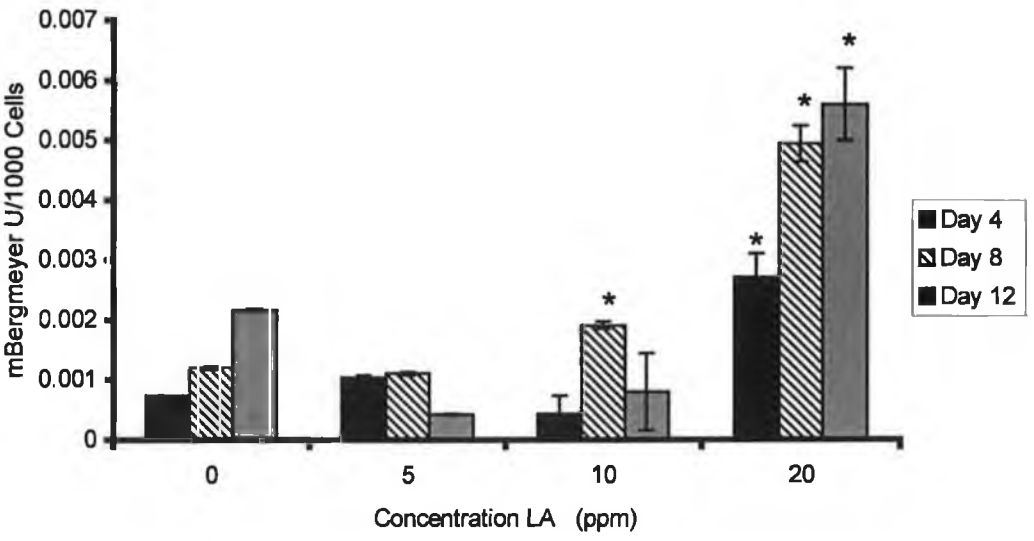

Figure 5.5 (a)

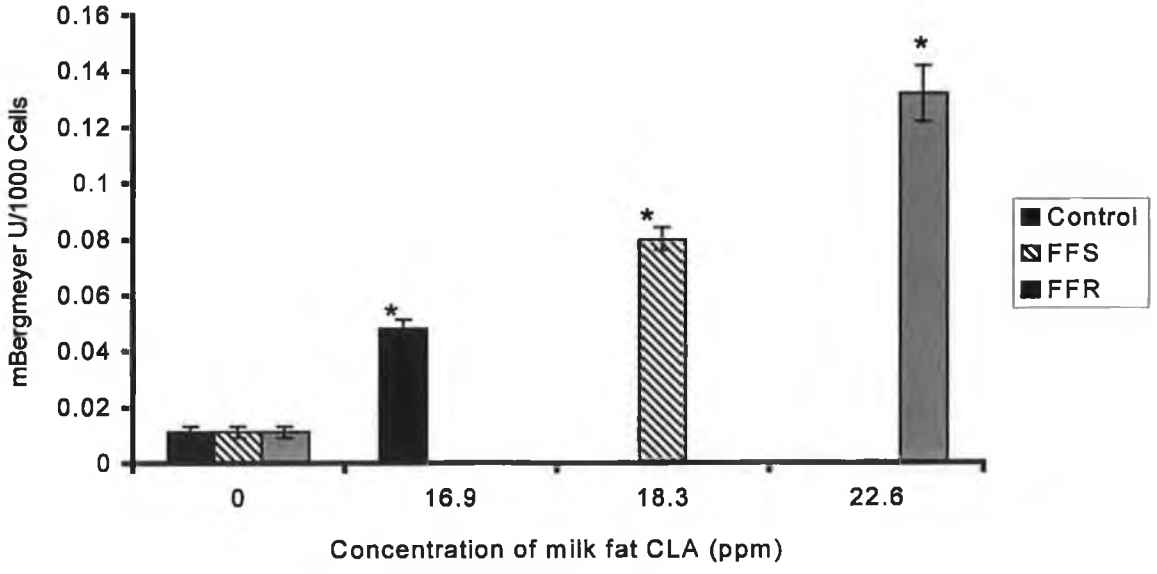

Figure 5.5 (b)

Figure 5.5. Catalase activity in MCF-7 cells treated with LA (0 - 20 ppm) (a) and milk fat CLA (16.9 – 22.6 ppm) (b). * Denotes results which are significantly ($p < 0.05$) increased compared with controls.

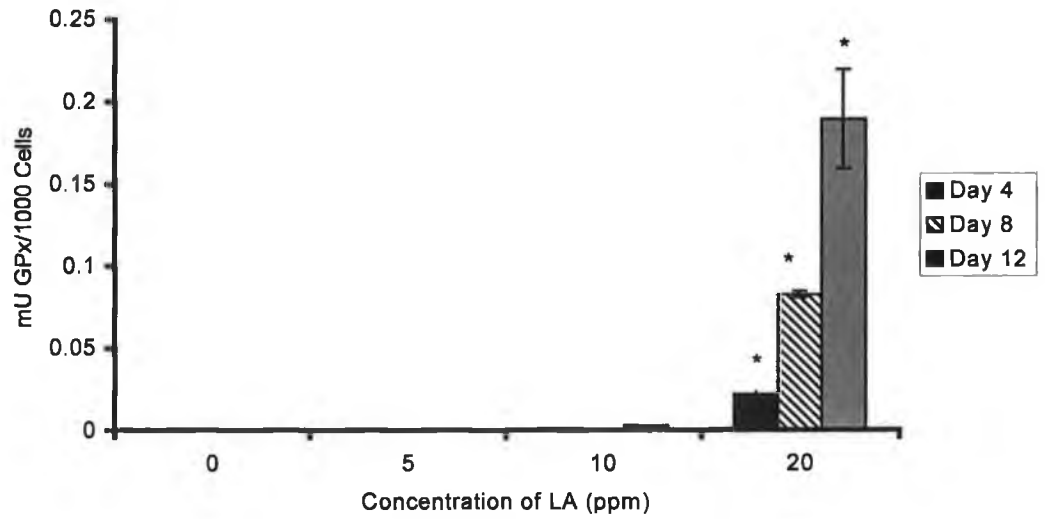

Figure 5.6 (a)

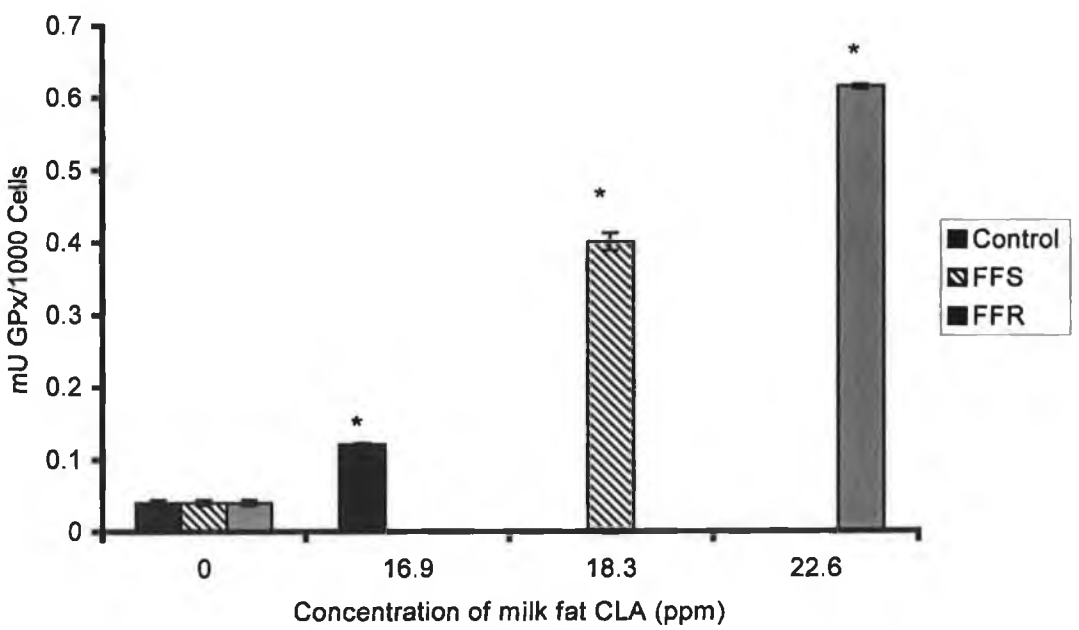

Figure 5.6 (b)

Figure 5.6. Glutathione Peroxidase activity in MCF-7 cells treated with LA (0 - 20 ppm) (a) and milk fat CLA (16.9 – 22.6 ppm) (b). * Denotes results which are significantly ($p < 0.05$) increased compared with controls.

