NEW SOURCES OF POLYUNSATURATED FATTY ACIDS

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Go raibh mile maith agaibh go lear agus, sin é!

ABSTRACT

Two groups of essential fatty acids (n-6 and n-3) are needed in a healthy human diet. Current advice suggests an optimal ratio of about 4:1 for these polyunsaturated fatty acids (PUFAs) in the diet but in Western countries it is usually 10-20:1. The n-3 PUFAs are synthesized in photosynthetic organisms from where they move up the food chain. Fish in particular, are a rich source of twenty and twenty two carbon n-3 PUFAs which are particularly effective for humans. Fish oils have been shown to be beneficial in a variety of chronic inflammatory diseases. However, fish stocks are under threat. Therefore, fish farming has increased recently and offers some opportunities for viable sources of n-3 PUFAs, especially since disposal of fish farm waste is expensive and environmentally problematic.

In this project, we investigated trout (*Oncorhynchus mykiss*) as a potential source of n-3 PUFAs. The lipid composition of different tissues was analysed by combinations of TLC and GLC. This work identified the differences between various individual tissues and pin-pointed those with high eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) content. Two possible industrial lipid extraction methods were compared with a standard laboratory procedure. These studies included a comparison of yields for offal which had been stored for various times. The rendering method, although not as efficient as the laboratory procedure (Garbus), was concluded to allow oil yield for nutraceutical development although tissues could not be stored for greater than two days without compromised quality. Finally, oil extracts from trout (containing 2.5% EPA and 10.5% DHA) were tested in a model system (bovine cartilage explants) as treatment for osteoarthritis. The trout oil preparation significantly reduced cytokine-induced glycosaminoglycan release indicating a protective action. However, it was not as effective as pure EPA or DHA. Moreover, the trout oil preparation was not able to reduce mRNA levels of inflammatory genes (e.g., COX-2, IL-6, ADAMTS-4, -5 and MMP-3) which were lowered by pure EPA or DHA supplementation. We conclude that trout waste is a viable source of n-3 PUFAs for nutraceutical development but that processing will probably be needed to produce a dietary supplement with potent anti-inflammatory properties.

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ABBREVIATION LIST

AA	Arachidonic Acid
ACC	Acetyl-Co A Carboxylase
ADAMTS	A Disintegrin And Metalloproteinases with Thrombospondin Motifs
AHA	American Heart Association
ALA	α-Linolenic Acid
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CD14	Cluster Designation-14
cDNA	Complimentary Deoxyribonucleic Acid
CS	Chondroitin Sulphate
CVD	Cardiovascular Disease
1,2-DAG	1,2 Diacylglycerol
1,3-DAG	1,3 Diacylglycerol
DHA	Docosahexaenoic acid
DMMB	Dimethylmethylene Blue
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
DPG	Diphosphatidylglycerol
DS	Dermatan Sulphate
EPA	Eicosapentaenoic acid
FACIT	Fibril Associated Collagens with Interrupted Triple Helices
FAME	Fatty Acid Methyl Esters
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde Phosphate Dehydrogenase
GLC	Gas Liquid Chromatography
H_2O_2	Hydrogen Peroxide
HA	Hyaluronate
HCl	Hydrochloric Acid
Hep	Heparan
HPETE	Hydroperoxy eicosatetraenoic acids

HS	Heparan Sulphate
ICES	International Council for Exploration of the Seas
IGD	Interglobular Domain
IκB	Inhibitory (subunit) Kappa B
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
KCl	Potassium Chloride
$KHCO_3$	Potassium Hydrogen Carbonate
KS	Keratan Sulphate
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LT	Leukotrienes
Lyso-PC	Lyso-Phosphatidylcholine
$MgCl_2$	Magnesium Chloride
MMP	Matrix Metalloproteinase
mRNA	Messenger Ribonucleic Acid
NADPH	Nicotinamide Adenine Dinucleotide Phosphate-Oxidase
NADH	Nicotinamide Adenine Dinucleotide Oxidase
NEFA	Non-Esterified Fatty Acid
NFκB	Nuclear Factor Kappa B
NFκBRE	Nuclear Factor Kappa B Receptor
NIH	National Institute of Health
OA	Osteoarthritis
PC	Phosphatidylcholine
PCR	Polymerase Chain Reaction
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PS	Phosphatidylserine
PG	Prostaglandin
PLA_2	Phospholipase A_2
PPAR	Peroxisome Proliferator Activated Receptor
PUFA	Polyunsaturated Fatty Acid

RA	Rheumatoid Arthritis
RNA	Ribonucleic Acid
RPE	Retinal Pigment Epithelium
RT	Reverse Transcription
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SLRPs	Small Leucine Rich Proteoglycans
SPH	Sphingomyelin
TAG	Triacylglycerol
TLC	Thin-Layer Chromatography
TLR	Toll-like Receptor
TNF-α	Tumour Necrosis Factor Alpha
TX	Thromboxane
VCAM-1	Vascular Cell Adhesion Molecule 1

CHAPTER 1

General Introduction

1.1 ARTHRITIS

Arthritis is a generic term that refers to the destruction of cartilage in joints. Inflammation of the joints together with tenderness, swelling, stiffness, restriction of movement and warmth accompany arthritic diseases. There are over 200 diseases which may lead to arthritis, including gout, synovitis, rheumatoid and osteoarthritis.

1.1.1 Rheumatoid arthritis

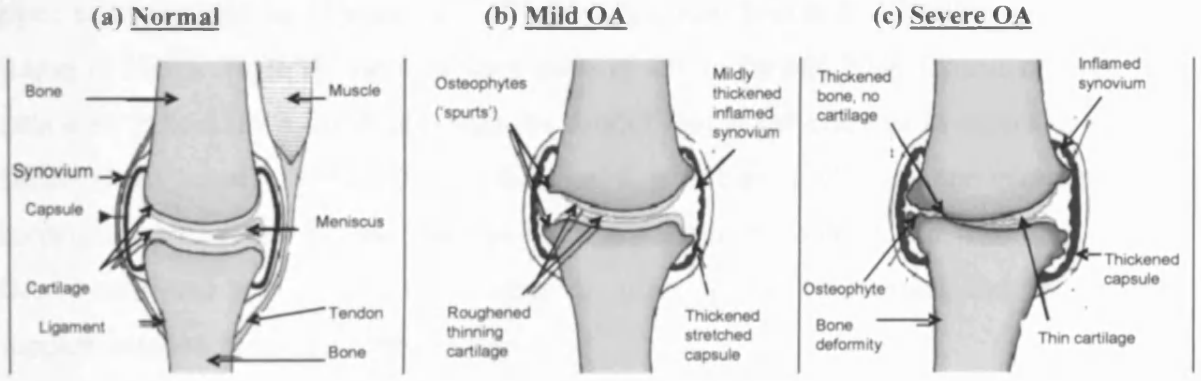
Rheumatoid arthritis (RA) is chronic, multi-system, inflammatory condition affecting the peripheral synovial joints leading to inflammation and swelling coupled with destruction of articular cartilage. The specific trigger for the development of RA is still elusive but it is considered to have an autoimmune basis and, in some cases, a genetic component (Buch and Emery, 2002).

RA is classified by inflammation of the synovium and infiltration of the synovium with immune cells, namely activated T-lymphocytes, macrophages and B-lymphocytes (Reines, 2004). Continued proliferation of the synovium gives rise to a granulated membrane, the pannus, which covers the surface of articular cartilage (Rayman and Callaghan, 2006). Inflammatory cells invade the pannus which facilitates the invasion of articular cartilage by these cells, thus promoting articular cartilage destruction (Rowan, 2001). The T-lymphoctyes (which some consider to be the initiator cells of RA (Panayi, 1993b)) together with the macrophages, mast cells and B-lymphocytes (in addition to elevated levels of cytokines) erode and degrade the articular cartilage resulting in loss of mobility, swelling, deformity and pain (Panayi, 1993b). Synovitis may reduce for short periods before re-appearing and it is believed that chronic destruction of the joint occurs during the inflammatory episodes (Panayi, 1993a). Lesions which develop in the joint are permeated with macrophages and antibody-secreting B-lymphocytes which enter the synovium. These cells, together with synoviocytes, give rise to new blood vessels contributing to the development of the pannus. The erosion of cartilage is thought to be primarily due to altered chondrocyte metabolism coupled with cytokine-and prostanoid-mediated induction of destructive enzymes such as matrix metalloproteinases and aggrecanases (Calder and Zurier, 2001).

1.1.2 Osteoarthritis

Osteoarthritis (OA) is one of the most common conditions associated with aging populations across the Western world (section 1.1.3). Affecting millions of people, this complex disease stems from both biomechanical and biochemical aetiology and is a leading cause of morbidity and disability. Once difficult to define, this degenerative disease of aging populations is now defined as "a degenerative disease of joints resulting from wear of the articular cartilage. Both mechanical and biological events destabilize the normal coupling of degradation and synthesis of articular cartilage chondrocytes, extracellular matrix (ECM), and subchondral bone (Fig. 1.1). OA may be initiated by multiple factors, including genetics, development, metabolic and traumatic events. Ultimately, osteoarthritic diseases are manifested by morphologic, biochemical, molecular and biomechanical changes of both cells and matrix which lead to softening, fibrillation, ulceration, loss of articular cartilage, sclerosis and eburnation of subchondral bone, osteophytes and subchondral cysts. When clinically evident, osteoarthritic diseases are characterized by joint pain, tenderness, limitation of movement, crepitus, occasional effusion and variable degrees of inflammation without systemic effects" (Goldberg and Kuettner, 1994).

Fig. 1.1: Schematic representation of (a) normal joint, (b) mild osteoarthritic joint and (c) severe osteoarthritic joint. Osteoarthritis (OA) of the joint results in cartilage depletion and progressive inflammation.



1.1.3 Prevalence of Osteoarthritis

Due to increases in life expectancies the burden of OA on medical and public health systems has increased exponentially. It is estimated that in England and Wales alone 1.3 – 1.75 million people are known sufferers of OA whilst in France it is believed that approximately 6 million cases of OA are diagnosed annually (Reginster, 2002). In the USA, a report published in 1997 by the American Centre for Disease Control and Prevention estimated that 43.3 million persons were afflicted with arthritis and it supported predications that over the next decade 60 million people will be diagnosed including 11.6 million people whose activity will become severely limited due to arthritic disease (Centre for Disease Control and Prevention, 2001).

A recent review concluded that 21% of the US population had doctor-diagnosed arthritis which included an estimated 27 million people suffering chronic clinical OA (Helmick *et al.*, 2007; Lawrence *et al.*, 2007). The burden of this disease is not solely restricted to prevalence and quality of life. The costs associated with OA are increasing astronomically, placing extra pressure on government health and medical systems. The economic burden of such a multi-factorial and far reaching disease takes both direct (hospitals, drugs, medical costs etc) and indirect (chronic and short-term disability, mortality) expenses into consideration. It is believed that for countries including the USA, Canada, the UK, France and Australia costs incurred by musculoskeletal disorders, particularly OA, are 1 – 2.5% of the gross national product (March and Bachmeier, 1997). A study conducted in 2003 revealed that in the USA alone costs incurred by OA are estimated to range from $3.4 to $13.2 billion per year (Lubeck, 2003). In 1998, the combined costs of OA to the UK NHS Executive were estimated to be £569 million, although the overall cost in the UK was in excess of £1 billion (Scott *et al.*, 1998). Due to the disabling effects of OA and the economic burden caused by this disease there is huge interest in prevention and treatment of OA. Nutritional intervention, for example by glucosamine, hyaluronate and fish oil supplementation is one such area of research.

Before the pathogenesis and development of OA is discussed (section 1.3) a review of joint morphology and composition is given.

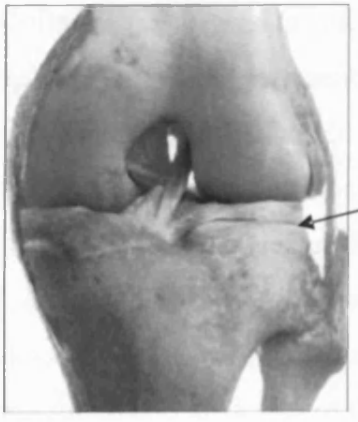
1.2 JOINT MORPHOLOGY AND COMPOSITION

1.2.1 Articular Cartilage

Articular cartilage is a unique form of connective tissue found at the end of the diarthrodial bones (Fig. 1.2). It plays an important role in synovial joints due to its ability to absorb water and compress under pressure, therefore allowing friction-free movement and, hence, preventing wear and tear of the joint (Kuettner, 1992). The ECM of articular cartilage is composed of an intricate network of collagen fibers intersected with a high concentration of aggregating proteoglycans along with other minor matrix proteins (Hardingham and Bayliss, 1990). The collagen fibers are responsible for the high tensile strength whilst the high concentration of proteoglycans determine the compressive nature of articular cartilage (Buckwalter and Mankin, 1998).

Chondrocytes are the main cells found in articular cartilage. They are embedded in the ECM and are responsible for the maintenance and synthesis of the components of the ECM (Rowan, 2001). As a member of the connective tissue family, articular cartilage is unique in that it contains no nerve, blood or lymphatic supply and lacks the presence of separating basement membranes (Kuettner, 1992). Due to the absence of a blood supply, nutrients are delivered by diffusion via the synovial fluid (synthesised by fibroblasts) (Rowan, 2001).

Fig. 1.2: The metacarphangeal joint is outlined below. The cartilage found at the end of long bones is termed articular cartilage and it functions to allow friction free movement and joint compressibility.



1.2.1.1 Articular Cartilage Zones

Articular cartilage may appear homogenous but it is actually organised in terms of both structure and composition. The structural and cellular components of articular cartilage can be divided into four identifiable zones. These include the superficial (tangential) zone; middle (transitional) zone; radial (deep) zone and a region of calcified cartilage and these zones are classified depending on depth from the surface (Tsou *et al.*, 2006) (Fig. 1.3). Each zone can be further divided into three regions, the pericellular, territorial and the inter-territorial region.

The pericellular region is the immediate area surrounding the chondrocyte membrane and is rich in proteoglycans and hyaluronic acid with a low collagen fiber content (Kuettner, 1992). The territorial region is an extension of the pericellular matrix. Here thin collagen fibers encapsulate chondrocytes or chondrons (groups of chondrocytes) in a basket-like fashion to provide protection for the cells. The inter-territorial region is furthest from the chondrocyte and the highest levels of proteoglycan and collagen fibrils are located here (Newman, 1998). As seen from the organisation of the zones and compartments, the ECM of articular cartilage varies in terms of collagen, proteoglycan and water concentrations and the chondrocytes vary with regard to shape and orientation.

1.2.1.2 The Superficial Zone

The superficial (tangential) zone is located at the surface and is bathed in synovial fluid. It is the thinnest of all four zones but provides the greatest tensile strength making it extremely important in combatting the compressive forces during joint loading (Poole *et al.*, 2001). Collagen fibrils tend to run parallel to each other and the articular surface. The proteoglycan, aggrecan, concentration is lowest in this zone although other minor leucine-rich proteoglycans such as decorin and biglycan are found in abundance here (Newman, 1998). Chondrocytes found here are flat and ovoid in shape and tend to run parallel with collagen fibrils (Aigner and McKenna, 2002). Chondrocytes found in the superficial zone synthesise a multi-functional domain proteoglycan protein known as the superficial zone protein (SZP) (Kuettner and Cole, 2005). The SZP is unique to chondrocytes of the superficial zone and is not

secreted by chondrocytes found in deeper zones, though it is also produced by synovial cells and plays an important role in joint lubrication (Poole *et al.*, 2001). It is also known to possess potential growth-promoting, cytoprotective and matrix-binding properties (Flannery *et al.*, 1999).

1.2.1.3 The Middle Zone

The largest compartment of articular cartilage is the middle (transitional) zone. It is also the area furthest away from the chondrocyte. The middle zone is much thicker than the superficial zone and it contains most of the proteoglycans and collagen fibrils. These are organised along lines which enable articular cartilage to perform its mechanical role (Newman, 1998).

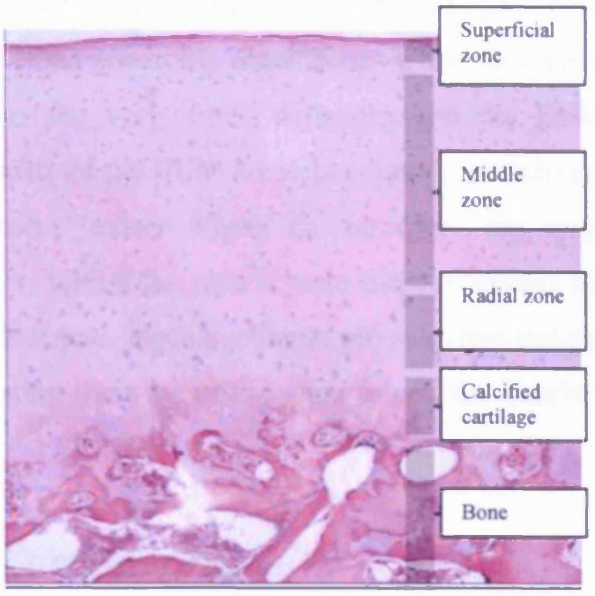
1.2.1.4 The Radial Zone

Chondrocytes of the radial (deep) zone are spherical in shape and are organised into vertical columns. They are also the most metabolically-active chondrocytes found in articular cartilage, even more so than those found in the superficial zone (Temenoff and Mikos, 2000). The collagen fibers found here have the largest diameter and are organised into upright columns perpendicular to the cartilage surface. Proteoglycan content is high in the radial zone whereas water is at its lowest (Buckwalter *et al.*, 2005; Tsou *et al.*, 2006). The radial zone is separated from the region of calcified cartilage by a thin basophile line, the tidemark.

