How can palm oil be modified to give improved dietary benefits?

By

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This thesis is dedicated

to my parents,

and my children

Farah, Faten and Farhan

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ABSTRACT

Arthritis is a chronic inflammatory disease which can be partly alleviated by anti-inflammatory compounds (such as non-steroidal anti-inflammatory drugs) or dietary components such as *n-3* polyunsaturated fatty acids (*n-3* PUFAs). Bovine chondrocyte cell cultures represent an experimental system that can be used to study arthritis in *vitro* and this was used in the work reported here. The relative effectiveness of different fatty acids in reducing inflammatory responses was studied using these cultures. Eicosapentaenoic acid (EPA) proved to be most effective *n-3* PUFA compared to docosahexaenoic (DHA) or α-linolenic acid (ALA), in suppressing the levels of mRNA for pro-inflammatory proteins (COX -2, IL-1α, IL-1β, TNF-1α), aggrecanases (ADAMTS-4 and ADAMTS-5) and matrix metalloproteinases (MMP-3 and MMP-13) in the bovine monolayer cultures which had been induced by IL-1α. Arachidonic acid (AA), an *n-6* PUFA, had no effect on these mRNA levels. Similarly, hydrolysed palm olein had no consistent effect, showing that neither of these fatty acid preparations could be regarded as anti-inflammatory.

Microscopic examination of the cells in culture showed some evidence for destructive effects on IL-1α stimulation and this was reduced by EPA. Moreover, this was confirmed when GAG release was examined. The latter was increased by IL-1α exposure and this was reduced by *n-3* PUFAs with EPA being the most effective.

To increase the potential value of palm olein products, the *n-3* PUFAs, α-linolenic acid and EPA were incorporated into palm olein through lipase-catalyzed interesterification reactions. Lipozyme *IM 60* was the most effective enzyme tested and its reaction was studied under various incubation conditions in order to find those suitable for an effective modification of its composition. Hexane was used as solvent, 55°C as temperature and 10 % (g/g) of enzyme to substrate. Palm olein, which had been modified with EPA, was tested for its anti-inflammatory properties. It was found to reduce GAG release, and the levels of mRNA for various inflammatory proteins (COX-2, TNF-α, IL-1α, IL-1β) and the proteinase ADAMTS-4. These results showed that it is possible to modify palm olein by interesterification to yield an oil with improved nutritional properties.

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ABBREVIATION LIST

AA	Arachidonic acid
ADAM	A Disintegrin And Metalloproteinase
ADAMTS	A Disintegrin And Metalloproteinase with ThromboSpondin motifs
ALA	α-linolenic acid (18:3n-3)
ATP	Adenosine triphosphate
A_w	Water activity
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CLA	Conjugated linoleic acid
CM	Cartilaginous matrix
CPO	Crude palm oil
COX	Cyclooxygenase
DAG	Diacylglycerol
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dNTP	Deoxynucleoside triphosphate
DGLA	Dihomo-γ-linolenic acid (20:3n-6)
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DHA	Docosahexaenoic acid (22:6n-3)
DMEM	Dulbecco's modified Eagle's medium
DMMB	Dimethylmethylene blue
EFA	Essential fatty acids
EPA	Eicosapentaenoic acid (20:5n-3)
FBS	Fetal bovine serum
FLAP	5-lipoxygenase activating protein
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

GC	Gas chromatography
GlcUA	Glucuronic acid
GaINAc	N-acetylgalactosamine
HETE	Hydroxyeicosatetraenoic acid
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid
HPETE	Hydroperoxyeicosatetraenoic acid
HPOo	Hydrolysed palm olein
HPLC	High performance liquid chromatography
IFN	Interferon
IE	Interesterification
IGF	Insulin -like growth factor
IL-1	Interleukin-1
IL-1Ra	Interleukin-1 receptor antagonist
IL-1α	Interleukin-1 alpha
IL-1β	Interleukin-1 beta
IV	Iodine value
LA	Linoleic acid (18:2n-6)
LOX	Lipoxygenase
LP	Link protein
MAG	Monoglycerides
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
ND	Not determined
NO	Nitric oxide
NSAIDS	Non-Steroidal Anti-Inflammatory Drugs
OA	Osteoarthritis
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PG	Prostaglandin
PGG_2	Prostaglandin G_2

PGH_2	Prostaglandin H_2
PGE_2	Prostaglandin E_2
POo	Palm oil olein
PUFA	Polyunsaturated fatty acids
RA	Rheumatoid arthritis
RBD POo	Refined bleached deodorized palm oil olein
RNA	Ribonucleic acid
RNase	Ribonuclease
rRNA	Ribosomal ribonucleic acid
RT	Reverse transcriptase
RT-PCR	Reverse transcription polymerase chain reaction
rpm	Revolutions per minute
SD	Standard deviation
TIMPs	Tissue inhibitor metalloproteinases
TAG	Triacylglycerols
TBE	Tris-borate-ethylenediaminetetracetic acid
TGF	Transforming growth factor
TGF-β	Transforming growth factor beta
TNF-α	Tumour necrosis factor-alpha
v/v	Volume to volume
w/v	Weight to volume
g/g	Gram to gram

CHAPTER ONE

General introduction

1.1 Introductory remarks

The inflammatory joint diseases, rheumatoid arthritis and osteoarthritis, are very common. They rank second (Harris and Firestein., 2001) as the most prevalent chronic diseases after heart disease and are more common in women than men. Almost 9 million people in the UK (19% of the population) have long-term health problems due to arthritis. Alarming statistics in Malaysia estimate that 60% of the population will have some form of arthritis (www.afm.org.my) by the age of 60, particularly osteoarthritis. Rheumatoid arthritis, on the other hand, affects 1% of the Malaysian population and can affect all age groups.

Many types of arthritis show signs of joint inflammation; swelling, stiffness, tenderness, redness or warmth. One of the important pathological processes contributing to cartilage erosion during degenerative joint disease is loss of the aggregating cartilage proteoglycan, aggrecan. Catabolism of aggrecan is increased by the proteolytic activity of aggrecanases, ADAMTS-4 and ADAMTS-5. When the cartilage loss is great, there may be severe pain in the joint involved, during use or even at rest. IL-1α and TNF-α are the two pro-inflammatory cytokines that have been regarded as pivotal mediators in arthritis that can increase aggrecanase activity in the cartilage. Furthermore, the initiation of cartilage breakdown in arthritis may arise from mechanical damage or be influenced by inflammatory cytokines which stimulate the release of arachidonic acid from the cell membranes of chondrocytes. Arachidonic acid is a metabolic precursor of prostaglandins (PGs) and

leukotrienes which are well known mediators of inflammation. Moreover, prostaglandin E_2 can also stimulate normal chondrocytes to release proteinases, which are capable of degrading the proteoglycans and collagens of the cartilage matrix. PGs are synthesized from arachidonic acid following the action of cyclooxygenases (the constitutive COX-1 and inducible COX-2).

In addition, diet seems to play an important role in immune cell functions. Fish oil supplements, rich in *n-3* PUFA such as eicosapentaenoic acid, have proved to be beneficial in arthritis through suppression of the immune system and its cytokine repertoire (Curtis *et al.*, 2000; Curtis *et al.*, 2002). β-Carotene may also slow the progression of rheumatoid arthritis since it has excellent antioxidant properties that help neutralize free-radicals (Heliovaara *et al.*, 1994). The anti-oxidant vitamin E is also beneficial in modulating immune reactions and the onset of autoimmunity. Since palm oil is one of the world's richest natural plant sources of carotenes in terms of retinol (pro-vitamin A) equivalents and contains high amounts of vitamin E, it would be expected to have anti-inflammatory effects that might be useful in the diet. The effects of palm oil fractions and other edible oil components, such as *n-3* polyunsaturated fatty acids, on a model system for studying arthritis forms the main topic for this thesis.

1.2 Structure of joints

1.2.1 Articular cartilage

A joint is where two bones meet. Joints are designed to allow the bones to move in certain directions, to be strong enough to take body weight and to lock into positions which enable upright stance (Mow *et al.*, 1992). A diathrodial joint is an encapsulated system, which encloses its articulating surfaces and lubricant. The end of each bone is covered with a protective layer of articular cartilage which has smooth slippery surfaces that serve to reduce

contact stress in the joint as shown in (Figure 1.1) (Setton *et al.,* 1993). The joint is surrounded by a membrane, called the synovial membrane, which produces a thick synovial fluid (Mow *et al.,* 1992). This is a clear, viscous fluid which lines the joint space and acts as a lubricant and shock absorber. The articular cartilage lacks blood vessels and nerves and is dependent upon diffusion of nutrients from synovial fluid. The synovial membrane serves several purposes: it regulates the amount and content of the synovial fluid, it permits the removal of waste materials from the synovial fluid, it allows nutrients to enter the synovial capsule and it secretes synovial fluid and other macromolecules for lubrication of the joint (Palastanga *et al.,* 1998). The synovium has a tough outer layer called the capsule that holds the joint in place and stops it from moving too much (Shipman, 1985).

Cartilage and bone are specialized connective tissues sharing a common characteristic: cells embedded in an extracellular matrix and permeated by a network of fibres. Cartilage is supplementary to bone, being formed whenever strength, rigidity and some elasticity are required. In foetal development, cartilage is often a temporary tissue being later replaced by bone. However, in many places cartilage persists throughout life. Articular cartilage has very important properties. It distributes the load to avoid high stresses on small load-bearing areas within the joint. It must also provide a low-friction bearing surface for the gliding and rolling of joint surfaces against one another (Mow *et al.,* 1995). There are three major types of cartilage in the body: (1) hyaline cartilage (2) fibrocartilage and (3) elastic cartilage. Hyaline cartilage has large amounts of both collagens and proteoglycan while fibrocartilage has more collagen than proteoglycan. Elastic cartilage has elastin, collagen, and proteoglycan (Palastanga *et al.,* 1998). Hyaline cartilage is the most abundant of the three types of cartilage and is a smooth, glistening, white tissue that covers the surface of all the diarthrodial joints in the human body (Sokoloff , 1980).

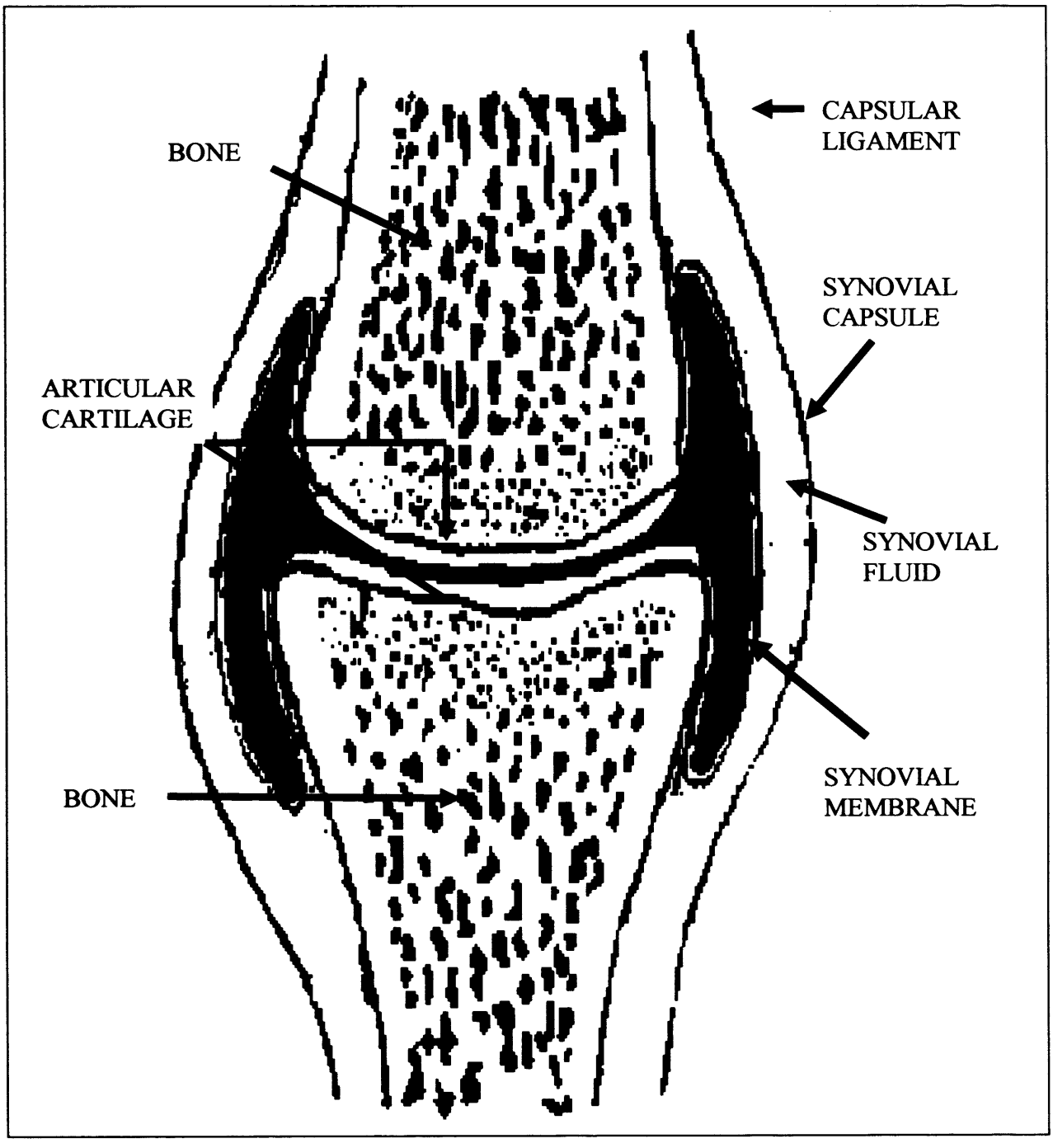


Figure 1.1: Synovial joint diagram (adapted from Shipman, 1985)

With increasing age, hyaline cartilage tends to become calcified and sometimes ossified (Palastanga *et al.,* 1998). As a special form of connective tissue, cartilage acts as a shock absorber between the opposing bones, which make up the joint and prevents impact damage to the ends of the bone (Kiani *et al.,* 2002). Articular cartilage is not an homogeneous tissue but has a complex composition and architecture that permits it to maintain proper biomechanical function over the majority of a human lifespan (Kuettner *et al.,* 1991). Cartilage receives nutrients from the synovial fluid, which itself is synthesised by fibroblasts (Palastanga *et al.,* 1998). Synovial fluid has three functions: it lubricates the articulating surfaces, carries nutrients to chondrocytes and transports waste products away from the cartilage (Kiani *et al.,* 2002). Synovial fluid is primarily composed of long-chain glycosaminoglycans such as hyaluronate and is weakly alkaline e.g. normal human knee fluid is pH 7.29 – 7.45 (Sokoloff, 1980).

1.2.2 Gross structure of articular cartilage

Cartilage is divided into four zones; superficial layer (or tangential layer or outer layer), middle layer (or transitional layer), deep layer (or radial layer) and the calcified cartilage layer. Figure 1.2 illustrates the organisation of these layers (Mow *et. al.,* 1995).

At the outer surface, the cartilage contains fine collagen fibres arranged parallel to the articulating surface of the joint and the chondrocytes are closely spaced. This region is the superficial tangential zone where the collagen fibres serve mainly to support the tensile stresses generated when compressive loads are applied to the cartilage. The superficial layer is the thinnest zone with the highest content of collagen and the lowest concentration of proteoglycans. Consequently, it has a good ability to resist shear stresses and serves as a

gliding surface for joints (Mow *et al.*, 1992). The superficial zone is the first to show changes in osteoarthritis (Sokoloff, 1980).

The middle layer zone contains around 40 – 60 % of total collagen, which are arranged more randomly and the chondrocytes are randomly dispersed. This zone is involved in the transition between the shearing forces of the surface layer to compression forces. It is also composed almost entirely of proteoglycans (Kiani *et al.*, 2002). The deep radial zone is characterized by collagen fibrils which are woven together and aligned perpendicular to the articulating surface. It distributes loads and resists compression (Mow *et al.*, 1992). The calcified zone is the region that connects the cartilage to the subchondral bone. Fibres nearer the bone are progressively more mineralized and the cartilage and bone are interlaced in a mesh (Sokoloff, 1980). The calcified layer contains the tidemark layer, which straddles the boundary between calcified and uncalcified cartilage. Thus, it separates hyaline cartilage from subchondral bone (Mow *et al.*, 1992).

1.2.3 Composition of articular cartilage

Articular cartilage has a unique structure and composition that allows it to cushion the joint from external stress. It consists of two distinct phases, a fluid phase composed of water (68 - 85 %) and electrolytes and a solid phase (Mow *et al.*, 1992). The solid phase of cartilage is composed of single cells, the chondrocytes and extra-cellular matrix (Sokoloff, 1980). Chondrocytes are the cells that produce cartilage matrix. The extracellular matrix predominates and determines the cartilage's mechanical properties. Collagen and proteoglycan (see section 1.2.6-1.2.7) are the major components (Kiani *et al.*, 2002). The water content of cartilage varies with its age and functional state (Sokoloff, 1980). Immature cartilage is slightly more hydrated than adult tissue.

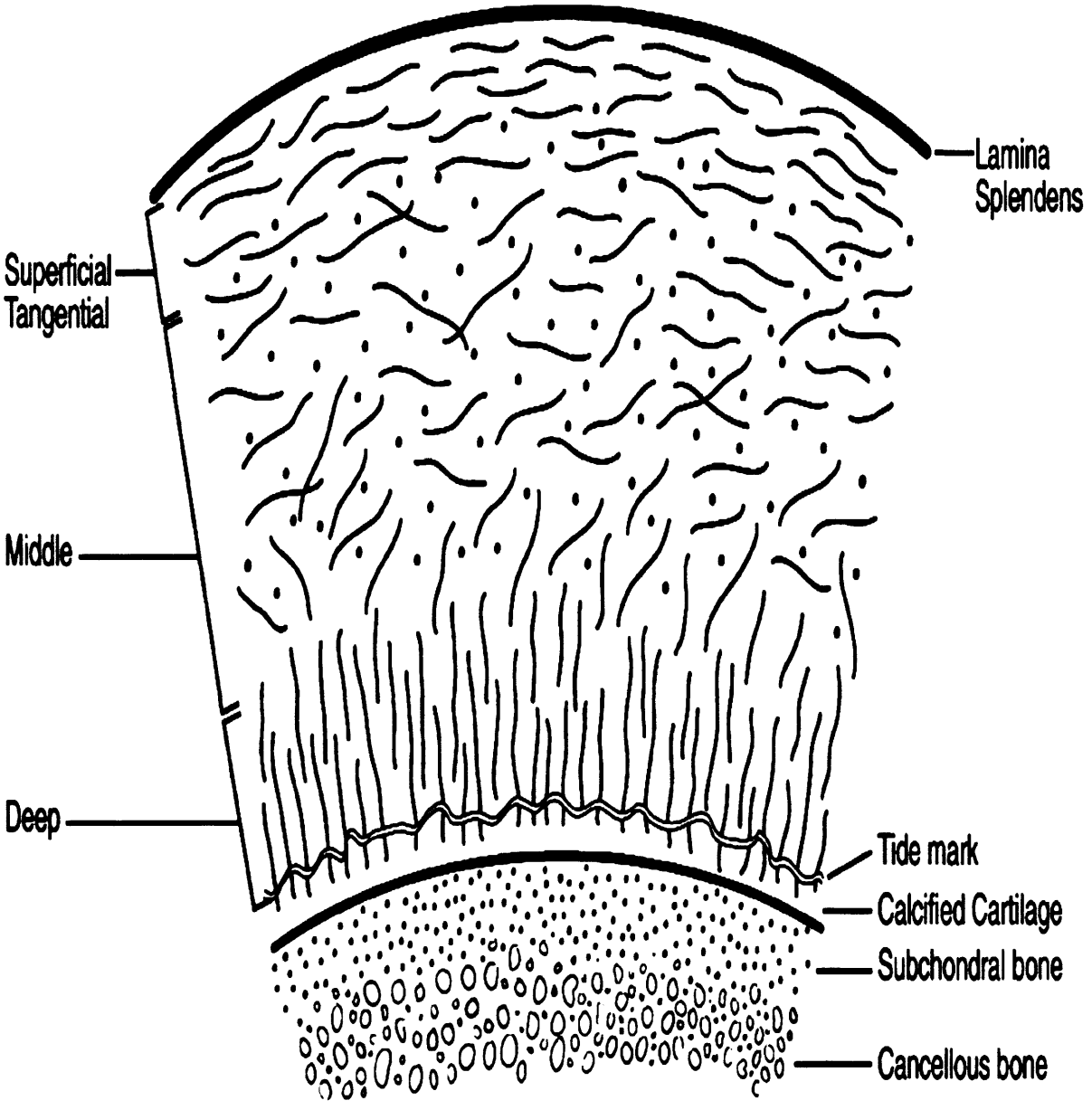


Figure 1.2: Schematic representation of hyaline cartilage with four different zones (taken from Alford and Cole, 2005).

The water is both intra- and extracellular but, since the cells occupy only 1-10 % of the cartilage volume, the intracellular compartment is relatively small. In articular cartilage there are changes in water content during joint activity and loading.

1.2.4 Chondrocytes

Chondrocytes are formed from chondroblasts (Buckwalter and Mankin, 1998). They are surrounded by the matrix and are notable because they manufacture unique extracellular matrix molecules (Watanabe *et al.,* 1998). Chondrocytes are important in matrix turnover, e.g. producing collagen, proteoglycans and enzymes for cartilage metabolism (Sokoloff, 1980). The properties of cartilage and the physical functions of joints are critically dependent on the integrity of the matrix (Kiani *et al.,* 2002) which, in turn, depends on the chondrocytes. Glucosamine produced by chondrocytes is used to make glycosaminoglycans (GAG) and proteoglycans (Buckwalter and Mankin, 1998). Chondrocytes are involved in both anabolism and catabolism of proteoglycan and collagen, which are assembled outside the cell into a mesh of collagen interwoven with aggregated proteoglycan molecules called aggrecan (Abbaszade *et al.,* 1999). Although chondrocytes are metabolically active, they do not divide after adolescence. They exist in a hypoxic environment and, thus, their metabolism is mostly anaerobic. Chondrocyte numbers decrease with age, e.g. about 133,000 cells/mm^3 in cartilage from young cattle and about 34,000 cells/mm^3 in old cattle (McLean and Urist, 1968).

1.2.5 Extracellular matrix

The unique biologic and mechanical properties of articular cartilage depend on the design of the tissue and the interactions between the chondrocytes and the matrix that maintain

the tissue (Buckwalter and Martin, 1998). The gross appearance of the matrix is homogeneous, but actually it has an elaborate structure. It comprises water (75%), type II collagen (10 - 20%) and proteoglycan (5 - 10%) (Mow *et al.,* 1992) as well as enzymes, growth factors and adhesives (fibronectin, chondronectin). Collagen provides tensile strength to resist stretching, while large proteoglycans (aggrecan) provide complementary functions, resisting compression and serving as space-fillers (Albert *et al.,* 1997). Small proteoglycans, including decorin, biglycan and fibromodulin, bind to other matrix macromolecules and, thereby, help to stabilize the matrix. They may also influence the function of the chondrocytes and bind growth factors (Buckwalter and Mankin, 1998). A network of collagen and brush-like proteoglycan molecules trap water in the material as a gel (Figure 1.3). This gel resists pressure and enables cartilage to support relatively high loads. As well as a material framework, the collagen network provides an ideal surface for sliding (Newman, 1998). Anchorin C11, a noncollagenous protein, appears to help to anchor chondrocytes to the matrix (Buckwalter and Mankin, 1998).

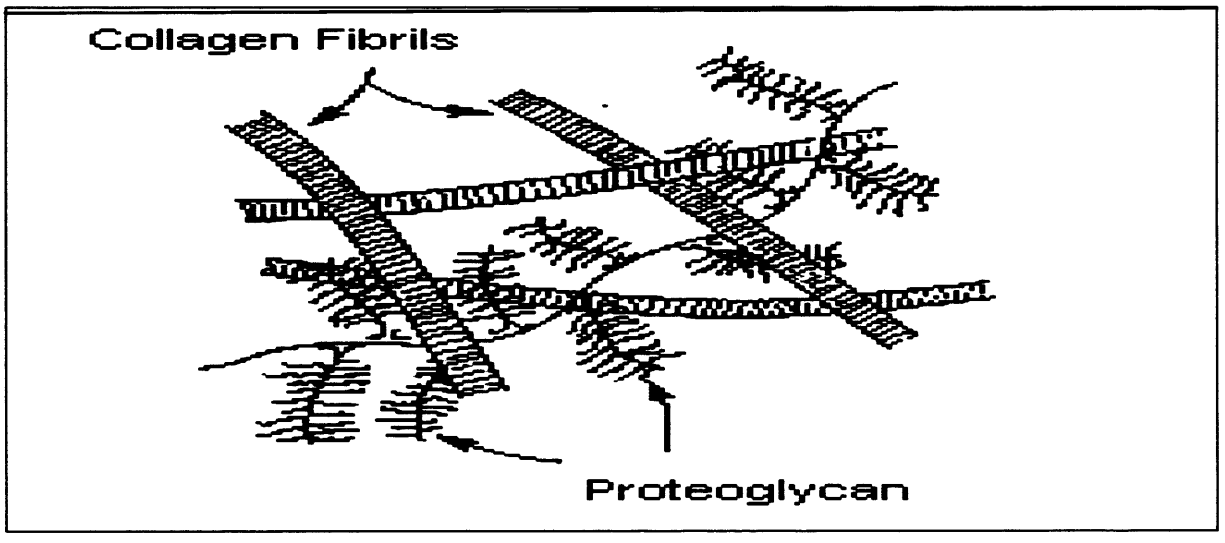


Figure 1.3: Organization of structural elements of cartilage extracellular matrix (taken from Hascall and Hascall, 1981).

1.2.6. Collagen

Collagen is a major component of cartilage. It provides the tensile strength and stiffness of the matrix. The predominant collagen in articular cartilage is type II collagen (90 - 95%) (Buckwalter and Mankin, 1998). The characteristic feature of a typical collagen molecule is its long, stiff, triple-stranded helical structure in which three collagen polypeptide chains are wound around one another in a rope-like super helix (Figure 1.4). These molecules in turn assemble into collagen fibrils, which are cables 10-300nm in diameter and many micrometers long, and which can pack together into still thicker collagen (Mow *et al.,* 1992).

The connective tissue cells that inhabit the matrix and manufacture it, are given various names according to the tissue. Chondrocytes are single cells in the articular cartilage, fibroblasts are found in skin and tendon, while osteoblasts occur in bone (Buckwalter and Mankin, 1998). All of these cell types make collagen and the other organic components of the matrix. Outside the cell, they will assemble into huge, cohesive aggregates. To avoid premature assembly the cells secrete collagen molecules in a precursor form (procollagen). Additional domains on procollagen are removed by collagenase before the extra-cellular assembly (Albert *et al.,* 1997).

Collagen comes in many varieties. At least 14 types of collagen have been described. The most important types are the fibril collagen types I, II, III, IV, V and VI. Type I collagen is the most abundant collagen type (90%) and is found in blood vessel walls, tendon, bone and skin. It may be synthesized by fibroblasts, smooth muscle cells (around blood vessels) and osteoblasts (bone-forming cells). It provides tensile strength to bones, skin, tendons and ligaments. Type II collagen fibres are unique to articular cartilage and intervertebral discs and they are synthesized by chondrocytes (Albert *et al.,* 1997).

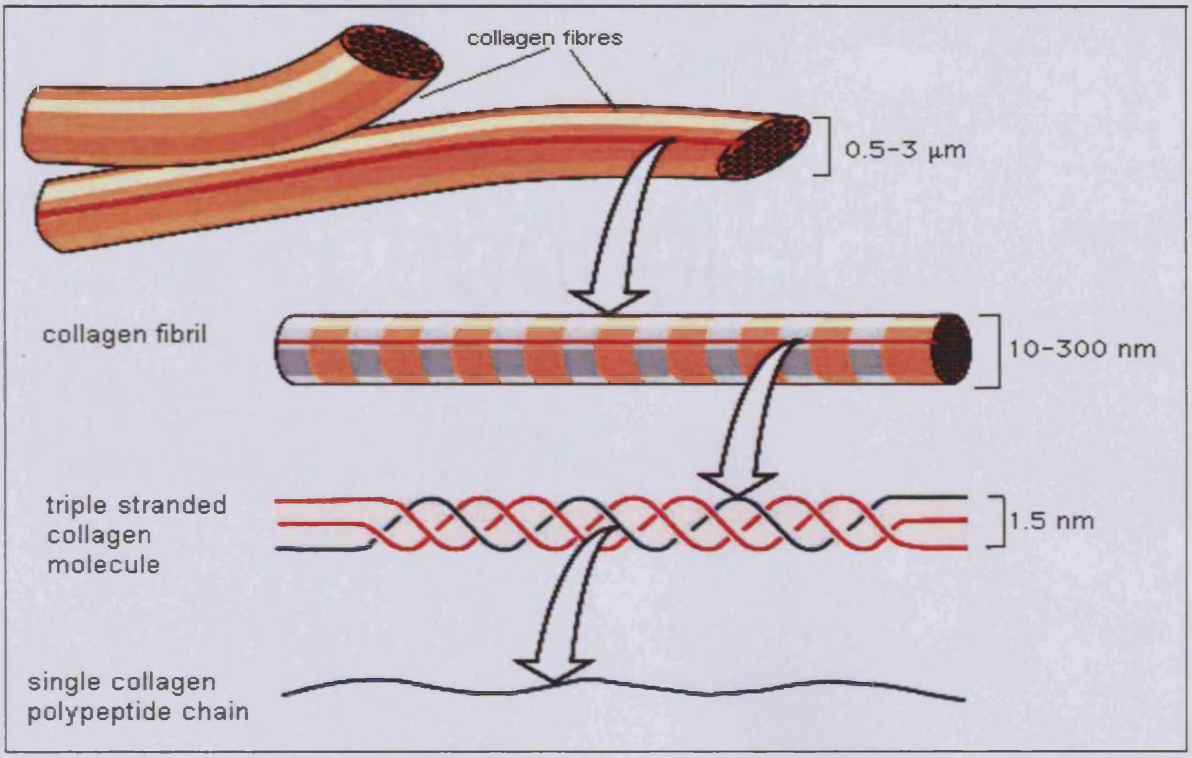


Figure 1.4: The organization and structure of collagen (taken from Albert *et al.*, 1997).

1.2.7 Proteoglycans

Proteoglycans are composed of a protein (core protein) with glycosaminoglycan (GAG) chains covalently attached (Figure 1.5) (Buckwalter and Mankin, 1998). GAGs are amino sugar chains of various lengths made up of repeating disaccharide subunits. Proteoglycan represents around 10 -15% of wet weight in articular cartilage. It is produced by chondrocytes, is secreted into the matrix and has a half-life of about 3 months. The structural diversity of proteoglycans suggests numerous biological functions in addition to simply providing hydrated space around cells. They can bind growth factors and other proteins that serve as signals for cells. They also can block, encourage or guide cell migration through the matrix (Albert *et al.*, 1997). Proteoglycan is composed mainly of two types of GAG, namely chondroitin sulphate and keratan sulphate. The protein core is normally about 1900 amino

acids long with as many as 100 chondroitin sulphate and 60 keratan sulphate chains attached. The negative charges of the sulphate groups cause the glycosaminoglycans to remain separated when attached to the core protein (Newman, 1998).

Biomechanical interactions take place between the fluid (water), proteoglycan molecules, collagen and various electrostatic charges. Proteoglycans are hydrophilic (attract water). There are electrostatic attractions between the positive charges along the collagen molecules and the negative charges that exist along the glycosaminoglycans (Buckwalter and Mankin, 1998). On the other hand, the glycosaminoglycan molecules repel each other if forced together.

The three sulphate-containing GAGs, chondroitin sulphate, keratan sulphate and dermatan sulphate contain negative charges which attract water (Albert *et al.*, 1997). Compression forces the negatively charged chains nearer together and expels fluid; release of pressure then allows expansion and the proteoglycans return to their fully extended state (Newman, 1998). The combination of collagen and proteoglycan together forms a fibres-reinforced composite, much like concrete. The collagen framework limits the ability of proteoglycans to swell. This creates a swelling pressure within the cartilage. This swelling pressure helps to keep cartilage turgid, thus resisting compressive forces. Indeed, a major role of proteoglycans in cartilage is to provide compressive stiffness (Mow *et al.*, 1992).

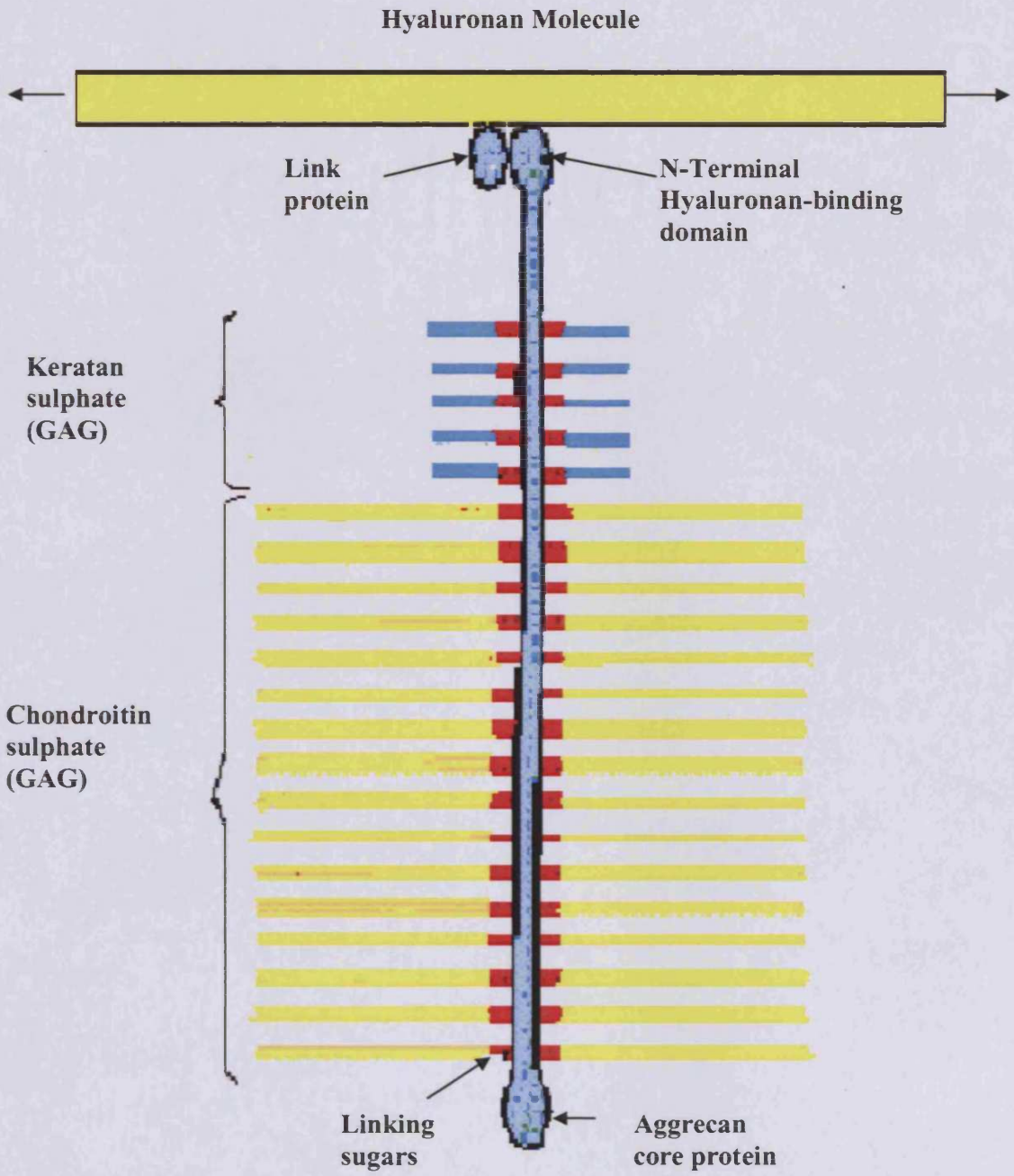


Figure 1.5: Structure of a cartilage proteoglycan aggregate including the structure of an aggrecan monomer (see Figure 1.6). The N-terminal domain of the core protein binds to a hyaluronan (HA) molecule. Binding is facilitated by a link protein, which binds to both the hyaluronan disaccharide and the aggrecan core protein. Keratan and chondroitin sulphates are the main types of glycosaminoglycans (GAGs) found in proteoglycan (taken from Lodish *et al.*, 2000).

1.2.8 Aggrecan

Aggrecan is one of the major structural components of cartilage (Figure 1.6). It represents up to 10% of the dry weight of cartilage. Aggrecan is the shortened name of the large aggregating proteoglycan containing chondroitin sulphate (Abbaszade *et al.,* 1999). Aggrecan is important in the proper functioning of articular cartilage because it provides a hydrated gel structure via its interaction with hyaluronan and link protein that endows the cartilage with load-bearing properties (Kiani *et al.,* 2002). Many individual monomers of aggrecan bind to hyaluronic acid and link protein to form huge aggregates. It is the monomer which is termed aggrecan. Aggregates are comprised of up to 100 monomers attached to a single chain of hyaluronic acid (HA) (Arner *et al.,* 1999). The function of link proteins is to help the formation and stabilization of the hyaluronan proteoglycan bonding or aggregate formation (Abbaszade *et al.,* 1999). In the presence of link proteins, one hyaluronan molecule can bind three times more aggrecan than in its absence. Two different types of link proteins have been isolated with molecular masses of 44 kDa and 49 kDa (Caterson *et al.,* 1985).

The aggrecan monomer contains a core protein with different oligosaccharide and glycosaminoglycan chains linked. The core protein of aggrecan has a molecular mass of 230 kDa (Watanabe *et al.,* 1998) and is composed of 3 globular domains (G1, G2, G3) and two glycosaminoglycan attachment domains containing chondroitin sulphate (100-150 monomer) and keratan sulphate chains (30-60 monomer). These domains play various roles in maintaining cartilage structure and function. The G1 (331 amino acids) and G2 (200 amino acids) domains are separated by a short interglobular domain (IGD) (Kiani *et al.,* 2002). The G1 domain forms a triple-loop structure that is able to bind non-covalently to hyaluronan

(Abbaszade *et al.*, 1999). It comprises the amino terminus (N) of the core protein and has the same structural motif as link protein.

Functionally, the G1 domain interacts both with hyaluronan acid and link protein, forming stable ternary complexes in the extracellular matrix. G2 is homologous to the tandem repeats of G1 and of link protein and is involved in product processing. G3 makes up the carboxyl terminus (COOH) of the core protein. It enhances glycosaminoglycan modification and product secretion (Ilic *et al.*, 1998). Although the G2 domain has high homology with the G1 domain it, obviously, cannot participate in the interactions between the aggrecan and hyaluronan.

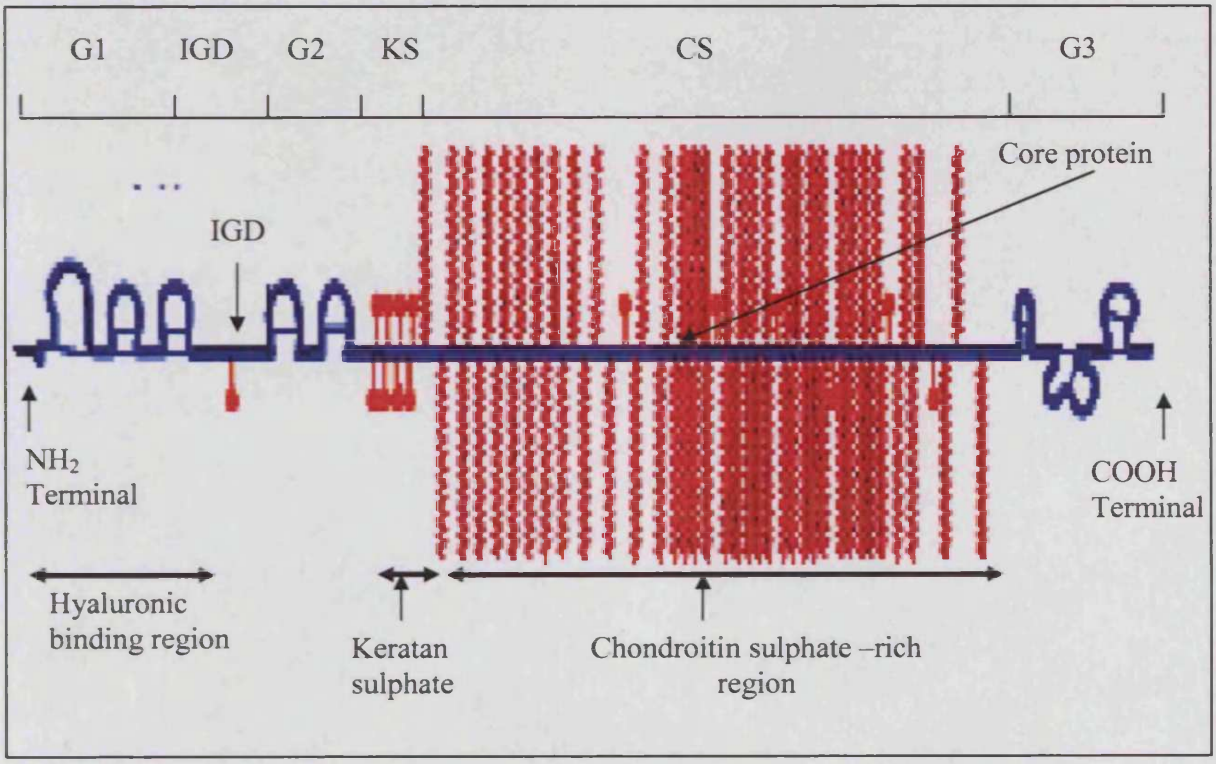


Figure 1.6: Schematic drawing of aggrecan. G1 domain forms a triple-loop structure that is able to bind non-convalently to hyaluronan. The GI and G2 domains are separated by a short interglobular domain (IGD). Proteolysis in the IGD releases the entire glycosaminoglycan-containing portion of aggrecan and is detrimental for articular cartilage function (taken from Wight *et al.*, 1991).

The G3 domain (222 amino acids) has several cysteine residues and has two disulphide bridges. In the IGD, between domains G1 and G2, several N-glycosylated linked oligosaccharides can be bound as well as to the globular domains G1, G2 and G3.

The major part of the aggrecan oligosaccharide and glycosaminoglycan chains are located between the globular G2 and G3 domains. Chondroitin sulphate chains tend to be located near the C-terminal while the keratan sulphate is preferentially located towards the N-terminal (Sandy *et al.,* 2001). The primary role of aggrecan appears to be a physical one, as it brings about an osmotic swelling and maintains the high levels of hydration in the cartilage extracellular matrix (Watanabe *et al.,* 1998). During resting, such as sitting down and reading, the osmotic swelling is at a maximum. However, during loading, such as when we stand up or walk, the weight of body is supported by the cartilagenous ends of the long bones. In this state, the weight compresses the cartilage, literally squeezing water out. This continues until the osmotic swelling generates a force equal to the compressive force generated by the weight, which it supports. When the load is removed, the cartilage swells fully due to the properties of the glycosaminoglycan chains attached to the aggrecan core (Mow *et al.,* 1992).

1.2.9 Glycosaminoglycan (GAG)

GAGs are a group of unique carbohydrate structures composed of repeating disaccharide units of a hexosamine and an uronic acid or galactose (Lodish *et al.,* 1999). Usually one sugar is an uronic acid (either D-glucuronic or L-iduronic) and the other is either N-acetylgalactosamine (GalNAc) or N-acetylglucosamine (GlcNAc). One or both sugars contain sulphate groups (the only exception is hyaluronic acid). GAGs are highly negatively charged which is essential for their function. There are 6 types of GAGs, hyaluronic acid, dermatan sulphate, chondroitin sulphate, heparin, heparan sulphate and keratan sulphate

(Figure 1.7) (Wright *et al.,* 1991). Hyaluronan is always present as a free carbohydrate chain but can form large aggregates with certain proteoglycans. Chondrocytes make GAGs by two pathways. Firstly, they use glucose molecules which can undergo phosphorylation and epimerization reactions. This modified sugar then receives an amino group donated from glutamine to yield glucosamine 6-phosphate, which then undergoes additional reactions in GAG synthesis. A second pathway is for chondrocytes to produce GAGs using preformed glucosamine from, for example, dietary sources (Hascall and Hascall, 1991). Glucosamine makes up 50% of the hyaluronan and keratan sulphate GAG molecules. It is also the immediate precursor of galactosamine, which makes up 50% of the chondroitin sulphate and dermatan sulphate GAG molecules (Wright *et al.,* 1991). Without adequate glucosamine, GAG synthesis would essentially shut down. In turn, this would cause proteoglycan synthesis to cease. There must be adequate glucosamine, whether from supplements, internal production, or dietary sources (gristle), in order for cartilage to be healthy (Hascall and Hascall, 1991). Glucosamine may also work as a stimulating agent. Thus, as chondrocytes increase GAG production, fibrotic articular tissues begin to heal. This improves the diffusion of glucose and amino acids through joint capsules and synovial membranes (Hascall and Hascall, 1991).

About 80% of the total amount of GAG in cartilage is chondroitin sulphate. This molecule consists of disaccharides containing a glucuronic acid (GlcUA) and N-acetylgalactosamine (GalNAc). Disaccharides are often sulphated in the position 4 or 6 of GalNAc (Mourao *et al.,* 1976). Biological variability in the sulphation position exists, *e.g.* chondroitin 4-sulphate forms a larger proportion than chondroitin 6-sulphate of the total chondroitin sulphate in young, immature articular cartilage (Saamanen *et al.,* 1989).

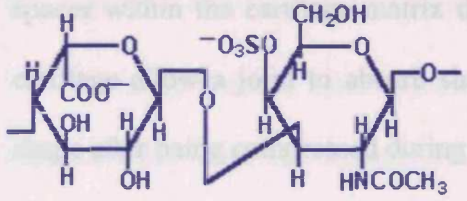
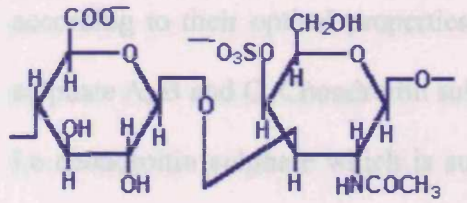
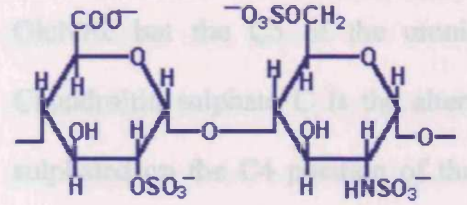
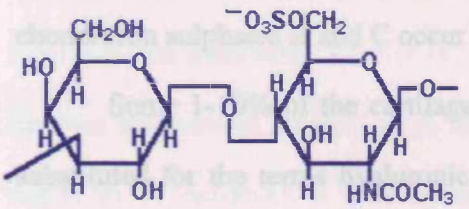
	**Dermatan sulphates:** composed of **L**-iduronate (many are sulphated) + GalNAc-4-sulphate linkages are β(1, 3)
	**Chondroitin 4- and 6-sulphates :** composed of **D**-glucuronate + GalNAc-4- or 6-sulphate linkage is β(1, 3) (the figure shows GalNAc 4-sulphate)
	**Heparin and Heparan sulphates:** composed of **D**-glucuronate-2-sulphate (or iduronate-2-sulphate) + *N*-sulfo-**D**-glucosamine-6-sulphate linkage is α(1, 4)
	**Keratan sulphates:** composed of galactose + GlcNAc-6-sulphate linkage is β(1, 4)

Figure 1.7: The structure of some GAG components (taken from King, 2004).

In osteoarthritis, chondroitin sulphate sulphation patterns may change in the cartilage (Plass *et al.,* 1998) and in synovial fluid (Lewis *et al.,* 1999). The sulphate group at position 6 is more freely oriented, which probably increases its interactions with collagens and other extracellular molecules (Shibata *et al.,* 1992). Chondroitin sulphate's main function is to act as a structural component in connective tissue. It is strongly negatively charged, which forces each molecule of chondroitin sulphate slightly apart from other nearby molecules to create

spaces within the cartilage matrix that can be filled with water. These pockets of water in cartilage allow a joint to absorb shock while the negative charges help restore the original shape after being compressed during normal body movements (Soldani and Romagnoli, 1991).

Three types of chondroitin sulphate are present in mammalian tissue designated according to their optical properties and enzyme reactions; these are designated chondroitin sulphate A, B and C. Chondroitin sulphate A is the alternative name for chondroitin 6-sulphate i.e chondroitin sulphate which is sulphated on the C6 position of the GlcNAc. Chondroitin sulphate B is the alternative name for dermatan sulphate. It is sulphated on the C4 position of GlcNAc but the C5 of the uronic acid has undergone epimerisation to iduronic acid. Chondroitin sulphate C is the alternative name for chondroitin 4-sulphate i.e CS which is sulphated on the C4 position of the GlcNAc. Only chondroitin sulphate A and C occur in cartilage. Chondroitin sulphate A seems to be peculiar to hyaline cartilage, whereas chondroitin sulphates B and C occur in other fibrous connective tissues (Sokoloff, 1980).

Some 1-10% of the cartilage GAG is hyaluronan. Recently, the term hyaluronan has substituted for the terms hyaluronic acid and hyaluronate. Its structure is the simplest of all GAG (Toole, 1997). Hyaluronan is an unsulphated GAG, made of repeating disaccharide units of GlcUA (glucuronic acid) and GalNAc (N-acetylgalactosamine). In articular cartilage, hyaluronan and aggrecan form large aggregates with the overall structure being important for the function of cartilage. Besides aggrecan, several other molecules can also bind hyaluronan, such as link protein, versican and neurocan (Baker and Caterson, 1979). A significant property of hyaluronan is its capacity to bind huge amounts of water (1000-fold of its own weight). In the joints, hyaluronan probably has an important role as a lubricant between the joint surfaces. The production of hyaluronan increases during inflammation and the resulting viscous solutions may inhibit cell activities (Fraser *et al.,* 1997).

1.3 Arthritis diseases/degenerative joint disease

The word arthritis literally means joint inflammation ('arth' – means joint, 'itis' means inflammation). Arthritic disease involves the destruction of articular cartilage and increased catabolism of the cartilage proteoglycan aggrecan (Rowan, 2001). This leads to the loss of normal joint function seen in patients suffering from the disease. Many types of arthritis show signs of joint inflammation, e.g. swelling, stiffness, tenderness or redness (Kineret, 2003). These diseases usually affect the area in or around joints such as muscle and tendons. The most common age for the disease to start is between 30 to 50 and women are more commonly affected than men (Harris and Firestein, 2001). Most forms of arthritis are chronic and are characterized by a persistent joint inflammation and concomitant joint destruction. The two most common forms of joint disease are rheumatoid arthritis and osteoarthritis (Krug, 1997).

Osteoarthritis (OA) and rheumatoid arthritis (RA) must be distinguished in terms of diagnosis and treatment (Ross *et al.,* 1997). Osteoarthritis is a mainly a degenerative process, whereas rheumatoid arthritis is an inflammatory process (Krug, 1997). Specific criteria have begun to be assigned to these diseases although RA and OA are still referred to as an inflammatory autoimmune disease and an erosive disease associated with old age, respectively. This classification is obviously oversimplified and, indeed, some patients may present with a combination of both diseases (Rowan, 2001).

1.3.1 Rheumatoid arthritis (RA)

Rheumatoid arthritis (RA) is a specific type of the arthritis where inflammation of the membrane lining of the joints is caused by abnormalities in the body's own immune system (Krug, 1997). The affected joint can lose its shape and alignment, resulting in pain and loss of

normal movement (Sun and Yokota, 2001). Areas that may be affected include the joints of the hands, wrists, neck, jaw, elbows, knees, feet, and ankles (Kineret, 2003). RA is 2-3 times more common in women than in men and generally affects people above the age of 20. However, young children can develop a form of RA called juvenile rheumatoid arthritis. Some experts classify rheumatoid arthritis as Type 1 or Type 2. Type 1 (less common), lasts a few months at most and leaves no permanent disability. Type 2 is chronic and lasts for years, often for life (Harris and Firestein, 2001).

RA is a disease driven primarily by the synovium, which undergoes proliferation to generate the 'pannus' that then invades articular cartilage and promotes its destruction. In rheumatoid arthritis, an abnormal immune system produces destructive molecules that cause continuous inflammation of the synovium (Rowan, 2001). Collagen is gradually destroyed, narrowing the joint space and eventually damaging bone. If the disease develops into a form called progressive rheumatoid arthritis, destruction of the cartilage accelerates (Feldman and Giannini, 1996). Fluid and immune system cells accumulate in the synovium to produce a pannus, a growth composed of thickened synovial tissue. The pannus produces more enzymes that destroy nearby cartilage, aggravating the situation and attracting more inflammatory white cells, thereby perpetuating the process. This inflammatory process not only affects cartilage and bones but can also harm other organs. Cytokines, as essential mediators of inflammation, are also known to be involved in the pathogenesis of RA. Inflammatory cytokines such IL-1, TNF and IL-6 are found in increased amounts in the affected tissues, in synovial fluids of affected joints and in the circulation of RA patients (Berg and Bresnihan, 1999).

1.3.2 Osteoarthritis (OA)

OA is a degenerative disease of the joints and is often thought to be more benign than RA. OA falls into two categories, according to the presence or absence of certain factors. Primary OA occurs in middle age or elderly patients where an active disease process is often presumed to be a consequence of joint 'wear and tear'. It remains a major health problem in the elderly (Scott *et al.,* 1998). Secondary OA occurs at any age as a result of trauma or disease (Rowan, 2001). The major causes of secondary OA are biomechanical (joint instability) or biochemical (increased matrix degradation) alterations in the joint environment (Mow *et al.,* 1995). In both situations, the central feature is loss of articulate cartilage and a reduced capacity for repair (Rowan, 2001). Although chondrocytes increase matrix synthesis in response to loss of extracellular matrix, they cannot keep up with the rate of depletion. Early signs of OA include altered cartilage matrix organization, increased hydration, thickening of the walls of the synovial capsule and biochemical changes in the joint proteoglycans (Sokoloff, 1980). Once the destabilization occurs, the proteoglycans become more accessible to the water in the joint because the limiting effect of the collagen is reduced. Thus, the cartilage as a whole swells, becomes softer and loses its compressive stiffness and tensile strength. The normal smooth surface of the cartilage begins to crack and fibrillate and the whole becomes thinner, causing joint space narrowing and bone exposure (Mow *et al.,* 1992).

Degenerative joint disease can occur even after relatively low loads on joints if the forces are applied impulsively and repetitively. This may occur because loads are applied too rapidly to permit normal cartilage fluid movement and this can result in microscopic injury to the matrix (Scott *et al.,* 1998). The cyclical disease course of OA has also been proposed to be

the result of sequential cytokine effects on collagenase activity. Although not classified as an inflammatory disease, orthopaedic surgeons often comment on the marked synovial infiltration seen during joint replacement surgery of OA patients, further increasing the evidence for the involvement of pro-inflammatory cytokines such as IL-1 and TNF-α (Rowan, 2001).

1.3.3 Cartilage proteoglycan catabolism

In normal adult cartilage, a steady state exists where the rate of synthesis of matrix components is equal to their rate of degradation (Rowan, 2001). An imbalance of the anabolic and catabolic activity of resident chondrocytes with excessive catabolism results in loss of joint cartilage. The anabolic function is to produce collagen and proteoglycans while chondrocytes produce degradative enzymes for catabolism. Two families of metalloproteinases, namely, MMPs (matrix metalloproteases) and the ADAMTSs (aggrecanases), are responsible for the degradation of the major components of the matrix (Mort and Bilington, 2001).

Aggrecanases are primarily responsible for the catabolism and loss of aggrecan from articular cartilage in the early stages of arthritic joint diseases that led to disruption of connective tissue integrity. At later stages, when collagen catabolism is taking place, there is evidence for MMP-mediated degradation of the small proportion of aggrecan remaining in the tissue, but this occurs independently of continued aggrecanase activity (Caterson *et al.,* 2000).

1.3.4 Aggrecanase/ ADAMTS protein

The principal pathological process, which leads to the degeneration of articular cartilage in arthritic joint diseases, is the increased catabolism of the cartilage proteoglycan aggrecan (Caterson *et al.,* 2000). The depletion of cartilage aggrecan due to increased

proteolytic cleavage within the interglobular domain is the earliest change observed in arthritis (Abbaszade *et al.,* 1999). Two major sites for cleavage of aggrecan have been shown to occur at Asn^{341} – Phe^{342} and Glu^{373} –Ala^{374} within the interglobular domain (IGD), which is responsible for formation of high molecular weight products (Arner *et al.,* 1999). The former cleavage site, (Asn^{341} –Phe^{342}), can be cleaved by matrix metalloproteinases (MMP-1, -2,-3,-7,-8,-9 and -13) (Hughes *et al.,* 1998). On the other hand, cleavage at the latter site, Glu^{373} – Ala^{374}, is responsible for the increased aggrecan degradation observed in inflammatory joint disease (Sandy *et al.,* 2001). A novel proteolytic activity, termed 'aggrecanase' was shown to be responsible for cleavage at the Glu^{373} –Ala^{374} site (a marker of aggrecanase activity), with this enzyme playing a pivotal role in the cartilage damage associated with OA and RA joint disease (Watanabe *et al.,* 1998). Thus, aggrecanase activity plays a key role in the early phase of cartilage destruction (Nagase, 2001). Products resulting from cleavage at the Glu^{373} –Ala^{374} site have been shown to accumulate in cartilage explants and chondrocyte cultures treated with catabolic agents such as IL-1, TNF-α and retinoic acid (RA) and in the synovial fluid of patients with OA and inflammatory joint disease (Abbaszade *et al.,* 1999).

Two aggrecanases that can cleave aggrecan at the Glu^{373} -Ala^{374} site have been isolated from interleukin -1α (IL-1α)-treated bovine nasal cartilage and they were named aggrecanase 1 (or ADAMTS-4) and aggrecanase 2 (or ADAMTS-5) (Arner *et al.,* 1999). Both enzymes are members of the disintegrin and metalloprotease (ADAMTs) family of zinc metalloproteinases. Typical ADAMTs have an epidermal growth factor repeat domain (EGF repeat), a transmembrane domain and a cytosolic tail (Nagase, 2001). ADAMTSs lack a transmembrane domain and cytosolic tail. Instead, they contain unique thrombospondin motifs that are responsible for extracellular matrix binding (Abbaszade *et al.,* 1999). Therefore, ADAMTS stands for A Disintegrin And Metalloproteinase with ThromboSpondin motif's (Kuno *et al.,*

2000). Recombinant human ADAMTS-5 and ADAMTS-4 cleave aggrecan at Glu^{1480} – Gly^{1481}, Glu^{1667} – Gly^{1668}, Glu^{1771} –Ala^{1772} and Glu^{1871} –Leu^{1872} bonds more readily than at the Glu^{373} –Ala^{374} bond. Cleavage of aggrecan by ADAMTS-5 or ADAMTS-4 at the five common aggrecanase-sensitive sites results in the formation of six terminal fragments (Figure 1.8).

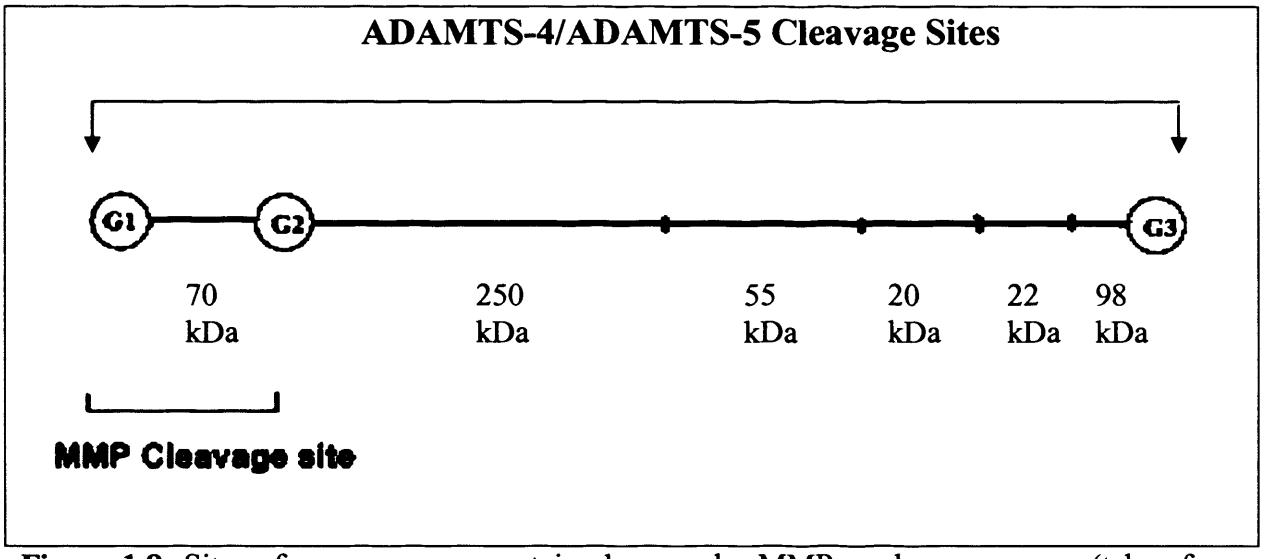


Figure 1.8: Sites of aggrecan core protein cleavage by MMPs and aggrecanases (taken from Tortorella *et al.*, 2001).

Furthermore, there are a few differences between ADAMTS-4 and ADAMTS-5. In young bovine cartilage, ADAMTS-4 is the primary enzyme responsible for aggrecan cleavage while ADAMTS-5 participates in the breakdown of aggrecan. Even though the enzymes cleave aggrecan at the same sites, they may do so at different rates. It has been reported that ADAMTS-5 cleaves aggrecan at the Glu^{373} –Ala^{374} and Glu^{1667} –Gly^{1668} sites approximately two–fold slower than ADAMTS-4 (Tortorella *et al.*, 2002). Both ADAMTS-4 and ADAMTS-5 have been detected at the message level in chondrocytes and cartilage after IL-1 and TNF stimulation. Interestingly, ADAMTS-5 message was detected in control cartilage, whereas

ADAMTS-4 message was not. This suggests that ADAMTS-5 is constitutively expressed in bovine articular cartilage, whereas ADAMTS-4 is induced (Curtis *et al.,* 2000; Tortorella *et al.,* 2001). Since ADAMTS-4 is more active than ADAMTS-5, the expression of ADAMTS-4 may be more detrimental to the health of cartilage and is, thus, responsible for the majority of degradation (Tortorella *et al.,* 2001).

1.3.5 Matrix metalloproteinases

Chondrocytes and synovial cells respond to a variety of inflammatory cytokines and growth factors that stimulate the production of destructive proteinases (Rowan, 2001). These proteinases are active in degrading collagens as well as the cartilage proteoglycan, aggrecan (Mort and Billington, 2001). In arthritis, the specific sources of the proteinases are dependent upon the type of disease. In OA, there are few inflammatory cells present and so proteinases produced by chondrocytes play a major role. By contrast, in a highly inflamed rheumatoid joint many other cell types are present, such as synovial cells and macrophages, which also produce proteinases and mediators that play a major part in joint destruction (Cawston, 1998).

Proteinases can be classified into four main groups according to the amino acid or chemical groups at the catalytic centre of the enzyme. All four major classes of proteolytic enzymes (aspartic, cysteine, serine and metallo) are involved in normal turnover as well as pathological destruction pathways (Konttinen *et al.,* 1998). Proteolytic pathways can be divided into (1) intracellular pathways, catalyzed by cysteine and aspartate proteinases, which cleave proteins at low pH inside the cell (2) extracellular pathways, catalysed by serine and metalloproteinases, which act at neutral pH outside the cell. These pathways all play a part in the turnover of collagen and proteoglycan. The matrix metalloprotinases (MMPs) are important enzymes involved in joint destruction (Mort and Billington, 2001). MMPs are a

family of neutral zinc endoproteinases that collectively degrade all the protein components of the extra-cellular matrix (Malemud and Goldberg, 1999) and they are divided into four main groups which differ in size and in the proteins they digest. These are called the stromelysins, collagenases, gelatinases and the membrane-bound MMPs (Mort and Billington, 2001).

They all contain a zinc atom at their catalytic center and are all synthesized as inactive proenzymes, which require activation, before they can degrade the matrix (Malemud and Goldberg, 1999). Others are produced by connective tissue cells following stimulation by cytokines such as IL-1 and TNF-α (Cawston, 2002). The first class of MMPs are the stromelysins, so named because of their broad substrate specificity. There are two highly homologous enzymes, stromelysin -1 (MMP-3) and stromelysin –2 (MMP-10). Stromelysin -1 (MMP-3) can degrade aggrecan, link protein, Type IV collagen, collagen cross-links and procollagens and also participates in the activation of other MMPs. MMP-3 is not normally widely expressed, but can be readily induced by growth factors, cytokines and tumor promoters in chondrocytes and connective tissue fibroblasts. The pH optimum for MMP-3 is surprisingly low and activation of this enzyme may occur after exposure to low pH conditions (Cawston, 1998).

There are three collagenases; interstitial collagenase (MMP-1), neutrophil collagenase (MMP-8) and collagenase-3 (MMP-13) (Cawston, 1998). The enzymes also differ in their specificity for different collagens. The two gelatinases cleave denatured collagens, type IV and V collagen and elastin. Tissue inhibitors of metalloproteinases (TIMPs) act against MMPs and thus, help to prevent cartilage and bone destruction. MMP inhibition has also been the subject of much research in an attempt to find highly specific and bioavailable inhibitors (Malemud and Goldberg, 1999).

1.4 Mediators of inflammation

Inflammation is a complex natural process involving cellular interactions modulated by numerous inflammatory mediators. Major mediators of inflammation, which are direct relevance to this project, are cytokines and lipid-derived compounds.

1.4.1 Eicosanoids

Eicosanoids are a group of biologically-active oxygenated fatty acids derived from twenty carbon PUFAs such as arachidonic acid (Calder, 2001). Eicosanoids are chemical messengers which can have varying inflammatory activity. Pro-inflammatory eicosanoids function as paracrine mediators of inflammation and have been implicated in arthritis and asthmatic disease (Henderson, 1994). Diet can directly affect the availability of eicosanoids. This is because the latter's synthesis often begins with the plant-derived components linoleic acid or α-linolenic acid. Humans cannot synthesize these fatty acids but they are essential to the body. With the help of the Δ-6-desaturase enzyme, linoleic acid is converted to γ-linolenic acid (GLA). γ-linolenic acid is elongated to dihomo-γ-linolenic acid (DGLA). DGLA can give rise to prostaglandin PGE_1. A Δ-5-desaturase enzyme converts DGLA into arachidonic acid as shown in Figure 1.9 (Calder, 1998). An analogous pathway is also used for the metabolites which derive from α-linolenic acid, except that the conversion of eicosapentaenoic acid (EPA) to docosahexaenoic acid (DHA) relies on the Sprecher pathway involving 24C intermediates (Figure 1.9).

Eicosanoids include prostaglandins (PGs), thromboxanes, leukotrienes (LTs), lipoxins, hydroperoxyeicosatetraenoic acids (HPETE) and hydroxyeicosatetraenoic acids (HETE). The 20C fatty acid precursor for eicosanoid synthesis is released from cell membrane phospholipids, by the action of phospholipase A2 which is activated in response to a cellular

stimulus. Since the membranes of most immune cells contain large amounts of arachidonic acid (AA), this fatty acid is usually the principal precursor for eicosanoid synthesis (Calder, 2001).

PGs are found in animals as primitive as the coelenterates and are present in a wide variety of human tissues. PGs not only play a central role in inflammation, but also regulate other critical physiological responses. In humans, PGs are involved in diverse functions, including bone metabolism, reproduction and kidney function as well as immune responses (Dubois *et al.,* 1998). PGs are members of the prostanoids, which are formed by the oxidative cyclization of the central 5 carbons within 20 carbon PUFA (Figure 1.10) (Smith *et al.,* 1996). Prostanoids include prostaglandins, prostacyclin and thromboxanes as shown in Figure 1.11. PGs are produced by cyclooxygenase (COX) action on suitable unesterified PUFAs to form a key cyclic intermediate (PGH_2 for arachidonic acid). The key cyclic intermediate can be further metabolised into various prostaglandins (depending on cell type).

From PGH_2, these include PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 and thromboxane (TX) A_2 (Dubois *et al.,* 1998). In inflamed tissues, the PGs produced by COX cause a variety of effects including fever, swelling, redness, accumulation of white blood cells and stimulation of bone resorption (Vane *et al.*, 1998).

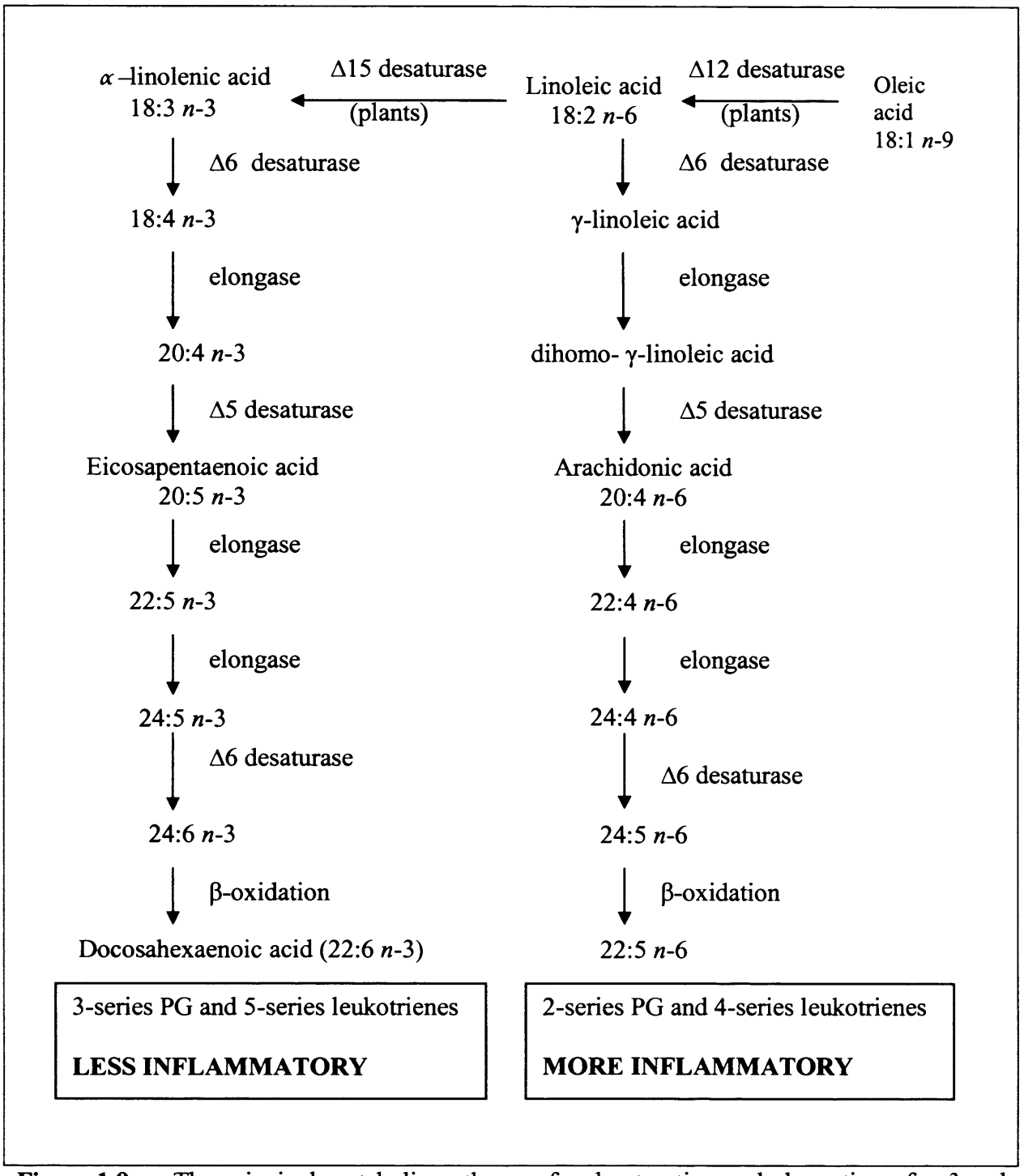


Figure 1.9: The principal metabolic pathways for desaturation and elongation of *n-3* and *n-6* fatty acids (adapted from Calder, 1998). Docosahexaenoic acid does not give rise to eicosanoids activity (see section 1.4.4).

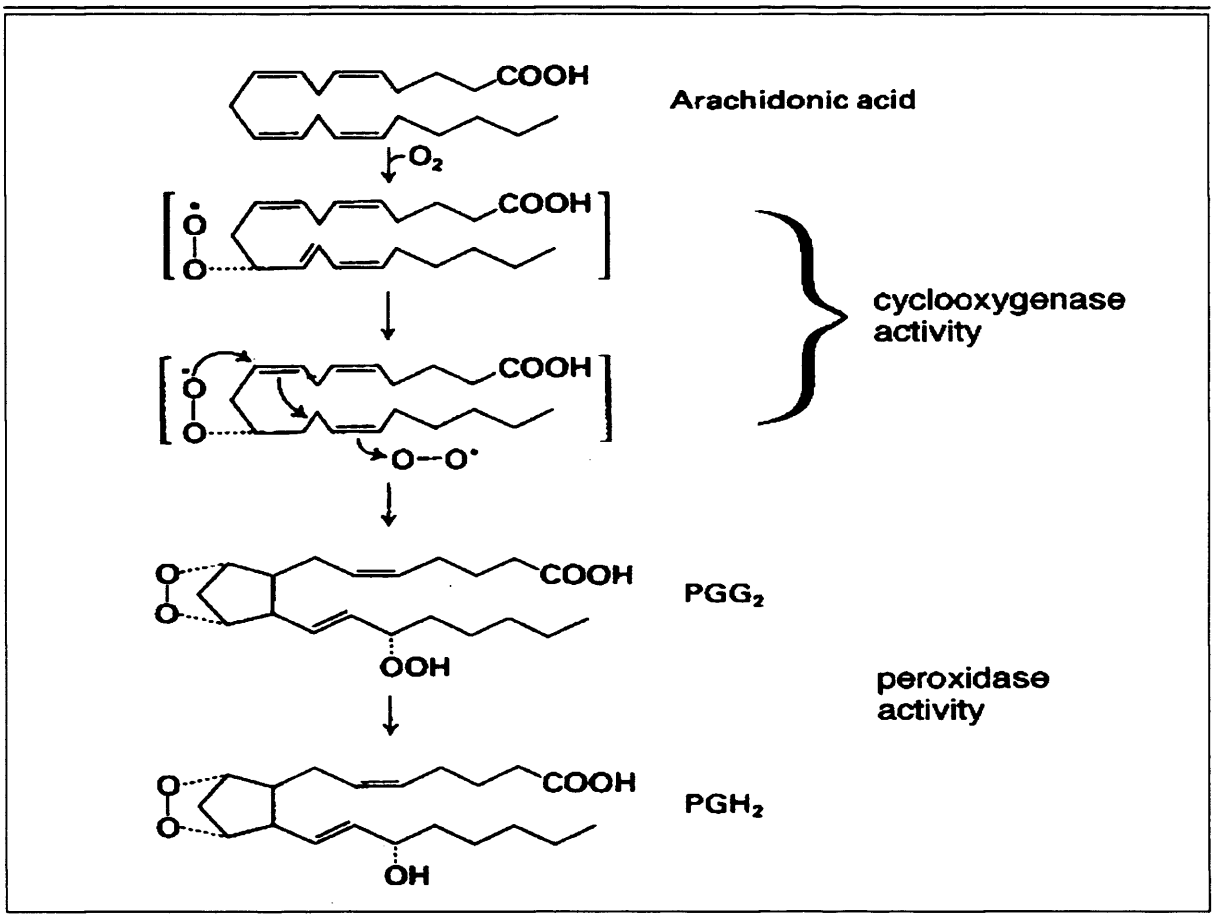


Figure 1.10: Mechanism of biosynthesis of the cyclic endoperoxide, PGH_2 as shown by Gurr *et al.*, 2002.

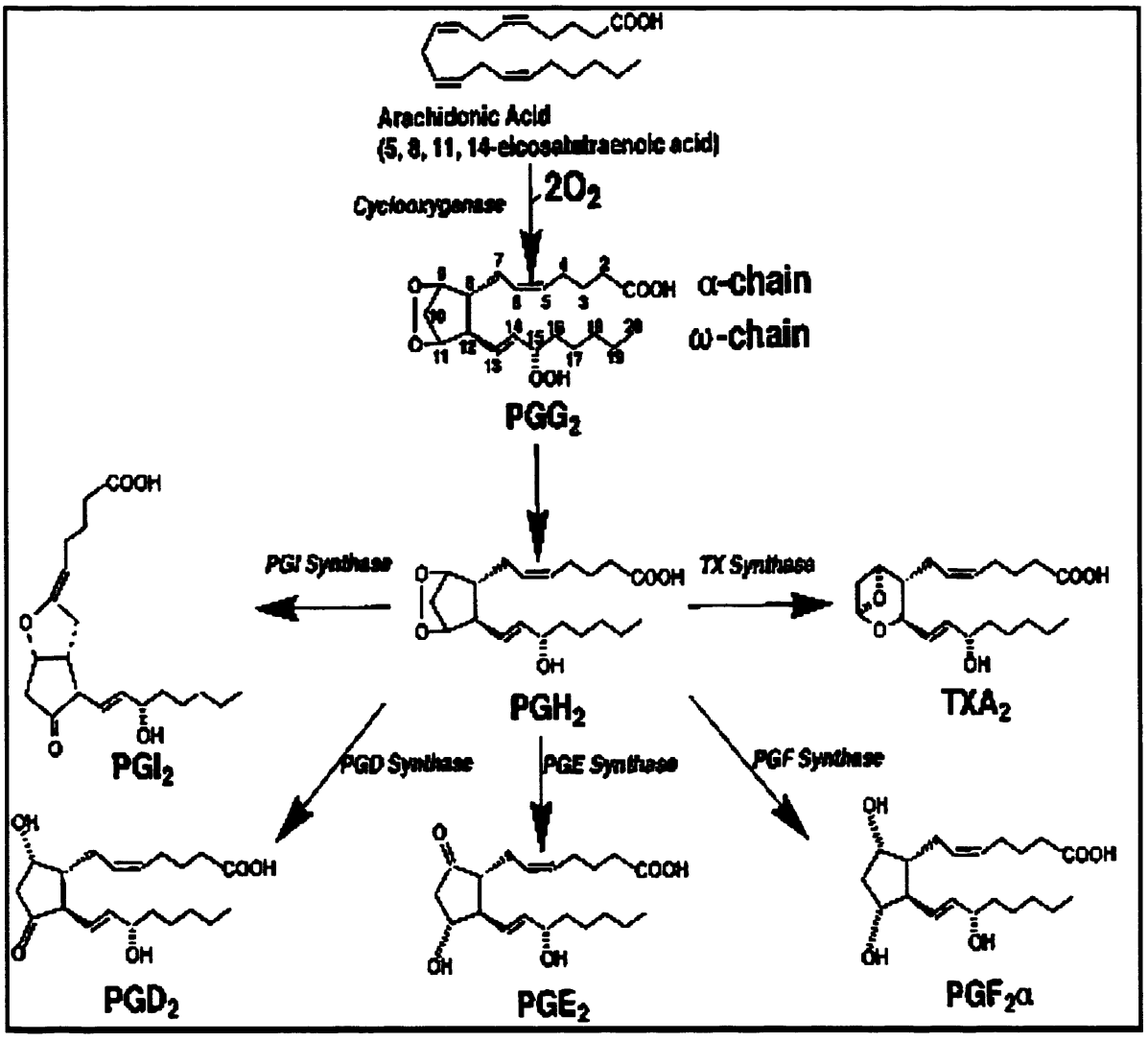


Figure 1.11: Biosynthetic pathways for prostanoids. Formation of series 2 prostaglandins (PG), PGD_2, PGE_2, $PGF_{2\alpha}$, PGG_2, PGH_2, and PGI_2, and a thromoboxane (Tx), TxA_2, from arachidonic acid is shown. The first 2 steps of pathway, i.e., conversion of arachidonic acid to PGG_2 and then to PGH_2, are catalyzed by cyclooxygenase and subsequent conversion of PGH_2 to each PG is catalyzed by a respective synthase as shown (adapted from Narumiya *et al.*, 1999).