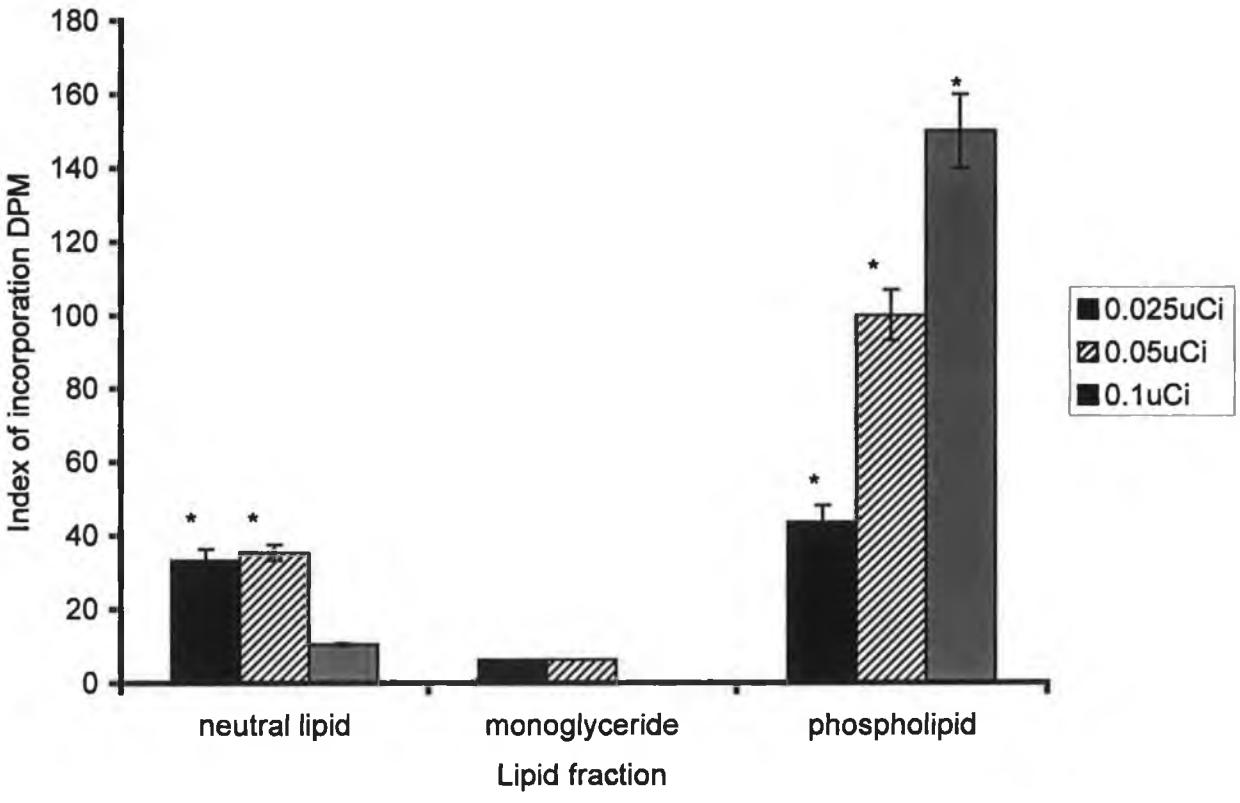

Figure 5.7

Figure 5.7. Incorporation of ^{14}C-CLA into MCF-7 cellular lipid classes. * Denotes results which are significantly ($p < 0.05$) increased compared with controls.

Table 5.2. Fatty acid composition of total cell lipids of MCF-7 cells, incubated in the presence of the pure *c*9, *t*11 CLA isomer (Matreya) (20 ppm) and with control and FFR milk fat samples containing 20 ppm CLA.

	MCF-7 Fatty Acids (g /100g FAME)			
Fatty Acid	Untreated Controls	Matreya *c*9, *t*11 CLA	Control Milk Fat	FFR Milk Fat *P < 0.05
$C_{14:0}$	2.62 ± 0.18	2.65 ± 0.15	8.85 ± 0.041*	7.26 ± 0.05*
$C_{16:0}$	26.25 ± 0.13	29.16 ± 0.12*	28.75 ± 0.22*	26.90 ± 0.61
$C_{16:1}$	1.45 ± 0.04	0	2.12 ± 0.02*	1.6 ± 0.03*
$C_{18:0}$	16.74 ± 1.36	18.39 ± 0.99	18.60 ± 0.61	21.71 ± 0.94*
$C_{18:1}$	20.78 ± 0.62	27.43 ± 0.7*	18.70 ± 0.66*	21.10 ± 0.59
$C_{18:1}$ vaccenic	0	0	1.32 ± 0.03*	1.5 ± 0.14*
$C_{18:2}$	1.87 ± 0.32	1.57 ± 0.03	1.49 ± 0.02*	2.21 ± 0.32
$C_{18:3}$	0.06 ± 0.02	0.68 ± 0.04*	0.43 ± 0.02*	0.54 ± 0.06*
$C_{18:2}$ CLA	0.26 ± 0.03	0.28 ± 0.03	1.10 ± 0.06*	1.57 ± 0.32*

5.5 Discussion

We have previously reported a cytotoxic effect of synthetic CLA isomers (Nu Chek Prep) in human MCF-7 and SW480 cell lines, and provided evidence for inhibition of protein and nucleic acid biosynthesis by CLA and increased peroxidation leading to activation of the cellular antioxidant defence enzymes (Chapter 4). In this chapter we have demonstrated similar effects to those previously reported, using milk fat enriched with CLA and a pure *c*9, *t*11 CLA isomer (Matreya). Thus, milk fat CLA is as effective an anticancer agent as the synthetic Nu Chek prep mixture of isomers. An opposite proliferative effect of LA was shown, which supports previous studies (Cunningham *et al.,* 1997, Shultz *et al.,* 1992a,b) showing LA can stimulate cancer cell growth. The cytotoxic effects of CLA are well documented (Cunningham *et al.,* 1997, Ip *et al.,* 1999a, Shultz *et al.,* 1992a,b), with the effects on lipid peroxidation also previously noted (Shonberg and Krokan, 1995). The increase in lipid peroxidation observed in CLA-treated cells suggests that CLA may reverse the resistance to oxidative stress characteristic of cancer cells (Meyer *et al.,* 1998, Muller *et al.,* 1993, Santillo *et al.,* 1996).

The data reported suggests that milk fat CLA when present in a food matrix is as effective as the synthetic CLA used in culture and animal studies to date (Belury *et al.,* 1996, Ip *et al.,* 1994, 1996, Liew *et al.,* 1995). When the concentration of milk fat in these treatments varied from 0.8 to 1.2 mg /ml to yield a final CLA concentration of 20 ppm, similar growth effects were observed for all treatments, suggesting that CLA alone

may be responsible for the effect. This supports the findings of Ip *et al.* (1996) where CLA was shown to act independently of the level or type of fat present in the diet. When the fat concentration of each sample was maintained at 1 mg /ml to yield varying levels of CLA (16.9 – 22.6 ppm) cell numbers decreased as CLA concentration increased and peroxidation was stimulated as observed previously (Chapter 4). The cells incubated with a synthetic mix (CLA/LA) that represented the CLA/LA concentration of the three milk fat samples showed similar effects to those obtained with milk fat CLA or synthetic CLA.

The *c*9, *t*11-CLA isomer (Matreya) was as effective as the mixture of CLA isomers (Nu Chek Prep) and both were significantly more effective than the *t*10, *c*12 CLA, suggesting that the *c*9, *t*11 CLA is the active isomer in terms of anticarcinogenic activity in these cells. The mixture of CLA isomers at 20 ppm, yielded only 6 ppm *c*9, *t*11 CLA but showed a similar effect to the pure *c*9, *t*11 CLA at 20 ppm, suggesting that the concentration of the *c*9, *t*11 isomer required for optimal activity was $\leq$ 6 ppm, and/or that other isomers, which are present in the mixture were also exhibiting anticarcinogenic activity towards MCF-7 cells. A growth stimulatory effect was obtained, upon incubation of MCF-7 cells with LA (20 ppm), but co-incubation with equimolar amounts of CLA caused a significant growth inhibitory effect compared with LA alone. Milk fat CLA also induced the activities of SOD, catalase and GPx to a similar extent as observed previously with the synthetic CLA mixture (Nu Chek Prep) (Chapter 4).

The effects of incubation of MCF-7 cells with LA in this study displayed very different effects to those seen previously with CLA (Chapter 4). Incubation of MCF-7 cells with LA (20 ppm) had a negligible effect on MDA levels following 8 days of incubation. Similarly, both SOD and catalase activities were 5-fold less in LA-treated cells compared with CLA-treated cells, while GPx was reduced 10-fold in the LA-treated cells compared to the CLA-treated cells. These results suggest a prooxidant effect in milk fat treated cells as observed previously with the mixture of isomers (Nu Chek Prep) (Chapter 4), and growth stimulation in LA treated cells was associated with negligible peroxidation.

CLA was preferentially incorporated into the phospholipid fraction of the MCF-7 cell lipids. CLA uptake was more proficient from milk fat than from synthetic sources, supporting previous work by Ip *et al.* (1999a). This may suggest the possible formation of CLA from *t*11-$C_{18:1}$ present in milk fat by the action of Δ-9 desaturase as previously suggested (Griinari and Bauman, 1999). The incorporation of CLA was 1.4-fold higher in FFR treated cell lipids compared with those treated with control milk fat. This increase is comparable to the difference in CLA levels of both milk fat samples, suggesting that CLA incorporation from milk fat may be dose-dependent. The *t*11-$C_{18:1}$ content of the FFR milk fat sample was 1.4 fold higher than in the control milk fat sample, which may be relevant in terms of endogenous synthesis of CLA.