1.2.1.5 Region of Calcified Cartilage

A thin region of calcified cartilage separates the subchondral bone from the soft cartilage. Chondrocytes located here are much smaller than those found elsewhere in articular cartilage and possess little or no endoplasmic reticulum and Golgi apparatus (Temenoff and Mikos, 2000). Within some areas of this region, the chondrocytes appear to be fully surrounded by the calcified matrix and, hence, have very low metabolic activity (Buckwalter and Mankin, 1998).

Fig. 1.3: Articular cartilage is divided in four distinct zones, the superficial (tangential) zone; middle (transitional) zone; radial (deep) zone and the region of calcified cartilage.



1.2.2 Chondrocytes

Chondrocytes, the architects of articular cartilage, are derived from the mesenchymal stem cells found in the bone marrow. During development, these spheroidal-shaped cells produce and secrete components of the ECM, collagen fibrils, proteoglycans and non-collagenous proteins organising them into a highly ordered structure in order to expand and remodel the articular surface. However, in mature cartilage, they do not substantially change matrix volume but replace degraded matrix macromolecules, maintaining its integrity (Muir, 1995). Their metabolic activity differs in relation to their zonal location, with cells of the middle zone having a greater concentration of synthetic organelles and, hence, greater metabolic activity. Chondrocytes in the region of calcified cartilage possess few or no synthetic organelles and have a very low rate of metabolic activity (Buckwalter and Mankin, 1998). In addition to synthetic organelles, chondrocytes may possess lipid and glycogen stores and secretory vesicles. They may also possess cilia radiating from the cell surface. These cilia are thought to be involved in determining mechanical changes in the ECM (Buckwalter and Mankin, 1998; Temenoff and Mikos, 2000).

Chondrocytes lack cell to cell contact. Occasionally they exist as clusters of cells, chondrons. As articular cartilage is non-vascularised the cells obtain their nutrients via diffusion through the ECM from the synovial fluid. As a consequence of the avascular nature of cartilage, chondrocytes are able to withstand low oxygen concentrations and rely on anaerobic metabolism to fulfill their energy needs (Muir, 1995). Chondrocytes are very much dependent on the ECM and, in turn, the components and integrity of the ECM are dependent on chondrocyte function. Hence, a symbiotic relationship exists where chondrocytes are responsible for matrix synthesis and turnover, whilst the matrix state directly affects chondrocyte function. These cells receive electrical, mechanical and physicochemical signals from the ECM and respond by adjusting their metabolic state to suit the matrix needs (Huber *et al.*, 2000).

1.2.3 The Extracellular Matrix

The ECM of articular cartilage is composed of a tangled network of collagen fibers of varying length and diameter. Entrapped within these fibers exists the hydrated proteoglycan, aggrecan. The collagen fibrils are responsible for providing the tensile strength whereas the proteoglycans, mainly aggrecan, provide the compressibility for cartilage. The ECM is organised into three different zones depending on depth from surface, and also into specific regions defined by distance from chondrocytes or chondrons. All components of the ECM are produced by the chondrocytes (Dudhia, 2005; Ottani *et al.*, 2001). The various components of the ECM are discussed below.

1.2.3.1 Collagens of the Extracellular Matrix

Collagen, a fibrous protein which is a principal component of connective tissues is found in tendons, bones, teeth, cartilage, skin and blood vessels. It is the single most abundant protein found in mammals (~30% of total proteins) (Burgeson and Nimni, 1992). The high tensile strength of collagen allows such activities as running, jumping or other activities that put severe stress on a joint. In higher vertebrates, there are at least 27 types of collagens which are the products of ~40 genes (Eyre, 2004).

Synthesis of collagen begins with a precursor molecule termed procollagen. These precursor molecules possess several N- and C- terminal peptides which are processed in the ECM by post-translational modification. The resulting molecule, collagen, is then integrated into the ECM (Burgeson and Nimni, 1992). Each collagen molecule is composed of three intertwined polypeptide chains which are about 100 amino acids in length. These intertwined chains give rise to a left-handed α-helix. Each collagen molecule is composed of the repeating amino acid sequence of glycine-X-Y, where X is frequently proline and Y, hydroxyproline. The amino acid 5-hydroxylysine is also present. This amino acid sequence is unique to the collagen three-dimensional structure and physical properities. Proline and hydroxyproline compose of ~30% of the residues of collagen. It is interesting to note that both 4-hydroxyproline and 5-hydroxylysine are formed from proline by the action of two enzymes (propyl hydroxylase and lysyl hydroxylase) after collagen polypeptides have already been synthesised. These hydroxylation processes are known as post-translational modifications of collagen (Brodsky and Persikov, 2005; Engel and Prockop, 1991; Prockop and Kivirikko, 1995; van der Rest and Garrone, 1991).

The triple helix of collagen is not as traditional a structure as α-helices or β-pleated sheets. This structure is formed as a result of the unique amino acid sequence. Within the triple helix every third residue faces the centre of the structure. As mentioned, the glycine-X-Y sequence is unique to collagen and it is the glycine residues in contact with the centre of the helix that confers this structure as this is the only amino acid residue which will fit. Therefore, this amino acid sequence is conserved for physical and functional reasons. There are approximately 3.3 residues per turn of each of these helices (Muir, 1995; Prockop and Kivirikko, 1995).

Another effect of the 'stacking' of the glycine residues along the centre of the collagen molecule is the generation of hydrogen bonding which contributes further to the molecule stability. The N-H of each glycine residue lies adjacent to each X (proline) residue and, hence, hydrogen bonds can form (Engel and Prockop, 1991; van der Rest and Garrone, 1991).

There are several different forms of collagen found within articular cartilage, collagen types II, IX, X, XI, and VI. However, it is collagen type II which is the predominant form.

1.2.3.2 Collagen Type II

Type II collagen comprises approximately 90% of adult articular cartilage. Type II is part of the fibril-forming collagen family (i.e. a class I collagen molecule) (Kuettner, 1992). This class of molecules contains large triple helical domains with about 1000 amino acids (Prockop and Kivirikko, 1995). Type II collagen is composed of three identical α1 polypeptide chains which compose a 300 nm long triple helix (Mendler *et al.*, 1989). It is synthesised as procollagen and before it is incorporated into the ECM to form fibrils and the non-collagenous extensions are removed (van der Rest and Garrone, 1991). As suggested by its family name (fibril-forming), type II is the main fibril-forming collagen found in articular cartilage. It integrates within the ECM and forms fibrils. Fibril thickness and density change in accordance with location within the ECM. For example, thicker fibrils are found in the deeper layers whereas finer fibrils are located close to the surface (Hall, 1983; Mayne, 1989). The tensile strength of articular cartilage is attributed to type II collagen which is also necessary for maintaining the volume and shape of the tissue. Covalent intermolecular crosslinks formed between collagen fibrils also increase tensile strength (Kuettner, 1992).

1.2.3.3 Other Collagens of Articular Cartilage

The remaining 10% of collagen within articular cartilage is composed of several different types of genetically distinct collagens, types IX, X, XI and, to a much lesser extent, VI.

Type IX collagen is a class III collagen molecule, (fibril-associated collagens with interrupted triple helices (FACIT)). This collagen does not form fibrils itself but attaches to the surface of pre-existing fibrils of type II collagen. The short triple helical domains, interrupted by short noncollagenous domains, characterise type IX collagen (Vaughan *et al.*, 1985). The globular regions are thought to attribute more flexibilty to the molecule. Since type IX collagen possesses both collagenous

domains and covalently-bound GAGs it has been considered that it mediates contact between the collagens and proteoglycans (Bruckner *et al.*, 1985; Muller-Glauser *et al.*, 1986).

Type X collagen is a macrofibril-associated collagen synthesised by chondrocytes located in the region of calcified cartilage (Schmid and Conrad, 1982). It is found in the pericellular region and belongs to a family of network-forming collagens (Habuchi *et al.*, 1985). Though as yet unclear, it is thought that collagen type X may play a role in structural support of long bones through the process of calcification (Grant, 2007).

Type XI collagen, another minor constitutent of articular collagens, is localised on or within macrofibrils formed by type II collagens. It belongs to the same family as type II collagen (fibril-forming family). Interactions between collagen type XI and proteoglycans have also been documented and it is thought that these interactions may influence proteoglycan distribution in cartilage (Mayne, 1989; Smith *et al.*, 1985).

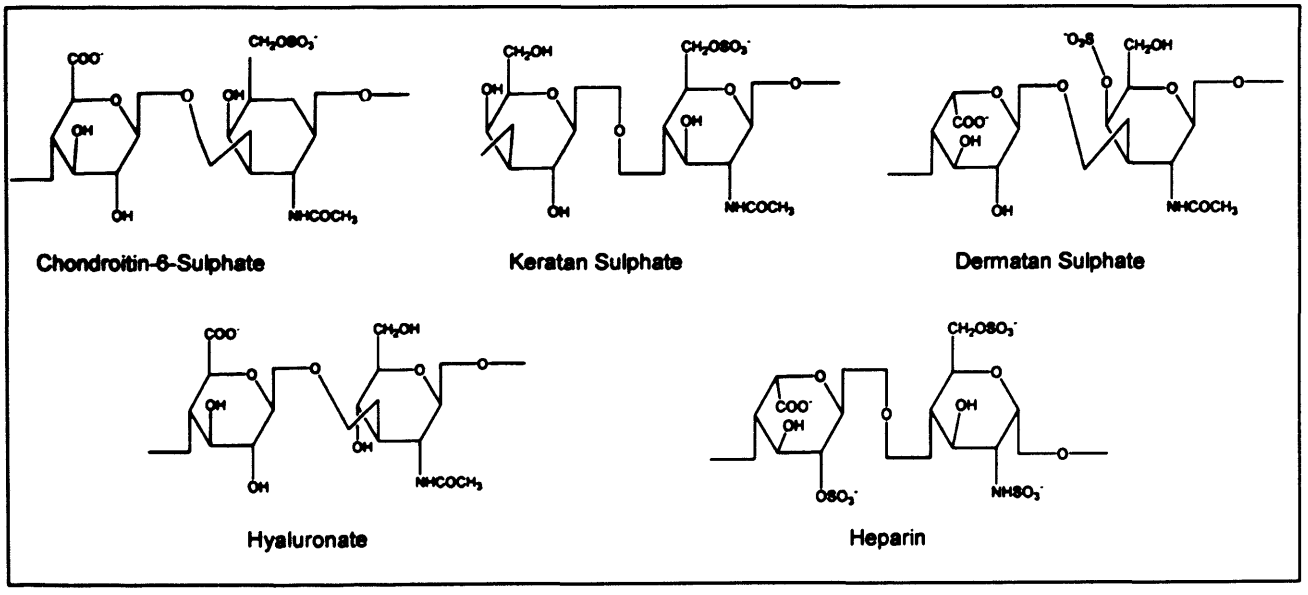
Type VI is a member of the beaded-filament forming collagen. The three α chains contain short triple helices and the remainder of the molecule is composed of large N-terminal and C-terminal globular domains (Jander *et al.*, 1984). Type VI collagen forms microfibrils in the pericellular region and it is believed that it may play a role in chondrocyte attachment to the ECM (Engel *et al.*, 1985; Jander *et al.*, 1984; Poole *et al.*, 1988).

1.2.4 Glycosaminoglycans

Glycosaminoglycans (GAGs) consist of linear chains of repeating disaccharides units. Each disaccharide consists of one hexosamine (D-galactosamine or D-glucosamine), which can be N-acetylated or N-sulphated, and one uronic acid (D-glucuronic acid, GlcA or L-iduronic acid, IdoA) or neutral hexose (D-galactose, Gal) (Vynios *et al.*, 2002). GAG chains possess either sulphate or carboxylate groups which confer a high negative charge to these molecules, a property which has great importance in the ECM, where GAG chains have a high affinity for water, thereby allowing articular cartilage the ability for reversible deformation (Dudhia, 2005; Kuettner, 1992).

In the ECM of articular cartilage, GAGs are covalently linked to proteoglycans, major macromolecules of the ECM. Proteoglycans have important structural functions (as seen in the ECM) but can also be found on the cell surface where they have a variety of roles varying from cell differentiation and tissue morphogenesis (perlecan, a basement membrane proteoglycan) to cell proliferation (heparan and heparan sulphate) and matrix organisation (aggrecan, decorin and biglycan) (Iozzo, 1998; Schwartz, 2000). The diversity of function is thought to be due to the multitude of GAG side chains (GAG type, size, composition and structural domain arrangement). There are five GAGs known to occur in articular cartilage, 1) hyaluronate, 2) chondroitin sulphate, 3) dermatan sulphate, 4) keratan sulphate; 5) heparin and heparan sulphate. All GAGs are sulphated to different degrees except for hyaluronate, which is not sulphated and is not found covalently linked to a core protein (Schwartz, 2000). Fig. 1.4 outlines the structures of the main GAGs found in articular cartilage.

Fig. 1.4: Chemical structures of the major glycosaminoglycans found in articular cartilage.



1.2.4.1 Hyaluronate

Hyaluronate (HA) is distinct from all other GAGs in that it is not sulphated and is not attached to a core protein. However, structurally it is similar to other GAGs and consists of repeating disaccharides of N-acetylglucosamine and glucuronic acid (Kakehi *et al.*, 2003). Even though HA can be regarded as the simplest GAG, it can have molecular weights from $10^5 - 10^7$ kDa. The highest concentrations of this large, negatively-charged molecule are found in the ECM of connective tissues. In the ECM, HA provides the backbone for the predominant aggregating proteoglycan, aggrecan, via binding of a link protein (Laurent and Fraser, 1992). HA is synthesised by hyaluronan synthase in the plasma membrane. This is in contrast to all other GAGs which are synthesised by enzymes located in the Golgi apparatus (Vynios *et al.*, 2002). HA is not unique to connective tissues. It can also be found in most tissues and fluids. As well as interacting with aggregating proteoglycans, it possesses other functions as diverse as a role in tissue morphogenesis, angiogenesis and the cancer cell microenvironment.

1.2.4.2 Chondroitin Sulphate

Chondroitin sulphate (CS) is composed of 30-50 repeating disaccharides of N-acetylgalactosamine and glucuronic acids in a β-1-3 linkage. It can be sulphated on either the 4 or 6 position giving rise to chondroitin-4-sulphate or chondroitin-6-sulphate (Hay, 1991). CS plays an important role within the ECM of articular cartilage. Due to its arrangement within the joint (i.e. attached to aggrecan) each negative molecule of CS repels the other, creating space between each neighbouring CS molecule. This space is filled with water which absorbs shock when pressure is applied to the joint. When pressure is removed, the negative charge confers the ability for the joint to return to its original shape (Bali *et al.*, 2001).

1.2.4.3 Dermatan Sulphate

Dermatan sulphate (DS) is similiar to CS except it contains iduronic acid instead of glucuronic acid. The disaccharide is often sulphated in position 2 of iduronic acid and position 4 of N-acetylgalactosamine. In contrast to CS and keratan sulphate, DS

monomers are usually smaller and do not bind to HA to form large aggregates but self-associate to form relatively small but unstable aggregates (Laurent and Fraser, 1992).

1.2.4.4 Keratan Sulphate

Keratan sulphate (KS) is a linear molecule composed of β-1-3 linked N-acetylgalactosamine disaccharide units. These units are sulphated to different degrees on the sixth carbon of either glucosamine or galactose residues (Lindahl *et al.*, 1996). Within the ECM, KS binds to aggrecan in a similiar fashion to CS. The combined effect of both CS and KS binding to aggrecan influences the resistance of articular cartilage to compressive forces (Roughley, 2001).

1.2.4.5 Heparin and Heparan Sulphate

Heparin (Hep) and heparan sulphate (HS) differ from other GAGs in terms of their different repeating disaccharides. Due to their structural similarity Hep and HS are often confused but the difference in these GAGs lies in their degree of sulphation. Hep is believed to be more heavily sulphated (approximately 70% N-sulfation) whilst HS is thought to contain approximately 50% or less N-sulphation (Volpi, 2006). They are both composed to D-glucuronate-2-sulphate (or iduronate-2-sulphate) and N-sulpho-D-glucosamine-6-sulphate connected via α-1-4 linkage (Vynios *et al.*, 2002). Hep is known to possess the highest charge density compared to any known biological molecule and exhibits diverse biological functions including antithrombotic activity (Powell *et al.*, 2004). HS is commonly found on the cell surface as well as the ECM and is believed to interact with other ECM molecules as well as growth factors, viruses and proteins (Bernfield *et al.*, 1999).