1.4.2. Cyclooxygenases (COXs)

Cyclooxygenases (COXs) are the key enzymes that catalyze the conversion of long chain PUFAs, such as arachidonic acid, to various prostanoids. This enzyme is also known as prostaglandin endoperoxide H synthase (PGHS) (Vane *et al.,* 1998). COX catalyses 2 reactions – peroxidation and cyclization (Figure 1.10). Thus, arachidonic acid is converted to prostaglandin G_2 (PGG_2) within the cyclization site, which is reduced to prostaglandin H_2 (PGH_2) by the peroxidase site (Smith *et al.,* 1998). First, prostaglandin endoperoxide synthase inserts two molecules of oxygen to yield a 15 hydroperoxy-9,11-endoperoxide with a substituted cyclopentane ring (PGG). This is the cyclooxygenase activity of the enzyme. The peroxidase activity then reduces PGG to its 15-hydroxy analogue, PGH (Gurr *et al.,* 2002). Further reactions lead to biologically important derivatives such as prostaglandin E_2 (PGE_2), prostacyclin, thromboxane A_2 (TXA_2), and prostaglandin $F_2\alpha$ ($PGF_2\alpha$) (Figure 1.11). PGE_2 is a major mediator of the inflammatory response (Brooks *et al.,* 1999).

Two major cyclooxygenase (COX) isoforms have been identified and are referred to as COX-1 and COX-2. COX-1 was first purified in 1976 and cloned in 1988 (Dubois *et al.,* 1998). In 1991, a second isoform of COX was discovered and named COX-2 (or PGHS-2) (Kalgutkar *et al.,* 2000). The two isoforms of COX are almost identical in structure but have important differences in substrate and inhibitor selectivity and in their intracellular locations. A major difference in COX isoforms lies in their physiological functions (Vane *et al.,* 1998). COX-1 (the 'good' COX) is constitutively expressed in almost all cells of the human body as a housekeeping enzyme (Dubois *et al.,* 1998). Its products stimulate normal body functions such as stomach mucous production, regulation of gastric acid and kidney water excretion. COX-1 is believed to protect against gastric damage (Marnett and Kalgutkar, 1999). In contrast, COX-

2 (the 'bad' COX), is highly inducible and usually absent in normal tissues (Hawkey, 1999) which have low or undetectable levels of COX-2 mRNA (Dubois *et al.,* 1998).

COX-2 is considered central to the inflammation that accompanies inflammatory responses. Therefore, reducing the production of COX-2 activity can prevent the joint pain that occurs in arthritis (Fiorucci *et al.,* 2001). Production of COX-2 is stimulated by inflammatory cytokines and growth factors (Dubois *et al.,* 1998).

Pro-inflammatory agents such as the cytokines IL-1 and TNF-α, have been shown to induce COX-2 expression in various tissues. On the other hand, anti-inflammatory cytokines (IL-4, IL-10, IL-13) will decrease induction of COX-2 (Vane *et al.,* 1998). Recently, it was reported that *n-3* fatty acid supplementation significantly reduced the IL-1α, TNF-α and COX-2 mRNAs in articular chondrocyte membranes and human explants stimulated with IL-1α (Curtis *et al.,* 2000; 2002).

COX-1 and COX-2 exist as homodimers with a molecular mass of 71kDa per monomer (Kurumbail *et al.,* 2001). Each dimer has three independent folding units: an epidermal growth factor-like domain, a membrane-binding domain and an enzymatic/catalytic domain (Brooks *et al.,* 1999). Both isoforms are almost identical in length. However, the human COX-2 gene at 8.3 kb is small, whereas human COX-1 originates from a much larger 22 kb gene (Vane *et al.,* 1998).The cyclooxygenase active site is located at the end of a long hydrophobic channel that is near the membrane-binding domain (the lobby) and narrows as it extends toward the interior of the protein. The peroxidase active site is located on the surface of the protein near the heme cofactor. The cyclooxygenase active site can be subdivided into the main (substrate-binding) channel, which is largely hydrophobic, and a smaller amphipathic side pocket (Kurumbail *et al.,* 2001).

Significant differences between COX-1 and COX-2 appear to be in their ability to use different substrate pools and in inhibitor selectivity (Dubois *et al.,* 1998). The latter is caused by the size and shape of the inhibitor binding sites (Brooks *et al.,* 1999). Moreover, COX-2 will accept a wider range of fatty acids as substrates than will COX-1 (Otto and Smith, 1995). Thus, although both enzymes can utilize arachidonic and dihomo γ–linolenic acids equally well, COX-2 oxygenates other substrates, such as eicosapentanoic acid, γ-linolenic acid, α-linolenic acid and linoleic acid more efficiently than does COX-1 (Vane *et al.,* 1998). This is due to the volume of the COX-2 active site compared to that of COX-1, especially differences in the side pocket to the main hydrophobic channel (Kurumbail *et al.,* 1996). Another striking difference is that COX-2 is located on the nuclear membrane as well as on the endoplasmic reticulum (ER), while COX-1 is found attached only to the ER (Dubois *et al.,* 1998). Although emphasis has been placed here on the differences between COX-1 and COX-2, the isoforms have similar catalytic mechanisms for the metabolism of PUFAs (Brooks *et al.,* 1999).

Recently, a third distinct COX isoenzyme, COX-3 has been reported. Like the other COX enzymes, COX-3 is involved in the synthesis of prostaglandins and plays a role in pain and fever. However, unlike COX-1 and COX-2, COX-3 appears to have no role in inflammation. COX-3 possesses glycosylation-dependent cyclooxygenase activity. COX-3 is made from the COX-1 gene but retains intron 1 in its mRNAs. In humans, COX-3 mRNA is most abundant in cerebral cortex and heart (Chandrasekharan *et al.,* 2002).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used to treat inflammation and pain including arthritis. The NSAIDs achieve their effects by blocking the activity of cyclooxygenase. Most NSAIDs currently used show little selectivity between COX-1 and COX-2 (Smith *et al.,* 1998). This non-selectivity leads to various unwanted side effects. Thus,

aspirin and other similar NSAIDs can cause excessive production of stomach acid (Vane *et al.*, 1998). In general, the undesirable side effects of NSAIDs are due to the inhibition of COX-1, whereas the beneficial effects are related to COX-2 inhibition (Mitchell and Warner., 1999).

Recent research has been directed at finding inhibitors selective for COX-2 over COX-1. Identifying drugs selective to COX-2 requires the consideration of the hydrophilic side pocket since this is where the isoforms vary slightly in their structure (Kurumbail *et al.*, 2001). Several new drugs (e.g. cerebrex) are on sale that show selectivity towards COX-2 and these have been reported to be effective against inflammation with no gastrointestinal or renal problems (Dubois *et al.*, 1998). Interestingly, COX-3, is inhibited by the drug acetaminophen, which has little effect on the other two COX enzymes. COX-3 is also inhibited by other analgesic/antipyretic drugs such as dipyrone, and is potently inhibited by some NSAID drugs. Thus, inhibition of COX-3 could represent a primary central mechanism by which these drugs decrease pain and possibly fever (Chandrasekaran *et al.*, 2002). However, despite their promise, COX-2 inhibitors have very recently been withdrawn from the market due to unforeseen side-effects which increase the risk of cardiovascular complications. On September 30, 2004, Merck & Co., Inc. announced a voluntary withdrawal of Vioxx (rofecoxib) from the U.S. and worldwide market due to safety concerns of an increased risk of cardiovascular events (including heart attack and stroke) in patients taking Vioxx. Other COX-2 inhibitors such as Valecoxib were withdrawn by Pfizer & Co., Inc. as requested by the U.S. Food and Drug Administration (FDA) in April 2005 (Drazen, 2005).

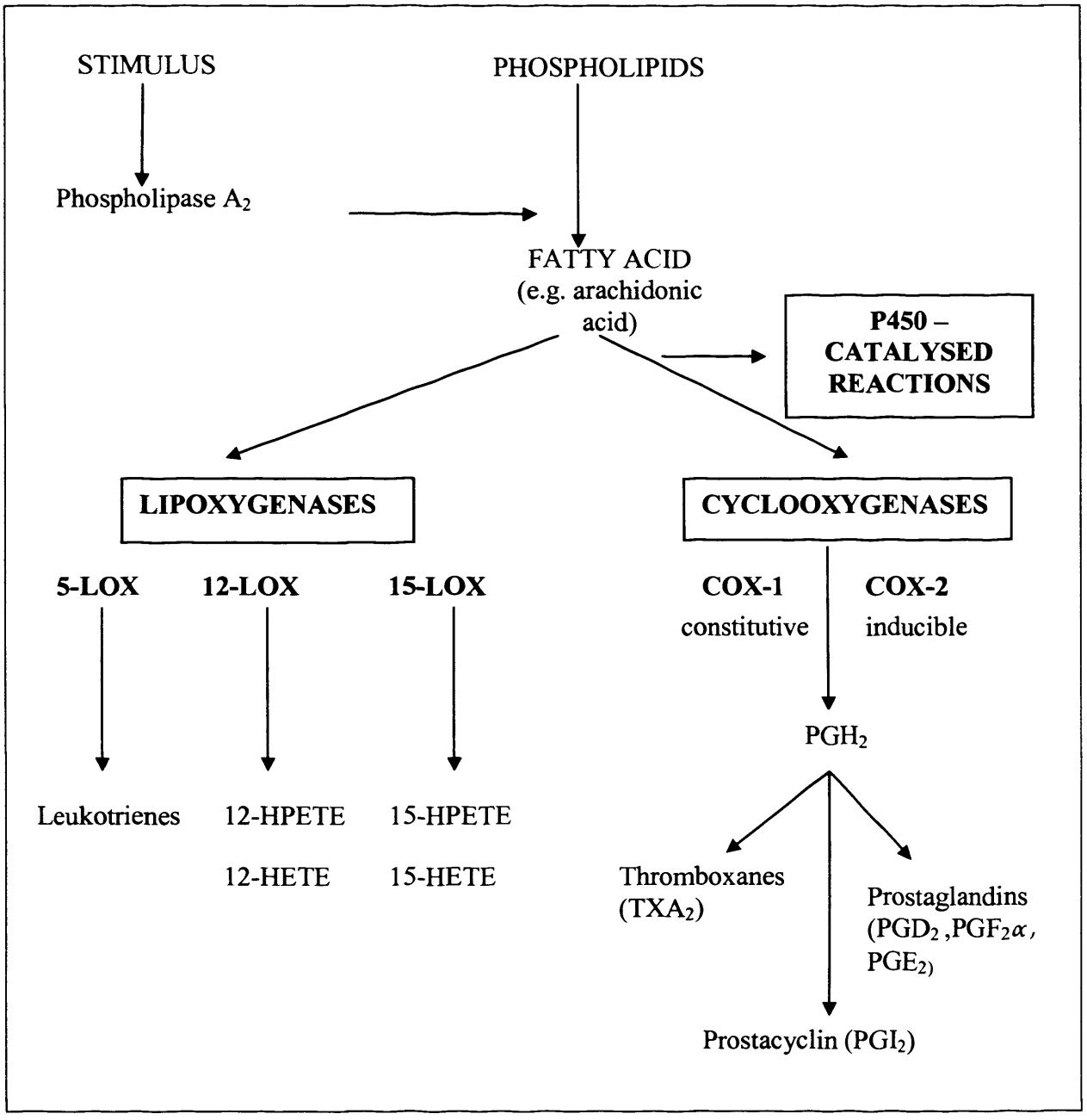


Figure 1.12: Schematic pathway for conversion of essential fatty acids into eicosanoids involving phospholipase A2, cyclooxygenases and lipoxygenases (adapted from Gurr *et al.*, 2002).

1.4.3 Leukotrienes and lipoxygenases

Leukotrienes, together with the prostaglandins and other related compounds (Figure 1.12), are derived from 20C PUFAs (Gurr *et al.,* 2002) and are thus another class of eicosanoids. They all have in common 4 double bonds, 3 of which are in conjugated structure (O'Donnell, 1999). Leukotrienes are produced from membrane lipids (Figure 1.13) by one of several distinct pathways that utilize lipoxygenase activity. The first steps in the generation of leukotrienes are oxidation of PUFA to hydroperoxy fatty acids (which for arachidonic acid are hydroperoxy eicosatetraenoic acids - HPETEs) by the lipoxygenase enzymes (LOXs). LOXs possess regiospecificity and are designated as 5-, 8-, 12- and 15- LOX (Fiorucci *et al.,* 2001).

The HPETEs can undergo three reactions. The hydroperoxy group can be reduced to an alcohol, thus forming a hydroxy-eicosatetraenoic acid (HETE). Alternatively, a second lipoxygenation yields a dihydroxy-eicosatetraenoic acid (diHETE) or dehydration produces an epoxy fatty acid. Epoxy fatty acids, such as leukotriene A4 can undergo non-enzymatic reactions to various diHETEs, can be specifically hydrated to a given diHETE or can undergo ring opening with glutathione *S*-transferase (GSH) (Gurr *et al.,* 2002). The primary products from arachidonic acid are 5S-, 8S-, 12S-, or 15S- hydroperoxyeicosatetraenoic acids (5-, 8-, 12- or 15–HPETEs), which can be reduced to the hydroxy forms 5-, 8-, 12-, 15-HETEs, respectively, as well as the epoxy route giving rise to leukotrienes (Funk, 2001).

Leukotrienes are potent biological mediators of inflammation and allergic disorders. For example leukotriene B_4 has been shown to be upregulated in arthritic tissues (Funk, 2001). A summary of the properties of different types of lipoxygenases is presented in Table 1.1.

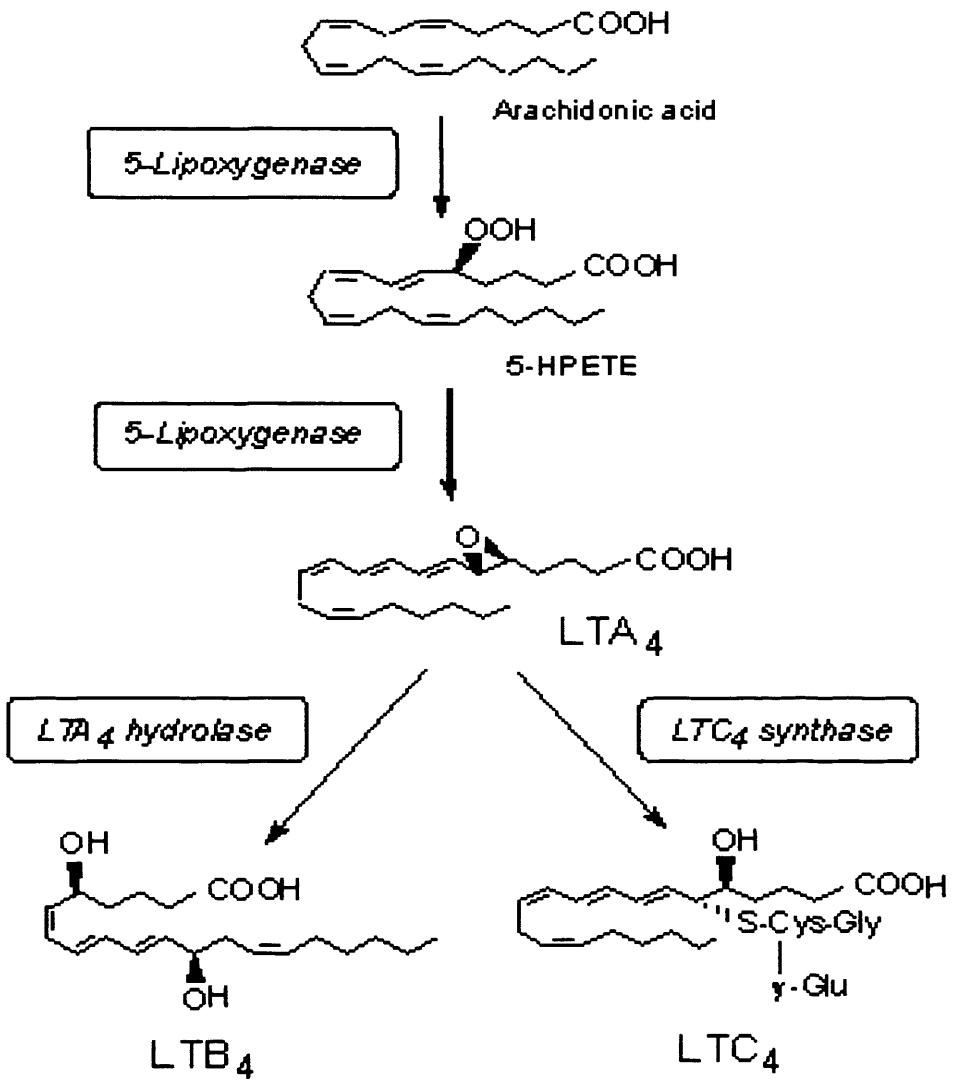


Figure 1.13: The main pathways to the formation of the leukotrienes from arachidonic acid catalysed by 5-lipoxygenase into the unstable epoxide leukotriene (LT) A_4. LTA_4 may be hydrolyzed into LTB_4 by the enzyme *LTA_4 hydrolase (LTA4H)*, or conjugated with GSH to form LTC_4, a reaction catalyzed by a specific *LTC_4 synthase (LTC4S)* (taken from www.mbb.ki.se/research/kemi_2/leuchron/LT/ltreview.htm)

Enzymes	**Typical Product**	**Properties**	**Physiological Role**
5-LOX	5-HPETE Leukotriene A_4 Leukotriene B_4 Leukotriene C_4 Leukotriene D_4 Leukotriene E_4	Needs activating enzyme, FLAP to catalyze the insertion of oxygen onto the 5-carbon of arachidonic acid	Inflammatory cell proliferation such as in arthritis and asthma
8-LOX	8-HPETE 8-HETE	Expressed in the skin after irritation or treatment with tumor promoters	Correlated with carcinogenesis and cancer growth.
12-LOX	12-HPETE 12-(S)-HETE	3 forms identified: i) leukocyte-type ii) platelet-type. Both 12-(S) LOXs can convert arachidonic acid to 12-(S)-HETE iii) epidermal form-12(LOX).	Correlated with tumor cell metastasis and stimulate prostate cancer cell migration.
15-LOX	15-HPETE 15-HETE 13-S-HODE	2 types: i) 15- LOX-1(preferred substrate is linoleic acid and this is metabolized to 13-(S)-HODE). ii) 15-LOX-2 (preferred substrate is arachidonic acid and this is metabolized to 15-HETE).	15-LOX correlated with cancer development. 13-S-HODE enhances colonic tumor genesis.

Table 1.1: Summary of the properties of lipoxygenases (O'Donnell, 1999; Funk, 2001; Shureiqi and Lippman, 2001).

1.4.4 New bioactive derivatives from polyunsaturated fatty acids.

PUFA such as EPA and DHA are widely believed to act by means of several possible mechanisms, such as preventing conversion of arachidonate to proinflammatory eicosanoids or by serving as an alternative substrate producing less potent products (Arita *et al.*, 2005). Recently, novel mediators generated from EPA and DHA that displayed potent bioactions were identified in resolving inflammatory exudates and in tissues enriched with DHA. The trivial names Resolvins (resolution phase interaction products) and docosatrienes were introduced for the bioactive compounds belonging to these novel series because they demonstrate potent anti-inflammatory and immunoregulatory actions. The compounds derived from EPA carrying potent biological action are designated the E series (given their EPA precursor) and denoted as Resolvins of the E series (Resolvin E1 or RvE1) and those biosynthesized from the precursor DHA are Resolvins of the D series (Resolvin D1 or RvD1). Bioactive members from DHA with conjugated triene structures are docosatrienes (DT) that are immunoregulatory and neuroprotective and are also termed neuroprotectins (Serhan *et al.*, 2002).

The specific receptors for these novel bioactive products from *omega-3* EPA and DHA are Resolvin D receptors, Resolvin E receptor and neuroprotectin D receptors, respectively, in recognition of their respective cognate ligands (Serhan *et al.*, 2004). Resolvin E1 (RvE1) is endogenously biosynthesized from EPA in the presence of aspirin during the spontaneous resolution phase of acute inflammation where specific cell-cell interactions occur by triggering endogenous formation of the 17R-D series Resolvins and docosatrienes (Serhan *et al.*, 2004; Arita *et al.*, 2005).

1.4.5 Cytokines and growth factors

Cytokines are proteins that are made by cells of the immune system and they are very important in regulating cell activation and differentiation and also affect other cellular functions. Cytokines and growth factors are produced in large amounts during inflammation by the synovial membrane, and diffuse into the cartilage through the synovial fluid (Cimpean *et al.,* 2000). They activate the chondrocytes, regulating metabolism and can drive the inflammatory response. The latter can be divided into pro-inflammatory and anti-inflammatory effects (Rowan, 2001). Pro-inflammatory cytokines accelerate inflammation by inducing cell migration and production of TNF-α while anti-inflammatory cytokines reduce the production of interferon-γ, IL-1β and TNF-α in synovium (Feldmann and Gianni, 1996).

1.4.6 Pro-inflammatory cytokines

The major pro-inflammatory cytokines for arthritis are IL-1 and TNF-α. IL-1 is the pivotal cytokine at early and late stages of disease and TNF-α is involved only at the onset of arthritis (Rowan, 2001). IL-1 is much more potent than TNF-α in inducing cartilage destruction in vivo. Tiny amounts of IL-1 are sufficient to cause inhibition of proteoglycan synthesis in chondrocytes, whereas a roughly 100 -1000 fold higher level of TNF-α is needed to obtain the same effect (Loo and Berg, 1990). Other pro-inflammatory agents are interleukins -6,-8,-10,-11,-13,-17,-18, granulocyte–macrophage colony-stimulating factor (GM-CSF) and transforming growth factor β (TGF–β) (Rowan, 2001).

IL-1 dominates the effects of cartilage thinning and destruction and increased matrix metalloproteinase (MMP) mRNA expression in immortalized and primary human chondrocytes (Goldring *et al.,* 1994). It also increased COX-2 expression and induces the

release of aggrecanase (Berg, 2001). In addition, IL-1 stimulates production of prostaglandin E_2 (PGE_2) and nitric oxide (NO) (Suffys *et al.,* 1987). It also decreases the cartilage-specific, type II collagen and causes the rapid loss of proteoglycans.

IL-1 has two isoforms, IL-1α and IL-β, which are translated as precursor polypeptides (31kDa). They only share about 30 % homology in their amino acid sequence, yet both of them interact with cells via the same IL-1 receptors (Dayer and Bresnihan, 2002). IL-1α is a major cytokine in early stages of inflammation in humans, whereas IL-β is the more dominant cytokine in advanced inflammation (Berg and Bresnihan, 1999). The action of IL-1 is inhibited by a protein named interleukin receptor antagonist (IL-1Ra). IL-1Ra binds to the IL-1 receptors on target cells and blocks the binding of IL-1. IL-1Ra is produced by activated monocytes and macrophages (Lotz, 2001). The beneficial consequences of blocking IL-1 activity with IL-1Ra are 2-fold: a decrease in the production of proteolytic enzymes as well as a reversal of the inhibitory effects of IL-1 on the repair process (i.e. new synthesis of proteoglycan and collagen) (Dayer and Bresnihan, 2002). The amount of IL-1Ra synthesized in arthritis cartilage may not be sufficient to inhibit the effects of IL-1 totally (Cimpean *et al.,* 2000).

TNF-α is produced by many different cells, especially monocytes and macrophages in a similar way to IL-1 (Dayer and Bresnihan, 2002). Over production of TNF-α as result of age or genetic defects has been implicated in many inflammatory diseases such as in chronic arthritis, although it is a far less potent cytokine for joint damage than IL-1 (Berg, 2001). TNF-α alone is neither arthritogenic nor destructive and exerts its arthritogenic potential mainly through the induction of IL-1 (Berg, 2001). During inflammation, TNF-α stimulates production of IL-1 which, in turn, stimulates release of IL-6. TNF-α shares many functions

and biological actions with IL-1. It is capable of stimulating COX-2 activity thus causing increased production of prostaglandin E_2 (Martel-Pelletier *et al.*, 1999).

TNF exists in two forms, TNF-α and TNF-β. TNF-β is less potent than TNF-α (Konisberg and Magnius, 1999). TNF-α exerts its biological effects on cell function by binding to specific cell-surface receptors. The cleavage of TNF-α at cellular surfaces appears to occur via a TNF-α converting enzyme (TACE) belonging to an ADAM family and being designated ADAM-17. TACE represents a potential target for the development of therapeutic agents for inflammation and related diseases (Amour *et al.*, 1998). Soluble TNF-α receptors act as inhibitors of TNF-α either by blocking its action directly or through competition with cell surface receptors (Feldmann and Giannini, 1996).

Not all the harmful catabolic activity in arthritic articular tissue can be attributed to IL-1α, IL-1β and TNF-α; other cytokines such as IL-6 and IL-8 may also involved. Nitric oxide (NO) is also an important mediator of cartilage destruction. NO inhibits the synthesis of cartilage matrix macromolecules and enhances MMP activity (Martel-Pelletier *et al.*, 1999).

1.4.7 Anti-inflammatory cytokines

The effects of anti-inflammatory cytokines include the promotion of matrix synthesis and repair, induction of protective enzyme inhibitors such as TIMPS, up regulation of IL-1Ra, down regulation of destructive enzymes and reduction in the levels of pro-inflammatory cytokines (Cawston, 1998). Three important anti-inflammatory cytokines are IL-4, IL-10, and IL-13 (Martel-Pelletier *et al.*, 1999).

1.5 Lipids

Lipids are a diverse group of biological substances mainly characterized by their hydrophobic nature and, hence, solubility in organic solvents. They have four basic roles in

natural organisms. First, they form the basic bimolecular structure of biological membranes. Second, they are often a major form of energy storage, particularly as triacylglycerols. Third, they may be major components of the surface coverings of different organisms, skin in mammals, cutin or suberin in plants, cell walls in bacteria. Finally, there are a diverse number of lipids, which have acute biological activity, such as in cell signalling (Harwood, 1999).

1.5.1 Fatty acids

Many lipids contain acyl groups and the fatty acids they contain are generally long-chain molecules with even carbon numbers. Non-esterified (free) fatty acids do not usually occur in significant quantities. Over 500 fatty acids have been found but only a few of them are important quantitatively (Harwood and Russell, 1984). Some of the more important naturally occurring straight chain saturated acids and unsaturated acids are shown in Table 1.2. They can be categorized depending on the number of double bonds; saturated (SFA), monounsaturated (MUFA) or polyunsaturated (PUFA). Essential fatty acids (EFAs) are fatty acids which cannot be synthesized by humans but are considered necessary for good health (Gurr *et al.*, 2002).

Saturated fatty acids have general formula $CH_3\ (CH_2)_nCOOH$ where n is usually an even number and the acid has n + 2 carbon atoms. They can be described as short chain saturated acids (<8C), medium chain saturated acids (C8-C12), long chain acids (C14 -C18) and very long chain acids (>18) (Gunstone, 1997). The most common saturated fatty acids

Chain Length	**Trivial name**	**Fatty Acid Subclass**	**Fatty acid structures**
4:0	Butyric Acid	Saturated	$CH_3(CH_2)_2CO_2H$
6:0	Caproic Acid	Saturated	$CH_3(CH_2)_4CO_2H$
8:0	Caprylic Acid	Saturated	$CH_3(CH_2)_6CO_2H$
10:0	Capric Acid	Saturated	$CH_3(CH_2)_8CO_2H$
12:0	Lauric Acid	Saturated	$CH_3(CH_2)_{10}CO_2H$
14:0	Myristic Acid	Saturated	$CH_3(CH_2)_{12}CO_2H$
16:0	Palmitic Acid	Saturated	$CH_3(CH_2)_{14}CO_2H$
16:1	Palmitoleic Acid	Monounsaturated (*n*-9)	$CH_3(CH_2)_5CH{=}CH(CH_2)_7CO_2H$
18:0	Stearic acid	Saturated	$CH_3(CH_2)_{16}CO_2H$
18:1	Oleic acid	Monounsaturated (*n*-9)	$CH_3(CH_2)_7CH{=}CH(CH_2)_7CO_2H$
18:2	Linoleic acid	Polyunsaturated (*n-6*)	$CH_3(CH_2)_3(CH_2CH{=}CH)_2(CH_2)_7CO_2H$
α-18:3	α–linolenic acid	Polyunsaturated (*n*-3)	$CH_3(CH_2CH{=}CH)_3(CH_2)_7CO_2H$
γ-18:3	γ-linolenic acid	Polyunsaturated (*n-6*)	$CH(CH_2)_3(CH_2CH{=}CH)_3(CH_2)_4CO_2H$
20:0	Arachidic acid	Saturated	$CH_3(CH_2)_{18}CO_2H$
20:4	Arachidonic acid	Polyunsaturated (*n-6*)	$CH_3(CH_2)_3(CH_2CH{=}CH)_4(CH_2)_3CO_2H$
20:5	Eicosapentaenoic acid	Polyunsaturated (*n*-3)	$CH_3CH_2(CH{=}CHCH_2)_5(CH_2)_2COOH$
22:6	Docosahexaenoic acid	Polyunsaturated (*n*-3)	$CH_3CH_2(CH{=}CHCH_2)_6CH_2COOH$

Table 1.2: Some saturated and unsaturated fatty acids (adapted from Gunstone, 1997; Lodish *et al.*, 2000).

include lauric (C12:0), myristic (14:0), palmitic (16:0) and stearic (18:0) acids (Harwood and Russell, 1984). They are very stable to oxidation (Gurr *et al.*, 2002). The short-chain saturated fatty acids are found in products made from milk fat. Some seed oils contain high levels of medium chain saturated fatty acids e.g. coconut oil and palm kernel oils. Palmitic acid (C16:0) is the most widely-occurring saturated acid and it is present in fish oils (10-30%), in milk and body fats of land animals (up to 30%), and in most vegetable fats at levels of between 5-50%. Palm oil contains between 41-48% palmitic acid (Gunstone *et al.*, 1986). Saturated fatty acids, together with *trans*-fatty acids, are often considered 'the bad fats'. However, this is an over-simplication because different individual dietary saturated fatty acids have variable absorptions and physiological effects. Indeed, mammalian milk contains a high saturated acid content and yet it is considered an ideal food source for young mammals (Gunstone, 1997; Gurr *et al.*, 2002).

Unsaturated fatty acids (UFA) are fatty acids with one or more double bonds and they can be categorized into monounsaturated or polyunsaturated. Some of the polyunsaturated fatty acids are also classed as essential fatty acids. The general structure of monounsaturated fatty acids are $CH_3\ (CH_2)_n CH{=}CH(CH_2)_m COOH$ (Gunstone, 1997) with one double bond along the carbon chain, such as oleic acid (18:1 *n*-9). Olive and canola oils contain large amounts of monounsaturated fatty acids mainly in the form of oleic acid (Gunstone, 1994). All plants and animals, including humans can synthesize oleic acid. In other words, oleic oil and other members of the *n*-9 family can be produced in the body (Gurr *et al.*, 2002). Polyunsaturated fatty acids (PUFA) contain two or more double bonds along the carbon chain. PUFA exits in two major families, depending upon the site of the first double bond, *n-3* (omega-3) and *n-6* (omega-6). *n-3* fatty acids have the first double bond at carbon number 3

counting from the methyl terminus while *n-6* fatty acids have their first double bond at carbon number 6 counting from the methyl terminus (Calder, 2004).

1.5.2 Essential fatty acids

The *n- 3* (omega-3) and *n-6* (omega-6) are termed essential fatty acids because they are important nutrients for health but cannot be synthesized by humans. Therefore, they must be obtained from the diet (Gurr *et al.,* 2002). Without EFAs, deficiency symptoms such as skin defects, organ damage and neurological and reproductive dysfunction will develop. Desaturase enzymes are needed for the biosynthesis of unsaturated fatty acids and mammals do not produce the desaturase enzymes needed to insert double bonds between an existing double bond (usually at carbon 9) and the methyl end of the chain. In contrast, plants contain abundant amounts of Δ12- and Δ15- desaturases which convert oleic into linoleic and linolenic acids. Thus, plant tissues and plant oils tend to be rich sources of linoleic and α-linolenic acids. Once consumed in the diet, linoleic acid can be converted via γ-linolenic (18:3 *n-6*) and dihomo -γ-linolenic (DGLA; 20:3 *n-6*) acids to arachidonic acid (ARA; 20:4 *n-6*) as shown in Figure 1.9 (Calder, 1998). Using the same pathway dietary α-linolenic acid can be converted into eicosapentaenoic acid (EPA; 20:5 *n*-3) and then, via extra reactions, to docosahexaenoic acid (DHA; 22:6 *n*-3).

The *n-3* fatty acids include α-linolenic acid (ALA), stearidonic acid, EPA and DHA. Dietary α-linolenic acid [18:3 *n*-3] is the precursor compound of the *n-3* family of PUFAs (Gurr *et al.,* 2002) and from it humans can produce stearidonic acid, EPA and DHA (Connor, 2000). Marine fish oils have a high content of *n-3* fatty acids and these are mostly in the form of EPA and DHA while some seed and nut oils contain high amounts of α- linolenic acid (Gunstone, 1997).

The *n-6* fatty acid family contains linoleic acid [18:2 *n-6*], γ-linolenic acid [18:3 *n-6*], dihomo-γ linolenic acid [20:3 *n-6*] and arachidonic acid [20:4 *n-6*]. Of all essential lipids, our body has the highest daily requirements for linoleic acid [18:2 *n-6*] which is the precursor of the *n-6* PUFA family. When ingested and metabolized in the body, linoleic acid is metabolized to arachidonic acid. γ-Linolenic acid [18:3 *n-6*] can be found in a few edible seed oils e.g. evening primrose (Gunstone, 1994). γ - and α-Linolenic acids are almost identical because they have 18 carbon atoms in their fatty acid chain and have 3 double bonds. However, in γ-linolenic acid, the first double bond located between carbons 6 and 7 whereas in α-linolenic acid, this double bond is between carbons 3 and 4 (numbering from the methyl end) (Okuyama *et al.,* 1997). Arachidonic acid [20:4 *n-6*] is a minor component of many fish oils but attains higher levels in animal phospholipids e.g. egg yolk (Gunstone, 1997).

1.5.3 Dietary fatty acids and arthritis

The amount and type of dietary fatty acids can alter a number of immune and inflammatory responses; *n-6* PUFAs are regarded as generally inflammatory while *n-3* PUFAs are generally anti-inflammatory. This is due to the fact that *n-3* and *n-6* PUFAs produce different types of eicosanoids that have distinct effects on the immune cells (Harwood, 1999) and, in addition, the fatty acids compete for the enzymes involved in their metabolism.

Because of increased consumption of meat and vegetable oils rich in *n-6* fatty acids and reduced consumption of foods rich in *n-3* fatty acids (Calder, 2001), there is thought to be an imbalance in the ratio of *n-6* to *n-3* PUFAs in the diet. The World Health Organization (FAO/WHO, 1993) and others (The British Nutrition Foundation, 1992) now recommend a ratio of between 3:1 and 4:1 for *n-6* to *n-3* fatty acids (Horrocks and Yeo, 1999) whereas the average ratio in Western diet is 10-20:1. The excess of *n-6* fatty acids stimulates the formation of arachidonic acid, which is the principal precursor for "inflammatory" eicosanoid synthesis.

Although some arachidonic acid is essential, the present high ratio may be responsible for the increased incidence of arthritis and other chronic inflammatory diseases (Gurr *et al.,* 2002). Arachidonic acid is converted to series-2 prostaglandins and thromboxanes by COX enzymes. The prostaglandins, particularly prostaglandin E_2 (PGE_2), produced by arachidonic acid metabolism are inflammatory mediators. In addition, metabolism of arachidonic acid by the 5-LOX pathway gives rise to hydroxyl and hydroperoxy derivatives (5-HETE and 5-HPETE) and the 4-series leukotrienes which are also inflammatory (Calder, 2001). By contrast, the *n*–3 PUFAs seem to promote better health by decreasing the level of inflammation found in the body so that, for example, EPA is converted to prostaglandins of the 3-series and leukotrienes of the 5-series, which do not have such powerful pro-inflammatory effects (Calder, 2001).

Diets containing significant levels of DHA and EPA have been shown consistently to reduce both the number of tender joints on physical examination and the amount of morning stiffness in patients with arthritis (Simopoulos, 1999). Fish oils are the most important dietary sources of EPA and DHA and increased levels of these acids in the diet of persons with rheumatoid arthritis are recommended to alleviate the pain and inflammation in their joints (Calder, 2001). The first known clinical test using fish oil was reported by Samuel Key, a physician at the Manchester Infirmary in 1752 to 1784. He demonstrated that cod liver oil gave relief to patients suffering from RA as well as other ailments (see Haraldsson and Hjaltason, 2001). Moreover, it has shown in the past that populations which consume a large amount of fish in their diets seem to have a low incidence of inflammatory and autoimmune disorders. Thus, for example, epidemiological studies of Greenland Inuits (Eskimos) who have a diet rich in *n-3* fatty acids have revealed a low incidence of arthritis (Connor, 2000). Since then, a number of studies, including, placebo-controlled clinical trials and animal studies, have

been carried out to try to ascertain whether dietary supplementation with fish oils can be used successfully in the treatment of arthritis (Kremer, 2000).

There is a significant amount of literature showing that dietary *n-3* PUFAs decrease TNF-α and IL-1 production when given at doses ≥ 5.2 g EPA + DHA per day while lower doses (about 1 g EPA + DHA per day) have little effect on these cytokines (Calder, 2001). Studies by Curtis *et al.,* (2000; 2002) using *in vitro* models of normal and accelerated aggrecan catabolism suggested some specific molecular mechanisms by which supplementation with *n-3* fatty acids can modulate the expression and activity of degradative enzymes and inflammatory factors that cause cartilage destruction during arthritis.

1.5.4 Triacylglycerols

Triacylglycerols (TAG), often termed fats, are one of the simplest lipids. In plant storage tissue, triacylglycerols form the major proportion of lipids. In most animals, triacylglycerols are also the major form of stored lipid which, in mammals, accumulates in the adipose tissues. Since triacylglycerols contain three esterified fatty acids, a whole variety of molecular species are possible. There is often a tendency for the acyl chain at the sn-2 position to be more unsaturated than that at the sn-1 position (Figure 1.14). However, it is extremely rare for the same fatty acid to present at all three positions (Gunstone, 1997).

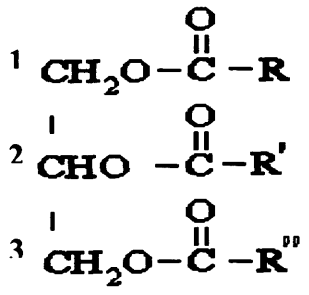


Figure 1.14: Triacylglycerol structure.

1.5.5 Palm oil

Palm oil is one of the major oils and fats produced and traded in the world. Malaysia is the world's largest exporter of palm oil, accounting for about 61% of the total exports at 17.37 million tonnes in 2001 (www.mpopc.org.my). In terms of exports, palm oil is the most widely traded oil accounting for 45.6% of the world's export of 176 documented oils and fats in 2001 (Oil World, 2004).

Palm oil is extracted from the oil palm (*Elaies guineensis)* which originated from West Africa. The oil palm produces two distinct oils: palm oil from the fleshy mesocarp, which is used mainly for edible purposes, and palm kernel oil from the fruit kernel, which has a wide application in the oleochemical industry (Sambanthamurthi *et al.,* 2000). Oil palm fruit contains 22-25% oil and palm kernel oil contains 2-4% oil (Berger, 2001). Crude palm oil is extracted from the mesocarp either by centrifugation or hydraulic pressing after it has been digested. Digestion involves mashing of the palm fruits under steam-heated conditions to inactive fungal lipases, which can rapidly hydrolyse the oil (Pantzaris, 1988). Crude palm oil from the presses consists of a mixture of palm oil (35-45%), water (45-55%) and fibrous materials in varying proportion (Ma, 1994). The crude oil is deep red in color due to carotenoids (500-700ppm) (Choo, 1994). The oil is bleached after neutralization using bleaching earth or by heat bleaching at temperatures of 240-270 °C. Neutralized bleached oil then proceeds to the next stage where the free fatty acid content and colour are further reduced and, more important, it is deodorized to produce a product, which is stable and bland in colour. The oil is now termed as neutralized, bleached and deodorized palm oil (Pantzaris, 1988).

1.5.6. The applications of palm oil

The usage of palm and palm kernel oils can be extended by fractionation which separates the various triacylglycerols into one or more fractions by using the different solubility of triacylglycerols, which, in turn, is caused by their molecular masses and degree of unsaturation (Timms, 1997). Palm oil gives a more valuable olein–liquid fraction and a less valuable stearin–solid fraction (Gunstone, 1997) as shown in Figure 1.15.

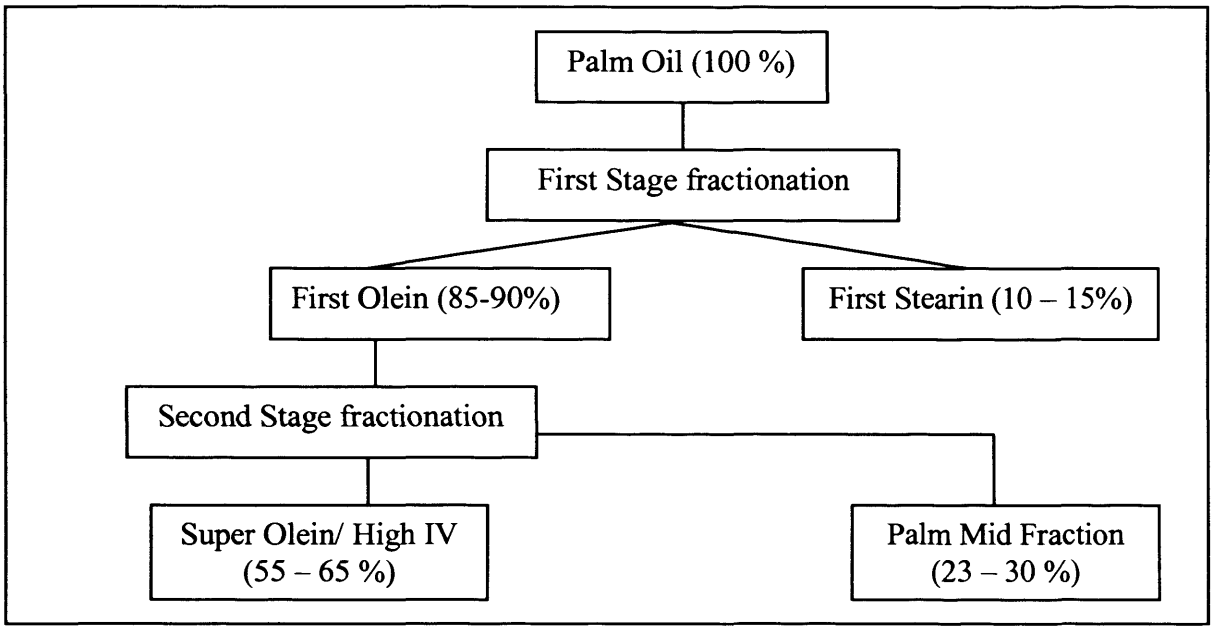


Figure 1.15: Flow diagram of simple two-stage solvent fractionation (taken from Chong, 1994).

Palm olein is used as frying oil because it is very stable to heat, whilst palm stearine is used in biscuits, in non-hydrogenated margarine and oleochemicals. Palm oil resists oxidation and rancidity, which means products made using palm oil have extended shelf lives. The oils and their fractions can be further modified by blending with other oils, by partial hydrogenation, or by interesterification in order to modify a fat for any specific purpose. Hydrogenation, interesterification and fractional crystallization modify the relationship between the solid content and the melting temperature of the fat. Further fractionation of the

palm oil fractions by solvent fractionation, yields palm mid fraction (PMF) which contains high levels of stearic-oleic-stearic (SOS) species and is used in cocoa butter equivalents (Timms, 1997).

Palm oil is characterized by high levels of carotene, tocopherols and tocotrienols (vitamin Es). Around 90% of palm oil production is used in the food industry (Pantzaris, 1988). Palm oil consists mainly of triacylglycerols containing a range of fatty acids as shown in Table 1.4. It contains almost equal amounts of saturated acids (44% palmitic, 5% stearic) and unsaturated acids (40% oleic, 10% linoleic acid) (Berger, 2001).

	% of total acids	
Fatty acid	**Range**	**Mean**
Lauric (C12:0)	0.1 – 1.0	0.2
Myristic (C14:0)	0.9 - 1.5	1.1
Palmitic (C16:0)	41.8 - 46.8	44.0
Palmitoleic (C16:1)	0.1 - 0.3	0.1
Stearic (C18:0)	4.2 - 5.1	4.5
Oleic (C18:1)	37.3 - 40.8	39.2
Linoleic (C18:2)	9.1 - 11.0	10.1
α-Linolenic (18:3)	0.5 - 0.3	0.5
Arachidic acid (C20:0)	0.0 - 0.6	0.4
Arachidonic acid (C20:4)	0.2 - 0.7	0.4

Table 1.4: Fatty acid composition of Malaysian palm oils (taken from Berger, 2001).

Palm oil is considered to contain adequate amounts of *n-6* essential fatty acids but only small amounts of *n-3* PUFA. Over 95 % of palm oil consists of mixtures of TAGs (Pantzaris, 1988). As expected from the high levels of palmitic and oleic acids in this oil, the TAGs

containing these two acids, palmitic-oleic-palmitic (POP) and palmitic-oleic-oleic (POO) are the dominant molecular species (Gunstone, 1997). Other components in palm oil include small proportions of diacyglycerols (DAGs) (up to 7.6%) and monoacylglycerides (MAGs) (below 1%) (Sambanthanmurthi *et al.,* 2000).

Palm oil contains 3 species of DAG namely dipalmitoylglycerol or PP (12.6%), palmitoyloleoylglycerol or PO (54.4%), and dioleoglycerol or OO (33.0%). DAGs are rather difficult to remove by refining because of their low volatility. The DAG in palm oil affects physical properties such as crystallization so that during fractionation the palm oil may become cloudy at low temperatures (Siew and Ng, 1995). Palm oil also contains other minor constituents, such as free fatty acids and non-glyceride components. These constituents may affect the oil's chemical and physical characteristics (Choo, 1994). Palm oil has distinctive properties, which enable it to meet some of the demanding quality requirements of edible-fat products. The proportion of palmitic and oleic acids in the major TAG species lead to stability of the β'crystals in edible fats. These are very desirable in the production of margarines and shortenings. As a frying fat, palm oil has a high resistance to oxidation and does not leave an unpleasant room odour, due to the low amounts of linolenic acid in its composition. Natural palm oil also contains a high level of tocopherols (vitamin E) which are powerful natural antioxidants (Berger, 2001).

1.5.7 Nutritional properties of palm oil and its components

Palm oil is frequently confused with palm kernel oil or coconut oil, which are highly saturated. In contrast, palm oil contains a balance of polyunsaturated, monounsaturated and saturated fatty acids. In addition, palm oil contains nutritionally- important substances such as linoleic acid, tocopherols and tocotrienols. The latter can act as natural anti-oxidants against

damaging free-radicals. In contrast to palm oil, high dietary concentrations of coconut or palm kernel oils may raise serum cholesterol levels (Sundram *et al.*, 1997).

There are two types of lipoprotein carrying cholesterol: low density lipoprotein (LDL) and high density lipoprotein (HDL). LDL cholesterol is often considered the 'bad' cholesterol since high amounts may be deposited on the arterial walls thus contributing to plaques. More and more plaques will narrow the blood vessel's lumen and block blood flow. HDL, on the other hand, takes excess cholesterol away and carries it back to the liver to be metabolised. It may also remove some of the cholesterol already present on the artery walls. Therefore, HDL is considered beneficial as high levels of HDL in the blood can decrease the risk of heart disease (Mensink and Katan, 1990). Lauric and myristic (saturated) fatty acids, which are found abundantly in coconut oil and palm kernel oil have been shown to increase LDL levels in various epidemiological studies. In contrast, a high palm oil diet did not raise serum total cholesterol or LDL-cholesterol. Moreover, it caused a significant increase in HDL levels (Bonanome and Grundy, 1988).

Palm oil is not genetically modified and provides *trans*-fat free solid products for food production. Because palm oil is solid at room temperature there is no need to use hydrogenation, a technique which hardens liquid oil but also produces potentially damaging *trans* fatty acids. In comparison, most vegetable oils such as corn oil, soy oil etc. require hydrogenation, which gives rise to *trans* fatty acids (Mensink and Katan, 1990).

Palm oil diets have been shown to be relatively benign in terms of arterio-vascular disease (Nesaretnam and Muhammad, 1994). There has also been some evidence that diets containing high levels of palm oil could be of benefit in reducing the occurrence of cancer.

This may be due to the tocotrienols present in palm oil inhibiting oxidation and, hence, onset or growth of a cancer. In addition, β-carotene has long been postulated to be beneficial as an anti-cancer agent (Nesaretnam *et al.,* 2000) and this is at significant levels in palm oil. Some of the findings of the human and animal studies on the nutritional properties of palm oil and its components are summarized in Table 1.5.

1.5.8 Palm oil carotenoids

Palm oil contains the highest known concentration of carotenoids of all plant oils examined. Indeed, unprocessed palm oil has a rich orange-red colour due to its high content of carotenes which is in the range 700-1000 ppm (Choo *et al.,* 1994). The major carotenoids in palm oil are α- and β-carotenes which constitute about 80-90% of the total carotenoids. It is well recognized that carotenoids are more stable and best absorbed in the presence of fat, which acts as a carrier. β–Carotene accounts for about 55 % of carotenoids in palm oil and α-carotene around 35 % (Yap *et al.,* 1991). A process to prepare carotene-enriched palm oil involves degumming of the oil with phosphoric acid, followed by treatment with bleaching earth. More than 80% of the carotenoids originally present in the crude palm oil are retained. The oil produced from this process is known as red palm oil (Choo, 1994). β-Carotene is converted into retinol in the body but plays a beneficial role in human health, beyond its pro-vitamin A function. Retinol is one of the most active forms of vitamin A. Palm oil carotene concentrate may prevent the development of hormone-dependent breast cancers (Nesaretnam *et al.,* 2000), atherosclerotic effects and blindness.

Parameter	Effect	Source
Cholesterol	Dietary palm oil has the effect of decreasing total blood cholesterol and LDL- cholesterol and increasing the level of HDL-cholesterol, when compared to other oils such as corn oil.	Kris-Etherton *et al.* (1984); Qureshi *et al.* (1991); Ng *et al.* (1991).
Atherosclerosis	The consumption of a diet with high proportions of palm oil did not result in an increase in arterial lesions compared to other commonly utilized fat blends.	Hornstra *et al.* (1991); Kritchevsky *et al.* (2002).
Arterial thrombosis	Palm oil diets either increase the production of prostacyclin that prevents blood-clotting or decrease the formation of thromboxane that contributes to blood clotting.	Sundram *et al.* (1997); Tomeo *et al.* (1995); Rand *et al.* (1988).
Cancer	Diets containing a high level of palm oil did not promote mammary cancer in rats when fed at high levels in the diet unlike most other dietary fats and oils. One study investigated the effect of palm carotene supplementation on the tumorigencity of human breast cancer cells which were injected into athymic nude mice. Palm oil carotene was able to modulate the immune system and suppress tumorigenesis.	Guthrie *et al.* (1997); Nesaretnam *et al.* (2000).

Table 1.5: Summary of experiments on the nutritional effects of palm oil

Carotenes may also slow the progression of rheumatoid arthritis. They have excellent antioxidant properties that help neutralize free radicals which have been implicated as mediators of tissue damage in patients with arthritis (Knekt *et al.,* 2000). β-Carotene together with α-tocopherol have been studied for association with a reduced risk of RA. The results were consistent with the hypothesis that a low antioxidant level is a risk factor for RA because β–carotene and α–tocopherol reduced the incidence (Heliövaara *et al.,* 1994).

1.5.9 Palm vitamin E

Crude palm oil is a rich source of vitamin E which ranges between 600-1000 ppm. Vitamin E is a fat-soluble vitamin, which comprises 2 major groups of compounds, known as tocopherols and tocotrienols. Each group comprises 4 different isomers, designated as alpha (α), beta (β), gamma (γ) and delta (δ) (Eldin and Appelqvist, 1996). The total vitamin E in crude palm oil is a mixture of tocopherols (18-22%) and tocotrienols (78-82%). About 70% of it remains in bleached deodorized palm oil after refining i.e. the latter contains about 420-700 ppm total vitamin E (Gapor *et al.,* 1981). Tocopherols and tocotrienols have great similarities in their structures, which consist of a head (chroman ring) and a tail (Papas, 1999). Tocotrienols differ only on the tail. The tocopherols are structurally characterized by a saturated side chain on the chroman ring whereas the tocotrienols possess an unsaturated phytyl side chain. The chroman ring carries the active antioxidant group. Tocotrienols, like tocopherols, are excellent antioxidants. However, tocotrienols are more potent than tocopherols as antioxidants (Eldin and Appelqvist, 1996).

The major tocotrienols occurring in palm oil in terms of total vitamin E are α-tocotrienol (22%), γ-tocotrienol (46%), and δ-tocotrienol (12%). These antioxidants in palm oil prevent damage to cells by reacting with free radicals before any other molecule can

become a target. By this means, they counteract inflammation. Thus, the vitamin E antioxidants may slow the rate of joint deterioration in arthritic patients and reduce the symptoms of arthritis (Traber and Packer, 1995). Vitamin E has been reported to cause a much slower rate of joint deterioration, particularly in the knees, compared with people eating foods containing lower amounts of antioxidants (McAlindon *et al.,* 1996). Vitamin E supplementation significantly enhances lymphocyte proliferation and decreases PGE_2 production by reducing COX activity (Serafini, 2000). In placebo-controlled studies, patients taking vitamin E (800 IU/day supplementation) showed relief from symptoms of rheumatoid arthritis (Kolarz *et al.,* 1990).

1.6 Lipase-catalysed synthesis of modified lipids

Natural fats and oils are mixtures of triacylglyerols. Their functional properties as ingredients in prepared foods are directly related to the type of triacylglyerols contained. These properties are determined by the fatty acid composition and the distribution of the fatty acids on the glycerol backbone of the triacylglyerols (Quilan and Moore, 1993). Interesterification is one of three important fat modification processes in the fats and oils industry (Rozendaal, 1992). Other methods are fractionation (to separate fats into solid and liquid fractions) and hydrogenation which is used to reduce the unsaturation of fats (thereby raising their melting points). Interesterification (IE) or ester-ester exchange or transesterification is a process during which the fatty acids of triacylglyerols can be exchanged, thereby altering the overall chemical composition and physical properties. It is a general term for reactions between an ester and a fatty acid, an alcohol, or other esters and includes acidolysis, alcoholysis and ester-ester exchange (Xu, 2000).

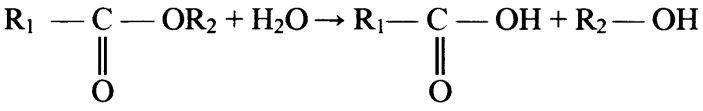
There are two main types of interesterification; chemical interesterification and enzymatic interesterification. Chemical interesterification requires alkaline catalysts to randomize all of the fatty acids in a triacylglyerol mixture while enzymatic interesterification usually uses a certain enzyme to accomplish the exchange of fatty acids at the external positions (*sn* 1- , *sn* 3-) of the triacylglyerols while leaving the fatty acid composition at the 2-position unchanged (Posorske *et al.,* 1988). Enzymatic IE is now used to produce high-value-added structured fats and oil products. Useful glyceride mixtures that cannot be obtained by chemical IE processes are possible by exploiting the specificity of lipases. In all glyceride reactions, lipases catalyse either the removal or the exchange of fatty acyl groups on the glycerol backbone. Different lipases can show preferences for positions where the fatty acid groups are esterified on the glycerol backbone. In general, lipases have been deployed for catalysing three major types of reactions, namely, hydrolysis, transesterification (also called interesterification), and esterification as shown in Figure 1.16.

1.6.1 Reaction systems

The effects of reaction parameters such as reaction time, temperature, substrate molar ratio, enzyme content, water content, incubation time, specificity (including regiospecificity) of enzyme, substrate type and chain length, are factors that can affect enzymatic activity and product yield (Akoh, 1998; Xu, 2000). The reactants for enzymatic interesterification processes are usually mixtures of TAGs or TAGs plus free fatty acids (FFAs) or their esters.

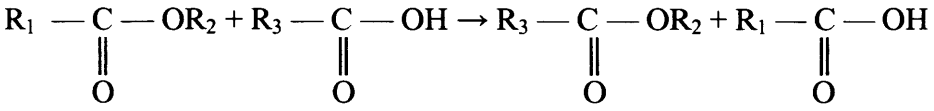
The main problems with using esters are their higher cost and the inevitable occurrence of some hydrolysis during enzymatic interesterification processes. This gives small amounts of FFA in addition to esters, TAGs and DAGs as reaction products and complicates product recovery and by-product recycling procedures (Rozendaal and Macrae, 1997).

(1) Hydrolysis

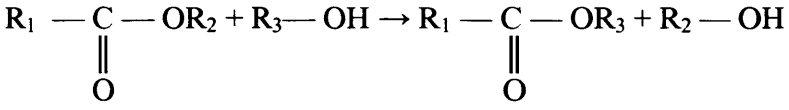


(2) Transesterification (interesterification) Reactions

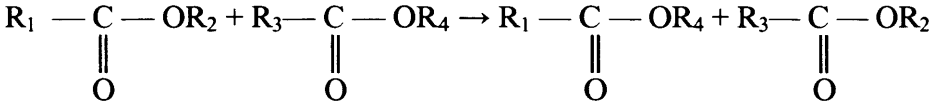
(a) Acidolysis



(b) Alcoholysis



(c) Ester Exchange



(3) Ester Synthesis

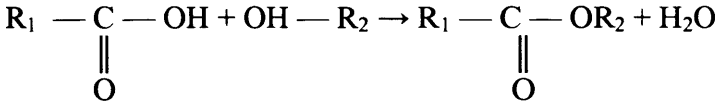


Figure 1.16: Various lipase mediated reactions (taken from Gandhi, 1997).

Enzymatic IE can be carried out in organic solvents, where substrates are soluble and hydrolysis can be minimized. The type of organic solvent employed can affect the reaction kinetics and catalytic efficiency of an enzyme. Hydrophilic or polar solvents can penetrate into the hydrophilic core of proteins and alter their functional structure. Hydrophilic refers to a physical property of a molecule that can transiently bond with water (H_2O) that can transiently through hydrogen bonding. This is thermodynamically favourable, and makes these molecules soluble not only on water, but also in polar solvents (http://en.wikipedia.org/wiki). They also strip off the essential water of the enzyme. Hydrophobic solvents are less likely to cause enzyme inactivation in esterification reactions (Akoh, 1998).

Microbial lipases vary greatly in their activity in interesterification reaction systems. The reactions are run under conditions of low water activity (A*w*) and one reason for the variation in interesterification activity lies in the different responses of the enzymes to low Aw (Halling, 1994). Aw is the relative availability of water in a substance. It is defined as the vapour pressure of water divided by that of pure water at the same temperature. Therefore, pure distilled water has a water activity of exactly one (http://en.wikipedia.org/wiki/Water_activity). Some immobilized lipases have good catalytic activity at low A*w* (<0.2), whereas others are essentially inactive under these conditions and express high catalytic activity only when A*w* is raised to >0.5. For interesterification catalysts, enzymes in the former category, e.g. *Rhizomucor miehei* lipase, are preferred (Rozendaal and Macrae, 1997).

Fatty acid methyl or ethyl esters can also be used instead of FFAs. The advantages of using esters rather than the free acids are that, with some lipases, faster reaction rates are obtained and, because the esters have lower melting points, lower reaction temperatures are possible (Macrae, 1992). The main problems with using esters are their higher cost and the

inevitable occurrence of some hydrolysis during enzymatic interesterification processes. This gives small amounts of FFA in addition to esters, TAGs and DAGs as reaction products and complicates product recovery and by-product recycling procedures (Rozendaal and Macrae, 1997).

Reaction temperature is also important and different lipases may have different useful temperature ranges. In the range 40-70°C, temperature is not usually a crucial factor for the reaction performance provided that the viscosity of the substrates is sufficiently low. Temperature and reaction time are two parameters that influence the equilibrium of acyl migration. Because reaction time correlates with temperature, a shorter time is needed to reach equilibrium when a high reaction temperature is applied. Both factors correlate positively with acyl migration, i.e. a longer reaction time or higher temperature lead to increased acyl migration (Xu, 2000). Immobilized lipases show good thermal stability at temperatures required to process most oils and fats and are, therefore, suitable for bio-modification of triacylglycerols. For example, Lipozyme IM from *Rhizomucor miehei* and Novozyme SP435 from *Candida antartica* are highly active and stable at temperatures in the range of 60-80°C (Bornscheuer *et al.,* 2003).

1.6.2 Lipase applications

Immobilized lipases can be used to impart new and improved physical and nutritional properties to triacyglycerols (Macrae, 1983). In relation to physical properties, enzyme interesterification can improve structuring and/or melting behaviour of fats for application in products such as spreads, non-dairy creams, and confectioneries. It can also improve organoleptic properties (for example, to reduce waxiness or to impart cool melting) and reduce the total saturated fat content of a product (Rozendaal, 1992). Thus, high value TAGs used in

the formulation of confectionery fats can be produced from cheaper oils such as palm oil or high oleate sunflower oil by an interesterification reaction with stearic acid using a 1, 3-regiospecific lipase *Rhizopus delemar* as shown in Figure 1.17 (Holmberg and Osterberg, 1989). The reaction in this transesterification can be illustrated as follows (Figure 1.7):

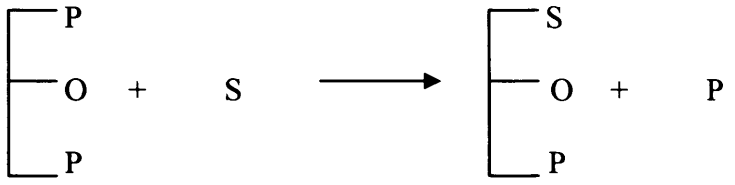


Figure 1.17: S is stearyl acid, P is palmitoyl acid and O is oleyl acid. A mixture of stearyl acid and triacylglycerols with palmitoyl acid enriched at the *sn*-1- and *sn*-3 positions and oleyl acid concentrated at position-2 is converted into a mixture of palmitoyl acid and new triacylglycerols where stearyl acid can now be at positions -1 or -3 (Holmberg and Osterberg, 1989).

Because of possible relationships between stereospecific fatty acid locations and lipid nutrition then it has been suggested that the process of interesterification could be used to improve the nutritional value of certain TAGs (Osborn and Akoh, 2002). For example, enzymatic interesterification is used to produce a human milk fat substitute for use in infant formula. Interesterification of a mixture of tripalmitin and unsaturated fatty acids using a 1,3–regiospecific lipase as catalyst gives TAGs derived entirely from vegetable oils that are enriched in palmitate at the *sn*- 2 position with unsaturated fatty acyl groups in the -1 and -3 positions. These TAGs closely mimic the fatty acid distribution found in human milk and when they are used in infant formula instead of conventional fats, the presence of palmitate at the 2-position of the TAGs was shown to improve digestibility of the fat and absorption of other important nutrients such as calcium (Rozendaal and Macrae, 1997).

Enzymatic interesterification can also be used for production of oils and fats containing nutritionally important PUFAs, such as eicosapentaenoic acid (EPA) and docosahexaenoic

acid (DHA). EPA and DHA are considered of high importance for human nutrition. Imbalances in the relative amounts of these fatty acids in the body have been attributed to the prevalence of many chronic disease states (Calder, 2002). Lipases provide a gentle route for synthesising polyunsaturated triacyglycerols and are suitable for the production and modification of lipids containing such PUFAs, because PUFAs often form potentially toxic products when exposed to oxidative stresses, as encountered in more conventional processing methods (Osborn and Akoh, 2002).

Interesterification of blends of palm and canola oils using the 1, 3 regiospecific *Rhizopus delemar* lipase as catalyst gave oils with improved fluidity compared with the original blends of chemically interesterified products. Because of their higher fluidity the enzymatically interesterified oils were effective as frying oils (Rozendaal and Macrae, 1997). Conversely, interesterification of blends of palm with cottonseed or rapeseed or soybean oils using 1, 3 regiospecific lipases produces fats with a low *trans* acid content and which are suitable as margarine hard stock. With these enzymatically interesterified fats, margarine could be formulated without using any hydrogenated fats (Rozendaal and Macrae, 1997).

In conclusion, enzymatic interesterification of fats and oils provides a safe, easy and cost-efficient alternative to chemical interesterification and hydrogenation. The process gives a more natural product, free of *trans* fatty acids and other undesirable by-products which might be produced under more vigorous conditions.

1.7 Objectives of the study

There were four main objectives of the present study:

1. To investigate the comparative effectiveness of different types of *n-3* PUFAs in reducing the inflammatory responses and cartilage degradation in chondrocyte cultures. Previous studies have shown that all types of *n-3* fatty acids used can reduce aggrecan catabolism and inflammatory responses. However, the difference in efficacy of these *n-3* fatty acids had not been investigated and, therefore, the most effective fatty acid had not yet been identified. Therefore, three major types of dietary *n-3* fatty acids were investigated; α-linolenic acid (ALA, 18:3), eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6). An *n-6* fatty acid (arachidonic acid, 20:4) was used for comparison.
2. To investigate the effect of palm oil fractions (particularly palm olein) on both inflammatory mediators and degradative enzymes in the test system.
3. To add value to palm olein by the incorporation of potentially beneficial *n-3* PUFAs, α-linolenic acid and EPA into its triacylglycerols by determining the ability of different lipases to catalyze the interesterification reaction of palm olein with these acids and to optimize the reaction conditions for incorporation.
4. To evaluate the resulting oils as potential sources of dietary fats with anti-inflammatory properties by using chondrocyte cultures as a model system for arthritis.

CHAPTER TWO

Materials and methods

2.1 Materials

2.1.1 Cell culture

Bovine metacarpo- and metatarsophalengeal joints were obtained from a local abattoir. Collagenase type II, prepared from *Clostridum histolyticum* was obtained from Worthington Biochemical Corporation (New Jersey, USA). Pronase (*Streptomyces griseus*) was from Boehringer Mannheim, Germany. Dulbecco's modified Eagle's medium (DMEM) with 4500mg/ml of glucose and fetal calf serum (FCS) were both obtained from Gibco-BRL (Paisley, U.K). Refined, bleached, and deodorized (RBD) palm oil olein (Iodine value 60) was obtained from the Malaysian Palm Oil Board (MPOB), Malaysia. α-Linolenic acid, eicosapentaenoic acid, docosahexaenoic acid, linoleic acid, arachidonic acid, palmitic acid and oleic acid were all obtained from Nu-Check Prep, Elysian, MN, USA. All fatty acids were a minimum of 99 % purity. Fatty acid-free bovine serum albumin (fraction V) was obtained from Sigma-Aldrich Co (Dorset, UK). Recombinant human interleukin (IL-1α) was from Totam Biologicals (Cambridge, UK). All cell culture plates and filter units were obtained from Corning Costar (Buckinghamshire, UK) and Millipore (Watford, UK) respectively.

2.1.2 Lactate assay

Lactate standard solution from Sigma-Aldrich Co. was L (+) lactic acid at 40mg/dl. Lactate reagent was also from Sigma-Aldrich Co. Lactate reagent buffer was composed of 0.1M citric acid, 1mg/ml BSA, 0.1% $CaCl_2$ and 0.02 % sodium azide, adjusted with 1M Na_2HPO_4 to pH 6.0. Lactate reagent, when reconstituted, contained microbial lactate oxidase (400 U/L), peroxidase (horseradish 2400U/L) and chromogen precursors (ABTS, 2,2'-azino-di[3-ethylbenzthiazonine sulphonate). The microplate (EL-312 Bio-kinetics reader) reader used in the experiments was manufactured by Elkay company, UK.

2.1.3 Fatty acid analysis

Pentadecanoic acid (used as an internal standard) and gas liquid chromatography standard mixtures were both obtained from Nu-Check Prep, Elysian, MN, USA.

2.1.4 Dimethylmethylene blue glycosaminoglycan assay

All general chemical reagents used to make up the dimethylmethylene blue (DMMB) solution and the chondroitin sulphate C from shark cartilage were obtained from Sigma-Aldrich Co, UK.

2.1.5 RNA extraction and reverse transcriptase – polymerase chain reaction

Tri-Reagent was obtained from Sigma-Aldrich Co. RNA isolation was carried out using RNEASY minicolumns from Qiagen (Crawley, UK). RT-PCR was carried out using the RNA PCR core kit from Applied Biosystem (Warrington, UK). The core kit consists of GeneAmp 10 x PCR buffer 11 (100mM-Tris/HCI pH 8.3; 500mM KCl), $MgCl_2$ (25mM solution), dNTPs (dATP, dCTP, dGTP, dTTP, all at 10mM each), RNase inhibitor, MuLV reverse transcriptase and oligo dT. For hot start PCR, Ampliwax PCR gem 100 wax beads

were also obtained from Applied Biosystems (Warrington, U.K). Taq DNA polymerase (from *Thermus acquaticus* strain YTI) was from Promega (Southampton, UK). Electrophoresis agarose was from ICN Biomedical (Ohio, U.S.A). The Wizard PCR preps DNA purification system was also obtained from Promega.

2.2 Methods

2.2.1 Hydrolysis of palm oil fractions

An aliquot of palm oil fraction (15-30mg) was pipetted into a glass methylating tube. Methanolic NaOH (0.3N, 0.5 ml) was added. The mixture was refluxed for 2 hours at 70°C in a thermal block to hydrolyse acyl lipids. HCL (6N, 0.3ml) was added to acidify the mixture and the free fatty acids were extracted with 3 - 4 portions (5ml) of petroleum ether.

2.2.2 Isolation and culture of bovine chondrocytes

Bovine articular chondrocytes were harvested aseptically from the metacarpo- and metatarsophalangeal joints of 7 day-old calves. For monolayer and agarose cultures, cartilage was removed from the joint surfaces under sterile conditions. The tissue was digested in 0.1% pronase in DMEM containing 5% (w/v) fetal calf serum (FCS) for 3 h at 37°C with agitation on the roller. It was further digested with 0.04% (w/v) collagenase in DMEM with 5% FCS and left overnight at 37°C with agitation to isolate the chondrocytes. After 24 h, cells were filtered through a 40μm Nitex filter (Falcon, UK) before cell numbers were estimated by counting under the microscope. Cells were resuspended at 6 x 10^6 cells/ml in DMEM. Monolayer cultures were established in 60 mm dishes by plating 1ml/ dish of a suspension of 6 x 10^6 chondrocytes/ml DMEM (to give a density of approximately 2 x 10^5 cells/cm^2). Experimental medium (4ml/plate DMEM containing 50μg/ml gentamicin [0.5% antibiotic/antimycotic solution -100x; Gibco Life Technologies]) was then added to cultures,

which were maintained at 37°C under 5% (v/v) CO_2 for 6 hours or until the monolayer cultures established. Polyunsaturated *n-3* fatty acids (α18:3 [α-linolenate] or 20:5[eicosapentaenoate] or 22:6 [docosahexaenoate]) or the *n-6* fatty acid arachidonic acid, or hydrolysed palm olein were added (10 - 300 μg/ml) in a complex with bovine serum albumin (3.5g/L). The fatty acid mixtures used above were first prepared by incubation for 16 hours at 37°C in Tyrode-HEPES buffer (20 mM HEPES, 140mM NaCl, 4.5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 11 mM glucose, pH 7.4) containing 3.5g/L fatty acid-free bovine serum albumin (Fraction V, Sigma-Aldrich Co.) before addition to the culture medium. All cultures were maintained for 8 hours in the presence or absence of fatty acids to allow the latter's incorporation into membrane lipids.

Bovine serum albumin contains binding sites for fatty acids (Curry *et al.,* 1999) and this preincubation is commonly used to make fatty acid supplementation more physiological. After 8 hours incubation, the fatty acid-BSA complexes were removed from the culture medium by washing three times with DMEM without FCS and replaced with fresh medium (without fatty acid supplement) and culture was continued with or without 10ng/ml IL-1α.

2.2.3 Determination of lactate concentration in chondrocyte culture media

The purpose of this analysis was to evaluate the metabolic state of chondrocyte cultures and the concentration of lactate in the culture medium was measured using a commercially available lactate assay kit (Sigma-Aldrich Co). The principle of this lactate assay is that lactic acid (end product of glycolysis) is converted to pyruvate by lactate oxidase. Then, in the presence of the hydrogen peroxide, peroxidase catalyzes the oxidative condensation of chromogen precursors (like ABTS, 2, 2'-azino-di [3-ethylbenzthiazonine

sulfonate]) to produce a coloured dye with an absorption maximum at 540nm. The lactate concentration is then calculated from a standard curve of known lactate concentration. The increase in absorbance is directly proportional to the lactate concentration in the sample. A decrease in lactate concentration indicates a decrease in the metabolism of the chondrocytes and was taken to be a measure of decreased cell metabolism and, possibly, viability.

Varying concentrations of lactate standard solutions were made up (0 - 400µg/ml) and 5µl of these and appropriately diluted media samples (1:10) were added to individual wells of a 96 multiwell plate. Lactate reagent was prepared by dissolving the reagent powder in double distilled water (10ml). Lactate reagent (250µl per well) was added and incubated for 10 minutes at room temperature (after which colour development was stable) and absorbance was measured at 540nm. The reaction equations are shown as follows (Figure 2.1):

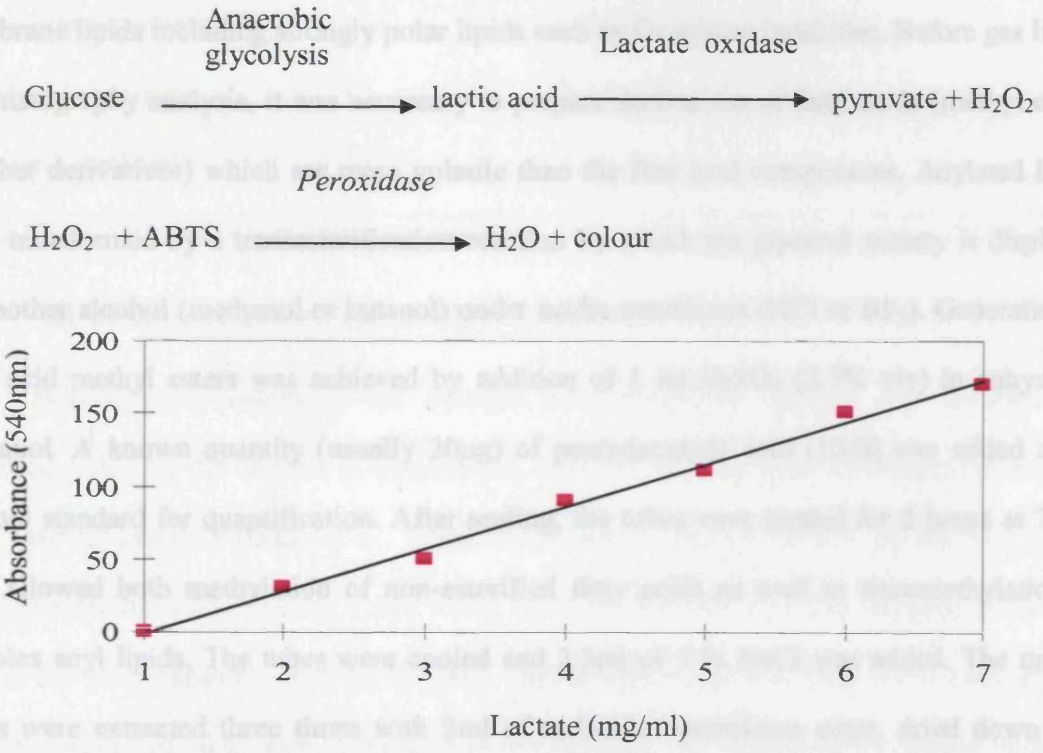


Figure 2.1: A typical lactate standard curve

2.2.4 Fatty acid analysis of chondrocytes

Chondrocyte cell layers and associated matrix were harvested and washed three times in phosphate-buffered saline using repeated centrifugation for 10 minutes at 10,000 rpm (4416 g min). The cell pellet was resuspended in 1ml deionized water and sonicated for 30 minutes in an ultrasonic water bath. Once complete lysis of the cells had been achieved, extraction of the lipids was performed using a previously established method (Garbus *et al.*, 1963). Briefly, 1ml of cell lysate was mixed with 3.75 ml of chloroform/ methanol (1:2 v/v) and incubated for 30 minutes at 20°C. Following the addition of 1.25ml chloroform and 1.25ml Garbus solution (2M KCl in 0.5 M potassium phosphate buffer, pH 7.4) and mixing, two phases were created and the lower (chloroform) phase (containing lipids) was collected and dried down under a stream of nitrogen.

The Garbus procedure is especially useful for ensuring quantitative extraction of most membrane lipids including strongly polar lipids such as the higher inositides. Before gas liquid chromatography analysis, it was necessary to prepare derivatives of fatty acids (methyl esters or other derivatives) which are more volatile than the free acid components. Acylated lipids were transformed by a transesterification reaction by which the glycerol moiety is displaced by another alcohol (methanol or butanol) under acidic conditions (HCl or BF_3). Generation of fatty acid methyl esters was achieved by addition of 1 ml H_2SO_4 (2.5% v/v) in anhydrous methanol. A known quantity (usually 20μg) of pentadecanoic acid (15:0) was added as an internal standard for quantification. After sealing, the tubes were heated for 2 hours at 70°C. This allowed both methylation of non-esterified fatty acids as well as transmethylation of complex acyl lipids. The tubes were cooled and 2.5ml of 5 % NaCl was added. The methyl esters were extracted three times with 3ml of redistilled petroleum ether, dried down in a

stream of nitrogen and then redissolved in chromatographically pure petroleum ether. The methyl esters were analyzed using an Autosystem XL gas liquid chromatogram (Perkin Elmer, Connecticut, USA), with the yields of fatty acid being calculated from the known amount of internal standard. A glass column (1.5m x 4mm internal diameter) packed with a polar packing material, operated isothermally at 180°C, was used. This procedure separates fatty acids according to their chain length as well as their degree of unsaturation. Preliminary identification of fatty acids in samples was achieved by co-chromatography with a temperature programmed method as shown in Table 2.1.

Temperature program	**Conditions**
Initial oven temperature (°C)	185 °C
Holding time (min)	3.0 min
Ramp rate (°C/min)	2.0/min to 240°C
Final oven temperature (°C)	260°C
Holding time (min)	1.0 min
Total run time (min)	30.50 min

Table 2.1: Temperature program method

2.2.5 Extraction and quantification of proteoglycans

Proteoglycan content in the medium of chondrocyte cultures was measured as sulphated GAG by the amount of polyanionic material reacting with 1,9-dimethylmethylene blue (DMMB assay) (Farndale *et al.,* 1986). In this assay, DMMB binds to the sulphate groups on glycosaminoglycans forming a dye: GAG complex. Formation of this complex produces a shift in the colour absorbance (blue to pink). Chondroitin sulphate C from shark cartilage was used as a standard. Standards ranging from 0 - 40μg/ml and appropriately diluted unknown

samples were added to a 96 well multiplate. Aliquots 200µl of DMMB solution (32mg 1,9 DMMB, 20ml ethanol, 59ml 1M sodium hydroxide, 7ml 98 % formic acid and made up to 2 litres with water) was added to the samples and the absorbance at 525nm read immediately (Figure 2.2).