Other anticarcinogens are also present in milk fat and these may act in conjunction with CLA, such as butyric acid, sphingomyelin, ether lipids and metabolites of tumour supressor lipids (Parodi *et al.,* 1997). The milk fat samples used in this study (FFR, FFS,

control (Lawless *et al.*, 1998) and soft fraction (Chapter 3)) demonstrate similar effects to the synthetic CLA mixture of isomers. It is envisaged that the practice used to produce these CLA-enriched milk fats may be applied at a larger scale to produce CLA-enriched products, such as milk, butter, cheese and yoghurt. Consumption of such products may afford a chemoprotective effect- without the additional cost of oral supplements or the need for dietary changes.

5.6 Summary

The relationship between growth and the antioxidant enzyme defence system in human MCF-7 cancer cells treated with bovine milk fat enriched with CLA was studied. Milk enriched in CLA was obtained from cows on pasture supplemented with full fat rapeseeds and full fat soyabeans. Cell number decreased up to 90 % ($p < 0.05$) and lipid peroxidation increased 15- fold ($p < 0.05$) following incubation of MCF-7 cells for 8 days with increasing levels of milk fat yielding CLA concentrations between 16.9 and 22.6 ppm. Growth suppression and prooxidant effects of milk fat CLA were independent of the variable composition of the milk fat samples, suggesting that CLA was the active ingredient in milk fat responsible for the cytotoxic effect. Mixtures containing isomers of CLA (*c*9, *t*11-, *t*10, *c*12-, *c*11, *t*13- and minor amounts of other isomers) and LA at similar concentrations to the milk fat samples were as effective at inhibiting growth and stimulating peroxidation of MCF-7 cells as the milk fatty acids. Incubation of the cells with the *c*9, *t*11 CLA isomer (20 ppm) or the mixture of CLA isomers (20 ppm) for 8 days resulted in a 60 % decrease ($p < 0.05$) in viability compared with untreated controls and was significantly ($p < 0.05$) more effective than incubation with the *t*10, *c*12 CLA

isomer (20 ppm), which caused only a 15 % decrease in cell numbers under similar conditions. A 25 % increase ($p < 0.05$) in cell proliferation occurred when LA (20 ppm) alone was incubated with MCF-7 cells for 8 days. ^{14}C-CLA was preferentially incorporated into the phospholipid fraction of the MCF-7 cell membranes in a dose-dependent manner and CLA accumulated in cell membranes more efficiently when the cells were incubated in the presence of milk fat than the *c*9, *t*11 synthetic CLA isomer. SOD, catalase and GPx activities were induced in MCF-7 cells exposed to milk fat (containing 16.9-22.6 ppm CLA) over 8 days. The data indicate that milk fat triglyceride-bound CLA, consisting primarily of the *c*9, *t*11 isomer, was cytotoxic towards MCF-7 cells.

CHAPTER 6

Differential effects of conjugated linoleic acid isomers on Ras farnesylation in human mammary MCF-7 and colon SW480 cancer cell lines.

6.1. Introduction

Chemopreventive agents act by inhibiting either the acquisition of mutations or the neoplastic processes that occur subsequent to mutagenesis (Clarke and Shankel, 1975, Ferguson, 1994, Hayes *et al.,* 1997) and exert anticarcinogenic effects by mediating diverse oxidant-induced signal transduction pathways that culminate in cell differentiation, inhibition of oncogene activation, inhibition of proliferation in initiated cells and enhancement of apoptosis (Clarke and Shankel, 1975, Ferguson, 1994, Hayes *et al.,* 1997). We have previously observed a dose- and time-dependent growth suppressive effect and an induction of antioxidant enzymes in CLA-treated mammary and colon cancer cells as an adaptive response to induction of lipid peroxidation by synthetic CLA (Chapter 4) and milk fat CLA (Chapter 5).

Mutations in the dominant oncogene *ras* represent the most commonly found gene mutations in human cancer cells. This is mainly due to the central role played by the *ras* encoded protein in membrane-to-nucleus signal transduction (Ames *et al.,* 1995). Ras oncoprotein is a membrane-bound GTPase required for a variety of signal transduction pathways involved in cellular proliferation and differentiation. More than 50 low molecular weight proteins categorised into the Ras-related superfamily act positively to stimulate a cascade of kinase-driven phosphorylation events that culminate in the activation of nuclear transcription (Ames *et al.,* 1995). Because of its central role in membrane-to-nucleus signal transduction, it has been proposed that modulation of the

Ras superfamily of proteins may provide a novel means by which growth of cancer cells may be inhibited (Gibbs *et al.,* 1994).

One of the strategies being devised to impair the dominant activity of *ras* is to inhibit farnesylation of Ras oncoprotein (Gibbs *et al.,* 1994). Farnesylation of Ras proteins by farnesyl protein transferase (PFTase) is an essential step for their binding to internal plasma membranes and for activation of events downstream of Ras (i.e. activation of Raf kinase, MAPK kinase, MAP kinase and phosphorylation of *fos* and *jun*) leading to cell proliferation (Stacey *et al.,* 1991). Non-farnesylated mutants of oncogenic Ras that cannot appropriately localise into the plasma membrane remain no longer transforming and have been shown to display a dominant inhibitory phenotype to antagonise the activity of membrane-bound oncogenic Ras (Stacey *et al.,* 1991).

Components of dietary fat have been shown to exert differential effects on oncogenic *ras* p21 expression. In an experimental model of colon carcinogenesis, rats fed a high fat diet composed of corn oil (59 % omega-6 and 1.2 % omega-3 fatty acids) had higher levels of *ras* p21 in colonic mucosa than rats fed a high fat fish oil diet (8 % omega-6 and 31 % omega-3 fatty acids) (Singh *et al.,* 1997). Higher expression of *ras* correlated with increased incidence and multiplicity of grossly visible colon tumours. By contrast, lower expression of *ras* in those animals fed the high fish oil diet correlated with decreased incidence and a lower multiplicity of colon tumours (Singh *et al.,* 1997). Analysis of Ras p21 levels in cytosol and plasma membrane revealed that the dietary fish oil resulted in increased accumulation of Ras p21 in cytoplasm with a concomitant decrease in

membrane-bound *ras* p21 levels. The type and amount of dietary fat was a potent modifier of farnesylation of Ras during the promotion and progression stages of colon cancer (Singh *et al.,* 1998). PFTase activity in the azomethane-induced rat colon cancer model was enhanced by a diet high in corn oil but reduced by a diet containing fish oil. It is therefore apparent that individual components of fat, in particular n-3 polyunsaturated fatty acids, may inhibit cancer cell growth by interfering with posttranslational modifications and membrane localisation of Ras p21 (Singh *et al.,* 1997, 1998). A previous study revealed that dietary CLA did not alter the proportion of mutant *ras* versus wild-type *ras* in mammary carcinogenesis (Ip *et al.,* 1997), suggesting that CLA did not affect events upstream of farnesylation.

6.2 Objectives

The aim of this study was to determine if CLA as a collection of isomers, shown to be anticarcinogenic (Ip *et al.,* 1999a, Shonberg and Krokan 1995, Shultz *et al.,* 1992ab), or in the pure form of *c*9, *t*11 and *t*10, *c*12, inhibited cell growth via modulation of PFTase activity in MCF-7 mammary and SW480 colon cancer cell lines.

6.3. Materials and methods

Human breast (MCF-7) and colon (SW480) cancer cell lines were obtained from the American Type Culture Collection, (Rockville, MD). Culture media and supplements were purchased from GIBCOBRL (Paisley, Scotland). The MCF-7 cells were maintained in Dulbecco's Minimum Essential Medium supplemented with 5 % v/v fetal bovine serum, 0.2 mM L-glutamine, 1 mM HEPES, and 1 unit/ml penicillin and streptomycin. The SW480 cells were maintained in the same medium as for MCF-7 cells containing, in addition, 10 mM sodium pyruvate. [^{3}H] Farnesyl pyrophosphate ([^{3}H] FPP; 15μCi/mmol), farnesyl pyrophosphate (FPP), H-Ras (recombinant: expressed in *E.coli*), Triton X 100, linoleic acid (LA), and docosahexanoic acid (DHA), (99 % purity) were all purchased from Sigma (Chemical Co., St. Louis, USA). Whatman phosphocellulose p81 paper was purchased from Whatman (Whatman Int., Maidstone, England). A mixture of CLA isomers was obtained from Nu Chek Prep (Inc., Elysian, MN), and individual *c*9, *t*11 and *t*10, *c*12 CLA isomers were obtained from Matreya (Matreya, Pleasant Gap, PA).

6.3.1 Cell culture

The MCF-7 and SW480 cells were cultured and maintained as previously described (Chapter 4). The effects of incubation of Nu Chek Prep CLA, LA, DHA, pure *c*9, *t*11 and pure *t*10, *c*12 CLA isomers for 4 days on the growth and PFTase activity of cell lines were compared. Initially, 1 x 10^6 cells were plated in flasks and cultured for 24 h in 5 ml of media allowing the cells to attach to the substratum. The medium was then replaced

with medium alone containing ethanol to a final concentration of 0.1 % (v/v) or medium supplemented with 20 ppm CLA, LA, DHA, *c*9, *t*11 and *t*10, *c*12 CLA dissolved in ethanol. Following 4 days of incubation, cells were harvested as described previously (Chapter 4).