1.2.5 Proteoglycans

Proteoglycans are a diverse group of heterogenous complexes of macromolecular glycoconjugates consisting of various glycoaminoglycans (GAGs) covalently-bound to a protein core (Huber *et al.*, 2000). They are widely distributed throughout the ECM of connective tissues or associated with the cell surface where they have the potential to bind to other cellular or matrix components (Schwartz, 2000). Functionally, proteoglycans are quite diverse with roles ranging from modulation of growth and growth-factor activities, biological lubrication, matrix organisation and cell adhesion (Iozzo, 1998). Aggregating proteoglycans are those which have the potential to form large aggregates via non-covalent interactions with the GAG, HA. Of these aggregating proteoglycans, aggrecan, is the best studied. Initially thought to be a cartilage-specific proteoglycan, it was later identified in the aorta, cornea and tendon (Huber *et al.*, 2000).

1.2.5.1 Aggrecan

Aggrecan is the main proteoglycan present in cartilage, accounting for 90% of the proteoglycan content of articular cartilage. It is a multi-domain proteoglycan with distinctly characterised functional regions (Caterson *et al.*, 2000). It is composed of globular domains with intervening heavily sulphated and glycosated regions. There are three distinct globular domains, G1, G2 and G3. The G1 domain located at the N-terminal of the molecule has a high affinity for HA and binds non-covalently to HA. This permits the formation of large aggregates. A link protein is also bound here and this aggrecan-HA-link protein interaction stabilises the molecule (Dudhia, 2005) (Fig. 1.5).

The G1 and G2 domains are separated by the interglobular domain (IGD). The function of this IGD region is still relatively unknown (Kiani *et al.*, 2002). Within this highly conserved IGD there are two specific sites for aggrecan cleavage. These sites are located at the following peptide bonds, Asn^{341}-Phe^{342} and Glu^{373}-Ala^{374}. Cleavage of these sites compromises the functional and structural integrity of the

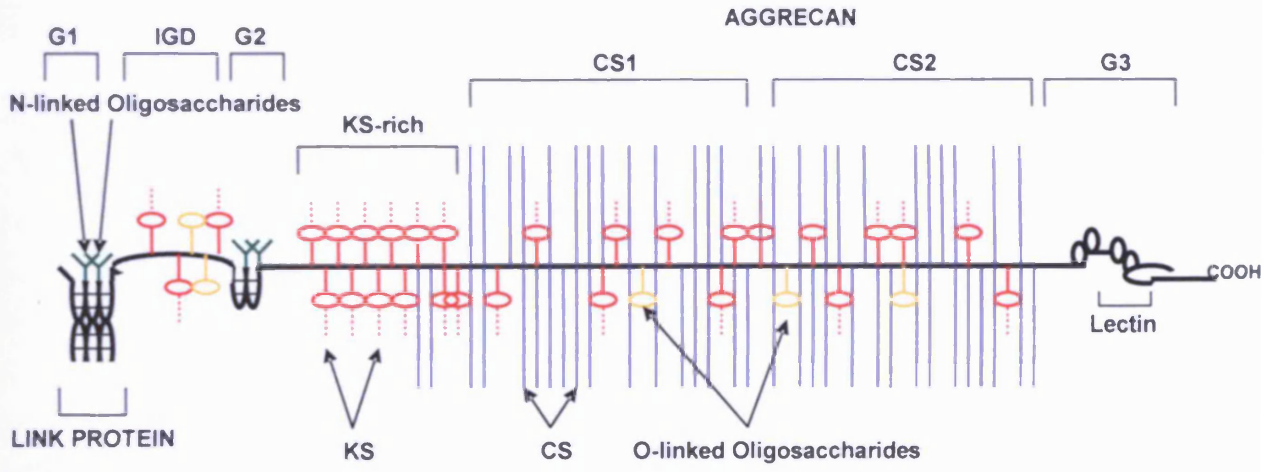
cartilage matrix. Matrix metalloproteinases (MMPs) and the 'A Disintegrin and Metalloproteinase with Thrombospondin Motifs' (ADAMTS) are responsible for this cleavage which is seen in osteoarthritic tissue (Caterson *et al.*, 2000). (See section 1.13).

The G2 domain, which is unique to aggrecan, is homologous to the tandem repeats found in the G1 domain. The function of G2 is still relatively unknown but there is evidence suggesting it may play a role in inhibiting the secretion of aggrecan before it has undergone GAG substitution by the Golgi (Luo *et al.*, 1996).

The area between the G2 and G3 domain is where the majority of GAG side chains, CS and KS attach. As the attachment of CS and KS is unique to aggrecan (as is the G2 domain), there is a hypothesis that the G2 domain may influence GAG binding (Dudhia, 2005). CS tends to bind nearer the C-terminal of the molecule whereas the KS is found nearer the N-terminal (Hardingham and Bayliss, 1990). The C-terminal is located in the G3 domain. This region is very different from both the G1 and G2 domains but it is highly conserved among species (Iozzo, 1998).

The major role of aggrecan lies in distributing load in weight-bearing joints. The association of aggrecan with HA and link protein as well as the GAG side chains (CS and KS) allows the formation of aggregates. The high negative charge conferred on aggrecan by the GAG side chains (CS and KS) allows these aggregates to be hydrated allowing reversible osmotic swelling of the tissue. When the joint is at rest the aggregates are fully hydrated but when stress is placed on the joint the osmotic pressure is depleted and water is essentially 'squeezed' out of the joint and the weight is distributed evenly (Watanabe, 2004; Watanabe *et al.*, 1998) (Fig. 1.6).

Fig. 1.5: Schematic drawing of aggrecan structure. The three globular domains are identified (G1, G2 and G3) as well as the interglobular domain (IGD). The glycosaminoglycan (GAG) side chains are outlined: KS, keratan sulphate; CS, chondroitin sulphate 1 and 2 and the carboxy-terminal at the third globular domain (G3). The domain sturcture of the link protein is also outlined. This is similiar to the G1 domain of aggrecan. In order to form aggregates the aggrecan binds to hyaluronate via a link protein which functions to stabilise this interaction. Blue, CS side chains; red, KS side chains; yellow, *O*-linked oligosaccharides; green, N-linked oligosaccharides.



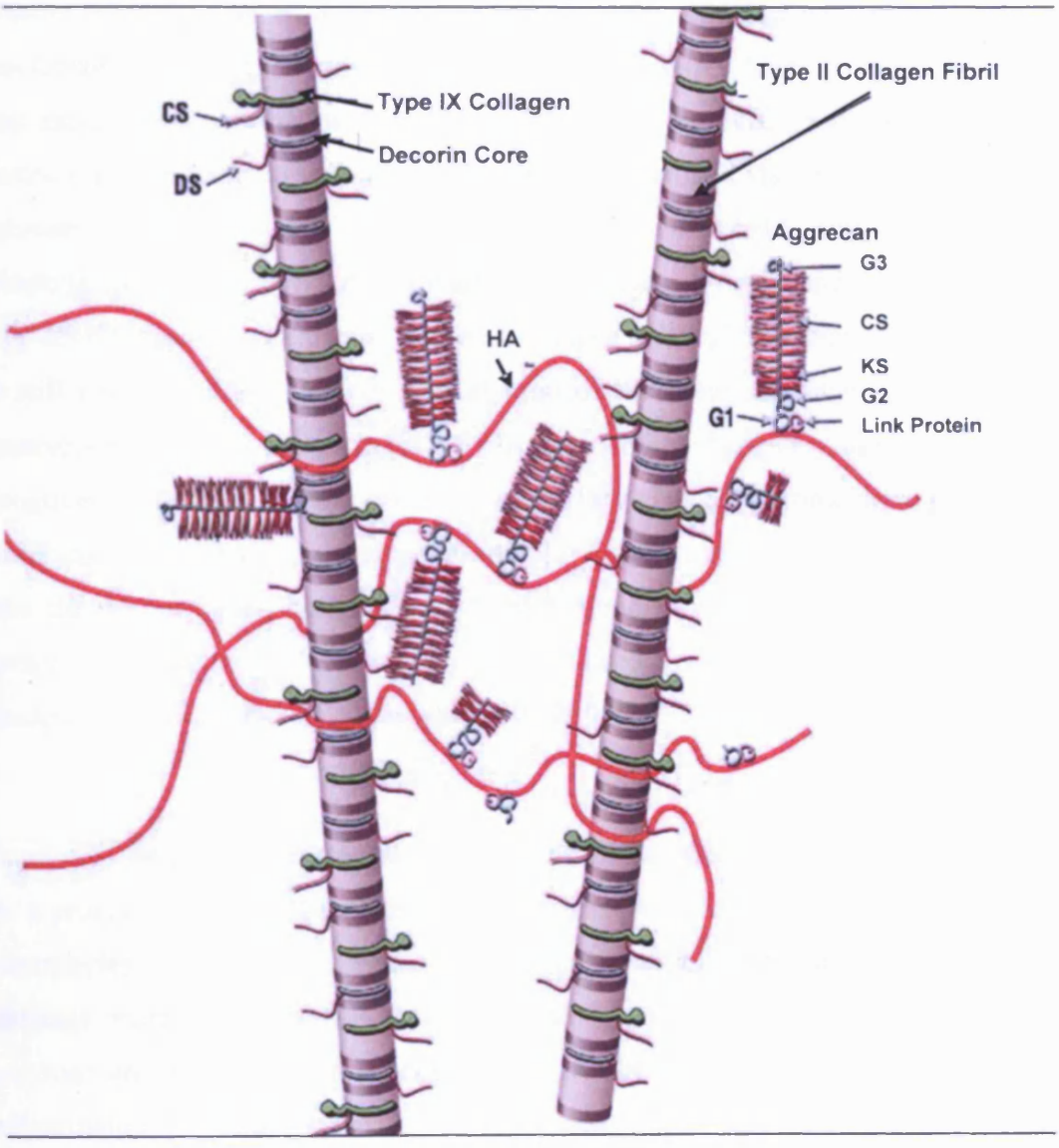
1.2.5.2 Small Leucine-Rich Proteoglycans

Within the ECM of articular cartilage exist other minor proteoglycan components known as small leucine-rich proteoglycans (SLRPs). These were coined 'non-aggregating' proteoglycans due to their inability to interact with HA. Decorin, biglycan and fibromodulin compose this group of SLRPs. These are all characterised by a central domain containing leucine-rich repeats with small cysteine-clusters at each end. They also possess DS within their structure (Hardingham and Fosang, 1992).

Decorin and biglycan are normally found throughout the life cycle in articular cartilage. However with age, biglycan is seen to undergo proteolytic cleavage of the amino terminal resulting in loss of the DS side chains. Decorin, located in the interterritorial matrix, is normally found 'decorating' the surface of collagen and is known to contain either CS or DS as side chains (Iozzo, 1998).

Biglycan, on the other hand, is normally situated in the pericellular matrix and does not interact with collagen fibrils (Iozzo, 1998; Roughley, 2001). Its precise function is still under investigation. Fibromodulin is known to possess up to four KS side chains and, like decorin, it can adorn the surface of collagen fibrils. It is thought to play a role in regulation of collagen fibril diameter (Knudson and Knudson, 2001).

Fig. 1.6: Diagrammatic representation of the macrofibrillar collagen network and aggrecan superaggregate with hyaluronic acid (HA). CS, chondroitin sulphate; KS, keratan sulphate; DS, dermatan sulphate; HA, hyaluronate (Poole *et al.*, 2001).



1.3 PATHOGENESIS AND DEVELOPMENT OF OSTEOARTHRITIS

OA is a degenerative joint disease which involves not only the progressive loss of cartilage but also affects the synovium, joint capsule and underlying bone. The disease involves loss of articular cartilage integrity coupled with remodelling and thickening/hardening (sclerosis) of the subchondral bone, development of bone cysts and osteophytes together with inflammation of the synovial joint resulting in pain, restriction of movement and joint deformity (Hedbom and Hauselmann, 2002). In an osteoarthritic joint, a number of catabolic processes override any repair processes resulting in overall degradation of articular cartilage (Aigner and McKenna, 2002). The development of OA occurs in specific stages although the sequence of each stage is still unclear. These include perturbation of the bone, cartilage zone and matrix destruction. In the early stages of OA the surface becomes rough with surface irregularities becoming more obvious. As surface changes become more pronounced, fibrillation of the superficial zone of articular cartilage is initiated and this continues into the deeper zones until the subchondral bone is visible. Joint inflammation is present throughout the progression of the disease (Aigner and McKenna, 2002; Buckwalter *et al.*, 2001; Kuettner and Cole, 2005).

Continued wear of the subchondral bone reduces the bone into a hard, ivory-like mass by a process of eburnation. Here bone hypertrophy occurs with the development of osteophytes (projections of bone). In conjunction with the fibrillation of cartilage, the cartilage matrix is degraded due to improper matrix metabolism by a series of enzymes and the condition is exacerbated by the action of cytokines due to joint inflammation. The presence of cells associated with the immune system, macrophages, monocytes and neutrophils, results in the over-expression of IL-1β, IL-6 and TNF-α (Guerne *et al.*, 1990; Sarzi-Puttini *et al.*, 2005). Such cytokines are thought be responsible for matrix destruction in cartilage and are believed to be involved in the inflammation seen in OA (section 1.3.2).

Loss of the main proteoglycan, aggrecan and reduction in integrity of the collagen network is thought to be mediated by an upregulation of catabolic enzymes. The enzymes belong to the metalloproteinase family of which there are two distinct types.

Aggrecan destruction, which is believed (though not confirmed) to occur prior to collagen depletion, is mediated by members of the ADAMTS family (Dudhia, 2005) (see section 1.3.1).

Collagen is responsible for forming the highly organised fibrillar network of the ECM and, hence, its destruction during the pathogenesis of OA is detrimental to joint integrity. Due to the action of a second class of matrix proteinases, the MMPs which are upregulated in arthritic disease, a progressive loss of collagen, most especially type II, results in the collapse of the ECM (Caterson *et al.*, 2000) (see section 1.3.1).

Loss of proteoglycan and the collagen networks of the ECM are the principal factors involved in OA. However, chondrocytes are also implicated in disease pathogenesis. In the early stages of OA, chondrocyte metabolism is increased in an attempt to repair the ECM. However, catabolism of the ECM over-rides any attempt at repair and, as disease progresses, chondrocytes release increased amounts of catabolic proteins and inflammatory cytokines (Goldring, 2000). In late stage OA, chondrocyte metabolism becomes mis-directed and often contributes to matrix degradation and down-regulation of anabolic pathways necessary for repair, before cell death (Goldring and Goldring, 2007).

1.3.1 Proteoglycan and Collagen Degradation

The loss of aggrecan from the ECM is one of the earliest pathophysiological hallmarks of OA (Hughes *et al.*, 1998). Cartilage degradation involves proteolytic cleavage of both aggrecan and collagen (Pratta *et al.*, 2003). Depletion of proteoglycan is due to an increased rate of aggrecan degradation which can be attributed to proteolytic cleavage within the IGD located between the G1 and G2 globular domains of aggrecan (Arner *et al.*, 1998). Degradation of aggrecan leads to increased concentrations of GAGs (KS and CS) in the synovial fluid.

Two related enzyme families are responsible for degradation of proteoglycan, the MMPs and the ADAMTS proteinases. The MMPs are zinc-dependent enzymes and, collectively, they can degrade all components of the ECM (Rowan, 2001). MMPs are

inhibited by tissue inhibitors of metalloproteinases (TIMPs) and a fine balance between MMPs and the TIMPs determine whether matrix components are catabolised or not. A disruption of this balance, as seen in OA gives rise to destruction of the ECM (Cawston, 1998) Of the MMP family several collagenases exist, MMP-1 (interstitial collagenase/collagenase-1), MMP-8 (neutrophil collagenase) and MMP-13 (collagenase-3) and all can aggressively degrade collagens of the ECM. Another important member of the MMP family is MMP-3 which is responsible for activation of the other collagenases (Woessner and Nagase, 2000).

The other proteinase family involved in ECM degradation are the aggrecanases (ADAMTSs). Though multiple members of the aggrecanases family exist it is believed that the two main members responsible for the cleavage of aggrecan and loss of matrix integrity (although research continues) are ADAMTS-4 and -5 (aggrecanases-1 and -2, respectively) (Abbaszade *et al.*, 1999; Flannery, 2006; Tortorella *et al.*, 1999). Though not strictly MMPs, the ADAMTS members may be inhibited by high concentrations of TIMPS (Rowan, 2001).

These two enzyme systems (MMPs and aggrecanases) have specific aggrecan cleavage sites within the IGD of the aggrecan molecule (Hughes *et al.*, 1998). In humans, the aggrecanases cleave aggrecan in the IGD at the peptide bond Glu^{373} – Ala^{374} whilst the MMPs cleave at Asn^{341} – Phe^{342} (Flannery *et al.*, 1992; Sandy *et al.*, 1992). Cleavage results in removal of the GAG side chains from the N-terminal hyaluronan-binding G1 domain and loss of aggrecan into synovial fluid. Evidence indicates that the aggrecanases are responsible for primary cleavage of the aggrecan IGD during cartilage degradation, while cleavage of the Asn^{341} – Phe^{342} by the MMPs can occur as a late event in cartilage degeneration (Caterson *et al.*, 2000).