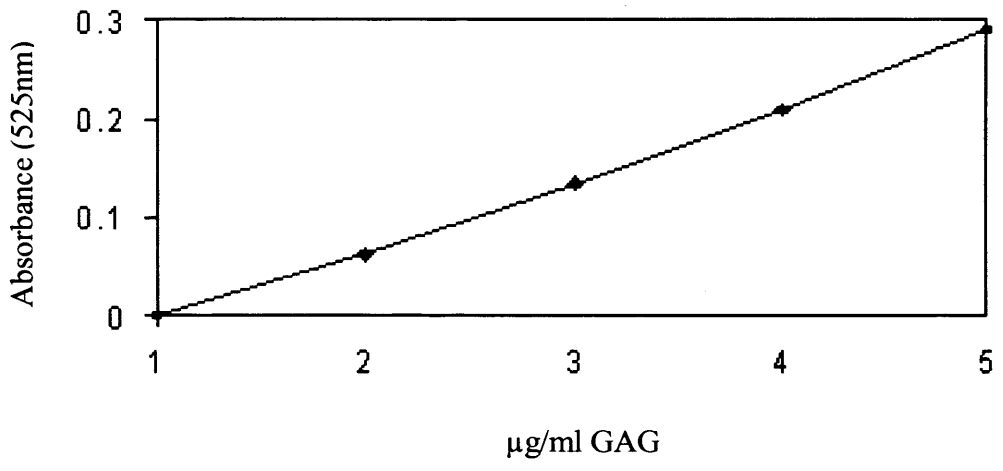


Figure 2.2: Standard curve for chondroitin sulphate C.

2.2.6 RNA extraction and isolation

Total RNA was extracted from cultured cartilage chondrocytes by direct addition of a 1ml aliquot of Tri-reagent. Chloroform (0.2ml/ml) was added to the Tri-reagent, samples were vortexed and left to stand for 15 minutes at room temperature. Samples were centrifuged at 16,000 rpm (11305.2 g min) for 15 minutes and the upper aqueous phase removed and mixed with an equal volume of 70% ethanol. Total RNA was isolated using RNeasy minicolumns and reagents (Qiagen Ltd, Crawley, U.K) according to the manufactures protocol and eluted in sterile water. The technology of the RNeasy columns uses the binding properties of a silica-gel-based membrane with microspin technology. When the sample is applied to the RNeasy

mini spin column, the total RNA binds to the membrane and contaminants are washed away. RNA is finally eluted in 30µl of sterile water. The RNeasy minicolumn procedure will isolate all RNA molecules longer than 200 nucleotides.

The purity of RNA was assessed by calculating the ratio of the absorbances at 260 (A_{260}nm) and 280nm (A_{280}nm). Pure RNA has an A_{260}:A_{280}nm ratio of 1.8-2.0, impurities may either increase or decrease this ratio. To be suitable for RT-PCR, a ratio between 1.8 and 2.2 is required. Outside these values, contamination may interfere in the assessment of the concentration and with the reverse transcription reaction.

2.2.7 Primer design

For construction of cDNA oligonucleotides, a number of primer design criteria were considered. They were: (i) the ideal size should be 20-25 nucleotides long, (ii) melting temperatures should be 50 - 65°C, and (iii) the GC content should be 40-60%. In addition, primers selected for optimal T_m (melting temperature) at the given primer length, were stringently assessed for possible sites of self-complementarity to the second primer (especially 3' complementarity), mainly to reduce the risk of primer-dimer formations. Sequences for genes for which primers were to be designed were found using Genbank. Primers were ordered from Invitrogen, U.K. The primers used are listed in Table 2.2.

2.2.8 Reverse transcriptase-polymerase chain reaction (RT-PCR)

To perform PCR using RNA as a starting template, the RNA has first to be reverse transcribed into cDNA in a reverse transcription (RT) reaction. Reverse transcription –PCR was performed using the RNA PCR core kit (Applied Biosystems). First strand cDNA was synthesized by reverse transcription using MuLV reverse transcriptase. MuLV reverse

Target Template	PCR Primers Sequences (5' -3')	Product size for bovine product (bp)	Annealing temperature (°C)
GAPDH	5'- TGG CAT CGT GGA AGG GCT CAT 5' - ATG GGA GTT GCT GTT GAA GTC	370	50.0
IL-1α	5'- AAGGAGAATGTGGTGATGGTG 5' – CAGAAGAAGAGGAGGTTGGTC	470	53.2
IL-1β	5' - GCTCTCCACCTCCTCTCACAG 5' – TACATTCTTCCCTTCCCTTCT	454	54.5
TNF-α	5' – CTCAAGCCTCAAGTAACAAGC 5' – GCAATGATCCCAAAGTAGACC	454	57.6
COX-1	5' – GCCCAACACTTCACCCATCAG 5' – CCAGGAAGCAGCCCAAACACT	287	59.0
COX-2	5' – GCTCTTCCTCCTGTGCCTGAT 5' – CATGGTTCTTTCCCTTAGTGA	229	52.3
ADAMTS-4	5' – TGGATCCTGAGGAGCCCTG 5' – TGGCGGTCAGCATCATAGTC	151	55.5
ADAMTS-5	5' – GGCCTCTCCCATGAYGATTCC 5' – TGAGCGAGAACACTGGCCCCA	498	59.2
AGGRECAN	5' - CGCTATGACGCCATCTGCTAC 5' – GCCTGCTTGGCCTCCTCAAA	497	57.0
COLLAGEN TYPE II	5' – GAATTCGGTGTGGACATAGG 5' – TACAGAGGTGTTTGACACAG	429	53.0
MMP-3	5' – CTTTTGGCGAAAATCTCTCAG 5' – AAAGAAACCCAAATGCTTCAA	404	50.0
MMP-13	5' – TTCTGGCACACGCTTTTCCTC 5' – GGTTGGGGTCTTCATCTCCTG	273	53.0
TIMP-1	5' – CCACCTTATACCAGCGTTAT 5' – CCTCACAGCCAACAGTGTAGG	282	54.0
TIMP-2	5' – GTGGACTCTGGAAACGACAT 5' – TCTTCTTCTGGGTGGTGCTCA	265	54.0
TIMP-3	5'– GGGAAGAAGCTGGTAAAGGAG 5'– GCCGGATGCAGGCGTAGTGTT	418	54.0

Table 2.2: Oligonucleotide primers used for RT-PCR purchased from Invitrogen, UK. All products gave a single product band of the predicted size whose sequence was verified using an ABI sequencer (see Appendix and section 2.2.9). In one case, a letter is used to specify a mixed base pair (e.g. Y = C and T).

transcriptase is a RNA-dependent DNA polymerase that uses single stranded RNA as a template in the presence of a primer to synthesize a complementary DNA strand. The 20µl RT reaction contained a RNA template which was based on the determination of RNA in the total RNA sample to give 50 ng RNA, oligo dT primer, MuLV reverse transcriptase, dNTPs, 10 x PCR buffer and a reverse inhibitor as shown in Table 2.3. The reaction mixture was incubated for 45 minutes at 42°C and generates a single-stranded DNA molecule complementary to the RNA (cDNA). The reaction was terminated by heating at 94°C for 2 minutes and the mixture could then be stored at -80°C.

Reagents	**Volume**
Template (cDNA)	X (the volume of sample to give 50ng RNA)
10 x PCR buffer	2µl
$MgCl_2$ (25 mM)	1.6µl
dNTPs (2.5mM)	1.6µl
Oligo dT primer	1µl
MuLV reverse transcriptase	1µl
Reverse inhibitor	1µl
Sterile water	11.8µl – X
Total volume	20µl

Table 2.3. RT- reagent mix where X is the appropriate volume of RNA.

The cDNA serves as a template in the PCR reaction. Components of the PCR, in addition to the cDNA, included $MgCl_2$, dNTPs, 10 X PCR buffer, thermostable DNA polymerase (Taq polymerase) and primers specific for the gene of interest (gene specific primers). The cDNA was amplified exponentially via cycles of denaturation, annealing and extension. PCR amplification was performed using oligonucleotide primers corresponding to cDNA sequences as shown in Table 2.2.

Reagents	Volume
10 x PCR buffer	5µl
$MgCl_2$ (50 mM)	3µl
dNTPs (40 mM)	4µl
Taq polymerase (5 units/µl)	0.3µl
Forward primer (10µM)	1µl
Reverse primer (10µM)	1µl
Sterile water	35.7µl
cDNA	1µl
Total	50µl

Table 2.4: PCR reagents for cold start

A master reagent mix was prepared to ensure tube-to-tube consistency in the experiment and reduce enzyme loss on pipette tips. Approximately, 1 µl of cDNA was incorporated into a 50µl PCR mix of primers, buffer, dNTPs and Taq polymerase (Promega Ltd, UK) (Table 2.4). The cycle programme employed a denaturing step at 95°C for 30 seconds to separate the DNA into single strands prior to the cycling stage. The cycle involved an denaturing step of 95°C for 30 seconds, a primer annealing temperature for 45 seconds, an extension step of 72° C for 45 seconds, and a final extension step at 72°C for 5 minutes. The cycle was repeated 35 - 45 times depending upon the length of the expected product. When reactions were complete, 5µl of the PCR product was analysed by electrophoresis using a 2% (w/v) agarose gel.

In those cases where a single product band was not produced, a hot start PCR technique was used to try and produce a single product band. The hot start PCR technique prevents complete mixing of PCR reactants until the reaction has reached a temperature at which non-specific annealing of primers to non-target DNA is minimized and the primer

oligomerization rate is reduced. In a hot start reaction, a solid wax layer is formed over a subset of PCR reactants. The remaining reactants (10x PCR buffer II, Taq polymerase, reverse transcribed sample) were then added above the wax layer (see Table 2.5). In the first thermal cycle, rapid heating to the denaturation temperature (95°C) melts the wax, allowing the layers to mix. Thermal convection suffices to mix completely the united upper and lower aqueous layers, while the melted wax serves as a vapour barrier for the rest of the amplification.

If the above reaction still produced non-specific bands, the annealing temperature was increased in 2°C increments, in order to increase the stringency of primer binding. In cases where there was insufficient product, the concentration of primers and dNTPs were altered over the following ranges to produce the optimum amount of product. The primers were increased by 0.1 μM increments to a maximum of 0.5μM. The dNTPs were increased by 25μM increments up to 125μM. In cases where these approaches still failed to optimise the reaction, then the $MgCl_2$ concentration was increased to optimize conditions for particular primers. The $MgCl_2$ was increased in 0.5mM increments from 1.5mM up to 3.5mM. Once the reaction was complete, the reaction mixture could be temporarily stored at 4°C. Reactions were performed in a programmable thermocycler (Primus 25 He, MWG-Biotech) using the annealing temperature of the primers. Following PCR, the reaction products were size-fractionated by agarose gel electrophoresis followed by visualization under UV light with ethidium bromide staining of the gel. RT-PCR was repeated three times to confirm the results.

Lower PCR Mix	**volume**
10 x PCR buffer	1.25µl
$MgCl_2$ (50 mM)	3µl
dNTPs (40 mM)	4µl
Forward primer (10µM)	1µl
Reverse primers (10µM)	1µl
Water	2.25µl
Total	*12.5µl*
Wax bead	One wax bead per tube
Upper PCR Mix	
10 x PCR buffer	5µl
Taq polymerase	0.3µl
cDNA	1 µl
Water	31.2µl
Total	*37.5µl*

Table 2.5: PCR reagents for hot start

2.2.9 Purification of PCR products and verification of nucleotide sequences

Wizard PCR Preps DNA purification system (Promega, UK) was used to purify the PCR products following the manufacturer's guidelines. The PCR products were then sequenced to check template specificity of each primer by using an Applied Biosystems AB1310 Genetic Analyser. This was followed by the alignment of nucleotide sequences using the MacDNASIS sequence analysis software package (Hitachi Software, San Francisco, USA). This work confirmed that the primers were identifying the correct bands on RT-PCR (see Appendix 1).

2.2.10 Agarose gel electrophoresis

The concentration of agarose in the gels was decided by the size of fragments to be separated. The PCR products were usually run on a 2% agarose gel. The agarose was mixed with 0.5X Tris Borate buffer (TBE) (0.04 M Tris Borate, 0.01 M EDTA pH 8.0) and heated in a microwave on full power for 1 -2 min until the agarose melted and the liquid began to boil. Once the solution had begun to cool, ethidium bromide (1% µg/ml) was added. Ethidium bromide, a fluorescent dye that is used to detect DNA in agarose and polyacrylamide gel, reduces the electrophoretic mobility of linear DNA by about 15%. The gel was allowed to cool to 60°C and poured onto a gel support before it was cast.

The PCR products (5µl) were mixed with 1µl of 6x gel loading buffer (Cambio,UK) (0.05% w/v bromophenol blue, 40% w/v sucrose, 0.1M EDTA pH 8.0, 0.5% w/v SDS) and 5µl samples loaded into the wells of the gel alongside a 50 - 1000bp ladder standard. The sizes of the fragments resolved by electrophoresis were determined by comparison with standard DNA molecular weight markers (PCR marker 50-1000 bp: Cambio, UK). Electrophoresis was carried out in a horizontal gel apparatus at a constant voltage of 120V, for approximately 45 minutes. The running buffer was 0.5X TBE. Following electrophoresis, the DNA was visualized using an ultraviolet transilluminator and photographed using a UVP transilluminator camera (Syngene Ltd, Cambridge, UK).

2.2.11 Statistical analysis.

Statistical evaluation was performed by Students' *T-test* using Microsoft Excel software. Data are expressed as mean ± standard deviation. Each means is from at least three samples. *P* values less than 0.05 were considered to represent statistically-significant differences.

CHAPTER THREE

The effect of fatty acid supplementation on cartilage metabolism by a cell culture system

3.1 Introduction

The aim of these experiments was to determine whether different concentrations of exogenous fatty acids could alter the composition of chondrocyte cell lipids, to discover the optimal concentration for such changes and to ensure that the fatty acids did not affect general cell metabolism and were not toxic to the chondrocytes. Fatty acid supplementations were assessed for altered cartilage metabolism as measured by both the release of degradative products as well as the expression of relevant proteins. The latter was relevant to the general aim of this thesis in determining the possible involvement of lipids during an inflammatory response in a cell culture system which could be a model for arthritis. Most cells in culture can synthesise lipids from glucose and amino acids available in the medium, but when lipids are also present in the medium, it has been shown that *de novo* biosynthesis of fatty acids is inhibited and the cells take up lipids from the medium (Martinez-Cayuela *et al.,* 2000). The effect of the different fatty acids on aggrecan and collagen degradation and the inflammatory response in the chondrocyte cultures was also assessed. However, before the initiation of such detailed studies to investigate the relative efficacy of various fatty acids in changing inflammatory responses and cartilage degradation in chondrocyte cultures, it was also necessary to optimise the conditions for RT-PCR.

3.2 Experimental strategy

As mentioned in the **Introduction**, previous studies have shown that dietary supplementation, with *n-3* polyunsaturated fatty acids (PUFAs) suppress the inflammatory symptoms involved in the pathogenesis of arthritis (see refs. in Curtis *et al.*, 2000, 2002). The aim of this thesis was to confirm these findings using a model experimental system and to investigate the relative efficacy of variety of fatty acids, including those from hydrolysed palm olein, different *n-3* fatty acids and an *n-6* fatty acid, in modifying both inflammatory mediators and degradative responses.

In this chapter, articular chondrocytes from 7 day-old calf metacarpo- and metatarsophalangeal joints were isolated using the previously described (Section 2.2.2) and maintained as monolayer cultures. Cultures were then pre-treated with varying doses of *n-3* fatty acids; EPA, DHA, ALA, complexed to fatty acid-free bovine serum albumin in Tyrode-HEPES buffer, for a period of 8 hours after which cultures were rinsed free of exogenous fats and treated with or without IL-1α (10ng/ml) for 72 hours. Fatty acid complexed to bovine serum albumin was at a concentration range of 25-300 μg/ml. IL-1α was used to stimulate inflammatory and degradative responses in the cultures. Hydrolysed palm oil fractions (Section 2.2.1) were also complexed to BSA. Media were collected and the glycosaminoglycan (GAG) proteoglycan content in the medium was measured using 1,9-dimethylmethylene blue (DMMB) as previously described in Section 2.2.5 (Figure 3.1).

Total RNA was extracted from cultured chondrocytes as previously described (Section 2.2.6) using RNeasy mini columns and reagents. RT-PCR was used to reveal mRNA levels for collagen and aggrecan. The reverse transcription-polymerase chain reaction is a powerful tool for the determination of mRNA levels in cultured cells. Using this approach, reverse transcription (RT) produces the cDNA (complementary DNA) and is subjected to a

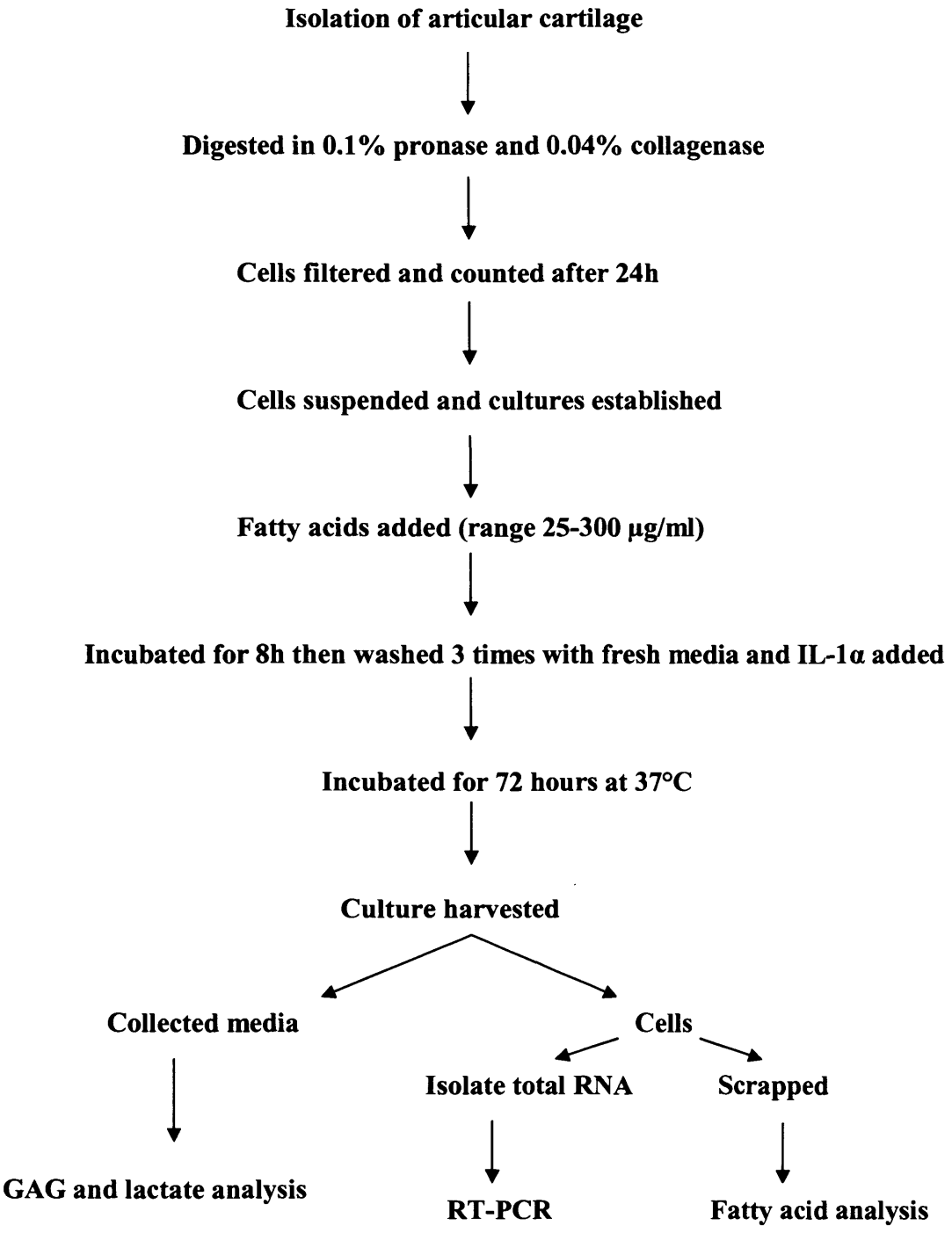
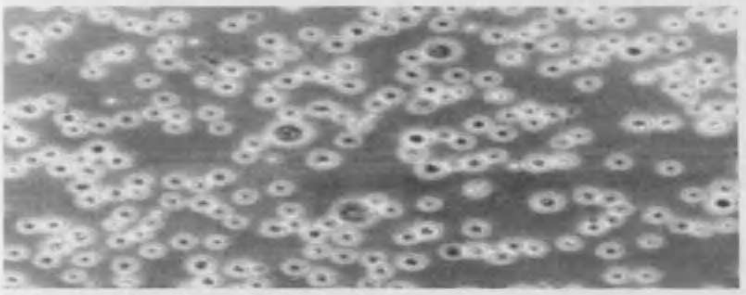


Figure 3.1: Flow chart showing the experimental strategy used for the various techniques applied in the model cartilage cell culture systems.

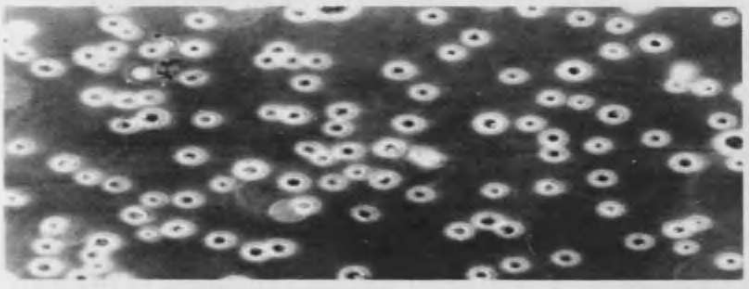
polymerase chain reaction (PCR) to amplify the cDNA in these samples. Subsequently, the internal control or housekeeping genes like GAPDH were used to normalise sample data. During the study, the composition of various fatty acids added to the monolayer cultures and the cell contents after treatment were analysed using the gas-liquid chromatography as previously described (Section 2.2.4).

3.3 Morphology of the cells

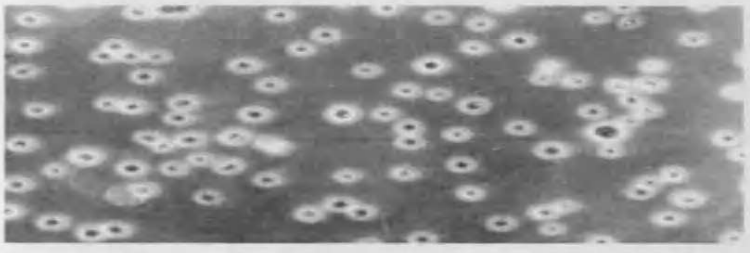
A fine balance between the continuous processes of degradation and synthesis of extra cellular matrix components is needed for the integrity of normal cartilage. However, in arthritis, this dynamic equilibrium is broken and catabolic processes become prevalent, resulting in cartilage destruction (Cimpean *et al.,* 2000). One of the factors is the presence of cytokines. IL-1α is considered an important cytokine involved in the degradation of cartilaginous extracellular matrix components. Using monolayer cultures of bovine chondrocytes, we studied the ability of IL-1α to affect cell morphology with and without fatty acids, during 4 days incubation. To initiate differentiation, the monolayer chondrocyte cultures were exposed to 10ng IL-1α with or without EPA supplements of 100 μg/ml and 300 μg/ml. The morphology of the cells were then examined at day 0 and days 1 to 4 following the initiation of the differentiation programme. The results for days 0 - 4 are shown in Figures 3.2 – 3.5. As shown in Figure 3.2, when the chondrocytes were isolated from OA cartilage and plated at high density ($1x10^6$), the typical spherical morphology were observed. This remained the same after 24 hours of culture and over the next 4 days. At day 4, the cells were actively dividing and they began to lose their spherical morphological shape and became more stretched out and flattened.



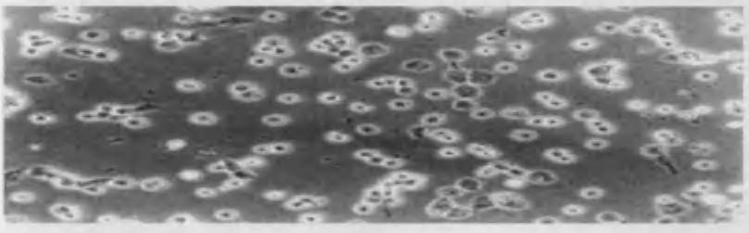
Control - day 0



Control - day 1



Control - day 2



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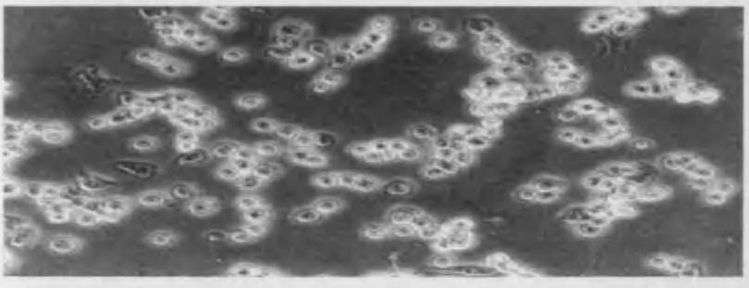


Control - day 4

Figure 3.2: Morphology of control chondrocytes cultured for 4 days. Cells were observed using light microscopy on the days indicated and photographed.

Some of the cells were hemispherical in pairs following cell divisions. The cell density is lower than that displayed by the corresponding growing chondrocytes on the first day.

The effect of IL-1α was determined by culturing the cartilage cells in the monolayer plate in the presence of 10 ng/ml IL-α for 4 days (Figure 3.3). This treatment changed the cell shape within 24 hours. Monolayer chondrocytes treated with IL-1α were heterogenous and their cell shapes becoming spindle-like sharp or stellate following disruption to their matrix substrate. This variation in cellular response to IL-1α may result from the heterogeneity reported among articular chondrocytes (Aydellotte *et al.,* 1992). By day 2, some cell debris can be seen which accumulated and increased after day 3. After incubation for 4 days, cell deaths were easily observed, due to degradation of extracellular matrix components as a result of IL-1α treatment. A significant proportion of the chondrocytes underwent modulation to a stellate morphology. It was an evident that IL-1α kills cells and disrupts the cartilaginous extracellular matrix components.

Following *n-3* PUFA treatment with EPA, monolayer chondrocytes displayed important morphological alterations compared to untreated cells (Figure 3.4 – 3.5). They looked healthier, some were stellate-shaped, with long cell processes, and well attached to each other. This may be explained by cell activation induced by the exogenous fatty acids. By day 4, some cell debris was start found in the medium with 100 (Figure 3.4) and 300 μg/ml (Figure 3.5) EPA supplementation stimulated with IL-1α. However, less cell deaths were observed in 300 μg/ml EPA supplementation compared with 100 μg/ml EPA supplementation and cell densities were comparable to control cultures, even at 4 days. With EPA, less cell death occurred than for IL-1 treatment alone. This confirmed that fatty acid supplementation was able to protect the extracellular matrix components from the effect of IL-1α.

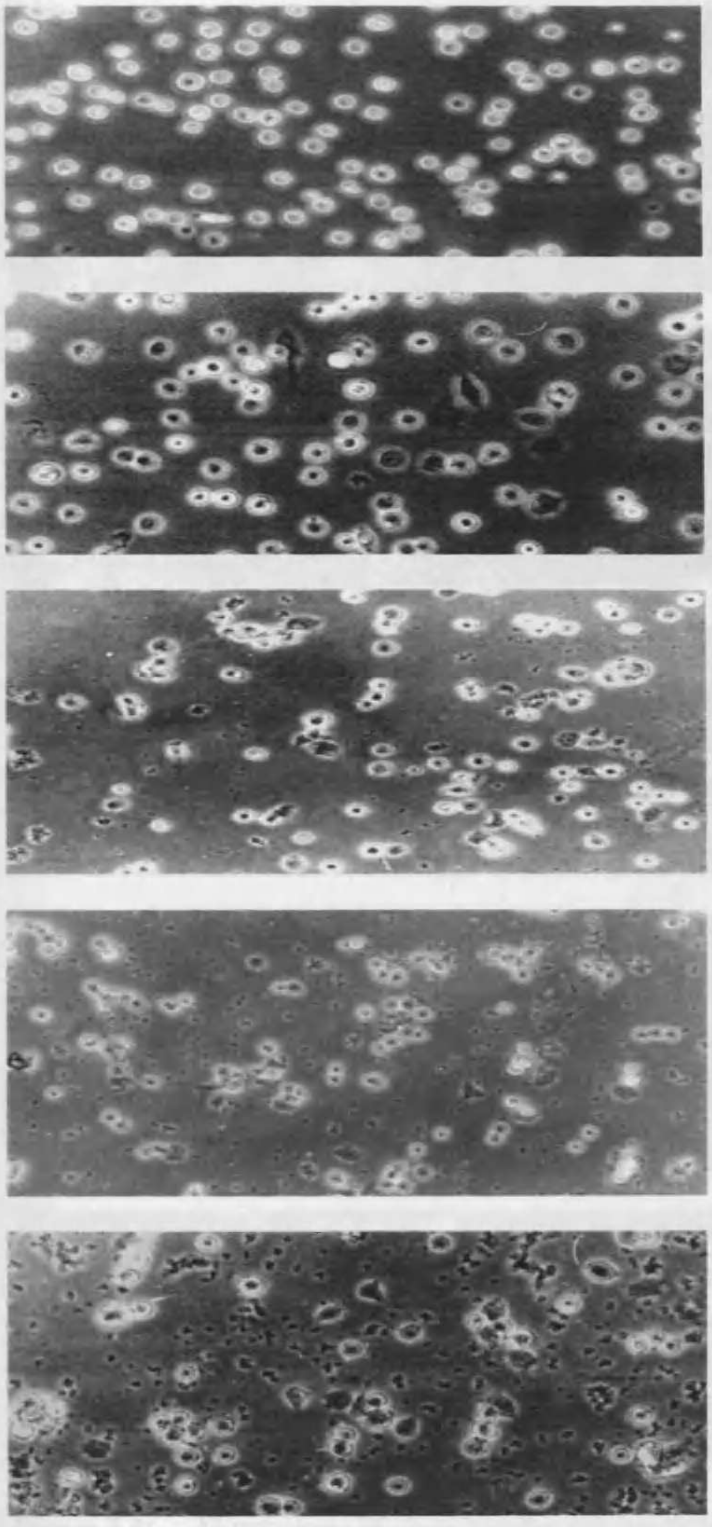
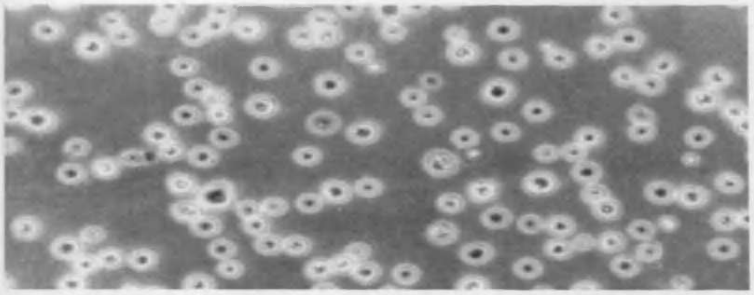
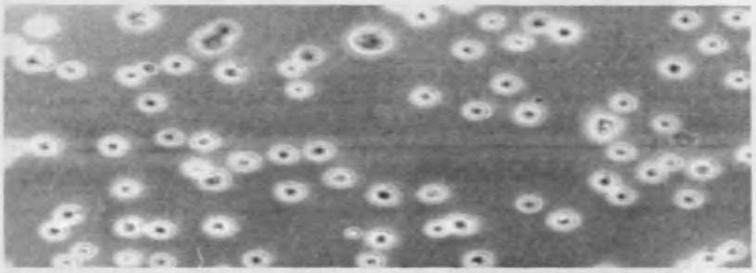


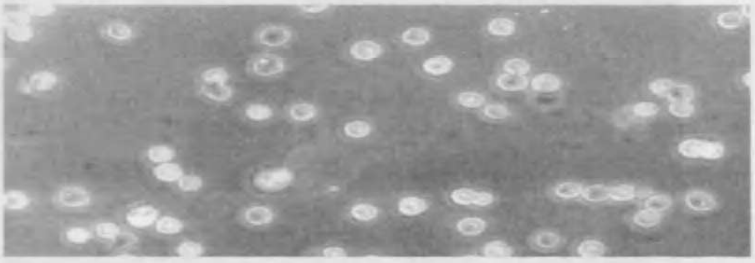
Figure 3.3: Morphology of chondrocytes cultured in the presence of IL-1α for up to 4 days. Cells were observed using light microscopy on the days indicated and photographed.



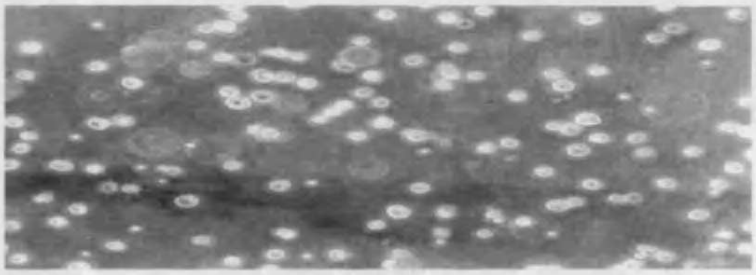
Control – 0 day



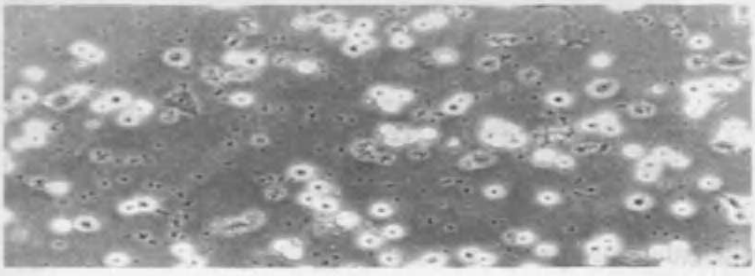
EPA (100) + IL-1 – 1 day



EPA (100) + IL-1 – 2 day



EPA (100) + IL-1 – 3 day



EPA (100) + IL-1 – 4 day

Figure 3.4: Morphology of chondrocytes pre-incubated with 100 μg/ml EPA and then incubated with IL-1α for 4 days. Cells were observed using light microscopy on the days indicated and photographed.

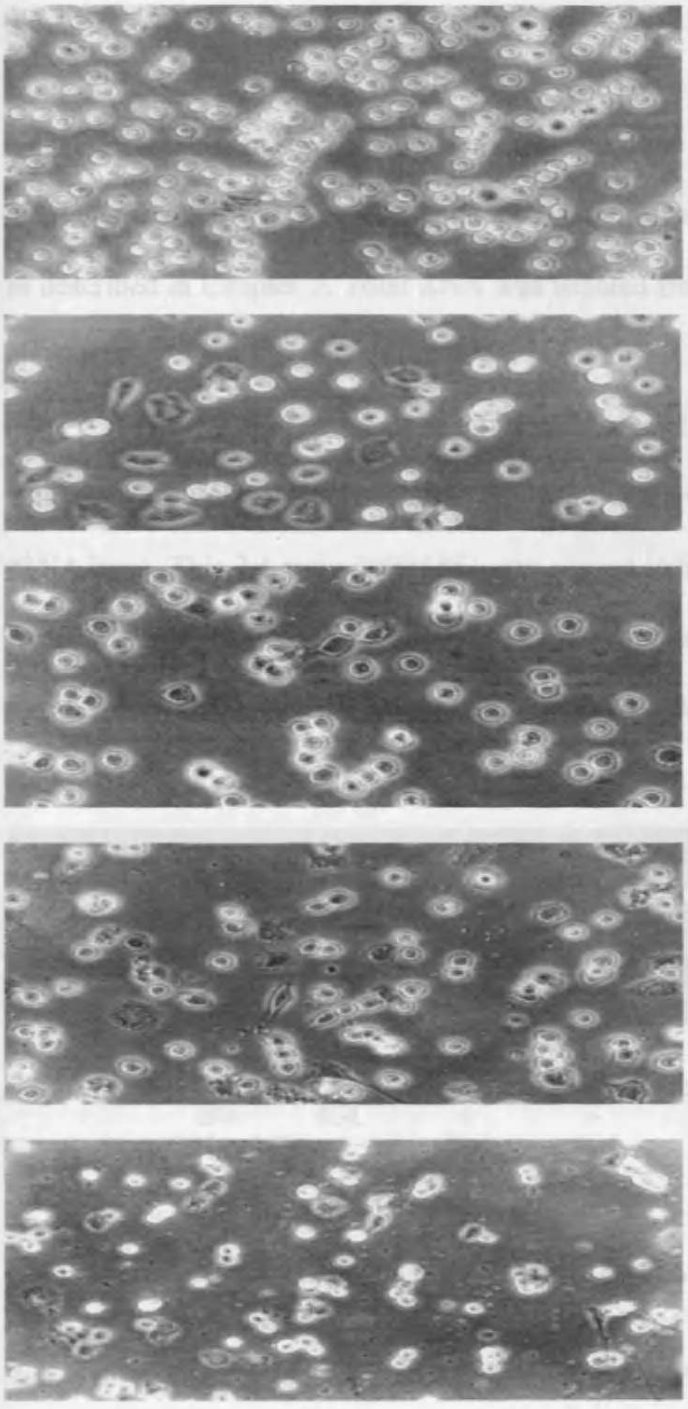


Figure 3.5: Morphology of chondrocytes pre-incubated with 300 μg/ml EPA and then cultured with IL-1α for 4 days. Cells were observed using light microscopy on the days indicated and photographed.

3.4 Isolation of total RNA from cells

Regardless of the downstream application, the integrity of isolated RNA was checked before gene expression analysis. Total RNA contents were determined using UV_{260} absorbance as described in Chapter 2. Total RNA was isolated from each sample using a Qiagen RNeasy kit (Section 2.2.6). Isolated total RNA was visualized on a 1.5% agarose gel (Figure 3.6) and clear 28S and 18S rRNA bands were seen. The quality of RNA was judged by the ratio of 28S:18S ribosomal RNA. The 28S rRNA band was approximately twice as intense as the 18S rRNA band. This 2:1 ratio (28S:18S) was a good indication that the RNA was largely intact. Partially-degraded RNA would have a smeared appearance, lack the sharp rRNA bands, and /or would not exhibit the 2:1 ratio of high quality RNA.

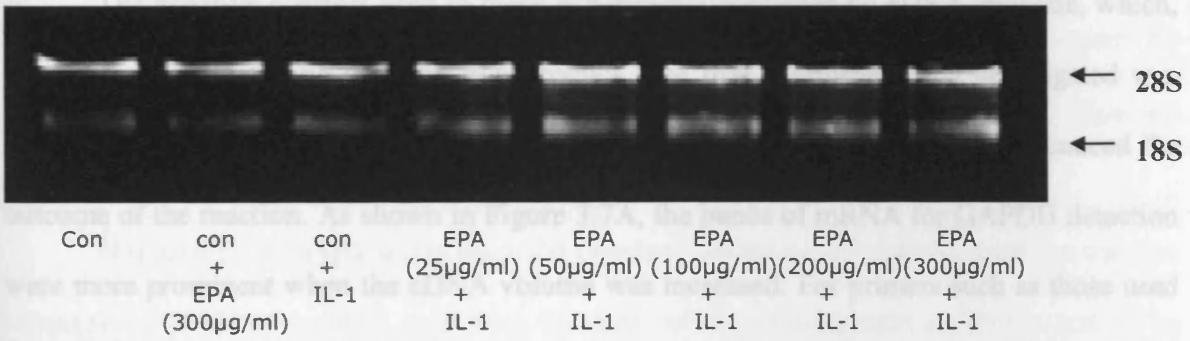


Figure 3.6: Representative samples of total RNA isolated from monolayer chondrocyte cultures treated with different concentrations of EPA, followed by IL-1α addition. Approximately 1 µg of each sample was run on a 1.5% agarose gel and stained with ethidium bromide. The 28S and 18S ribosomal RNA bands are clearly visible in the RNA samples.

3.5 RT-PCR analysis –optimisation of PCR conditions

RT-PCR was employed initially to examine the levels of chondrocyte mRNAs in cultured cells in response to pro-inflammatory stimulation and/or after fatty acid exposure. Prior to a full RT-PCR analysis, optimisation experiments were carried out with primers chosen as described in Section 2.2.8. The most important parameters that are known to influence reaction specificity are the annealing temperature, the cycling regime and the buffer composition. This section describes various modifications made to the RT and PCR steps, for mRNA detection and quantification. In the cell, DNA is copied to form mRNA, which in turn, is used by ribosomes to make protein. For RT-PCR, mRNA is used to produce cDNA (complementary DNA) by reverse transcription (RT) and then the specific cDNA is amplified by the polymerase chain reaction (PCR).

The negative controls used in these experiments contained no cDNA template, which, as expected, produced no amplification product. The first parameter to be investigated was cDNA concentration. Results show that the amount of DNA template strongly influenced the outcome of the reaction. As shown in Figure 3.7A, the bands of mRNA for GAPDH detection were more prominent when the cDNA volume was increased. For primers such as those used for ADAMTS-4 and ADAMTS-5 where their mRNAs have a low copy numbers, higher amounts of cDNA were used in the PCR.

Adjusting the annealing temperature can alter the specificity of pairing between template and primer. The generic protocol, for the PCR reactions described in Chapter 2, did not suit all the primers for the different mRNAs studied, and the reactions had, therefore, to be optimised for each mRNA. Gradient PCR was used to determine the optimum annealing temperature. The annealing temperature was usually increased by 0.5°C or 1°C increments in

order to increase the stringency of primer binding. The GAPDH and ADAMTS-4 primers used in this work showed clear results when the temperature was decreased from the manufacture's recommended temperatures of 50 to 51°C and 55.5 to 56.5°C, respectively, as shown in Figure 3.7 (B) and 3.7 (C).

$MgCl_2$ amount was also changed. Altering the amount or concentration of $MgCl_2$ may lead to one primer/template pair behaving significantly differently from another under otherwise identical conditions. Mg^{++} affects the annealing of the oligo to the template DNA by stabilising the oligo-template interaction and it also stabilises the replication complex of polymerase with template-primer. The optimal conditions identified by these experiments were 3µl of 25mM $MgCl_2$ with 4µl of 2.5 mM dNTP for 50ng RT samples (Figure 3.8A).

Extension time or cycles also play an important role in adjusting the outcome of the PCR reaction. The cDNA templates of GAPDH were amplified using PCR programs with 20, 30, 40, 44 and 45 cycles per program. Approximately, 40 cycles were adequate for GAPDH (Figure 3.8B) but for COX-2 (Figure 3.8C), ADAMTS-4 or ADAMTS-5 (data not shown), about 45 cycles were needed to visualize bands adequately on the gel.

Hot start PCR, which relies upon the physical separation of reagents until the reaction temperature has been reached, was used for some of the primers such as TNF-α and IL-1α where the reactions proved difficult to manage successfully. However, this technique was not suitable for processing large numbers of samples due to the time involved in making additions to individual tubes; the 'loss-of – concentration' phenomenon leading to failure to add enzyme to one or more tubes and the opportunity for contamination due to the need to open the tubes (McPherson *et al.,* 2000).

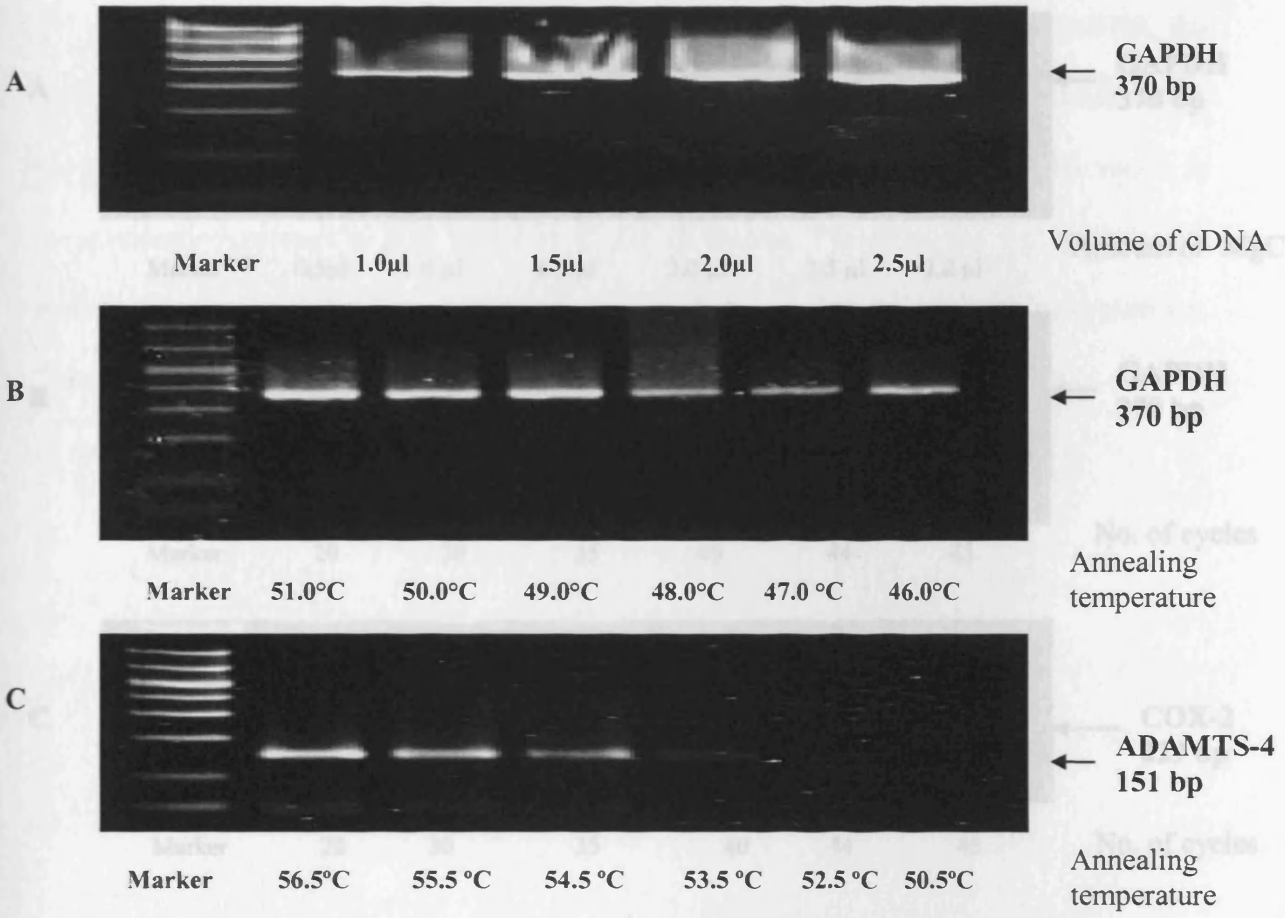


Figure 3.7: Optimisation of PCR reactions. The RT-PCR reactions for GAPDH and ADAMTS-4 were studied by: **A) Increasing the amount cDNA template**. **B) and C) Optimisation of temperature.** The PCR reaction was carried out with GAPDH and ADAMTS-4 primers using different annealing temperatures. Samples were separated on a 2% agarose gel, alongside a 50-1000 bp markers.

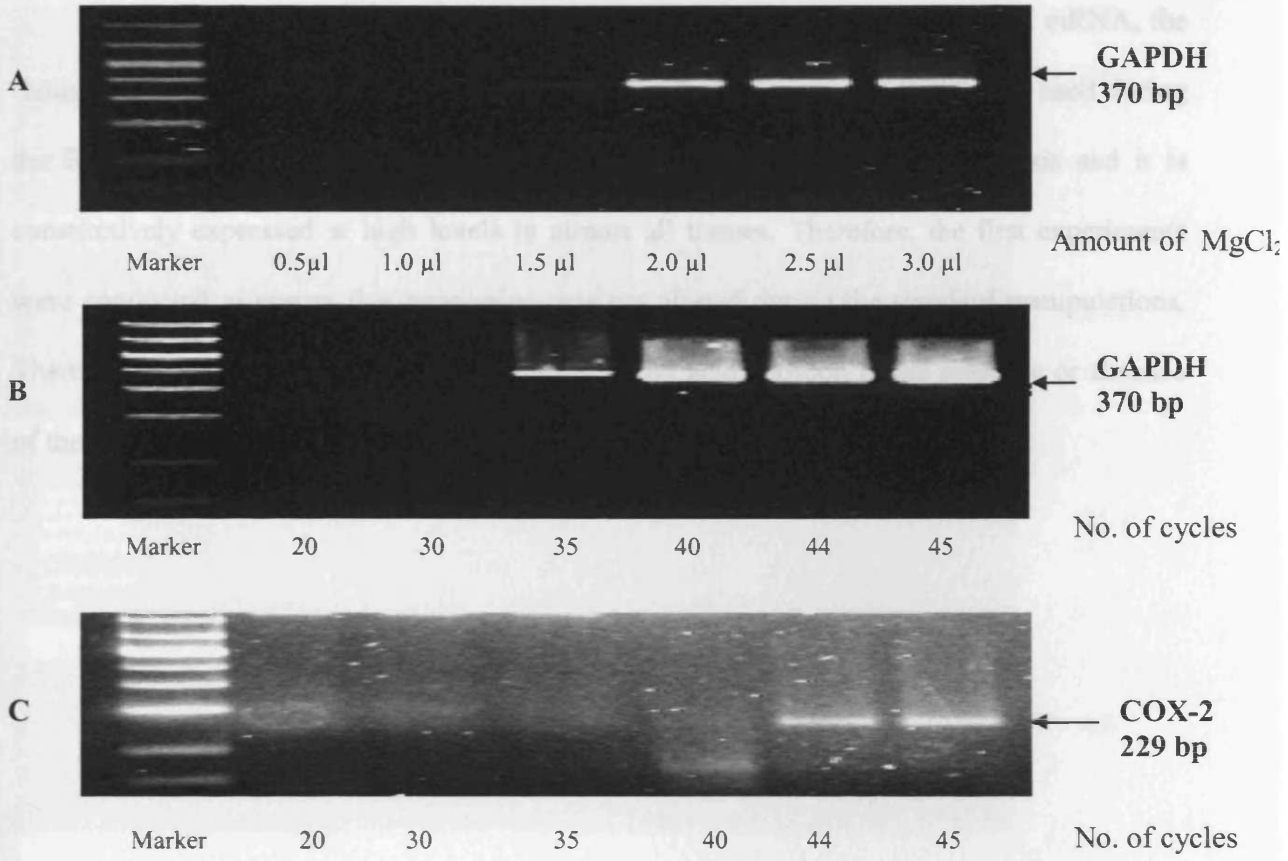


Figure 3.8: Optimisation of PCR reactions. A) Optimisation of 25 mM $MgCl_2$ volume. RT-PCR using the template cDNA from RNA of monolayer chondrocyte cell was optimised by changing the $MgCl_2$ (25 mM) volume used. The PCR reaction was initially carried out with GAPDH primer using an annealing temperature of 51.0°C. **B) and C) Optimisation of PCR cycles.** RT-PCR using template cDNA from RNA of monolayer chondrocyte cells was optimised by changing the PCR cycles used. The PCR reaction was initially carried out with GAPDH and COX-2 primers. The samples were separated on a 2% agarose gel, alongside a 50-1000 bp marker.

To ensure that all the RT-PCR reactions contained an equivalent content of mRNA, the 'housekeeping' gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used during the RT-PCR experiments. GAPDH is a catalytic enzyme involved in glycolysis and it is constitutively expressed at high levels in almost all tissues. Therefore, the first experiments were conducted to ensure that expression was not altered during the standard manipulations. There was no evidence that this was altered by fatty acid addition in the presence or absence of the inflammatory cytokine, IL-1α (Figure 3.9 and 3.10).

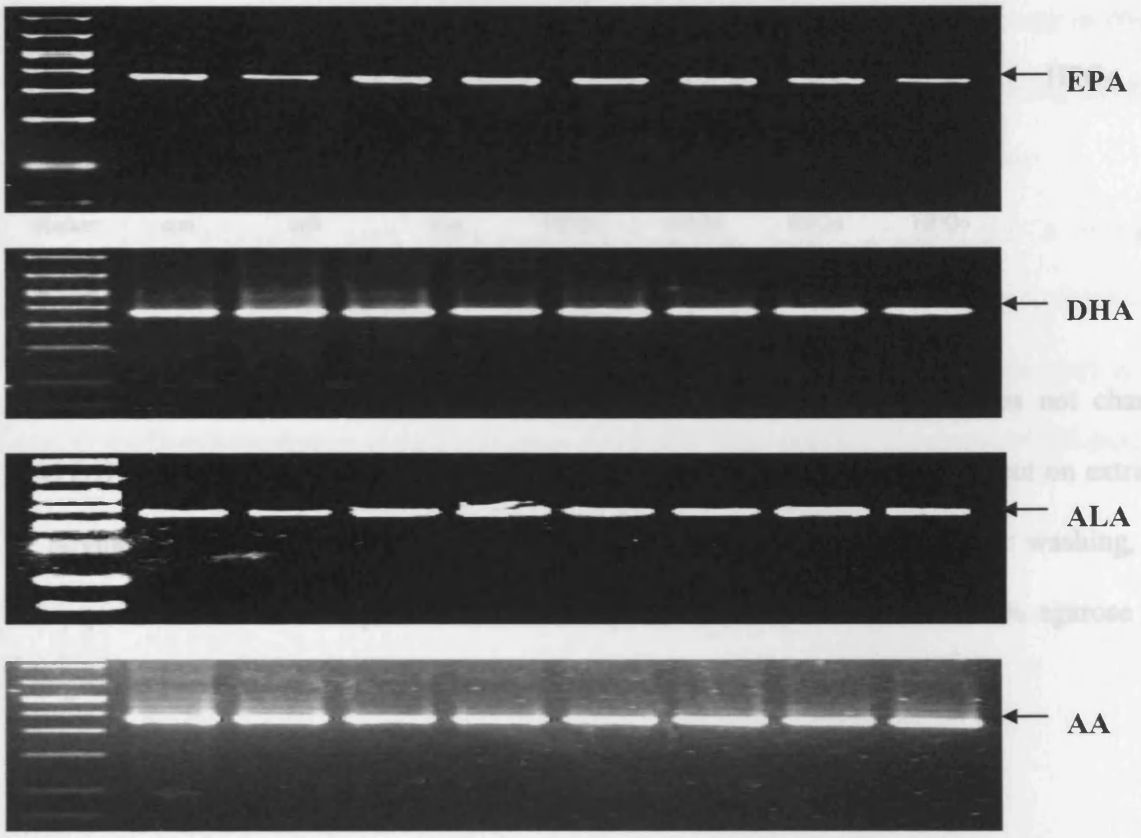


Figure 3.9: Fatty acid exposure does not change GAPDH mRNA levels. RT-PCR using GAPDH primers (370 bp) was carried out on extracts of bovine monolayer cultures treated with different fatty acids (FA), EPA, DHA, ALA and AA for 8 hours followed, after washing, by subsequent culture for 4 days with IL-1α (10ng/ml). Separations were on a 2% agarose gel alongside a 50-1000 bp marker.

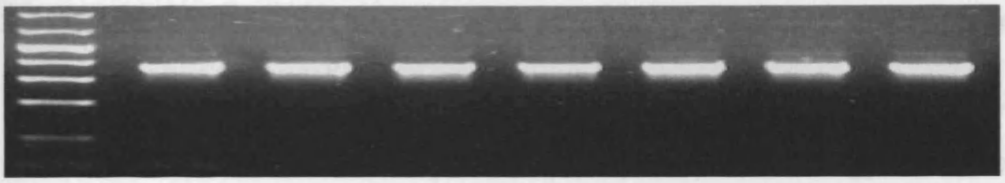


Figure 3.10: Exposure of cultures for hydrolysed palm olein (HPOo) does not change GAPDH mRNA levels. RT-PCR using GAPDH primers (370 bp) was carried out on extracts of bovine monolayer cultures treated with HPOo for 8 hours followed, after washing, by subsequent culture for 4 days with IL-1α (10ng/ml). Separations were on a 2% agarose gel alongside a 50-1000 bp marker.

3.6 Fatty acid incorporation into chondrocyte lipids

Cell cultures have been commonly used to analyse various characteristics of lipid metabolism since they are able to take up and utilize lipids supplied to them in the medium. Fatty acids are one example of exogenous lipids which have been extensively used. The fatty acids can be taken up and incorporated into membrane lipids and/or used as eicosanoid precursors.

In the present work, the lipid composition of chondrocyte cell cultures were modified by adding the fatty acids; EPA, DHA, ALA, AA or hydrolysed palm olein (HPOo) to the culture. Cell lipids were analysed by gas liquid chromatoraphy (GLC) to follow uptake. Even though previous experiments had shown that *n-3* PUFA suppressed the production of cytokines in the samples (Curtis *et al.,* 2000, Hurst *et al.,* 2002), the underlying mechanisms

for this and any variations between acids were poorly understood. It was necessary to obtain information on the amount of uptake of various fatty acids into the cells by analysing the fatty acid content of 'control' chondrocytes, before and after fatty acid supplementation.

PUFA standards were used to identify peaks in the samples and a standard chromatogram is shown in Figure 3.11. The individual fatty acids used for supplementations are shown in Figure 3.12. Chromatograms of the different individual fatty acids used in the culture medium experiments confirmed that, as stated by the supplier, they were >99% pure.

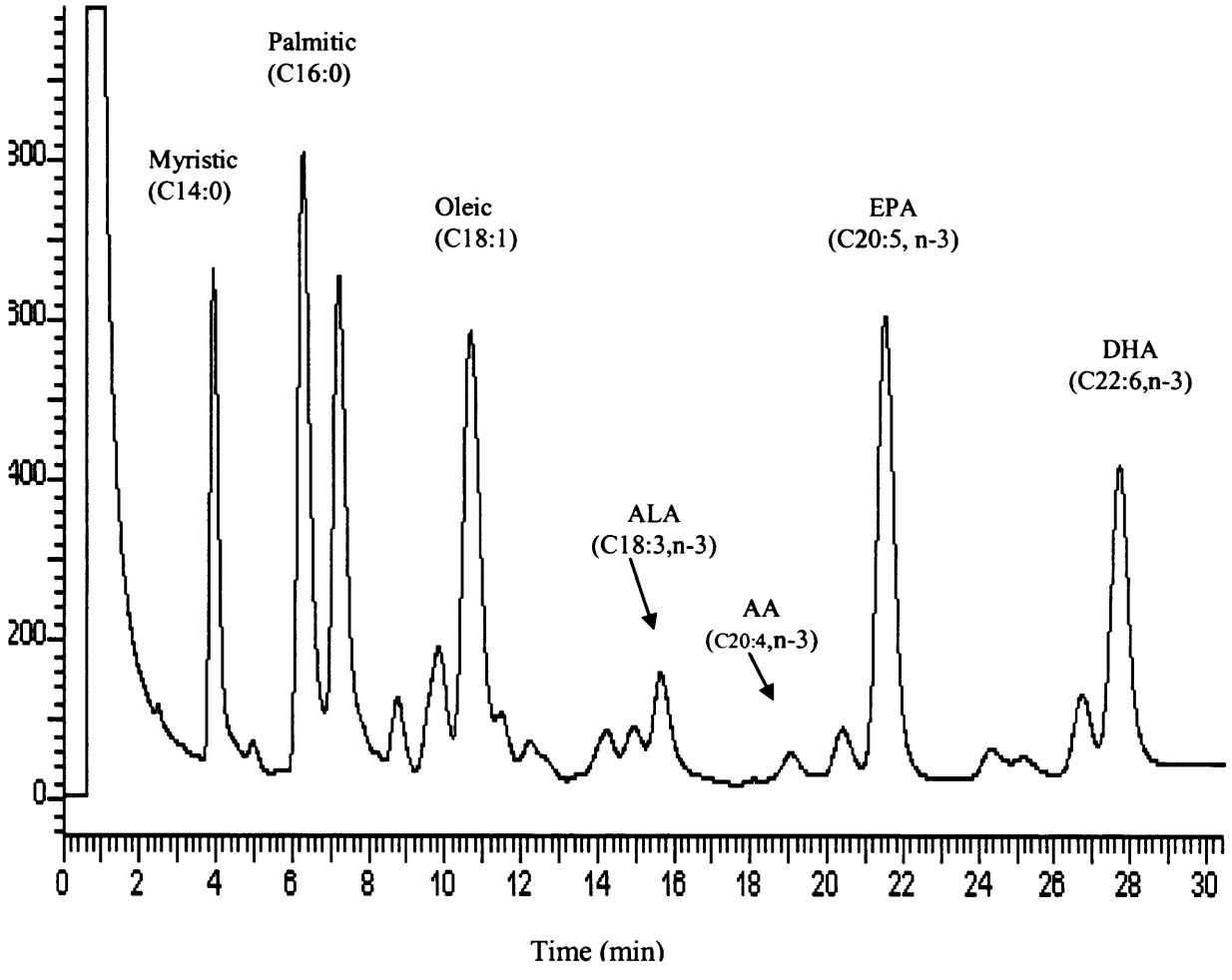


Figure 3.11: The chromatogram of the standards used in the experiments. Relevant fatty acid components are marked.

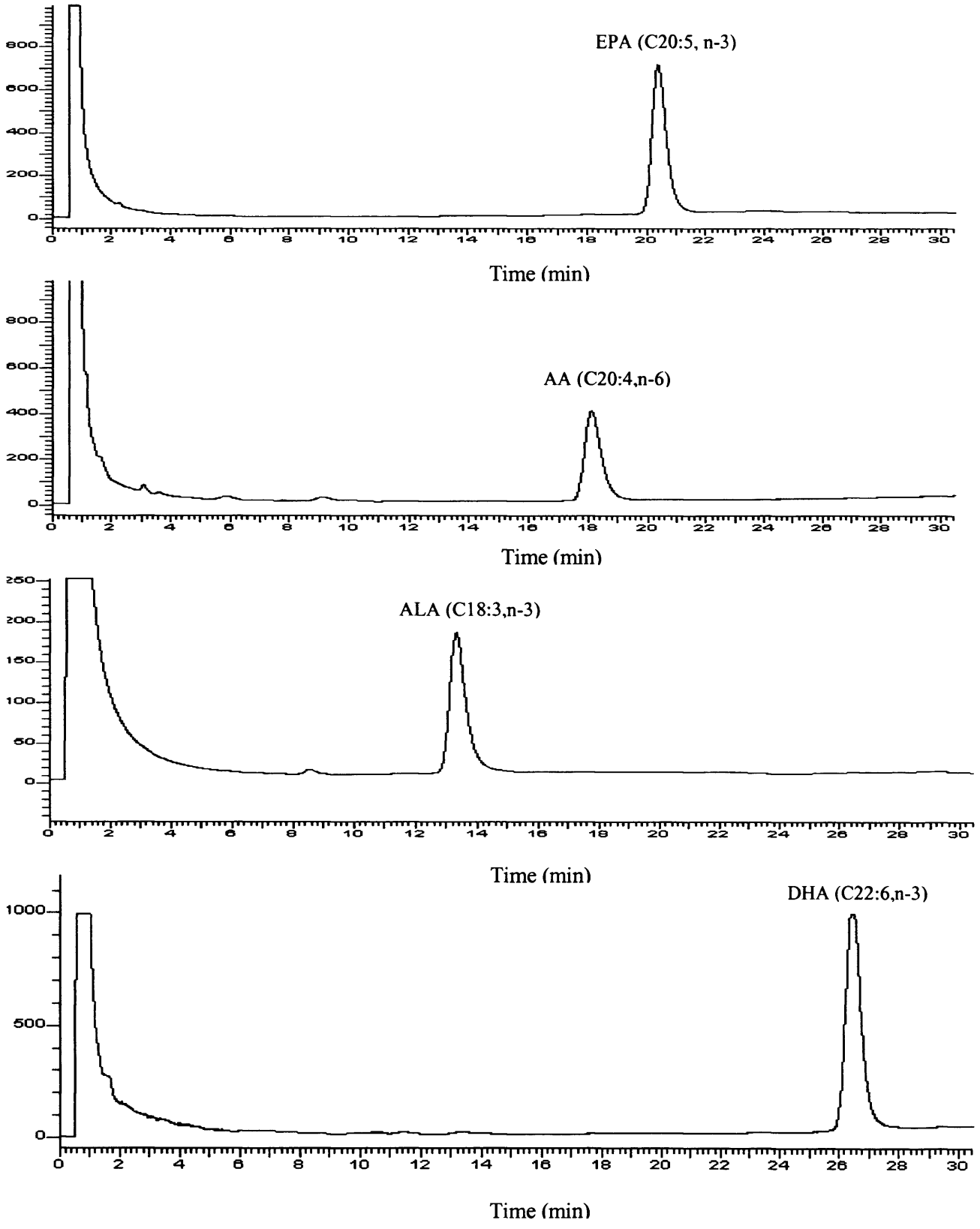
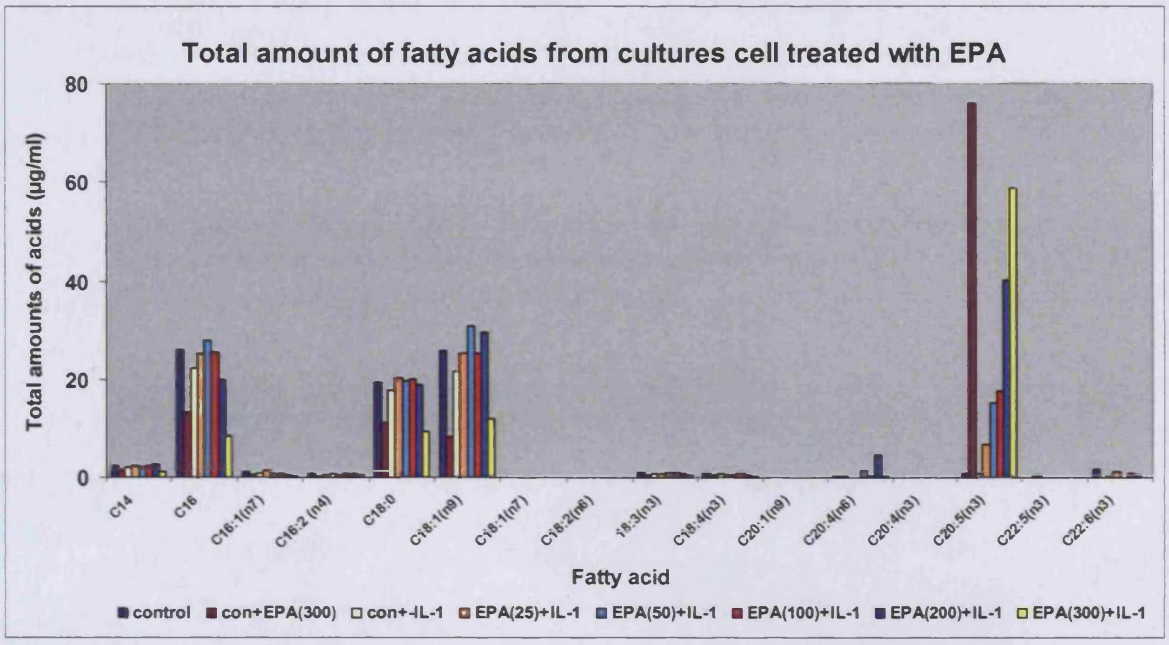


Figure 3.12: Chromatograms of the individual fatty acids used for supplementation.

It was also necessary to find out what the fatty acid composition of the cell culture was, in order to check the composition of the fetal bovine serum used. The control culture cell contained C18:1n-9 (34.0%) and C16:0 (19.7%) as the main fatty acids, followed by C18:0 (14.5%) and a lower percentage of C14:0. PUFAs were only minor components. The fatty acid content was quantified by reference to a C15:0 standard (data not shown).

In order to check that uptake was significant for all fatty acids used and, also, to see that this was proportional to the amount of exogenous acid used, several preliminary experiments were carried out. The uptake of a given fatty acid into total cell lipids was investigated when chondrocytes were cultured with different amounts of fatty acids for 8 hours as shown in Figures 3.13 – 3.20. Analysis of total lipids from the cultured chondrocytes supplemented with the different PUFAs showed the effective uptake of the individual exogenous fatty acids added.

The percentage of EPA in cultured cells clearly increased when the culture medium were supplemented with this fatty acid (Figure 3.13). Chondrocyte cell cultures were treated with or without 25 - 300 μg/ml EPA. The percentage of EPA increased from a base line level of 2.1% in non-supplemented chondroyctes to 67.4% in chondrocytes supplemented with the highest amount (300μg/ml) of the fatty acid. Figure 3.14 shows that the amounts of EPA taken up by the cells was approximately in proportion to the amount of EPA supplied. Furthermore, it also shows that subsequent treatment of the cell with IL-1α did not alter, to any significant extent, the total amount of EPA that had been previously taken up. In addition, IL-1α treatment alone did not alter the chondrocyte fatty acid composition.



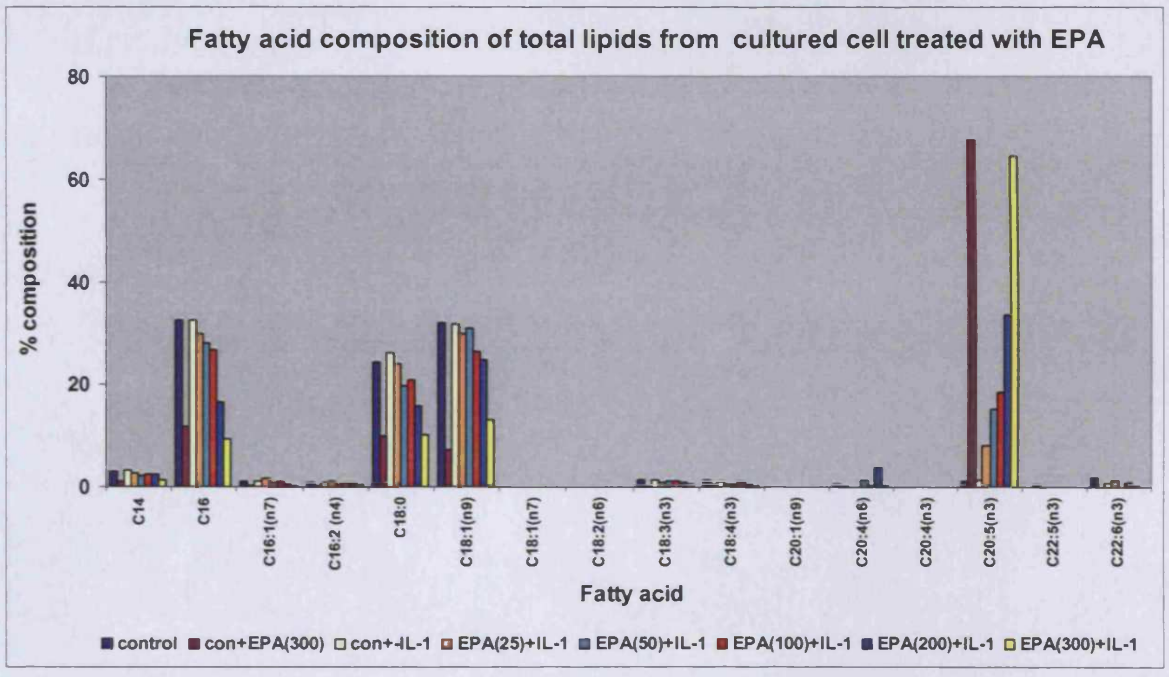


Figure 3.13: Effects of EPA on the total amounts of fatty acids and the percentages of fatty acid composition in total lipids from the chondrocyte cells in culture.

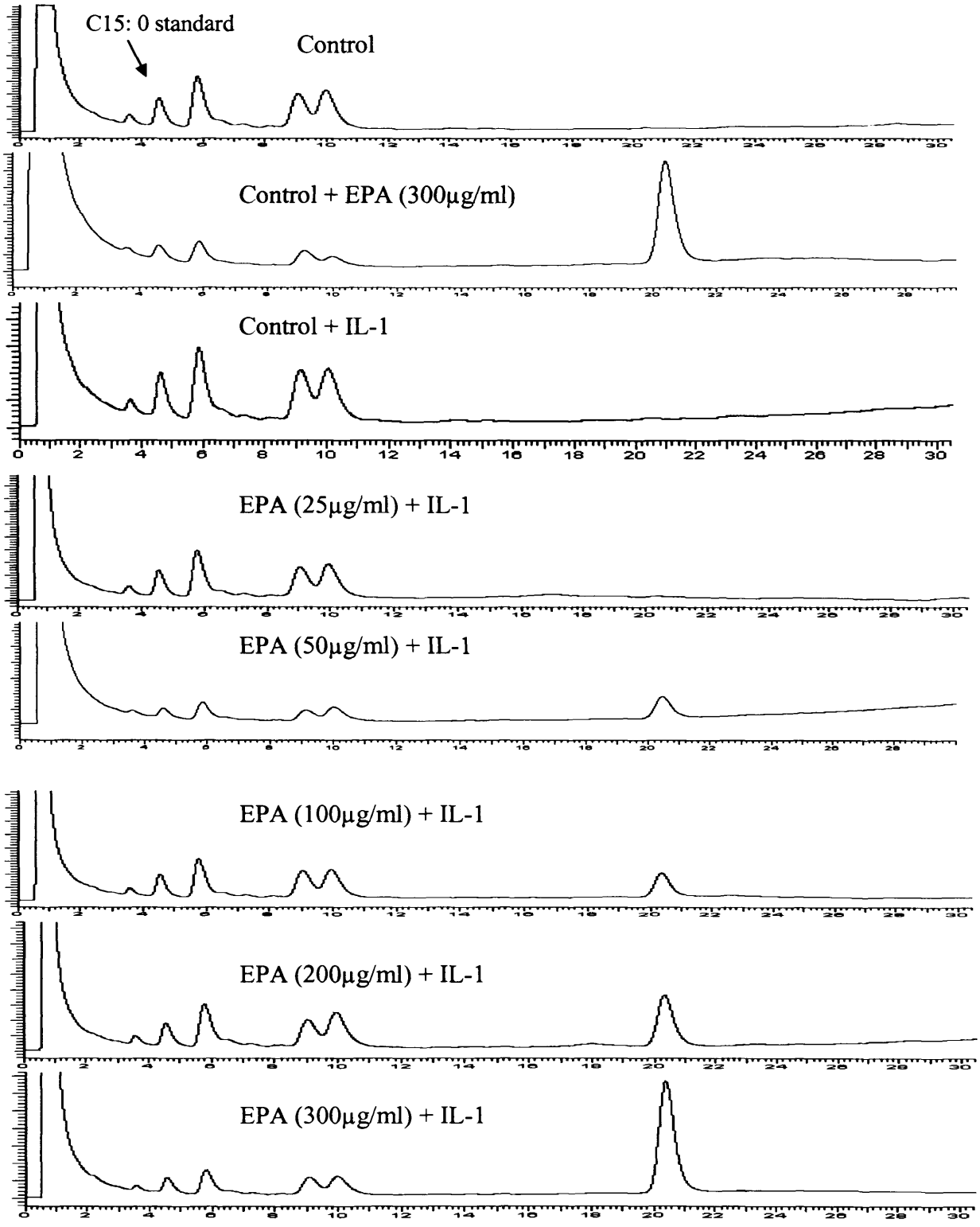


Figure 3.14: Gas chromatograms for FAMEs of chondrocyte cell lipids from cultures treated as above with or without the *n-3* PUFA, EPA.

Similar analyses of cultures following supplementations with the other n-3 PUFAs, α-linolenate and DHA, gave equivalent results. Progressive uptake of the fatty acids in proportion to the amounts added were found (Figure 3.15 – 3.20) and the total amount accumulated at 300μg/ml was not altered significantly by IL-1α treatment.

For AA supplementation, there seemed to be some variability in the total uptake. Thus, although the amount of AA accumulated after 300 μg/ml exposure was similar with or without IL-1α challenge, the accumulation of AA was not clearly proportional to exposure for example, the percentage of AA in the total fatty acids did not increase following 100 – 300 μg/ml addition to the medium.

Nevertheless, the overall conclusion of the above experiments is that exposure of the cell cultures to exogenous fatty acids for 8h was sufficient for adequate uptake. Furthermore, the uptake was broadly in proportion to the amount of fatty acid supplied. Thus, higher doses of added fatty acids in the medium resulted in higher total amounts taken up and, hence, those available for metabolism and /or action on gene expression etc.

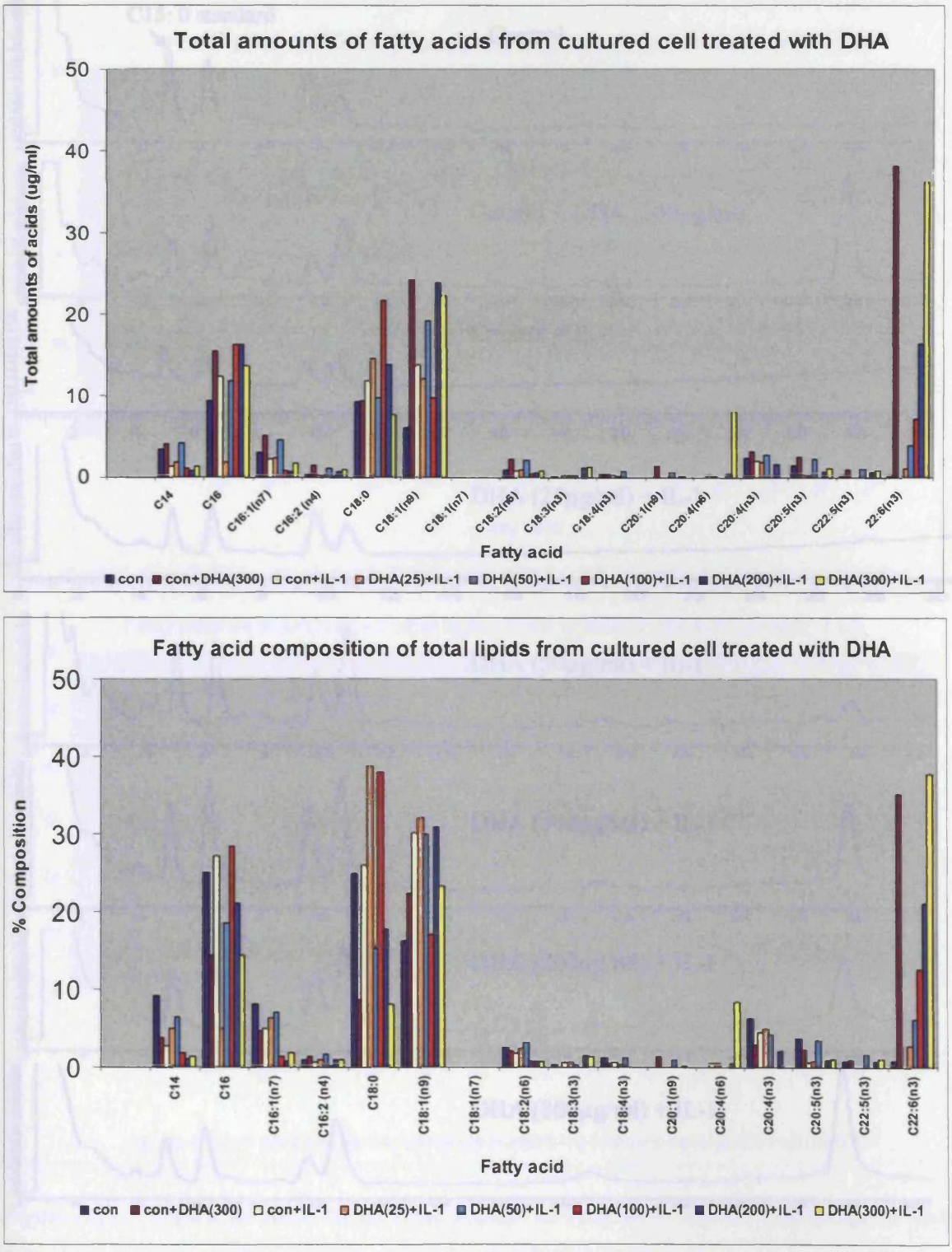


Figure 3.15: Effects of DHA on total amounts of fatty acids and the percentage of fatty acid composition in total lipids from chondrocyte cells in culture.