6.3.2 Preparation of cell extracts

Cell pellets of harvested cells from each flask were washed by resuspension in 3 ml of PBS (phosphate buffered saline), centrifuged at 800 g for 5 min and the supernatant was removed by aspiration. Cells were resuspended in 100 µl of PBS and lysed by sonication to release cytosolic extracts and 10 µl of the cell extract was used for determination of protein content using the Bradford assay (Bradford, 1976), and 10 µl of the cell extract was used for the PFTase assay, as described below.

6.3.3 PFTase assay

Using cytosolic extracts of MCF-7 and SW480 cells as the source of PFTase, H-ras as the source of farnesyl acceptor protein and [$^{1\text{-}3}$H] farnesyl pyrophosphate (FPP) as the farnesyl donor, the PFTase activity of cells was determined, using methods described by Harwood (1995) and Roskosi *et al.* (1994). A solution containing 50 mM Tris-HCL, 5 mM dithiothreitol and 20 µM $ZnCl_2$ was used as the PFTase assay buffer and a PFTase cofactor substrate solution was prepared using 2x PFTase assay buffer (400 µl) containing 50 µl of 20 µM [^{3}H] FPP, 100 µl of 100 mM $MgCl_2$ and 250 µl of water to a final volume of 800 µl. The PFTase reaction was initiated by the addition of 5 µl of 20

μM solution of H-Ras in PFTase assay buffer and 10 μl of PFTase cofactor substrate solution to 10 μl of each of the sonicated cell solutions or 10 μl of PFT assay buffer as controls and incubated for 60 min at 37 °C. The PFTase catalysed reaction reaction was terminated by addition of 200 μl of 10 % (v/v) HCL in ethanol for 15 min at 37 °C. Following incubation 300 μl of ethanol (HPLC grade) was then added to each sample and the entire sample (525μl) was immediately applied to 4 × 4 cm Whatman phosphocellulose paper strips. The samples were air dried for 30 min, then cut into 1 cm squares and immersed in 50 ml ethanol/phosphoric acid solution (37.5 mM) prepared by mixing equal volumes of 95 % ethanol and 75mM phosphoric acid. The samples were then vortexted for 10 min and, the ethanol/phosphoric acid solution was aspirated and replaced with 50 ml of fresh 37.5 mM ethanol/ phosphoric acid solution. This washing procedure was repeated 4 times as described above. The phosphocellulose squares were then air dried for 45 min and placed in 10 ml of scintillation fluid and counted in a Beckman LS 6500 scintillation counter. PFTase activity was converted to molar concentrations using the specific activity of [^{3}H] FPP (11034 dpm/pmol) and was expressed as picomoles of H-Ras farnesylated mg^{-1} min^{-1} protein.

6.3.4 Statistical analysis.

All analyses were carried out in triplicate except for milk fatty acid composition analyses, which were carried out in duplicate. The Student's *t* test was used to determine significance between treatments.

6.4. Results

6.4.1 Growth of MCF-7 cells

We have previously shown a dose dependent growth inhibitory effect of CLA in the MCF-7 cancer cell line (Chapter 4). High fat fish oil diets are known to inhibit tumour growth (Singh *et al.,* 1997, 1998, Bartsch *et al.*, 1999) and farnesylation (Singh *et al.,* 1998) in animal models of breast and colon carcinogenesis. The relative potency of DHA, a single n-3 fatty acid component of fish oil to inhibit growth and PFTase activity when present at an amount equivalent to CLA and LA in the culture medium of two cancer cell lines, MCF-7 (breast) and (SW480) colon was examined in this study. CLA significantly ($p < 0.05$) inhibited growth in MCF-7 cells following 4 days incubation at 20 ppm compared with control cells (Figure 6.1). The *c*9, *t*11 and *t*10, *c*12 isomers of CLA were significantly ($p < 0.05$) more effective than the CLA mixture of isomers at reducing cell numbers following incubation for 4 days. Incubation of MCF-7 cells with DHA (20 ppm) for 4 days also resulted in decreased cell numbers ($p < 0.02$) compared with untreated controls and was as effective a growth inhibitory agent as *c*9, *t*11-CLA and *t*10, *c*12-CLA isomers. Cell numbers following incubation in the presence of LA (20 ppm) were similar to untreated control cells following 4 days (Figure 6.1).

6.4.2 PFTase activity in MCF-7 cells

MCF-7 cells were incubated in the presence of Nu Chek Prep CLA, *c*9, *t*11- and *t*10, *c*12-CLA (Matreya), LA and DHA at 20 ppm for 4 days following which the effect on

cytosolic PFTase activity was assessed (Figure 6.2). Incubation with both the *c*9, *t*11 CLA and the mixture of CLA isomers reduced ($p < 0.05$) PFTase activity in MCF-7 cells compared with untreated controls but the *c*9, *t*11 CLA isomer was significantly ($p < 0.03$) more effective than the Nu Chek Prep mixture ($P < 0.05$) in this regard. Incubation of MCF-7 cells with the *t*10, *c*12 CLA isomer at similar concentrations (20 ppm) caused an increase of 15 % ($p < 0.05$) in PFTase activity in MCF-7 cells while LA also resulted in a stimulatory effect (Figure 6.2) yielding a 47 % increase in PFTase activity compared with untreated controls. Although, incubation of MCF-7 cells with DHA reduced PFTase activity by 10.5 %, this was not significant compared to control cells (Figure 6.2).

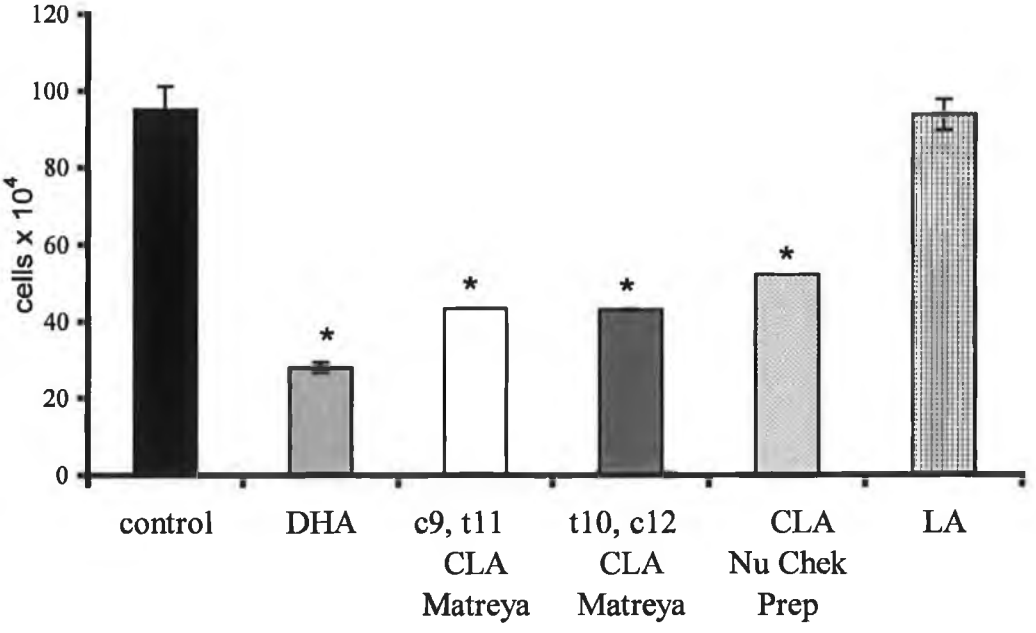

Figure 6.1. Cell viability for MCF-7 cells incubated with 20 ppm Nu-Chek Prep CLA, *c*9, *t*11 and *t*10, *c*12 pure CLA isomers (Matreya), LA and DHA for 4 days. * Denotes values that are significantly different ($p < 0.05$) compared with controls, values that are significantly different ($p < 0.02$) compared with controls.