1.3.2 Inflammation and Cytokines

Cytokines are soluble proteins that function as chemical messengers between cells and are believed to be involved in almost all biological process including cell growth, differentiation, inflammation, tissue repair/remodelling and immune response. By acting in either an autocrine or a paracrine manner, cytokines exert either pro- or anti-inflammatory effects (Buch and Emery, 2002) and oftentimes work synergistically. With regard to arthritic disease, two specific cytokines have received particular investigation, IL-1 and TNF-α. Both IL-1 and TNF-α stimulate a pro-inflammatory response in cells which, in turn, leads to the upregulation of catabolic and proteolytic enzymes (e.g. MMPs and aggrecanases), as well as chemotaxis together with T- and B- cell activation (Wood, 2006). However, IL-1 and TNF-α are not the only cytokines implicated in RA. IL-6, -8, -10, -11, -13 and -17 as well as macrophage-granulocyte colony-stimulating factor (GM-CSF) may also contribute. Lower levels of IL-2, -3, -12, oncostatin M and leukaemia inhibitory factor (LIF) have also been detected in the synovial fluid of arthritic patients (Rowan, 2001).

Some cytokines have anti-inflammatory properties (IL-4, -10) but, in the case of OA, the pro-inflammatory cytokines are thought to be present in greater concentrations than the anti-inflammatory cytokines resulting in little protection from the latter. Little, if any, of the chondro-protective cytokine, IL-4 is usually detected (Kardel *et al.*, 2003; Rowan, 2001). Eicosanoids are another series of pro-inflammatory mediators involved in the pathogenesis and progression of OA (see section 1.5.1.1).

Before the effects of n-3 PUFAs on the pathogenesis, progression and possible treatment of OA are discussed (section 1.6) a review of n-3 PUFA chemistry is outlined (section 1.4).

1.4 n-3 POLYUNSATURATED FATTY ACIDS

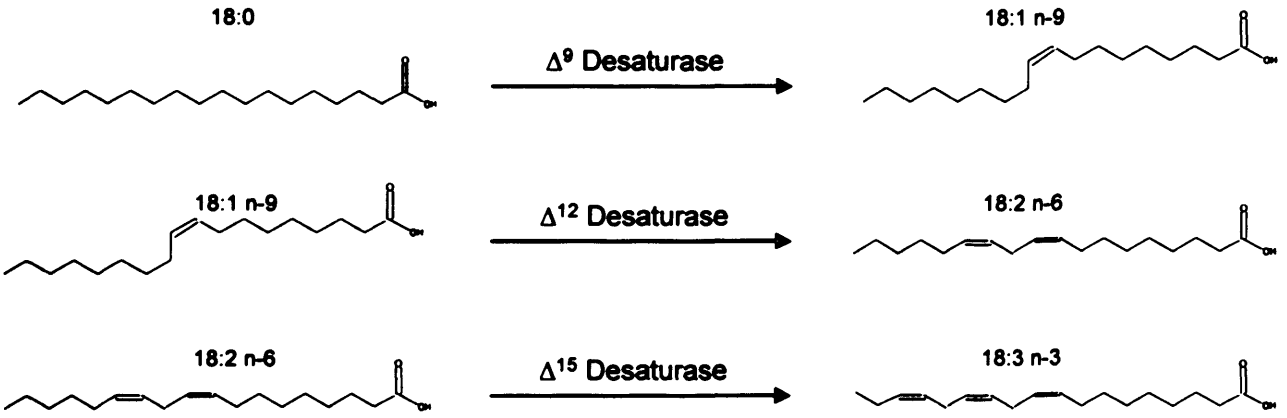
PUFAs are an unsaturated class of fatty acids which contain more than one double bond in their carbon chain. The two main families of PUFAs are the n-6 and n- 3 PUFAs and it is the position of the first double bond within the hydrocarbon chain that gives the n-6 and n-3 PUFAs their name and properties. The n-6 PUFAs have their first double bond on the sixth carbon of the methyl (non-carboxyl) end of the fatty acid molecule whereas in an n-3 PUFA the first double bond is located at the third carbon from the non-carboxyl end. PUFAs are usually methylene-interrupted meaning that there is at least one intervening carbon between the carbons involved in a double bond. The most important PUFAs in health and nutrition are 18:2 n-6 linoleic acid and 18:3 n-3 α-linolenic acid together with their respective very long chain derivatives 20:4 n-6 AA, 20:5 n-3 EPA and 22:6 n-3 DHA which play important roles in human health and disease (Gurr *et al.*, 2002).

De novo synthesis of fatty acids occurs in the mammalian cell cytosol and consists of a cyclic series of reactions which 'build-up' the acyl chain by the addition of two carbon units (Gurr *et al.*, 2002). Using the multi-functional proteins acetyl-CoA carboxylase and fatty acid synthase conversion of acetyl-Coenzyme-A to palmitic/stearic acids is achieved (Benyon, 1998). Despite the ability of mammals to adequately produce saturated fatty acids, the diet provides an abundant supply. Elongation and desaturation enzyme systems exist to insert double bonds at certain positions in the acyl chain to yield unsaturated fatty acids. However, mammals lack the necessary enzymes to yield the simplest members of the two main PUFA families, n-6 linoleic acid (18:2) and n-3 α-linolenic acid (18:3). Due to the inability of mammals to generate these PUFAs both 18:2 n-6 and 18:3 n-3 are classified as essential fatty acids (EFAs) (Cunnane, 2003).

In contrast to mammals, plants possess the necessary desaturases to convert the 18:0 fatty acid (stearic acid) to both 18:2 n-6 and 18:3 n-3 (Fig. 1.7). As 18:2 n-6 and 18:3 n-3 are EFAs they need to be provided by the diet. Essentiality of both these fatty acids was determined in 1927-1930 when experimental animals fed a semi-purified fat-free diet suffered impaired growth, scaliness of the skin, water retention, retarded growth, infertility and reproductive failure. These symptoms were reversed when the

EFAs were introduced to the diet (Burr and Burr, 1930; Evans and Burr, 1927). However, it should be mentioned that the term EFAs has come under review due to the hypothesis that certain non-EFA may be essential (termed conditionally essential) during certain periods of development (for review see Cunnane, 2003). One such example is the case of the saturated fatty acid, palmitic acid (16:0) for which there is evidence that it is required for normal expression of hedgehog proteins (e.g. sonic hedgehog) during embryonic development (and hence, could be seen as being conditionally essential during this period) (Chamoun *et al.*, 2001). Despite this ongoing debate, both 18:2 n-6 and 18:3 n-3 are considered the classical EFAs and need to be provided by the diet.

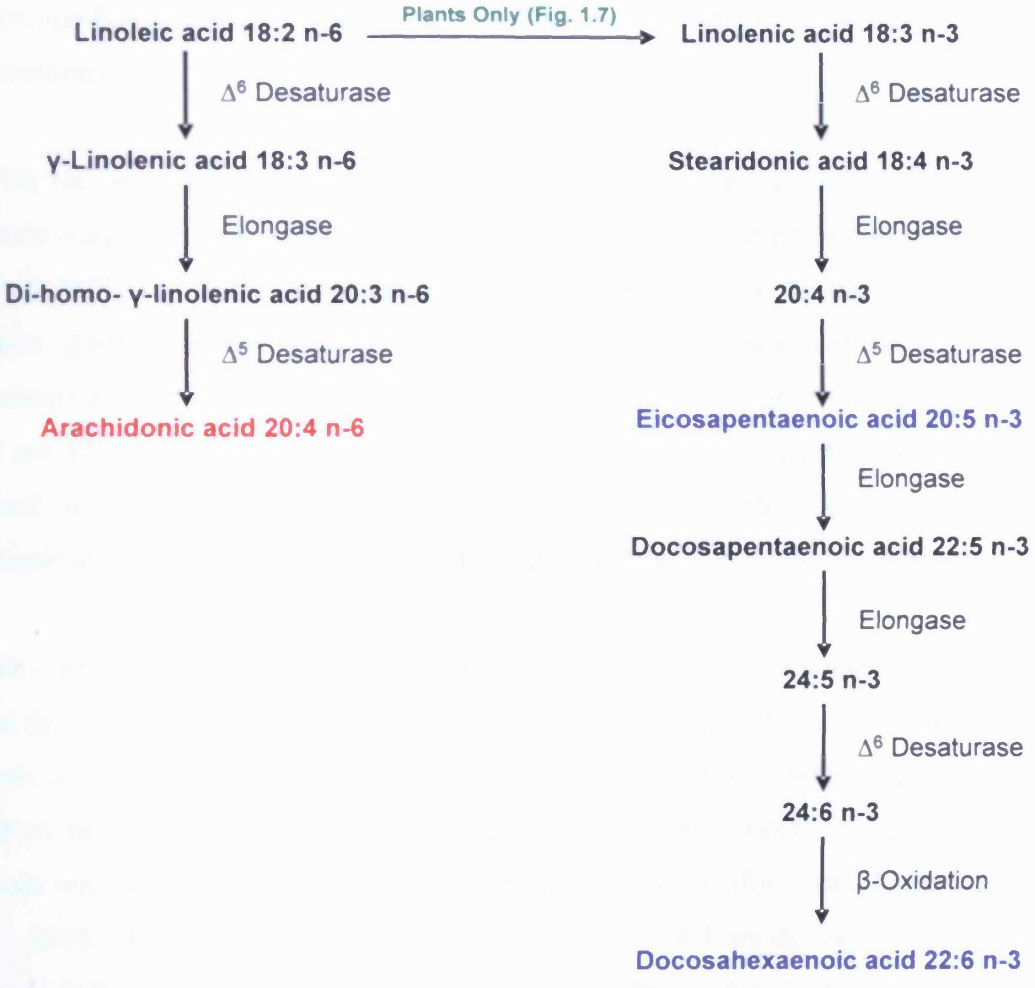
Fig. 1.7: Conversion of stearic acid (18:0) to linolenic acid (18:3 n-3) via oleic acid (18:1 n-9) and linoleic acid (18:2 n-6) by the action of specific desaturase enzymes (Δ^9, Δ^{12} and Δ^{15}).



Although 18:2 n-6 and 18:3 n-3 cannot be synthesized *de novo*, mammals can further metabolise them to their longer chain polyunsaturated fatty acids (PUFA) derivatives, arachidonic acid (AA) (n-6) and eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (n-3) by the action of desaturases and elongases (Fig. 1.8). In mammals, the pathway of desaturation and elongation occurs primarily in the liver. However, despite possessing the ability to convert the 18 carbon precursors to the longer chain derivatives the efficiency of this pathway is particularly low and it is also affected by the preferential β-oxidation of 18 carbon fatty acids (Cunnane, 2003; Gurr *et al.*, 2002). Therefore, the n-3 PUFAs, EPA and DHA, are often also considered EFAs.

The same enzymes are used in the metabolism of both the n-6 and n-3 fatty acids, hence, competition exits between the two families. The enzymes involved in the conversion of 18 carbon fatty acids favour the more unsaturated fatty acid, 18:3 n-3, but as 18:2 n-6 is more common in the diet there is more conversion of 18:2 n-6 to AA (Calder, 2004; Simopoulos, 2002a; Simopoulos, 1995). It is the generation and consumption of large quantities of n-6 fatty acids and the relative lack of n-3 fatty acids that have, in part, given rise to the extensive research on the role of n-3 and n-6 fatty acids in the diet and the resulting implications on health and disease.

Fig. 1.8: Conversion of linoleic (18:2 n-6) and linolenic acids (18:3 n-3) fatty acids to their respective very long chain derivatives arachidonic acid (20:4 n-6), eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3).



1.4.1 Balance of Essential PUFAs

Millions of years ago, when the human genome was being established the dietary ratio of n-6/n-3 fatty acids was thought to be approximately 1 (Simopoulos, 2002). During this stage of human development the diet consisted primarily of green leafy vegetables, fish, lean meats, fruits, berries and honey, meaning that it contained equal levels of n-3 and n-6 PUFAs (Simopoulos, 2002). However, since the arrival of the agricultural revolution dramatic changes have occurred in terms of the types and

quantities of foods consumed by humans and a large change in the n-3/n-6 ratio of dietary PUFAs has occurred. Despite the inherent changes of the diet the human genetics have not changed. The spontaneous mutation rate for nuclear DNA is thought to be 0.005% per million years. Therefore, not enough time has elapsed for the genome to have mutated in accordance with the drastic dietary changes (Simopoulos, 2002a). Therefore, this imbalance can be implicated in disease development.

With the development of technology which allowed cereal grains to become staple foods within the global food chain there has been a dramatic impact on the ratio of n-6/n-3 PUFAs. Cereal grains are a relatively recent addition to the human diet and cause great deviation from man's original diet (which was consumed when the genome was being established) (Simopoulos, 1995). Cereal grains are a major source of n-6 PUFAs and contain little or no n-3 PUFAs or antioxidants. Moreover, most plant oils have much more n-6 than n-3 PUFAs. Therefore, excessive intake of cereals and plant oils reduces the n-3/n-6 fatty acid ratio (Simopoulos, 2002a).

The current consensus on Western type diets is that they are abundant in n-6 PUFAs but deficient in n-3 PUFAs. In Western countries, dietary ratios are believed to be as high as 15-20/1 (n-6/n-3) (Crawford, 1968; Simopoulos, 1998) although a recent report by the NHANES committee suggest that improvements are gradually being made with a reported ratio of 10/1 in some countries (Griffin *et al.*, 2006; Sanders *et al.*, 2006). Current advice suggests that ratios of 3 – 4/1 are desirable (Kris-Etherton *et al.*, 2001). Excessive amount of n-6 PUFAs, a high n-6/n-3 ratio and a deficiency of n-3 PUFAs can promote the pathogenesis of many diseases, especially cardiovascular disease (CVD) and chronic inflammatory diseases. The n-6 PUFAs exert pro-inflammatory effects whereas the n-3 PUFAs are thought to express inhibitory effects on a range of cells involved in inflammation, particularly the immune cells, and have less-inflammatory properties (Calder, 2003; Sardesai, 1992a; Simopoulos, 2002b). CVD and chronic inflammatory dieseases such as arthritis, are a major cause of ill-health and mortality in the developed world.

High dietary intakes of n-6 PUFAs mean that the PUFA components of the phospholipids in cell membranes are mainly composed of the n-6 PUFA, AA (20:4 n-6). AA is metabolised by enzyme systems to produce eicosanoids which are active mediators of the immune system (section 1.5.1). The eicosanoids derived from AA are biologically-active in small quantities and, if they are formed in large amounts, they encourage the formation of thrombus and atheromas; allergic and inflammatory disorders, and the proliferation of cells. Previous research has proven that the n-6 derived eicosanoids are several times more biologically potent than the n-3 derived. One classic example is that of the inflammatory response invoked by leukotriene B4 (n-6 derived). Leukotriene B4 (n-6 PUFA derived) is believed to be 10 times more aggressive in promoting an inflammatory attack compared to leukotriene B5 (an n-3 PUFA-derived) (Calder, 2002). Due to this evidence, several investigators including Kris-Etherton *et al.* (2001), have concluded that a diet rich in n-6 PUFAs shifts the physiological state to one that is "pro-inflammatory (prothrombotic and proaggregatory, with increases in blood viscosity, vasopasm and vasoconstriction and decreases in bleeding time)". A reduction in bleeding is seen in most aspects of CVD from patients with hypocholesterolemia, myocardial infarction and diabetes.

Chronic inflammatory dieseases, such as CVD, are characterised by the formation of increased amounts of thromboxane A2, leukotriene B4, IL-1β, IL-6 and TNF-α (Mehra *et al.*, 2005). Unsurprising, increases in these mediators has been correlated with high intakes of n-6 PUFAs. By increasing the intake of n-3 PUFAs a balance of the n-6/n-3 ratio is achieved with beneficial effects particularly with regard to immune cells and inflammatory response (von Schacky, 2007). Such effects are achieved by the incorporation of EPA and DHA into cellular membranes at the expense of AA and, therefore, the production of less-inflammatory prostanoids (Grimble, 1998; Simopoulos, 2002b). In view of this, assessment of the role of fat quality and the balance of n-6/n-3 PUFAs is of considerable importance.

1.4.2 Biological Properties of Polyunsaturated Fatty Acids

The two classes of EFA (n-3 and n-6) are metabolically and functionally distinct, and often have opposing physiological functions (Simopoulos, 2002a). Dietary EFAs can be converted directly or indirectly into a class of twenty carbon oxygenated derivatives collectively known as eicosanoids. Eicosanoids are a group of chemical messengers which act locally and include prostaglandins (PG), thromboxanes (TX), leukotrienes (LT), lipoxins, hydroperoxyeicosatetraenoic acids (HPETE) and hydroxyeicosatetraenoic acids (HETE) (Bowman and Russell, 2000). They are potent regulators of cell function and act locally in tissues in which they are formed and are rapidly converted to their inactive form (Kinsella *et al.*, 1990). A new series of bioactive lipid compounds is currently being elucidated, the resolvins, docosatrienes and neuroprotectins. These compounds are produced from both EPA and DHA and possess potent immunoregulatory and anti-inflammatory actions (Serhan *et al.*, 2004a; 2004b) (see section 1.5.3).