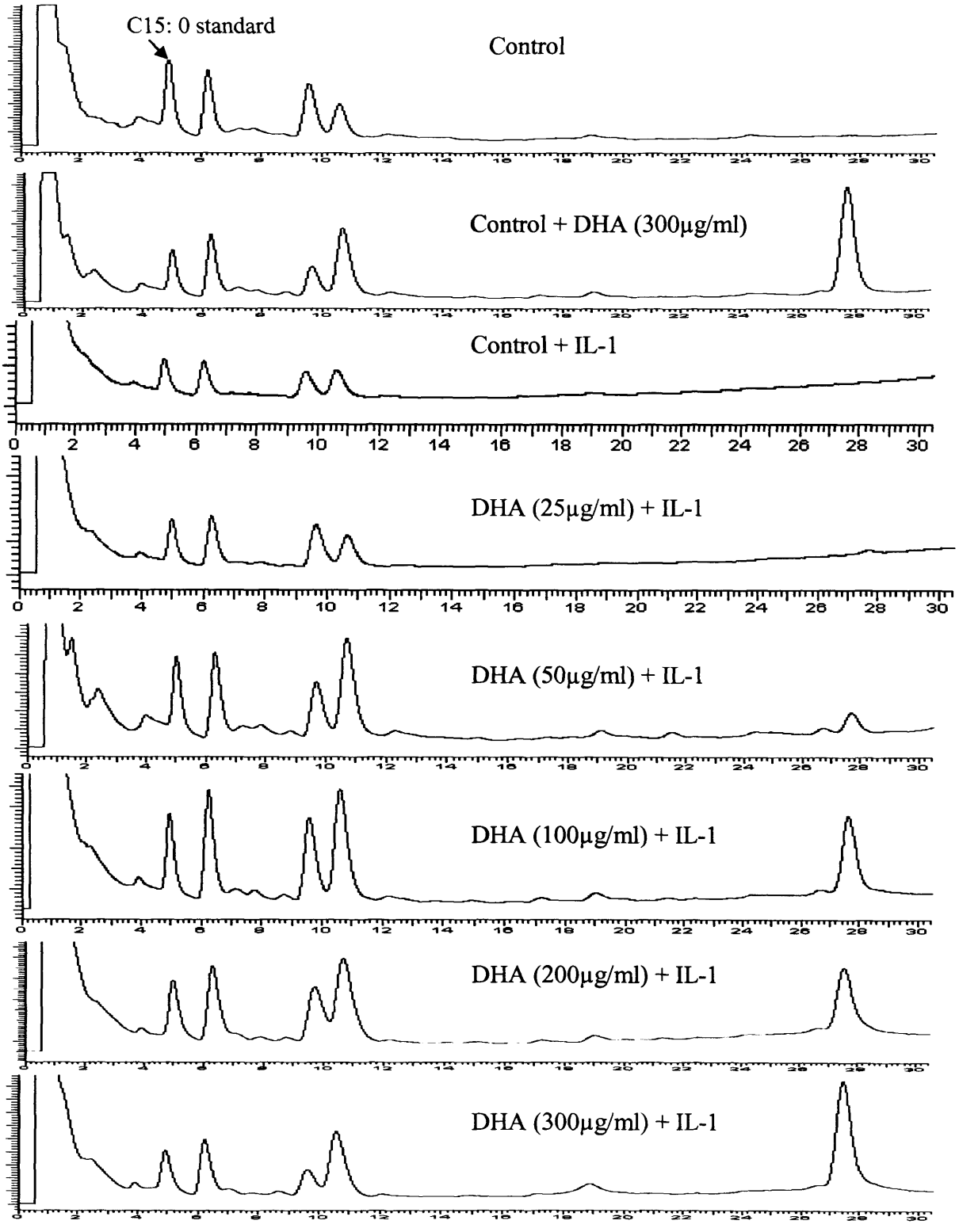


Figure 3.16: Gas chromatograms for FAMEs of chondrocyte cell lipids from cultures treated as above with or without the *n-3* PUFA, DHA.

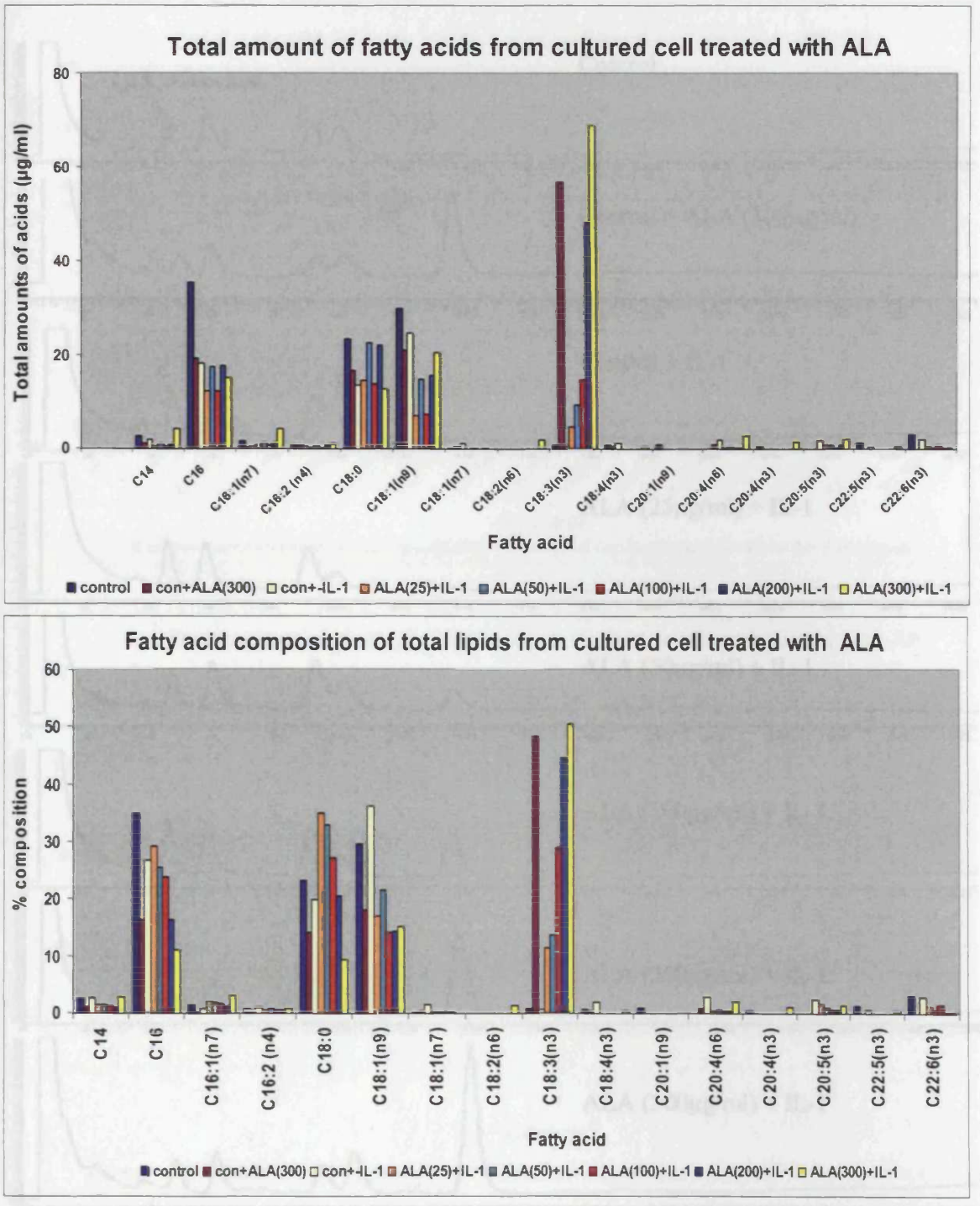


Figure 3.17: Effects of ALA on the total amount of fatty acids and the percentage of fatty acid composition in total lipids from chondrocyte cells in culture.

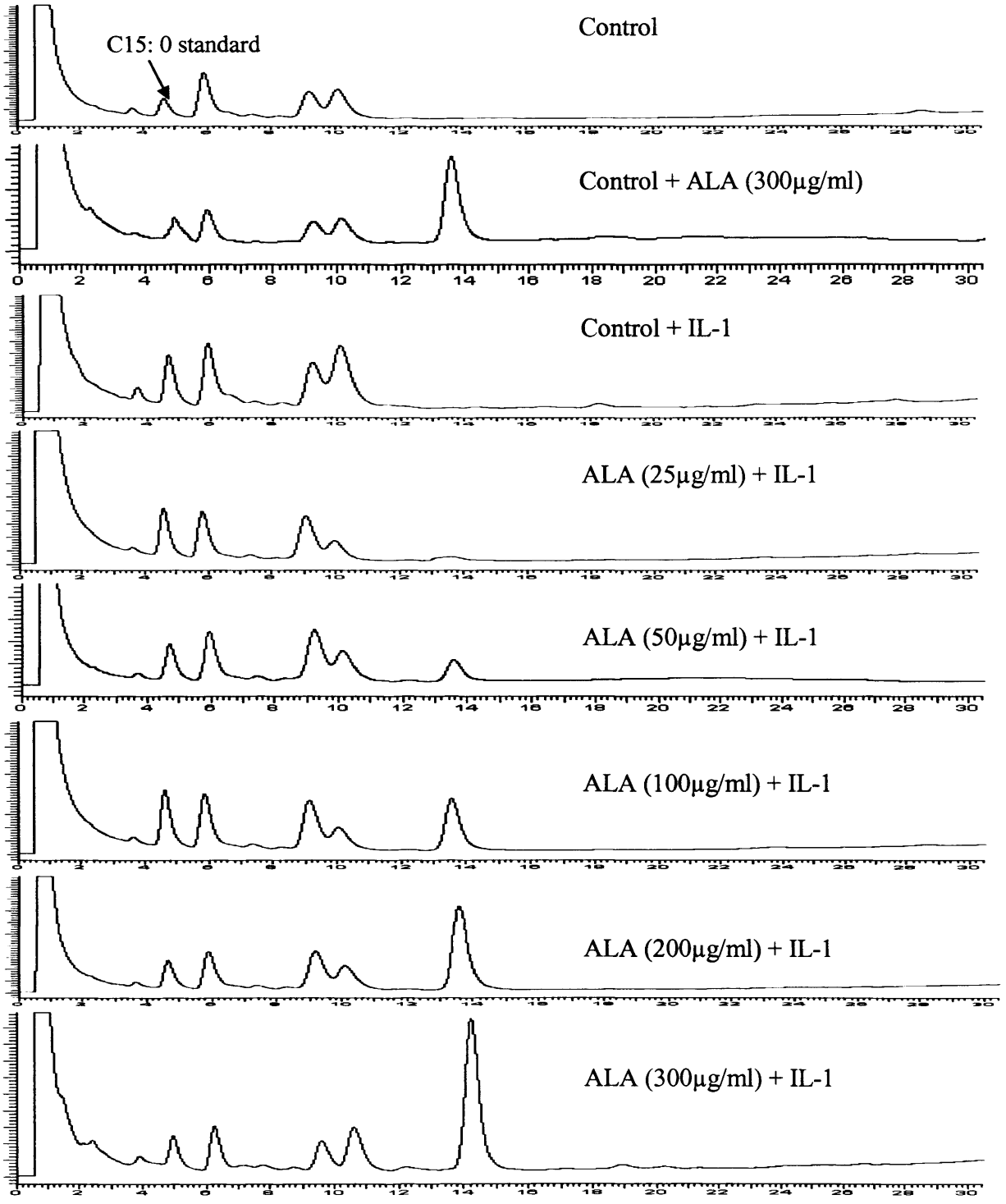


Figure 3.18: Gas chromatograms for FAMEs of chondrocyte cell lipids from cultures treated as above with or without the *n-3* PUFA, ALA.

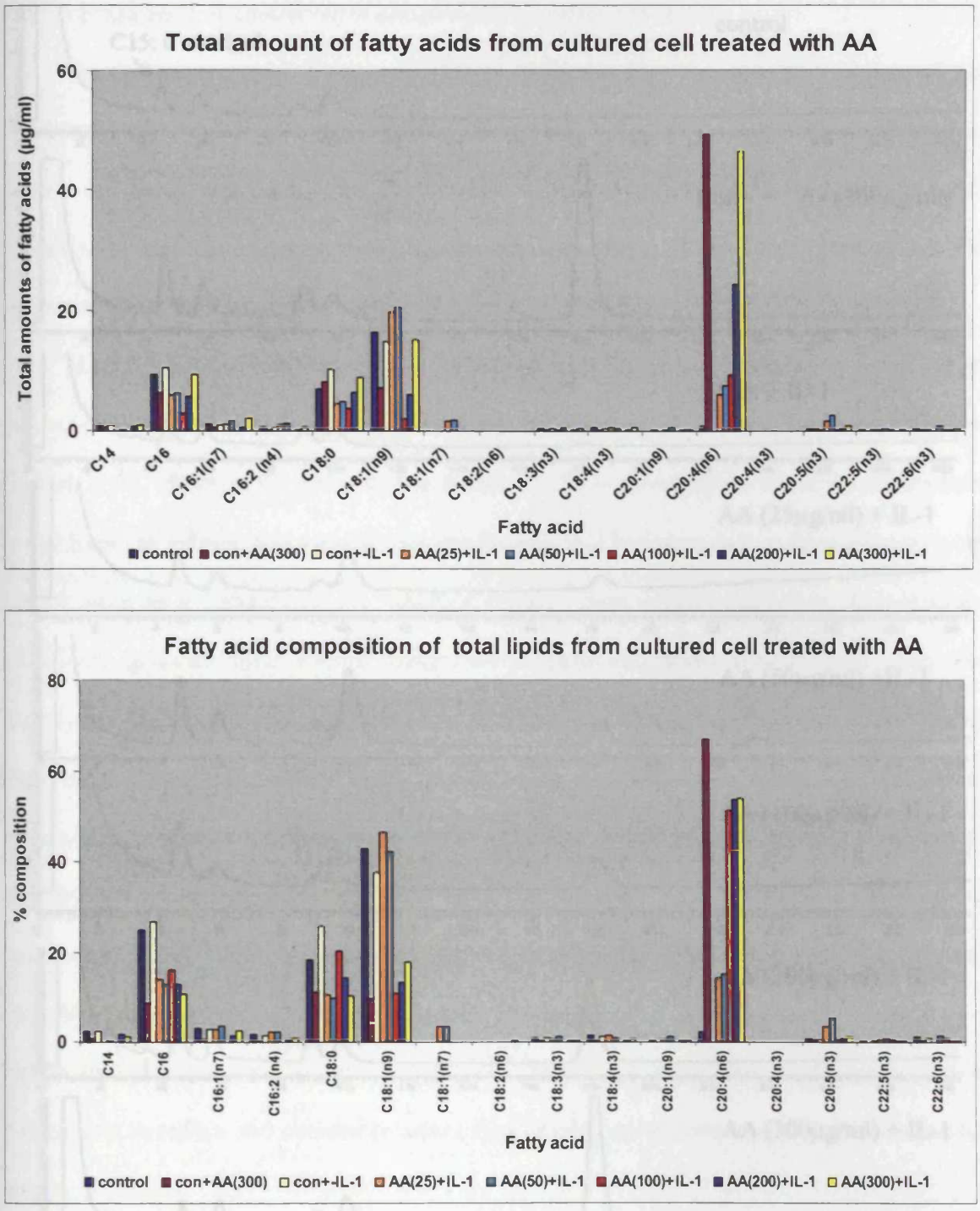


Figure 3.19: Effects of AA on the total amount of fatty acids and the percentage of fatty acid composition in total lipids from chondrocyte cells in culture.

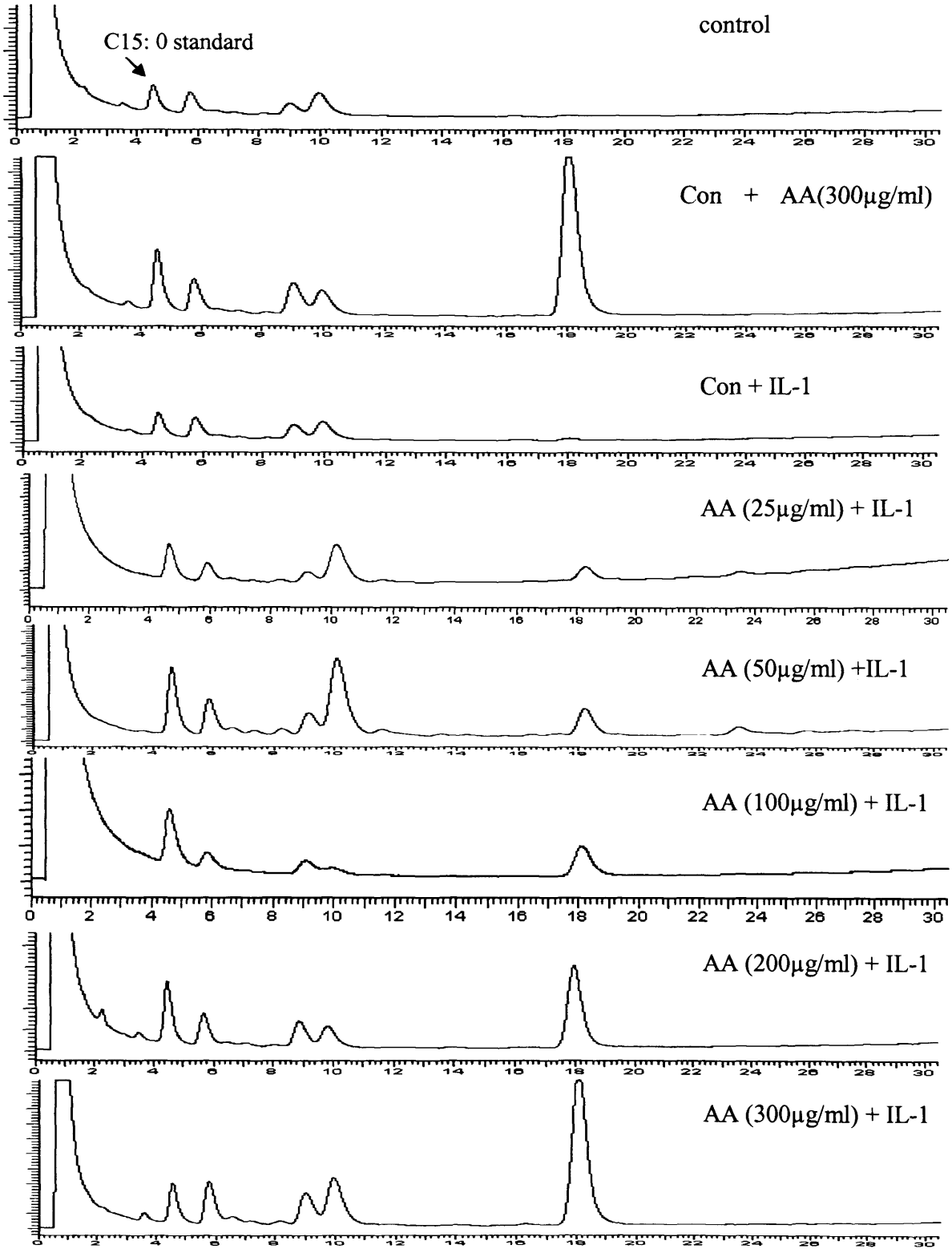


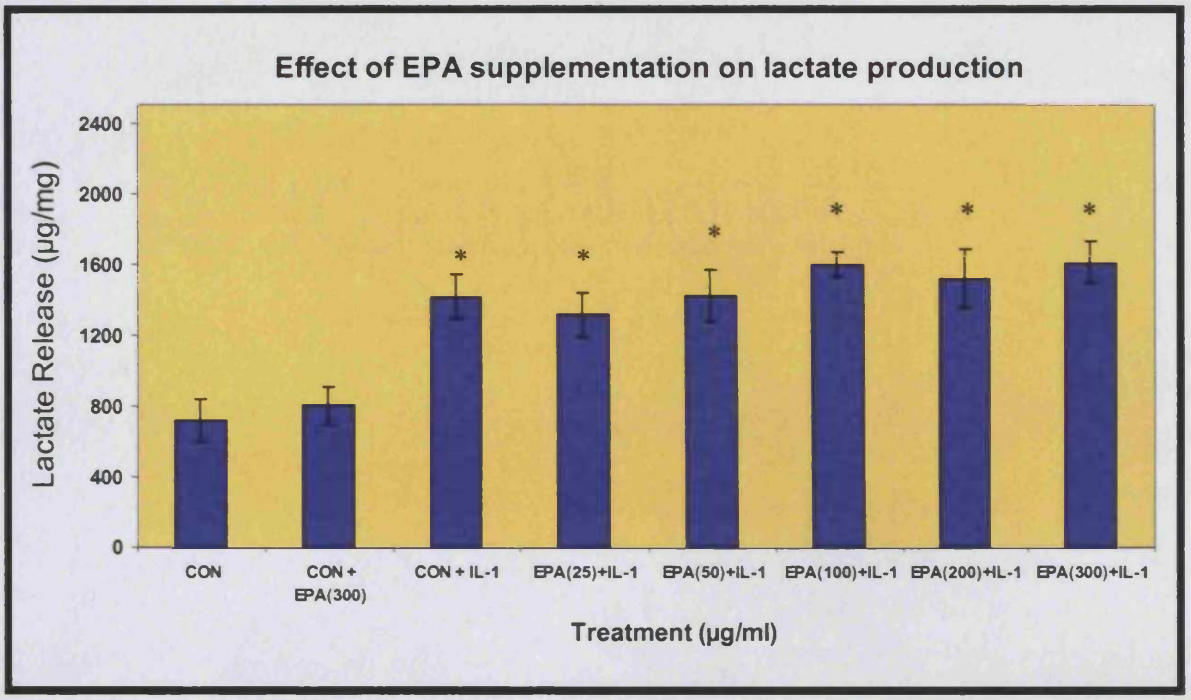
Figure 3.20: Gas chromatograms for FAME's of chondrocyte cell lipids from cultures treated as above with and without *n-6* AA.

3.7 Evaluation of chondrocyte metabolism by release of lactate

In our experiments, it was essential to determine that the experimental procedures involving various fatty acid and palm oil fractions did not affect chondrocyte viability or general cellular metabolism, before further studies were carried out. As a measure of general chondrocyte metabolism under the experimental conditions, lactate concentrations in the culture medium were measured using the commercially-available lactate assay kit (Sigma).

Lactate is a result of normal metabolism (glycolysis) and is produced by reduction of pyruvate, the direct end-product. It is normally produced in proportion to glucose consumption (Lodish *et al.,* 1999). In the body, excess lactate can be removed by the blood for metabolism in the liver. In culture, this clearly does not happen, but lactate excretion from chondrocytes can be used as a measure of the culture's status. Under culture conditions, chondrocytes effectively generate their energy under low oxygen conditions (i.e. through anaerobic glycolysis). In this pathway, one molecule of glucose is metabolized into two molecules of pyruvate and two molecules of ATP. The pyruvate is converted to lactate by the enzyme lactate dehydrogenase (LDH) to replenish NAD levels for further use in glycolysis (Lodish *et al.*, 2000). Lactate can then be transported out of the cell. Such mechanisms are of great importance in cells and tissues lacking high oxygen levels. Thus, lactate, which is released from chondrocytes into the culture medium during anaerobic glycolysis, can be used as a marker/indicator of chondrocyte metabolism. Moreover, cell/tissue death would cause metabolism to reduce and eventually cease, thus causing a commensurate reduction in lactate levels.

Chondrocytes were cultured in the presence or absence IL-1α, over a 4 day period following 8h exposure to fatty acids. As shown in Figures 3.21 and 3.22, IL-1α stimulated lactate production in all samples. Depending on the particular preparation of cells used, IL-1α



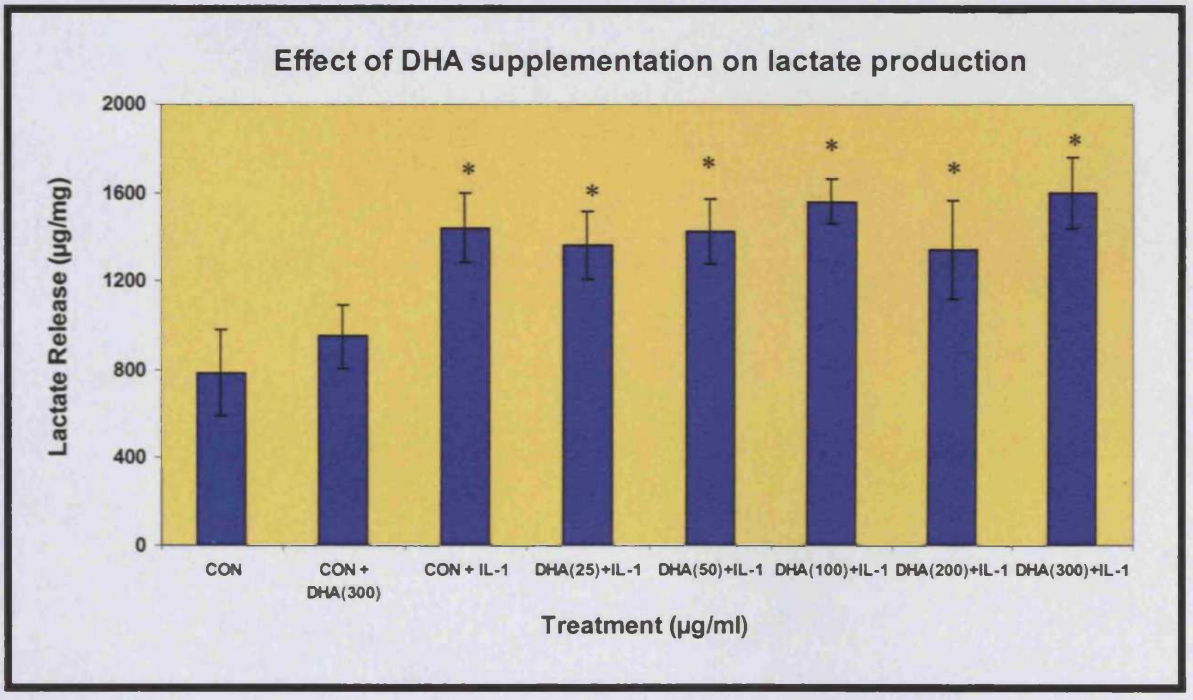
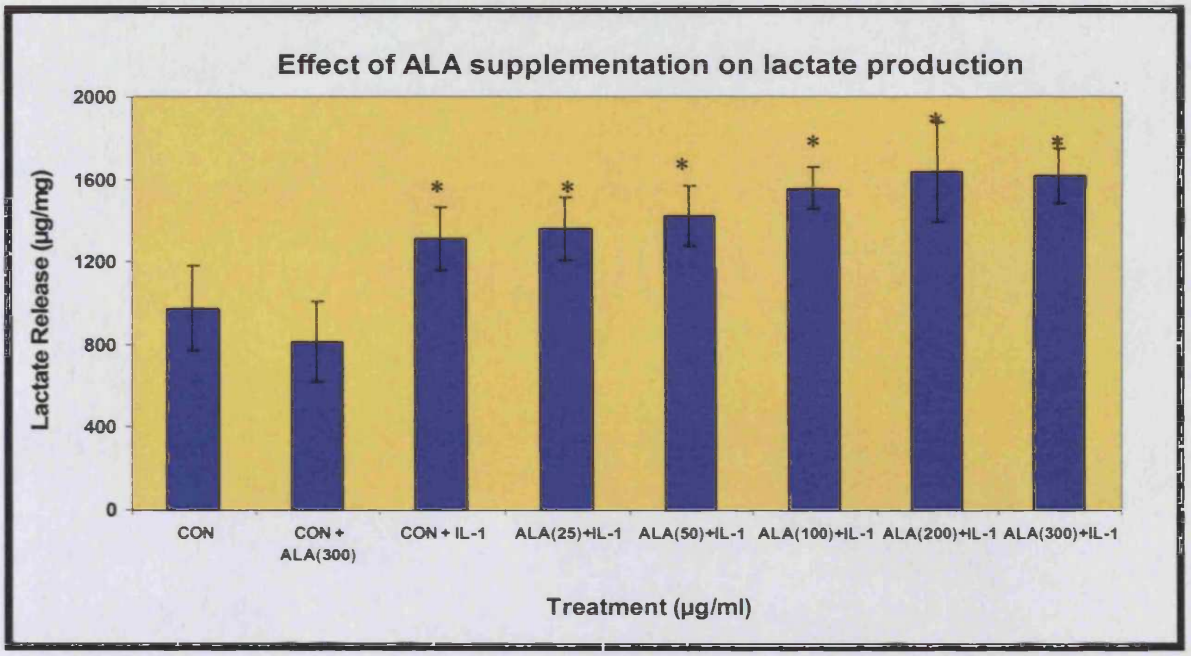


Figure 3.21: Effect on lactate release into the culture medium following incubation of cells with 25 – 300µg/ml of the *n-3* fatty acids; EPA and DHA for 8 hours and exposure to IL-1α for 4 days. Data as means ± S.D. (n=3) are shown. Significance difference using *T*-test given by * compared to control ($p<0.05$).



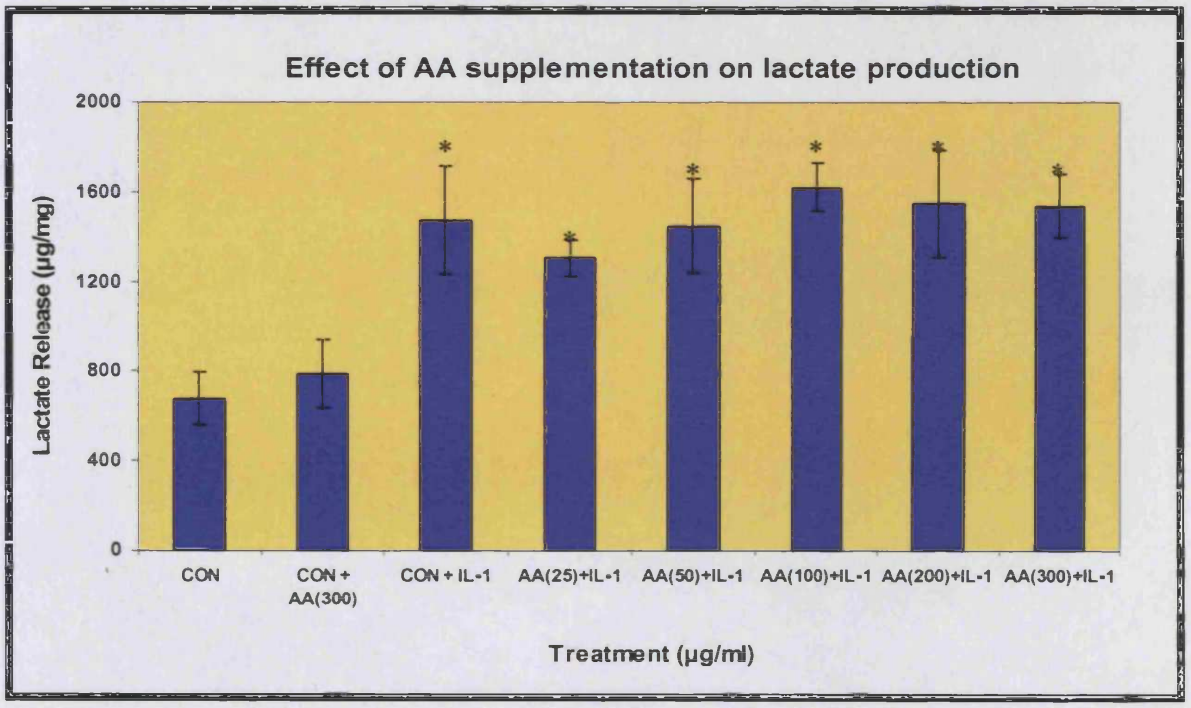


Figure 3.22: Effect on lactate release into the culture medium following incubation of cells with 25 – 300µg/ml of the *n-3* fatty acids; ALA and AA for 8 hours and exposure to IL-1α for 4 days. Data as means ± S.D. (n=3) are shown. Significance difference using *T*-test given by * compared to control ($p<0.05$).

exposure increased lactate release up to 3-fold compared with that in the untreated control cultures. This shows there was an increase in metabolism in cells when challenged with the cytokine. However, there were no statistically significant changes in lactate levels when fatty acids alone were added to control cultures. This result was also found for samples with high amounts of fatty acids (300μg/ml), compared to control cultures. This indicated that EPA, DHA, ALA and AA had no significant effect ($p < 0.05$) on lactate release, even at the highest dose and therefore, that they did not alter metabolism.

When cultures, which had been previously incubated with different concentrations of fatty acids, were stimulated with IL-1α, there were no differences in the lactate values. This was observed for concentrations of 25 - 300μg/ml of EPA, DHA (Figure 3.21), ALA, AA (Figure 3.22) and for fatty acids from hydrolysed palm olein (Figure 3.23). These data show that the fatty acids did not moderate the increase in lactate release (and, by inference, anaerobic glycolysis) caused by IL-1α addition. The results also confirm that fatty acids did not change existing metabolic rates under the conditions of the experiments.

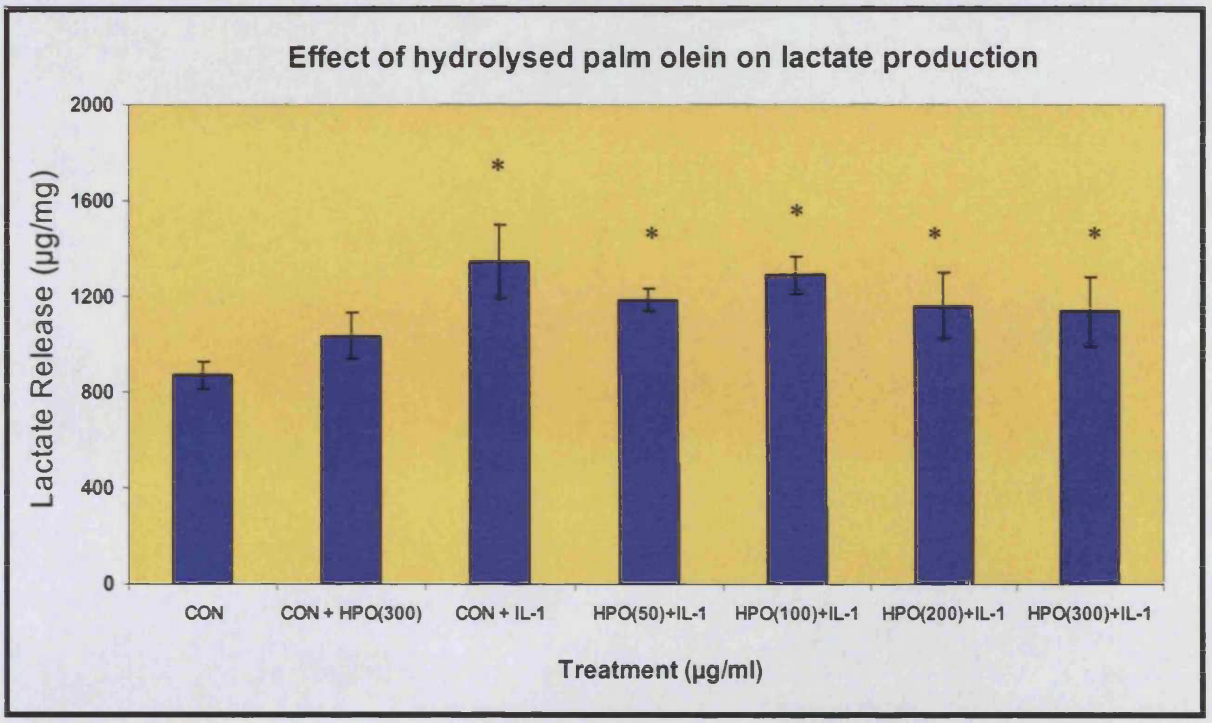


Figure 3.23: Effect on lactate release into the culture medium following incubation of cells with 50 – 300µg/ml hydrolysed palm olein for 8 hours and exposure to IL-1α for 4 days. Data as means ± S.D. (n=3) are shown. Significance difference using *T*-test given by * compared to control ($p<0.05$).

3.8 Effect of exogenous fatty acids on expression levels of Type II collagen and aggrecan in chondrocyte cultures

Cartilage degradation, leading to a loss of joint function in osteoarthritis, involves proteolytic cleavage of both aggrecan and collagen. Aggrecan is one of the first extracellular matrix components to undergo measurable loss in arthritic diseases. Indeed, there are several lines of indirect evidence suggesting that the presence of aggrecan may serve to protect type II collagen molecules from proteolytic degradation (Abbaszade *et al.*, 1999).

In this experiment, RT-PCR methods were employed to determine the expression of mRNAs for aggrecan and collagen type II in order to ensure that the chondrocyte phenotype was maintained during the different culture conditions employed for fatty acid supplementation. As shown in Figure 3.24, monolayer chondrocyte cultures were stimulated to undergo matrix degradation with the cytokine IL-1α. Under the conditions of this study, in which the cultures were exposed to fatty acid supplementation for 8 hours before adding IL-1α and culturing again for another 4 days, the mRNA signal levels attributable to Type II collagen were detectable in the samples supplemented with EPA, DHA, ALA and AA both with or without IL-1α. The intensity of the bands did not change on IL-1α exposure and was not affected by pre-incubation with any of the fatty acids. Thus, these acids in the range of 25 – 300μg/ml did not affect mRNA levels. This suggested that neither *n*-3 and *n*-6 fatty acids affected this basic aspect of chondrocyte metabolism.

Similarly, the levels of mRNA for aggrecan remained constant for all the samples regardless of exposure to EPA, DHA, ALA and AA for 8 hours in the range 25-300 μg/ml with or without IL-1α (Figure 3.26). Any changes seen were very minor and suggested that basic cartilage components such as aggrecan and type II collagen should be capable of being synthesised at the same rates as control cultures. When the cell cultures were incubated with a mixture of fatty acids from hydrolysed palm olein, no significant affects were found in the mRNA levels for Type II collagen (Figure 3.25) or for aggrecan (Figure 3.27).

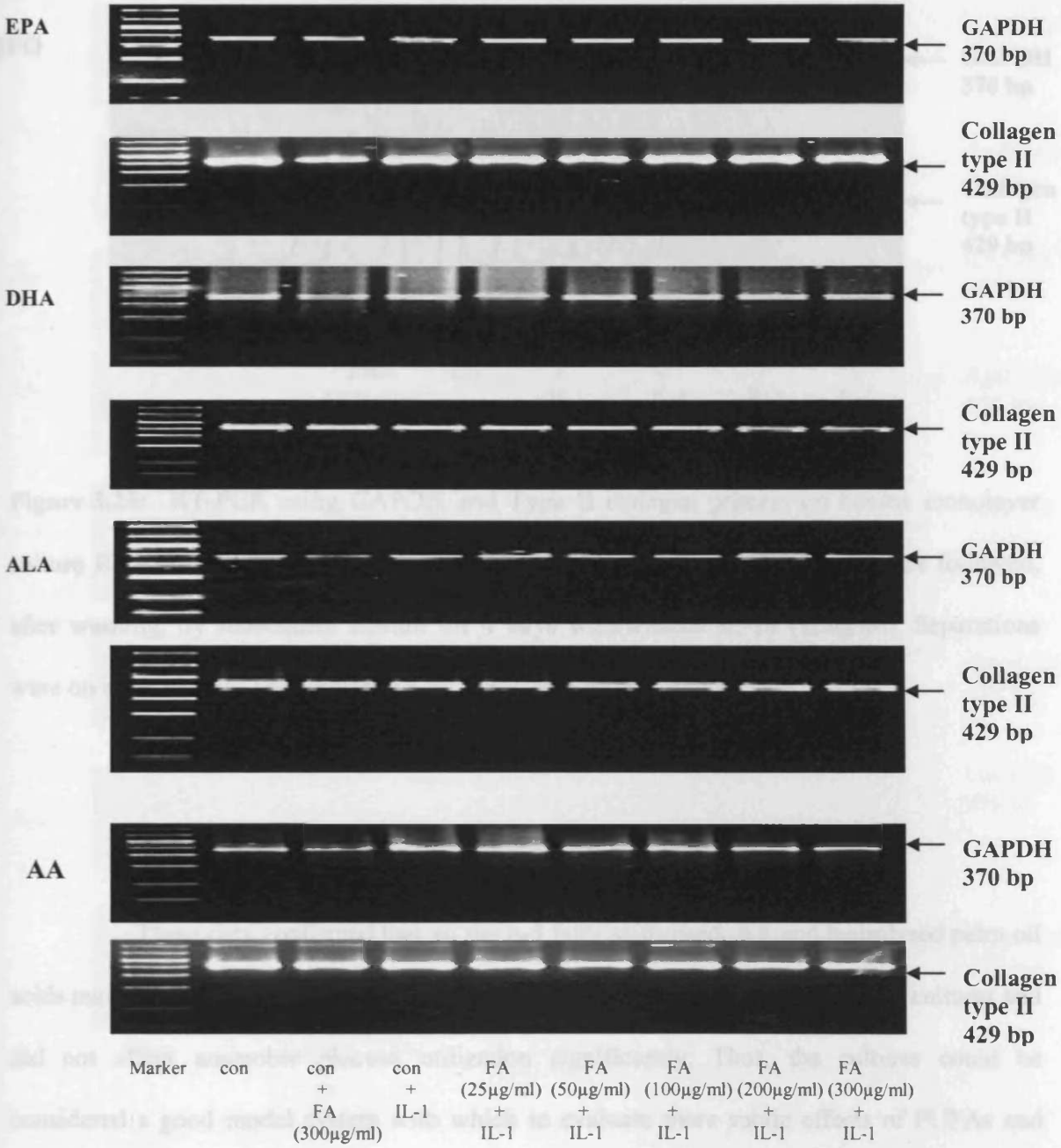


Figure 3.24: RT-PCR of GAPDH and Type II collagen mRNA levels in bovine monolayer cultures treated with different fatty acids (EPA, DHA, ALA) and AA for 8 hours followed, after washing, by subsequent culture for 4 days with/without IL-1α (10ng/ml). Separations were on 2% agarose gels alongside a 50-1000 bp marker.

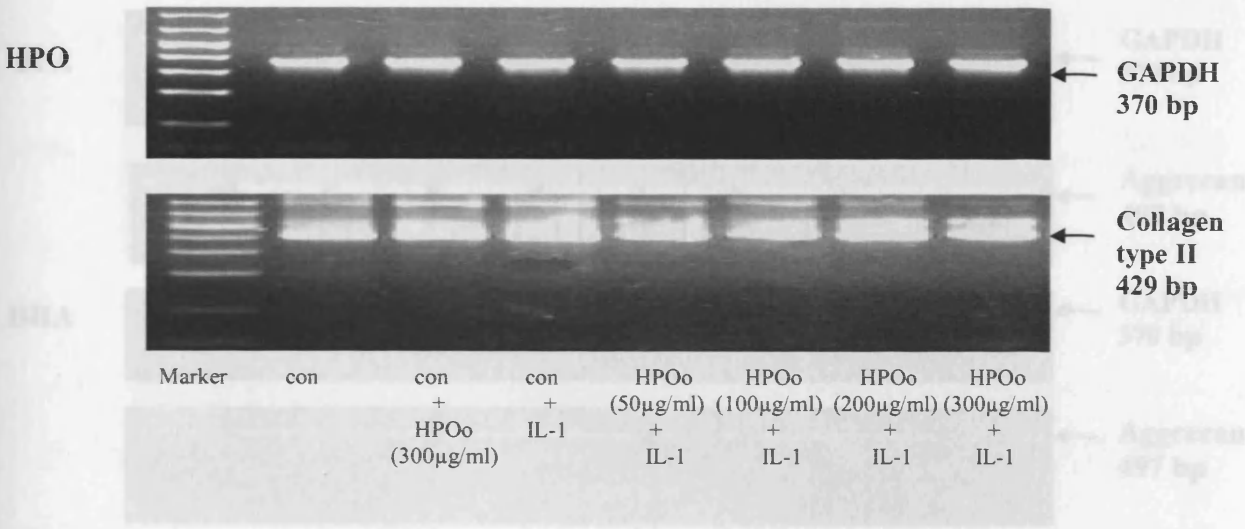


Figure 3.25: RT-PCR using GAPDH and Type II collagen primers on bovine monolayer culture RNA following treatment with hydrolysis palm olein (HPOo) for 8 hours followed, after washing, by subsequent culture for 4 days with/without IL-1α (10ng/ml). Separations were on a 2% agarose gel alongside a 50-1000 bp marker.

These data confirmed that all the *n-3* fatty acids used, AA and hydrolysed palm oil acids maintained characteristics of the chondrocyte phenotype in the experimental cultures and did not affect anaerobic glucose utilization significantly. Thus, the cultures could be considered a good model system with which to evaluate more subtle effects of PUFAs and other lipids on inflammatory responses.

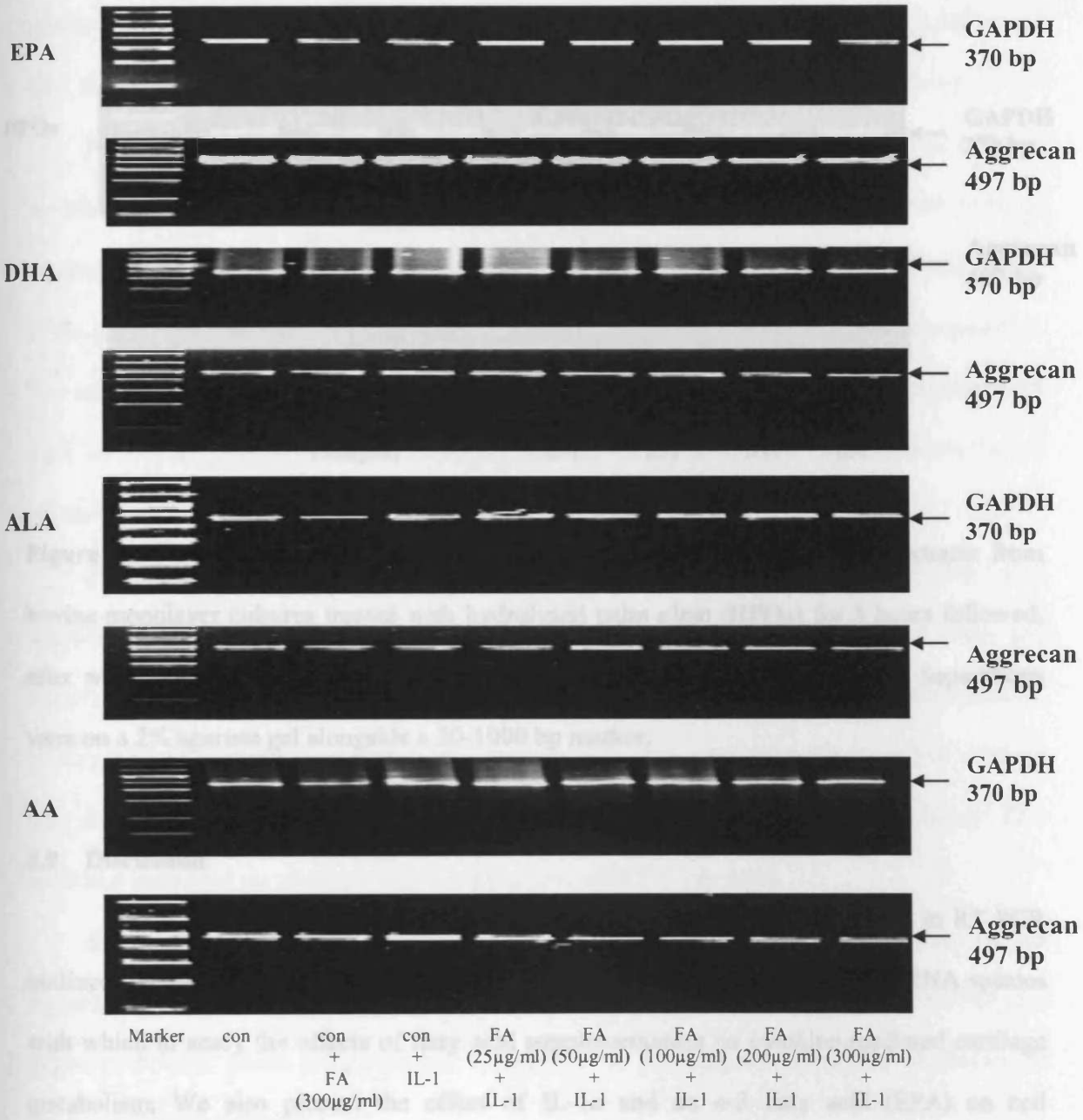


Figure 3.26: RT-PCR using GAPDH and aggrecan primers carried out on extracts from bovine monolayer cultures treated with different fatty acids (EPA, DHA, ALA and AA) for 8 hours followed, after washing, by subsequent culture for 4 days with IL-1α (10ng/ml). Separations were on 2% agarose gels alongside a 50-1000 bp marker.

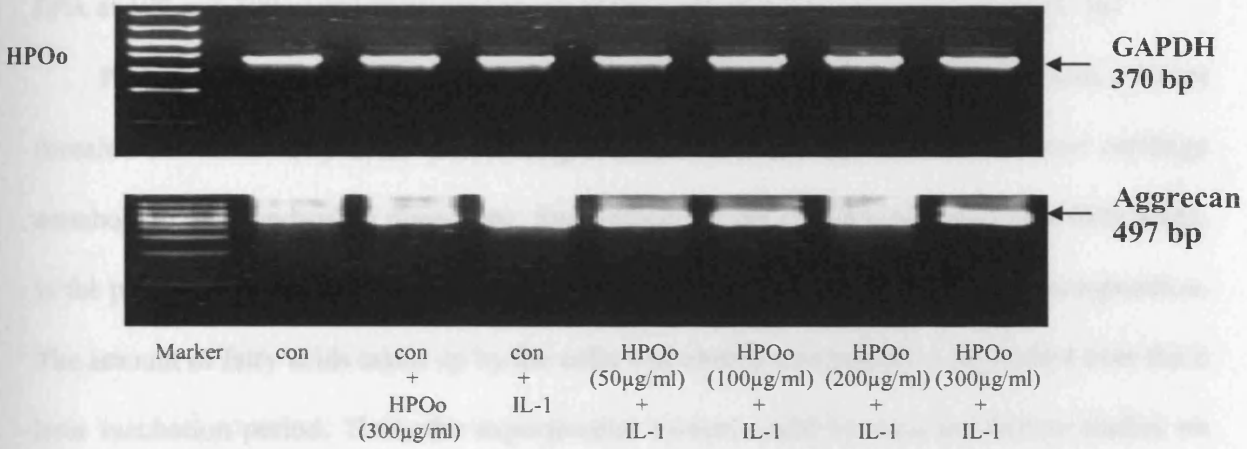


Figure 3.27: RT-PCR using GAPDH and aggrecan primers carried out on extracts from bovine monolayer cultures treated with hydrolysed palm olein (HPOo) for 8 hours followed, after washing, by subsequent culture for 4 days with/without IL-1α (10ng/ml). Separations were on a 2% agarose gel alongside a 50-1000 bp marker.

3.9 Discussion

In this study, we have successfully overcome the problems encountered in RT-PCR outlined in chapter 2 and developed a satisfactory RT-PCR assay for various mRNA species with which to study the effects of fatty acid supplementation on cytokine-mediated cartilage metabolism. We also present the effect of IL-1α and an *n-3* fatty acid (EPA) on cell morphology as found by examination of chondrocyte cultures with light microscopy. The morphology of cartilage cells is the result of an intensive reaction between cells and their matrix components. According to the results, treatment with IL-1α alone resulted in alteration of cell morphology compared to control. IL-1α differentially induced or stimulated the chondrocytes resulting in extracellular matrix degradation and, finally, alteration of cell-matrix

interaction and cell morphology, as found by examination by light microscopy. Addition of EPA at 100 and 300 μg/ml prevented much of the implied degradation caused by IL-1α.

Preliminary studies with addition of different fatty acids into chondrocytes cell cultures revealed that these fatty acids were incorporated into chondrocytes without altered cartilage metabolism or chondrocyte phenotype. Supplementing the cultures with different fatty acids, in the presence of bovine serum albumin, produced a drastic change in fatty acid composition. The amount of fatty acids taken up by the cells was clearly concentration-dependent over the 8 hour incubation period. Thus, the experimental system could be used for further studies on effects of exogenous fatty acids.

Furthermore, the data obtained from the lactate results revealed that fatty acid supplementation with EPA, DHA, ALA, AA or hydrolysed palm olein did not show any significant effect ($p<0.05$) on the production of lactate, even at the highest concentration (300μg/ml). In contrast, a considerable increase in lactate production compared to controls was observed when the cytokine, IL-1α was added to the chondrocyte cell cultures. This indicated enhanced metabolism of the cell cultures with this cytokine.

Reverse transcriptase-PCR analysis revealed that addition of fatty acids to the experimental chondrocytes cultures did not change the levels for Type II collagen mRNA and aggrecan mRNA which suggested that neither *n-3* or *n-6* fatty acids affected expression of these cartilage components.

3.10 Chapter summary

- RT-PCR conditions were optimized for subsequent studies of the effects of fatty acid supplementation on cytokine-mediated cartilage metabolism.
- Incubation of cultures for 8h with different fatty acids in the presence of bovine serum albumin allowed a concentration-dependent uptake into the chondrocytes.
- Supplementation with EPA, DHA, ALA, AA or hydrolysed palm olein gave no statistically significant change in lactate release compared to controls even at high concentrations (300μg/ml). Addition of the cytokine, IL-1α, increased lactate production significantly ($p<0.05$) compared to controls, indicating an increased metabolism in cells exposed to this cytokine. This increase was not affected by pre-incubation with fatty acids.
- mRNA levels for aggrecan and Type II collagen were unaffected by pre-exposure to different fatty acids or to the cytokine, IL-1α. Thus, these measures of cartilage formation were not affected under the experimental conditions.

CHAPTER FOUR

Effect of fatty acid supplementation on connective tissue metabolism in chondrocyte cultures

4.1 Introduction

The purpose of these experiments was to characterize some of the biochemical changes that could occur in chondrocytes monolayer cultures which might be relevant to cartilage degradation. The rationale behind this is that cartilage breakdown is known to be a major feature of both rheumatoid and osteo-arthritis. One of the major macromolecules to be lost early in these disease processes is the large aggregating proteoglycan called aggrecan which is composed of a protein core that contains a high level of sulphated glycosaminoglycans (GAGs) (Yamanishi *et al.,* 2002). In our experiments, the release of GAG was measured in order to gain an idea of the extent of proteoglycan degradation in the extracellular matrix, a process which, in *vivo*, would influence tissue stiffness and compression. Interleukin-1 (IL-1) was used to stimulate cartilage degradation in these experiments, because IL-1 (together with tumour necrosis factor, TNF-α) are the major cytokines thought to control this process *in vivo* (Martel–Pelletier *et al.,* 1999). Thus, IL-1 has been shown to inhibit extracellular matrix synthesis and induce aggrecanases and matrix metalloproteinase (MMPs) which increases the degradation of proteoglycans. In this study, the IL-1α isoform was used since it has been shown that much smaller doses of IL-1α compared to IL-1β are more effective with bovine articular cartilage (Legare *et al.*, 2002).

We used young bovine articular cartilage (7 days-old calves) in which IL-1α induces a characteristic spatially heterogeneous depletion of GAG. This young tissue still contains blood

vessels and it is in the perivascular regions that IL-1α induced-GAG depletion is most severe and the inherent ability of chondrocytes to replenish the matrix is relatively uniform (Williams *et al.*, 2003). Little or no cytokine mRNA expression was present in the young bovine cartilage (Curtis *et al.*, 2002). Therefore, the changes or deficiency of proteoglycan in the model can be investigated and extent of degradation measured. IL-1 degraded cartilage tissues and cell suspensions were employed as model systems to study cartilage metabolism under osteoarthritis-like conditions and to assess the efficacy of various therapies. Compounds (such as fatty acids) which might be able to inhibit the IL-1α mediated effects on matrix degradation may well have therapeutic activity in OA and RA, so decreases in the indicators of matrix catabolism were sought for.

Release of GAG-containing fragments from cartilage is generally believed to be due to the action of proteases released from the chondrocytes. Collagen and aggrecan-degrading proteases such as collagenases (MMP-13), stromelysin (MMP-3) as well as the aggrecanases (ADAMTS-4 and ADAMTS-5) are involved in cartilage degradation. In this study, the mRNAs for these proteases and aggrecanases were studied to see if their levels changed with IL-1α treatment and if exogenous fatty acids could modify any alterations seen.

4.2 Fatty acid effects on glycosaminoglycan (GAG) release from chondrocyte monolayer cultures

Proteoglycan release was determined by measuring glycosaminoglycan released into the culture medium using dimethylmethylene blue (DMMB) (Section 2.2.5). Changes in the release of GAG in response to IL-1α following exposure to EPA, DHA, ALA, and AA or hydrolysed palm olein were analysed and the results are presented in Figures 4.1-4.3.

As expected, the GAG production was increased in some cultures treated with IL-1α compared to controls. The largest increases were seen in the monolayer cultures used to examine DHA effects (Figure 4.1) when a 50% increase was found on IL-1α challenge ($p<0.05$). Pre-incubation of control cultures with EPA or DHA reduced GAG release, significantly ($P<0.05$; Figure 4.1). Increasing concentrations of all three *n-3* PUFAs progressively reduced the IL-1 stimulated GAG release. EPA seemed the most effective of these acids (Figure 4.1- 4.2). These results would suggest that dietary *n-3* PUFAs might have therapeutic use for the treatment of OA and RA, in agreement with clinical trials.

As shown in Figure 4.2, the results for GAG release, following treatment with the arachidonic acid in response to IL-1α were somewhat different to those found with *n-3* fatty acids. Supplementation with AA (arachidonic acid) was unable to reduce proteoglycan degradation, following IL-1α stimulation, even at concentrations as high as 300μg/ml. This would be in agreement with the general acceptance that an *n-6* PUFAs like arachidonic acid do not have utility for the treatment of OA or RA.

Figure 4.3 shows data for hydrolysed palm olein acids. Although a significant decrease in GAG release was found at the highest concentration compared with control samples, the results did not show concentration dependence following IL-1α stimulation and because of their variability, their reliability is in question. More experiments would be needed to further assess the effect.

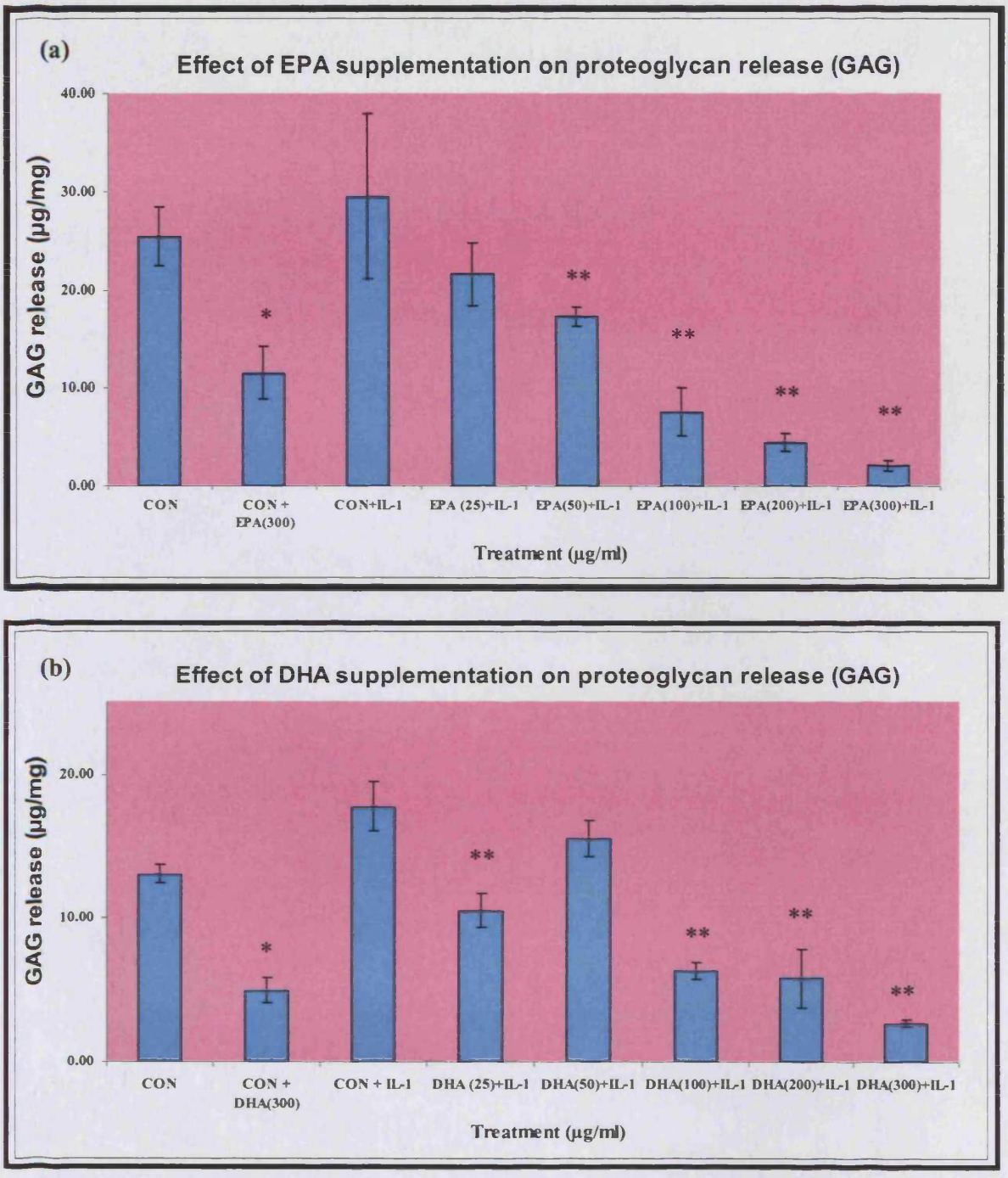


Figure 4.1: GAG release into the culture medium supplemented with and without (a) EPA (b) DHA prior to exposure to IL-1α. Supplementation with fatty acids was for 8h before washing and culturing with IL-1α for a further 4 days. Means ± S.D. (n=5) are shown. Significant difference was made using T-test at $p<0.05$.

* = Significantly different compared to control ($p<0.05$).

** = Significantly different compared to control + IL-1 ($p<0.05$).

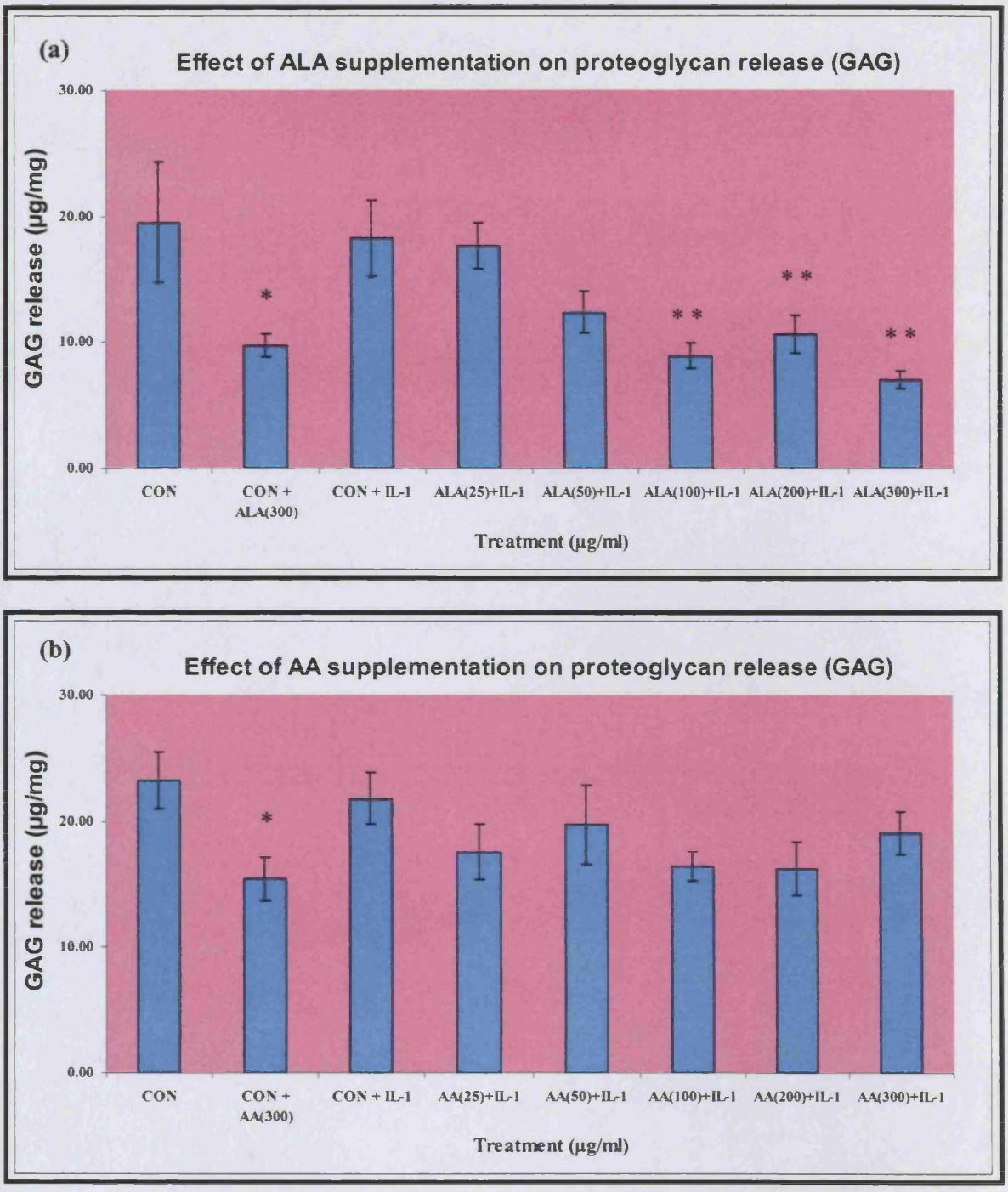


Figure 4.2: GAG release into the culture medium supplemented with and without A) ALA b) AA prior to exposure to IL-1α. Supplementation with fatty acids was for 8h before washing and culturing for a further 4 days. Means ± S.D. (n=5) are shown. Significant difference was made using T-test at $p<0.05$.

* = Significantly different compared to control ($p<0.05$).

** = Significantly different compared to control + IL-1 ($p<0.05$).

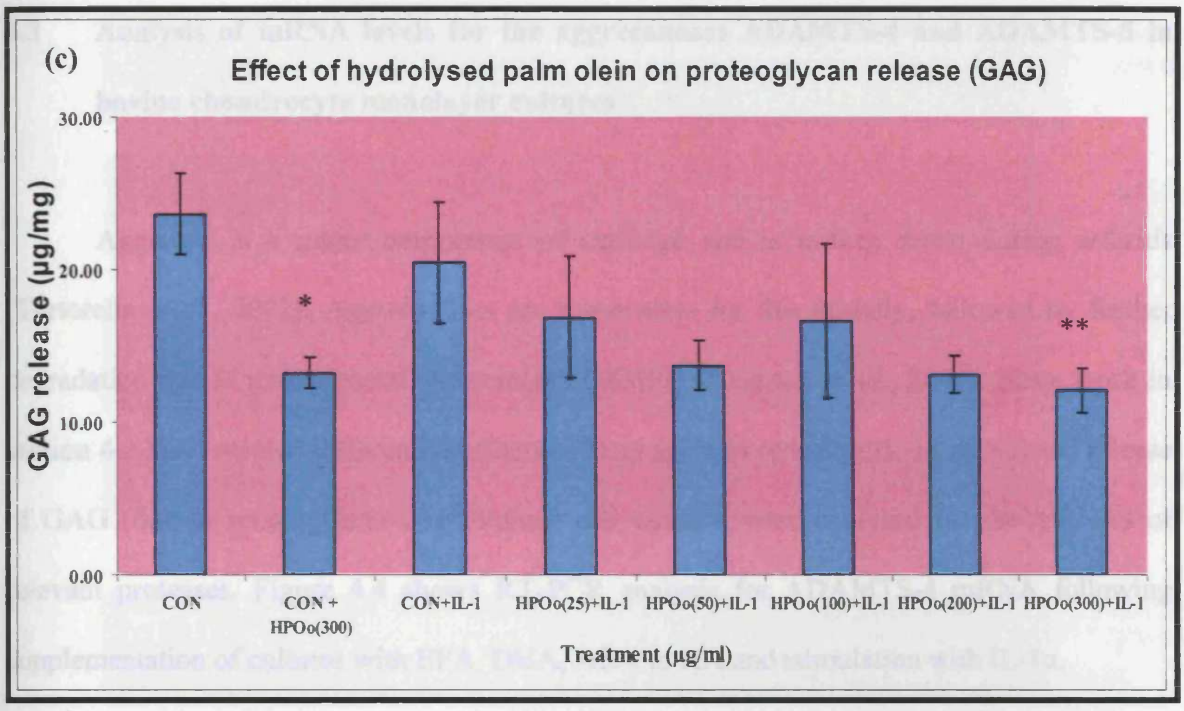


Figure 4.3: GAG release into the medium for cultures supplemented with and without hydrolysed palm olein prior to exposure to IL-1α. Supplementation with fatty acids was for 8h before washing and culturing for a further 4 days. Means ± S.D. (n=5) are shown. Means ± S.D. (n=5) are shown. Significant difference was made using T-test at $p<0.05$.
* = Significantly different compared to control ($p<0.05$).
** = Significantly different compared to control + IL-1 ($p<0.05$).

In summary, the results showed that all the *n-3* PUFAs (EPA, DHA, ALA) significantly inhibited IL-1α stimulated GAG release. This effect was dose-dependent. EPA seemed the most effective, followed by DHA and then ALA. In contrast, arachidonic acid did not significantly abrogate the increase in GAG release caused by IL-1α. These data led directly to RT-PCR studies to see whether the mRNA levels of relevant degradative proteinases were affected by the treatment of cultures with the inflammatory cytokine, IL-1α, and the various fatty acids.

4.3 Analysis of mRNA levels for the aggrecanases ADAMTS-4 and ADAMTS-5 in bovine chondrocyte monolayer cultures

Aggrecan is a major component of cartilage and is broken down during arthritis (Tortorella *et al.*, 2002). Aggrecanases are responsible for this initially, followed by further degradation due to matrix metalloproteinases (MMPs) (Caterson *et al.*, 2000). Since work in section 4.2 had revealed differential effects of fatty acids in reducing IL-1α stimulated release of GAG (due to proteoglycan degradation) cell extracts were analysed for the mRNAs of relevant proteases. Figure 4.4 shows RT-PCR analysis for ADAMTS-4 mRNA following supplementation of cultures with EPA, DHA, ALA or AA and stimulation with IL-1α.

Expression of ADAMTS-4 was clearly induced by IL-1α treatment, its mRNA being undetectable or at low levels in the control cultures. Pre-treatment of control cultures with high concentrations of the different acids did not significantly change mRNA levels. In contrast, all three *n-3* PUFAs lowered the expression, which was stimulated by IL-1α, in a dose-dependent manner. EPA seemed the most effective, followed by DHA and then by ALA. Exposure to AA did not have any obvious effect on ADAMTS-4 mRNA levels (Figure 4.4).

Bovine chondrocytes cultures were also incubated with hydrolysed palm olein in the absence or presence of IL-1α in a dose-dependent manner. As expected, pre-treatment with hydrolysed palm olein did not reduce the ADAMTS-4 mRNA after incubation with IL-1α. This contrasted clearly with the effects of the *n-3* PUFAs. Treatment of control cultures with HPOo did not affect mRNA levels either (Figure 4.5).

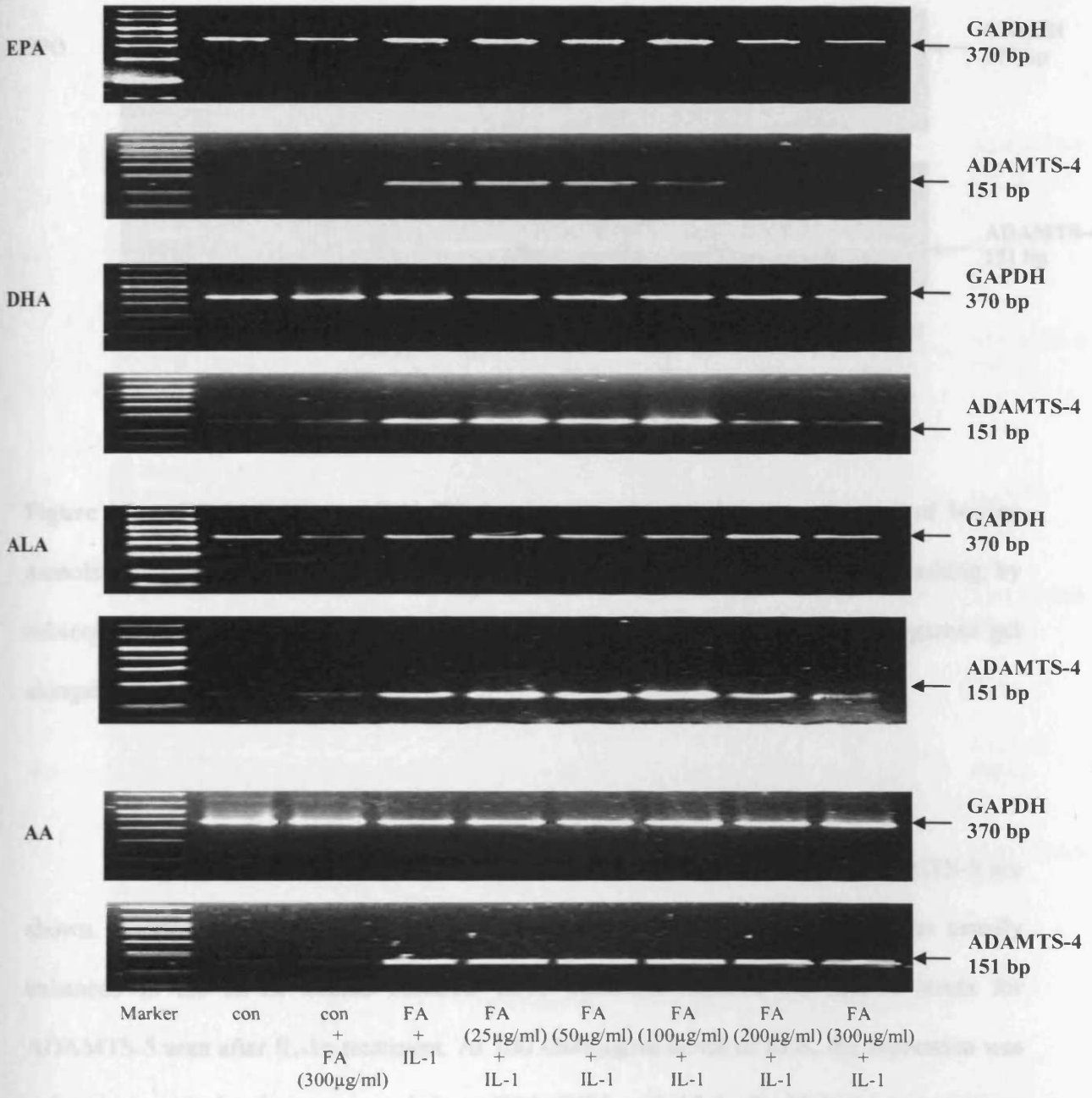


Figure 4.4: RT-PCR using ADAMTS-4 primers was carried out on extracts of bovine monolayer cultures treated with different fatty acids (FA) (EPA, DHA, ALA and AA) for 8 hours followed, after washing, by subsequent culture for 4 days with IL-1α (10ng/ml). Separations were on a 2% agarose gel alongside a 50-1000 bp marker.

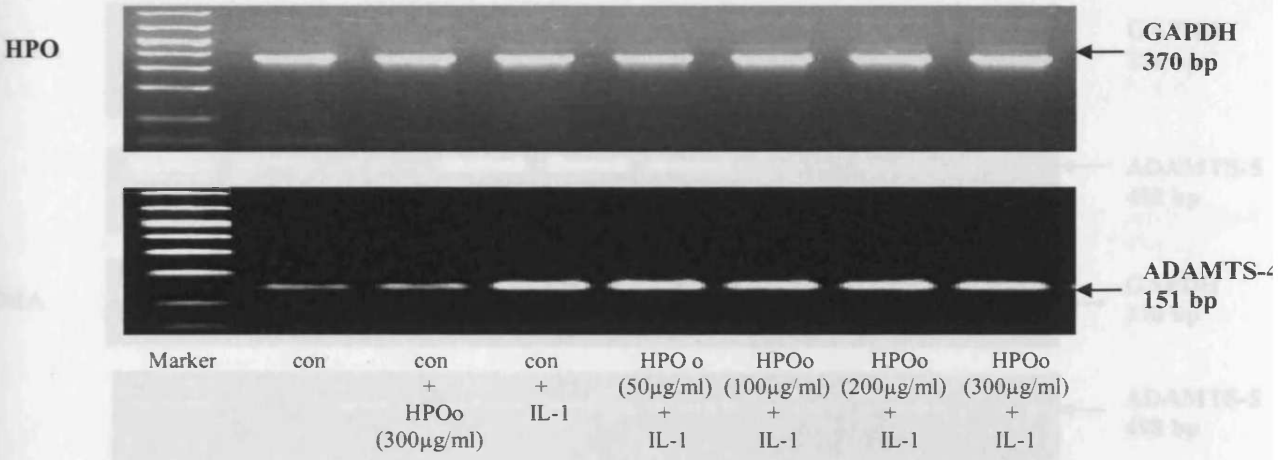


Figure 4.5: RT-PCR using ADAMTS-4 primers was carried out on extracts of bovine monolayer cultures treated with Hydrolysed palm olein for 8 hours followed, after washing, by subsequent culture for 4 days with IL-1α (10ng/ml). Separations were on a 2% agarose gel alongside a 50-1000 bp marker.

In Figure 4.6, the results of RT-PCR experiments using primers for ADAMTS-5 are shown. Control (untreated) samples showed low levels of expression but this was usually enhanced in the IL-1α treated cultures. EPA treatment reduced the mRNA levels for ADAMTS-5 seen after IL-1α treatment. At 100 -300 µg/ml levels of EPA, the expression was reduced to control values or even below. With DHA and ALA, the highest concentrations again (300µg/ml) reduced IL-1α – stimulated ADAMTS-5 mRNA levels. At this concentration (300µg/ml) of DHA, expression was comparable to that following EPA treatment. One *n-6* PUFA, arachidonic acid, was used. This may have reduced IL-1α – stimulated mRNA levels for ADAMTS-5 at the highest concentrations.