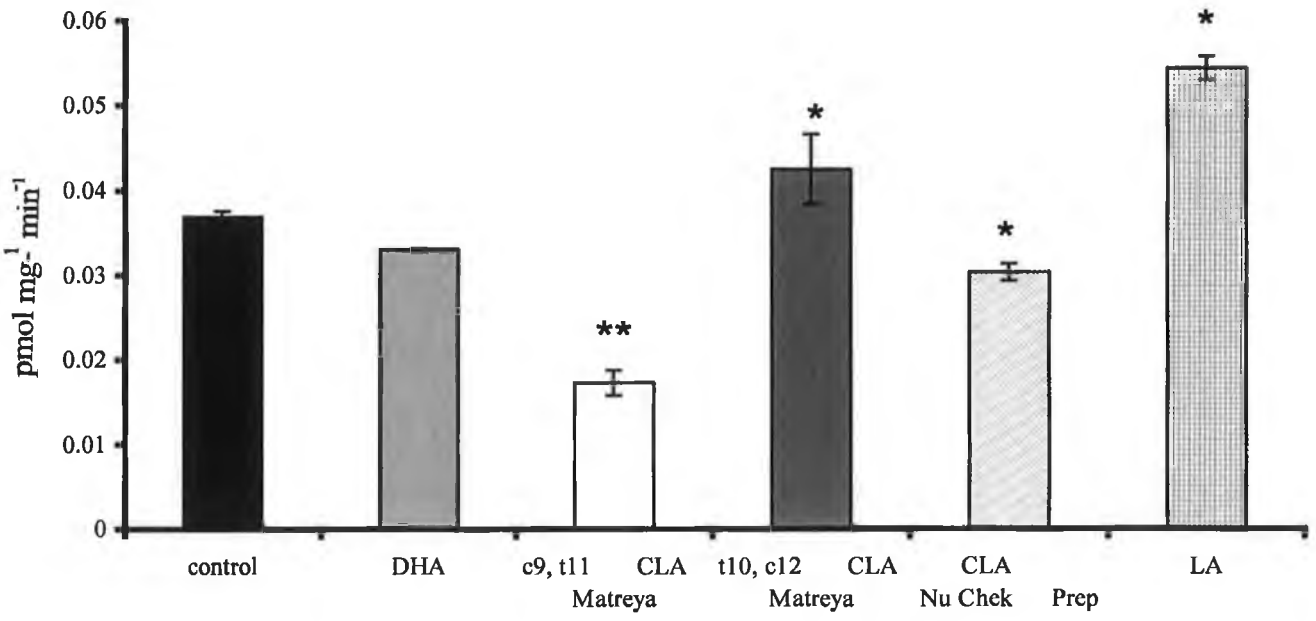

Figure 6.2. PFTase activity for MCF-7 cells incubated with 20 ppm Nu-Chek Prep CLA, *c*9, *t*11 and *t*10, *c*12 pure CLA isomers (Matreya), LA and DHA for 4 days. * Denotes values that are significantly different ($p < 0.05$) compared with controls, ** Denotes values that are significantly different ($p < 0.03$) compared with controls.

6.4.3 Growth of SW480 cells

As observed previously (Chapter 4) the Nu Chek Prep mixture of CLA isomers and the pure *c*9, *t*11- and *t*10, *c*12-CLA (Matreya) isomers significantly inhibited growth in SW480 cells following 4 days incubation at 20 ppm compared with control cells (Figure 6.3). The individual *c*9, *t*11-CLA isomer and the mixture of isomers were significantly ($p < 0.05$) more effective at reducing cell numbers than the *t*10, *c*12-CLA isomer. LA (20 ppm) had a negligible effect on growth of SW480 cells following 4 days. As observed in MCF-7 cells there was a significant ($p < 0.05$) decrease in cell numbers of DHA-treated SW480 cells compared with controls.

6.4.4 PFTase activity in SW480 cells

An investigation of PFTase activity of SW480 cells incubated in the presence of Nu Chek Prep CLA, *c*9, *t*11- and *t*10, *c*12-CLA (Matreya), LA and DHA at 20 ppm for 4 days showed a trend that was similar to that described in MCF-7 cells. However increased levels of PFTase activity were obtained in the SW480 cells and this may be a consequence of the more vigorous growth observed previously with these cells (Chapter 4) (Figure 6.3). The *c*9, *t*11-CLA and the Nu Chek Prep mixture of CLA isomers significantly reduced ($p < 0.05$) PFTase activity by 27 % and 30.7 % respectively, compared with control cells following 4 days of incubation. Incubation of MCF-7 cells with the *t*10, *c*12 CLA isomer at similar concentrations (20 ppm) caused an increase of 60 % ($p < 0.01$) in PFTase activity in SW480 cells while LA also resulted in a similar effect (Figure 2) yielding an 85 % increase ($p < 0.01$) in PFTase activity compared with

controls. Although, incubation of SW480 cells with 20 ppm DHA resulted in reduced PFTase activity following 4 days incubation this was not significantly different from control cells.

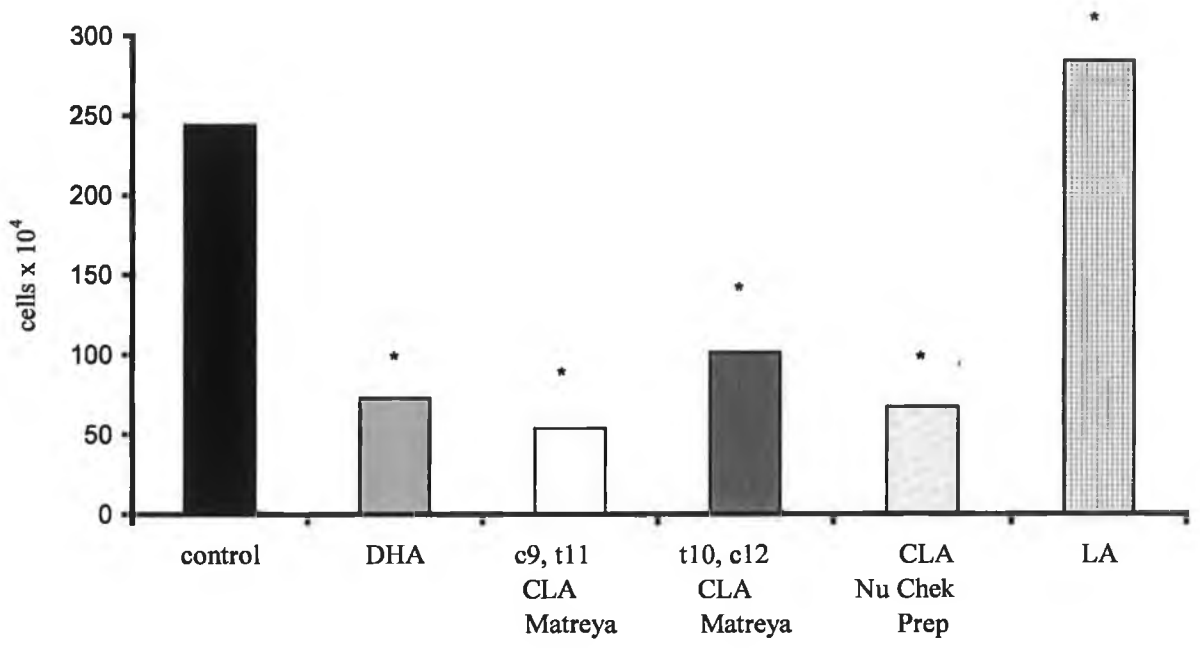

Figure 6.3. Cell viability for SW480 cells incubated with 20 ppm Nu-Chek Prep CLA, *c*9, *t*11 and *t*10, *c*12 pure CLA isomers (Matreya), LA and DHA for 4 days. * Denotes values that are significantly different ($p < 0.05$) compared with controls.

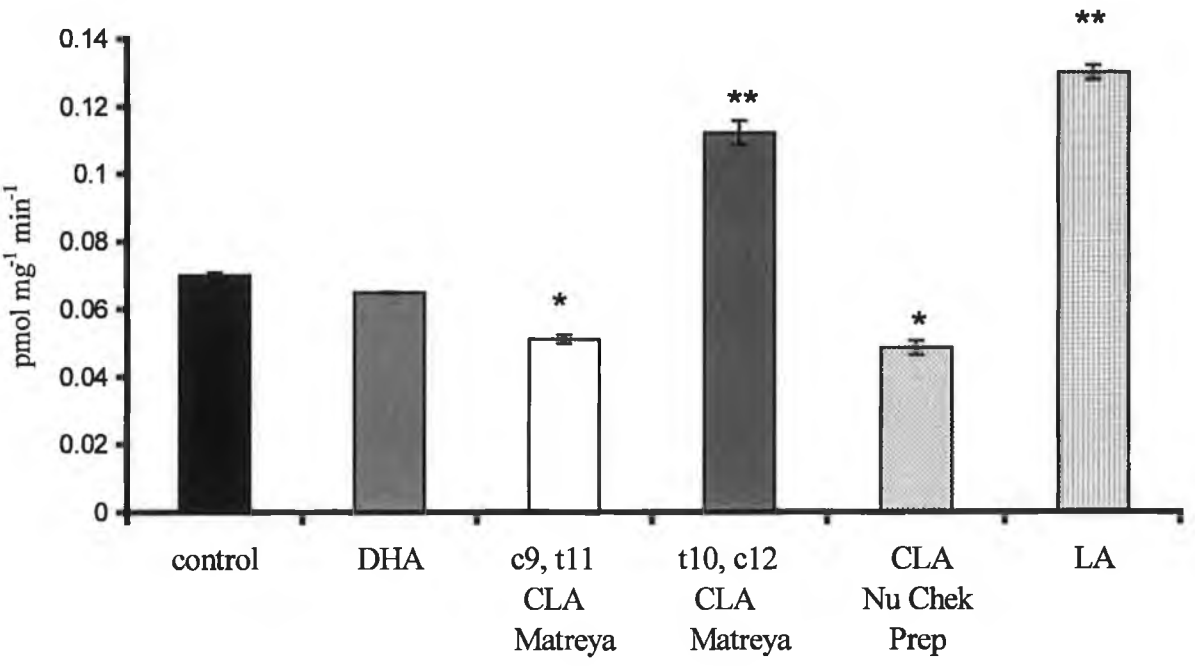

Figure 6.4. PFTase activity for SW480 cells incubated with 20 ppm Nu-Chek Prep CLA, *c*9, *t*11 and *t*10, *c*12 pure CLA isomers (Matreya), LA and DHA for 4 days. * Denotes values that are significantly different ($p < 0.05$) compared with controls, ** Denotes values that are significantly different ($p < 0.01$) compared with controls.