1.5 'ANTI'-INFLAMMATORY EFFECTS OF n-3 PUFAs

An abundance of scientific evidence indicates a role for n-3 PUFA in combating inflammation. In particular, α-linolenic acid (ALA), EPA and DHA have been extensively investigated over the last two decades and evidence now exists that the type of fat in the diet has a major impact on inflammation and other areas of immune function. It should be noted that the term 'anti'-inflammatory is used loosely as many n-3 PUFA-derived mediators of inflammation are mildly-inflammatory rather than strictly 'anti'-inflammatory.

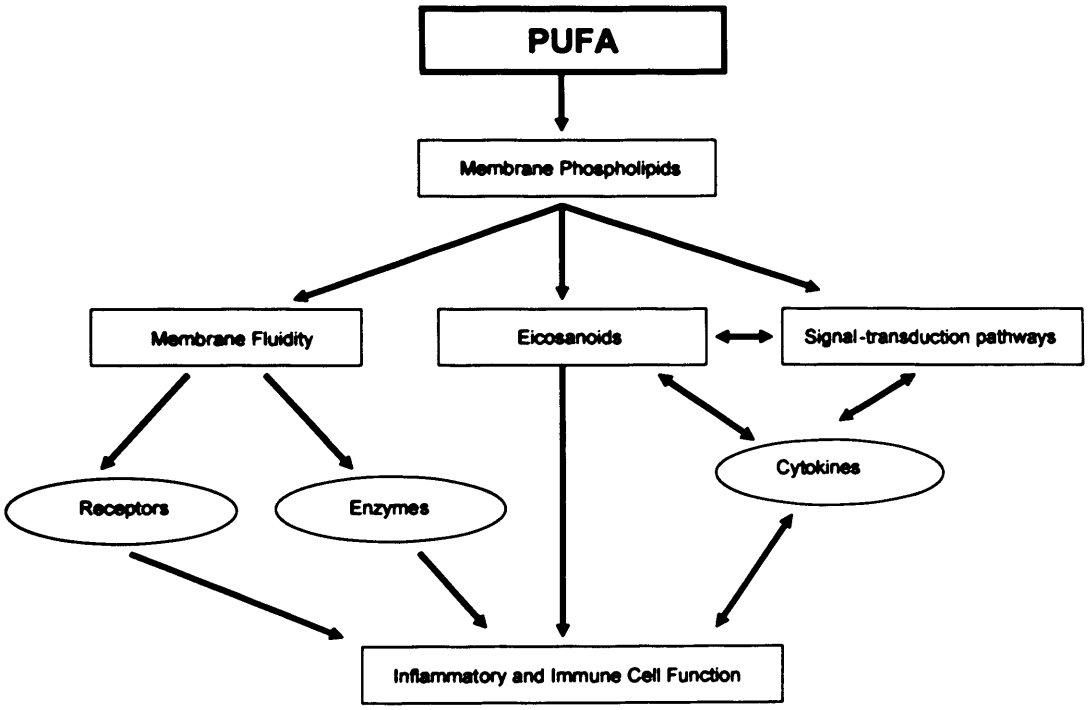
Investigation into the diets of Greenland Eskimos prompted speculation that a component of their diet may have protective properties due to their low incidence of CVD and other autoimmune and inflammatory disorders. It was concluded that, despite the high fat content of the diet, intakes of n-3 PUFAs were particularly high and offered protection again CVD and other inflammatory disease (Bang *et al.*, 1971; Bang and Dyerberg, 1980).

High levels of n-3 PUFAs were also observed in Japan where a low incidence of CVD is also found (Kromann and Green, 1980). Data indicated that it was the type of fat consumed which demonstrated protection again CVD and gave rise to an abundance of studies investigating the protective effects of n-3 PUFAs. More recently, the GISSI study (Italian Group for the Study of Streptokinase in Myocardial Infarction (Gruppo Italiano per lo Studio della Streptochinasi nell'Infarto Miocardico)) reported a significant reduction (45%) in sudden death in >11,000 patients with CVD, when taking almost a gram of n-3 PUFA per day (Marchioli *et al.*, 2002). Such reports led to extensive research using animal models, tissue culture and human studies which complemented the original observational studies.

From this research, some of the mechanisms by which n-3 PUFAs exert protective anti-inflammatory effects have been elucidated (Fig. 1.9). The main mechanisms identified include, the generation of active metabolites (e.g. eicosanoids, resolvins, docosatrienes) (Calder, 2003); effects on inflammatory gene expression (e.g. cyclooxygenase-2) (Calder, 2005); modulation of inflammatory cytokine production (e.g. TNF-α) (Simopoulos, 2002b) and influence over adhesion molecule expression

(e.g. E-selectin) (Calder, 1998). The effect of n-3 PUFAs on adhesion molecule expression is not a parameter examined in this project and, therefore, will not be discussed further.

Fig. 1.9: Mechanisms by which polyunsaturated fatty acids (PUFAs) may affect immune cell function.



1.5.1 Cyclooxygenase

Cyclooxygenase (COX), otherwise known as prostaglandin endoperoxide H synthase (PGHS), was initially purified from sheep seminal vesicles and was finally cloned in the late 1980s (DeWitt and Smith, 1988; Tanabe and Tohnai, 2002; Vane *et al.*, 1998). There are two known isoforms of COX, although recent developments have presented evidence of the existence of a third isoform, COX-3 as well as two smaller COX-1 derived proteins (partial COX-1 or PCOX-1 proteins) (Chandrasekharan *et al.*, 2002). COX-3 is an acetaminophen-sensitive splice variant of COX-1 which was first identified in canine tissues but its function is still relatively unknown (Dogne *et al.*, 2005).

COX enzymes are heme-containing proteins which possess both oxygenase and peroxidase activity (Smith *et al.*, 1996). The oxygenase and peroxidase sites are both physically and functionally separate (Smith *et al.*, 1996). The COX enzymes are membrane-bound and located on the endoplasmic reticulum (ER) membrane, the nuclear envelope or, in some cases, the plasma membrane. Thus, they exist in various locations within the same cell (Spencer *et al.*, 1999). It has been suggested that the prostaglandins produced in different locations function via two different classes of receptor systems (Lim *et al.*, 1999; Smith *et al.*, 2000). For example the prostaglandins produced by ER-bound COX are thought to both exit the cell and exert their function through G-protein coupled receptors which are linked to different cytoplasmic cell signalling pathways (Negishi *et al.*, 1995).

COX-1 is a constitutive enzyme expressed at a constant level in many mammalian cells. It is thought to function in 'housekeeping' events via prostaglandin formation. Maintenance of mucosal epithelium in the stomach, regulation of kidney function and vascular homeostasis are some of the normal physiological events COX-1 derived prostaglandins appear to regulate (Spencer *et al.*, 1999; Vane *et al.*, 1998). COX-2 is considered the inducible isoform as it is induced by a range of mitogenic agents such as cytokines (Bagga *et al.*, 2003; Tanabe and Tohnai, 2002). However, the labelling of COX-1 as constitutive and COX-2 as inducible is somewhat of an over-

simplification as COX-1 levels vary during development and some organs (brain, testes) constitutively express low levels of COX-2 (Smith *et al.*, 1996). It is believed that expression of COX-2 in response to stimuli increases and decreases rapidly. Fagan and Goldberg (1986) estimated the half life of COX-2 in skeletal muscle is less than 4 minutes which indicates that enzyme turnover is tightly regulated. COX-2 plays important roles in disease pathology. It is implicated in diseases ranging from arthritis (Martel-Pelletier *et al.*, 2003) to colon cancer (Eberhart *et al.*, 1994) and Alzheimer's disease (Hoozemans *et al.*, 2001; Hoozemans and O'Banion, 2005; Tzeng *et al.*, 2005).

The isolation and characterisation of the COX enzymes established them as targets for non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin and ibuprofen (Fig.1.10). NSAIDs function by acetylating amino acid residues (Ser530 in COX-1 and Ser516 in COX-2) near the active site of the COX enzyme. Acetylation results in a conformational change in enzyme structure therefore preventing the cyclic oxygenation of AA and, thus, preventing eicosanoid production (Fiorucci *et al.*, 2003; Marnett, 2002; Smith *et al.*, 1996). NSAIDs have the ability to inhibit both COX isoforms as these proteins are very similar in both structure and catalytic activity (60% homology) (Vane *et al.*, 1998). However, one of the most common NSAIDs, aspirin, is known to be a more potent inhibitor of COX-1 (10 – 100 fold) relative to COX-2 (Smith *et al.*, 1996). This is due to the substrate channel of the COX-2 isoform being larger and more flexible due to the presence of arginine and valine which are smaller and less bulky than the histidine and isoleucine found at the same positions (513 and 523 respectively) in the COX-1 isoform. Aspirin acetylated inhibition of COX-2 occurs at Ser516 whilst the selective COX-2 inhibitors target the hydrophobic channels surrounding the active site (Botting *et al.*, 2006).

There are currently two types of NSAIDs (a) classical NSAIDs (pre-1995) and (b) selective COX-2 inhibitors (Smith *et al.*, 2000). Once the pharmacological mechanism of acetyl-salicylic acid (aspirin) had been elucidated and its effects on COX-1 and COX-2 demonstrated, the development of classical NSAIDs ensued. As outlined earlier, COX-1 is constitutively expressed and plays important roles in normal physiological events, such as maintenance of gastric lining. Classical NSAIDs inhibit both COX-1 and -2 but, in spite of their anti-inflammatory effects,

they also give rise to toxicity issues (Wallace, 2006). Toxicity from NSAIDs includes the development of ulcers and/or other serious gastrointestinal complications as the prostaglandins necessary for gastric mucosal integrity are no longer formed due the inhibition of COX-1 (DeWitt, 1999).

The toxicity issues associated with classical NSAIDs together with the emergence of information regarding the difference in the active sites of COX-1 and -2 paved the way for the development of selective COX-2 inhibitors. As selective COX-2 inhibitors do not inhibit COX-1 it was believed that the risks for gastrointestinal toxicity would be much lower compared to the classical NSAIDs (DeWitt, 1999). However, selective COX-2 inhibitors were not problem-free. Several of these drugs (e.g. rofecoxib, celecoxib and valdecoxib), developed for the treatment of OA and acute-pain conditions, were withdrawn from the market following six placebo-controlled studies (e.g. VIGOR study) which reported increased cardiovascular risks (myocardial infractions, stroke and congestive heart failure) (Davies and Jamali, 2004; Scheen, 2004). One of these placebo-controlled studies which investigated the potential reduction in GI complications with the selective COX-2 inhibitor, rofecoxib, compared to classical NSAIDs reported a 4-fold increase in cardiovascular risk. Subsequently, rofecoxib was removed from the market by Merck & Co. Other studies carried out also reported no significant increase in gastrointestinal safety with selective COX-2 inhibitors compared to the classical NSAIDs (e.g. CLASS study) (for review see James *et al.*, 2007).

1.5.1.1 Eicosanoid Production and Polyunsaturated Fatty Acids

Eicosanoids are twenty carbon oxygenated derivatives derived from both the n-3 and n-6 family of PUFAs. The production of specific categories of eicosanoids is governed by fast-acting and quickly inactivated enzyme systems (Bowman and Russell, 2001). This group of short-lived lipid derivatives possess potent biological activity and link PUFAs to inflammation (Calder, 2005). They act locally in either an autocrine or a paracrine fashion, influencing numerous biological processes including inflammation (Gil, 2002).

Two membrane-bound enzyme systems are responsible for the formation of eicosanoids, the COXs and specific lipoxygenases (LOX) (the P450 oxygenase system also plays a role but this is not discussed further) (section 1.5.2). Precursors for eicosanoid synthesis are the unesterified PUFAs, AA and EPA (Sardesai, 1992b). Initiation of eicosanoid synthesis begins with mobilization of PUFAs found in the cellular membrane by the action of phospholipases (mainly phospholipase A_2). The phospholipases themselves are stimulated in a number of ways such as mechanical stimuli or by peptides and other chemical mediators of inflammation such as complement C_{5a} (Gil, 2002). The amounts and types of eicosanoids produced depends on a number of factors including, availability of the substrate (PUFA), activities of phospholipases and the various fast-acting COX, LOX or P450 enzyme systems. The cell type and the nature of the stimulus will also influence the eicosanoids produced (Calder, 1998).

Inflammatory cells typically contain a high proportion of the n-6 PUFAs, mainly AA and a low proportion of the n-3 PUFAs, especially EPA (Calder, 2005). Thus, AA is the primary substrate for eicosanoid synthesis. Metabolism of PUFAs to eicosanoids is mainly initiated by phospholipase A_2 cleaving off PUFAs from the *sn*-2 position of a phospholipid molecule, such as phosphatidylcholine (PC) (Calder, 1998). However, increasing the levels of n-3 PUFAs consumed in the diet reduces the n-6 PUFA levels in cellular membranes, (i.e. AA is replaced by EPA, which is a competing substrate for the COX and LOX systems) (Robinson *et al.*, 2001; Robinson and Field, 1998; Wallace *et al.*, 2001). Eicosanoids produced from EPA may exert the same effects as those from AA but possess much less potency. Hence, they are deemed to have 'anti-inflammatory' effects because they will reduce the amount of inflammation (Calder and Grimble, 2002). Regardless of whether the initial PUFA substrate is n-6 or n-3, the same families of eicosanoids are produced – the prostaglandins (PGs), thromboxanes (TXs) and leukotrienes (LTs), only the series differs (section 1.5.1.2).

The first step in the formation of many of the aforementioned eicosanoid families is the oxygenation of the precursor PUFA by the COX enzyme system. The cyclooxygenation occurs in a hydrophobic channel in the enzyme core and combines the precursor PUFA with two molecules of oxygen to yield prostaglandin G_2 (PGG_2). PGG_2 is then quickly reduced to the endoperoxide, PGH_2, from which the classical

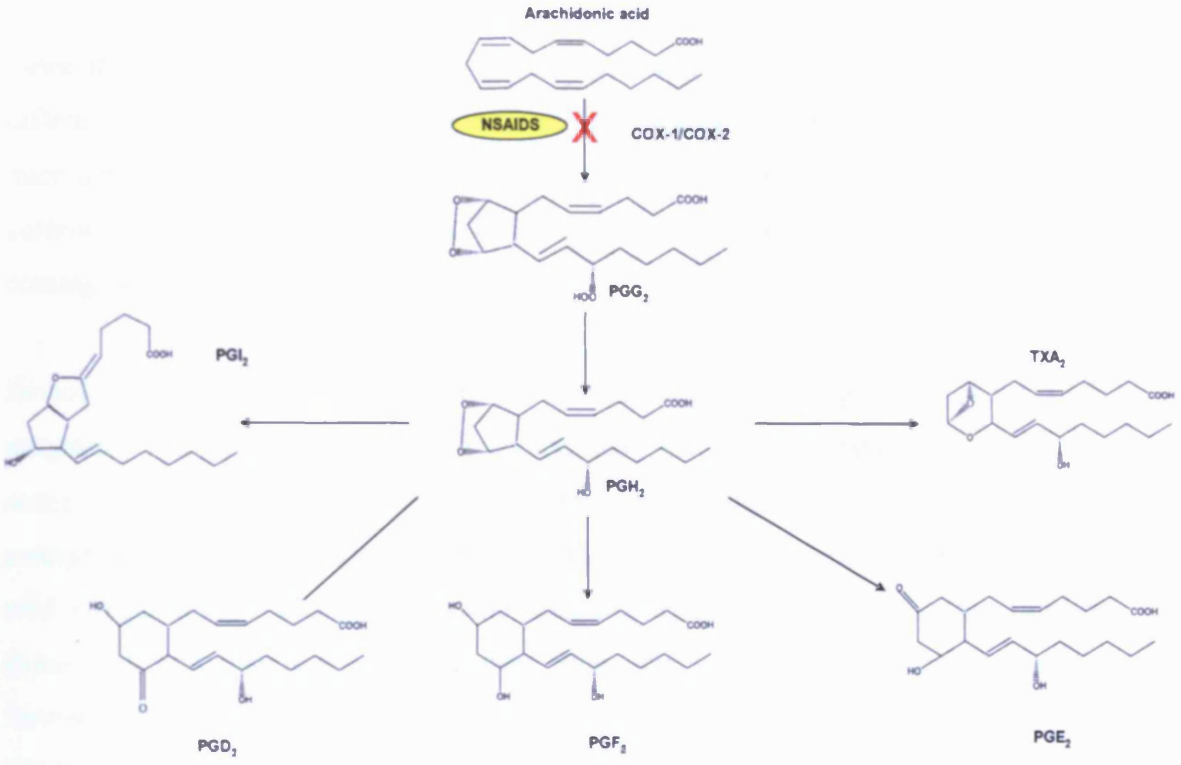
PGs, TXs or prostacyclins can be produced (Gurr *et al.*, 2002; Smith *et al.*, 2000; Smith *et al.*, 1996; Vane *et al.*, 1998). The resulting eicosanoids exit the cell via a carrier-mediated process to activate prostanoid G-protein-linked prostanoid receptors or may interact with nuclear receptors (Chan *et al.*, 1998; Lim *et al.*, 1999; Ushikubi *et al.*, 1998) (Fig. 1.10).