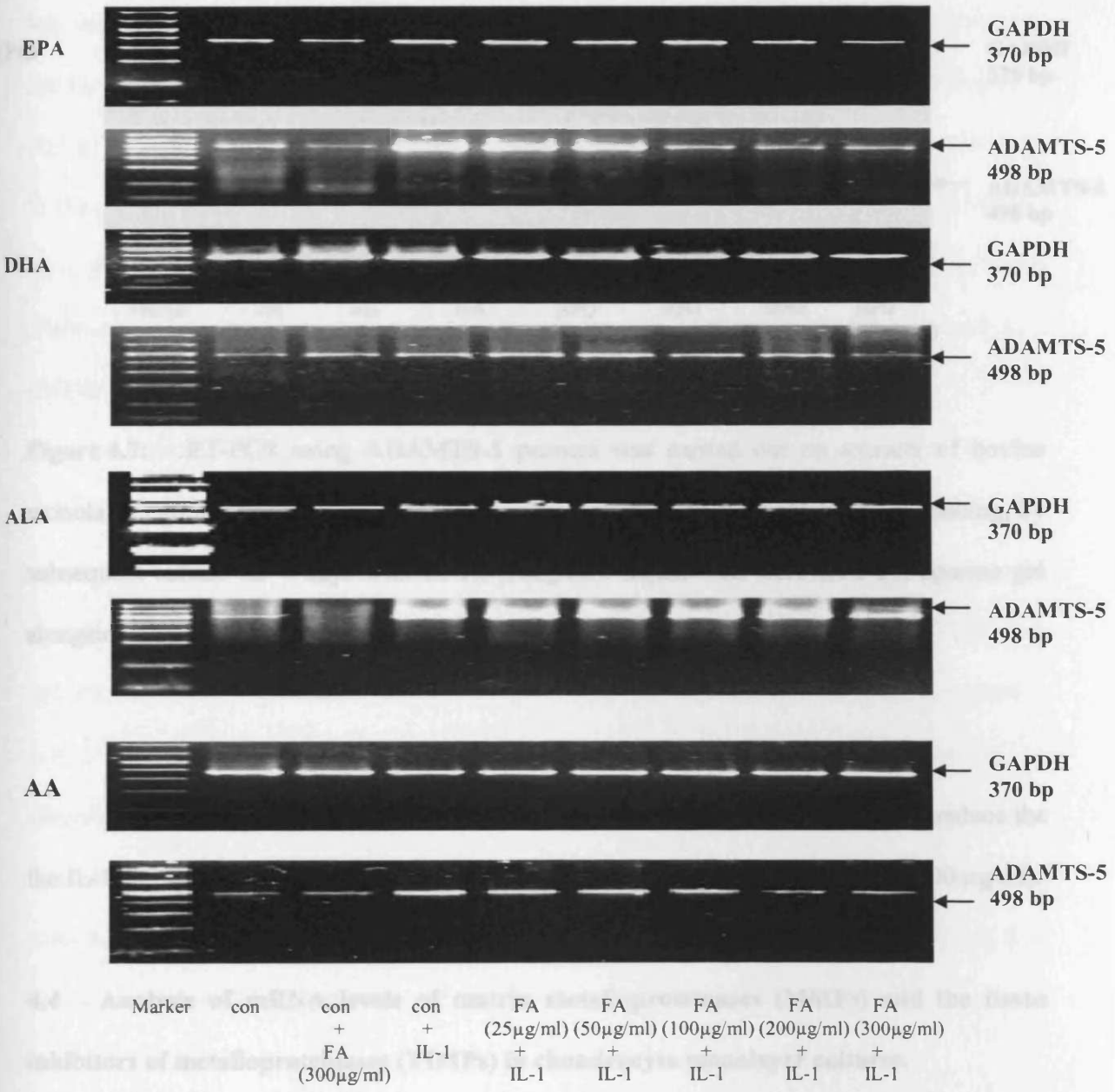


Figure 4.6: RT-PCR using ADAMTS-5 primer was carried out on extracts of bovine monolayer cultures treated with different fatty acids (FA) (EPA, DHA, ALA and AA) for 8 hours followed, after washing, by subsequent culture for 4 days with IL-1α (10ng/ml). Separations were on a 2% agarose gel alongside a 50-1000 bp marker.

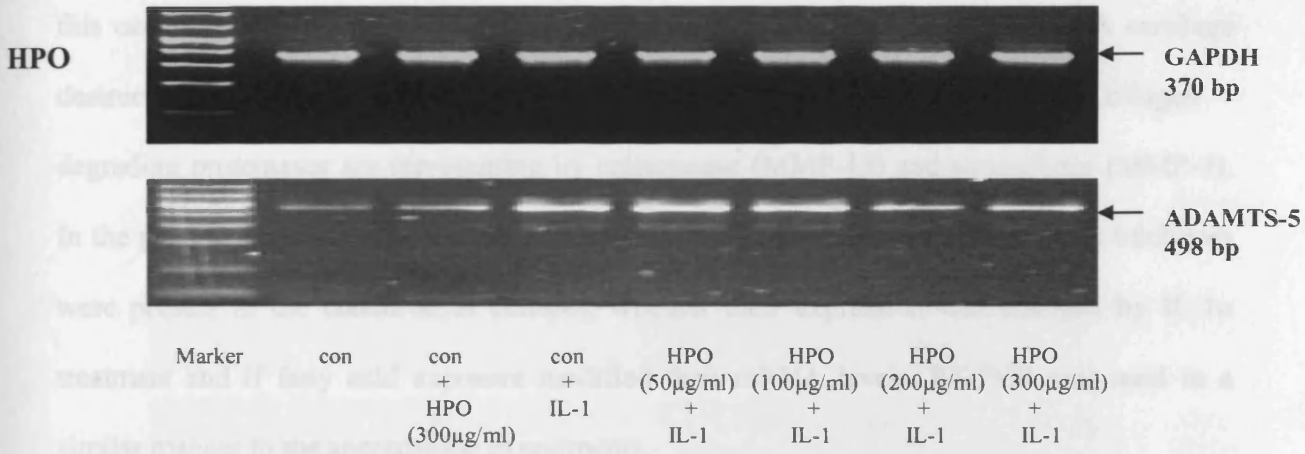


Figure 4.7: RT-PCR using ADAMTS-5 primers was carried out on extracts of bovine monolayer cultures treated with hydrolysed palm olein for 8 hours followed, after washing, by subsequent culture for 4 days with IL-1α (10ng/ml). Separations were on a 2% agarose gel alongside a 50-1000 bp marker.

As shown in Figure 4.7, incubation with hydrolysed palm olein appeared to reduce the the IL-1α stimulated expression of ADAMTS-s at the highest concentrations (200-300 µg/ml).

4.4 Analysis of mRNA levels of matrix metalloproteinases (MMPs) and the tissue inhibitors of metalloproteinases (TIMPs) in chondrocyte monolayer cultures.

Aggrecanase(s) is primarily responsible for the initial degradation of proteoglycan and loss of aggrecan from articular cartilage in the early stages of arthritic joint diseases that lead to overt collagen catabolism and disruption of the tissue integrity (Caterson *et al.*, 2000). At later stages, when degradation of soft connective tissue collagen is occurring, there is evidence for MMP-mediated degradation of the small proportion of aggrecan remaining in the tissue, but

this occurs independently of continued aggrecanase activity. Thus, complete OA cartilage destruction depends on both collagen and aggrecan degrading proteinases. Collagen – degrading proteinases are representing by collagenase (MMP-13) and stromelysin (MMP-3). In the present study, we examined if mRNAs for these enzymes and relevant tissue inhibitors were present in the chondrocyte cultures, whether their expression was changed by IL-1α treatment and if fatty acid exposure modified their mRNA levels. RT-PCR was used in a similar manner to the aggrecanase experiments.

As shown in Figure 4.8, message encoding MMP-3, was expressed at significant but somewhat variable levels in untreated monolayer cultures. MMP-3 mRNA levels were usually increased in response to treatment with IL-1α, further indicating a possible contribution by this enzyme to inflammatory disease processes in joint cartilage. Three *n-3*PUFAs were tested but only EPA (at 200 or 300μg/ml) was able to clearly reduce MMP-3 mRNA levels. Arachidonic acid had no effect on the levels of MMP-3 mRNA following IL-1α stimulation. In fact, as with α-linolenate, control levels of expression may have been increased following arachidonate treatment (Figure 4.8). Treatments of cultures with hydrolysed palm olein had no effect on mRNA levels for MMP-3 (Figure 4.9), although it has to be noted that, in this experiment, IL-1α treatment did not significantly increase the band intensity.

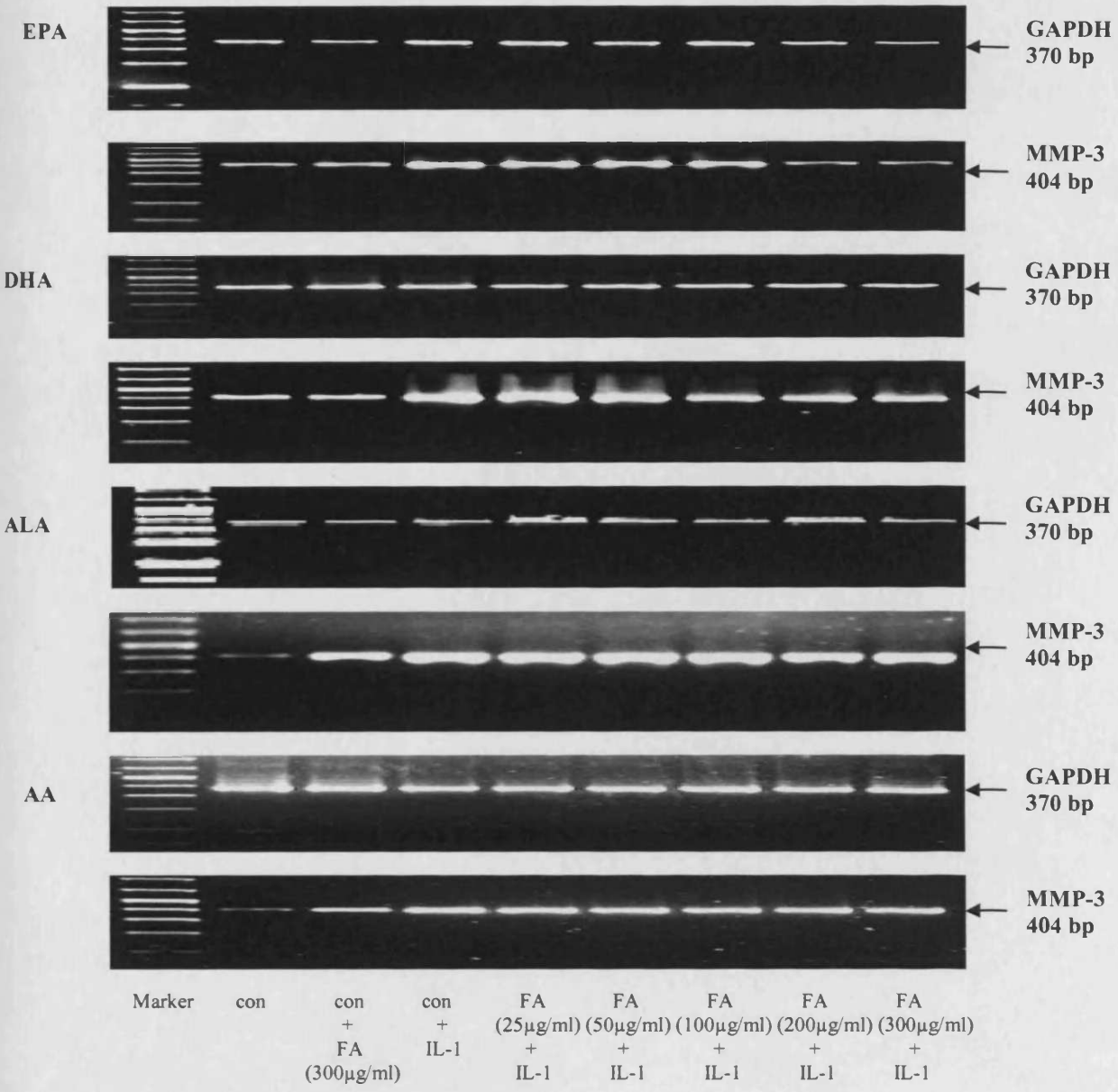


Figure 4.8: RT-PCR using MMP-3 primers was carried out on extracts of bovine monolayer cultures treated with different fatty acids (FA) (EPA, DHA, ALA and AA) for 8 hours followed, after washing, by subsequent culture for 4 days with IL-1α (10ng/ml). Separations were on 2% agarose gels alongside a 50-1000 bp marker.

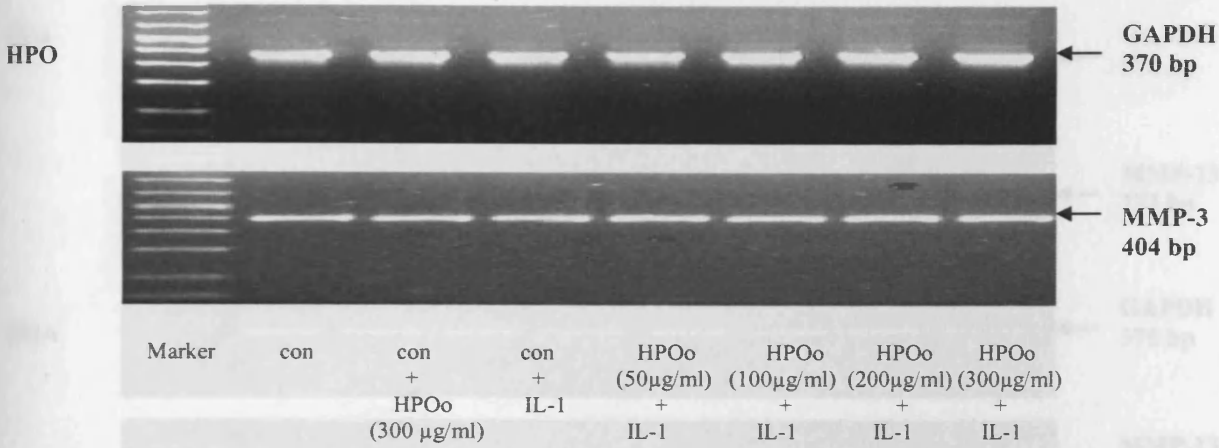


Figure 4.9: RT-PCR using MMP-3 primers was carried out on extracts of bovine monolayer cultures treated with hydrolysed palm olein for 8 hours followed, after washing, by subsequent culture for 4 days with IL-1α (10ng/ml). Separations were on 2% agarose gels alongside a 50-1000 bp marker.

Levels of MMP-13 mRNA were increased by IL-1α treatment in two of the experiments but remained at a constant, although substantial, level in the other two (Figure 4.10). Three *n-3* PUFAs and arachidonic acid were assessed for their ability to affect MMP-13 mRNA levels. No effect was found except for EPA. This fatty acid reduced IL-1α – stimulated mRNA levels when used at 200 or 300µg/ml. It might also have reduced basal expression in control cultures.

The bovine monolayer cultures supplemented with HPOo showed no significant decrease of MMP-13 mRNA in any sample (Figure 4.11), a similar result to that found for MMP-3 (Figure 4.9).

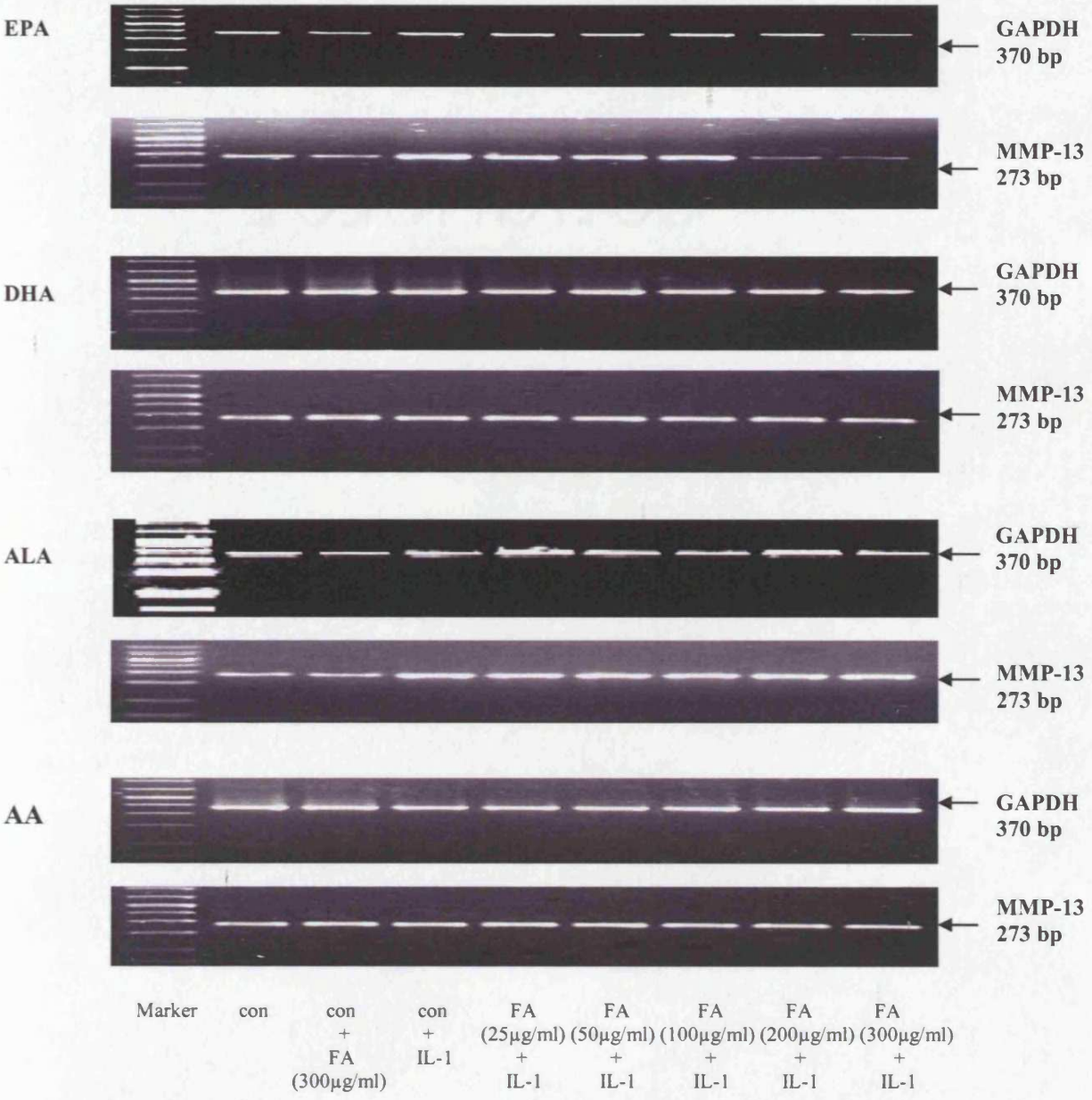


Figure 4.10: RT-PCR using MMP-13 primers was carried out on extracts of bovine monolayer cultures treated with different fatty acids (FA) (EPA, DHA, ALA and AA) for 8 hours followed, after washing, by subsequent culture for 4 days with IL-1α (10ng/ml). Separations were on 2% agarose gels alongside a 50-1000 bp marker.

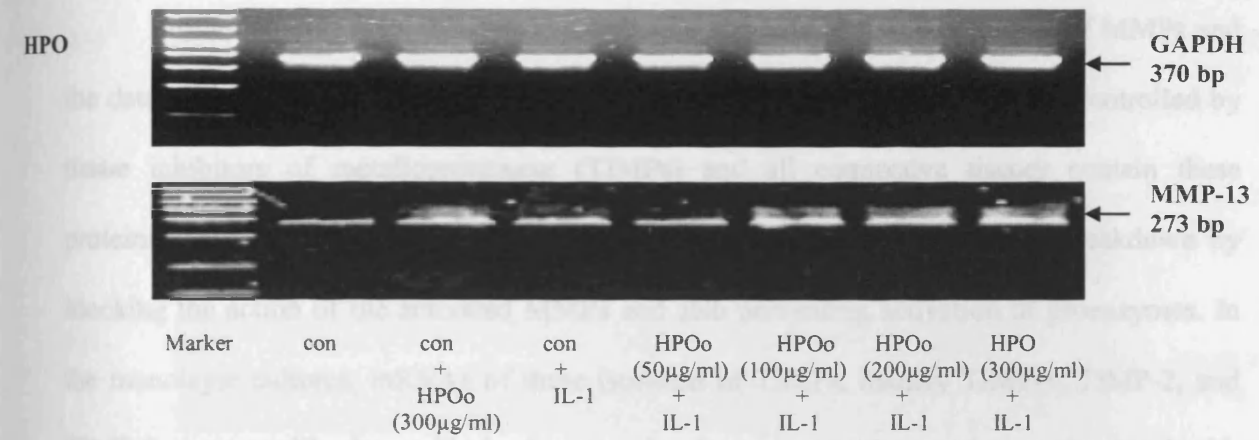


Figure 4.11: RT-PCR using MMP-13 primers was carried out extracts of the bovine monolayer cultures treated with hydrolysed palm olein for 8 hours followed, after washing, by subsequent culture for 4 days with IL-1α (10ng/ml). Separations were on a 2% agarose gel alongside a 50-1000 bp marker.

In summary, these data demonstrated that both MMP-3 and MMP-13 may contribute to the degradation of cartilage collagen since their expression could be stimulated by IL-1. From the results, however, the increase in MMP-3 was more consistent and obvious so that this enzyme may be given more importance for inflammatory responses. The mRNA levels for both enzymes were reduced by higher doses of EPA (200µg/ml and 300µg/ml) compared with cultures stimulated with IL-1α alone. The other fatty acids used did not produce any obvious effects. Assuming the mRNA changes are also reflected in the levels and activities of the proteinases, then EPA treatment might be useful in controlling damage due to MMP up-regulation.

Further studies were then made to examine the natural tissue inhibitors of MMPs and the data were shown in Figures 4.12 – 4.17. The activity of MMPs is normally controlled by tissue inhibitors of metalloproteinase (TIMPs) and all connective tissues contain these proteins. Thus, TIMPs play an important role in preventing unwanted tissue breakdown by blocking the action of the activated MMPs and also preventing activation of proenzymes. In the monolayer cultures, mRNAs of three isoforms of TIMPs, namely TIMP-1, TIMP-2, and TIMP-3, were readily detectable in the control cultures and in those cultures stimulated with IL-1α.

For TIMP-1, there was no change in mRNA that could be seen with any of the treatments (Figures 4.12, 4.13). Thus, IL-1α did not increase its expression nor did any of the pre-treatments with fatty acids alter the mRNA levels. For TIMP-2 (Figures 4.14, 4.15), there was again no change on IL-1α treatment.

For TIMP-3, the experiment with EPA showed a slight increase in mRNA levels with IL-1α treatment (Figure 4.16). This stimulated value was reduced back to control levels by the two highest concentrations of EPA (200μg/ml), 300 μg/ml). However, examination of these results in comparison to the GAPDH bands, suggested that loading was higher for the middle lanes. Thus, any effect of IL-1 or EPA was very slight if there was any effect at all. No changes were found for the other three treatments or for HPOo experiments (Figure 4.17).

Overall, the results indicate that TIMP mRNA levels remain unaffected by inflammatory conditions, at least under our experimental conditions, where the cytokine IL-1α was the mediator. Furthermore, treatment with fatty acids (whether *n-3* PUFAs, AA or HPOo) did not affect levels of the relevant mRNAs. Therefore, any effects that *n-3* PUFAs had on MMP degradation of connective tissue are likely to be on the enzymes themselves rather than on their tissue inhibitors.

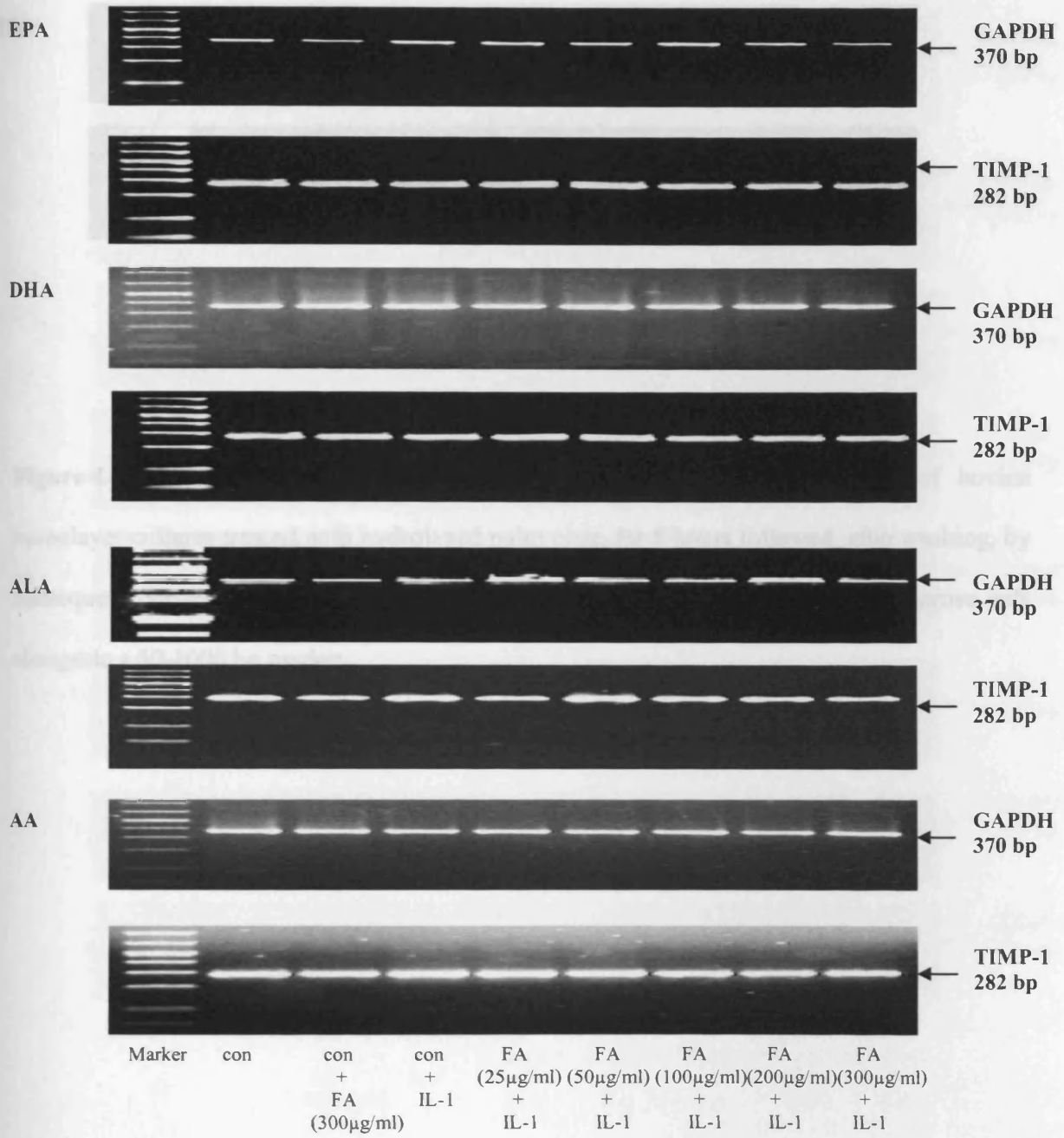


Figure 4.12: RT-PCR using TIMP-1 primers was carried out on extracts of bovine monolayer cultures treated with different fatty acids (FA) (EPA, DHA, ALA and AA) for 8 hours followed, after washing, by subsequent culture for 4 days with IL-1α (10ng/ml). Separations were on 2% agarose gels alongside a 50-1000 bp marker.

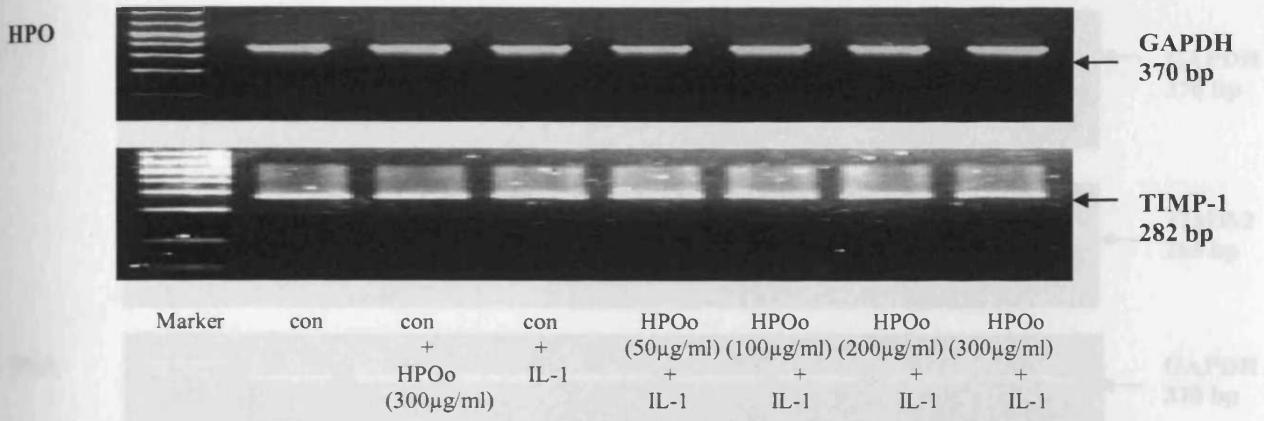


Figure 4.13: RT-PCR using TIMP-1 primers was carried out on extracts of bovine monolayer cultures treated with hydrolysed palm olein for 8 hours followed, after washing, by subsequent culture for 4 days with IL-1α (10ng/ml). Separations were on 2% agarose gels alongside a 50-1000 bp marker.

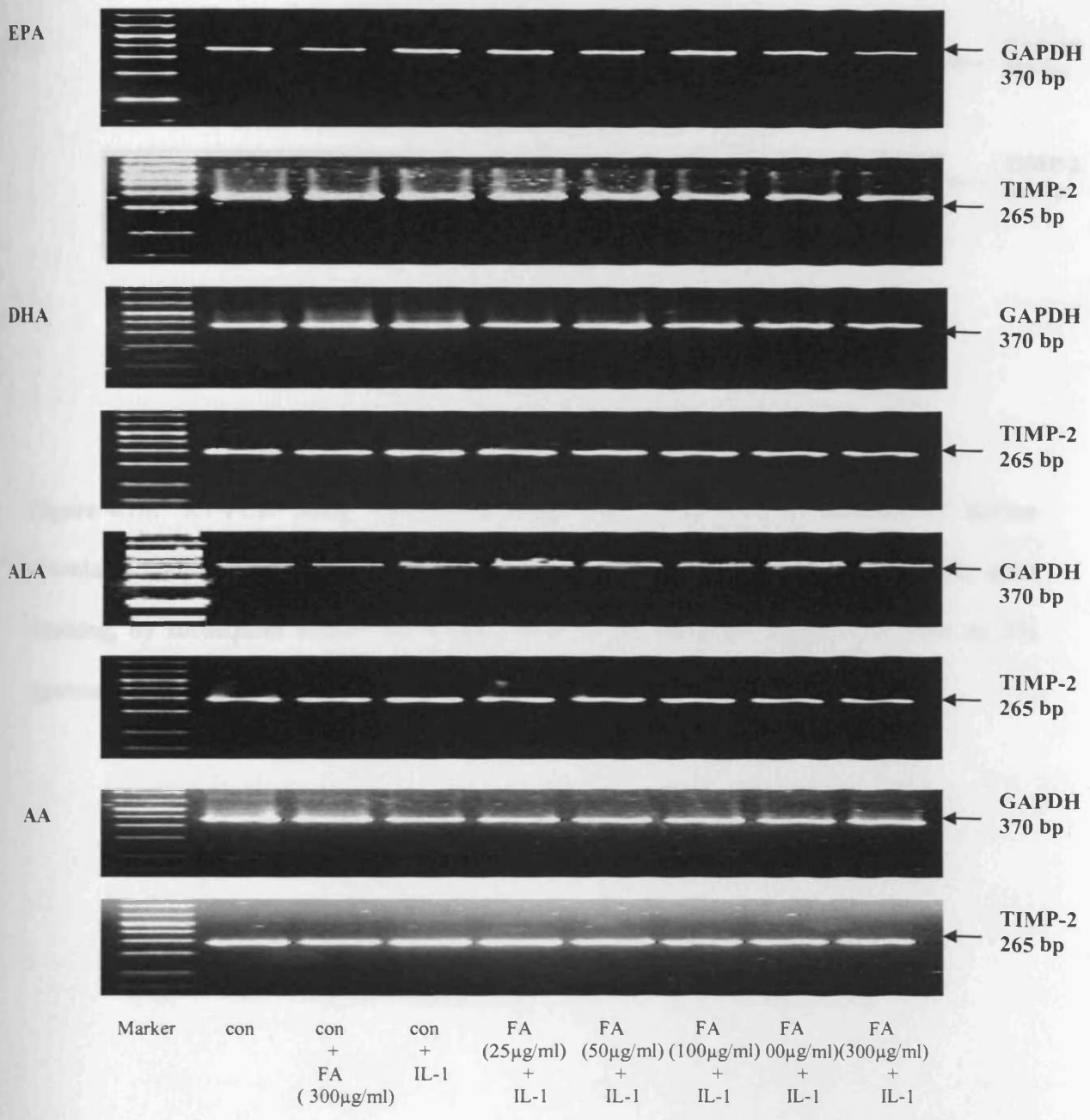


Figure 4.14: RT-PCR using TIMP-2 primers was carried out on extracts of bovine monolayer cultures treated with different fatty acids (FA) (EPA, DHA, ALA and AA) for 8 hours followed, after washing, by subsequent culture for 4 days with IL-1α (10ng/ml). Separations were on 2% agarose gels alongside a 50-1000 bp marker.

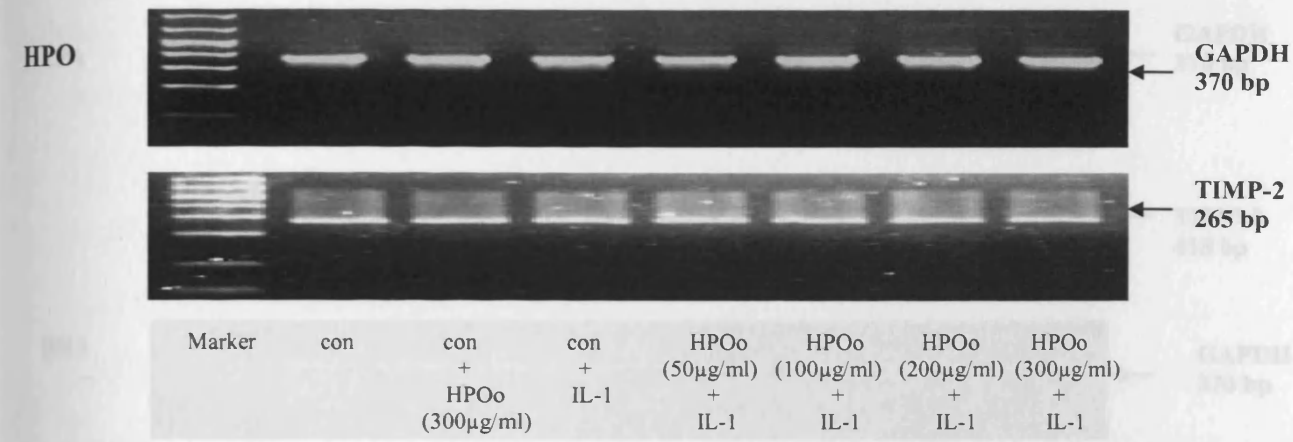


Figure 4.15: RT-PCR using TIMP-2 primers was carried out on extracts of bovine monolayer cultures treated with and hydrolysed palm olein for 8 hours followed, after washing, by subsequent culture for 4 days with IL-1α (10ng/ml). Separations were on 2% agarose gels alongside a 50-1000 bp marker.

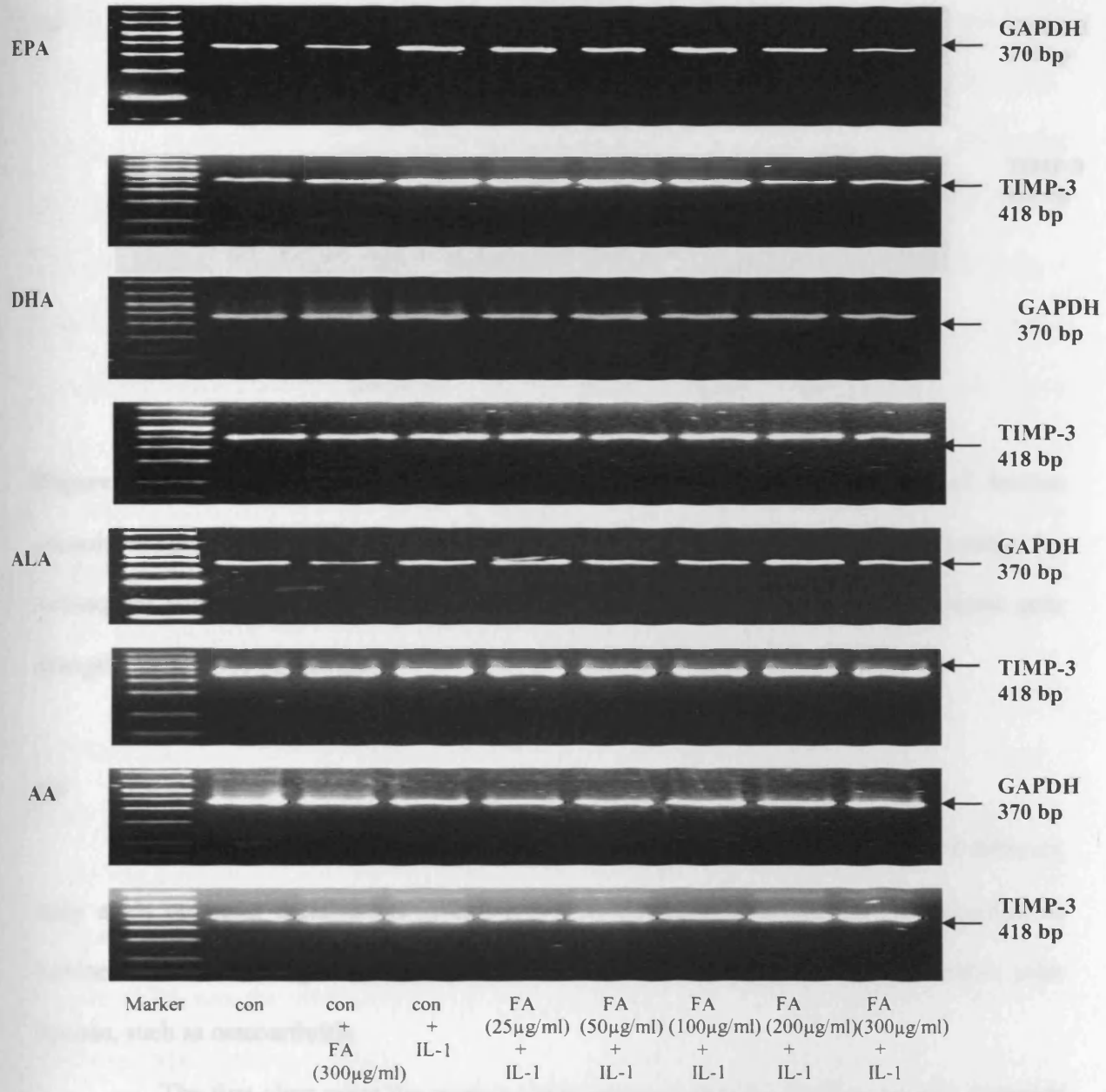


Figure 4.16: RT-PCR using TIMP-3 primers was carried out on extracts of bovine monolayer cultures treated with different fatty acids (FA), EPA, DHA, ALA and AA for 8 hours followed, after washing, by subsequent culture for 4 days with IL-1α (10ng/ml). Separations were on 2% agarose gels alongside a 50-1000 bp marker.

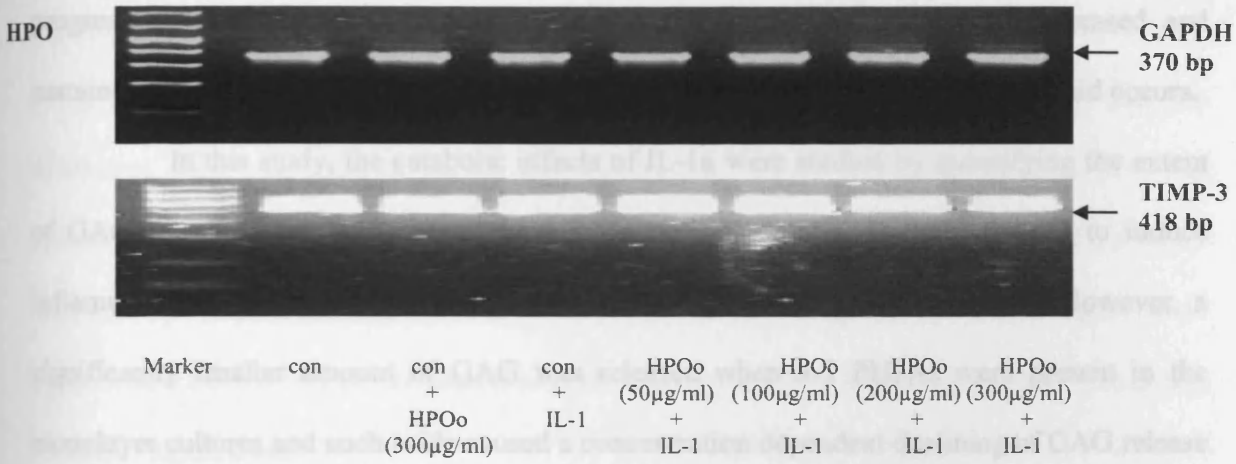


Figure 4.17: RT-PCR using TIMP-3 primers was carried out on extracts of bovine monolayer cultures treated with hydrolysed palm olein for 8 hours followed, after washing, by subsequent culture for 4 days with IL-1α (10ng/ml). Separations were on 2% agarose gels alongside a 50-1000 bp marker.

4.5 Discussion

This chapter presented the results of an investigation of the effects of different fatty acids on basal metabolism of cartilage and experimentally-induced inflammation in bovine articulate cartilage cultures, which mimic catabolic events during degenerative joint disease, such as osteoarthritis.

The first clear point the present study makes is that the cartilage usually responds to stimulation by IL-1α with increased GAG release. High concentrations of GAG are normally present in joint tissues, enabling the cartilage matrix to attract and absorb water molecules. Since these large molecules provide such a vital role in maintaining the biomechanical properties of cartilage, their depletion from the extracellular matrix leads to a reduction in its ability to recover from deformation in an effective manner. During the

progression of degenerative joint diseases and subsequent joint injury, an increased and sustained loss of aggrecan metabolites from the cartilage matrix into the synovial fluid occurs.

In this study, the catabolic effects of IL-1α were studied by quantifying the extent of GAG release from tissue maintained in monolayer culture. IL-1α was used to induce inflammation but this did not usually increase GAG release into the medium. However, a significantly smaller amount of GAG was released when *n-3* PUFAs were present in the monolayer cultures and such acids caused a concentration dependent declining of GAG release from IL-1α stimulated cultures. Bovine cartilage cultured with EPA reveal the most considerable ($p<0.05$) decline in the released of GAG, followed by DHA and ALA. This suggests that EPA has a protective effect in reducing cartilage degradation in the presence of IL-1α. AA and hydrolysed palm olein acids had little, if any, effect.

We found that mRNA for ADAMTS-5 was detectable in control cultures and that ADAMTS-4 was sometimes detected at low levels. ADAMTS-4 and ADAMTS-5 are the two most important proteinases responsible for the early degradation of aggrecan in arthritis. There was a significant increase in mRNA levels for ADAMTS-4 when IL-1α was added. This increased level of mRNA for ADAMTS-4 was reduced with *n-3* PUFAs in a dose-dependent manner. EPA was the most effective of the *n-3* PUFAs. AA and hydrolysed palm olein did not show any significant effects with ADAMTS-4 mRNA. ADAMTS-5 mRNA increased with IL-1α treatment but not as obviously as with ADAMTS-4. All three *n-3* PUFAs were able to reduce the ADAMTS-5 mRNA, with EPA again being the most effective.

The mRNA for MMP-3 was expressed at significant levels in the monolayer cultures. On challenge with IL-1α, the mRNA levels were usually increased but only EPA, at high concentrations, was able to reduce them. The MMP-13 mRNA was also expressed

significantly in untreated cultures. IL-1α had little effect, if any, on its levels and again, only EPA, at high concentrations, could cause any reduction. No fatty acid treatment had any detectable effect on mRNA levels for TIMPS -1, -2 or -3. These data suggest that inflammation induced by lL-1α only affected MMP expression and not that of their tissue inhibitors. Only EPA had any effect on MMP expression but, together with its strong effect on aggrecanase mRNA levels, the results suggest that this *n-3* PUFA could be of use in reducing cartilage degradation.

4.6 Chapter Summary

- All three *n-3* PUFAs tested, EPA, DHA and ALA reduced cartilage degradation as measured by GAG release ($p<0.05$). EPA was most effective and showed a clear dose-dependent response. The *n-6* fatty acid, AA and hydrolysed palm olein did not significantly change GAG release in the presence of IL-1α.
- Levels of ADAMTS-4 mRNA were increased by IL-1α and these were reduced by *n-3* fatty acids with EPA most effective. In contrast to ADAMTS-4, expression of ADAMTS-5 appeared constitutive. However, IL-1α usually increased its mRNA levels DHA and especially, EPA reduced the mRNA levels for ADAMTS-5.
- The matrix metalloproteinase MMP-3 and, to a lesser content, MMP-13 showed increases in their mRNAs on IL-1α stimulation. These were reduced by high concentrations of EPA. No other fatty acid treatment appeared to affect the mRNA levels for these proteinases.
- Supplementation with fatty acids had no effect on the level of tissue metalloproteinase inhibitor (TIMPs) mRNAs in either control or IL-1α treated cultures.

CHAPTER FIVE

Effects of different fatty acids on expression mRNA for COX-2 and inflammation – inducible cytokines in a chondrocyte culture system

5.1 Introduction

Arthritis is a chronic, systemic, inflammatory disease characterized by hyperplasia of synovial lining cells and extensive destruction of the articular cartilage. When a joint is injured, the body releases enzymes that may further breakdown the already damaged articular cartilage (Cimpean *et al.*, 2000). Arachidonic acid may be released into the cell when a cell membrane is injured and it can then be oxidized by cyclooxygenase (COX) enzymes. The by-products of this reaction, endoperoxides, are converted into prostaglandins (Dubois *et al.*, 1998). COX is a key regulatory enzyme in the synthesis of prostaglandins (PG), which are important mediators of inflammation in arthritis (Kurumbail *et al.*, 2001). Up regulation of COX-2 mRNA and protein is observed in explants of arthritic cartilage, together with induction of PGE_2 production. COX-2 is the enzyme responsible for the biosynthesis of inflammatory prostaglandins (Mitchell *et al.*, 1999). In contrast to COX-1, it is inducible and has been detected in the synovial tissue of patients with OA, but not in synovial tissue from normal patients. This is different to COX-1 mRNA which is constituently expressed in almost all cell types (Vane *et al.*, 1998).

The current recommended treatment of arthritis involves the use of pain relievers, including non-steroidal anti-inflammatory drugs (NSAIDs). These drugs prevent some aspects of inflammation by inhibiting cyclooxygenases, however, they do not reverse the

degenerative process in arthritis and show some adverse effects on cartilage metabolism (Kurumbail *et al.,* 2001). In fact, NSAIDs represent a major risk for mobility and mortality from gastrointestinal disturbances, perforation, ulcers and bleeding. Moreover, NSAIDs inhibit both COX-1 and COX-2 whereas inflammation involves COX-2. This has led to the development of COX-2 – specific NSAIDs (Dubois *et al.,* 1998), although their clinical use is in some doubt currently, due to unforeseen adverse reactions (Drazen, 2005).

Previous *in vitro* studies have shown that the pro-inflammatory cytokines interleukin-1 (IL-1) and tumour necrosis factor–α (TNF-α), strongly inhibit the expression of the cartilage extracellular matrix genes and also stimulate the production of COX-2 enzyme. The expression of cartilage-specific genes, such as those for collagen and aggrecan, is inhibited by both IL-1 and TNF-α (Badger *et al.,* 1999). These inhibitory effects have been implicated in the breakdown of cartilage in arthritis, as IL-1 and TNF-α are produced by synovial cells in arthritic lesions and are present at elevated levels in synovial fluid of osteoarthritic and rheumatoid arthritic patients (Cimpean *et al.,* 2000).

More recently, the potential benefits of *n-3* PUFAs have received a great deal of attention. Our laboratory has shown that *n-3* PUFAs, such as those from fish oil, (but not other classes of fatty acids) could reduce COX-2 mRNA levels (Hurst *et al.,* 2002) but not those of COX-1 and suppress the production of inflammatory cytokines that cause cartilage loss (Curtis *et al.,* 2000; 2002).

Moreover, the clinical benefits of dietary supplementation with *n-3* PUFAs have been suspected for a number of years and there is increasing evidence for their advantages (Calder, 2002). *n-3* PUFAs have also been shown to be chondroprotective and, thus, may stop the progression of cartilage degradation caused by cytokine-induced inflammatory mediators and degradative enzymes (Darlington and Stone, 2001).

The results presented in this chapter, were from experiments to investigate the relative efficacy of different fatty acids (such as EPA, DHA, ALA, AA and hydrolysed palm olein) on mRNA levels for cyclooxygenases (COX-2 and COX-1) and the inflammatory cytokines, IL-1 and TNF-α. This followed on from the basic studies on the metabolism of the bovine articular cartilage system as described in Chapter 3 and 4.

5.2 Effect of different fatty acids on the expression of mRNA levels of the cyclooxygenases, COX-1 and COX-2

COX-1 and COX-2 were investigated in bovine chondrocyte monolayer cultures supplemented with eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), α-linolenic acid (ALA), arachidonic acid (AA) or hydrolysed palm olein (HPOo). The differential levels of mRNAs for COX-1 and COX-2 in these cultures were characterized using reverse transcription-polymerase chain reaction (RT-PCR). IL-1α stimulated cultures were compared to unstimulated preparations in order to study the ability of the different fatty acids to reduce the inflammatory response induced.

As shown in Figures 5.1 and 5.2, the mRNA for COX-1 was detected in all samples. IL-1α addition had no apparent effect on its level and no fatty acid appeared to change the band intensities. Thus, COX-1 appeared to be constitutive in those cultures and was not altered by factors promoting or, possibly, reducing inflammation.

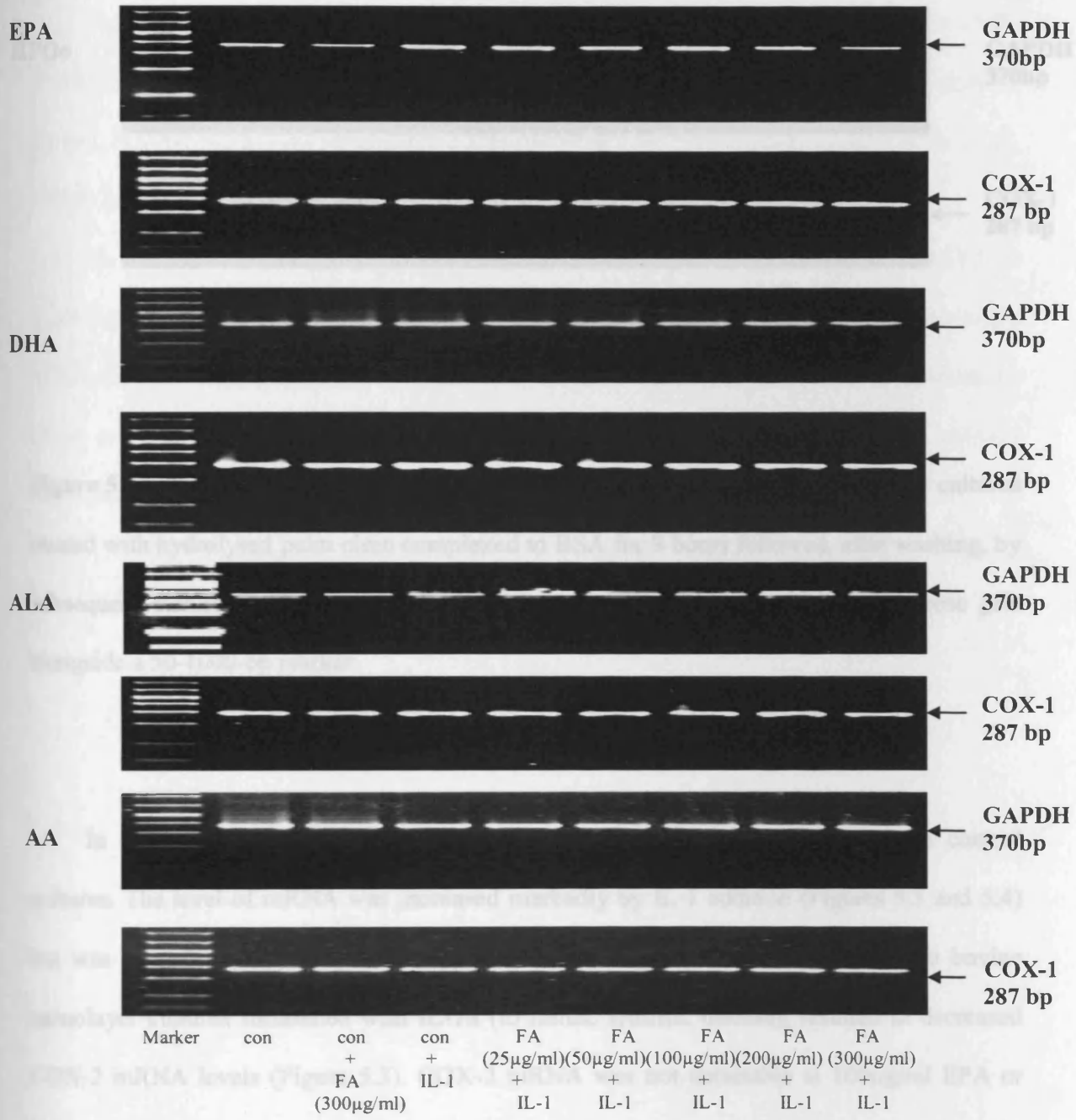


Figure 5.1: RT-PCR using COX-1 primers was carried out on bovine monolayer cultures treated with various fatty acid supplements (as detailed) complexed to BSA for 8 hours followed, after washing, by subsequent culture for 4 days with IL-1α (10ng/ml). Separations were on 2% agarose gels alongside a 50-1000 bp marker.

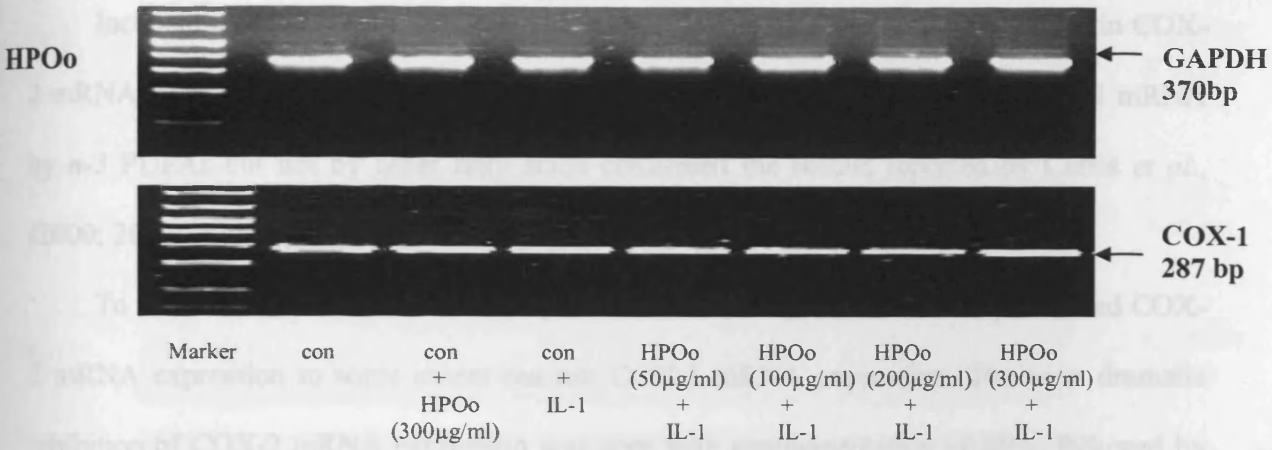


Figure 5.2: RT-PCR using COX-1 primers was carried out on bovine monolayer cultures treated with hydrolysed palm olein complexed to BSA for 8 hours followed, after washing, by subsequent culture for 4 days with IL-1α (10ng/ml). Separations were on 2% agarose gels alongside a 50-1000-bp marker.

In contrast to COX-1, the mRNA for COX-2 was usually undetectable in control cultures. The level of mRNA was increased markedly by IL-1 addition (Figures 5.3 and 5.4) but was not affected by fatty acid supplementation alone. EPA supplementation to bovine monolayer cultures stimulated with IL-1α (to mimic arthritic disease), resulted in decreased COX-2 mRNA levels (Figure 5.3). COX-2 mRNA was not detectable at 100µg/ml EPA or higher.

Two other *n-3* PUFAs were also tested, DHA and ALA. Both of these were also able to reduce COX-2 mRNA levels but only at the highest concentrations (300µg/µl) (Figure 5.3). DHA appeared to be more effective than ALA. In contrast, the *n-6* PUFA, AA, had no detectable effect on COX-2 mRNA levels.

Incubation of the bovine cultures with hydrolysed palm olein showed no effect in COX-2 mRNA levels with increasing concentrations (Figure 5.4). The reduction in COX-2 mRNA by *n-3* PUFAs but not by other fatty acids confirmed the results reported by Curtis *et al.,* (2000; 2002).

To summarize, all the three *n-3* fatty acids tested (EPA, DHA and ALA) inhibited COX-2 mRNA expression to some extent but not COX-1 mRNA expression. The most dramatic inhibition of COX-2 mRNA expression was seen with supplementation of EPA, followed by DHA and ALA. There was no inhibition of COX-2 mRNA expression in the cultures supplemented with AA or with hydrolysed palm olein.

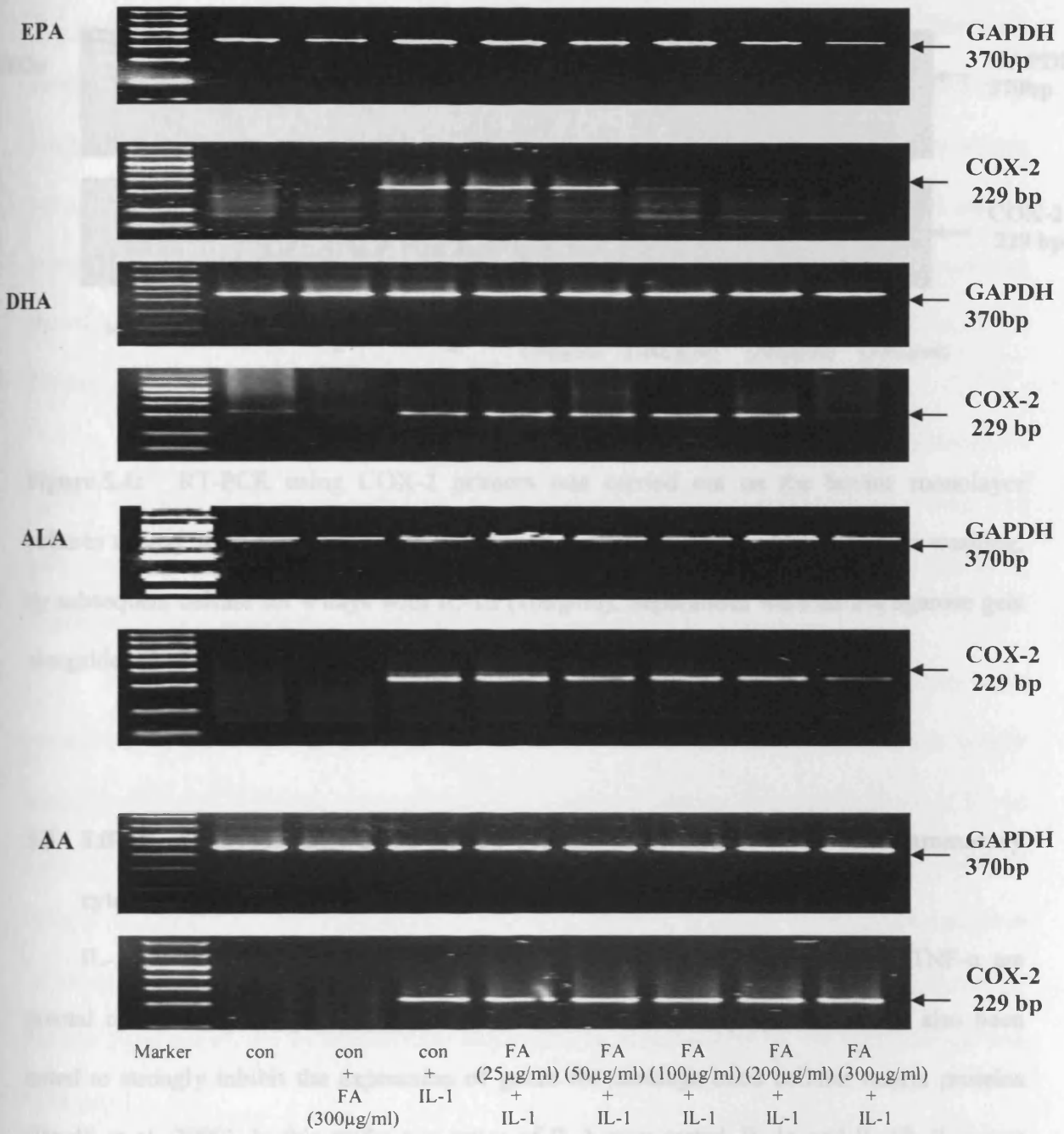


Figure 5.3: RT-PCR using COX-2 primers was carried out on bovine monolayer cultures treated with fatty acids complexed to BSA (as detailed) for 8 hours followed, after washing, by subsequent culture for 4 days with IL-1α (10ng/ml). Separations were on 2% agarose gels alongside a 50-1000-bp marker.

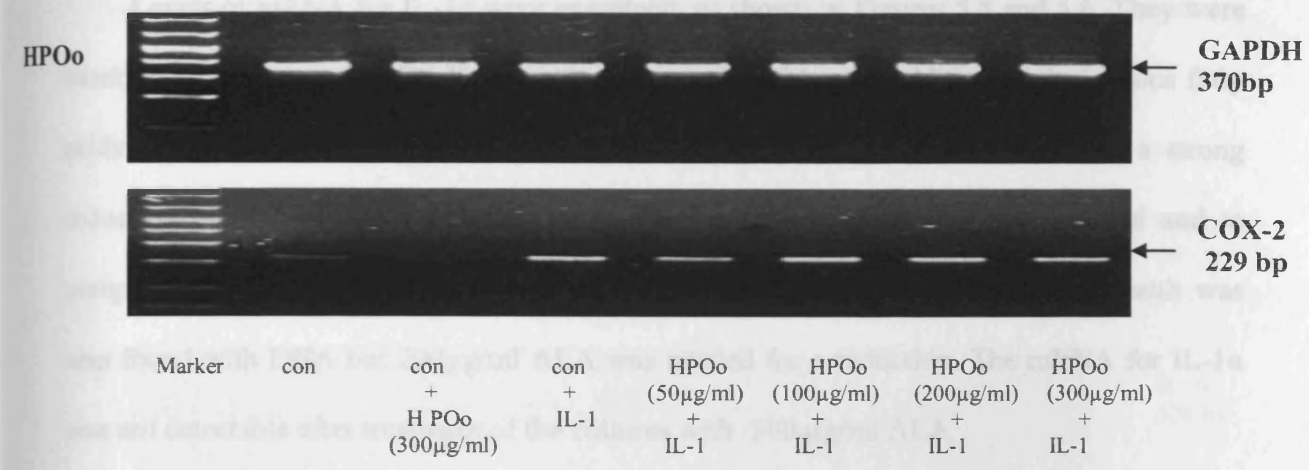


Figure 5.4: RT-PCR using COX-2 primers was carried out on the bovine monolayer cultures treated with hydrolysed POo complexed to BSA for 8 hours followed, after washing, by subsequent culture for 4 days with IL-1α (10ng/ml). Separations were on 2% agarose gels alongside a 50-1000-bp marker.

5.3 Effects of various fatty acids on the expression of mRNA levels of inflammatory cytokines (TNF- α, IL-1α, IL-1β).

IL-1 (IL-1α and IL-1β have varying effects, depending on the tissue) and TNF-α are pivotal cytokines in the process of arthritis. These inflammatory cytokines have also been noted to strongly inhibit the expression of genes for cartilage extra cellular matrix proteins (Bordji *et al.,* 2000). In this study, two types of IL-1 were tested, IL-1α and IL-1β. IL-α was used to induce inflammation in the bovine cultures because they are more sensitive to this isoform (Legare *et al.,* 2002). Both IL-1α and IL-1β mRNA were measured on the effect of fatty acid supplementation in bovine monolayer cultures treated with or without IL-1 α.

Levels of mRNA for IL-1α were examined, as shown in Figures 5.5 and 5.6. They were barely detectable in control cultures and were not altered by the addition of the various fatty acids complexed to BSA. However, IL-1α challenge to the cultures resulted in a strong induction of mRNA levels. EPA pre-treatment was able to reduce this at 50μg/ml and to completely abolish mRNA levels at higher concentration (Figure 5.5). The same result was also found with DHA but 200μg/ml ALA was needed for a reduction. The mRNA for IL-1α was not detectable after treatment of the cultures with 300 μg/ml ALA.

In contrast to the results with *n-3* PUFAs, AA did not reduce the IL-1α induced levels of mRNA (Figure 5.5). For the hydrolysed palm olein the results are unclear (Figure 5.6). The palm olein may have caused a slight decrease in IL-1α mRNA but, since this did not appear to be concentration-dependent, it is difficult to judge its reliability.

Even though the IL-1α is the most studied, IL-β is considered to play an important role in arthritis by its ability to stimulate chondrocytes to synthesize aggrecanases and matrix metalloproteinases (MMPs), although the effects are not usually as strong as those of IL-1α (Legare *et al.*, 2002). Prolonged exposure of articular tissue to IL-1β can provoke a variety of cellular inflammatory responses and IL-β has been reported to inhibit proteoglycan synthesis in chondrocytes in 3 day-old cultures (Mukundan *et al.*, 2002). In view of this background, the ability of *n-3* PUFA to suppress the expression of IL-1β mRNA in the chondrocytes was examined.

Little, if any, expression of IL-1β expression was observed in unstimulated cultures or in those incubated with fatty acids alone (Figures 5.7 and 5.8). However, incubation of the cultures with IL-1α induced a strong mRNA band. This was eliminated by pre-incubation with 200μg or 300μg/ml EPA and reduced by pre-incubation with 300μg/ml DHA and slightly with

300 μg/ml ALA (Figure 5.7). Use of AA did not reduce the mRNA levels of IL-1β following incubation of the cultures with IL-1α.

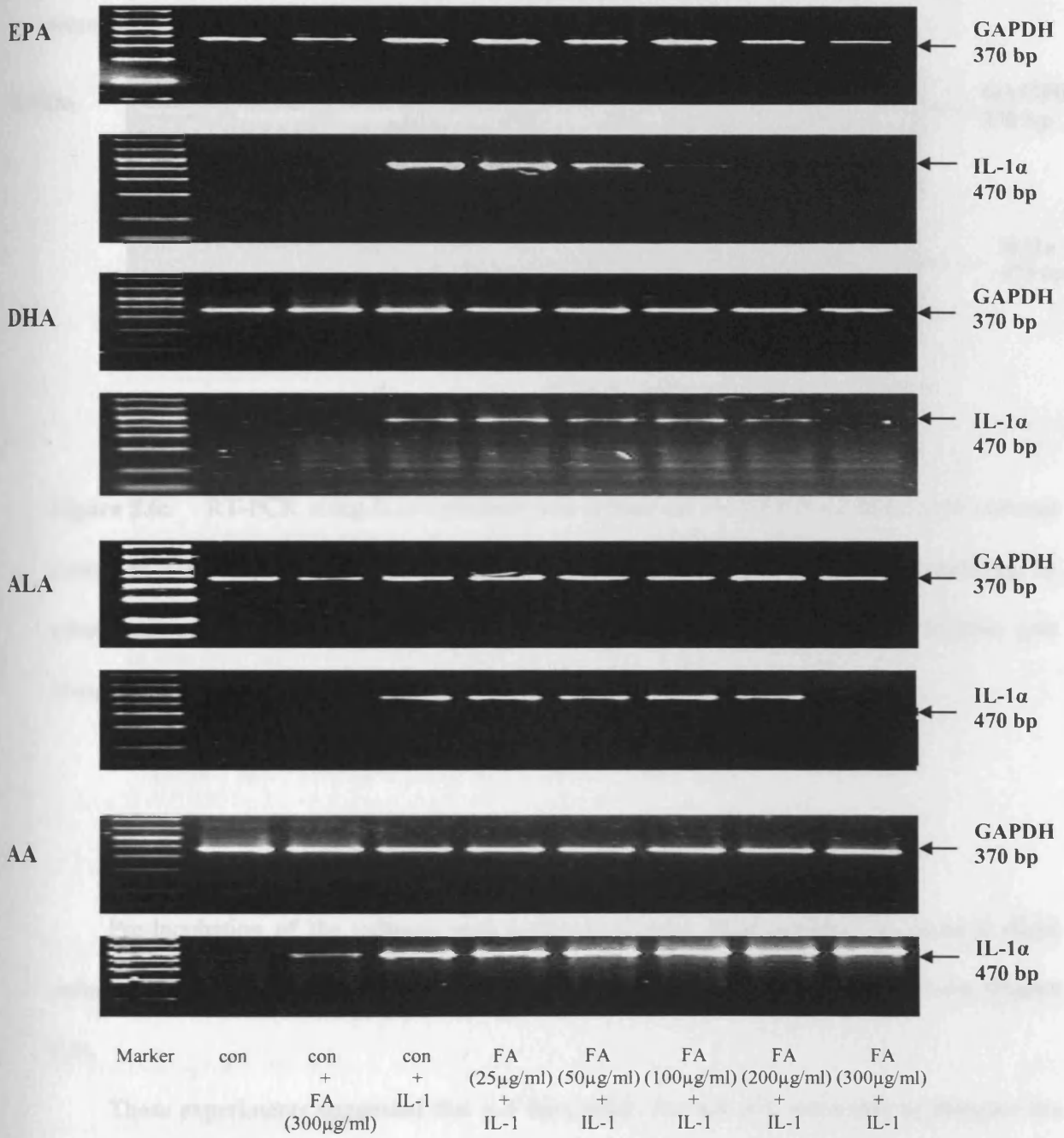


Figure 5.5: RT-PCR using IL-1α primers was carried out on the bovine monolayer cultures treated with different fatty acids, EPA, DHA, ALA, AA complexed to BSA for 8 hours followed, after washing, by subsequent culture for 4 days with IL-1α (10ng/ml). Separations were on 2% agarose gels alongside a 50-1000bp marker.

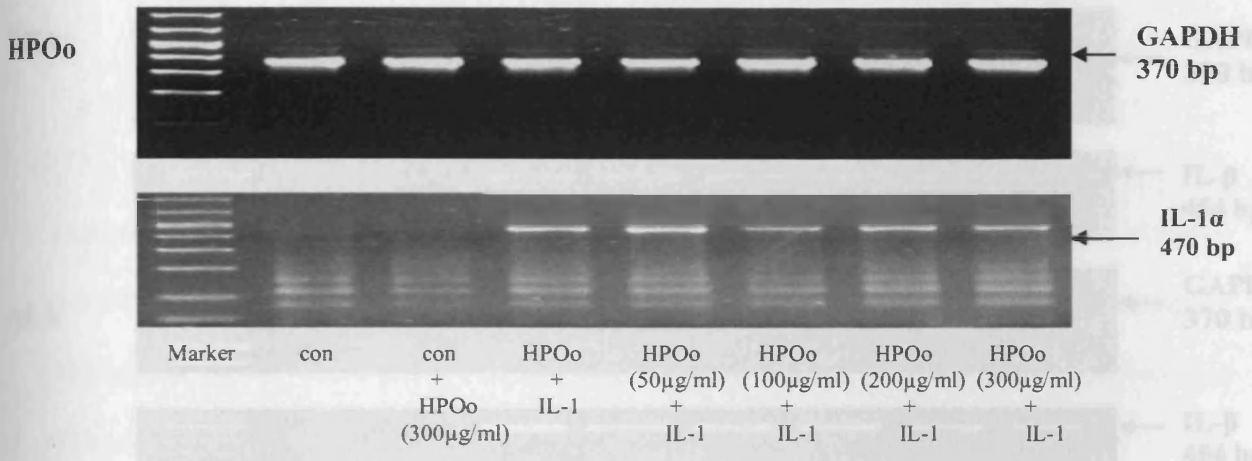


Figure 5.6: RT-PCR using IL-1α primers was carried out on the bovine monolayer cultures treated with hydrolysed palm olein complexed to BSA, for 8 hours followed, after washing, by subsequent culture for 4 days with IL-1α (10ng/ml). Separations were on 2% agarose gels alongside a 50-1000bp marker.

Pre-incubation of the cultures with hydrolysed palm olein appeared to cause a slight reduction in the mRNA for IL-1β following stimulation of the cultures with IL-1α (Figure 5.8).

These experiments suggested that *n-3* fatty acids, but not AA, were able to abrogate the IL-1α- stimulated expression of mRNA for both IL-1α and IL-1β. EPA seemed to be the most effective of the *n-3* PUFAs tested.

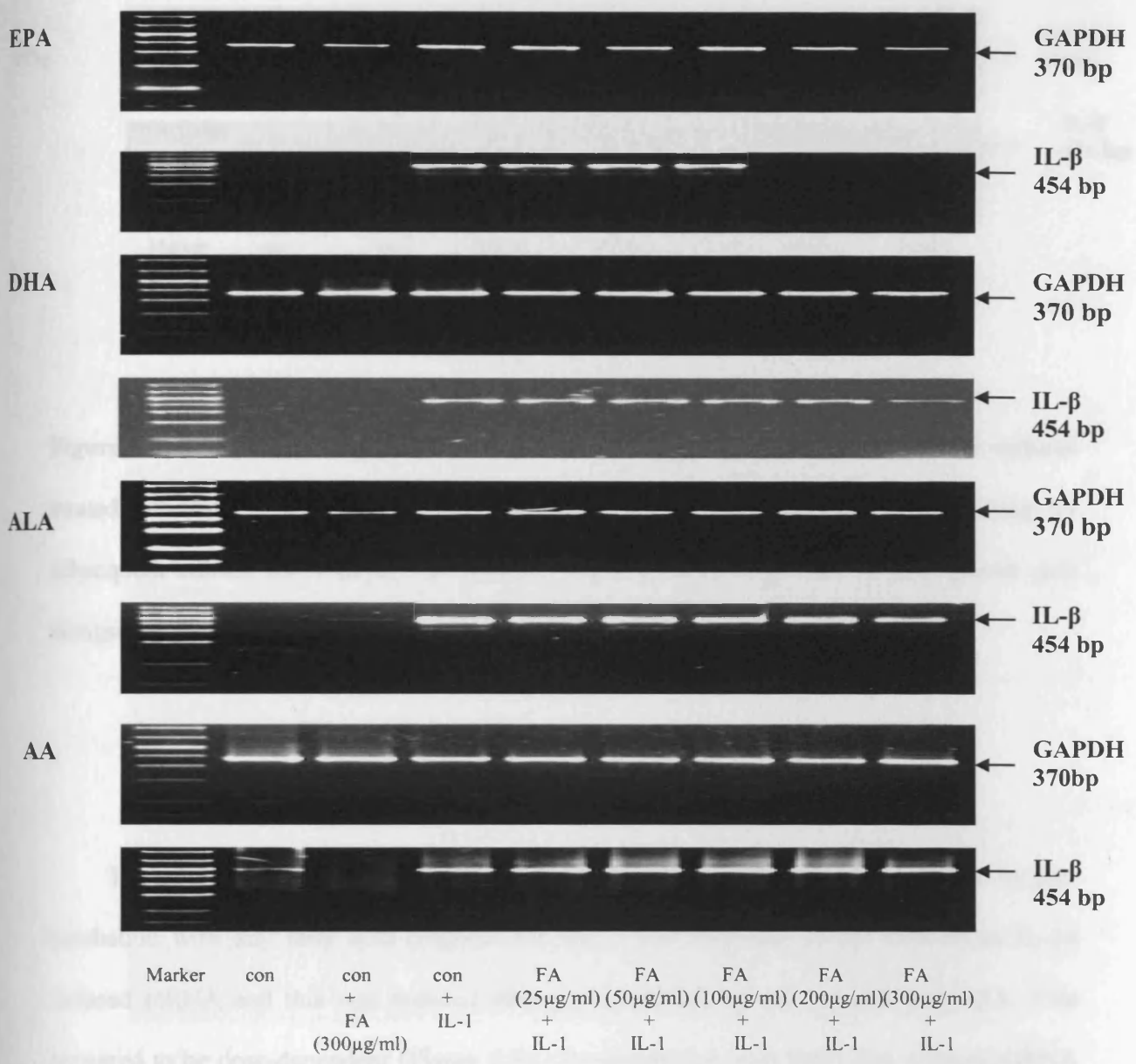


Figure 5.7: RT-PCR using IL-1β primers was carried out on bovine monolayer cultures treated with different fatty acids complexed to BSA for 8 hours followed, after washing, by subsequent culture for 4 days with IL-1α (10ng/ml). Separations were on 2% agarose gels alongside a 50-1000 bp marker.

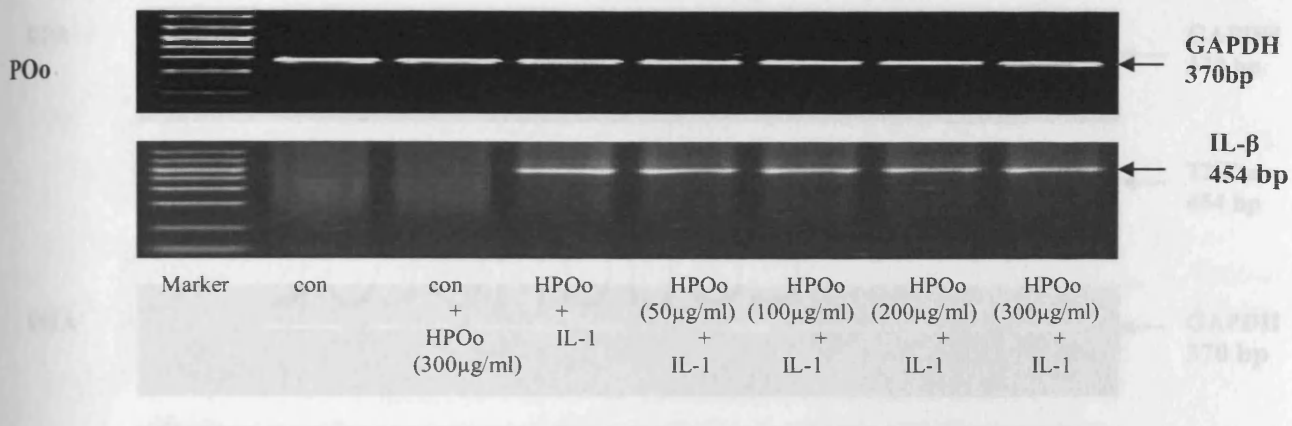


Figure 5.8: RT-PCR using IL-1β primers was carried out on the bovine monolayer cultures treated with hydrolysed palm olein complexed to BSA, for 8 hours followed, after washing, by subsequent culture for 4 days with IL-1α (10ng/ml). Separation was on 2% agarose gels alongside a 50-1000 bp marker.

The mRNA for TNF-α was not detectable in control cultures, nor was it induced by pre-incubation with any fatty acid (Figures 5.9 and 5.10). Exposure of the cultures to IL-1α induced mRNA and this was reduced after pre-incubation of the cultures with EPA. This appeared to be dose-dependent (Figure 5.9). Pre-incubation with DHA also reduced mRNA levels although this acid did not appear to be quite as affective as EPA. ALA also reduced mRNA levels for TNF-α but only at the two highest concentrations (200 and 300 µg/ml).