6.5 Discussion

There have been many possible mechanisms of action suggested for the anticarcinogenic effect of CLA, including a prooxidant effect (Schonberg and Krokan, 1995), inhibition of mutagen activation (Liew *et al.,* 1995) and modulation of eicosanoid production (Belury et al., 1997, Li and Watkins, 1998). In addition, it has been proposed that growth regulatory genes such as *c-myc* and *c-jun*, may be influenced by binding of CLA to peroxisomal proliferator activated receptors (Moya-Camarena *et al.,* 1999b). We have previously observed that CLA was incorporated into phospholipids and neutral lipids of MCF-7 cells (Chapter 5). As a result of incorporation into phospholipids CLA may modulate subsequent signal transduction pathways which depend on the proper membrane localisation of oncogene encoded proteins, for example Ras. As observed previously a high fat fish oil diet containing 31 % n-3 fatty acids has been shown to inhibit PFTase activity (Singh *et al.,* 1998). In this study we compared the effects of equal amounts (20 ppm) of DHA, CLA and LA on PFTase activity of MCF-7 and SW480 cell lines.

Data from this study suggest that specific isomers of CLA differentially modulate the action of Ras oncoprotein. An association between decreased farnesylation and growth suppression was observed in the MCF-7 and SW480 cells treated with the CLA mixture of isomers and with *c*9, *t*11 CLA. Diminished farnesylation suggests that the Ras remained in its biologically inactive form and prevented growth signals from reaching proliferation genes in the nucleus. Incubation with DHA caused a decrease in cell

numbers for both cell lines, however PFTase activity was negligible in these cells. This may be a consequence of the DHA concentration (20 ppm) used, as it has been reported that tumourigenic inhibition by fish oil is expressed at levels close to 100 times the level of CLA (Scimeca *et al.,* 1994). In addition, modulatory effects on tumourigenic inhibition and Ras farnesylation of fish oils were manifested by diets that contained a mixture n-3 fatty acids; therefore DHA may not be the sole modulator of farnesylation kinetics (Singh *et al.,* 1997, 1998). A 47 % increase in PFTase activity was observed in the LA treated MCF-7 cells, which may be responsible for the proliferative effect displayed by LA when incubated with these two cancer cell lines. The relationship between growth and farnesylation was more notable in SW480 cells treated with 20 ppm LA, where a 100 % increase in PFTase activity was associated with 14 % increase in growth. The proliferative effect of LA in this study supports previous studies (Shultz *et al.*, 1992ab, Cunningham *et al.,* 1997) showing LA can stimulate cancer cell growth. Inhibitory effects of CLA and *c*9, *t*11 CLA, on PFTase and stimulatory effects of the *t*10, *c*12 CLA isomer suggest that growth is only partly controlled by farnesylation

Incubation with the *t*10, *c*12 CLA isomer caused a 15 % increase in the activity of PFTase in MCF-7 cells and a 66 % increase in SW480 cells, yet was anti-proliferative in both cell lines. Stimulation of the farnesylation reaction in *t*10, *c*12 CLA and LA treated cells may have initiated different phosphorylation signaling cascades resulting in proliferation in LA-treated cells and growth suppression in *t*10, *c*12 CLA treated cells. However the differential effects of individual fatty acid treatments suggest that farnesylation is only partly responsible for growth modulatory effects observed in these

cells. This is evident from the observation that the large increase (100 %) in PFTase activity observed in LA treated SW480 cells was associated with only a marginal increase in growth (15 %). The *t*10, *c*12 CLA isomer may also target events downstream of Ras farnesylation and thereby inhibit proliferation. The *c*9, *t*11 CLA isomer at 20 ppm had a similar effect as a CLA mixture of isomers which contained only 6 ppm *c*9, *t*11 CLA. The mixture also contained approximately 6 ppm *t*10, *c*12 CLA, which in the pure form (20 ppm) was found to be stimulatory towards PFTase activity. This suggests that 6 ppm *c*9, *t*11 CLA may be sufficient to reduce PFTase activity, and that the *c*9, *t*11 isomer has a dominant effect over the *t*10, *c*12 isomer. However other isomers present in the mixture may also have an effect.

Farnesyl-pyrophosphate the substrate for Ras farnesylation by PFTase is formed as an intermediate in the mevalonate pathway responsible for cholesterol biosynthesis (Singh *et al.,* 1997, 1998). n-6 and n-3 fatty acids have been shown to differentially modulate the activity of 3-hydroxymethylglutaryl Co-enzyme A (HMG-CoA) reductase, the rate limiting enzyme in the mevalonate pathway (ElSohemy and Archer, 1997). Further studies examining the effects of CLA on HMG-CoA-reductase in cancer cells are warranted.

We have previously shown a 90 % inhibition of growth in cancer cells treated with milk fat to yield a CLA concentration of 20 ppm (Chapter 5). The *c*9, *t*11 form is the predominant (92 %) CLA isomer found in milk fat triglycerides (Chin *et al.,* 1992) and in view of the inhibitory effect of this CLA isomer on PFTase activity, it is possible that

milk fat CLA in its triglyceride bound form may also modulate cell growth in these cells by inhibition of ras farnesylation. Further studies to investigate the effects of milk fat CLA and individual CLA isomers on events downstream of Ras are warranted.

6.6 Summary

Ras oncoproteins have been implicated in a phosphorylation cascade which results in the activation of proliferation genes. The aim of this study was to determine if the anticarcinogenic fatty acid CLA inhibited the activity of PFTase, the rate limiting enzyme in the farnesylation of Ras in MCF-7 and SW480 cancer cell lines. A mixture of CLA isomers (*c*9, *t*11-, *t*10, *c*12-, *c*11, *t*13- and minor amounts of other isomers), *c*9, *t*11 CLA, *t*10, *c*12 CLA and DHA (all at 20 ppm) significantly ($p < 0.05$) suppressed growth of both cell lines following 4 days of incubation compared with untreated controls. Incubation of MCF-7 and SW480 cells with LA (20 ppm) had negligible effects on cell numbers after 4 days. Incubation of both cell lines with 20 ppm CLA (mixture of isomers) or 20 ppm *c*9, *t*11 CLA for 4 days resulted in significantly ($p < 0.05$) reduced PFTase activity compared with untreated controls. Although the pure *c*9, *t*11 CLA isomer was a more potent inhibitor ($p < 0.03$) than the mixture of CLA isomers ($p < 0.05$) of MCF-7 cell PFTase activity, there was no significant difference between these two treatments on the activity in SW480 cells. On the other hand PFTase activity was increased ($p < 0.05$) in both MCF-7 and SW480 cells incubated with similar concentrations (20 ppm) of pure *t*10, *c*12 CLA or LA compared with untreated control cells. DHA (20 ppm) had a negligible effect on PFTase activity in both cell lines. The results suggest that the mixture of CLA isomers and the pure *c*9, *t*11 CLA isomer down

regulate a *ras*-dependent signaling cascade resulting in growth suppression. Stimulation of the farnesylation reaction in the LA treated cells may have induced signaling events that initiated proliferation. The stimulation of PFTase activity due to incubation with the pure *t*10, *c*12 CLA isomer suggests that the two CLA isomers (*t*10, *c*12 and *c*9, *t*11) mediate growth suppressive effects in cancer cells via distinct mechanisms of action.

CHAPTER 7

Final Discussion and Conclusions

The field of cancer chemoprevention has experienced a rapid growth in the identification and characterisation of a vast number of anticarcinogenic substances that are present naturally in many food sources. Research is unveiling milk fat as a novel source of such anticarcinogenic substances; evidence contained within this thesis suggests that CLA in particular is a potent anticarcinogenic component of milk fat. Following a landmark study showing a powerful protective effect of CLA-enriched butter against risk of mammary cancer development in rodents (Ip *et al.*, 1999a), it is imperative that foods enriched in this novel fatty acid are made available. Many health enhancing components of milk are currently being exploited for the development of added-value 'functional food' ingredients or nutraceuticals, and is an area where biotechnology may provide new possibilities for the food industry. Biotechnology embraces a wide range of technologies over and above genetic manipulation and includes fermentation technologies, protein separation technologies and the use of enzymes for food processing. These techniques offer an opportunity to the dairy industry to promote dairy products and overcome the negative consumer image that exists due to the presence of cholesterol, saturated and *trans* fatty acids in these products. CLA is a *trans* fatty acid and in view of the potential harmful effects of TFA described in chapter 2 it is necessary to monitor the levels of TFA during fractionation and/or lipid modification procedures aimed at enrichment of products with CLA. The aims of the current study were 5-fold: (i) to validate methodology for the accurate quantification of TFA in edible oils and fats, (ii) to analyse the CLA and TFA contents of a range of food products, (iii) to investigate dry fractionation as a means of enriching the CLA content of milk fat, (iv) to investigate specific mechanisms of action in human cancer cell lines that may be responsible for the

anticarcinogenic effect of synthetic CLA and (v) to compare effects of synthetic CLA with those of milk fat triglyceride bound CLA.