1.5.1.2 Prostaglandins and Thromboxanes

Prostaglandin (PGs) and thromboxanes (TXs) are potent mediators of inflammation and, due to their transient nature, they have extremely short half lives, generally in the realm of seconds (Funk, 2001). Depending on the cell type where they are produced they can induce different responses in either an autocrine or a paracrine fashion. PGE_2 is probably the best studied of all the eicosanoids and it has many pro-inflammatory effects ranging from fever onset and the induction of pain and odema caused by other stimulatory agents such as histamine. It has also been demonstrated to suppress lymphocyte proliferation, natural killer cell activity and inhibit the production of IL-2 and interferon-γ (INF-γ). It has also been demonstrated to promote immunoglobulin E production by B-lymphocytes (Calder, 2002; Fantone *et al.*, 1980; Kunkel *et al.*, 1979). It is believed that PGE_2 exits the cell via facilitated transport through the prostaglandin transporter (PGT) which belongs to the organic anion transporter polypeptide family. The PGTs belong to three clusters within a distinct subfamily of the G protein-coupled receptor (GPCR) superfamily (Funk, 2001).

Of the thromboxanes (TXs), TXA_2 is the best documented. It is formed from the endoperoxide, PGH_2 via the action of thromboxane synthase. Roles for TXA_2 include vasoconstriction, vasodilation and platelet aggregation. It is also a smooth muscle mitogen. The production of TXA_2 is increased during the development of artherogenesis and its biosynthesis and biological activities are now being investigated as therapeutic targets in the treatment of this disease (Dogne *et al.*, 2005).

Fig. 1.10: Synthesis of eicosanoids from arachidonic acid and inhibition by NSAIDs. The metabolic cascade indicates prostaglandin (PG), thromboxane (TXA_2) and prostacyclin (PGI_2) generation from arachidonic acid via the cyclooxygenase (COX) enzyme system.



1.5.2 Lipoxygenase

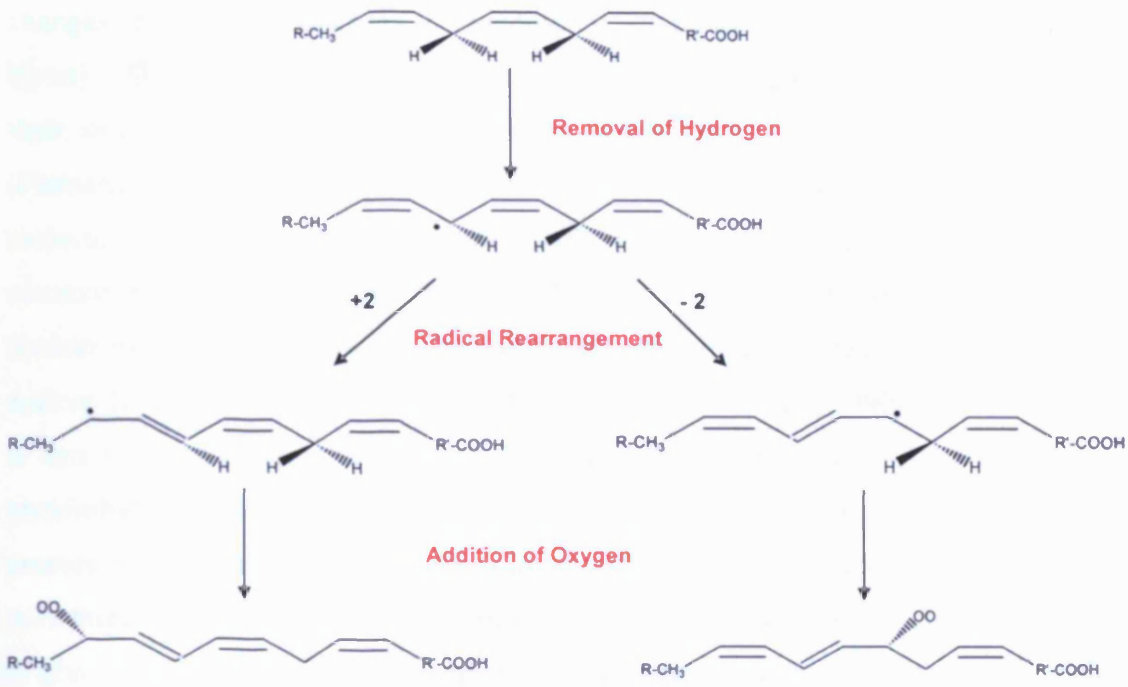
Lipoxygenases (LOX) are a group of enzymes which function in the biosynthesis of a group of inflammatory and cell signalling mediators, the leukotrienes and lipoxins (Prigge *et al.*, 1997). This family of lipid peroxidising enzymes is widely distributed throughout both the plant and animal kingdom. They have also been identified in lower marine organisms such as sea-urchins and starfish (Kuhn and Thiele, 1999). Up until the mid 1970s it was assumed that LOX enzymes were only to be found in the plant kingdom. Most of the early research into these enzymes was done on the soyabean LOX-1 and some other plant isoforms (Kuhn *et al.*, 2005). It was not until the 1970s that the presence of LOX in mammalian cells was established. Two important studies led to a resurgence of research in the LOX field. Hamberg and

Samuelsson (1974) documented the formation of 12-hydroxyeicosa-5,8,10,14-tetraenoic acid (12-HETE) from human thrombocytes whilst they were incubated with exogenous AA. Another important study was carried out in 1974 which identified the presence of 15-LOX in rabbit reticulocytes. This isoform was capable of oxidising membrane phospholipids to active metabolites (Schewe *et al.*, 1986).

Since then, 18 different LOX sequences have been determined representing seven different isoforms studied in seven mammalian species. Among the species studied, mice appear to have the greatest diversity of LOX enzymes. It is thought that the current six different isoforms established in mice is set to expand further over the coming years (Kuhn and Thiele, 1999).

Initially, much mystery surrounded the mechanistic action (Fig. 1.11) of the LOX enzymes. However, it is now accepted that the reaction takes place in three consecutive steps: (a) stereo-selective removal of hydrogen from a bisallylic methylene forming a carbon-centred fatty acid radical; (b) rearrangement of the fatty acid radical from a *cis-cis* methylene interrupted radical to a *trans cis*-conjugated diene and (c) insertion of molecular dioxygen (peroxidation) resulting in the formation of an oxygen-centred fatty acid radical (Gurr *et al.*, 2002; Ivanov *et al.*, 2005). LOX metabolism gives rise to 5-hydroperoxyeicosatetraenoic acid (5-HPETE) which rapidly undergoes dehydration coupled with isomerization of double bonds to yield the parent leukotriene, LTA_4.

Fig. 1.11: Mechanism of lipoxygenase (LOX) action from a trienoic precursor unsaturated fatty acid (adapted from Kuhn and Thiele, 1999).

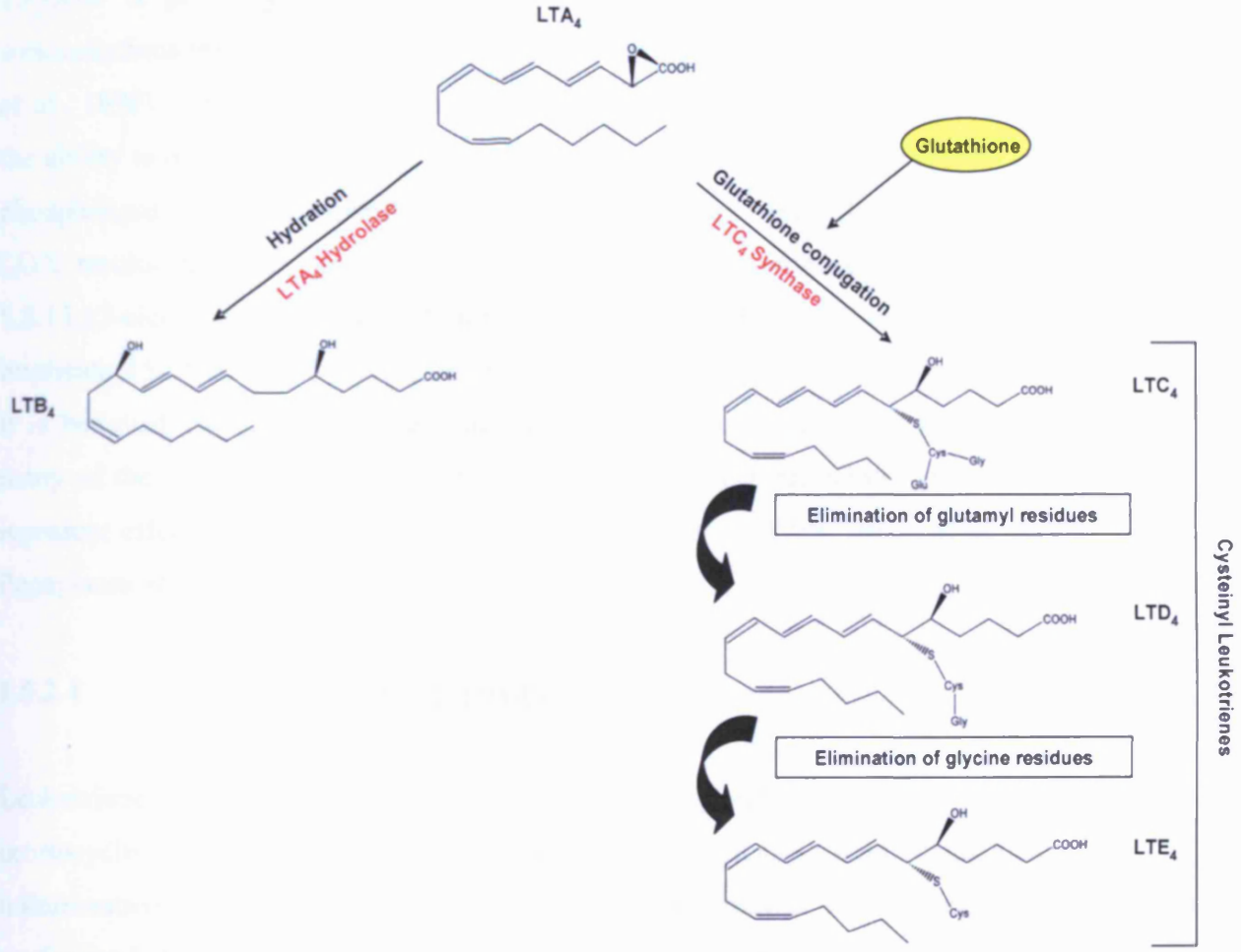


As mentioned, leukotrienes and lipoxins are the end products of LOX metabolism. However, they are produced by different isomers of LOX enzymes. The three main isoforms are: 5-LOX, 12-LOX and 15-LOX. In mammals, the LOXs are named in terms of their positional specificity of AA oxygenation (i.e. the carbon number which they oxygenate) (Ivanov *et al.*, 2005). Despite the substrate selectivity of the LOX enzymes (the PUFAs must possess a minimum of two *cis* double bonds interrupted by a methylene group) other PUFAs can act as substrates in LOX metabolism, for example, EPA (Gurr *et al.*, 2002). Also, the 15-LOX (see section 1.5.2.3) may use both free and esterified PUFAs, and perhaps, even more complex lipid assemblies such as lipoproteins (Brinckmann *et al.*, 1998; Calder, 2002).

1.5.2.1 5-Lipoxygenase

5-LOX is a non-heme iron dioxygenase located in either the nucleus or cytosol depending on the type of cell. The initiation of 5-LOX metabolism of AA begins with changes in cytosolic calcium in response to stimulation of the cell (e.g. trauma, burns). This mobilises phospholipase A_2 which cleaves phospholipid PUFAs from their *sn*-2 position. The free AA is then metabolised to produce the leukotrienes (Flamand *et al.*, 2006). When AA is metabolised by 5-LOX another important molecule, 5-LOX activating protein (FLAP), is necessary for the generation of the common precursor of all leukotrienes, LTA_4. FLAP is an 18 kDa protein bound to the nuclear membrane and plays an important role in the cellular regulation of 5-LOX-activity (it appears unique to the 5-LOX isoform) (Miller *et al.*, 1990). Its importance is derived from the fact that it acts in 'presenting' the activated AA to 5-LOX for metabolism (Dixon *et al.*, 1990; Wenzel, 2003; Yokomizo *et al.*, 2001). The early precursor of all leukotrienes, LTA_4, is an unstable allylic epoxide (a cyclic ether with only three ring atoms) and it undergoes rapid transformation (hydrolysis, conjugation to gluthatione or transcellular metabolism to generate bioactive eicosanoids) to yield the other leukotrienes. For example, LTC_4 is produced by gluthatione conjugation of LTA_4 whereas hydrolysis of LTA_4 produces LTB_4 (Flamand *et al.*, 2006). Fig. 1.12 outlines conversion of LTA_4 into the functional leukotrienes.

Fig. 1.12: Conversion of leukotriene A_4 into leukotriene B_4 and the cysteinyl leukotrienes C_4, D_4 and E_4.



1.5.2.2 12-Lipoxygenase

12-LOX oxygenates AA at carbon-12. There are two major locations of this isoform, 'platelet 12-LOX' and 'leukocyte 12-LOX'. The most notable difference between these two isoforms is due to their substrate selectivity – platelet 12-LOX can only metabolise AA to produce 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid (12-HPETE) whereas leukocyte 12-LOX can generate both 12- and 15-HPETEs from a variety of PUFAs (Yoshimoto and Yamamoto, 1995).

1.5.2.3 15-Lipoxygenase

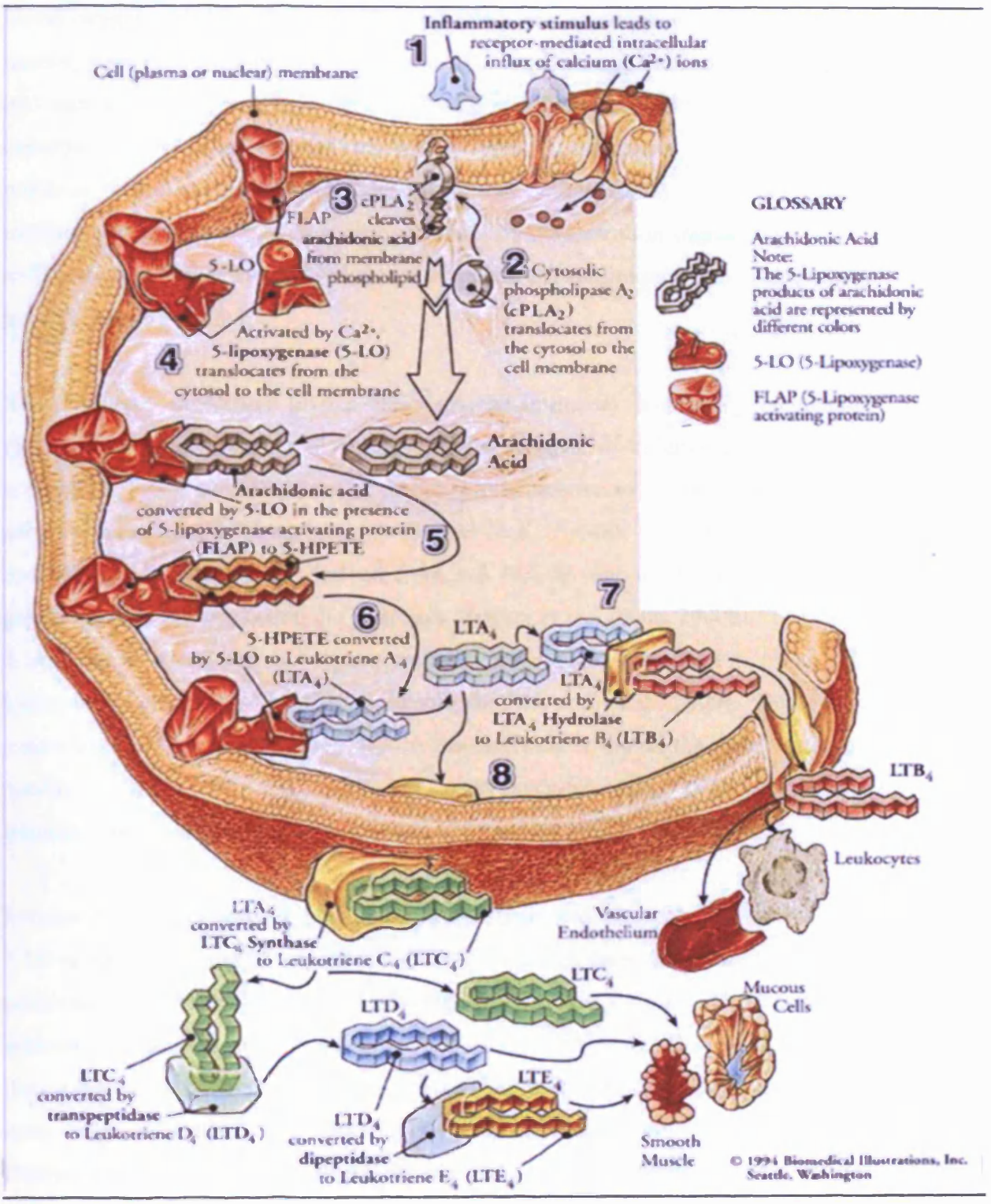
15-LOX is primarily found in the cytosol but increases in cytosolic calcium concentrations results in binding of 15-LOX to intracellular membranes (Brinckmann *et al.*, 1998). This isoform was initally identified in the rabbit reticulocytes and has the ability to oxygenate esterified polyenoic PUFAs whilst they are still present in the phospholipids of the cell membrane (Kuhn *et al.*, 2005). Metabolism of AA by 15-LOX results in the oxygenation of AA at carbon-15 to produce 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid (Gurr *et al.*, 2002). Several lines of evidence implicate 15-LOX in cell development and differentiation (Kuhn and Thiele, 1999). It is believed that actions of the generated lipoxins (see section 1.5.2.4) antagonize many of the responses of leukotrienes (and possibly other mediators) and that they represent effective inhibitors of inflammation (Badr *et al.*, 1989; Brady *et al.*, 1990; Papayianni *et al.*, 1996).