In contrast, pre-incubation with AA did not appear to have any effect on mRNA levels for TNF-α (Figure 5.9). The results for hydrolysed palm oil were equivocal and did not show any obvious reduction in the IL-1α induced mRNA levels (Figure 5.10).

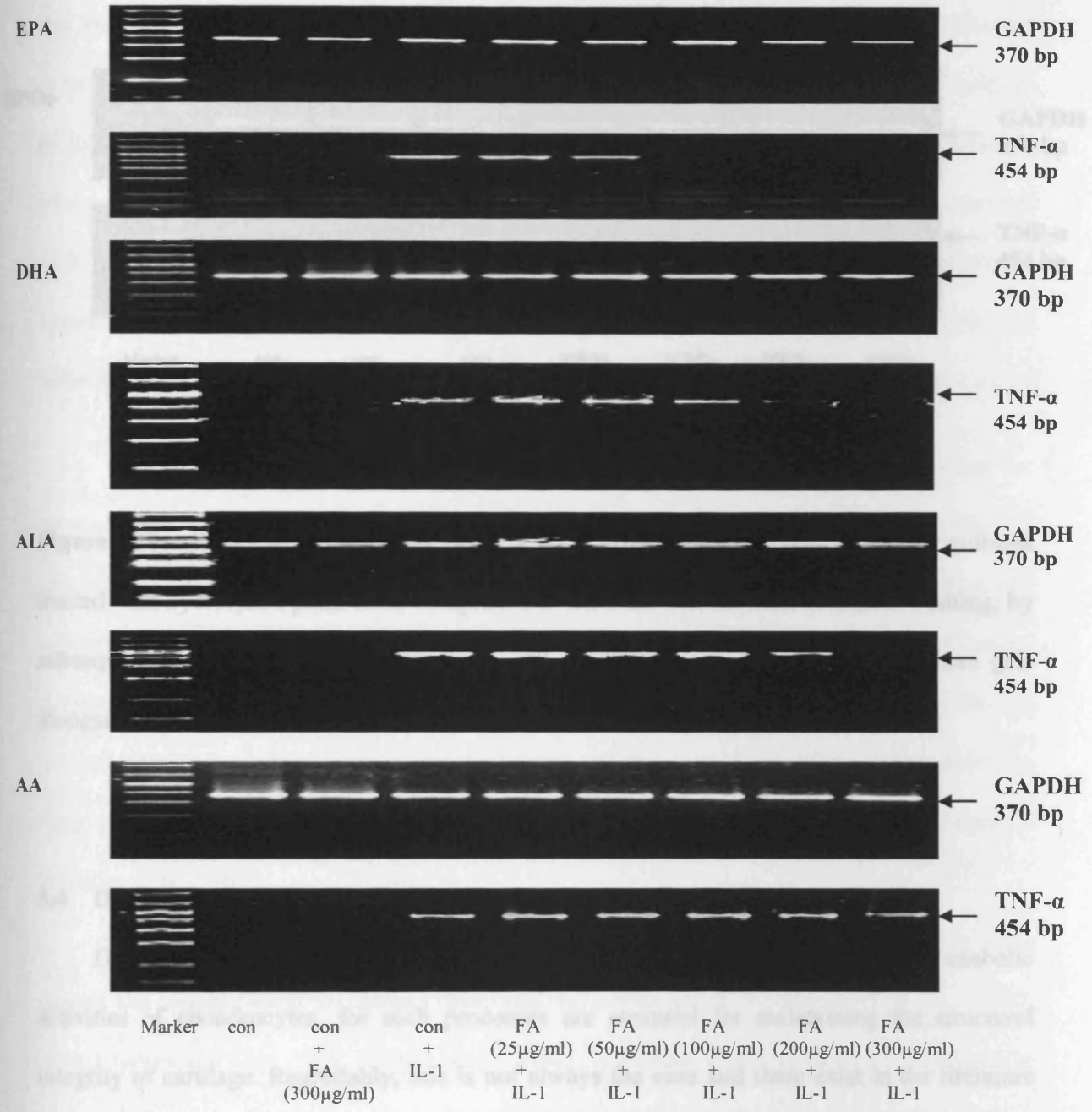


Figure 5.9 Comparison by RT-PCR using TNF-α primers was carried out on extracts from bovine monolayer cultures treated with different fatty acids complexed to BSA for 8 hours followed, after washing, by subsequent culture for 4 days with IL-1α (10ng/ml). Separation was on a 2% agarose gel alongside a 50-1000 bp marker.

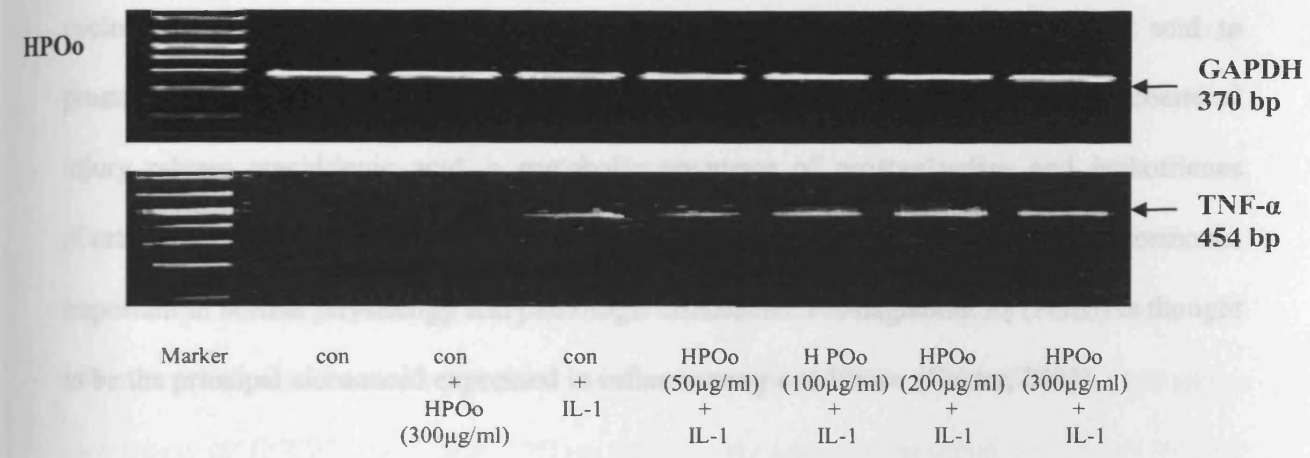


Figure 5.10: RT-PCR using TNF-α primers was carried out on bovine monolayer cultures treated with hydrolyzed palm olein complexed to BSA for 8 hours followed, after washing, by subsequent culture for 4 days with IL-1α (10ng/ml). Separation was on 2% agarose gels alongside a 50-1000 bp marker.

5.4 Discussion

Drugs used to suppress arthritis or synovial inflammation should not reduce the anabolic activities of chondrocytes, for such processes are essential for maintaining the structural integrity of cartilage. Regrettably, this is not always the case and there exist in the literature numerous *in vitro* and *in vivo* studies which indicate that some non-steroidal anti-inflammatory drugs (NSAIDs) cause effects which are detrimental to the preservation of the cartilage matrix (Raskin, 1999; Dingle, 1999).

Therapeutic agents are widely used in the treatment of arthritis patients. NSAIDs represent the most widely-prescribed drugs on the market and are effective for decreasing pain

and inflammation. Originally, their mechanism of action was discovered to be the inhibition of cyclooxygenase enzyme that was responsible for the conversion of arachidonic acid to prostaglandins (Dubois *et al.,* 1998) Chondrocytes subjected to mechanical and chemical injury release arachidonic acid, a metabolic precursor of prostaglandins and leukotrienes (Curtis *et al.,* 2002). Prostaglandins are short-lived substances that act as local hormones important in normal physiology and pathologic conditions. Prostaglandin E_2 (PGE_2) is thought to be the principal eicosanoid expressed in inflammatory conditions (Calder, 2001).

COX-1 is a cytoplasmic housekeeping enzyme which generates prostaglandins for physiological functions and it is the inhibition of this enzyme which produces the NSAID side-effects (Vane *et al.,* 1998). COX-2 is an inducible enzyme which appears during cell injury and synthesizes prostaglandins which function in the inflammatory response and, hence, inhibition of this enzyme produces the anti-inflammatory effects (Brooks *et al.,* 1999). During the inflammation process, the pro-inflammatory cytokines, IL-1 and TNF are produced in large amounts by synovial cells. Both of them also induce the production of COX-2 (Martel – Pelletier *et al.,* 1999).

Several animal-feeding experiments and studies with in *vitro* model systems have shown the effectiveness of dietary fish oils to reduce joint disease (Lee *et al.,* 1985; Venkatraman and Chu, 1999; Volker *et al.*, 2000). Therefore, there is a need for additional in *vitro* and in *vivo* studies to investigate the cellular and molecular mechanisms by which dietary *n-3* PUFAs might cause the beneficial effect of abrogating joint pathology in degenerative joint disease.

Work done by Curtis *et al.*, (2000, 2002) on the mechanisms involved in the chondrocytes gene transcription showed that the *n-3* PUFA present in fish oils, have a

beneficial effect on model arthritis systems. However, the use of different types of fatty acids in reducing the pro-inflammatory cytokines in cartilage is still not investigated yet. Cartilage bovine monolayers are an in *vitro* model system that mimics cartilage degradation in arthritis.

Results in this chapter show that supplementation with different types of *n-3* fatty acids in bovine chondrocyte cultures resulted in a dose-dependent decrease in COX-2 mRNA, but not COX-1 mRNA, and cytokine mediators involved in cartilage degradation and inflammation related to arthritis. EPA seems the most effective *n-3* PUFA for decreasing expression of COX-2, IL-1 and TNF-α mRNA. By contrast, supplementation of bovine cartilage with an *n-6* PUFA, AA, had no effect on COX-2 mRNA or pro-inflammatory, IL-1α, IL-1β and TNF-α mRNA.

Supplementation of the cartilage monolayer with hydrolysed palm olein seemed to have no effect on the mRNA levels described above even though HPOo contains a significant amount of vitamin E and beta-carotene, which are anti-oxidants and, therefore, in some cases anti-inflammatory (Heliovaraa *et al.,* 1994).

In conclusion, the 'anti-inflammatory' effects of *n-3* PUFAs in the above experiments confirm that these PUFAs may be a neutral useful therapy for diseases such as rheumatoid arthritis, which is characterized by altered immune function without the adverse effects often associated with NSAIDs. Moreover, the relative effectiveness of EPA, of all the *n-3* PUFAs tested has been identified.

5.5 Chapter Summary

- IL-1 treatment of cultures was able to induce mRNA for COX-2 and the cytokines IL-1α, IL-β and TNF-α.

- Addition of *n-3* PUFA to bovine cultures reduced, in a dose-dependent manner, the mRNA levels for COX-2 and the inflammatory cytokines TNF-α, IL-1α and IL-β which had been induced by IL-1α exposure.
- Pre-incubation with AA had no effect on the levels of the above mRNAs.
- EPA seemed the most effective *n-3* fatty acid in reducing these mediators of cartilage inflammation and degradation, perhaps because it could be directly converted to non-inflammatory eicosanoids.
- COX-1 mRNA, which is thought to be a constitutive enzyme, was present in all bovine monolayer cultures at a constant level and was not affected by IL-1α or fatty acid treatment.
- Hydrolyzed palm olein treatment had little, if any, effect.

CHAPTER SIX

Lipase-catalysed incorporation of *n-3* PUFAs into palm olein

6.1 Introduction

Palm oil is the major agricultural commodity of Malaysia and its production is increasing annually. This leads to the need to diversify the usage of palm oil and its fractions. Palm olein is a light, yellow edible oil obtained from palm oil by a fractionation process. Refined, bleached and deodorized, palm oil is separated into two fractions by partial crystallization to separate liquid parts (olein) from solid parts (stearin). The liquid fraction is called palm olein (Berger, 2001). Unlike palm oil, which is rich in palmitic acid (44%), palm olein (a lower melting fraction of palm oil), contains 32-36% palmitic acid (C16:0) and 44-46% oleic acid (C18:1). It is normally used in frying (Pantzaris, 1988).

With recent advances in bioreactor technology, many new and commercially important products can be produced from palm oil. Lipase-catalysed reactions, which represent an energy-efficient and environmentally-friendly process, have been identified for this purpose. Lipases, as immobilised forms, can be used repeatedly with little or no deterioration in their operating process (Gunstone, 1997). Any new combination of fatty acid molecules within the triacylglycerols of palm oil, or palm oil fractions, will result in alterations in the final product's profiles and give rise to potential new uses (Xu *et al.*, 2002).

Lipase-catalysed reactions are a combination of esterification and hydrolysis. Water must be continuously removed from the reaction medium in order to increase esterification reactions, while minimizing hydrolysis in order to obtain high conversion rates to products.

When excess water is present, hydrolysis predominates, resulting in the accumulation of glycerol, FFAs, MAGs and DAGs (Osborn and Akoh, 2002). However, enzymatic interesterification reaction systems must contain a small amount of water which is required by the enzyme catalyst for the maintenance of an active hydrated state (Gunstone, 1997).

α-Linolenic or ALA, is an essential fatty acid, which means that it is essential to human health but cannot be manufactured by the body. For this reason, ALA must be obtained from food. ALA, as well as the fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), belong to a group of fatty acids called fatty acids called *omega* or *n-3* PUFA (Calder, 1998, Gurr *et al.,* 2002). EPA and DHA are found primarily in fish while ALA is found in certain plants oils, such as flaxseed oil. Once ingested, the body converts ALA to EPA and DHA, the two types of fatty acids more readily used by the body (Donald, 2002). Consuming increased amounts of long chain *n-3* polyunsaturated fatty acids (found in oily fish and fish oils) results in a partial replacement of the arachidonic acid in cell membranes by eicosapentaenoic and docosahexaenoic acids. This leads to decreased production of arachidonic acid-derived mediators (Calder, 2004).

Omega-3 fatty acids have been shown to reduce inflammation and help prevent certain chronic diseases such as heart disease and arthritis (Gurr *et al.,* 2002; Simopoulos, 2002; Calder, 2005). They result in suppressed production of pro-inflammatory cytokines and can modulate adhesion molecule expression. These effects take place at the level of altered gene expression (Curtis *et al.,* 2000, 2002; Calder, 2005).

However, consumption of *n-3* PUFA also increases the requirement for antioxidants, as oxidised fatty acids can have harmful side effects. Free *n-3* PUFAs are easily oxidized and are less acceptable in food due to their fishy flavour and odour. Therefore, triacyglycerol (TAG) is considered to be the most desirable chemical form in food (Yamane, 1992).

Incorporation of *n-3* fatty acids into palm olein to produce new types of healthy fats and oils seems a viable means of supplying *n-3* PUFA to the diet. Moreover, palm olein contains significant amounts of natural antioxidants (Berger, 2001) which would be expected to reduce oxidative deterioration.

Because of the regioselectivity of lipase enzymes, they are well suited as biocatalysts to produce lipids with desirable fatty acids. By structuring TAG in different ways, the health benefits of PUFAs can be enhanced by the nutritional and metabolic advantages of palm olein, resulting in a combination that can provide special health properties. Thus, palm olein containing *n-3* PUFA would serve as a single rich source of *n-3* PUFA and vitamins A and E. Vitamin E is also a powerful antioxidant during the processing and storage period (Elisabeth, 1998). This improved oxidative stability with natural antioxidants adds to the value of the product. The modified enzymatically-produced interesterified health product could also be used for frying (Rozendaal and Macrae, 1997).

Several reports of enzymatic interesterification of oils with *n-3* PUFA are available in the literature (Li and Ward, 1993; Akoh *et al.,* 1996; Jennings and Akoh, 1999; Garcia *et al.,* 2000; Xu *et al.,* 2000). The use of palm oil with *n-3* PUFA, involving lipase-catalysed incorporation, has been reported in one study (Fajardo *et al.,* 2003) but the enrichment of palm olein with ALA (or other *n-3* fatty acids) has not yet been reported.

The main objective of this work was to add value to palm olein by the incorporation of potentially beneficial *n-3* PUFAs, α-linolenic acid and EPA into its triacyglycerols. Methyl linolenate and methyl EPA, more stable derivatives than the free acids, were used for the interesterification. In order to make more efficient and more economic use of the costly ALA feedstock in the interesterification reaction, a study to optimise the reaction conditions was conducted. In this study, we examined factors known to affect enzymatic activity and product

yield which include water content, the substrate ratio, enzyme amount, incubation time, temperature, solvent effects and amount of solvents.

6.2 Materials and methods

6.2.1 Materials

Refined, bleached, and deodorized (RBD) palm oil olein (Iodine value 60) was obtained from the Malaysian Palm Oil Board (MPOB), Malaysia. Methyl α-linolenic acid and methyl eicosapentaenoic acid (EPA) were purchased from Nu-check Prep, Elysian, Minnesota, U.S.A. The compositions of the oils used are shown in Table 6.1. *Lipozyme IM 60* (1, 3 specific) lipase from *Rhizomucor miehei* is an immobilized form and was purchased from Novo Nordisk A/S (Bagsvaerd, Denmark). Other immobilized lipases were a gift from Amano Enzyme Inc, Nagoya, Japan. All the solvents and reagents used for analyses were analytical grade or the best available grades.

6.2.2 Enzymatic modification reactions

The substrates used for interesterification consisted of 0.2 mg of RBD palm olein (POo) and 0.2 mg of methyl α-linolenate or methyl EPA, except when the substrate ratio was an experimental variable. When tested, the following weight ratios of palm oil to *n-3* PUFA were used: 4:1, 3:1, 2:1, 1:1, 1:2, 1:3, 1:4, 1:5 and 1:6. The lipases (in a dry form) were used at 10% of substrate weight (g/100g substrate) except when enzyme content was an experimental variable, when lipase contents of 1, 2, 5, 10, 20, 30, 40, 50 and 60% of substrate weight were used to catalyze interesterification at 55°C. Ten ml hexane/g of total substrate was used as an organic solvent unless the types and amount of the solvents used were an experimental variable. The enzymatic interesterifications were carried out in capped 20ml glass vials with

reciprocal shaking at 55°C for 24 hours at 250 rpm, except when the temperature and time reactions were experimental variables. All reactions were carried out in triplicate.

6.2.3 Analysis of products

Samples were taken and filtered through a column of glass wool. The lipid mixture was solvent-extracted and washed (section 6.2.2). The reaction products were separated by thin-layer chromatography (TLC) on pre-coated silica gel 60 plates using petroleum ether/ ethyl ether/acetic acid (80:20:1, by vol) as the solvent system. The TLC plates were sprayed with 0.2% 8-anilino-4-naphthalenesulphonic acid (ANSA) and bands visualized under UV light. The bands corresponding to methyl esters, TAG, FFA, DAG and MAG were scraped off the TLC plates and fatty acid methyl esters (FAME) formed in 2 ml of 2.5% H_2SO_4 in dry methanol/toluene (2:1) for 2 hours at 70°C. After this time, the methylation was stopped by adding 5 ml (5% wt/wt) NaCl and the fatty acid methyl esters extracted 3 times with petroleum ether (60-80°C fraction).

FAMEs were analyzed by a Perkin-Elmer Autosystem XL gas liquid chromatogram (Perkin Elmer, Connecticut, USA). A glass column (1.5m x 4mm internal diameter) packed with a polar packing material (10% SP-2330 on 100/120 Supelcoport), operated isothermally at 180°C was used. This procedure separates fatty acids according to their chain length as well as their degree of unsaturation. The carrier gas was nitrogen. FAMEs were provisionally identified by comparing their retention times (RT) with RTs obtained with standard mixtures of fatty acids, obtained from Nu-ChekPrep (Elysian, MN, U.S.A). The relative content of FAME was calculated by computer (Excell 2003 software) using Perkin-Elmer internal software and with 15:0 as an internal standard.

6.3 Effect of lipase-catalyzed interesterification of palm olein with methyl ALA

6.3.1 Fatty acid composition of substrates

The fatty acid profiles of TAG isolated from palm olein and ALA and EPA (methyl esters) before modification are given in Table 6.1. The fatty acid composition of palm olein was similar to reported values by the Chong (1994). As shown in Table 6.1, the sample of palm olein (Iodine value 60) contained high amounts of C16:0 (36.8%) and C18:1 (44.6%) and a smaller amount of C18:2 (13.7%). Meanwhile, the initial FA composition of the ALA (methyl ester) and EPA (methyl ester) used for transesterification was shown to have a good purity with only low amounts of other fatty acids as contaminants.

	Fatty acid composition (% total)									
	C14:0	**C16:0**	**C18:0**	**C18:1 (n9)**	**C18:2 (n6)**	**C18:3 (n3)**	**C20:1**	**C20:4**	**C20:5**	**C22:6**
Palm olein (PO)	1.1± 0.4	36.8± 1.2	3.2± 0.2	44.6± 0.2	13.7± 0.9	0.6± 0.3	ND	ND	ND	ND
Methyl ester α–linolenate acid (ALA)	tr	tr	tr	ND	ND	99.8± tr	ND	tr	ND	ND
Methyl ester α – eicosapentaenoic acid (EPA)	tr	tr	tr	tr	tr	ND	0.2± 0.1	tr	99.2 ± 0.1	0.5± 0.1

Table 6.1: Fatty acid compositions (%) of TAG from palm olein, methyl ALA and methyl EPA. Means ± S.D. (n=3) are shown. N.D = not detected. Values corrected to one decimal point. Tr = trace (< 0.05).

6.3.2 Screening of commercial lipases for transesterification activity

The average fatty acid composition of TAGs isolated from the reaction mixture, after lipase enzymatic interesterification of palm olein with α-linolenate acid (methyl-ester) at 55°C and using several enzyme preparations is shown in Table 6.2. Seven types of lipase, *Lipozyme-IM 60*, *Rhizopus oryzae (*Lip F*), Pseudomonas sp.* (Lip PS), *Candida lipolytica* (Lip AK), *Penicillium roqueforti* (Lip R), *Rhizopus niveus* (Lip N) and *Rhizopus delemar* (Lip D), respectively, were investigated for their ability to incorporate α-linolenate acid into palm olein. *Lipozyme IM 60* is a 1, 3-specific lipase from *Rhizomucor miehei* immobilized on a macroporous exchange resin. However, the other lipases used were added as free enzymes. All lipases showed, at least, some ability to transesterify palm olein with linolenate, although those from *Penicillium roqueforti* (Lip R) and *Rhizopus niveus* (Lip N) only showed low activity. Of the enzymes tested, *Lipozyme IM 60* incorporated the highest amount of ALA into the palm olein (Table 6.2). In fact, use of the enzyme under the reaction conditions used, resulted in virtually complete equilibration of the fatty acids available in the substrate mixture such that the final composition of the TAG fraction had an average composition of the original palm olein /methyl linolenate mixture. Because of its ability to incorporate ALA into palm olein, TAG and its immobilised physical form (which should confer stability), the *Lipozyme IM 60* lipase from *Rhizomucor miehei* was selected for use in subsequent experiments.

The differences in the ability of the lipases to incorporate ALA presumably reflected the substrate specificity of each type of lipase (Jenssen, 1996). Thus, not only does one need to consider the activity of enzymes in the microaqueous-reactor system but also any marked specificity for the substrates to be used (Rozendaal and Macrae, 1997).

	Fatty acid composition (% total)						
Lipase used	**C14:0**	**C16:0**	**C18:0**	**C18:1 (n9)**	**C18:2 (n6)**	**C18:3 (n3)**	**Other**
Lipozyme IM 60	0.4±0.1	20.0±0.5	2.1±1.6	23.7±0.4	5.9±0.4	47.5±3.1	0.4±0.1
Pseudomonas sp (Lip PS)	0.4±0.1	22.0±2.1	1.8±0.5	24.5±2.4	6.4±0.2	44.2±5.5	0.7±0.1
Rhizopus delemar (Lip D)	0.3±0.6	20.6±1.9	2.2±0.5	29.0±1.8	5.2±0.1	43.6±8.7	0.1±0.6
Rhizopus oryzae (Lip F)	0.5±0.1	27.1±2.8	2.7±0.7	31.8±2.9	8.2±0.5	29.7±5.0	ND
Candida lipolytica (Lip AK)	16.5±1.4	24.6±2.5	2.7±0.4	24.9±1.9	6.5±0.4	24.6±6.9	0.2±0.1
Rhizopus niveus (Lip N)	0.8±0.1	34.2±4.5	ND	36.0±2.5	11.1±0.9	17.9±7.4	ND
Penicillium roqueforti (Lip R)	0.4±0.1	38.5±4.5	ND	36.8±3.9	8.8±0.5	15.2±5.3	0.3±0.1

Table 6.2: Fatty acid composition of TAG product in the reaction mixture after 24 hour at 55°C was analyzed (Section 6.2.3). N.D = none detected. Data are shown as means ± S.D. (n=3).

6.3.3 Effect of enzyme concentration

Economic considerations, usually related to the high cost of enzymes have prevented more widespread commercial adoption of enzymatic synthesis procedures (Arcos *et al.,* 2000). Therefore, the amount of enzyme used in a reaction is of economic importance to any process of interesterification. Moreover, lipase load as well as reaction time and temperature have a strong influence on the degree of interesterification. In addition, the enzyme load may also have an effect on acyl migration because carriers of lipases, such as resin and silica, can induce acyl migration (Xu, 2000).

Figure 6.1 shows the effects of enzyme load on the lipase-catalyzed interesterification reaction of palm oil and ALA (methyl ester) in lipid class composition (%) profiles. Initially, the percentage of methyl ester and TAG declined gradually as the enzyme load was increased. The TAG % stabilised after about 10% w/w enzyme whereas the methyl ester fraction plateaued after about 30% w/w enzyme concentration. The reductions in methyl esters and TAG at higher enzyme loads may have been due to an increase in water from the enzyme preparation. This is suggested by an increase in free fatty acids (FFA). Thus, more water was brought in with increased enzyme concentration causing more hydrolysis.

In fact, it has been suggested that, for commercial purposes, the enzymes or the reaction mixture should be first dried, either under nitrogen or by using silica gel or molecular sieves, to reduce the water content and maintain water at $A_w = 0.5$ (Bloomer *et al.,* 1991). In this experiment, the water content of the enzyme was detected at an Aw = 0.54 while palm olein and ALA were found to have Aw = 0.28 and 0.29. The water content was measured by the Paw-Kit water activity meter from Decagon Devices, Pullman, Washington, U.S.A. Thus, from these data, the level of water in the enzyme and substrates should not be sufficient to cause major hydrolysis. The content of by-products such as MAG (%) and DAG (%) was not

greatly affected by the enzyme load. Initially, DAG increased but it remained at 7-10% for most enzyme concentrations. MAG was between 1-2%, at all enzyme loads.

Figure 6.2, shows the effect of enzyme concentration on the fatty acid composition of ALA (18:3) into TAG. As expected, the initial incorporation, catalysed by the lipase enzyme IM 60, was dependent on the amount of enzyme used at 55°C. Essentially, at between 5 and 60%, a maximum incorporation of ALA into the TAG fraction was achieved. Thus, immobilised enzyme contents of 5 or 10% (g/100 g substrate) were enough to achieve good incorporation rates of linolenate into the TAG fraction. This would be in keeping with a necessity to keep the potential cost of the enzyme needed for interesterification at a reasonable level. It can also be noted that, at the lowest level of enzyme used (1%), the TAG fraction had a composition close to that of palm olein, indicating that little transesterification had taken place.

Figure 6.3, shows the effect of enzyme content on the fatty acid composition of the methyl ester fraction following reaction of methyl ALA with palm olein. The ALA (18:3) content of this fraction decreased significantly with increasing enzyme content up to 10% and this paralleled the increase in ALA in the TAG fraction (Figure 6.2). The increase in the methyl esters of C16:0, 18:1 and other acids with increasing enzyme load showed that the interesterification was taking place for all fatty acids. There was no marked substrate selectivity for this enzyme. The composition of the methyl ester fraction at the highest enzyme concentrations showed an unexpected decrease in the linolenate content. However, the experiment would need to be repeated to be certain of this result.

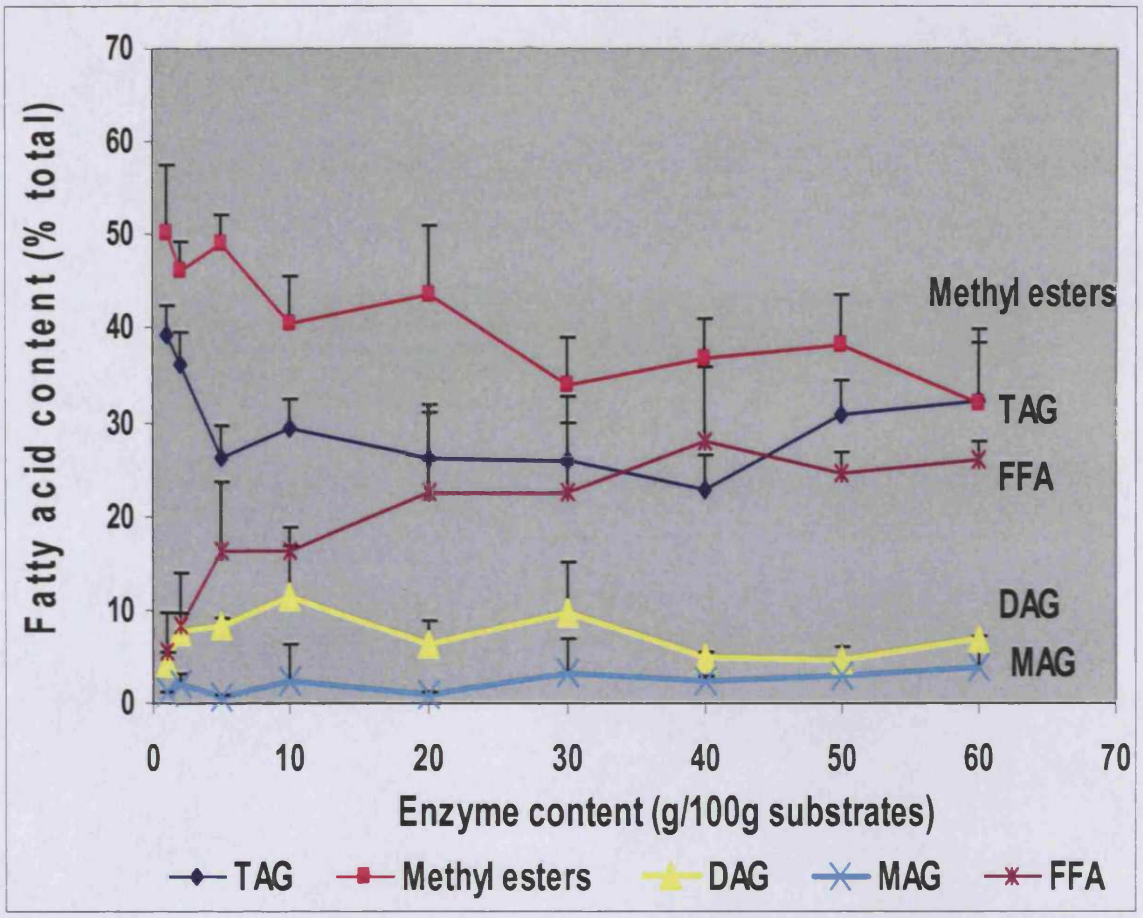


Figure 6.1: Effect of *Lipozyme IM 60* concentration on the lipid class composition (%) of the reaction products following enzymatic interesterification of palm olein with methyl linolenate at 55°C for 24 hours. Results show means + S.D (n=3).

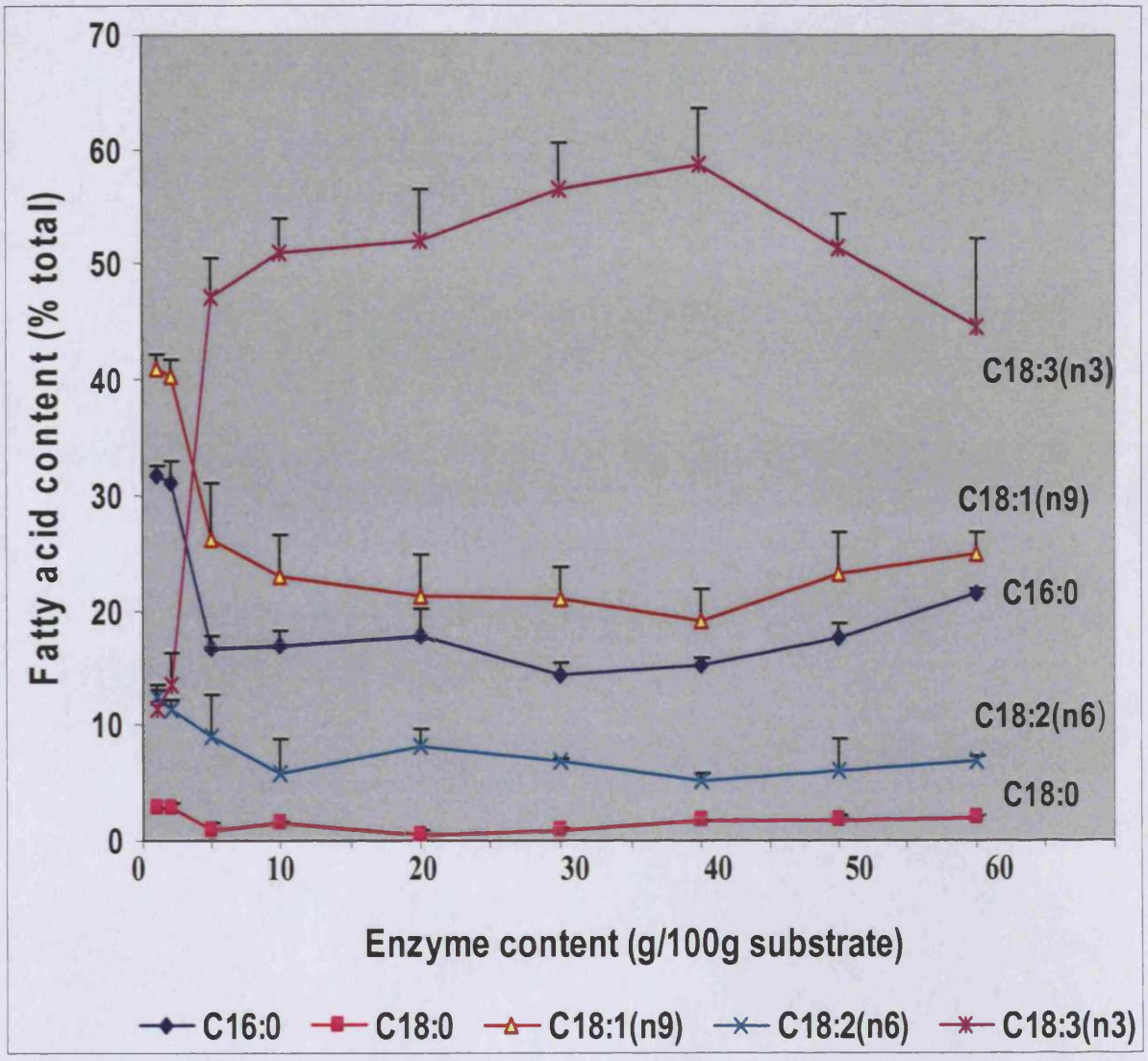


Figure 6.2: Effects of *Lipozyme IM 60* content on the fatty acid composition of the TAG fraction following enzymatic interesterification of palm olein with methyl linolenate for 24 h at 55°C. Results show means + S.D (n=3).

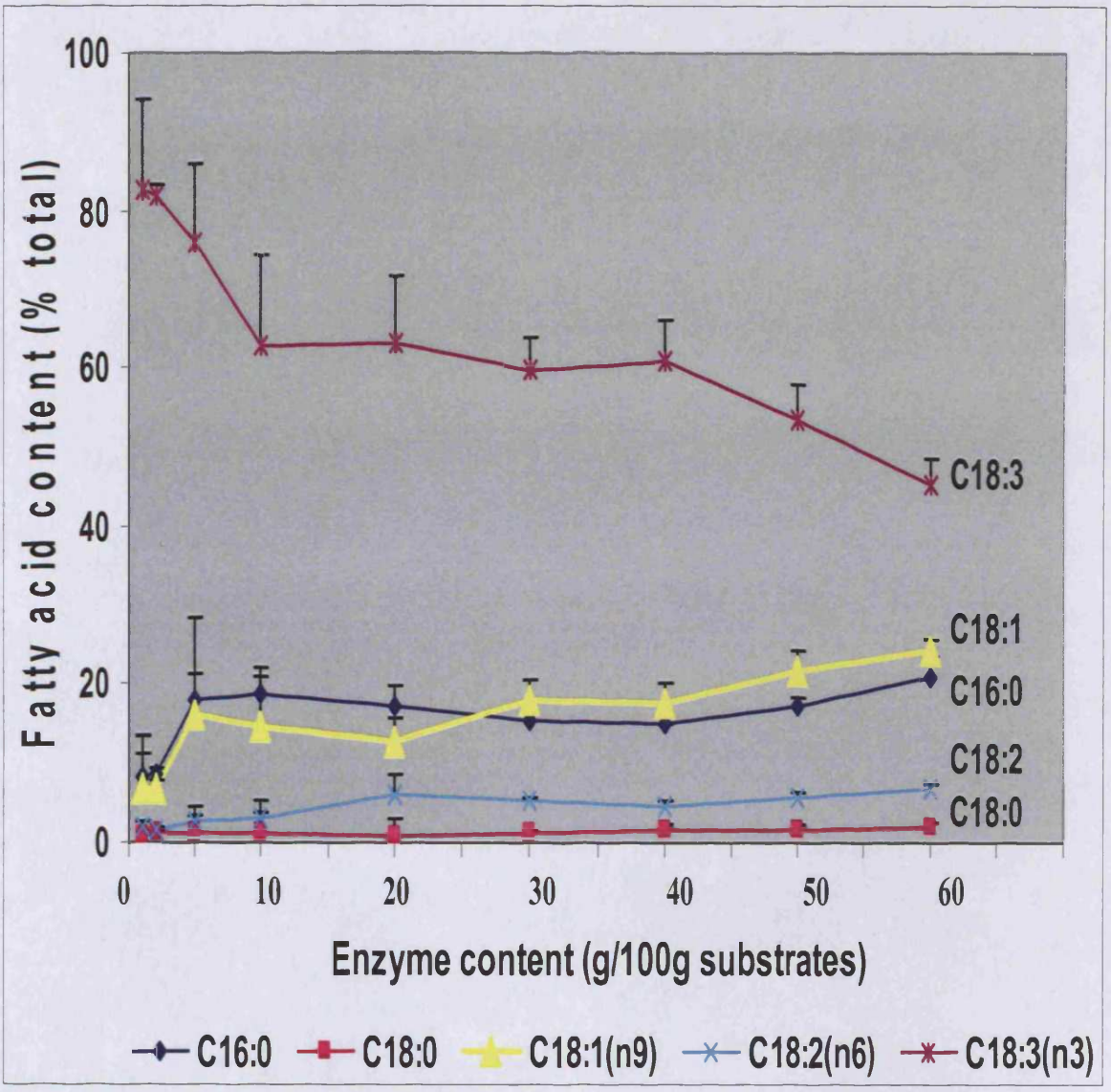


Figure 6.3: Effects of *Lipozyme IM 60* content on the fatty acid composition of the methyl ester fraction following enzymatic interesterification of palm olein with methyl linolenate for 24 h at 55°C. Results show means + S.D (n=3).

6.3.4 Effect of Organic Solvent

Lipases are largely hydrophilic proteins and water is their natural environment. Thus, lipase-catalysed reactions are normally carried out in an aqueous medium where hydrolysis will take place (Holmberg *et al.,* 1989). The amount of water can control the reversibility of the hydrolysis reaction and allow interesterification to be catalysed. Thus, control over the water/moisture content in the reaction matrix in which the enzyme is suspended facilitates the interesterification reaction. Moisture control is the key to success (Basheer *et al.,* 1995). It affects the rate of reaction, yield of product, product selectivity and the operational stability of the enzyme used (Yee and Akoh, 1996). Therefore, it is essential to utilise organic solvents with low water content in the reaction system to allow the bioconversions of lipophilic compounds effectively (Li and Ward, 1993).

In this study, we examined eleven solvents to determine those giving the highest incorporation of linolenate and the highest yield of TAG. A higher percentage of TAG in the final lipid mixture was observed with hexane, petroleum ether, acetone and chloroform (Figure 6.4). A high percentage of methyl esters were found with methanol, presumably because of the methylation of fatty acids released from the glycerides. DAG and MAG, which are by-products of the reaction, were observed in small quantities in all the solvents used (Figure 6.4). FFAs which are, clearly, undesirable by-products were formed in significant quantities with some solvents (such as chloroform) perhaps because these contained significant amounts of water.

The choice of solvent can also affect the substrate specificity and the stability of the enzymes (Jenssen *et al.,* 1996). Therefore, an important factor in the balance between stabilisation and inactivation was caused by the solvent. Solvents of lower polarity (i.e.

greater hydrophobicity) are less able to disrupt the structure of the tightly-bound water molecules

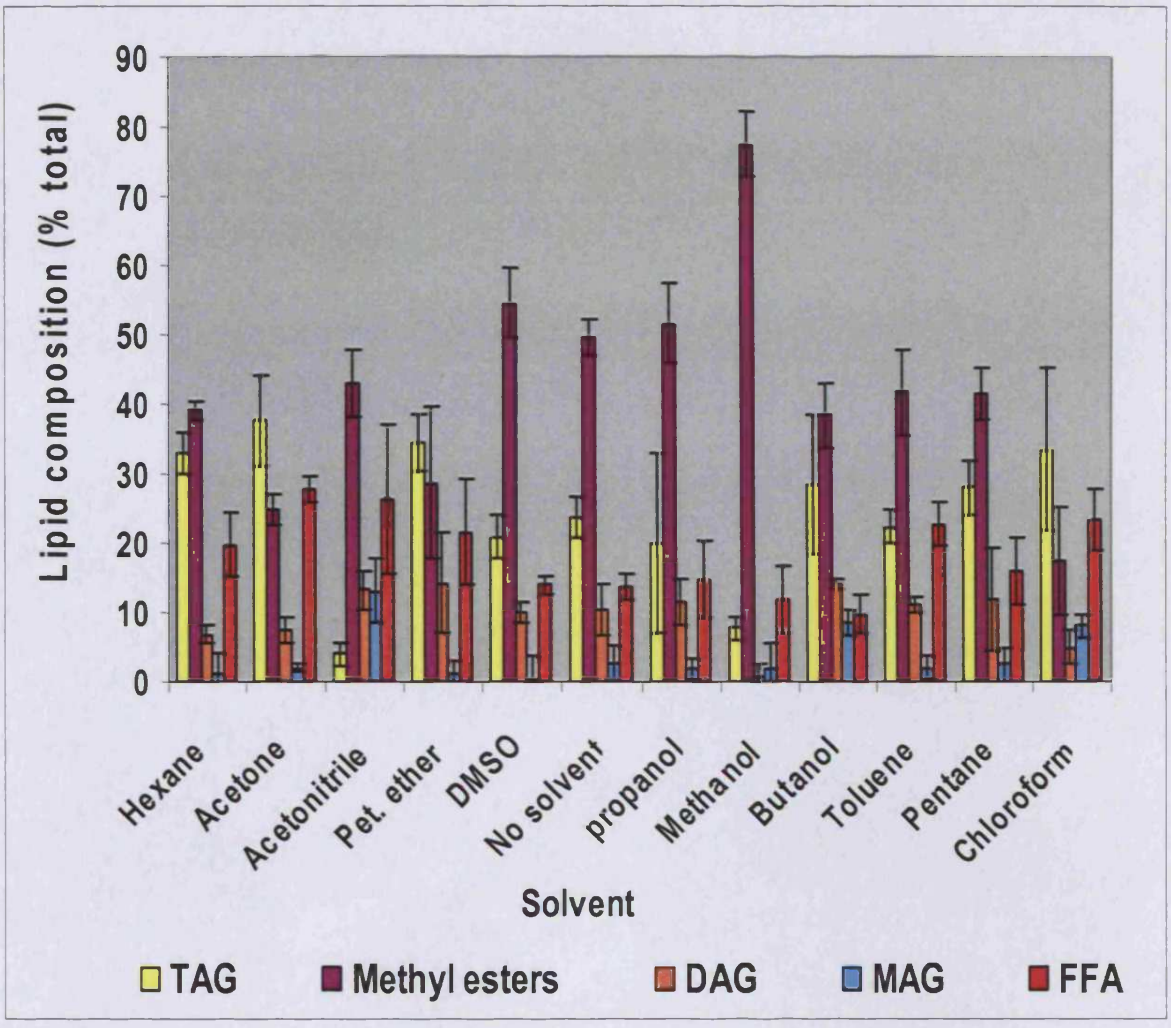


Figure 6.4: Effect of different organic solvents on the final lipid composition following enzymatic interesterification of palm olein with methyl linolenate by *Lipozyme IM 60*. Data show means ± S.D. (n=3).

(Chaplin and Bucke, 1990). The expression of Log P is normally used to predict the denaturing effect of a solvent on an enzyme (Halling, 1994). Log P is the partition coefficient of the organic liquid between *n*-octanol and water (Chaplin and Bucke, 1990). The higher the Log P, the more non-polar (hydrophobic) the solvent. In general, enzyme catalysis in organic solvents is governed by a few ground rules: (a) solvents with log P values of < 2 are not suitable for enzyme-catalyzed systems because they distort the essential water from the enzyme, thereby inactivating them; (b) solvents with log P values in the range of 2-4 are weak water distorters, and their effect on enzyme activity is unpredictable and (c) solvents with log P values of > 4 do not distort the essential water layer, thereby leaving the enzyme in an active state (Chaplin and Bucke, 1990; Akoh and Yee, 1997; Senanayake and Shahidi, 2002).

Figure 6.5 shows the effect of organic solvents on the fatty acid content of TAG in the final mixture following incubation. Under the conditions used, good transfer of ALA into TAG was formed with most solvents which also gave a good TAG yield (Figure 6.4). Thus, the use of organic solvents such as n-hexane (log P= 3.5), pentane (log P = 3.0), petroleum ether (log P = 3.5) and toluene (log P = 2.7) were found to increase the percentage of linolenate in TAG. Lowest yields were obtained in methanol (log P = -0.76), acetone (log P =-0.23), DMSO (log P = 1.3), propanol (log P = 0.28) and butanol (log P = 0.8). Although, chloroform (log P = 2.2) gave a high yield of TAG, the incorporation of linolenate was not as good as for the above solvents. Thus, while chloroform may be suitable for some applications, it may cause harmful effects in others. Moreover, water-miscible solvents such as ethanol cannot be used generally because of their denaturing effect on enzymes (Holmberg and Osterberg,, 1989).

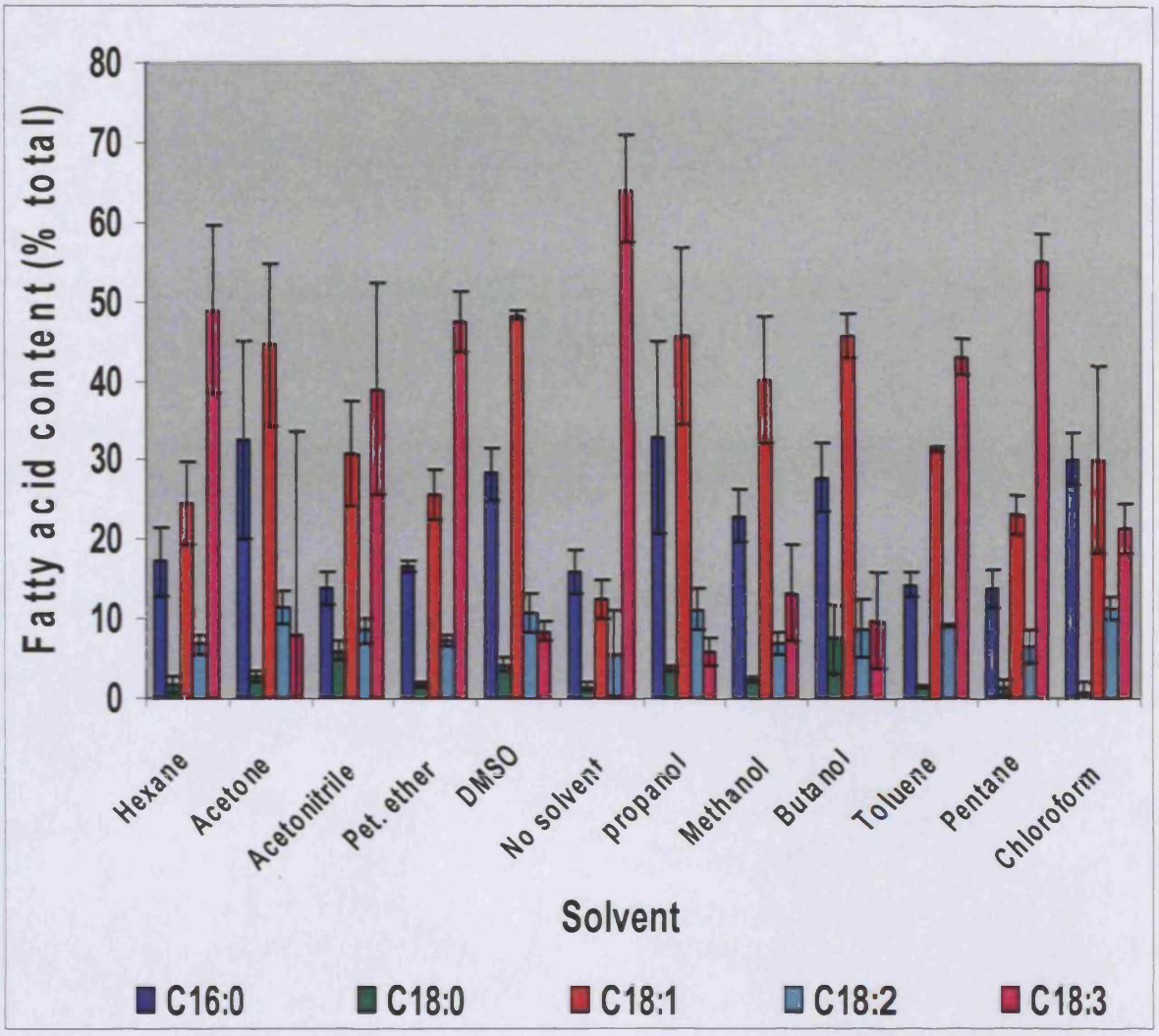


Figure 6.5: Effect of different organic solvents on the fatty acid composition of the TAG fraction after enzymatic interesterification of palm olein and methyl linolenate by lipase *Lipozyme IM 60*. Data show means ± S.D. (n=3).

The results obtained in this study were broadly compatible with the use of Log P as guideline or indicator to select a suitable solvent for interesterification of palm olein with methyl linolenate. Under the conditions used, pentane, petroleum ether, toluene and n-hexane allowed linolenate values in TAG to reach about 50 % which was the maximum expected for non-specific interesterification.

Interestingly, lipase interesterification in a solvent-free reaction mix gave the highest linolenate percentage in TAG. However, it should be noted that the actual yield of TAG (Figure 6.4) was not high. The enrichment of linolenate under these conditions implies that the enzyme had some selectivity towards ALA in absence of solvent. In fact, few studies involving lipases have been carried out in solvent-free systems (Lee and Akoh, 1998; Ergan *et al.*, 1991). Nevertheless, findings by Senanayake and Shahidi (2002) showed that when no-solvent was used EPA enrichment in borage oils was relatively high (21%), in agreement with our observations.

The fatty acid composition of the methyl ester fraction showed that ALA was still enriched for all solvents (Figure 6.6). These solvents giving the higher rates for transfer of linolenate into TAG (Figure 6.5) showed, as expected, lower % ALA values in the methyl ester fraction. From the data shown in Figures 6.4 - 6.6, petroleum ether and hexane appeared to be effective solvents. Organic solvents such as hexane have several functions, including shifting the reaction toward synthesis rather than hydrolysis (Senanayake and Shahidi, 2002).

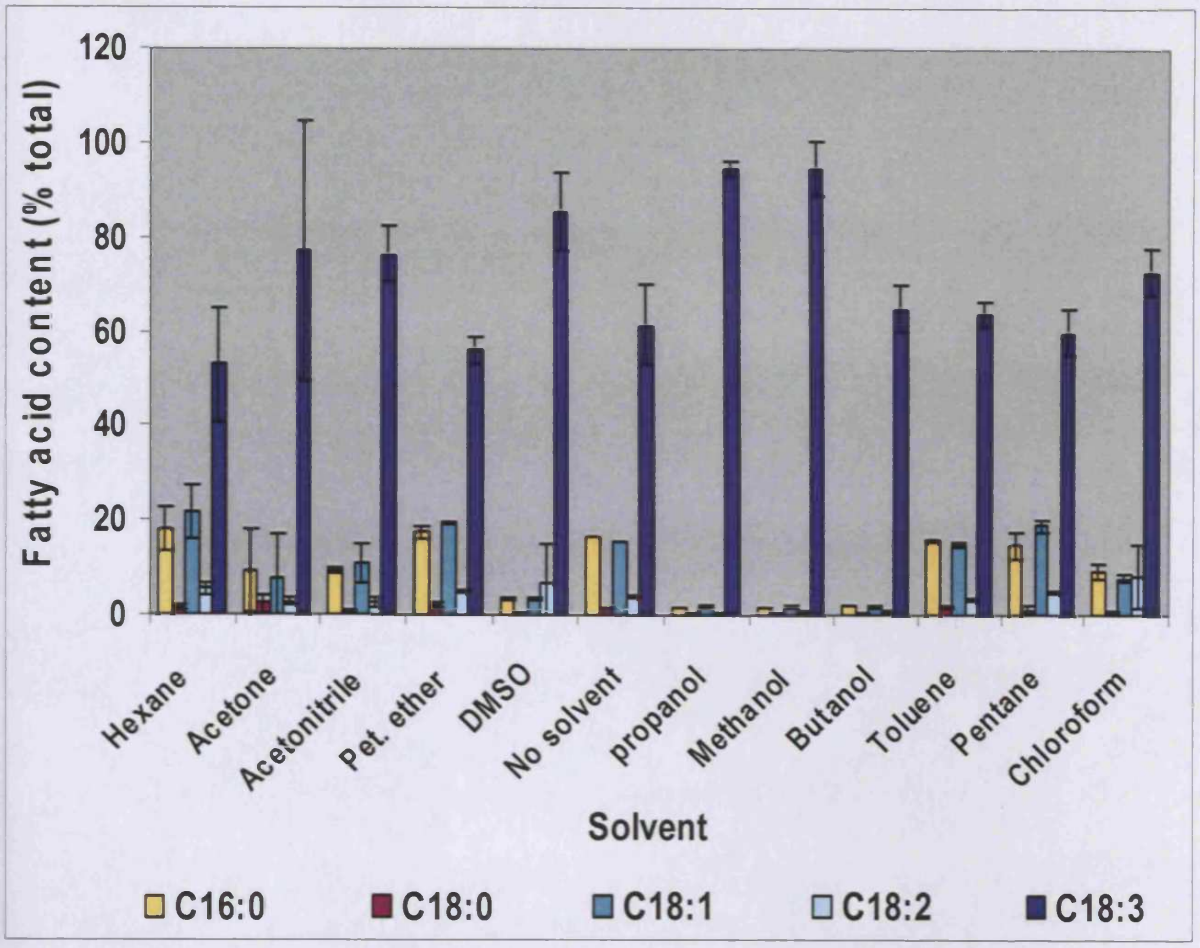


Figure 6.6: Effect of different organic solvents on the fatty acid composition of the methyl ester fraction after enzymatic interesterification of palm olein and methyl linolenate by *Lipozyme IM 60*. Means ±S.D. (n=3) are shown.

6.3.5 Effect of solvent volume

The most important problem when using lipases for synthesis is shifting the equilibrium towards esterification. Adding hexane has been found to have a significant effect on the equilibrium (Akoh, 1998). Adding a certain amount of solvent is an effective way to enhance the TAG content in the reaction mixture (by decreasing the viscosity of the reaction system, increasing the solubility of reaction products, and shifting the thermodynamic equilibrium towards synthesis rather than hydrolysis) (Gunnlaugsdottir and Sivik, 1995). Moreover, adding solvents to the reaction mixture can also help in dissolving more reactants and products in the medium (Yang *et al.,* 2003). On the other hand, the addition of water-miscible solvents has often been used to increase the solubility of polar reactants. Usually, addition of small amounts of a water-miscible solvent has little effect on the biocatalyst activity and stability. However, in some cases, modest concentrations of these solvents can enhance enzyme activity and stability (Halling, 1994).

In this study, the increasing amounts of hexane in the reaction resulted in a lower TAG content after 24 hours (Figure 6.7). Above 1.6 ml, an increase in methyl esters was found. A possible reason may be the dilution effect. Another factor might be the water content of the enzyme. A large amount of the solvent has been reported to strip away the enzyme's essential monolayer of water (Halling, 1994). A better process might be to add solvents continuously to the reaction mixture (Gunnlaugsdottir and Sivik, 1995). In our standard conditions 400 µl of hexane was used. As shown in Figure 6.8, this gave the highest value for ALA in TAG, as well as the highest TAG values in the reaction products (Figure 6.7) (though neither was statistically significant). Thus, additional amounts of hexane solvent did not confer any advantage over the usual experimental conditions.

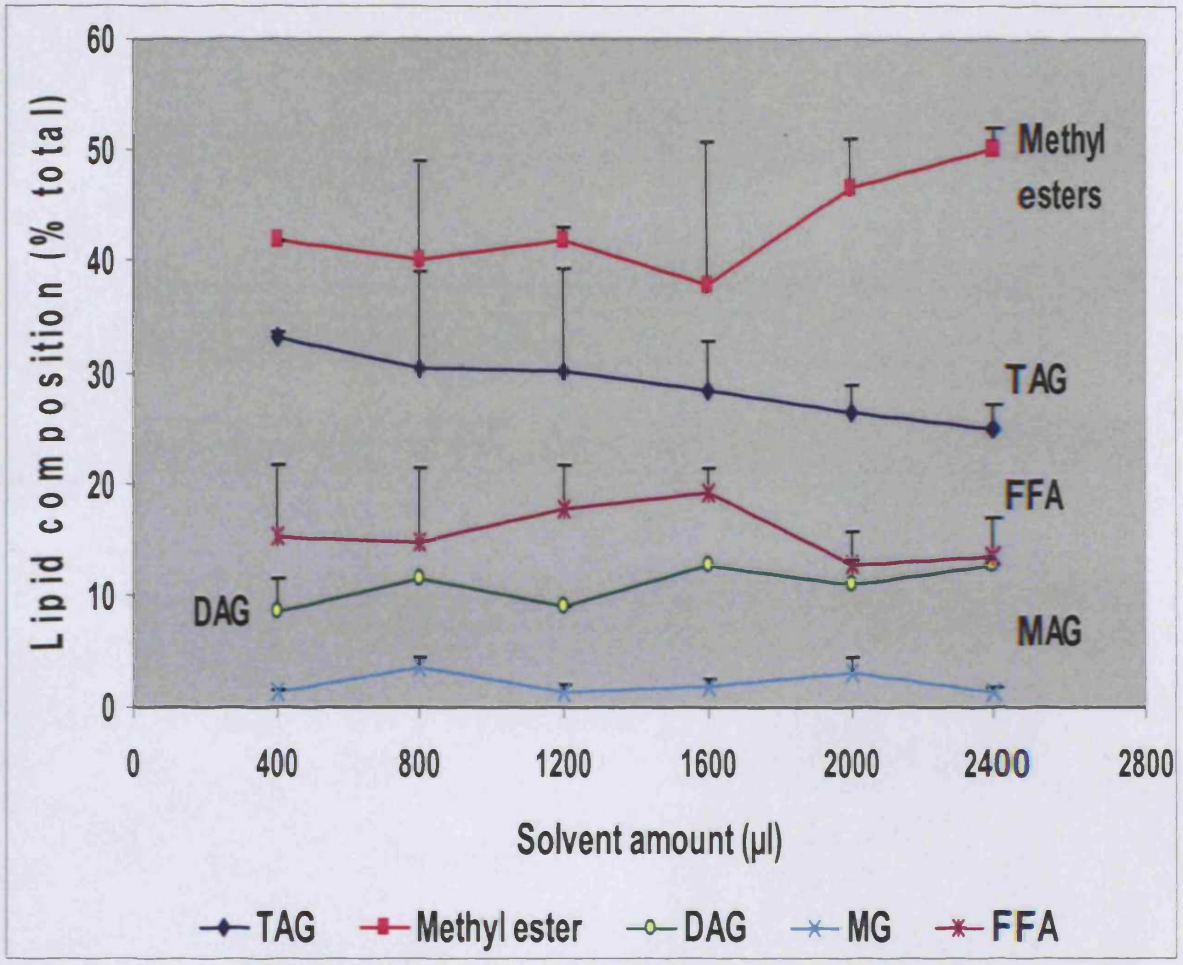


Figure 6.7: Effect of different amounts of hexane on the lipid composition of the products after enzymatic interesterification of palm olein and methyl linolenate by lipase *Lipozyme IM 60*. Data show means + S.D. (n=3). Lipid percentage is expressed in terms of fatty acid content.

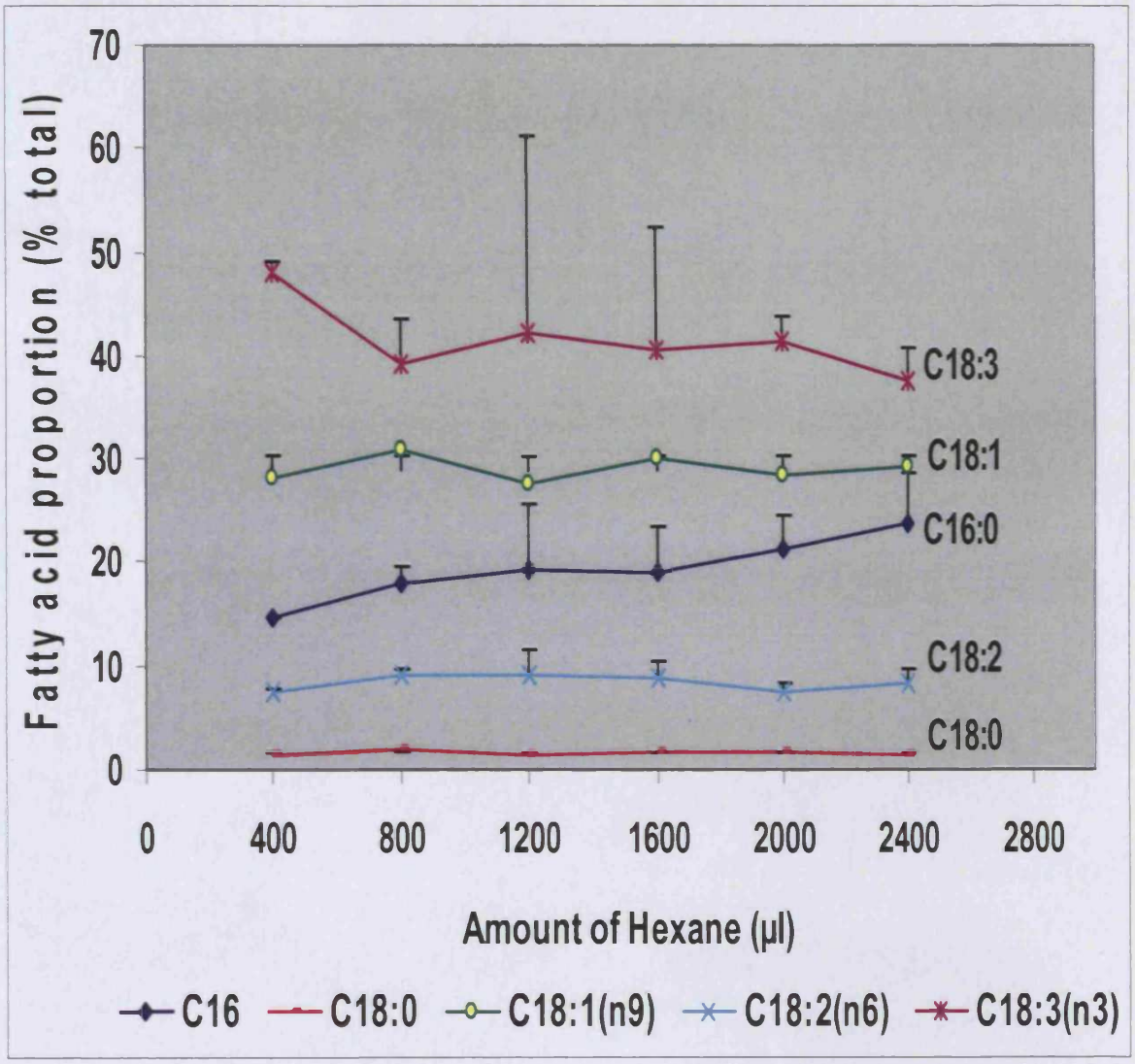


Figure 6.8: Effect of different amounts of hexane on the fatty acid composition of the TAG fraction after enzymatic interesterification of palm olein and methyl linolenate by lipase *Lipozyme IM 60*. Data show means + S.D. (n=3).

6.3.6 Effect of substrate ratio

The effect of varying the ratio of palm olein and methyl linolenate was investigated in this study. The effect on lipid class composition is shown in Figure 6.9. As expected, the final yields of TAG and methyl esters were dependent on the amounts of these substrates added.

Again, as could be predicted, the percentage of ALA in the TAG fraction increased as the proportion of methyl linolenate substrate was increased, athough beyond 1:1 there was only a small increase (Figure 6.10). A ratio of 1:1 of the two substrates seemed to represent a good compromise, enriching TAG with ALA without having to use huge amounts of (the expensive) methyl linolenate.

Huang *et al.*, (1994), who incorporated EPA into melon seed oil, showed that there was no economic advantage in using high substrate ratios. However, depending on the level of EPA incorporation desired in the final product, the substrate mole ratio could be manipulated to achieve it. Nevertheless, high fatty acid concentrations in the medium may indeed lead to substrate inhibition. Other observations also reported that a concentration – dependent substrate preference in lipase-acidolysis reactions can be attributed to acidification of the micro aqueous environment of the lipase and deabsorption of water by the fatty acid substrates (Kuo and Parkin, 1993). These effects should be lessened by the use of methyl ester substrates but, as pointed out above, high concentration of such compounds are not economically viable.

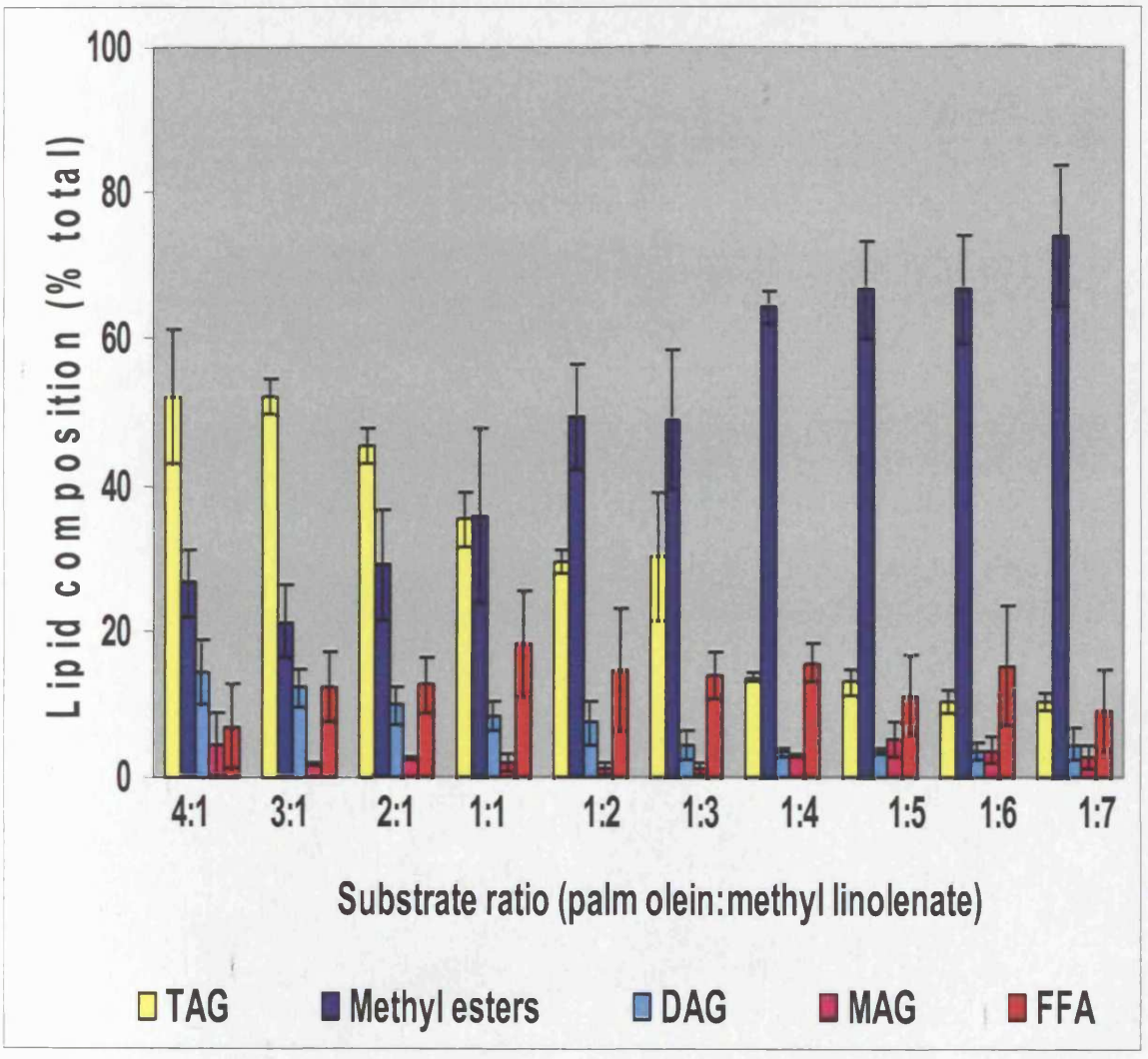


Figure 6.9: Effect of the substrate ratio of palm olein to methyl linolenate on the lipid class composition at the end of the transesterification reaction. The substrate ratios shown are palm olein: methyl linolenate (g/g). Means ± S.D. (n=3) are shown. Lipid amounts were estimated from the fatty acid content.

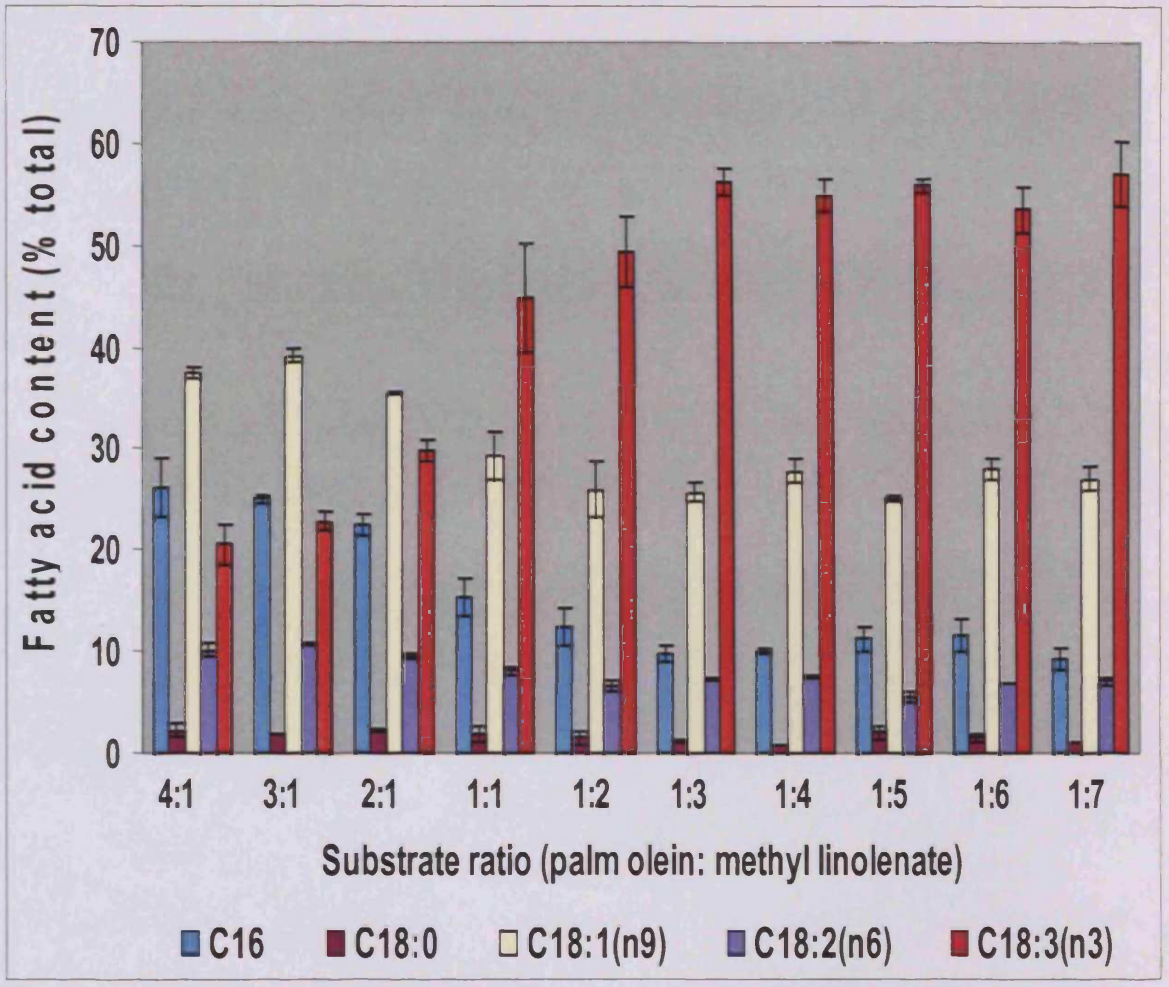


Figure 6.10: Effect of the substrate ratio of palm olein to methyl linolenate on the percentage of ALA in the TAG product. The substrate ratios shown are palm olein: methyl linolenate (g/g). Results show means ± S.D. (n=3).

6.3.7 Temperature

Six reaction temperatures were investigated as shown in Figure 6.11. They were chosen in the range 40-80°C to fit with the sort of temperatures typically used in lipase-catalysed interesterification reactions. Incubations lasting 24 h were used initially. The highest yields of TAG were found at 70-80°C. This temperature range also gave the lowest FFA products, although these were not statistically different from 55°C values. The proportions of the methyl esters did not seem to be affected by temperature. According to Li and Ward (1993), the high temperature optimum for the *Lipozyme IM 60* enzyme was probably partly due to the fact that immobilization conferred greater thermo-stability on the enzyme compared to other lipases.

The fatty acid composition of the TAG fraction produced at different temperatures is shown in Figure 6.12. Temperatures of 55 and 70°C gave high linolenate values which were not statistically significantly different from each other. Incubation at 80°C gave a lower percentage ALA although this was not statistically significant. Overall, different incubation temperatures had little effect on the fatty acid composition of the products, as expected for heat-stable enzymes.

As well as the small changes in the fatty acid composition of TAG with incubation temperature, a similar result was found into the methyl ester fraction (Figure 6.13). In short, the substrate selectivity of the lipase, *Lipozyme IM 60,* was not altered markedly by temperature. Our data suggested that the higher temperatures slightly increased the content of the desired TAG product in the interesterification products. However, high temperatures can also accelerate inactivation of the enzyme as well as increasing acyl migration. Because different incubation temperatures had rather little effect on product yields over a 24 h period and because of concerns about the long-term enzyme stability, we chose to use standard

('industrial') conditions with a reaction temperature at 55°C. Furthermore, higher temperatures can increase lipid oxidation rates, especially for polyunsaturated fatty acids. Therefore, temperatures between 50 and 60°C are recommended for polyunsaturated oils that have low melting points.

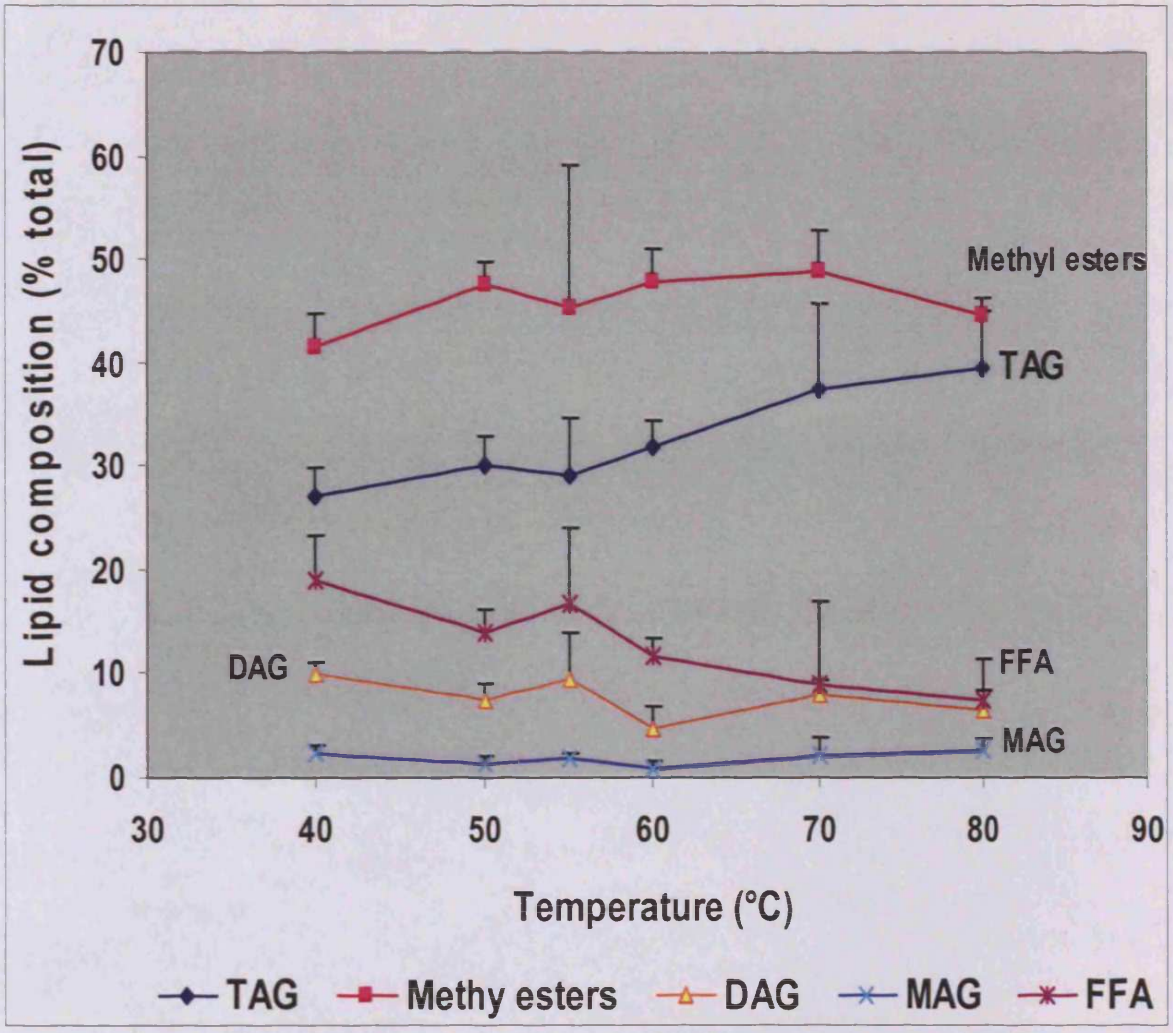


Figure 6.11: Effect of the temperature on % lipid composition of products in lipase-catalyzed interesterification of palm olein with methyl linolenate. Reactions were for 24 hours and 10% *Lipozyme IM 60* content in hexane. Data as means + S.D (n=3).