Analysis of TFA in the past has been hampered by the unavailability of suitable reference materials particularly those of minor, unusual *cis* and *trans* isomers (Ratnayake, 1990). Oil samples of known TFA content were supplied to European laboratories as part of an international collaborative study for the purpose of validating methodology leading to the accurate quantification and detailed compositional analysis of individual TFA isomers. The analysis of similar samples by different laboratories using differing techniques was critical in the development of a suitable method. This study validated a combined Ag-TLC and GLC methodology for the quantification of TFA in vegetable oils. Initially the accuracy and separation power of the method was established by the successive analysis of fatty acid standards until satisfactory repeatability and reproducibility was obtained. The base catalysed FAME procedure was shown to have no adverse effects on the TFA levels. Intercomparison of data obtained from analyses of common samples in different laboratories led to critical assessment and validation of the methodology for TFA analysis. Given that certified reference material for TFA are generally not available, this methodology may be used in the certification of reference materials containing TFA. Spreads containing hydrogenated vegetable fat only as the fat source contained relatively high amounts of TFA compared with products containing only milk fat as the fat source. CLA analysis of dairy products involved acid catalysed methanolysis (4 % HCL in methanol) of samples followed by separation of FAME on a Supelcowax-10 capillary GLC column (Stanton *et al.*, 1997a). Of all the dairy products analysed, butter was found

to be the richest source of CLA (12.3 – 14.2 mg/g fat), while the yoghurt and cheese samples, contained, on average, one and two-thirds, respectively, of the CLA content of butter. Evidence now indicates that specific biological effects may be due, in some cases, to the specific action of a single CLA isomer and in other cases, to a more general effect that may be produced by two or more CLA isomers. Accordingly, it is imperative to have standardised methodologies for separating and quantifying individual CLA isomers in complex mixtures such as foods, biological materials and manufactured products. In addition, as basic research reveals new information concerning the biological importance of specific CLA metabolites, it will be desirable to quantify their levels in foods that are derived from animals that naturally produce CLA (i.e. ruminants) and those fed CLA in either a natural or synthetic form. A study similar to that carried out in chapter 2 of this thesis would provide a standard method of CLA analysis and allow accurate quantification of individual CLA isomers in foods and biological materials.

No studies have yet been published that document typical CLA intake using the gold standard, food duplicate methodology. This approach involves combining estimates of CLA levels in duplicate portions of food with dietary records. Indirect methodologies relying on available CLA databases have been used. Because CLA concentrations within similar foods can vary greatly depending on the initial composition of raw food products, the accuracy and adequacy of these databases are questionable. Rigorous validation of these nutrient databases is necessary, and potential indicators of CLA intake, such as fasting plasma level or adipose tissue concentrations of CLA should be evaluated. Additionally, estimations of dietary intakes of CLA isomeric forms by infants, children

and adolescents have not yet been documented. It is important that estimations of CLA intake in children be investigated in view of the finding that dietary CLA was more effective in influencing anticarcinogenic activity when consumed during periods of active mammary gland development in the rodent model (Ip *et al.*, 1995). Age and dietary CLA intake may interact, such that increased CLA intake during infancy and adolescence might preferentially decrease the risk of cancer in later life. Examination of the relationships among dietary intake of CLA isomers, their concentrations in adipose tissue and plasma, and risk of various chronic degenerative diseases (e.g. cancer, diabetes and osteoporosis) is essential for scientists and public health officials to draw conclusions concerning the importance of dietary CLA isomers in human health.

Dry fractionation was examined as a means of increasing the CLA content of milk fat. The maximum CLA enrichment obtained by dry fractionation in this study was 63 % over the parent fat, yielding a milk fat fraction containing 2.22 g CLA /100g FAME. This enrichment in the CLA content represented approximately a 3-fold increase in the amount of CLA found in a range of milk fat containing-food products. Following milk fat fractionation, the CLA-enriched milk fat fraction was increased in *t*11-$C_{18:1}$ content by 28 %, compared with the parent fat. It has been proposed that *t*11-$C_{18:1}$ the major *trans* fatty acid in milk fat, may also act as a beneficial fatty acid (Ip *et al.*, 1999), since recent studies show that it is converted *in vivo* to the *c*9, *t*11-CLA isomer by the Δ9-desaturase enzyme (Griinari and Bauman, 1999, Santora *et al.*, 2000). To establish the importance of *t*11-$C_{18:1}$ as a precursor of endogenous synthesis of CLA, it is imperative to conduct animal and cellular studies to investigate cell and tissue responses to *t*11-$C_{18:1}$. Both

descriptive data (tissue sites) and quantitative studies (activities of Δ9-desaturase) should be undertaken. Such studies will increase not only our understanding of CLA metabolism in humans, but also will contribute to an appreciation of the importance of these fatty acids in the diet. Thus, milk fat enriched in CLA and *t*11-$C_{18:1}$, by dry fractionation may offer a means to generate a food ingredient with potential benefits to human health. Lipase mediated interesterification reactions to modify the fatty acid composition of milk fat triglycerides is a novel biotechnological approach to enriching dairy products with CLA (Garcia *et al.,* 1998). The use of starter cultures (e.g. lactic acid cultures) and additives (e.g. lactose and fructose) which promote CLA formation from linoleic acid are alternative possibilities leading to the natural production of food grade products enriched in CLA.

The anticarcinogenic effects of CLA were examined in this study using two human tumour cell lines. Mammary and colon tumour cell lines furnished *in vitro* models for the investigation of the anticarcinogenic effects of CLA. Evidence of CLA-induced cytotoxicity, lipid peroxidation and modification of Ras oncoprotein farnesylation in these two cell lines was provided. CLA-enriched milk fat and the pure *c*9, *t*11 CLA isomer (Matreya) exerted similar cytotoxic and prooxidant effects in these cell lines. An opposite proliferative effect was demonstrated in these model systems for LA, which supported previous studies (Cunningham *et al.,* 1997, Shultz *et al.,* 1992) showing LA can stimulate cancer cell growth. An excess of intracellular reactive oxygen species in relation to antioxidative systems is the driving force in several human malignancies. It results in an oxidative environment which may modulate gene expression or damage

cellular molecules via adduct formation. Data suggests that enhanced ability of tumour cells in scavenging free radicals by antioxidant enzymes and thiol compounds may, at least in part, contribute to the resistance of cancer cell death during chemotherapy (Suzuki *et al.*, 2000). High levels of glutathione during chemotherapy can reduce the effectiveness of a chemotherapeutic drug due to detoxification (Hercberg, 1992). Efforts made to treat cancer with chemotherapeutic agents are often hampered by intrinsic or acquired drug resistance of the tumours. Some tumours initially respond favourably to chemotherapy, but subsequently develop multi-drug resistance. Enrichment with polyunsaturated fatty acids makes leukemia cells more susceptible to lipid peroxidation and increases total lipid radical formation in response to oxidative stress. Polyunsaturated fatty acids such as DHA, eicosapentaenoic (EPA), gamma linolenic acid (GLA) and parinaric acid, have been shown to be cytotoxic to tumour cells by inducing oxidative stress and altering the activity of cell membrane bound enzymes such as sodium-potassium-ATPase, suggesting that essential fatty acids and their metabolites can reverse tumour cell drug resistance at least *in vitro* (Burns and Spector, 1994, Das *et al.*, 1997). The prooxidant activity of CLA observed in cancer cells may provide a means to reverse this resistance to chemotherapy. Future work must elucidate if the cytotoxic effect of CLA in cancer cell lines can be blocked by the addition of antioxidants.

The prooxidant effect observed in cancer cells treated with CLA in this study may not be solely responsible for the anticarcinogenic activity of CLA. CLA was preferentially incorporated into the phospholipid fraction of the MCF-7 cell membranes. CLA uptake was more proficient from milk fat than from synthetic sources, supporting previous work

by Ip *et al.* (1999a), suggesting possible enzymatic conversion of *t*11-$C_{18:1}$ present in milk fat to CLA by a cellular Δ9-desaturase. The presence of Δ9-desaturase in humans would offer potential for *t*11-$C_{18:1}$-enriched products allowing for endogenous synthesis of CLA. Indeed dietary *t*11-$C_{18:1}$ led to increased levels of CLA in carcass lipids (Santora *et al.*, 2000). This may be a more effective means of increasing CLA levels in tissues than dietary intake of CLA. Through its incorporation into membrane phospholipids CLA may modulate signal transduction pathways which depend on the proper membrane localisation of oncoproteins, for example Ras. Data from this study suggest that an association exists between decreased farnesylation and growth suppression in the MCF-7 and SW480 cells treated with the CLA mixture of isomers and with *c*9, *t*11 CLA. The *c*9, *t*11 form is the predominant (92 %) CLA isomer found in milk fat triglycerides (Chin *et al.*, 1992) and in view of the inhibitory effect of this CLA isomer on PFTase activity, it is possible that milk fat CLA in triglyceride bound form may also modulate cell growth in these cells by inhibition of *ras* farnesylation.