1.5.2.4 Leukotrienes and Lipoxins

Leukotrienes and lipoxins, together with prostaglandins, thromboxanes and prostacyclins, are major constituents of the biologically-potent mediators of inflammation, the eicosanoids. Leukotrienes are a powerful class of eicosanoids synthesised from PUFAs by various cells including mast cells, basophils and macrophages. The leukotrienes play a major role in inflammation, for example the cysteinyl-containing leukotrienes (LTC_4, LTD_4 and LTE_4) produced by glutathione conjugation from LTA_4 are potent bronchoconstrictors in the lung, are known to increase the permeability of post-capillary venules and stimulate the secretion of mucus (Samuelsson *et al.*, 1987) (Fig. 1.13). LTB_4 is regarded as being one of the most potent chemoattractants and activators of leukocytes and plays a major role in inflammatory diseases. This calcium ionophore induces adhesion as well as chemotactic movement of leukocytes. It is also known to stimulate aggregation and the release and generation of superoxide neutrophils (Dahlen *et al.*, 1981; Hoover *et al.*, 1984; Sha'afi *et al.*, 1981).

Lipoxins are any group of eicosanoids containing a conjugated tetraene structure and three hydroxyl groups. Also produced from PUFAs, lipoxins are short-lived endogenously-produced eicosanoids involved in signalling the resolution of inflammation following stimulation by IL-4 (Bonnans *et al.*, 2002). Lipoxins are functionally antagonistic to the actions of leukocytes. They control the entry of neutrophils to inflammatory sites (leukocytes promote neutrophils recruitment), reduce vascular permeability and stimulate macrophages to uptake apoptotic neutrophils. They exert potent chemoattractant effects on monocytes which play a significant role in wound healing (Schwab and Serhan, 2006).

Fig. 1.13: Leukotriene (LT) formation in inflammatory cells and their sites of action (Henderson, 1994).



1.5.3 Novel n-3 PUFA Derived Inflammatory Mediators – Resolvins and Protectins

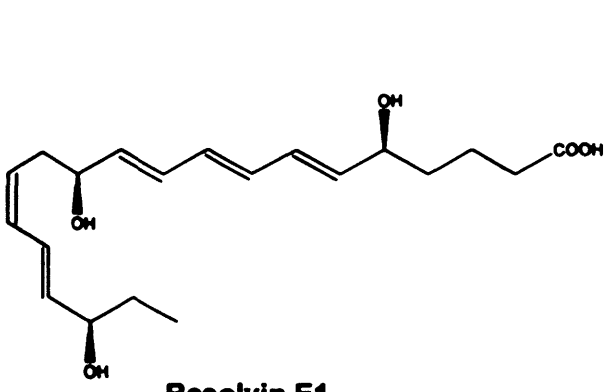
Novel oxygenated derivatives of EPA and DHA, the resolvins and protectins, have recently been identified as inflammatory mediators possessing both anti-inflammatory and immunoregulatory effects (Serhan, 2005b). Mediators derived from EPA are classified as the E-series resolvins whilst those derived from DHA are termed resolvins or protectins of the D-series (Ariel and Serhan, 2007) (Fig. 1.14). These mediators were discovered in a murine model of inflammation (dorsal skin pouch) and it was shown that they played an active role in the resolution of the inflammatory process (Serhan, 2005a).

Resolvins and protectins invoke their anti-inflammatory and immunoregulatory functions at the pico/nano molar range via the removal of inflammatory cells (e.g. neutrophils) and neutralisation of some pro-inflammatory prostanoids, thereby, allowing restoration of tissue integrity (Serhan *et al.*, 2004a). As mentioned, both the resolvins and protectins are derived from n-3 PUFAs and the presence of aspirin greatly facilitates these metabolic pathways (Serhan *et al.*, 2000a, 2000b). The COX-2 enzyme is acetylated by aspirin and this allows the introduction of an 18*R* hydroperoxy-group into the EPA molecule (Serhan and Clish, 2000). Subsequent metabolism of the EPA-derived 18*R*-hydroperoxy-EPA yields the resolvins, most notably resolvin E1 (5*S*, 12*R*, 18*R*-trihydroxy-6*Z*, 8*E*, 10*E*, 14*Z*, 16*E*-eicosapentaenoic acid) (Fig. 1.14) (Serhan and Chiang, 2007).

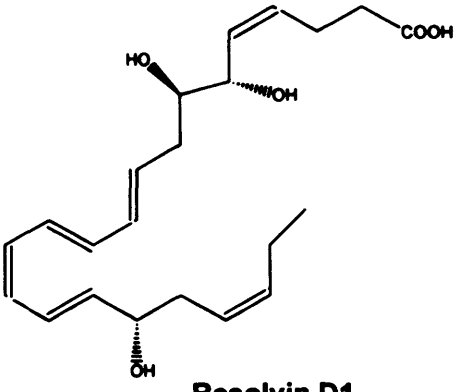
Formation of DHA-derived 17*R* and 17*S* resolvins and protectins (D-series) (Fig. 1.14) is also facilitated by aspirin via COX-2, although these mediators can also be produced in the absence of aspirin, although in that case, they are stereochemically different. Neuroprotectins were initially discovered in neural tissues of the brain (hence the name) but further research proved that their production is not localised to brain tissue. Therefore, they are simply referred to as protectins (Serhan, 2005a). Protectins are generated via LOX metabolism and contain conjugated triene structures (three conjugated double bonds) (Serhan *et al.*, 2004b). Various protective and anti-inflammatory properties have also been reported for these compounds, for example it has been confirmed that neuroprotectin D1 protects retinal pigment epithelial (RPE)

cells from oxidative stress-induced apoptosis and, possibly, offers protection to neurons. Therefore, this lipid mediator may indirectly contribute to photoreceptor cell survival as well (Serhan *et al.*, 2004a, 2004b). By this mechanism, neuroprotectin D1 may play an important role in age-related macular degeneration (AMD), a leading cause of blindness in the developed world. It has been suggested that, given the role of n-3 PUFAs in the generation of resolvins and protectins, dietary supplementation with EPA and DHA in conjunction with aspirin may reduce inflammation associated with many chronic diseases including CVD, cancer and arthritis.

Fig. 1.14: Structural representation of EPA-derived Resolvin E1 and DHA-derived Resolvin D1.



Resolvin E1



Resolvin D1

1.5.4 Inflammatory Gene Expression and n-3 Polyunsaturated Fatty Acids

The collection of epidemiological, experimental and clinical studies demonstrating a protective role of long chain n-3 PUFAs on inflammatory gene expression is currently expanding, with particular attention being paid to the mechanisms as to how n-3 PUFAs exert their effects on gene expression (Deglon *et al.*, 1995; Ntambi and Bene, 2001; Sampath and Ntambi, 2005; Tebbey and Buttke, 1993). It has been demonstrated that diets enriched with n-6 PUFAs stimulate the growth of different cell types and promote metastasis whereas the n-3 PUFAs do not demonstrate such effects but, on the contrary, promote curative effects in inflammatory/autoimmune diseases (Denys *et al.*, 2005).

Zhao *et al.* (2004) reported decreased expression of TNF-α in a human monocytic cell line when the cells were pre-incubated with EPA followed by stimulation with bacterial lipopolysaccharide (LPS). This suppression was also demonstrated when cells were pre-treated with DHA. Addition of the n-6 PUFAs, AA and LA or of the monounsaturated acid, oleate, resulted in a decrease in TNF-α expression but to a much lesser extent. This study concluded that a significant down-regulation of TNF-α production was specific for n-3 PUFAs (Zhao *et al.*, 2004). A study by de Caterina *et al.* (1994) showed that on increasing the cellular levels of DHA there was a reduction in cytokine-induced expression of endothelial leukocyte adhesion molecules, secretion of inflammatory mediators and leukocyte adhesion to cultured endothelial cells. DHA also decreased cytokine-stimulated endothelial cell expression of E-selectin and intercellular adhesion molecule-1 expression of constitutive surface molecules. This effect was shown to be eicosanoid-independent as inhibition of COX did not quench this effect indicating that the effect may be based on gene expression (de Caterina *et al.*, 1994). Another study reported that fish oil supplementation of splenocytes resulted in partial suppression of virus-induced overproduction of serum immunoglobulins (IgG and IgM). The same study also reported that LPS-stimulated production of LTB_4, TNF-α and IL-1β were all suppressed with fish oil supplementation. When compared to maize oil, fish oil also resulted in partial suppression of activation of NFκB (Xi *et al.*, 2001). In a study where the anti-inflammatory action of dietary fish oil and calorie restriction was tested in mice the authors determined that dietary fish oil (together with a 40% reduction in calorie restriction) effectively suppressed age-related inflammatory processes by modulating pro-inflammatory COX-2, iNOS gene expression and other related inflammatory genes (Kim *et al.*, 2006).

1.5.4.1 Mechanisms of the Effects of n-3 Polyunsaturated Fatty Acids on Gene Expression

The anti-inflammatory effects of eicosanoids are well documented. However, some of the effects exerted by n-3 PUFAs on gene expression are thought to be eicosanoid-independent. It is thought that these effects on gene expression are due to n-3 PUFA direct effects on intracellular signalling pathways which in turn lead to the activation of transcription factor(s) (Fig. 1.15). The most well documented transcription factors studied with relation to n-3 PUFAs and gene expression are NFκB and peroxisome proliferator-activated receptors (PPAR) which are discussed below. However, these are not the sole transcription factors involved. Hepatic nuclear factor-4α (HNF-4α), sterol regulatory element binding protein-1c (SREBP-1c), liver X receptors (LXRs) (α and β) and retinol X receptor (RXR) are also documented to be involved in the effects of n-3 PUFAs and gene expression (Jump, 2002).

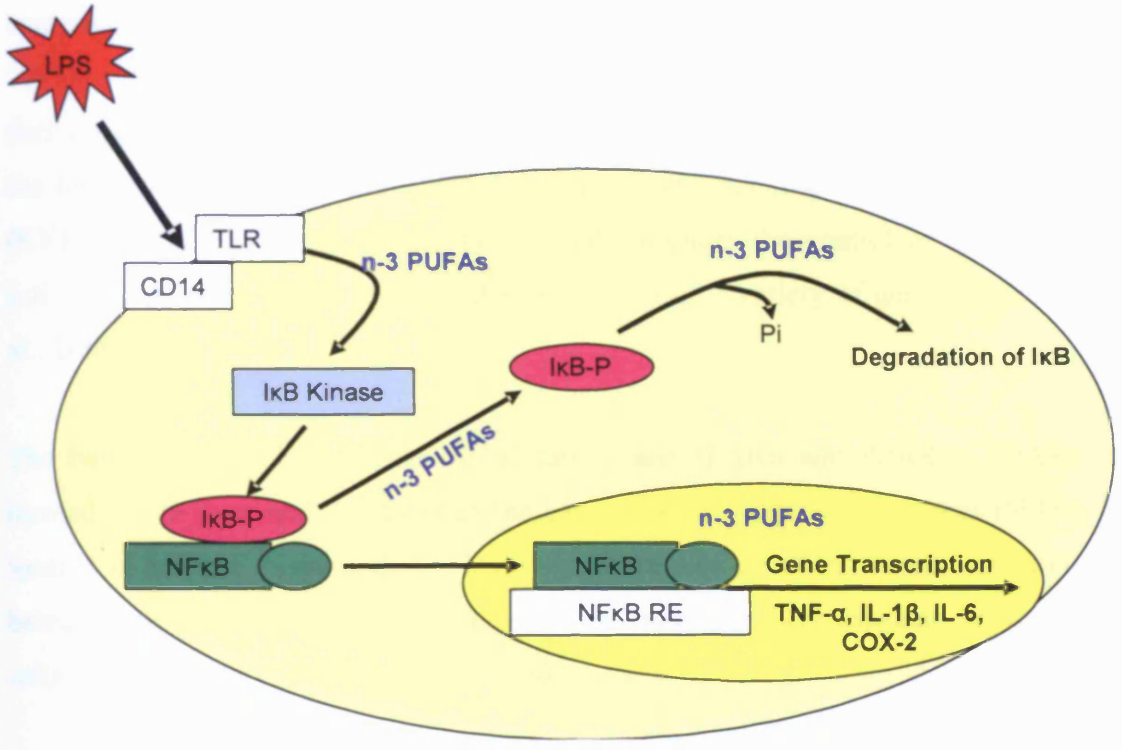
1.5.4.2 Nuclear Factor Kappa-B

Nuclear factor kappa-B (NFκB) is an inactive heterotrimer located in the cytosol of most, if not all cells. One of the three subunits is an inhibitory subunit (IκB). On stimulation of NFκB by a range of inflammatory stimuli (e.g. LPS), a signalling cascade is set in motion activating a protein complex termed IκB kinase. Activated IκB kinase results in phosphorylation of the IκB subunit which dissociates resulting in the active heterodimer of NFκB. The activated heterodimer moves to the nucleus where it binds to NFκB response elements and initiates the transcription of various inflammatory genes (Christman *et al.*, 1998). The phosphorylated IκB subunit then undergoes polyubiquitination, resulting in IκB degradation.

NFκB is thought to be one of the links between n-3 PUFA and inflammatory gene expression. Several studies have documented the ability of n-3 PUFAs to down-regulate the activity of NFκB. One study reported that feeding mice a fish oil-enriched diet resulted in a lower level of NFκB in the nucleus of LPS stimulated lymphocytes compared to mice fed a maize oil-enriched diet (Xi *et al.*, 2001). This study also demonstrated that fish-oil fed mice had lower levels of NFκB in the nucleus than the mice fed maize oil when the mice were infected with murine AIDS.

Chen and Zhao (2001) incubated human monocytes with EPA. When the monocytes were stimulated by LPS the NFκB response was reduced (see Calder 2002). This response was associated with decreased phosphorylation of IκB kinase indicating that the EPA effect was elicited on the signalling process leading to the activation of IκB kinase. Another study showed an n-3 PUFA effect at a different step during NFκB activation. Pancreatic cells incubated with EPA followed by exposure to TNF-α resulted in up-regulated degradation of IκB. This effect may either be due to inhibition of phosphorylation of IκB, hence preventing degradation or, alternatively, inhibiting the degradation process itself (Ross, 1999). Fig. 1.15 outlines the mechanisms by which n-3 PUFAs may regulate inflammatory gene expression via NFκB.

Fig. 1.15: Nuclear factor kappa-B (NFκB) stimulation by lipopolysaccharide (LPS) and the possible sites where n-3 polyunsaturated fatty acids (PUFAs) may regulate inflammatory gene expression. TRL, toll-like receptor; CD14, cluster designation-14; TNF-α, tumour necrosis factor-α; IL-1β, interleukin-1β; IL-6, interleukin-6; COX-2, cyclooxygenase 2.



Increases in n-3 PUFA concentrations may results in less NFκB in the nucleus, increased phosphorylation of IκB and degradation of IκB and a reduction in IκB kinase activity thereby affecting inflammatory gene expression.

1.5.4.3 Peroxisome Proliferator Activated Receptors

Peroxisome proliferator activated receptors (PPARs) belong to the steroid hormone nuclear receptor superfamily of ligand-activated transcription factors. There are three isoforms of PPARs currently known, PPAR-α, PPAR-β/δ and PPAR-γ, each of which is encoded by a different gene (Daynes and Jones, 2002; Yaqoob, 2003). PPARs are key regulators of lipid and glucose homeostasis and are activated by several diverse compounds including PUFAs, eicosanoids and hypolipidaemic drugs (Kliewer *et al.*, 1997). PPARs are 'molecular sensors' of endogenous fatty acids and their derivatives. Following binding of a ligand PPAR activate transcription resulting in the formation of a heterodimer coupled to a co-activator (e.g. retinoic acid receptor (RXR)). The expression of variety of genes is under the control of these ligand-activated receptors which regulate the expression of a variety of genes (Lefebvre *et al.*, 2006).

The two main members of the PPAR family are PPARα and PPARγ. PPARα is located in the liver and functions in the catabolism of fatty acids whereas PPARγ is located in adipose tissue and it is involved in the storage of fatty acids. As well as being located in the liver and adipose tissue both members can also be found in inflammatory cells (Devchand *et al.*, 1996).