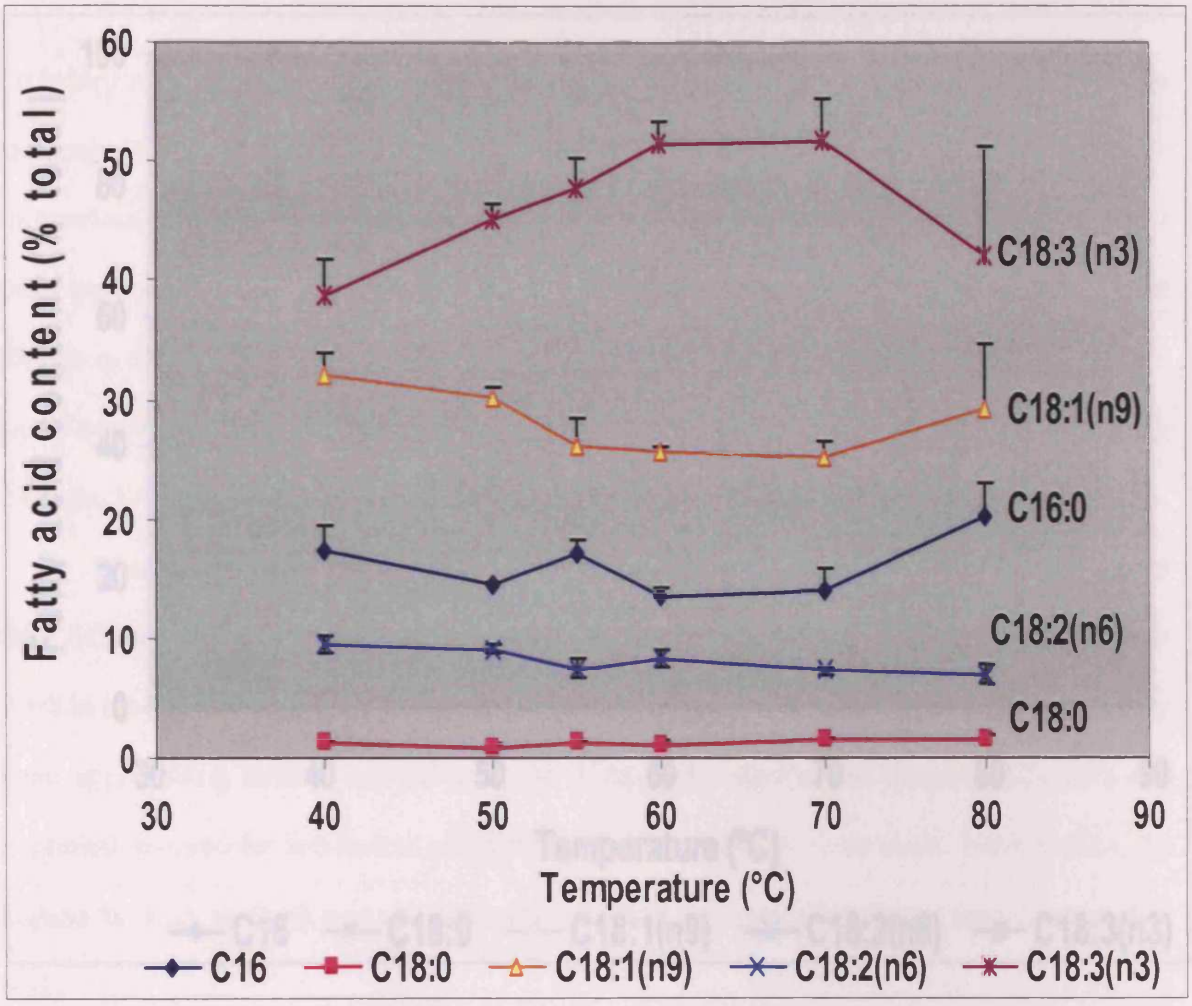


Figure 6.12: Effect of temperature on the fatty acid composition of the TAG fraction following lipase-catalyzed interesterification of palm olein with methyl linolenate. Incubations were for 24 hours and 10% *Lipozyme IM 60* content in hexane. Results show means + S.D. (n=3).

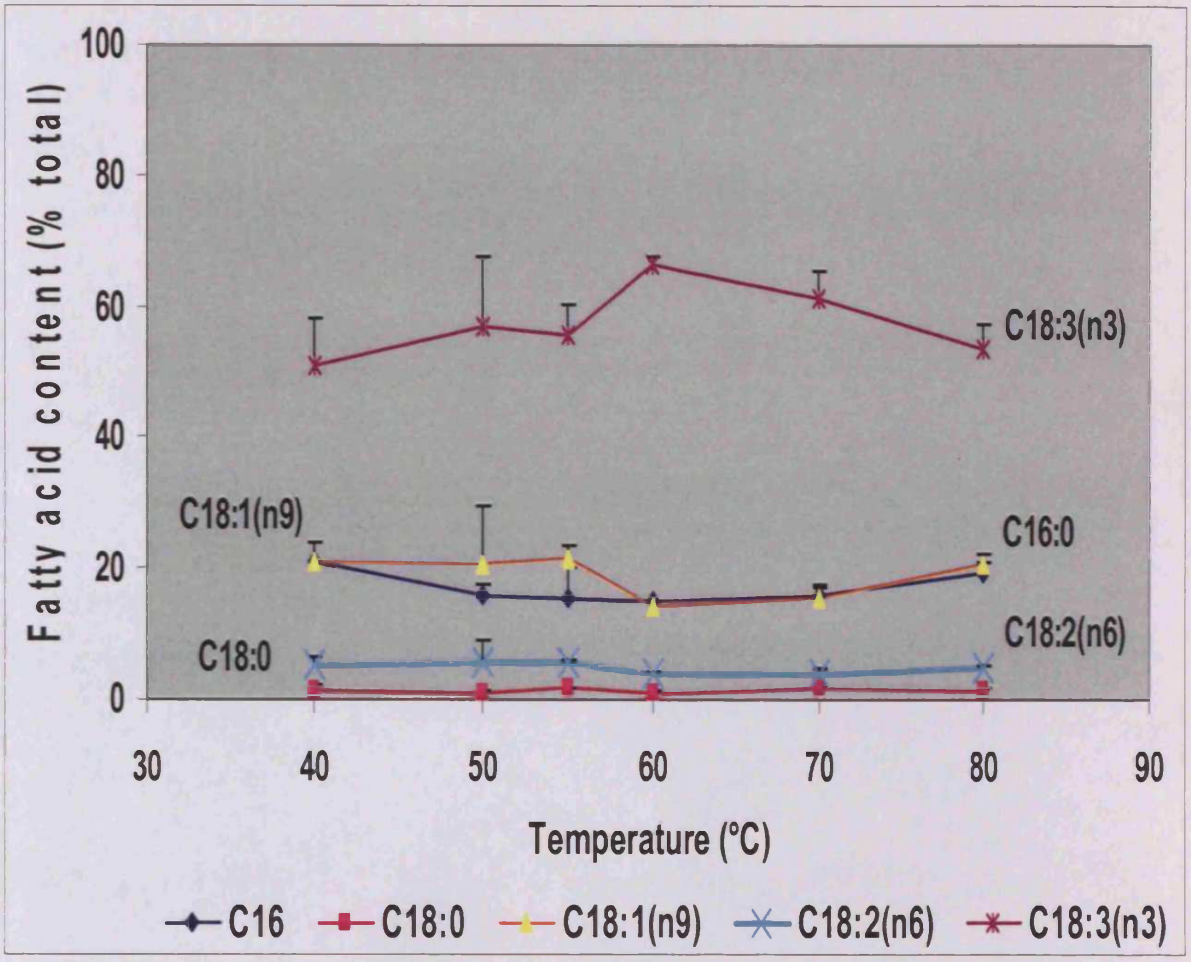


Figure 6.13: Effect of temperature on fatty acid composition of the methyl ester fraction following lipase-catalyzed interesterification of palm olein with methyl linolenate. Incubations were for 24 hours and 10% *Lipozyme IM 60* content in hexane. Results show means + S.D. (n=3).

6.3.8 Time course

We followed the time course of the reaction in order to find the shortest, adequate time necessary to obtain good yields. For an industrial process this would also minimize process expenses (Yee *et al.,* 1997). The time course of a typical experiment is shown in Figure 6.14. In previous work, incorporation of acyl donors was affected more by residence time than by other parameters in small-scale experiments (Xu *et al.,* 1998). As expected, there was little change in the pattern of products. With time there was a tendency for the methyl ester fraction to reduce. Also during the first 30 min, there may have been some hydrolysis of fatty acids from the TAG fraction.

However, more importantly, Figure 6.15 shows the exchange of fatty acids between the TAG and the methyl ester fraction with time. There was a rapid incorporation of ALA into TAG in the first 30 min, followed by a much more gradual rise to 32 % after 2 h. These values were approaching those achieved after 24 h in some experiments (Figures 6.5, 6.9) and suggested that shorter incubation could be used, if required, in some cases. Nevertheless, the highest % ALA in TAG was achieved with the 24h (standard) incubation time (Figures 6.7, 6.11).

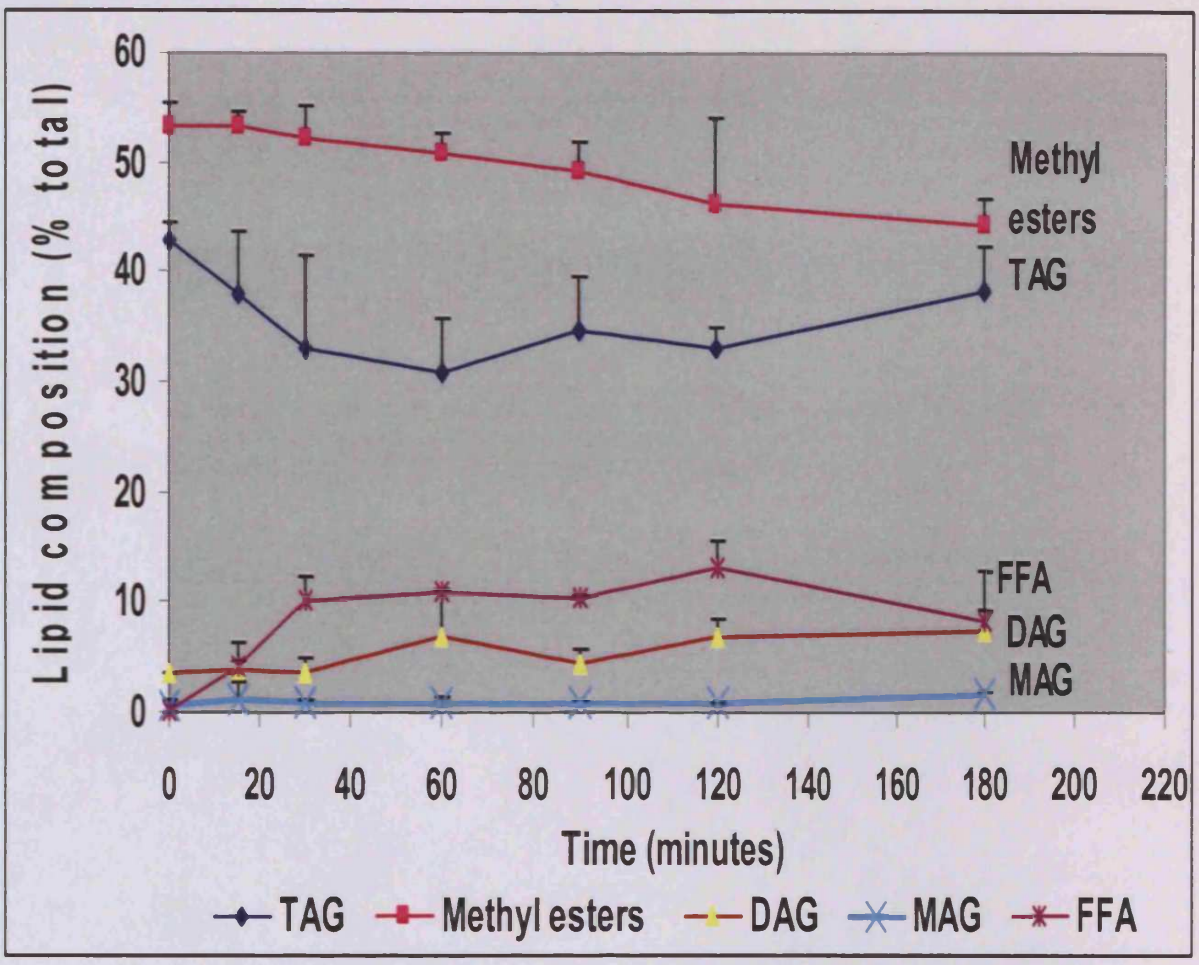


Figure 6.14: Effect of the reaction time on the reaction products (%) following lipase-catalyzed interesterification at 55°C, and 10% *Lipozyme IM 60* content in hexane as reaction media. Results show means + S.D. (n=3).

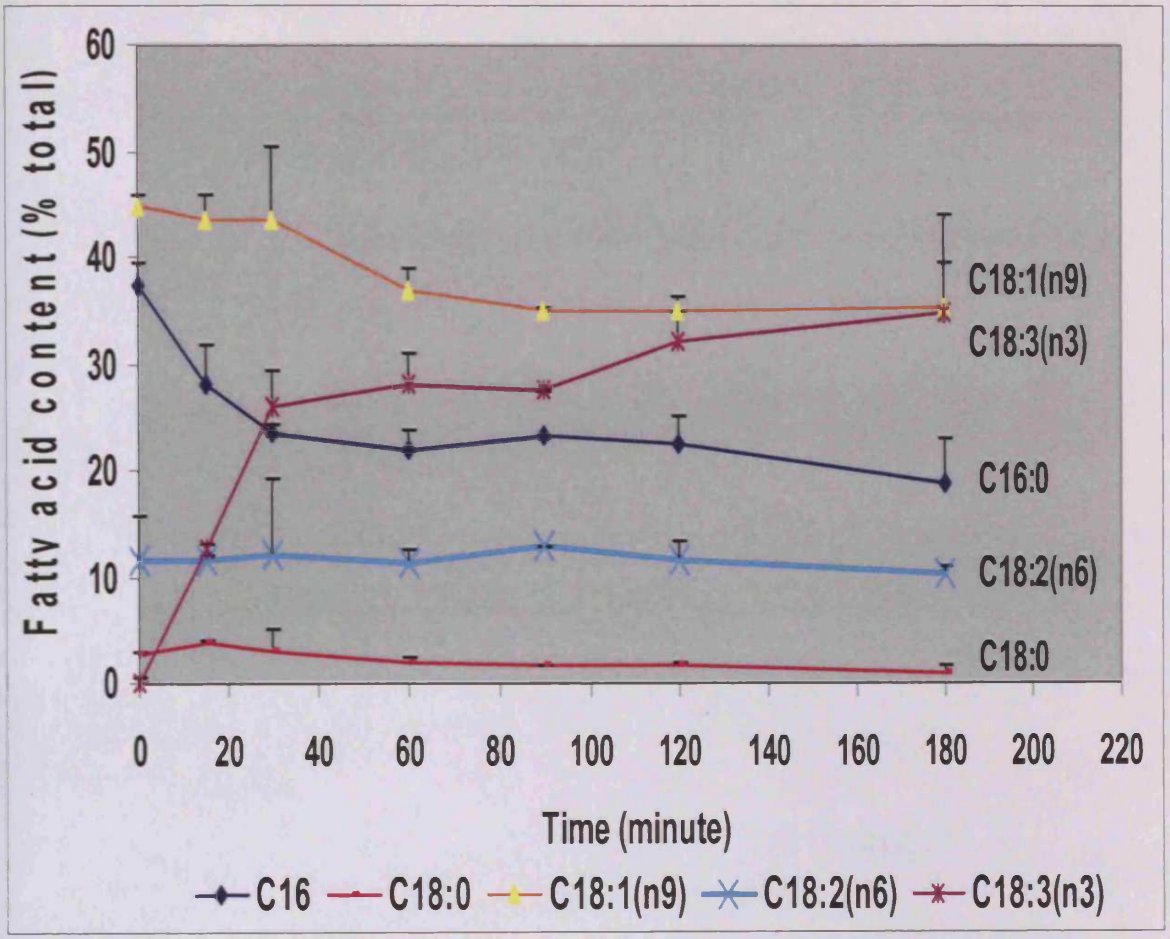


Figure 6.15: Effect of the reaction time on the incorporation of ALA into TAG (%) following lipase-catalyzed interesterification at 55°C and 10% *Lipozyme IM 60* content in hexane as reaction media. Results show means + S.D. (n=3).

6.3.9 Reusability of enzymes

The reusability of *Lipozyme IM 60* was investigated by recovering the lipase after each esterification and re-using it in subsequent experiments. The experimental conditions for reusability were chosen as follows; the enzyme was used at 10g/100g substrate, at a temperature of 55° C and with a reaction time of 3 h. Three batch recycles were conducted. The immobilized lipase was separated from the reaction products, by filtration, using glass wool, after each incubation. The lipase preparation was washed with hexane twice and dried under vacuum before reusing in the stability study. As can be seen from Figure 6.16, no statistically significant decrease in the activity of the enzyme was observed after these cycles of use. However, the slight decrease in TAG proportion, accompanied by increased FFA proportions (Figure 6.16) may indicate a limit to the amount of re-use possible.

Nevertheless, the enzyme was able to transesterify ALA effectively into TAG at all stages in the re-use cycle (Figure 6.17). It was obvious that *Lipozyme IM 60* is stable as an enzyme catalyst at least over a period of 3 reaction cycles (each of 3 hours) at 55 °C. More reusable cycles should be performed at longer times to observe any significant deterioration in the activity of *Lipozyme IM 60* in the lipase-catalyzed interesterification of palm olein and methyl linolenate.

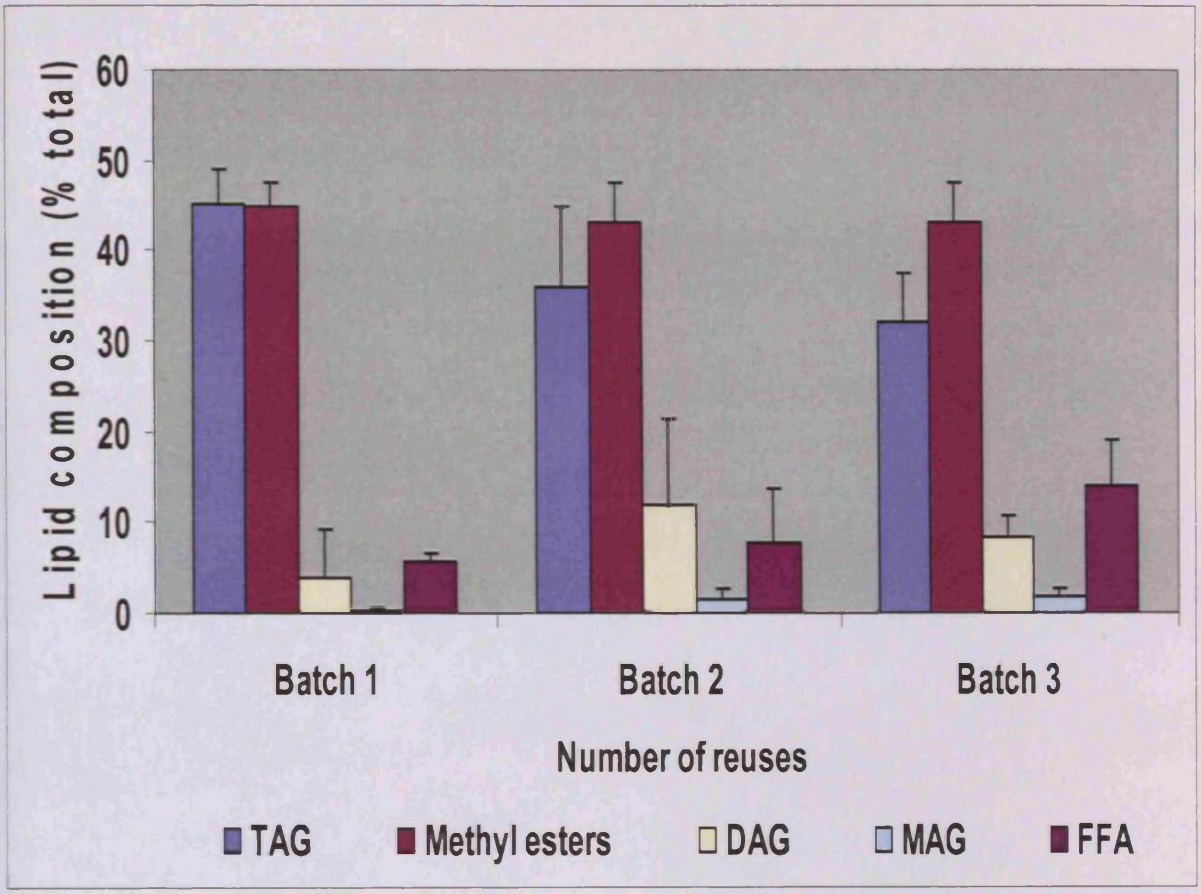


Figure 6.16: Effect of enzyme re-use on the lipid products following lipase-catalyzed interesterification of palm olein with ALA. For details see text. Results show means + S.D. (n=3). Quantification of lipids was from their fatty acid content.

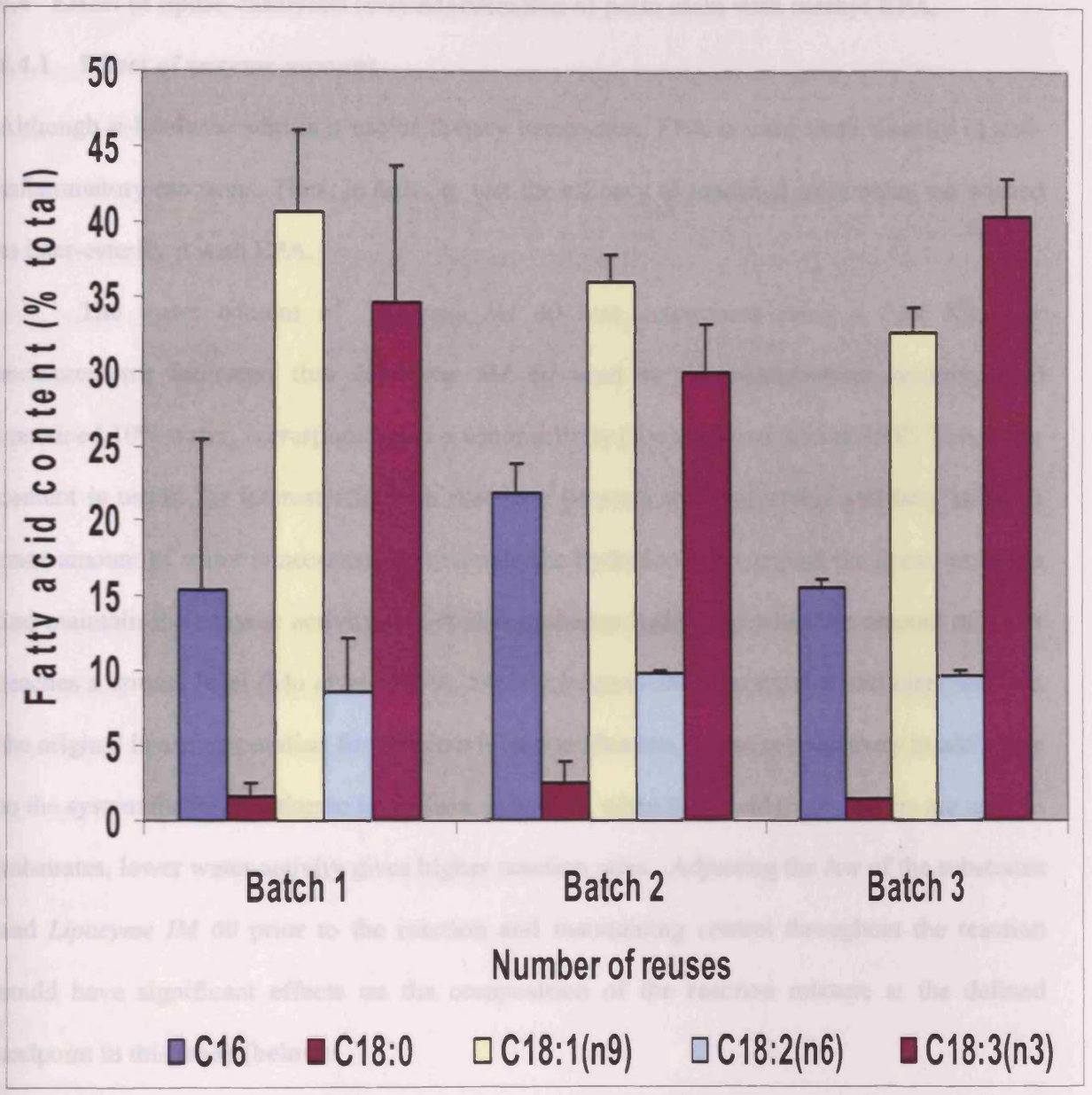


Figure 6.17: Effect of enzyme re-use on the incorporation of ALA into TAG (see text for details). Results show means + S.D. (n=3).

6.4 Effect of lipase-catalysed interesterification of palm olein with methyl EPA.

6.4.1 Effect of enzyme amount

Although α-linolenic acid is a useful dietary component, EPA is used more directly in anti-inflammatory reactions. Thus, in order to test the efficacy of modified palm olein, we wished to inter-esterify it with EPA.

The water content of *Lipozyme IM 60* was determined using a Paw Kit. Our measurements indicated that *Lipozyme IM 60* used as the manufacturer recommended contained 10% water, corresponding to a water activity (Aw) value of 0.55 at 25°C. This water content is useful for interesterification reactions between triacylglycerols and fatty acids. A trace amount of water is necessary to maintain the hydration layer around the lipase molecule and maintain the enzyme activity, but it also promotes hydrolysis when the amount of water reaches a critical level (Mu *et al.,* 1999). Since *Lipozyme IM 60* contained sufficient water in the original lipase preparation for efficient interesterification, it was not necessary to add water to the system for the reaction to take place. However, when fatty acid methyl esters are used as substrates, lower water activity gives higher reaction rates. Adjusting the Aw of the substrates and *Lipozyme IM 60* prior to the reaction and maintaining control throughout the reaction could have significant effects on the composition of the reaction mixture at the defined endpoint in this study (below).

Thus, the results of interesterification of methyl linolenate and palm olein with increasing enzyme amounts conducted without control-dried enzymes (Aw = 0.55) showed an increasing FFA content as shown in Figure 6.1. This would agree with the premise that the nature of the lipid products would be related to Aw; where increasing water levels led to greater hydrolysis and, thus, a higher FFA content. The water content measured in palm olein, methyl linolenate and methyl EPA were 0.23, 0.25 and 0.24 Aw, respectively.

In an experiment conducted using methyl EPA instead of methyl linolenate as the substrate, the release of FFA at higher enzyme values but under the same conditions as the methyl linolenate experiments, produced somewhat lower FFA yields (Figure 6.18). However, in this study, samples (100mg) of dried *Lipozyme IM 60* preparation (Aw= 0.22) were used. They had been equilibrated overnight at room temperature (25°C) with dry silica gel. Thus, the FFA yield appeared to be decreased when dried enzyme was used. Overall, as with the transesterification using methyl linolenate, there were rather small changes in the proportions of the final lipid products with an increase in enzyme amount.

We examined the composition of the TAG product following interesterification in the presence of increasing amounts of lipase. Levels above 5% of added lipase gave EPA proportions in the range 45-52% in the TAG fraction (Figure 6.19). No major changes were observed at enzyme loads above the standard 10% value. These values of incorporation of EPA were similar to the values of ALA achieved in the TAG products (see Figure 6.12) and indicate that *Lipozyme IM 60* did not discriminate between either of these *n-3* PUFAs.

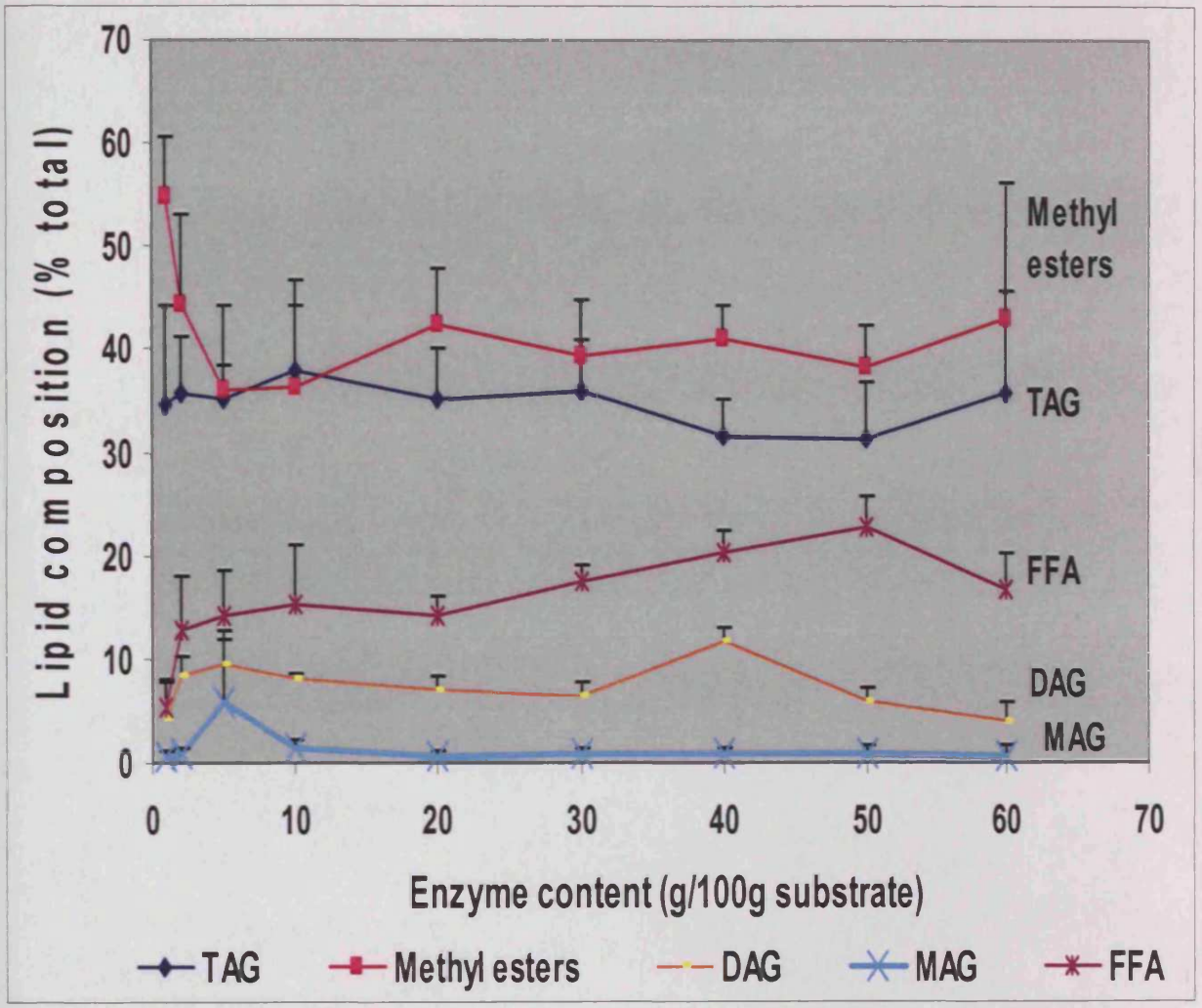


Figure 6.18: Effects of *Lipozyme IM 60* content (1-60%) on the lipid composition following lipase-catalyzed interesterification of palm olein with methyl EPA Standard conditions apart from enzyme amounts were used. Results show means +S.D. (n=3). Amounts of lipids were calculated as their fatty acid contents.

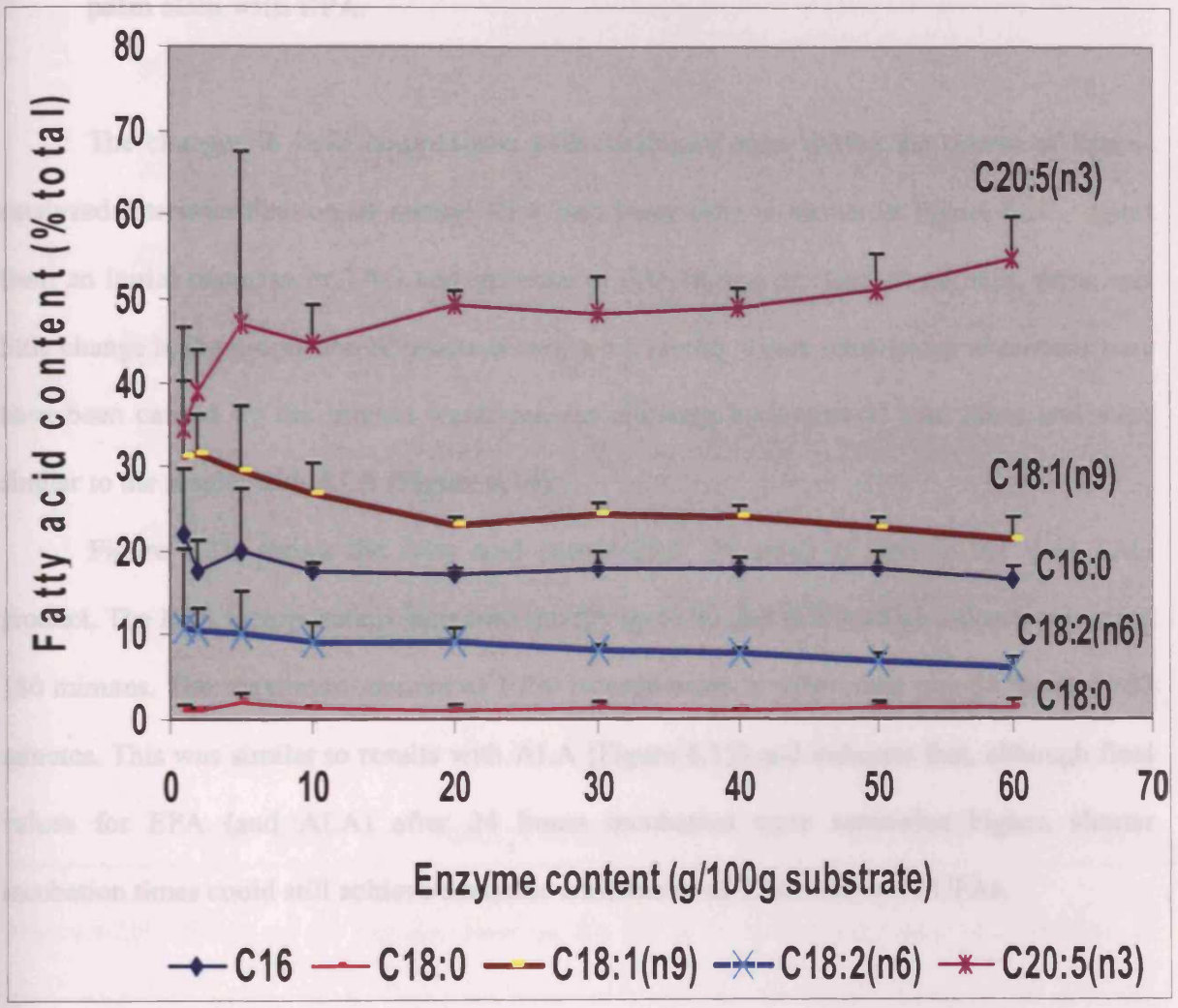


Figure 6.19: Effects of *Lipozyme IM 60* content on the incorporation of EPA into the TAG fraction following the lipase-catalyzed interesterification. Results show means +S.D. (n=3).

6.4.2 Comparison of short incubations on the lipase-catalyzed interesterification of palm olein with EPA.

The changes in lipid composition with incubation time during the course of lipase-catalyzed interesterification of methyl EPA into palm olein is shown in Figure 6.20. Apart from an initial decrease in TAG and increase in FFA during the first 20 minutes, there was little change in the proportion of products over a 3 h period. These small initial alterations may have been caused by the limited water present allowing hydrolysis to take place and were similar to the results with ALA (Figure 6.14).

Figure 6.21, shows the fatty acid composition (% total) of EPA in the final TAG product. The EPA incorporation increased rapidly up to 90 min followed by a slower rise up to 180 minutes. The maximum amount of EPA incorporation in palm olein was 34 % after 180 minutes. This was similar to results with ALA (Figure 6.15) and indicates that, although final values for EPA (and ALA) after 24 hours incubation were somewhat higher, shorter incubation times could still achieve adequate enrichment of TAG with *n-3* PUFAs.

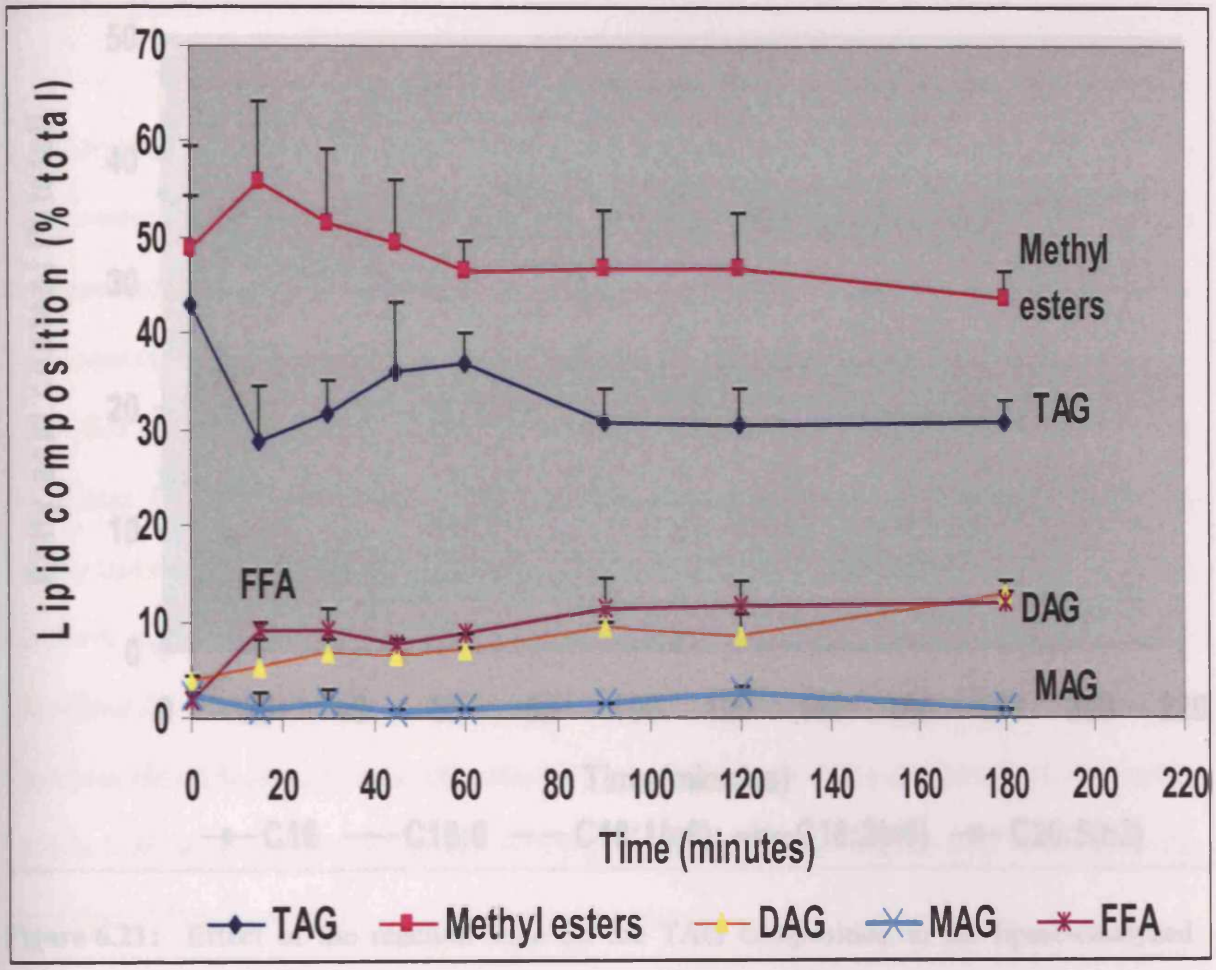


Figure 6.20: Effect of the reaction time on the products of the lipase-catalyzed reaction of palm olein with methyl EPA. Reaction times at 0, 15, 30, 45, 60, 90, 120 and 180 minutes, respectively, were used at 55°C and 10% *Lipozyme IM 60* content in hexane. Results show means + S.D. (n=3). Lipids were quantified as their fatty acid contents.

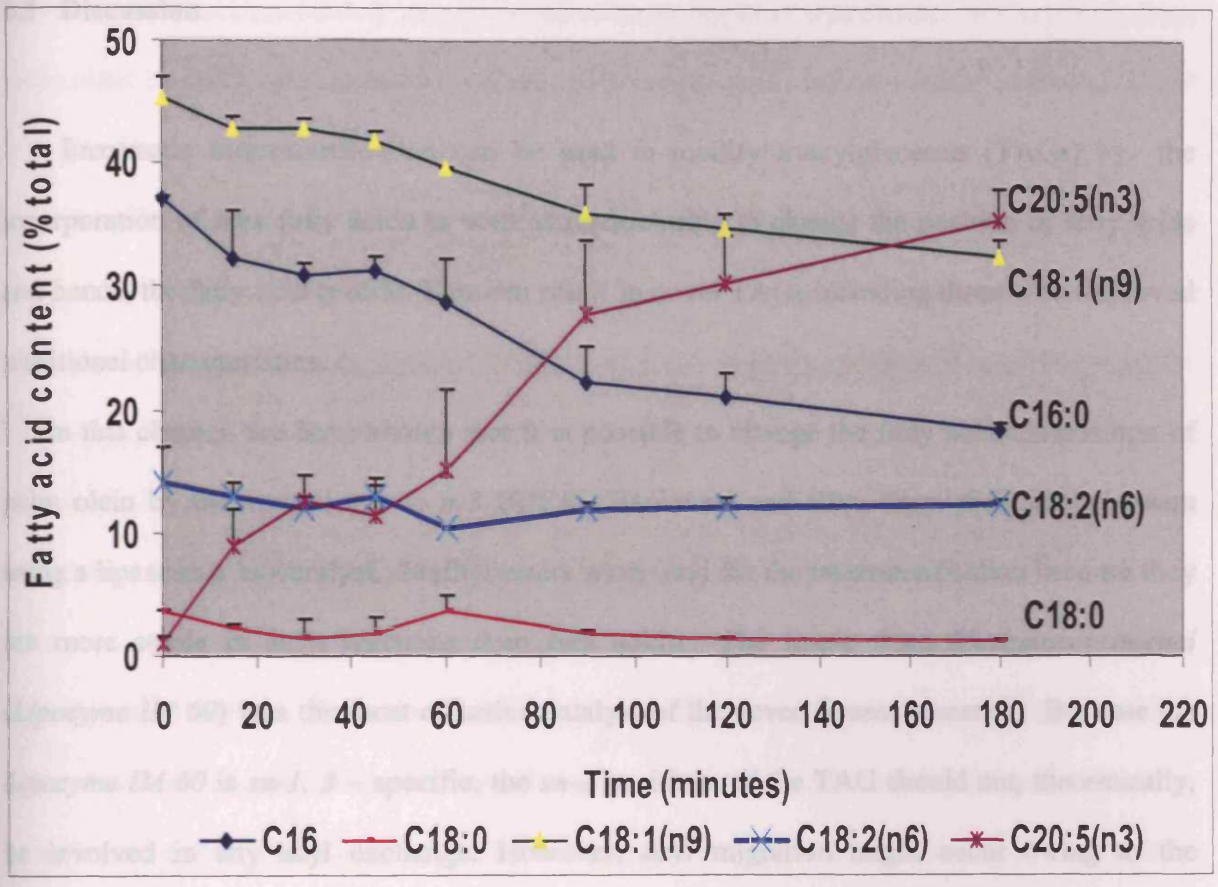


Figure 6.21: Effect of the reaction time on the TAG composition in the lipase-catalyzed reaction of palm olein with methyl EPA. Reaction times at 0, 15, 30, 45, 60, 90, 120 and 180 minutes, respectively, were used at 55°C and 10% *Lipozyme IM 60* content in hexane. Results show means + S.D. (n=3).

6.5 Discussion

Enzymatic interesterification can be used to modify triacylglycerols (TAGs) by the incorporation of new fatty acids as well as restructuring to change the position of fatty acids and hence, the fatty acid profile. This can result in novel TAGs including those with improved nutritional characteristics.

In this chapter, we have shown that it is possible to change the fatty acid composition of palm olein by incorporating two *n-3* PUFAs, linolenate and EPA, from their methyl esters using a lipase as a biocatalyst. Methyl esters were used for the interesterification because they are more stable in such reactions than free acids. The lipase from *Rhizomucor miehei* (*Lipozyme IM 60*) was the most effective catalyst of the seven lipases screened. Because the *Lipozyme IM 60* is *sn-1, 3* – specific, the *sn-2* positions of the TAG should not, theoretically, be involved in any acyl exchange. However, acyl migration might occur owing to the formation of DAG (or MAG) as reaction intermediates.

Enrichment of the *n-3* PUFAs at the *sn-1,3* positions might have a conceivable advantage in that the digestive enzyme pancreatic lipase has a specificity for these positions and this would result in the rapid hydrolysis (and absorption) of these acids during digestion (Gurr *et al.*, 2002).

We carefully checked the reaction conditions for interesterification in order to use appropriate parameters for effective incorporation of *n-3* PUFAs into the TAG from palm olein. The lipase used was stable to high temperatures, preferred non-polar solvents and worked well with an equal proportion of the substrates. While small amounts of water were necessary for enzyme activity, there was some evidence that it gave rise to a little hydrolysis. Nevertheless, it was possible to enrich the TAG fraction with up to 50% of either ALA or

EPA. These data show that it should be possible to produce value-added *n-3* products from palm olein by using similar incubations on an industrial scale. Moreover, the *Lipozyme IM 60* was suitable for re-use in its immobilized form.

Thus, the incorporation of ALA and EPA into palm olein was sucessfully made and the product may have potential health benefits. Selection of enzyme and control of enzyme content, substrate ratio, time period, temperature, water activity and organic solvent type were the major controlling factors for incorporation of *n-3* PUFAs into the TAG fractions. The efficiency of the immobilized lipase, *Lipozyme IM 60*, which could be used repeatedly, established the cost-effectiveness of the whole process. Thus, the bio-catalytic route described here could be considered economical, efficient and environmentally-benign for carrying out esterification reactions of commercial significance. Diets incorporating *n-3* PUFA-containing palm olein may be potentially more beneficial than diets with unmodified palm and fish oils. It could be classed as a speciality edible oil for specific nutritional and clinical needs, whereby it would serve as a single rich source of *n-3* PUFAs, provitamin A, vitamin E and oleic acid.

6.6 Chapter summary

- The most effective lipase for incorporation of ALA into palm olein was *Lipozyme IM 60* from *Rhizomucor miehie*.
- The highest incorporation of ALA into palm olein TAG was obtained with substrate ratios (wt/wt) of at least 1:1 (gram methyl ALA: gram palm olein).
- Temperatures in the range 55-70°C seemed to be the most suitable.
- Increasing the enzyme content above 10% (10g/100g substrates) did not significantly increase the yields of ALA and EPA in the TAG product.

- The interesterification reaction proceeded quickly with at least half of the incorporation of ALA occurring in 30 min and of EPA in 90 min. However, incorporation after 24h was higher than for shorter (up to 3h) incubations.
- The incorporation of ALA into palm olein did not show a significant decrease when the enzyme was re-cycled three times.
- Non-polar solvents such as hexane were the best for the interesterification reaction.
- Use of silica gel to dry the lipase had a marginal effect in reducing the free fatty acid production but did not significantly increase TAG yield or its *n-3* PUFA content.
- Thus, it was shown that *Lipozyme IM 60* could be used successfully to synthesise a modified palm olein, suitable as a speciality nutraceutical enriched in *n-3* PUFAs.

CHAPTER SEVEN

The effects of incorporation of EPA into palm olein on reducing inflammation in a cell culture system

7.1 Introduction

Palm oil (PO) is currently second (behind soybean oil) in world edible oil production tonnage. It is rich in 16:0 and 18:1(n-9) fatty acids, as well as carotenes, tocopherols and tocotrienols but has relatively low levels of 18:2(n-6) (Nesaretnam and Muhammad, 1994). Malaysian palm oil dominates the world market for palm oil and its derivatives. As the world's largest producer and exporter of palm oil, Malaysia accounts for more than 50% of world palm oil output and more than 60% of world palm oil exports (www.matrade.gov.my). Edible palm oil-based specialty fats are primary elements in processed food products such as margarine, vanaspati or vegetable ghee, shortening or non-dairy creamers. They are also useful for making cocoa butter substitutes in confectionery and biscuits (Berger, 2001).

The anti-inflammatory effects of *n-3* PUFAs from fish oil (rich in EPA and DHA) play an important role in the regulation of biological functions and prevention and treatment of a number of human diseases including arthritis, Alzheimer's disease, lung fibrosis and cardiovascular diseases (Gurr *et al.,* 2002). Two potent inflammatory eicosanoids are prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4), which are produced from arachidonic acid by the enzymes cyclooxygenase (COX) and 5-lipoxygenase (LOX) (Simopoulos, 2002). *n-3* PUFAs decrease the level of arachidonic acid in inflammatory cell membranes, thus

decreasing the production of arachidonic acid-derived eicosanoids. In addition, the *n-3* PUFAs give rise to a family of eicosanoid mediators that are analogs of those produced from arachidonic acid and are often less potent than those produced from arachidonic acid. Thus, the anti-inflammatory action of *n-3* PUFAs has long been viewed as being similar to the anti-aggregatory action, with altered patterns of eicosanoid production of differing potencies being the central mechanism of action (Calder, 2004). A significant sign of the anti-inflammatory actions of *n-3* PUFAs is their ability to reduce the production of cytokines involved in inflammation, like interleukin (IL)-1, tumor necrosis factor-alpha (TNF) and IL-6, as well as changing eicosanoid profiles (Martel-Pelletier *et al.,* 1999).

The health benefits to human consumers of reducing intake of *n*-6 PUFAs and increasing intake of *n-3* PUFAs has been well-documented (Simopolous, 1999; Curtis *et al.,* 2000; Calder, 2001; Curtis *et al.,* 2002). Recently, new-anti-inflammatory mediators such as resolvins and neuroprotectins have been discovered. While resolvin E1 is formed by a yet ill-defined pathway from EPA, resolvin D1 and neuroprotectins are biosynthesized from DHA. The latter, therefore, are biologically-active lipids derived from a 22 C precursor, in contrast to the eicosanoids (Serhan *et al.,* 2004).

Enriching available foods with EPA and /or DHA provides an option for increasing consumption of these fatty acids. Moreover, technological advances in oil refining permit the incorporation of fish oil into edible vegetable oil blends. However, high amounts of EPA and DHA in enriched foods may impart a fishy aroma or flavour because these highly unsaturated fatty acids are susceptible to oxidation. Thus, efforts to prevent oxidation during processing, cooling, and storage are necessary. Controlling oxidation, to prevent deterioration of PUFAs, is presently a major technological challenge for industry (Uauy and Valenzuela, 2000).

In this chapter, we modified palm olein with EPA through an enzymatic interesterification process, with the aim of producing a fat mixture which had anti-inflammatory properties. The new blend was tested using the chondrocyte cell culture system in order to evaluate its properties and to see if this could represent a value-added product for the palm oil industry.

7.2 Materials and methods

7.2.1 Materials

The materials used were the same as described in section 6.2.1.

7.2.2 Enzymatic modification reactions

For general interesterification of modified palm oil, 0.02g of refined, bleached, deodorized palm olein (POo) was mixed with 0.02g methyl EPA in 0.4 ml hexane. Immobilized lipase *IM 60* (0.12 g) was added and the mixture was incubated in an orbital shaking water bath at 55°C for 24 hour at 200 rpm.

7.2.3 Analysis of products

Samples were taken and filtered through a column of glass wool. The lipid mixture was solvent extracted and washed (section 6.2.2). The reaction products were separated by thin-layer chromatography (TLC) on pre-coated silica gel 60 plates using petroleum ether/ethyl ether/acetic acid (80:20:1, v/v/v) as the solvent system. The TLC plates were sprayed with 0.2% (w/v) 8-anilino-4-naphthalenesulphonic acid (ANSA) in methanol and bands visualized under UV light to reveal lipids. The bands corresponding to TAG were scraped off from the TLC plates and hydrolysed with 5.0ml of methanolic NaOH (0.3N). The mixture was refluxed for 2 hours at 70°C in a thermal block to hydrolyse acyl lipids, 0.3ml of 6N HCl was added to acidify the mixture and the free fatty acids were extracted with 3 - 4 portions (5ml each) of

petroleum ether. The fatty acids were incubated with fatty acid-free bovine serum albumin for 16 hours at 37°C in Tyrode-HEPES buffer (20 mM HEPES, 140mM NaCl, 4.5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 11 mM glucose, pH 7.4) (see section 2.2.2) before addition to the culture medium. All cultures were maintained for 8 hours in the presence or absence of fatty acids to allow the latter's incorporation into membrane lipids (see section 3.6).

7.2.4 Preparation and culture of bovine chondrocytes

Bovine monolayer cultures were prepared and cultured as described in section 2.2.1.

7.2.5 Determination of lactate concentration in chondrocyte culture media

Lactate concentration in chondrocyte culture media was determined as described in section 2.2.3.

7.2.6 Extraction and quantification of proteoglycans

The extraction and quantification of proteoglycan (GAG) in culture media was carried out as described in section 2.2.5.

7.2.7 RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis.

Total RNA was extracted from intact cartilage as described previously in section 2.2.6. RT-PCR was performed as described previously in section 2.2.8.

7.2.8 Statistical analysis.

Statistical evaluation was performed by *T-test* using Microsoft Excel software as described in section 2.2.10.

7.3. Results

7.3.1 Fatty acid composition of the TAG produced by enzymatic interesterification of palm olein with EPA

Studies on the optimization of interesterification of palm olein with *n-3* PUFAs such as methyl linolenic and methyl EPA (catalyzed by Lipozyme *IM 60)* were reported in Chapter 6. Starting with a palm olein and methyl EPA mixture (1:1 w/w) containing 30% lipase *IM 60,* a TAG product containing significant amounts of EPA was obtained as shown in Table 7.1.

Palm olein contained a high proportion of both palmitic (36.8%) and oleic acids (44.6%), a smaller proportion of linoleic acid (13.7%) and a small amount of linolenic acid (0.6%). The fatty acid composition of incorporation of TAG following interesterification of palm olein showed C16:0 and C18:1 and C20:5 as the main fatty acids (see Table 7.1). This composition was slightly different to the proportion of fatty acids obtained in Chapter 6 (see Figure 6.19) while using lipase at 30% of substrate weight (g/100g substrate) with the same conditions. However, as noted in Chapter 6, slightly different yields of products were found in successive but comparable experiments. One reason for this might have been due to the exact water content of the enzyme preparation, solvents and substrates used.

	Fatty acid									
	C14:0	**C16:0**	**C18:0**	**C18:1 (n9)**	**C18:2 (n6)**	**C18:3 (n3)**	**C20:1**	**C20:4 (n6)**	**C20:5 (n3)**	**C22:6**
Palm olein(PO)	1.1± 0.4	36.8± 1.2	3.2± 0.2	44.6± 0.2	13.7± 0.9	0.6± 0.3	ND	ND	ND	ND
Methyl ester α – eicosapentaenoic acid (EPA)	ND	ND	ND	ND	ND	ND	0.2± 0.5	0.1± 0.1	99.2 ± 0.6	0.5± 0.7
Modified palm olein	0.2± 0.1	27.9± 1.6	2.9± 0.8	35.0± 2.6	8.6± 4.4	0.2± 0.1	ND	ND	25.2± 7.4	ND

TABLE 7.1: Fatty acid compositions (%) of TAG from palm olein, methyl EPA and the TAG produced after interesterification of palm olein with EPA. Means ± S.D. (n=3) are shown. ND not detected.

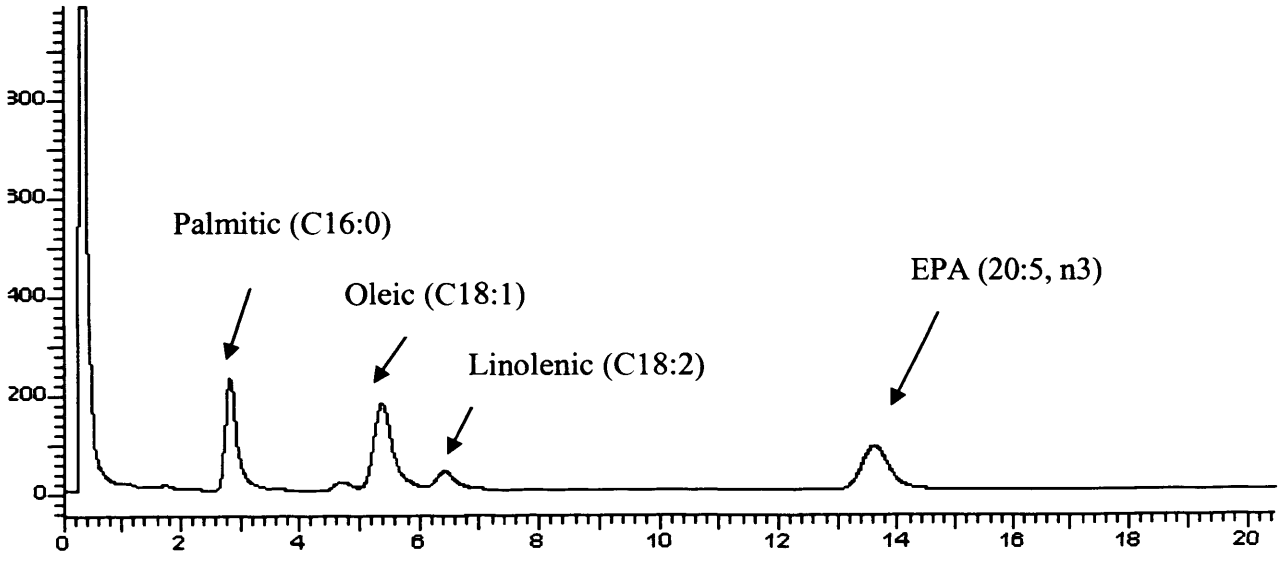
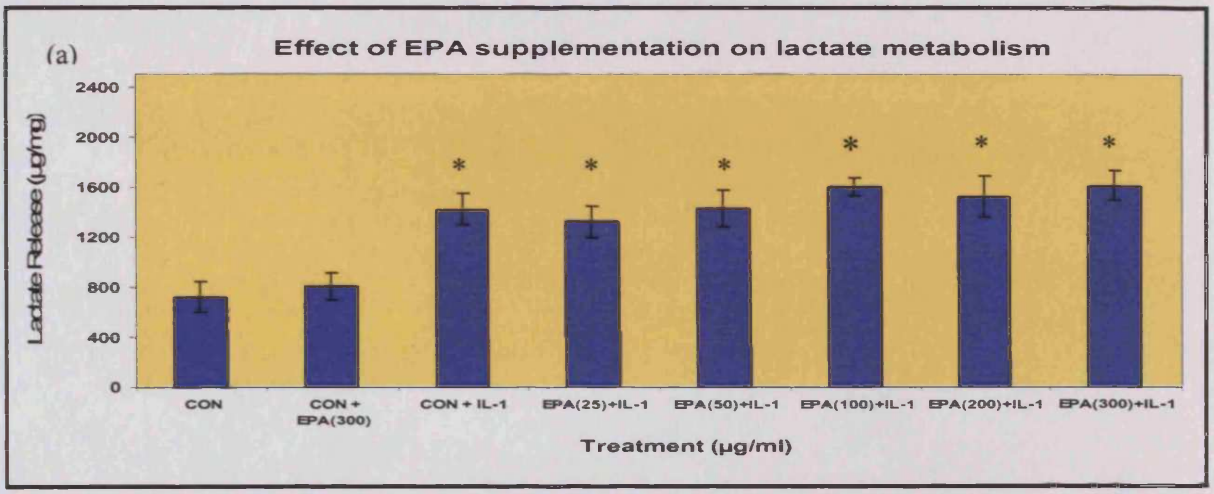


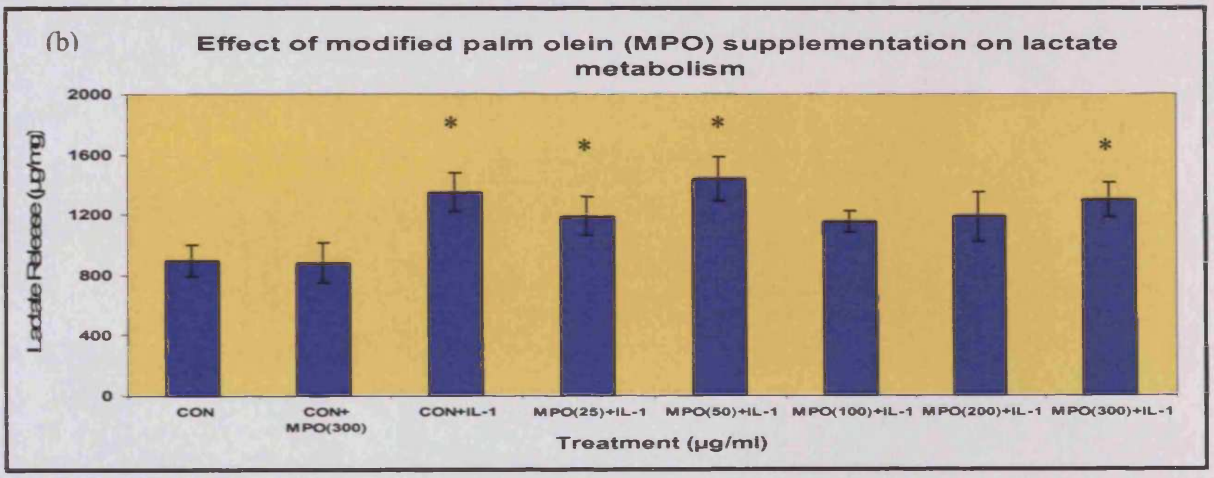
Figure 7.1: The chromatogram of the cells after treatment with modified palm olein.

7.3.2 Analysis of cell viability after enzymatic supplementation

Cell viability was used to determine if significant cell death had occurred in the monolayer chondrocyte cultures. As had been previously described in the Chapter 2, media collected from bovine monolayer cultures after supplementation with various fatty acids was analysed and lactate, which is a by-product of carbohydrate metabolism, was used as an indicator of cell metabolism, following its release into the culture medium. As shown in Figure 7.2, the results showed that there was an increase in lactate production when IL-1α was added compared to the control cultures, indicating an increase in non-aerobic cell metabolism on addition of this cytokine.

Further addition of the various fatty acids (EPA, hydrolysed palm olein, interesterified palm olein mixture) did not change the level of lactate release from that caused by IL-1α addition alone. These results showed that the cells remained viable during the time-course of the experiments and this was confirmed by the microscopic appearance of cultures (results not shown) and the yield of cells from the monolayer after 4 days.





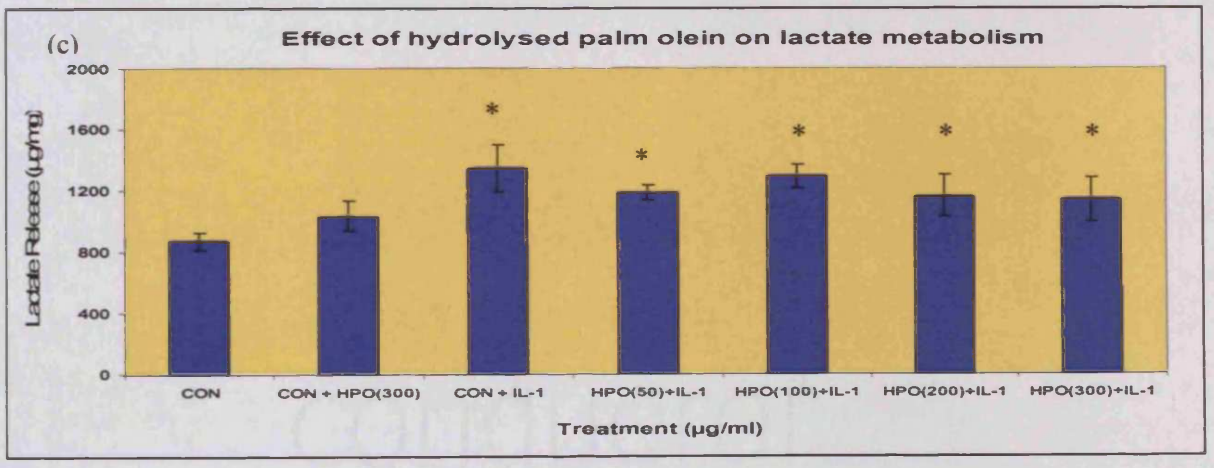


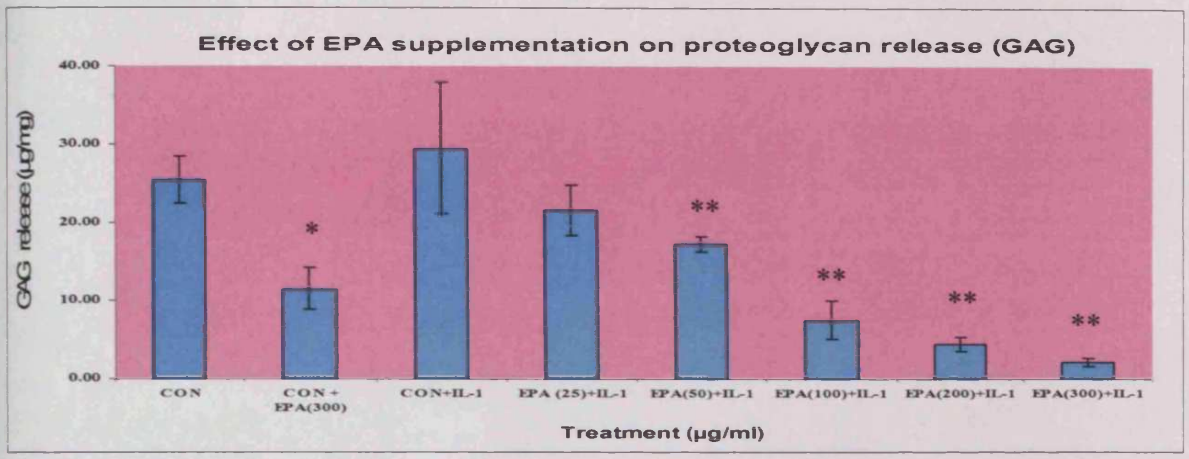
Figure 7.2: Effect on lactate release into the culture medium following incubation of cells with 25 – 300µg/ml EPA, modified palm olein (MPO) and HPOo for 8 hours followed by exposure to IL-1α for 4 days. Data as means ± S.D. (n=3) are shown. Significant difference using Students' T-test given by * compared to control ($p<0.05$).

7.3.3 Effect of fatty acid supplementation on GAG release in IL-1α stimulated chondrocytes

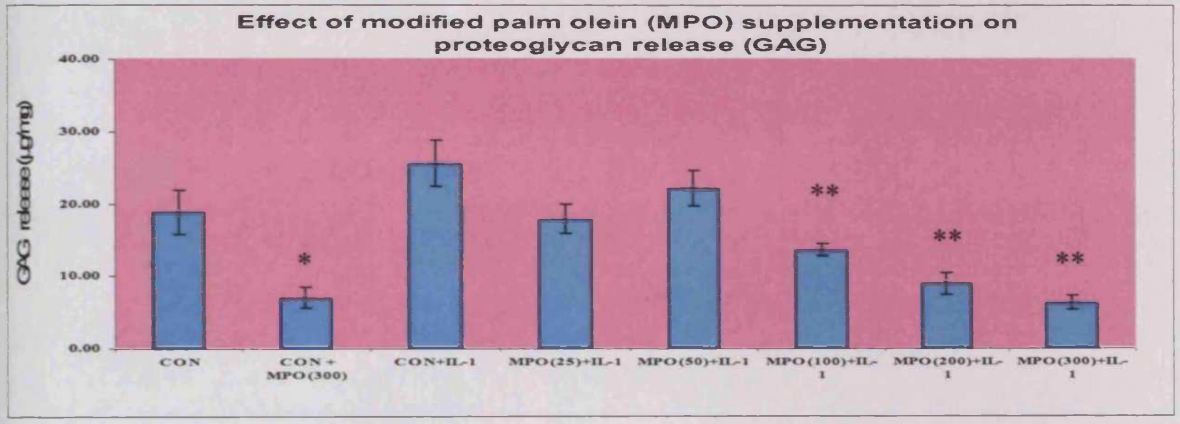
Media collected from the monolayer chondrocyte cultures after challenge with various fatty acids were measured for GAG release (see section 2.2.5). Basically, GAG is the main component in proteoglycan so, under the influence of pro-inflammatory cytokine IL-1α, more GAGs will be produced due to increased matrix degradation. Previous work in this thesis (and in the literature) has shown that *n-3* fatty acids have a beneficial effect in reducing the levels of GAGs released into the media following IL-1α addition. As shown in the Figure 7.2, bovine monolayer cultures showed higher GAG levels in the media when IL-1α had been added, compared to control. Increasing amounts of EPA caused a progressive reduction in GAG levels detected in the culture medium (Figure 7.3 (a)). Addition of the modified palm olein, containing about 25% EPA (see Table 7.1 and Figure 7.1); also produced a progressive decrease in the IL-1 stimulated GAG levels. This decrease was statistically significant with 200 and 300μg fatty acid /ml culture.

For hydrolysed palm olein, as remarked in previous chapters, the results were equivocal. The highest concentrations (300μg/ml) gave significantly lower GAG levels compared to IL-1 treatment alone. However, there was no obvious concentration-dependence. Interestingly, all three treatments reduced GAG released by the control cultures. This reduction was particularly marked for the modified palm olein and for EPA.

(a)



(b)



(c)

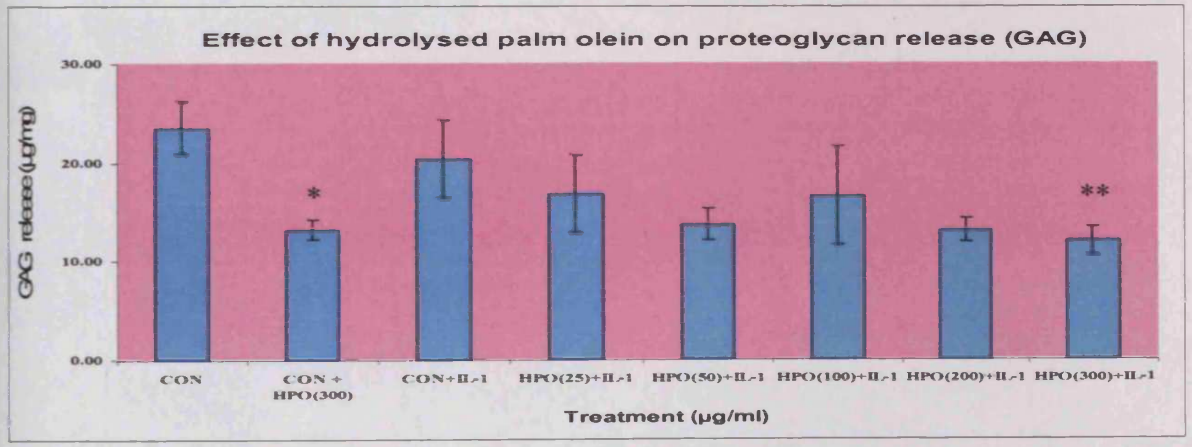


Figure 7.3: GAG release into the culture medium following supplementation with different fatty acids and with or without IL-1α. Supplementation with fatty acids was for 8h before washing and culturing for a further 4 days. Means ± S.D. (n=5) are shown. Significant difference was analysed using Students' *T-test* at $p<0.05$.

* = Significantly different compared to control ($p<0.05$).

** = Significantly different compared to control + IL-1 ($p<0.05$).

7.3.4 Effects of modified palm olein on the levels of cyclooxygenase-2 (COX-2) mRNA.

We next examined the effect of supplementation with the modified palm olein fatty acids on COX-2 mRNA in monolayer cultures. The results obtained were compared with the COX-2 mRNA levels in monolayer cultures after EPA or HPOo supplementation. As shown in Figure 7.4, chondrocyte COX-2 mRNA was detected in IL-1α treated samples but not appreciably in control cultures. Significantly, pro-inflammatory COX-2 mRNA expression in IL-1α-treated cultures was decreased by the modified palm olein products. The mRNA COX-2 was totally abolished at 300μg fatty acids/ml. This was similar to EPA supplementation of bovine monolayer cultures (Figure 7.4) although EPA may have been more effective. This was expected because the modified palm olein had about 25% EPA in its total acids and would, therefore, be expected to be less anti-inflammatory than EPA alone. In contrast, supplementation of HPOo had no effect on the level of COX-2 mRNA.

The results obtained show that the enzymatic interesterification of palm olein with EPA gave a product that had a significant effect in reducing the level of mRNA for the pro-inflammatory COX-2. Theoretically, COX-2 is inducible by inflammation. It is not present in non-inflammatory tissue, but increases in response to inflammation, including that seen in arthritis. COX-2 has 60% homology with COX-1 but the latter is present in most tissues as a constitutive enzyme.

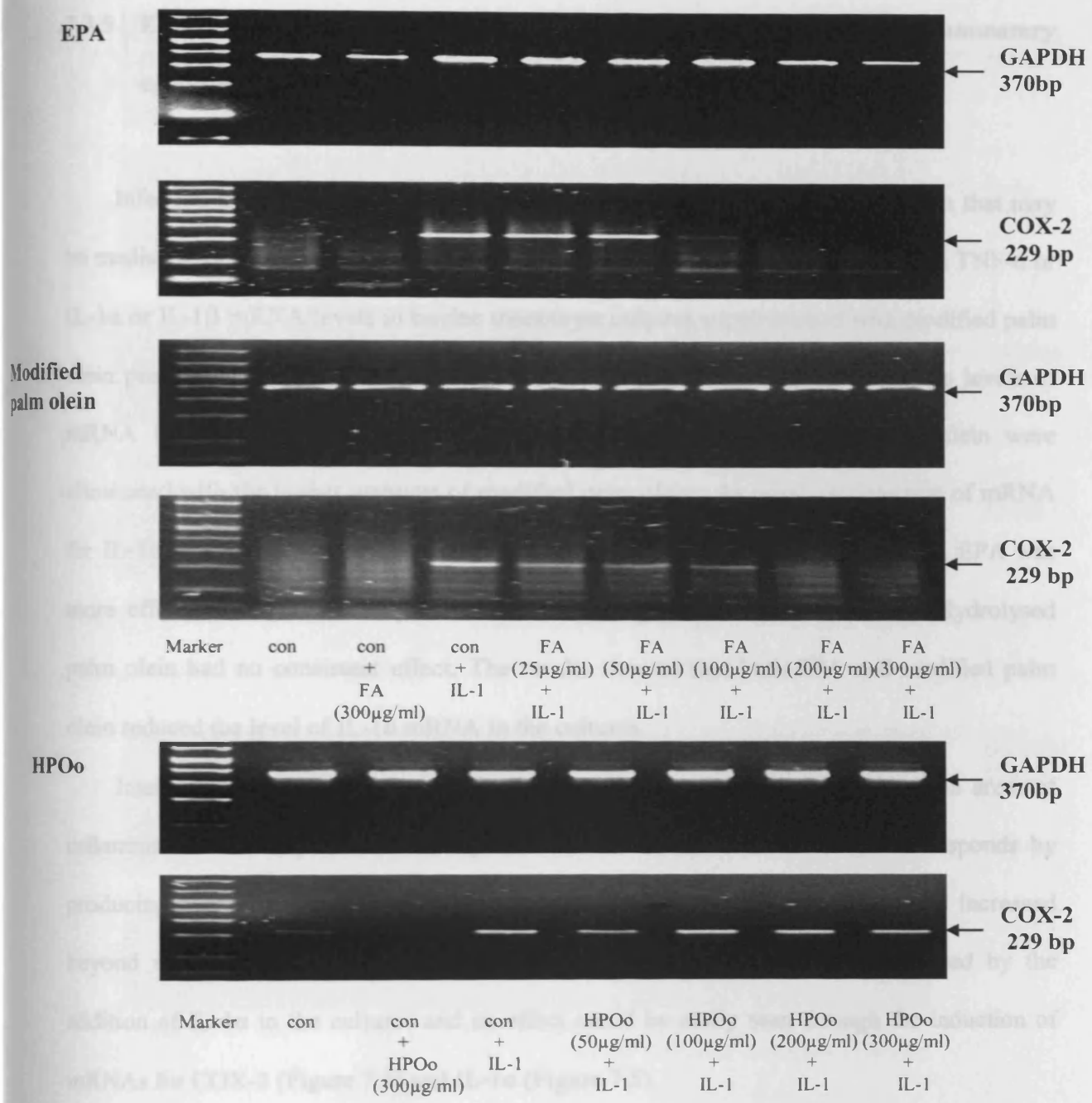


Figure 7.4: RT-PCR using COX-2 primers was carried out on bovine monolayer cultures treated with EPA, modified palm olein products or hydrolysed palm olein (HPOo) for 8 hours followed, after washing, by subsequent culture for 4 days with IL-1α (10ng/ml). Separations were on 2% agarose gels alongside a 50-1000 bp marker.

7.3.5 Effects of fatty acid supplementation on the level of mRNAs for pro-inflammatory cytokines (TNF- α, IL-1α, IL-1β).

Infection and inflammation are associated with alterations in lipid metabolism that may be mediated by cytokines such as TNF-α and IL-1. So we determined the effects on TNF-α or IL-1α or IL-1β mRNA levels in bovine monolayer cultures supplemented with modified palm olein products compared with EPA and hydrolysed palm olein (Figure 7.5). The levels of mRNA for IL-1α in chondrocyte cultures exposed with the modified palm olein were eliminated with the higher amounts of modified palm olein. As usual, no detection of mRNA for IL-1α was found in control cultures. As with the data for COX-2 (Figure 7.4), EPA was more effective than modified palm olein in reducing mRNA levels for IL-1α. Hydrolysed palm olein had no consistent effect. The results showed that both EPA and modified palm olein reduced the level of IL-1α mRNA in the cultures.

Interleukin-1α (IL-1α) is a specific inflammatory mediator that is produced in areas of inflammation. When part of the body becomes inflamed the immune system responds by producing cytokines. Because arthritic tissue is inflamed, production of IL-1α is increased beyond normal levels. In this experiment, an inflammatory reaction was induced by the addition of IL-1α to the cultures and its effect could be easily seen through the induction of mRNAs for COX-2 (Figure 7.4) and IL-1α (Figure 7.5).