CLA may have potential effects on other stages of carcinogenesis yet to be investigated such as angiogenesis. Neovascularisation, or angiogenesis, is essential for solid tumour growth (Folkman, 1990), and also provides the tumour cells with access to the vascular circulatory system, thus establishing the potential for metastatic disease progression. Angiogenic activity in primary breast cancers has been associated with metastasis (Heimann *et al.*, 1996), benign breast lesions or breast ductal carcinoma with a propensity for progression to malignant disease (Guinebreti *et al.*, 1994, Heffelfinger *et al.*, 1996). The process of neovascularization is promoted by a variety of angiogenic factors, which

include both prostaglandins (Form *et al.,* 1983) and 12-HETE (Tang *et al.,* 1995). Transfection of the MCF-7 human breast cancer cell line so that it stably overexpresses eicosanoids (12-LOX and 12-HETE), causes rapid growth with not only reduced apoptosis, but also high angiogenic activity (Connolly & Rose, 1998). Dietary CLA, because of its suppressive effects on eicosanoid biosynthesis, may prove to be antiangiogenic.

Although extensive research has now shown that a family of enzymes known as matrix-degrading metalloproteinases are implicated in early and late stages of tumour progression (Mc Donnell *et al.,* 1999), no study has yet related the antimetastatic effect of dietary CLA with these enzymes. Hence studies to examine the influence of CLA on metalloprotease secretion, angiogenic capacity and invasion, factors critical in the metastatic cascade seem warranted.

The possibility that CLA may be considered as a potential dietary component for use in nutritional prevention of breast cancer is an attractive issue. However, to date beneficial effects of CLA have been demonstrated in animal experimental models and *in vitro* systems only. Even though there is some epidemiological evidence in the literature linking CLA intake and mammary tumour prevention in humans, more studies are required to confirm this effect. The amount of dietary CLA required, the duration of intervention, as well as the most appropriate stage(s) in life for such an intervention are issues that are not yet known. Performing human dietary intervention studies with diets enriched in CLA, such as selected dairy products, followed by analysis of adipose tissue

fatty acids would provide more insight into these issues. The efficacy of CLA supplementation to inhibit tumour growth in cancer patients needs to be evaluated. Analysis of normal and malignant tissues post surgery would give a novel insight into the use of CLA as adjuncts to conventional therapies.

More mechanistic studies are required to tie the multifunctional benefits of CLA together. Future work needs to ascertain potential biomarkers of CLA exposure; such work is ideal for DNA array analysis. Multi array analysis is a new tool of functional genomics that has been developed to visualise changing patterns of gene expression in a more holistic fashion than was possible at any other time in biology. This technology will be required to give information on the effects of CLA on a large number of genes regulating for example differentiation, apoptosis and lipogenesis in CLA treated cells and tissues. Technologies such as differential display reverse transcriptase polymerase chain reaction (DD RT-PCR) offer a method of rapidly identifying many genes that are expressed in different amounts in two or more samples. Unlike subtraction–hybridisation, the DD RT-PCR method enables the detection of both up-regulated and down regulated mRNAs in the same experiment. The technique involves seven major steps that lead to the definition of specific gene(s) that are affected by a treatment. These steps are (1) extraction of total and mRNA from a biological sample, (2) cDNA synthesis with distinct one base-anchored oligo (dT) primers, (3) amplification of cDNAs with a combination of anchored and random primers in the presence of a radiolabeled deoxynucleotide triphosphate, (4) separation of the amplified radiolabeled cDNA fragments on polyacrylamide gel followed by exposure of the dried gel to an autoradiographic film, (5)

lane by lane comparison of the cDNA bands displayed on the film followed by identification and selection of differentially displayed bands, (6) excision of the selected bands from the gel followed by extraction of cDNA, amplification and purification, and (7) cloning and sequencing of the gel-purified cDNAs and verification of the specific mRNA change by Northern analysis. The possibility of identifying non homologous effects between two samples from the comparison of their entire gene sequences will allow for the identification of specific functions of individual genes. This technology would provide an exciting new approach to studying effects of CLA in conjunction with other beneficial fatty acids such as omega-3 fatty acids.

It is becoming increasingly recognised that, at least in the long term, future advances in clinical cancer research will come from an emphasis on prevention rather than the treatment of metastatic disease. Such research efforts will encompass epidemiological studies, laboratory experiments, and the execution of clinical preventive trials (Greenwald *et al.,* 1993). This would provide a sound scientific basis for investigating if CLA may be considered for use as a nutraceutical in functional foods for maintaining health and preventing certain diseases in the human population.

CHAPTER 8

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Appendix 1

Accuracy Testing

Relative RF values for the distributed test mixture (mean of 5 replicates). * Analysis in this laboratory

Lab	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$ (n-9)
1	1.028	1.014	1.000	1.019
2	0.920	0.966	1.000	1.006
3	1.048	1.020	1.000	1.001
4	0.995	0.989	1.000	0.996
5	1.009	1.001	1.000	1.008
6	1.072	1.034	1.000	1.023
7	0.948	0.977	1.000	1.024
8	1.035	1.008	1.000	1.010
9	0.979	1.004	1.000	1.010
10*	1.082	0.914	1.000	0.971
11	0.995	0.999	1.000	1.018

Appendix 2

Relative response factors of the distributed FAME mix (n=11)

	Mean	SD	Min	Max
$C_{16:0}$	1.005	0.037	0.936	1.062
$C_{18:0}$	1.000			0
$C_{18:1}$	0.992	0.014	0.962	1.006
$C_{18:2}$	1.022	0.023	0.986	1.055
$C_{18:3}$	1.058	0.038	1.001	1.121

Relative response factors of the distributed triglyceride mix (n=11)

	Mean	SD	Min	Max
$C_{16:0}$	0.985	0.040	0.898	1.029
$C_{18:0}$	1.000			
$C_{18:1}$	0.989	0.047	0.846	1.023
$C_{18:2}$	1.009	0.036	0.927	1.075
$C_{18:3}$	1.060	0.050	0.999	1.174

Appendix 3

Repeatibility (r) and reproducibility (R) of Ag-TLC/ GLC for quantification of methyl elaidate in soya oil FAME (gTFA/100 g FAME).

	FAME 1	FAME 2	FAME 3
RSD (r)	3.61	1.65	2.65
RSD(R)	6.97	3.61	3.37

Repeatibility (r) and reproducibility (R) of Ag-TLC/ GLC for quantification of trieladin in soya oil (gTFA/100 g FAME).

	Oil 1	Oil 2	Oil 3
RSD (r)	4.47	1.95	2.18
RSD(R)	6.19	2.90	3.28

Appendix 4

Comparison of precision data between 1st and 2nd intercomparison studies for 11 laboratories

Low level TFA

	$C_{18:1}$		$C_{18:2}$		$C_{18:3}$		Total	
Intercomparison	1st	2nd	1st	2nd	1st	2nd	1st	2nd
RSD(r)	247	167	115	62	55	25	53	36
RSD(R)	247	167	115	62	55	25	53	36

Medium level TFA

	$C_{18:1}$		$C_{18:2}$		$C_{18:3}$		Total	
Intercomparison	1st	2nd	1st	2nd	1st	2nd	1st	2nd
RSD(r)	6	5	8	4	29	15	5	4
RSD(R)	13	10	15	10	116	32	9	8

High Level TFA

	$C_{18:1}$		$C_{18:2}$		$C_{18:3}$		Total	
Intercomparison	1st	2nd	1st	2nd	1st	2nd	1st	2nd
RSD(r)	3	3	5	2	54	136	3	2
RSD(R)	15	7	13	9	179	245	13	5

Appendix 5

Intercomparison 2 data from 11 labs

Soya Oil

	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	Total
Mean	0.05	0.63	0.97	1.64
Min	0.00	0.51	0.49	1.13
Max	0.18	0.81	1.15	2.13
RSD(r)	84.58	9.82	4.72	5.24
RSD(R)	142.83	16.06	18.96	16.60

Rapseed Oil

	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	Total
Mean	0.06	0.16	0.57	0.79
Min	0.00	0.00	0.30	0.30
Max	0.28	0.31	0.75	1.33
RSD(r)	108.65	36.41	5.77	10.22
RSD(R)	167.36	62.32	25.32	35.61

7 % TFA Sample

	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	Total
Mean	4.75	2.51	0.23	7.50
Min	4.23	1.95	0.11	6.87
Max	5.50	2.85	0.34	8.68
RSD(r)	5.29	4.45	15.13	3.62
RSD(R)	10.46	10.39	31.69	8.37

20 % TFA Sample

	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	Total
Mean	13.51	6.24	0.02	19.77
Min	11.76	4.83	0.00	17.95
Max	14.65	6.94	0.11	20.92
RSD(r)	2.85	2.08	135.52	2.31
RSD(R)	6.82	8.94	245.26	5.03

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