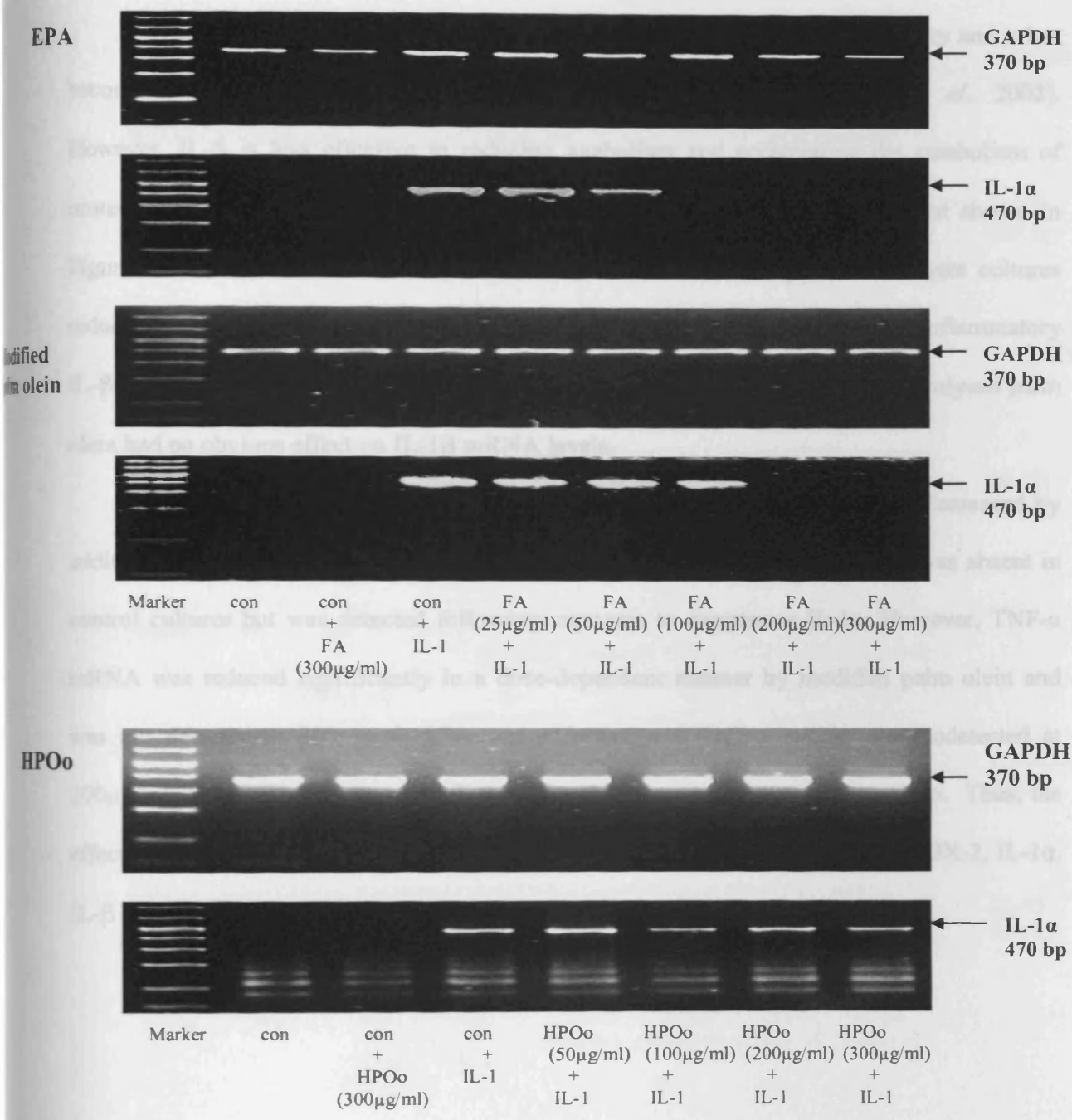


Figure 7.5: RT-PCR using IL-1α primers was carried out on bovine monolayer cultures treated with EPA, modified palm olein products or hydrolysed palm olein (HPOo) complexed to BSA for 8 hours followed, after washing, by subsequent culture for 4 days with IL-1α (10ng/ml). Separations were on 2% agarose gels alongside a 50-1000 bp marker.

IL-1β is another important mediator of inflammation and joint tissue injury and it has become a target of therapy for patients with rheumatoid arthritis (Furse *et al.,* 2002). However, IL-β is less effective in reducing anabolism and accelerating the catabolism of proteoglycans compared with IL-1α (Aydelotte *et al.,* 1992). In the experiment shown in Figure 7.6, addition of modified palm olein products in monolayer chondrocytes cultures reduces IL-1β mRNA following stimulation with IL-1α. This reduction in pro-inflammatory IL-β mRNA was seen at 300μg/ml. As before, EPA was more effective but hydrolysed palm olein had no obvious effect on IL-1β mRNA levels.

Significantly, TNF-α mRNA levels in IL-1α treated cultures were also decreased by addition of modified palm olein products (Figure 7.7). The mRNA for TNF-α was absent in control cultures but was detected following exposure to exogenous IL-1α. However, TNF-α mRNA was reduced significantly in a dose-dependent manner by modified palm olein and was not detected at 300μg/ml. EPA was effective and TNF-α mRNA was undetected at 200μg/ml. As usual, there was no effect on the cultures supplemented with HPOo. Thus, the effect of modified palm olein on TNF-α mRNA levels was similar to that on COX-2, IL-1α, IL-β and was somewhat less effective then EPA alone.

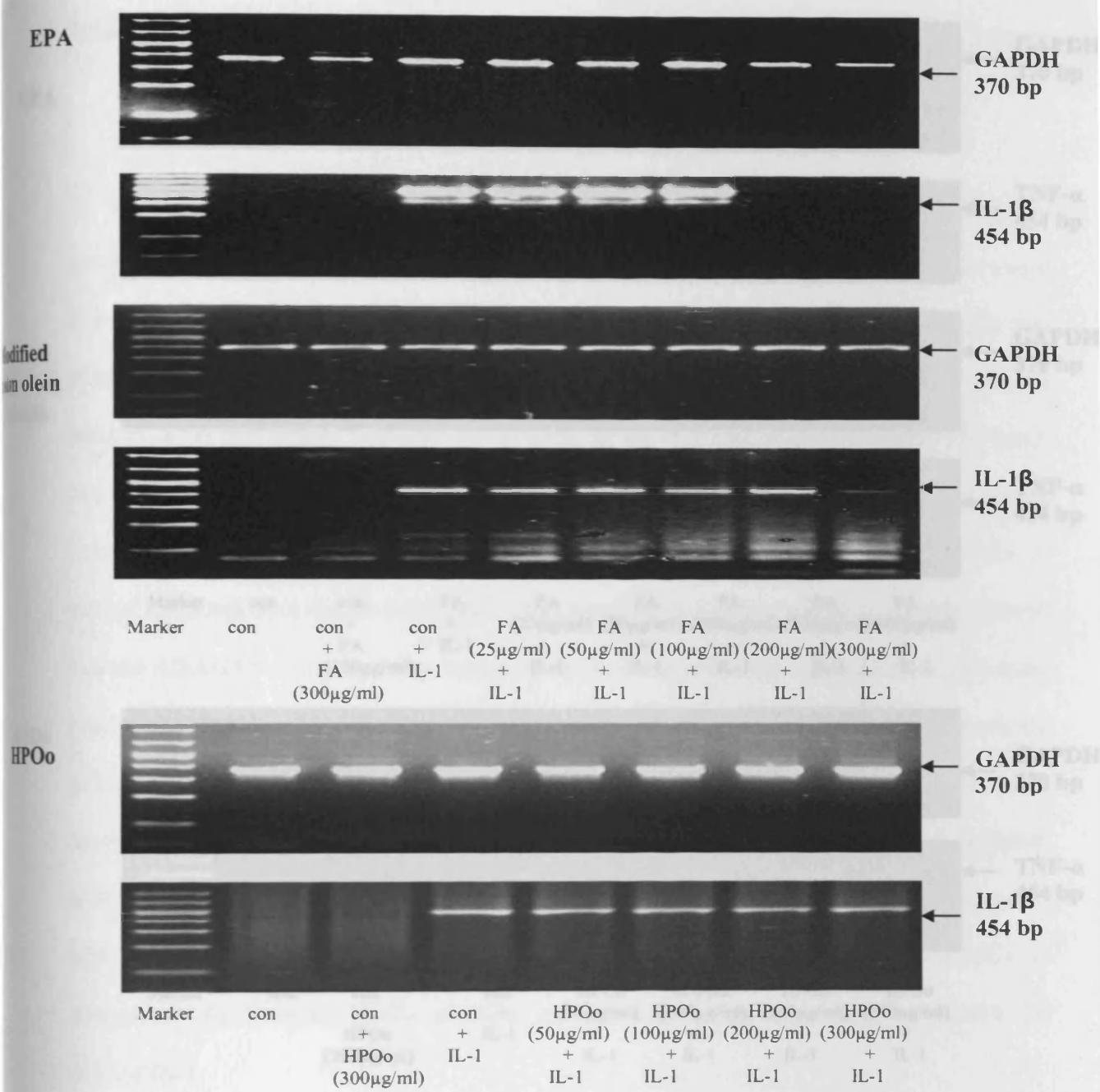


Figure 7.6: RT-PCR using IL-1β primers was carried out on bovine monolayer cultures treated with EPA, modified palm olein or hydrolysed palm olein (HPOo) complexed to BSA for 8 hours followed, after washing, by subsequent culture for 4 days with IL-1α (10ng/ml). Separations were on 2% agarose gels alongside a 50-1000 bp marker.

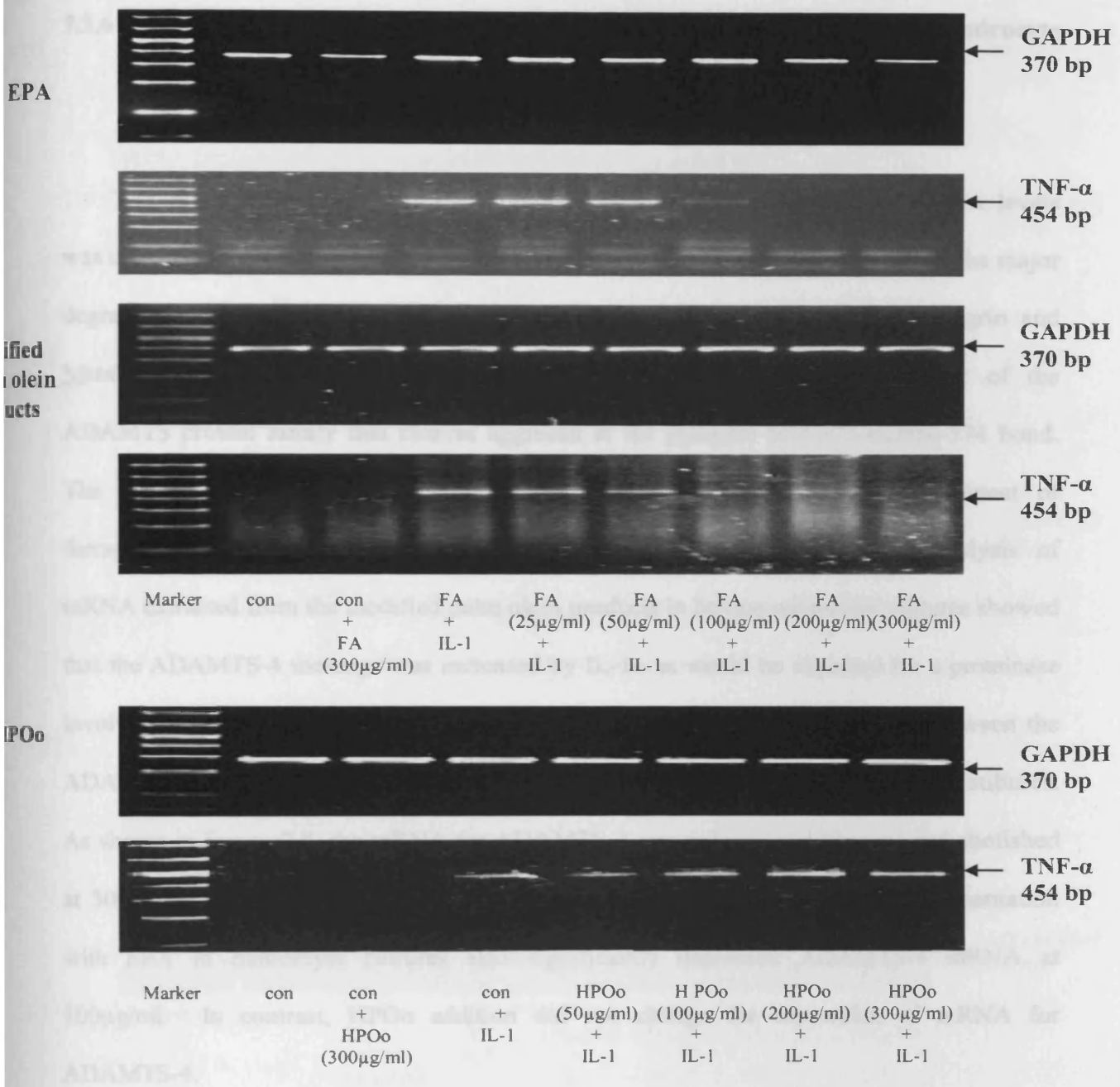


Figure 7.7: RT-PCR using TNF-α primers was carried out on bovine monolayer cultures treated with EPA, modified palm olein products or hydrolyzed palm olein (HPOo) complexed to BSA for 8 hours followed, after washing, by subsequent culture for 4 days with IL-1α (10ng/ml). Separations were on 2% agarose gels alongside a 50-1000 bp marker.

7.3.6 Analysis of mRNA levels for the aggrecanase ADAMTS-4 in bovine chondrocyte monolayer cultures

The effect of modified palm olein products on chondrocyte aggrecanase mRNA levels was then investigated. Aggrecanase is a protease that is thought to be responsible for the major degradation of cartilage aggrecan in arthritic diseases. Aggrecanase-1 [A Disintegrin and Metalloproteinase with ThromboSpondin Motifs-4 (ADAMTS-4)] is a member of the ADAMTS protein family that cleaves aggrecan at the glutamic acid-373-alanine-374 bond. The identification of this protease provides a specific target for the development of therapeutics to prevent cartilage degradation in arthritis (Tortorella *et al.*, 2002). Analysis of mRNA extracted from the modified palm olein products in bovine monolayer cultures showed that the ADAMTS-4 message was increased by IL-1α as would be expected for a proteinase involved in inflammation-induced cartilage degradation. Comparison was made between the ADAMTS-4 mRNA levels following EPA or HPOo supplementation in monolayer cultures. As shown in Figure 7.8, the mRNA for ADAMTS-4 was reduced at 100μg/ml and abolished at 300μg/ml in cultures supplemented with modified palm olein products. Supplementation with EPA in monolayer cultures also significantly decreased ADAMTS-4 mRNA at 100μg/ml. In contrast, HPOo addition did not change the expression of mRNA for ADAMTS-4.

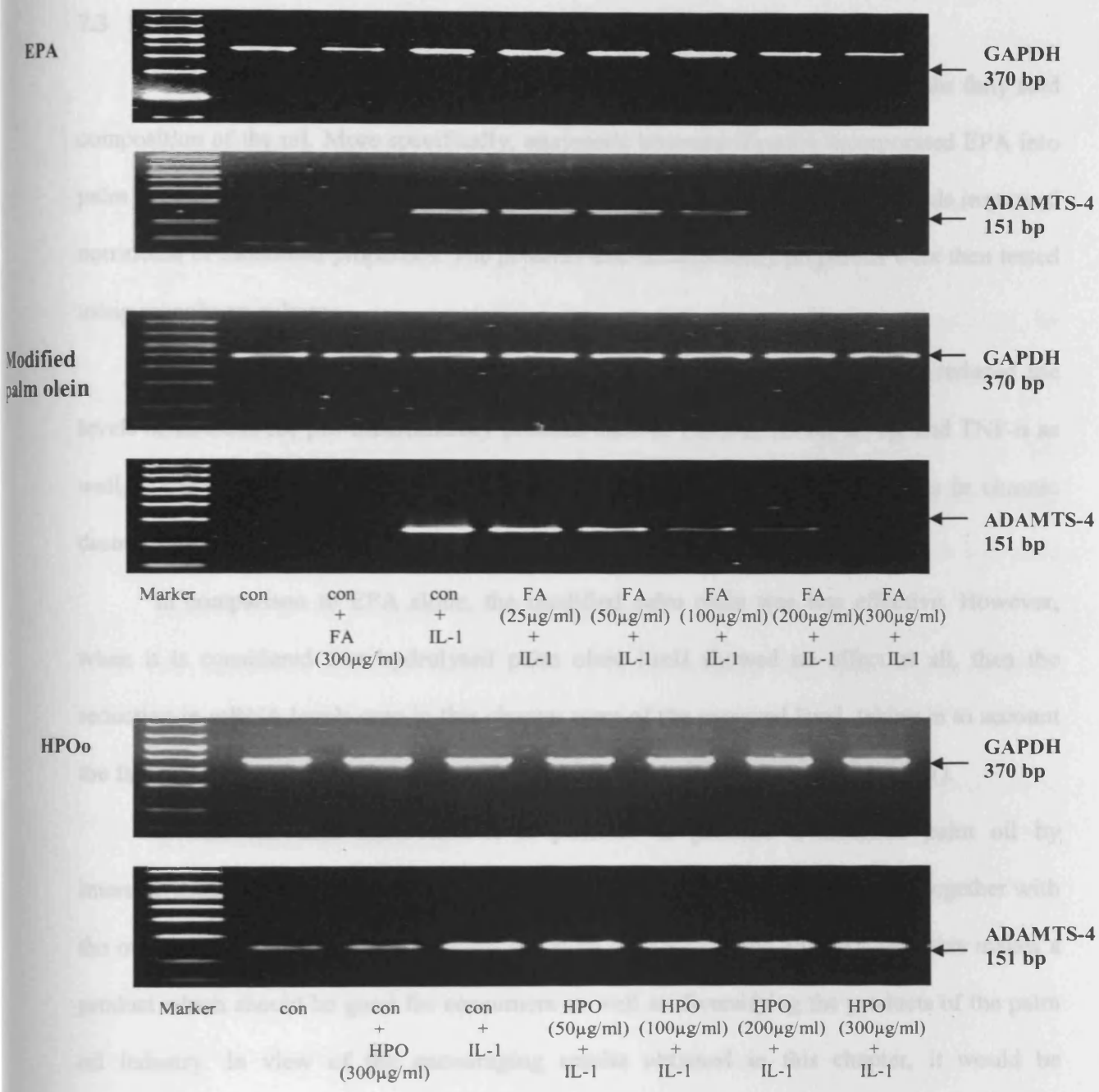


Figure 7.8: RT-PCR using ADAMTS-4 primers was carried out on bovine monolayer cultures treated with EPA, modified palm olein or hydrolysed palm olein (HPOo) complexed to BSA for 8 hours followed, after washing, by subsequent culture for 4 days with IL-1α (10ng/ml). Separations were on 2% agarose gels alongside a 50-1000 bp marker.

7.3 Discussion

Enzyme interesterification of palm olein with EPA successfully modified the fatty acid composition of the oil. More specifically, enzymatic interesterification incorporated EPA into palm olein to about 25% of the total fatty acids. It was hoped that this would provide improved nutritional or functional properties. The possible anti-inflammatory properties were then tested using monolayer cultures.

The results in this chapter showed that modified palm olein fatty acids reduced the levels of mRNAs for pro-inflammatory proteins such as COX-2, IL-1α, IL-1β, and TNF-α as well as the proteinase, ADAMTS-4. IL-1α and TNF-α are important cytokines in chronic destructive arthritis.

In comparison to EPA alone, the modified palm olein was less effective. However, when it is considered that hydrolysed palm olein itself showed no effect at all, then the reduction in mRNA levels seen in this chapter were of the expected level, taking in to account the fact that the modified palm olein contained 25% EPA (Table 7.1 and Figure 7.1).

Overall the data show that it is possible to produce a modified palm oil by interesterification and that such an oil has "value-added" nutritional properties. Together with the other desirable components in palm oil, such as vitamins and anti-oxidants, this makes a product which should be good for consumers as well as diversifying the products of the palm oil industry. In view of the encouraging results obtained in this chapter, it would be worthwhile to carry out feeding studies with the modified oil in order to confirm its nutritional effects.

7.5 CHAPTER SUMMARY

- Palm olein was modified by enzymatic interesterification to produce an oil with 25% EPA content.
- Modified palm olein had no effect on lactate production in a monolayer culture system and, therefore, did not appear to modify basic energy metabolism.
- IL-1α induced GAG release from bovine monolayer cultures was reduced by supplementation with the modified palm olein. In comparison to EPA alone, it was somewhat less effective.
- The modified palm olein also reduced the mRNA levels for COX-2, the inflammatory cytokines TNF-α, IL-1α and IL-β and the proteinase ADAMTS-4 which had been induced by IL-1α in bovine monolayer cultures.
- The reductions in mRNA levels produced by the modified palm olein were at about the expected level compared to EPA alone, as judged by RT-PCR.
- These data show that incorporation of EPA into palm olein changed its properties to an anti-inflammatory edible oil.

CHAPTER EIGHT

GENERAL DISCUSSION

Malaysia's position as the World's leading palm oil-producing country (Oil World, 2005) has allowed the industry to flourish in a way it never has before. Now, Malaysia is producing a wider variety of by-products as a result of continuous research and development efforts which are turning downstream manufacturing into an industry in itself. This has allowed Malaysia to stay ahead of its competitors. However, there is a need to continually identify and produce new products in order for this situation to be maintained.

Palm oil has a unique biochemical profile so this oil cannot be labelled saturated or unsaturated (Berger, 2001). It does not behave as a saturated oil, although palm oil contains some 50% saturated fatty acids. Furthermore, it would be presumptuous to say that it is harmful to health as there is no direct evidence for that. In general, long chain saturated fatty acids promote arterial thrombosis in rats but palm oil does not follow this overall rule. Moreover, in terms of its nutritional effect (as shown in Table 1.5), palm oil is neutral or beneficial in respect of cancer development and progression of chemically-induced tumours in rodents (Nesaretnam *et al.*, 2000).

Crude palm oil is a rich source of β-carotene (a precursor of vitamin A). However, this is normally removed during the refining process and so its availability is often restricted. Palm oil is also a rich source of vitamin E (tocopherols and tocotrienols), a powerful nutritional anti-oxidant necessary for protection against free-radical induced cellular damage (Choo, 1994).

Many of the beneficial properties of palm oil can be attributed to its high vitamin E content as well as other anti-oxidants. In addition, palm oil also contains linoleic acid, an essential fatty acid for man.

Some efforts to increase the market for palm oil include expansion of the use of palm oil and improving the health benefits of palm oil by modification of palm oil itself or of palm oil fractions. One of the main aspects of this thesis was to modify palm oil products with *n-3* PUFAs to give modified oil which might be of use to people suffering from inflammatory diseases such as arthritis. Before we investigated how to modify palm oil, we first carried out research to identify the most effective *n-3* PUFAs for reducing cartilage degradation and inflammatory responses using a model cell culture system for arthritis. Other fatty acids were also compared such as the *n-6* PUFA, arachidonic acid, and those from palm oil itself.

Numerous reports suggest that supplementation of the diet with omega-3 polyunsaturated fatty acids (*n-3* PUFAs) such as from fish oil, has beneficial effects in human diseases and laboratory animals (see Chapter 1). These have included lively discussions of the potential anti-inflammatory responses relevant in arteriosclerosis, arthritis and asthma (Panash, 1983; Simopoulos, 1991; Okuyama *et al.,* 1997; Calder, 1998; Calder, 2001; Cunnane, 2003; Calder, 2004). The first evidence for an important role of dietary PUFAs in inflammation was derived from epidemiological observations of the low incidence of autoimmune and inflammatory disorders in Eskimos. The 1980s were a period of expansion in our knowledge about PUFAs in general and *n-3* PUFAs in particular (Simopoulos, 2002). Research was carried out in animal models, tissue cultures and humans. The first study showing positive effects with *n-3* PUFA dietary supplementation was published in 1985 (Kremer *et al.,* 2000). Since then, feeding trials with animals and humans have reported many

significant benefits of *n-3* PUFAs and these have often been interpreted as being due to their anti-inflammatory properties (Kelly, 1991; Cleland and James, 1997; Venkatraman and Chu, 1999). However, these and other explanations offered have not always been generally accepted because of the lack of molecular evidence to explain the mechanisms involved and the high concentrations of *n-3* PUFAs required to achieve recognized "beneficial actions" in *vitro*. Thus, the exact cellular and molecular mechanism(s) for the protective actions of dietary *n-3* PUFAs in these studies remain largely unexplained.

Previous work carried out by Curtis *et al.,* (2000, 2002) and other researchers in our laboratory (e.g. Hurst, 2004) found that *n-3* PUFA (but not other fatty acids) produced potentially beneficial effects by reducing the expression and activity of inflammatory factors (COX-2, IL-1, TNF-α and 5-lipoxygenase) and of degradative enzymes (MMPs and aggrecanases [ADAMTS]) which jointly trigger the damage and destruction of cartilage, as found in arthritis.

Therefore, one of my objectives was to continue this work by examining the relative efficacy of various types of *n-3* PUFAs (EPA, DHA, α-linolenic acid) in dose-dependent experiments to see their effect in reducing inflammatory reactions and cartilage degradation in chondrocyte cultures. Studies with arachidonic acid (AA) and hydrolysed palm olein (HPOo) were carried out at the same time to compare the activity of these 'other' fatty acids and to provide background information for further experiments with modified palm olein.

Optimisation of RT-PCR was conducted for subsequent studies of the effects of fatty acid supplementation on cytokine-mediated cartilage metabolism before the initiation of such detailed studies (Chapter 3). In addition, the primers used had to be checked in order to

confirm that they were identifying the correct bands. Studies on the morphology of chondrocyte cell monolayers, following stimulation by IL-1α, indicated that supplementing with EPA protected the cells from damage caused by IL-1α exposure. Incubation of cultures for 8h with different fatty acids, in the presence of bovine serum albumin, allowed a concentration-dependent uptake into the chondrocytes. No statistically significant change in lactate release compared to controls, was found even at the highest concentration (300μg/ml) of the fatty acids, showing that such treatment did not affect (anaerobic) metabolism (as measured by lactate production) when EPA, DHA, ALA, AA or hydrolysed palm olein was added to the cultures. Furthermore, mRNA levels for aggrecan, type II collagen and the various inflammatory factors tested were also unchanged by pre-exposure to various fatty acids. These data showed that fatty acid supplementation of the culture medium did not, by itself, have any obvious detrimental effect. Furthermore, examination of cell morphology by light microscopic methods showed that high concentrations of EPA (the most effective *n-3* PUFA: see later), if anything, had a beneficial effect on cell growth.

Further studies of GAG release (induced by IL-1α exposure) found that EPA was the most effective *n-3* PUFA in decreasing the release of GAG, compared to DHA and ALA. In addition, EPA was also the most effective in reducing the effects of IL-1α on ADAMTS-4 and ADAMTS-5 mRNA levels compared to other *n-3* PUFAs, showing that the latter reduced cartilage catabolism. In comparison, AA and HPOo had no significant effect in reducing IL-1α stimulated GAG release or aggrecanase expression (see Chapter 4). ADAMTS-4 is the primary enzyme responsible for aggrecan cleavage in young bovine cartilage and it is inducible in such tissues. On the other hand, ADAMTS-5, which also participates in the breakdown of aggrecan, is constitutively expressed in bovine articular cartilage (Tortorella *et al.*, 2001) and as we found (Chapter 4).

High concentrations of EPA also seemed the most effective in reducing MMP-3 and MMP-13 mRNA levels compared to the other fatty acids tested. This result differs slightly from the report by Curtis (1999) which showed (in bovine monolayer cultures) that the preincubation of cultures with EPA had no effect on the mRNA levels of either MMP-3 or MMP-13. Both MMP-3 and MMP-13 are important collagenases in articular cartilage tissue responsible for the later stages of collagen catabolism (Caterson *et al.,* 2000). MMP-3 is important for the activation of collagenases while MMP-13 is responsible for cleavage of type II collagen (Cawston, 1998).

There are two main isoforms of COX (COX-1 and - 2) and these represent the classic sites of action for nonsteroidal anti-inflammatory drugs (NSAIDs). The two isoforms appear to serve separate physiologic and pathophysiologic roles in humans. Each COX isoform carries dual enzymatic activities, a cyclooxygenase and a peroxidase. Recently, a COX-3 isoenzyme has been found which may also be involved in the synthesis of prostaglandins. In a similar manner to other COX enzymes, it is also thought to be important for pain and fever (Chandrasekharan *et al.,* 2002). Specific inhibition of COX-2 has been a recent focus of research by several pharmaceutical companies, as selective inhibition of COX-2 without blocking COX-1 could reduce unwanted side effects associated with traditional NSAIDs. However, in September 2004, a few COX-2 inhibitors such as Vioxx and Valecoxib were withdrawn from the market due to a possible increased risk of cardiovascular events and other potential side effects (Drazen, 2005).

The results of this study show that the supplementation of cultures with *n-3* PUFAs, especially EPA, decreased the mRNA levels for COX-2 but not COX-1. Moreover, mRNA levels for the inflammatory cytokines TNF-α, IL-1α and IL-1β which had been induced by IL-1α exposure were also reduced in a dose-dependent way. Pre-incubation with AA had no

effect on the levels of the above mRNAs and hydrolysed palm olein treatment had only a slight, if any, effect (see Chapter 5). It might be expected that dietary lipids, particularly those containing *n-3* PUFAs, would influence the release of cytokines, since this is regulated by eicosanoids and in fact, dietary lipids are known to affect eicosanoid production. The effects of *n-3* PUFAs on cytokine release have been reviewed several times in recent years (e.g Calder, 1998).

Thus, the overall results from Chapters 3-5 showed the *n-3* PUFAs, but not the other fatty acids tested, were able to reduce mRNAs for inflammatory factors in a cell culture system which is a model for arthritis. In addition, these fatty acids reduced mRNAs for relevant cartilage degrading enzymes (such as aggrecanases and MMPs) and abrogated the GAG release induced by the IL-1α stimulation of inflammation. These data agree with, and extend, the results reported by Curtis *et al.*,(2000, 2002). Furthermore, comparison of the dose-dependent effects of different fatty acids showed that EPA was the most effective. In fact, Simopolous (2002) has reported that, among the *n-3* PUFAs, EPA and DHA are more biologically potent than ALA. Therefore, this result agreed with deductions from feeding studies (Cunnane, 2003) and would be in keeping with the logic that EPA can be directly converted to eicosanoids (Calder, 2004) and /or that it may have a particular action on gene expression (Clarke, 2001). The effects of fatty acids on gene expression have received considerable attention because this represents a direct route for fatty acids to regulate gene function (Jump, 2002). In addition, the results obtained in this thesis are supported by several other recent *in vitro* studies that add additional information to the epidemiological and clinical surveys that have showed significant advantages of dietary *n-3* PUFAs especially in treatment of the inflammation and pain in human arthritic disease (Cleland and James, 2001; Calder and Zurier, 2001).

Taken together, the data provides confirmatory information on the biological and molecular mechanisms by which *n-3* PUFAs act. They also show the relative effectiveness of the three *n-3* PUFAs tested. In order, they were EPA > DHA > ALA as measured by their ability to reduce mRNA levels of inflammatory proteins and cartilage degradation in the model cell culture systems. Recently, an endogenous lipid mediator that exhibits potent anti-inflammatory effects was discovered to be formed from EPA by an ill-defined enzymatic route. The novel mediator (from EPA) is termed E-series resolvin and those biosynthesized from the precursor docosahexaenoic acid are D-series resolvins (Serhan *et al.,* 2004). These compounds open a new chapter in lipid signalling molecules that can be formed from fatty acids.

Since preliminary studies showed that HPOo supplements in bovine monolayer cultures had little effect in reducing pro-inflammatory factors in arthritis, we continued our study by examining the incorporation of potentially beneficial *n-3* PUFAs (α-linolenic acid and EPA) into palm olein triacylglycerols through lipase-catalysed interesterification reactions (see Chapter 6). This study was notable because most of the previous studies (Li and Ward, 1993; Akoh *et al.,* 1996; Jennings and Akoh, 1999; Garcia *et al.,* 2000; Xu *et al.,* 2000; Fajardo *et al.,* 2003) on the enzymatic interesterification of palm or other edible oils with *n-3* PUFAs were to improve the physical and thermal characteristics of solid fats rather than to produce modified fats with nutritional benefits. Moreover, none of the previous studies showed any specific molecular evidence either *in vivo* or *in vitro* to prove any particular beneficial effects.

The first part of this work was to optimise the reaction conditions in order to make more efficient use of the costly *n-3* PUFA feedstock for the interesterification of palm olein.

Of the seven lipases that were tested in the initial screening, Lipozyme *IM 60* from *Rhizomucor miehie* resulted in the highest incorporation of ALA and EPA into palm olein. This enzyme was studied further with regard to the influence of enzyme load, temperature, incubation time, solvent effects and amount of solvents. Products were analyzed by TLC and GLC. Lipozyme *IM 60*, from *Rhizomucor miehei,* is a 1, 3–selective lipase immobilized on a macroporous anion exchange resin. Among many regioselective lipases, tested for their ability to incorporate fatty acids into triacylglycerols, the best results have been found with lipases from *Rhizomucor miehei* (Lipozyme *IM 60*), *Rhizopus delemar* and *Candida antartica* (Huang and Akoh, 1996; Soumanou *et al.,* 1999). The maximum incorporation reported for most studies was in the range 40-65 mol %. Lipozyme *IM 60* has been used intensively for the incorporation of *n-3* PUFA into edible oil (Senanayake and Shahidi, 1999; Xu *et al.,* 1999; Fajardo *et al.*, 2003) due to its high activity and stability in the temperature range of 60 – 80°C (Bornscheuer *et al.*, 2003).

The highest incorporation of ALA into palm olein TAG was observed with substrate ratios (wt/wt) of at least 1:1 (gram methyl ALA: gram palm olein) and occurred at 55-70°C which seemed to be a suitable temperature range. Increasing the enzyme content above 10% (10g/100g substrates) did not significantly increase further the yields of ALA and EPA in the TAG product. Time course studies indicated that the interesterification reaction proceeded quickly with at least half of the incorporation of ALA occurring within 30 min and of EPA in 90 min. However, incorporation after 24h was always higher than for shorter (up to 3h) incubations showing that long incubations gave the best yield. The incorporation of ALA into palm olein did not show a significant decrease when the enzyme was re-cycled three times emphasizing the stability of the enzyme preparation. Among the solvents examined, *n*-hexane

served best for the interesterification reactions. A study by Akoh and Huang (1995) on the effect of solvent polarity on the synthesis of triaclyglycerol using Lipozyme *IM 60* showed that nonpolar solvents, such as isooctane and hexane, produced 40% mole of a disubstituted structure TAG, while a more polar solvent such as acetone produced only 1.4% of the same structured lipid.

After 24 h incubation in hexane, the fatty acid composition of palm olein was changed to contain up to 52% ALA or 45% EPA under the standard reaction conditions used. Thus, it was concluded that Lipozyme *IM 60* could be used successfully to synthesise a modified palm olein, suitable as a speciality neutraceutical fat enriched in *n-3* PUFAs. Studies by Fajardo *et al.* (2003) using Lipozyme *IM 60* for the modification of the FA composition of palm oil by the incorporation of *n-3* PUFAs, gave an average incorporation of 20.8% EPA and 15.6% DHA into palm oil despite using a substrate mole ratio of 1:3 (TAG:FA). This result showed that we were successful in obtaining a higher incorporation of *n-3* PUFAs into palm olein at a substrate ratio of 1:1 but using similar parameters to those utilised by Fajardo *et al.*, (2003).

Several authors have investigated the incorporation of PUFA into vegetable oils to obtain the possible health benefits associated with *n-3* PUFA. Huang and Akoh (1994) studied the ability of immobilised lipases from *Mucor miehei* (*1,3*-specific) and *Candida antarctica* (non-specific) to increase the amount of EPA and DHA in soybean oil. *Mucor miehei* lipase gave higher incorporation of EPA than did *Candida antarctica* lipase (10.5 mol% versus 6.8 mol% EPA). However, using ethyl esters of EPA and DHA, *Candida antarctica* lipase was found to be more efficient (35% versus 29% for EPA ester and 33% versus 14% for DHA ester) than the *Mucor miehei* lipase. Like ours, other studies also used

high ratios of *n-3* PUFAs to edible oils in order to get a better incorporation of PUFAs (Lee and Akoh, 1996; Huang and Akoh, 1994; Schmid *et al.*, 1998; Xu, 2000).

Studies with the EPA-modified palm olein (see Chapter 7) showed that its fatty acids were able to reduce the mRNA levels for COX-2 and the inflammatory cytokines TNF-α, IL-1α and IL-β. The modified palm olein was not as effective as EPA alone but was clearly better than palm olein itself which had no effect on mRNA levels. It also reduced ADAMTS-4 mRNA levels and GAG released from the cultures. Thus, the modified palm olein was shown to possess moderate anti-inflammatory properties and to be a potential 'valued-added' new product for the palm oil industry. As pointed out before, palm oil contains useful levels of vitamins A and E which function as anti-oxidants against cellular damage. Indeed, benefit from vitamin E treatment has been claimed from several small studies of human OA (Doumerg, 1969; McAlindon and Felson, 1997). Furthermore, many of the beneficial properties of palm oil can be attributed to its high vitamin E content, as well as other anti-oxidants (Chandrasekharan, 2002).

Thus, by modifying palm oil fractions which already contain significant amounts of vitamins, it may be possible to produce oils which have several beneficial effects for human health. Moreover, interesterification reactions, such as we have used, are the sort of processes which could be used to modify palm oil without damaging unstable components such as vitamins or PUFA.

Overall, the results presented in this thesis show clearly that exposure of bovine monolayer cultures to *n-3* PUFAs (especially EPA) reduces the IL-1α induced degradative enzymes and factors associated with cartilage destruction and inflammation. This provides

further evidence for the generally-accepted benefits for *n-3* PUFAs in the diet (Calder and Zurier, 2001; Simopolous 2002). Although we did not study them in this work, the newly discovered anti-inflammatory derivatives (resolvin D1, neuroprotectins) from DHA (Serhan *et al.*, 2002; Arita *et al.*, 2005) may provide part of the explanation as to why DHA was also effective in reducing the inflammatory responses.

In addition, these basic studies on successful modification of palm olein to produce an *n-3* PUFA enriched product with anti-inflammatory properties has laid some groundwork for the future development of such oils in Malaysia and other palm oil-producing nations. Therefore, several areas should be explored in the course of future research. Clinical trials using modified palm olein with EPA (or other *n-3* PUFAs) produced by enzymatic interesterification should be carried out to investigate the potential benefits of dietary supplementation on inflammation in general and specific diseases such as arthritis. Moreover, there are several different types of palm oil fractions (such as palm oil, red palm olein, palm carotene, palm tocopherol and double fractionated palm olein with a higher amount of linolenic acid like olive oil) which could also be tested for their effect (with or without interesterification with *n-3* PUFAs) on a bovine monolayer culture system that mimics cartilage degradation in arthritis. To begin with, the parameters tested in the present study could be evaluated in order to look for anti-inflammatory effects. Such work could then be followed by animal feeding studies as well as clinical evaluation of different disease states. The experiments could be expanded to evaluate different *n-3* PUFAs or combinations thereof.

Such future work should also look at some other key effectors, such as eicosanoid levels as well as including analysis of newly discovered molecules such as the resolvins.

By these means, the work presented in this thesis could be developed to give further information about the biochemistry involved in the activity of *n-3* PUFAs as well as

developing nutritionally-beneficial edible oils. The latter are a key requirement for Malaysia not only in sustaining its economic growth but also in improving the well-being of its population.

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213. www. matrade.gov.my. Malaysia External Trade Development Corporate.

APPENDIX

Verification of nucleotide sequences.

The forward primer is indicated in red and the reverse primer is indicated in blue.

Bovine COX-1 Primers (CLC 9/10)

5′-GCCCAACACTTCACCCATCAG-3′
5′-CCAGGAAGCAGCCCAAACACT-3′

gi|2957179|gb|AF004943.1|AF004943 Bos taurus cyclooxygenase-1 (COX-1) mRNA, partial cds
Length=777

```
 Score = 42.1 bits (21),  Expect = 0.068
 Identities = 21/21 (100%), Gaps = 0/21 (0%)
 Strand=Plus/Plus

Query  1    GCCCAACACTTCACCCATCAG  21
            |||||||||||||||||||||
Sbjct  244  GCCCAACACTTCACCCATCAG  264


 Score = 42.1 bits (21),  Expect = 0.068
 Identities = 21/21 (100%), Gaps = 0/21 (0%)
 Strand=Plus/Minus

Query  22   CCAGGAAGCAGCCCAAACACT  42
            |||||||||||||||||||||
Sbjct  530  CCAGGAAGCAGCCCAAACACT  510
```

Full Gene Sequence With Primers Identified:

AF004943

tccaaccttatccccagccctcccacctacaacgtagcgcatgactacatcagctg
ggagtccttctccaatgtgagttattatactcgcattctgccctccgtgccccgag
actgtcccacgcccatgggcaccaaagggaagaagcagttgccagatgcggagttt
ctgagtcgtcgcttcctgctcaggaggaagttcatccccgaccctcaaggcaccaa
cctcatgtttgccttctttgcccaacacttcacccatcagttcttcaaaacttctg
gcaagatgggtcctggcttcaccaaggcgttgggccacggggtaggcctcggccac
atttatggagacaatctggaacgtcggtatcagctgcggctctttaaggatgggaa
actcaagtaccagatgctcaatggagaggtgtaccagccatcggtggaagaggctc
ccgtgctgatgcactacccccggggcatcccgccccagagccagatggccgtgggc
caggaagtgtttgggctgcttcctgggctcatggtctacgccacgatatggctgcg
tgagcacaaccgcgtgtgtgacctgctgaaggctgagcagccgacctggggcgacg
agcagctcttccagacggcccgcctcatcctcatcggggagaccatcaagattgtg
atagaggagtatgtgcagcagctgagcggctacttcctgcagctcaagttcgaccc
agagctgctgtttggggcccaattccagtaccgcaaccgcatcgccatg

Bovine COX-2 Primers (CLC 11/12)

5′-GCTCTTCCTCCTGTGCCTGAT-3′
5′-CATGGTTCTTTCCCTTAGTGA-3′

gi|2957181|gb|AF004944.1|AF004944 Bos taurus cyclooxygenase-2 (COX-2) mRNA, partial cds
Length=449

Score = 42.1 bits (21), Expect = 0.068
Identities = 21/21 (100%), Gaps = 0/21 (0%)
Strand=Plus/Plus

```
Query  1    GCTCTTCCTCCTGTGCCTGAT  21
            |||||||||||||||||||||
Sbjct  97   GCTCTTCCTCCTGTGCCTGAT  117
```

Score = 42.1 bits (21), Expect = 0.068
Identities = 21/21 (100%), Gaps = 0/21 (0%)
Strand=Plus/Minus

```
Query  22   CATGGTTCTTTCCCTTAGTGA  42
            |||||||||||||||||||||
Sbjct  325  CATGGTTCTTTCCCTTAGTGA  305
```

Full Gene Sequence With Primers Identified:

AF004944

tccagatcacatttgattgacagtccaccaacttataatgtgcactacagctataa
aagctgggaagccttttctaacctgtcttattataccaga***gctcttcctcctgtgc***
ctgatgactgcccaacacccatgggtgtgaaagggaggaaagagcttcctgattca
aaagaagttgtgaaaaaagtacttctaagaagaaagttcattcctgatccccaggg
cacaaatctgatgtttgcattctttgcccagcacttcacccatcaatttttcaaga
cagattttgaacgaggaccagctt***tcactaagggaaagaaccatg***gggtggactta
agtcacatttatggtgaatctttagagagacagcataagctgcgcctttcaagga
tggaaaaatgaaatatcagatgattaatggagagatgtatcctcccacagtcaaag
a

Bovine IL-1α Primers (CLC 3/4)

5′-AAGGAGAATGTGGTGATGGTG-3′
5′-CAGAAGAAGAGGAGGTTGGTC-3′

gi|444|emb|X12497.1|BTIL1AR Bovine mRNA for interleukin-1 alpha
Length=1003

Score = 42.1 bits (21), Expect = 0.068
Identities = 21/21 (100%), Gaps = 0/21 (0%)
Strand=Plus/Plus

```
Query  1    AAGGAGAATGTGGTGATGGTG  21
            |||||||||||||||||||||
Sbjct  234  AAGGAGAATGTGGTGATGGTG  254
```

Score = 42.1 bits (21), Expect = 0.068
Identities = 21/21 (100%), Gaps = 0/21 (0%)
Strand=Plus/Minus

```
Query  22   CAGAAGAAGAGGAGGTTGGTC  42
            |||||||||||||||||||||
Sbjct  703  CAGAAGAAGAGGAGGTTGGTC  683
```

Full Gene Sequence With Primers Identified:

BTIL1AR

tgttgctagctcggttcagcaaagaagtgaagatggccaaagtccctgacctctttgaagacctgaagaactgttacagtgaaaatgaagactacagttctgaaattgaccacctctctctcaatcagaagtccttctatgatgcaagctatgagccacttcgtgaggaccagatgaataagtttatgtccctggatacctcggaaacctctaagacatccaagcttagcttc***aaggagaatgtggtgatggtg***gcagccagtgggaagattctgaagaagagacggttgagtttaaatcagttcatcaccgatgatgacctggaagccatgccaataatacagaagaagaaatcatcaagcccagatcagcacattacagcttccagagtaacgtgaaatacaactttatgagagtcatccaccaggaatgcatcctgaacgacgccctcaatcaaagtataattcgagatatgtcaggtccatacctgacggctactacattaaataatctggaggaggcagtgaaatttgacatggttgcttatgtatcagaagaggattctcagcttcctgtgactctaagaatctcaaaaactcaactgtttgtgagtgctcaaaatgaagacgaacccgtcttgctaaaggagatgcctgagacacccaaaatcatcaaagatga***gaccaacctcctcttcttctg***ggaaaagcatggctctatggactacttcaaatcagttgcccatccaaagttgtttattgccacaaagcaagaaaaattggtgcacatggcaagtgggccgccctcgatcactgactttcagatattggaaaaatagccttgactgtgcactctacttacttgtaaagtggtgaccatccgtatgtactatgtac

atgaaggagtcgagcccttcactgttagtcactcgctgagcatgtgctgagctttt
gtaattctaaatgaatgtttactctctttgtaagagagaacacaaagtcc

Bovine IL-1β Primers (CLC 5/6)

5′-GCTCTCCACCTCCTCTCACAG-3′
5′-TACATTCTTCCCTTCCCTTCT-3′

>gi|448|emb|X12498.1|BTIL1BR Bovine mRNA for interleukin-1 beta
Length=944

Score = 42.1 bits (21), Expect = 0.068
Identities = 21/21 (100%), Gaps = 0/21 (0%)
Strand=Plus/Plus

```
Query  1    GCTCTCCACCTCCTCTCACAG  21
            ||||||||||||||||||||
Sbjct  465  GCTCTCCACCTCCTCTCACAG  485
```

Score = 42.1 bits (21), Expect = 0.068
Identities = 21/21 (100%), Gaps = 0/21 (0%)
Strand=Plus/Minus

```
Query  22   TACATTCTTCCCTTCCCTTCT  42
            ||||||||||||||||||||
Sbjct  918  TACATTCTTCCCTTCCCTTCT  898
```

Full Gene Sequence With Primers Identified:

BTIL1BR

attctctccagccaaccttcattgcccaggtttctgaaacagccatggcaaccgta
cctgaacccatcaacgaaatgatggcttactacagtgacgagaatgagctgttatt
tgaggctgatgaccctaaacagatgaagagctgcatccaacacctggacctcggtt
ccatgggagatggaaacatccagctgcagatttctcaccagttctacaacaaaagc
ttcaggcaggtggtgtcggtcatcgtggccatggagaagctgaggaacagtgccta
cgcacatgtcttccatgatgatgacctgaggagcatcctttcattcatctttgaag
aagagcctgtcatcttcgaaacgtcctccgacgagtttctgtgtgacgcacccgtt
cagtcaataaagtgcaaactccaggacagagagcaaaaatccctggtgctggctag
cccatgtgtgctgaag***gctctccacctcctctcacag***gaaatgaaccgagaagtgg
tgttctgcatgagctttgtgcaaggagaggaaagagacaacaagattcctgtggcc
ttgggtatcaaggacaagaatctatacctgtcttgtgtgaaaaaaggtgatacgcc
caccctgcagctggaggaagtagaccccaaagtctaccccaagaggaatatggaaa
agcgctttgtcttctacaagacagaaatcaagaatacagttgaatttgagtctgtc
ctgtaccctaactggtacatcagcacttctcaaatcgaagaaaggcccgtcttcct
gggacattttcgagctggccaggatataactgacttcagaatggaaaccctctctc

cctaaagaaagccatacccagggagtccacgtgggctgaataaccccgaggactgg
c***agaagggaagggaagaatgta***gctgcagcctgaacttcactgttgtc

Bovine TNF-α Primers (CLC 7/8)

5′-CTCAAGCCTCAAGTAACAAGC-3′
5′-GCAATGATCCCAAAGTAGACC-3′

gi|27806786|ref|NM_173966.1| Bos taurus tumor necrosis factor, alpha (TNF superfamily, member 2) (TNF), mRNA
Length=705

```
 Score = 42.1 bits (21),  Expect = 0.069
 Identities = 21/21 (100%), Gaps = 0/21 (0%)
 Strand=Plus/Plus

Query  1    CTCAAGCCTCAAGTAACAAGC  21
            |||||||||||||||||||||
Sbjct  245  CTCAAGCCTCAAGTAACAAGC  265


 Score = 36.2 bits (18),  Expect = 4.2
 Identities = 18/18 (100%), Gaps = 0/18 (0%)
 Strand=Plus/Minus

Query  25   ATGATCCCAAAGTAGACC  42
            ||||||||||||||||||
Sbjct  695  ATGATCCCAAAGTAGACC  678
```

Full Gene Sequence With Primers Identified:

NM_173966.1

atgagcaccaaaagcatgatccgggatgtggagctggcggaggaggtgctctccga
gaaagcaggggggcccccagggctccagaagttgcttgtgcctcagcctcttctcct
tcctcctggttgcaggagccaccacgctcttctgcctgctgcacttcggggtaatc
ggcccccagagggaagagcagtccccaggtggcccctccatcaacagccctctggt
tcaaacactcaggtcctctt***ctcaagcctcaagtaacaagc***cggtagcccacgttg
tagccgacatcaactctccggggcagctccggtggtgggactcgtatgccaatgcc
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aacatcctgtctgccatcaagagcccttgccacagggagaccccagagtgggctga
ggccaagccctggtacgaacccatctaccagggaggagtcttccagctggagaagg
gagatcgcctcagtgctgagatcaacctgccggactacctggactatgccgagtct
gggca***ggtctactttgggatcat*cg***ccctgtga

* The primer has an additional base (T) at this position. Sequence above is for Bos Taurus, however, the primer does match 21/21 bases in the gene for Bos sp. Shown below:

gi|755701|emb|Z48808.1|BSPTNFA Bos sp. mRNA for tumor necrosis factor alpha. Length=558

Bovine/Human GAPDH Primers (CRF 3/4)

5′-TGGCATCGTGGAAGGGCTCAT-3′
5′-ATGGGAGTTGCTGTTGAAGTC-3′

>gi|37590766|gb|BC059110.1| Rattus norvegicus glyceraldehyde-3-phosphate dehydrogenase, mRNA (cDNA clone MGC:72650 IMAGE:6919611), complete cds
Length=1307

Score = 42.1 bits (21), Expect = 0.069
Identities = 21/21 (100%), Gaps = 0/21 (0%)
Strand=Plus/Plus

```
Query  1    TGGCATCGTGGAAGGGCTCAT  21
            |||||||||||||||||||||
Sbjct  574  TGGCATCGTGGAAGGGCTCAT  594
```

Score = 42.1 bits (21), Expect = 0.069
Identities = 21/21 (100%), Gaps = 0/21 (0%)
Strand=Plus/Minus

```
Query  22   ATGGGAGTTGCTGTTGAAGTC  42
            |||||||||||||||||||||
Sbjct  943  ATGGGAGTTGCTGTTGAAGTC  923
```

N.B. The primers are 100% homologous to the Rat GAPDH gene

Full Gene Sequence (Rattus) With Primers Identified:

BC059110.1

ggggctctctgctcctccctgttctagagacagccgcatcttcttgtgcagtgcc
agcctcgtctcatagacaagatggtgaaggtcggtgtgaacggatttggccgtatc
ggacgcctggttaccagggctgccttctcttgtgacaaagtggacattgttgccat
caacgacccttcattgacctcaactacatggtctacatgttccagtatgactcta
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tccatgacaactt***tggcatcgtggaagggctcat***gaccacagtccatgccatcact
gccactcagaagactgtggatggcccctctggaaagctgtggcgtgatggccgtgg

ggcagcccagaacatcatccctgcatccactggtgctgccaaggctgtgggcaagg
tcatcccagagctgaacgggaagctcactggcatggccttccgtgttcctaccccc
aatgtatccgttgtggatctgacatgccgcctggagaaacctgccaagtatgatga
catcaagaaggtggtgaagcaggcggccgagggcccactaaagggcatcctgggct
acactgaggaccaggttgtctcctgt***gacttcaacagcaactcccat***tcttccacc
tttgatgctggggctggcattgctctcaatgacaactttgtgaagctcatttcctg
gtatgacaatgaatatggctacagcaacagggtggtggacctcatggcctacatgg
cctccaaggagtaagaaaccctggaccacccagcccagcaaggatactgagagcaa
gagagaggccctcagttgctgaggagtccccatcccaactcagcccccaacactga
gcatctccctcacaattccatcccagaccccataacaacaggaggggcctggggag
ccctcccttctctcgaataccatcaataaagttcgctgcaccctcaaaaaaaaaaa
aaaaaaaaaaaaaaaaaa

Full Gene Sequence (Bovine) With Primers Identified:

U85042

cgcatcgggcgcctggtcaccagggctgcttttaattctggcaaagtggacatcgt
cgccatcaatgacccccttcattgaccttcactacatggtctacatgttccagtatg
attccacccacggcaagttcaacggcacagtcaaggcagagaacgggaagctcgtc
atcaatggaaaggccatcaccatcttccaggagcgagatcctgccaacatcaagtg
gggtgatgctggtgctgagtatgtagtggagtccactggggtcttcactaccatgg
agaaggctggggctcacttgaagggtggcgccaagagggtcatcatctctgcacct
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cctcaagattgtcagcaatgcctcctgcaccaccaactgcttggccccctggcca
aggtcatccatgaccactt***tggcatcgtgga*gggacttatgaccactgtccacgc***
catcactgccacccagaagactgtggatggcccctccgggaagctgtggcgtgacg
gccgaggggctgcccagaatatcatcctgcttctactggcgctgccaaggccgtg
ggcaaggtcatccctgagctcaacgggaagctcactggcatggccttccgcgtccc
cactcccaacgtgtctgttgtggatctgacctgccgcctggagaaacctgccaagt
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ctaggctacactgaggaccaggttgtctcctgc***gacttcaacagc***gat***actc***a***c***tc
ttctaccttcgatgctggggctggcattgccctcaacgaccactttgtcaagctca
tttcctggtacgacaatgaatttggctacagcaaacagg

* the primer sequence has an A here, but is absent in the gene sequence
... bases highlighted in yellow are different from the rat sequence and, hence, primer sequences

MMP-3 Primers (CRF 5/6)

5′-CTTTTGGCGAAAATCTCTCAG-3′
5′-AAAGAAACCCAAATGCTTCAA-3′

>gi|19424169|ref|NM_133523.1| Rattus norvegicus matrix metallopeptidase 3 (Mmp3), mRNA
Length=1771

```
 Score = 42.1 bits (21),  Expect = 0.069
 Identities = 21/21 (100%), Gaps = 0/21 (0%)
 Strand=Plus/Plus

Query  1     CTTTTGGCGAAAATCTCTCAG  21
             ||||||||||||||||||||
Sbjct  990   CTTTTGGCGAAAATCTCTCAG  1010


 Score = 42.1 bits (21),  Expect = 0.069
 Identities = 21/21 (100%), Gaps = 0/21 (0%)
 Strand=Plus/Minus

Query  22    AAAGAAACCCAAATGCTTCAA  42
             ||||||||||||||||||||
Sbjct  1393  AAAGAAACCCAAATGCTTCAA  1373
```

N.B. The primers are 100% homologous to the Rat GAPDH gene

Full Gene Sequence (Rattus) With Primers Identified:

NM_133523.1

```
aaggaggcagcaaagaacccgctgagagcagtgcagaactgtgggaagccagtgga
aatgaaagggctcccagtcctgctgtggctgtgtacggctgtgtgctcatcctacc
cattgcatggcagtgaagaagatgctggcatggaggttctgcagaaatacctagaa
aactactatggtcttgaaaaggatgtgaagcagtttactaagaaaaaggacagtag
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ggaagctggactcgaacactatggagctgatgcacaagccccggtgtggtgttccc
gacgtcggtggcttcagtacctttccaggttcacccaaatggaggaaaaaccacat
ctcctacaggattgtgaattatacactggatttaccaagagagagtgtggattctg
ccattgagagagctttgaaggtctgggaggaggtgaccccactcacattctccagg
atctctgaaggagaggctgacataatgatctcctttgcagttgaagaacatggaga
ctttatccctttgatgggcctggaatggtcttggctcatgcctatgccctggac
cagggaccaatggagatgctcactttgatgatgatgaacgatggacagatgatgtc
acaggtaccaacctattcctggttgctgctcatgaacttggccactccctgggtct
ctttcactcagccaatgctgaagctttgatgtacccagtctacaagtcctccacag
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```

ggacctcccacagaatcccctgatgtcctcgtggtacccaccaaatctaactctct
ggaccctgagaccttaccaatgtgtagctctgctttgtccttcgatgcagtcagca
ccctgcggggagaagtcttgttctttaaagacaggca***ctttttggcgaaaatctctc***
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attcacactctgggcctccctgaaaccgtccagaagatcgatgcagccatttcttt
aaaggaccaaaagaagacgtacttctttgtagaggacaaattctggagatttgatg
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tcagctgaccctgatagctcttcctctgaaacttggcgcactgaagtggtttcctt
actctagcatgtgctatggcagagcaaaatgggagctacatatggcaccagtcaac
ctcaagttgtcgaaggacattcagaagcactgcttgtcttatactgtgtcaaaggg
agaggagaaaacacactcctgggctacagacaagtaactgtctctgtgtagatggt
ttgttttatttaataaaatggtgtgtcatttattt

Full Gene Sequence (Bovine) With Primers Identified:

(sequence is unavailable on Pubmed)

Full Gene Sequence (Human) With Primers Identified:

NM_002422.

acaaggaggcaggcaagacagcaaggcatagagacaacatagagctaagtaaagcc
agtggaaatgaagagtcttccaatcctactgttgctgtgcgtggcagtttgctcag
cctatccattggatggagctgcaaggggtgaggacaccagcatgaaccttgttcag
aaatatctagaaaactactacgacctcaaaaaagatgtgaaacagtttgttaggag
aaaggacagtggtcctgttgttaaaaaaatccgagaaatgcagaagttccttggat
tggaggtgacggggaagctggactccgacactctggaggtgatgcgcaagcccagg
tgtggagttcctgatgttggtcacttcagaacctttcctggcatcccgaagtggag
gaaaacccaccttacatacaggattgtgaattatacaccagatttgccaaaagatg
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ctccctgggtctctttcactcagccaacactgaagctttgatgtacccactctatc
actcactcacagacctgactcggttccgcctgtctcaagatgatataaatggcatt
cagtccctctatggacctccccctgactccccctgagacccccctggtacccacgga

acctgtccctccagaacctgggacgccagccaactgtgatcctgctttgtcctttg
atgctgtcagcactctgaggggagaaatcctgatctttaaagacaggcactttggc
gcaaatcctcaggaagcttgaacctgaattgcatttgatctcttcattttggcc
atctcttccttcaggcgtggatgccgcatatgaagttactagcaaggacctcgttt
tcatttttaaaggaaatcaattctgggccatcagaggaaatgaggtacgagctgga
tacccaagaggcatccacaccctaggtttccctccaaccgtgaggaaaatcgatgc
agccatttctgataaggaaaagaacaaaacatatttctttgtagaggacaaatact
ggagatttgatgagaagagaaattccatggagccaggctttcccaagcaaatagct
gaagactttccagggattgactcaaagattgatgctgtttttgaagaatttgggtt
cttttatttctttactggatcttcacagttggagtttgacccaaatgcaaagaaag
tgacacacactttgaagagtaacagctggcttaattgttgaaagagatatgtagaa
ggcacaatatgggcactttaaatgaagctaataattcttcacctaagtctctgtga
attgaaatgttcgttttctcctgcctgtgctgtgactcgagtcacactcaagggaa
cttgagcgtgaatctgtatcttgccggtcatttttatgttattacagggcattcaa
atgggctgctgcttagcttgcaccttgtcacatagagtgatctttcccaagagaag
gggaagcactcgtgtgcaacagacaagtgactgtatctgtgtagactatttgctta
tttaataaagacgatttgtcagttgtttt

... bases highlighted in yellow are different from the rat sequence and, hence, primer sequences

MMP-13 Primers (CRF 1/2)

5′-TTCTGGCACACGCTTTTCCTC-3′
5′-GGTTGGGGTCTTCATCTCCTG-3′

gi|62653209|ref|XM_343345.2| PREDICTED: Rattus norvegicus matrix metallopeptidase 13 (Mmp13), mRNA
Length=1648

```
 Score = 42.1 bits (21),  Expect = 0.069
 Identities = 21/21 (100%), Gaps = 0/21 (0%)
 Strand=Plus/Plus

Query  1    TTCTGGCACACGCTTTTCCTC  21
            |||||||||||||||||||||
Sbjct  562  TTCTGGCACACGCTTTTCCTC  582

 Score = 42.1 bits (21),  Expect = 0.069
 Identities = 21/21 (100%), Gaps = 0/21 (0%)
 Strand=Plus/Minus

Query  22   GGTTGGGGTCTTCATCTCCTG  42
            |||||||||||||||||||||
Sbjct  834  GGTTGGGGTCTTCATCTCCTG  814
```

N.B. The primers are 100% homologous to the Rat GAPDH gene

Full Gene Sequence (Rattus) With Primers Identified:

XM_343345.2

cattcaacatgcattcagctattctggccaccttcttcttgttgagttggactcac
tgttggtccctgccccttccctatggtgatgatgatgatgatgacttgtctgagga
agatcttgagtttgcagagcactacttgaaatcatactaccatcctgtgactcttg
cgggaatcctgaagaagtctacagtgacctctacagttgatagactccgagaaatg
cagtctttctttggcttagatgtgactggcaaacttgatgatcctaccttagacat
catgagaaaaccaagatgtggagtgcctgatgtgggtgaatacaatgttttccctc
gaacactcaaatggtcccaaacaaacttaacttacagaattgtgaactacacccct
gatatatcccattctgaagtggagaaggccttcagaaaagccttcaaggtctggtc
tgatgtgacacctctgaattttaccaggatccatgatggcactgctgatatcatga
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c***ttctggcacacgcttttcctc***ctggaccaaaccttggcggggacgcccattttga
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agagcttagttctgtgaacaagcttcagtaagttatctttgagcatgcagtatcta
catgactatgcgtggctggaaccacattgaagaattgtaaagtactgaaattgaga
gccccaaggatcacctgattcttg

Full Gene Sequence (Bovine) With Primers Identified:

NM_174389

atgcacccaagggtcctggctggcttcctcttcttcagctggacggcttgttggtc
tctgcccttcccagtgatggagattctgaagacttgtccgaggaagacttccagt
ttgcagagagctacctgaaatcatactactatcctcagaatcctgctggaatcctg
aagaaaactgcagcaagctctgtgattgacaggcttagagaaatgcagtcatttt
tggcttagaggtgactggcagacttgatgataacaccttagacatcatgaaaaaac
caagatgtggggtccctgatgtgggtgaatacaatgttttcctcgaactctcaag
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cagtttgaatacagcatctggagtaaccgtattgttcgagtcatgacaacaaattc

cttattgtggtgttaaaagtgtttttaaaatattgttatttaaatcctggaaagca
tttgagataacacttccagacatttgggggtgagttatgggagaaggagagatat
cggg

... bases highlighted in yellow are different from the rat sequence and, hence, primer sequences

Full Gene Sequence (Human) With Primers Identified:

NM_002427

caacagtccccaggcatcaccattcaagatgcatccaggggtcctggctgccttcc
tcttcttgagctggactcattgtcgggccctgccccttcccagtggtggtgatgaa
gatgatttgtctgaggaagacctccagtttgcagagcgctacctgagatcatacta
ccatcctacaaatctcgcgggaatcctgaaggagaatgcagcaagctccatgactg
agaggctccgagaaatgcagtctttcttcggcttagaggtgactggcaaacttgac
gataacaccttagatgtcatgaaaaagccaagatgcggggttcctgatgtgggtga
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gccttcaaagtttggtccgatgtaactcctctgaattttaccagacttcacgatgg
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tcttcatcttcagaggtagaaaattttgggctcttaatggttatgacattctggaa
ggttatcccaaaaaaatatctgaactgggtcttccaaaagaagttaagaagataag
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tctggagatatgatgatactaaccatattatggataaagactatccgagactaata
gaagaagacttcccaggaattggtgataaagtagatgctgtctatgagaaaaatgg
ttatatctatttttcaacggacccatacagtttgaatacagcatctggagtaacc
gtattgttcgcgtcatgccagcaaattccattttgtggtgttaagtgtcttttaa
aaattgttatttaaatcctgaagagcatttggggtaatacttccagaagtgcgggg
taggggaagaagagctatcaggagaaagcttggttctgtgaacaagcttcagtaag
ttatctttgaatatgtagtatctatatgactatgcgtggctggaaccacattgaag
aatgttagagtaatgaaatggaggatctctaaagagcatctgattcttgttgctgt
acaaaagcaatggttgatgatacttcccacaccacaaatgggacacatggtctgtc
aatgagagcataatttaaaaatatatttataaggaaatttacaagggcataaagt
aaatacatgcatataatgaataaatcattcttactaaaaagtataaaatagtatga
aaatggaaatttgggagagccatacataaaagaaataaaccaaaggaaaatgtctg
taataatagactgtaacttccaaataaataattttcattttgcactgaggatattc
agatgtatgtgcccttcttcacacagacactaacgaaatatcaaagtcattaaaga

caggagacaaaagagcagtggtaagaatagtagatgtggcctttgaattctgttta
attttcacttttggcaatgactcaaagtctgctctcatataagacaaatattcctt
tgcatattataaaggataaagaaggatgatgtctttttattaaaatatttcaggtt
cttcagaagtcacacattacaaagttaaaattgttatcaaaatagtctaaggccat
ggcatccctttttcataaattatttgattatttaagactaaaagttgcattttaac
cctattttacctagctaattatttaattgtccggtttgtcttggatatataggcta
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cccttgagatatggaaggatgccttttttttctctgcatttaaaaaaatcccccagc
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aggcaaagaaaatgaaatgcatatttgcaaagtgtattaggaagtgtttatgttgt
ttataataaaaatatattttcaacagaaaaaaaa

... bases highlighted in yellow are different from the rat sequence and, hence, primer sequences

TIMP-1 Primers (CRF 63/64)

5′-CCACCTTATACCAGCGTTAT-3′
5′-CCTCACAGCCAACAGTGTAGG-3′

>gi|4507508|ref|NM_003254.1| Homo sapiens tissue inhibitor of metalloproteinase 1 (erythroid potentiating activity, collagenase inhibitor) (TIMP1), mRNA
Length=782

Score = 44.1 bits (22), Expect = 0.017
Identities = 22/22 (100%), Gaps = 0/22 (0%)
Strand=Plus/Minus

```
Query  20   TCCTCACAGCCAACAGTGTAGG  41
            ||||||||||||||||||||||
Sbjct  508  TCCTCACAGCCAACAGTGTAGG  487
```

Score = 40.1 bits (20), Expect = 0.27
Identities = 20/20 (100%), Gaps = 0/20 (0%)
Strand=Plus/Plus

```
Query  1    CCACCTTATACCAGCGTTAT  20
            ||||||||||||||||||||
Sbjct  226  CCACCTTATACCAGCGTTAT  245
```

Full Gene Sequence (Human) With Primers Identified:

NM_003254

aggggccttagcgtgccgcatcgccgagatccagcgcccagagagacaccagagaa
cccaccatggccccctttgagccctggcttctggcatcctgttgttgctgtggct
gatagccccagcagggcctgcacctgtgtcccaccccacccacagacggccttct
gcaattccgacctcgtcatcagggccaagttcgtggggacaccagaagtcaaccag
a***ccaccttataccagcgttat***gagatcaagatgaccaagatgtataaagggttcca
agccttaggggatgccgctgacatccggttcgtctacacccccgccatggagagtg
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cagcctgagcttagctcagcgccggggcttcaccaaga***cctacactgttggctgtg***
aggaatgcacagtgtttccctgtttatccatcccctgcaaactgcagagtggcact
cattgcttgtggacggaccagctcctccaaggctctgaaaagggcttccagtcccg
tcaccttgcctgcctgcctcgggagccagggctgtgcacctggcagtccctgcggt

cccagatagcctgaatcctgcccggagtggaactgaagcctgcacagtgtccaccc
tgttcccactcccatctttcttccggacaatgaaataaagagttaccacccagc

Full Gene Sequence (Bovine) With Primers Identified:

NM_174471

Ggagatgcatccttcgcagctcaggccctgccgccatcgccgcagacccagcgccc
agagaggctacaccagagaacgcaccatggccccctttgcacccatggcctctggc
atcctgttgctgctgtggctcacagcccccagccgagcctgtacctgcgtcccacc
ccacccacagacggccttctgcaactccgatgtcgtcatcagggccaagttcgtgg
ggaccgcagaagtcaatgaaa*ctg**ccttataccagcgttat*gagatcaagatgact
aagatgttcaaagggttcagtgccttgagggatgcccctgacatccggttcatcta
cacccctgccatggagagcgtctgcggatacttccacaggtcccagaaccgcagtg
aggagtttctcatagctggacaattgtcgaatgggcacctgcacatcaccacctgc
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ga*ccta*tg*ctg*c*tgg*t*tgtgagg*aatgcacagtgtttccctgctcatccatcccct
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gacaaggggttccagagccgccacctggcctgcctgccccgggagccagggctgtg
cacctggcagtccttgcgggcccagatggcctgagtcctgcccccagcagaagctg
aagcccgcacagtgttcgcccctgtttccactcccacctttttttcttcaagagga
tgaaataaagaactaccacccagc

... bases highlighted in yellow are different from the human sequence and, hence, primer sequences

TIMP-2 Primers (CRF 65/66)

5′-GTGGACTCTGGAAACGACAT-3′
5′-TCTTCTTCTGGGTGGTGCTCA-3′

>gi|52632388|ref|NM_003255.3| Homo sapiens tissue inhibitor of metalloproteinase 2 (TIMP2), mRNA
Length=3669

```
 Score = 42.1 bits (21),  Expect = 0.069
 Identities = 21/21 (100%), Gaps = 0/21 (0%)
 Strand=Plus/Plus

Query  1    GTGGACTCTGGAAACGACATT  21
            |||||||||||||||||||||
Sbjct  465  GTGGACTCTGGAAACGACATT  485


 Score = 42.1 bits (21),  Expect = 0.069
 Identities = 21/21 (100%), Gaps = 0/21 (0%)
 Strand=Plus/Minus

Query  21   TCTTCTTCTGGGTGGTGCTCA  41
            |||||||||||||||||||||
Sbjct  729  TCTTCTTCTGGGTGGTGCTCA  709
```

Full Gene Sequence (Bovine) With Primers Identified:

NM_003255

cgcagcaaacacatccgtagaaggcagcgcggccgccgagaaccgcagcgccgctc
gcccgccgccccccaccccgccgccccgcccggcgaattgcgccccgcgcccctcc
cctcgcgcccccgagacaaagaggagagaaagtttgcgcggccgagcggggcaggt
gaggagggtgagccgcgcgggaggggcccgcctcggccccggctcagcccccgccc
gcgcccccagcccgccgccgcgagcagcgcccggaccccccagcgcggcccccgc
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agaagaacatcaacgggcaccaggccaagttcttcgcctgcatcaagagaagtgac
ggctcctgtgcgtggtaccgcggcgcggcgcccccaagcaggagtttctcgacat

cgaggacccataagcaggcctccaacgcccctgtggccaactgcaaaaaaagcccc
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ctctgatgctttgtatcattcttgagcaatcgctcggtccgtggacaataaacagt
attatcaaagagaaaaaaaaaaaaaaa

Full Gene Sequence (Bovine) With Primers Identified:

NM_174472

Gaattccggcttctatggagcactcgggaccaggtccgcggcgcgcgcactcgctc
gctcgccgccccccagccagctctcgcttccgcgccgccagccgcgccccgcgcct
cctcgctgcaccccgcgacctagagccaagaaagtttgtgtggcgagtgagggccg
gagaggagagcgcgcccgcggagtgccgtccagaccagcgcggccccggcggagag
gggagcgccccgagcccaggcggcggcggctagcccgagtccgcgacccccgcccc
tccgcccgccatgggcgccgccgcccgcagcctgccgctcgcgttctgcctcctgc
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caacaggcgttttgcaatgcagacatagtgatcagggccaaagcagtcaataagaa
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agcaggccaccaggactcctggggccaattgacagtgtccaagagttcagactggt
ccagctccgacatcccttcctggacacagcatgaataaa

... bases highlighted in yellow are different from the human sequence and, hence, primer sequences

TIMP-3 Primers (CRF 67/68)

5′-GGGAAGAAGCTGGTAAAGGAG-3′
5′-GCCGGATGCAGGCGTAGTGTT-3′

>gi|495251|emb|X76227.1|HSTIMP3 H.sapiens TIMP3 mRNA for tissue inhibitor of metalloproteinases-3
Length=1021

Score = 44.1 bits (22), Expect = 0.017
Identities = 22/22 (100%), Gaps = 0/22 (0%)
Strand=Plus/Plus

```
Query  1    GGGAAGAAGCTGGTAAAGGAGG  22
            ||||||||||||||||||||||
Sbjct  212  GGGAAGAAGCTGGTAAAGGAGG  233
```

Score = 42.1 bits (21), Expect = 0.069
Identities = 21/21 (100%), Gaps = 0/21 (0%)
Strand=Plus/Minus

```
Query  22   GCCGGATGCAGGCGTAGTGTT  42
            |||||||||||||||||||||
Sbjct  629  GCCGGATGCAGGCGTAGTGTT  609
```

Full Gene Sequence (Human) With Primers Identified:

HSTIMP3

cccgccggcggcgcgcacggcaactttggagaggcgagcagcagccccggcagcgg
cggcagcagcggcaatgacccctggctcgggctcatcgtgctcctgggcagctgg
agcctgggggactggggcgccgaggcgtgcacatgctcgcccagccacccccagga
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Full Gene Sequence (Bovine) With Primers Identified:

NM_174473

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Aggrecan Primers (CRF 9/10)

5′-CGCTATGACGCCATCTGCTAC-3′
5′-GCCTGCTTGGCCTCCTCAAA-3′

>gi|31343114|ref|NM_173981.2| Bos taurus aggrecan 1 (chondroitin sulfate proteoglycan 1, large aggregating proteoglycan, antigen identified by monoclonal antibody A0122) (AGC1), mRNA
Length=7447

Score = 42.1 bits (21), Expect = 0.069
Identities = 21/21 (100%), Gaps = 0/21 (0%)
Strand=Plus/Plus

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Query  1     CGCTATGACGCCATCTGCTAC  21
             |||||||||||||||||||||
Sbjct  1387  CGCTATGACGCCATCTGCTAC  1407
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Score = 40.1 bits (20), Expect = 0.27
Identities = 20/20 (100%), Gaps = 0/20 (0%)
Strand=Plus/Minus

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Query  22    GCCTGCTTGGCCTCCTCAAA  41
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Sbjct  1883  GCCTGCTTGGCCTCCTCAAA  1864
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Full Gene Sequence (Bovine) With Primers Identified:

NM_173981

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Collagen IIα1 Primers (CRF 17/18)

5′-TGCCTGGTGCTCCTGGTCTGA-3′
5′-CTTCTCCCTTCTCGCCGTTAG-3′

>gi|30040|emb|X16711.1|HSCOL2A1R H.sapiens COL2A1 mRNA for alpha1 (II) collagen
Length=3480

Score = 44.1 bits (22), Expect = 0.017
Identities = 22/22 (100%), Gaps = 0/22 (0%)
Strand=Plus/Minus

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Query  21    ACTTCTCCCTTCTCGCCGTTAG  42
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Sbjct  2210  ACTTCTCCCTTCTCGCCGTTAG  2189
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Score = 42.1 bits (21), Expect = 0.069
Identities = 21/21 (100%), Gaps = 0/21 (0%)
Strand=Plus/Plus

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Query  1     TGCCTGGTGCTCCTGGTCTGA  21
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Sbjct  1658  TGCCTGGTGCTCCTGGTCTGA  1678
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Full Gene Sequence (Human) With Primers Identified:

HSCOL2A1R

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gcccgaga

Full Gene Sequence (Bovine) With Primers Identified:

(sequence is unavailable on Pubmed)

ADAMTS-4 Primers (BCK 5/6)

5′-TGGATCCCGAGGAGCCCTGGT-3′
5′-TGGCGGTCAGCGTCGTAGTCC-3′

>gi|31324243|gb|AF516915.1| Bos taurus aggrecanase-1 (ADAMTS-4) mRNA, complete cds
Length=3720

Score = 42.1 bits (21), Expect = 0.069
Identities = 21/21 (100%), Gaps = 0/21 (0%)
Strand=Plus/Plus

```
Query  1     TGGATCCCGAGGAGCCCTGGT  21
             |||||||||||||||||||||
Sbjct  1595  TGGATCCCGAGGAGCCCTGGT  1615
```

Score = 42.1 bits (21), Expect = 0.069
Identities = 21/21 (100%), Gaps = 0/21 (0%)
Strand=Plus/Minus

```
Query  22    TGGCGGTCAGCGTCGTAGTCC  42
             |||||||||||||||||||||
Sbjct  1745  TGGCGGTCAGCGTCGTAGTCC  1725
```

Full Gene Sequence (Bovine) With Primers Identified:

AF516915

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ADAMTS-5 Primers (CLC 50/51)

5'-GGCCTCTCCCATGAYGATTCC-3'
5'-TGAGCGAGAACACTGGCCCCA-3'

(N.B. Y= a combination of 50:50 C or G)

>gi|5901887|ref|NM_007038.1| Homo sapiens a disintegrin-like and metalloprotease (reprolysin
type) with thrombospondin type 1 motif, 5 (aggrecanase-2)
(ADAMTS5), mRNA
Length=5533

```
 Score = 42.8 bits (21),  Expect = 0.046
 Identities = 21/21 (100%), Gaps = 0/21 (0%)
 Strand=Plus/Minus

Query  22    TGAGCGAGAACACTGGCCCCA  42
             |||||||||||||||||||||
Sbjct  1868  TGAGCGAGAACACTGGCCCCA  1848

 Score = 38.8 bits (19),  Expect = 0.74
 Identities = 20/21 (95%), Gaps = 0/21 (0%)
 Strand=Plus/Plus

Query  1     GGCCTCTCCCATGAYGATTCC  21
             ||||||||||||| ||||||
Sbjct  1371  GGCCTCTCCCATGACGATTCC  1391
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Full Gene Sequence (Human) With Primers Identified:

NM_007038

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>gi|6319133|gb|AF192771.1|AF192771 Bos taurus aggrecanase-2 mRNA, partial cds

```
          Length=621

 Score = 40.8 bits (20),  Expect = 0.18
 Identities = 20/20 (100%), Gaps = 0/20 (0%)
 Strand=Plus/Minus

Query  23   GAGCGAGAACACTGGCCCCA  42
            ||||||||||||||||||||
Sbjct  539  GAGCGAGAACACTGGCCCCA  520


 Score = 38.8 bits (19),  Expect = 0.74
 Identities = 20/21 (95%), Gaps = 0/21 (0%)
 Strand=Plus/Plus

Query  1    GGCCTCTCCCATGAYGATTCC  21
            ||||||||||||| ||||||
Sbjct  43   GGCCTCTCCCATGACGATTCC  63
```

Partial Gene Sequence (Bovine) With Primers Identified:

AF192771

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ggaag

... bases highlighted in yellow are different from the human sequence and, hence, primer sequences