As mentioned previously, gene expression is regulated by PPAR dimerisation with the RXR and binding appears to be under regulation by PUFAs and eicosanoids (Kliewer *et al.*, 1997). Devchand *et al.*, (1996) reported that mice deficient in PPARα had a prolonged response to an inflammatory stimulus indicating that PPARα activation may have anti-inflammatory properties. Evidence of the ability of n-3 PUFAs in conjunction with PPARs to influence gene expression is growing in abundance. Several studies indicate that activators of PPARα and γ can inhibit the activation of a range of inflammatory genes such as TNF-α, iNOS, IL-1β, IL-6 and COX-2 (Jackson *et al.*, 1999; Jiang *et al.*, 1998; Ricote *et al.*, 1998).

Donnellan *et al.* (2000) demonstrated that feeding mice fish oil increased the expression of PPARα in the liver and PPARγ in adipose tissue. Expression of PPAR-inducible genes in these tissues were also increased. Therefore, there is a suggestion that n-3 PUFAs might act by increasing the level of these anti-inflammatory transcription factors in immune cells thus implicating n-3 PUFAs in regulating inflammatory gene expression via PPARs.

1.6 n-3 PUFAs AND OSTEOARTHRITIS

Clinical studies on dietary supplementation with the n-3 PUFAs, EPA and DHA, have demonstrated a reduction of many inflammatory symptoms involved in the pathogenesis of RA (Calder and Zurier, 2001, James *et al.*, 2003). In a population of RA patients consuming 2.8 mg of n-3 PUFAs/day Geusens *et al.* (1994) reported significant clinical benefits together with a reduction in anti-rheumatic medication. Nielsen *et al.* (1992) investigated the effect of dietary supplementation of n-3 PUFAs on six clinical parameters in patients of RA. They reported significant improvement in two out of the six parameters in the experimental group compared to the placebo group. In another study, Kremer *et al.* (1995), investigated whether supplementation of n-3 PUFA in a population of RA patients would result in a reduction of NSAID therapy and the biological efficacy of n-3 PUFAs in this population. Sixty-six patients took part in this double blind, placebo-controlled, prospective study. Patients were divided into two groups and consumed either a n-3 PUFA supplement or placebo whilst taking diclofenac (over-the-counter NSAID). The results indicated that the experimental group showed significant improvements in tender joint, morning stiffness and scored better on the physician's and patient's global arthritis activity scale. Also, the levels of IL-1β were reduced in the n-3 PUFA supplemented group (Espersen *et al.*, 1992). Some, though not all, patients of the supplementted group were also able to discontinue NSAID therapy. As well as these studies, it has been documented that EPA and DHA compete with AA for incorporation into the phospholipids of cell membranes (Yaqoob *et al.*, 2000) which, as discussed previously, (section 1.5.1, 1.5.2), has advantageous effects in terms of the inflammatory response associated with arthritic conditions (Calder, 2001; Sperling, 1991).

Eicosanoids derived from AA are of the 2-series PGs and TXs and the 4-series LTs and hydroxy fatty acids and are considered to be pro-inflammatory. On the other hand, metabolism of EPA by the COX and LOX enzymes systems gives rise to a different series of eicosanoids, the 3-series PGs and TXs and the 5-series of LTs and hydroxy fatty acids. The functional significance of this is that the mediators formed from EPA are believed to be less potent and less-inflammatory (Calder, 2005; van der Tempel *et al.*, 1990). Furthermore, as discussed earlier (section 1.5.1, 1.5.2) AA and

EPA will compete with each other for COX, LOX and other relevant enzymes. Supplementation with n-3 PUFAs has been effective in changing the balance of eicosanoids produced from pro-inflammatory to less-inflammatory (Calder, 2001; Calder and Zurier, 2001; Sperling, 1991).

As previously discussed (section 1.5.1.1), increasing dietary intakes of n-3 PUFAs gives rise to competition between the n-3 and n-6 PUFAs for enzymes involved in the conversion of 18 carbon fatty acids to AA and EPA. As outlined earlier, (section 1.5.1, 1.5.2) dietary supplementation of n-3 PUFAs is thought to affect three significant pathways involved in the synthesis of lipid mediators known to play a role in the inflammatory response, the COX pathway, the 5-LOX pathway and the synthesis of platelet activating factor (PAF) (James *et al.*, 2000; Sperling, 1991). PAF is known to be a potent platelet aggregator and leukocyte activator and strongly promotes the metabolism of AA. PAF is not a lipid signalling molecule investigated in the course of this work and, hence, will not be discussed further.

Another benefit of dietary supplementation of n-3 PUFAs in terms of OA is the suppressive effects exerted on inflammatory cytokines (Babcock *et al.*, 2002; Endres *et al.*, 1989; Priante *et al.*, 2002; Sundrarjun *et al.*, 2004). TNF-α, IL-1 and IL-6 are the most important cytokines produced by cells of the immune system (monocytes, macrophages and neutrophils) (Wood, 2006). Appropriate amounts of TNF-α, IL-1 and IL-6 are beneficial in combating infection but a disruption in their metabolism that yields unnecessary and inappropriate levels are implicated in the pathological responses that occur in OA (Simopoulos, 2002). Supplementation with n-3 PUFAs has demonstrated suppressive effects on these cytokines, whereas no effect was observed with other classes of fatty acids (Endres *et al.*, 1989; Calder and Zurier, 2001; Priante *et al.*, 2002).

In terms of the mechanistic actions of n-3 PUFAs, evidence demonstrates that n-3 PUFAs may exert effects on inflammatory gene expression through direct actions on the intracellular signalling pathways that lead to activation of one or more transcription factors such as nuclear factor kappa B (NF-κB). Previous studies have shown that n-3 PUFAs can down-regulate the activity of the nuclear transcription factor NF-κB suggesting a direct effect of long-chain n-3 PUFAs on inflammatory

gene expression through the inhibition of NF-κB (section 1.5.4.2, Fig. 1.11) (Lo *et al.*, 1999; Novak *et al.*, 2003).

Therefore, there are numerous mechanisms in which n-3 PUFA supplementation may exert protective effects against inflammation such as that seen with OA. As rich sources of n-3 PUFAs (e.g. cod liver) are in decline, the importance of identifying new, viable and long-term sources of n-3 PUFAs is underscored. This would allow continued use of n-3 PUFAs in the dietary treatement of inflammatory diseases such as OA whilst also alleviating environmental pressure on the sources of n-3 PUFAs.

1.7 SOURCES OF n-3 PUFAs – THE ROLE OF AQUACULTURE

Although ALA is present in plants, the conversion of ALA to the longer chain PUFAs, EPA and DHA, is inefficient. Therefore, EPA and DHA are often recommended in the diet (section 1.4). The main dietary sources of such very long chain n-3 PUFAs include fish and fish oils (Abrami *et al.*, 1992). Currently the recommended intake for EPA and DHA in the UK are 450 mg EPA and DHA/day (Givens and Gibbs, 2008). However, many studies investigating the anti-inflammatory properties of n-3 PUFAs use between 3 - 6 g EPA and DHA per day and therefore, this amount is often recommended for anti-inflammatory benefits (Rayman and Callaghan, 2006). Despite this, these requirements are not often met by dietary consumption of fish, and fish oil capsules are often taken as an adjunct to the diet (Rayman and Callaghan, 2006).

However, despite the beneficial effects of fish and fish oil consumption, over the past two decades the marine fishing industry has come under extensive pressure because of declining fish stocks caused by over-fishing, mis-management of stocks, inaccurate data on time-scales for stock replenishment and government policies (FAO Department of Fisheries, 2004; Myers and Worm, 2003; Rice *et al.*, 2003; Zheng *et al.*, 2004). Because of the problems faced by marine fisheries there has been a subsequent increase in the aquaculture industry (FAO Department of Fisheries, 2004; Naylor *et al.*, 2000). A report by the Food and Agriculture Organisation estimated that global production of farmed-fish more than doubled in the last 15 years (Food and Agricultural Organisation, 1999). As the world population continues to expand and, due to the growing problems encountered by the traditional marine fishing industry, the reliance on aquaculture is likely to expand. Aquaculture involves the cultivation of aquatic populations under controlled seawater or fresh water conditions and is a tightly regulated industry with growing economic value (SEPA, 2003).

As outlined above, the main source of n-3 PUFAs is fish and fish oils and, needless to say, the aquaculture industry supplies fish for dietary consumption. However, the waste materials (e.g. viscera, skin, bones) are commonly disposed of and disposal systems are strictly monitored (www.fao.org). Fish waste was extensively used in fish feed products but, due to the bovine spongiform encephalopathy (BSE) crisis, a

review of disposal regulations restricted the used of fish waste in numerous products (www.defra.gov.uk). Nowadays, the aquaculture industry has to adhere to strict regulations regarding waste disposal. A common method of disposal is incineration (DEFRA, 2003).

Disposal of fish waste is seen as a major expense (especially for smaller farms) and, in some instances, disposal regulations are not adhered to, resulting in inappropriate disposal of waste products which in turn can have environmental consequences (e.g. pollution of rivers). However, more recently there have been initiatives to utilise waste products for several different end products. Work by the Portland Shellfish Inc. is one such example. Circa 1999 approximately 3.9 million pounds of crab waste was generated annually. This waste was formerly added to soil fertilizers but a change in regulations outlawed this practice. Faced with growing costs for correct waste disposal, the Portland Shellfish Inc., invested in research to convert the waste products into a marketable gardening product via a composting procedure (www.epa.gov/jtr/docs/me/me.htm). Despite preliminary difficulties, an appropriate system for composting was established and resulted in the development of a highly marketable product of economic value. There are numerous other examples of waste products being transformed into other viable products, such as in the whey industry.

However, despite success stories such as Portland Shellfish Inc., the aquaculture industry commonly disposes of waste products. For the UK alone, the Fish Foundation reports approximately 40,000 tonnes of salmon waste annually. This waste contains approximately 30% fat and has a potential market value of £5 million. The fat content of trout waste is reported to be even greater, approximately 50%. Therefore, finding an effective method to transform farmed fish waste into a viable product would not only reduce disposal costs and environmental implications but also result in the generation of revenue. Due to the abundance of scientific evidence reporting the health benefits of n-3 PUFAs, fish oil capsules are commonly taken by today's populations. Therefore, an opportunity exists to determine whether farmed fish waste could be a plentiful source of n-3 PUFA for the nutraceutical industry.

AIMS AND OBJECTIVES:

The overall objective of this project was to assess the potential of offal from an example of a farmed, fresh-water fish species, *Oncorhynchus mykiss* (rainbow trout), as a potential source of n-3 PUFAs for nutraceutical development. The project was divided into three distinct phases as described below.

a) Determination of Lipid and Fatty Acid Composition of *Oncorhynchus mykiss* Tissues

Waste by-products from fish farms are currently disposed of at economic cost and this process is stringently regulated by government policies. We set out to determine if the n-3 PUFA content in farmed fish waste was adequate to allow the offal to be processed into products (e.g. dietary supplements) for human/animal consumption. We used *Oncorhynchus mykiss* offal from Crucorney Trout Farm, Abergavenny, to test for the levels of n-3 PUFAs. We set out to report qualitative and quantitative data of the complex acyl lipids and fatty acids, with specific interest in EPA and DHA, in tissues of *Oncorhynchus mykiss.*

b) Determination of the Effect of Storage Time and Extraction Method on Lipid Yields from Different Sources of *Oncorhynchus mykiss* Offal

Fish farm waste is generated on a daily basis and normally stored on-site for some time prior to treatment and disposal. As the waste is currently not processed, these storage conditions are generally not suitable for the storage of lipid-saturated tissues because they can result in the oxidation of the unsaturated compounds especially EPA and DHA. We conducted research to determine the effect of storage time on the qualitative and quantitative composition of fish lipids and PUFAs. Also, the effect of processing was determined by comparison of a laboratory-based extraction procedure with two adaptations of industrial-based procedures.

c) Testing of Trout-Derived Oil for Beneficial Effects using an *in vitro* Model of Osteoarthritis

As previously outlined, n-3 PUFAs have been identified as having beneficial effects for human health and can alleviate diseases such as arthritis. EPA and DHA have previously been suggested to elicit protective effects in patients suffering from rheumatoid arthritis and, more recently, for *in vitro* models of OA. We sought to determine if a trout oil preparation could induce protective effects on a model tissue culture system of OA. Parameters measured included the effect of trout-derived fatty acids in comparison to n-3 PUFAs on mRNA levels for degradative and inflammatory enzymes known to be involved in OA pathology and on connective tissue degradation.

CHAPTER 2

General Materials and Methods

2.0 Lipid Extraction

Lipids are present in tissues in numerous physical forms for example; the non-polar lipids (mainly triacylglycerols) tend to be found as part of large deposits in storage tissues whereas the more complex lipids tend to be constituents of membranes where they are closely associated with proteins. In order to efficiently extract lipids from tissues it is necessary to select solvents that will dissolve the lipids present while overcoming the interactions between the lipids and other cellular components. When correctly done lipid extraction separates cellular lipids from all other cellular constituents while preserving these lipids for further analysis.

Total lipid was extracted from all organs by the method of Garbus *et al.* (1963). This method is a modification of an earlier procedure of Bligh and Dyer (1959) and employs a two-phase extraction procedure using chloroform/methanol (2:1 by vol.) and a 2 M potassium chloride (KCl) salt solution in phosphate buffer. The high salt solution forces phospholipids into the organic phase of the extraction mixture and the phosphate buffer combines with calcium allowing the extraction of those lipids which are more resistant to extraction, such as the phosphoinositides, which are known to form calcium protein complexes reducing the extraction efficiency. Specific details (volumes etc) for lipid extraction from different tissues are outlined in sections 3.3, 3.4.

2.1 Lipid Analysis

2.1.1 Individual Lipid Class Separation by Thin Layer Chromatography

Thin-layer chromatography (TLC) is a well-established method used to separate complex lipid mixtures into their component lipids. It employs the use of a stationary phase which consists of an adsorbent material, silica gel being the most common. The sample to be analysed is applied to the adsorbent which is immobilised onto a glass support. The TLC plate is then placed into a chamber containing a particular solvent system. As the solvent system migrates on the TLC plate and passes through the sample the analytes partition between stationary and moving phases. When using silica-based TLC plates the separation of the individual components is dependent on numerous components such as the external polarity of the stationary and moving

phases. With regard to silica-based plates, the less polar the compound the greater the distance travelled by the individual components of the analyte. The distance travelled by the analyte is known as the R_f value which is the distance travelled by the components divided by the distance travelled by the solvent as measured from the origin. Comparison of R_f values is one method used in identifying the components. Although other adsorbents can be used, silica gel is the most common. A pore size of 60 μm was used to separate individual lipids.

Polar and non-polar lipids were separated by (TLC) on silica gel G 60 plates (Merck, Germany) which had previously been activated by heating them to 100 °C for 1 h. The water content is an important factor in TLC as it determines the polarity of the adsorbent. Therefore, in order to decrease the water content and 'activate' the plates, heating to 100 °C for 1 h was used prior to analysis of the mixture. Appropriate solvent systems for the separation of polar and non-polar lipids were used (section 2.1.2, 2.1.3, Table 2.1).

2.1.2 Polar Lipids

Initially, a two-dimensional solvent system was prepared to separate the polar lipids present in each extract. Solvent I consisted of the chloroform/methanol/water (65:25:4 by vol.) and solvent system II consisted of chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5 by vol.). The lipid extract was applied as a spot in one corner of the activated TLC plate which was then inserted into a TLC chamber which contained solvent system I. When the solvent approached the top of the TLC plate, the plate was removed and any residual solvent was allowed to evaporate under nitrogen before the plate was rotated 90° and inserted into another TLC chamber containing the second solvent system. When the solvent had run to completion the plate was removed. Any residual solvent was evaporated and the plates were dried. Plates were then stained with lipid-specific dyes for lipid identification (Kates, 1986) or with a non-destructive agent such as 4-amino-3-hydroxyl-1-naphthalenesulfonic acid (ANSA) (see section 2.1.4).

2.1.3 Non-Polar Lipids

A suitable non-polar two-dimensional solvent system was employed to separate the non-polar lipids of a sample. Solvent system I consisted of toluene/hexane/formic acid (140/60/1 by vol.) and the solvent system II consisted of hexane/diethyl ether/formic acid (60/40/1 by vol.). The same procedure used for the polar lipids was also employed here. Identification of lipids was done by co-chromatography using authentic non-polar standards (Sigma, UK) and by the use of specific colour reagents (Kates, 1986).

As outlined a two-dimensional system was used to identify all lipids present in samples. The advantage of two-dimensional TLC is that only small amount of lipid is required for identification purposes. However, once all major polar and non-polar lipids in each sample had been appropriately determined a one-dimensional TLC system was employed.

2.1.4 Identification of Lipids

Since lipids separated are usually colourless, specific stains were used to locate the lipid spots and also to identify individual lipids.

Ninhydrin reagent (0.25 g reagent grade ninhydrin in acetone (100ml), prepared fresh) reacts with lipids and phosphatides containing primary amino groups and, therefore, was used for the verification of phosphoethanolamine (PE), phosphatidylserine (PS) and their lyso derivatives. Where present, these lipids appeared as pink/mauve spots.

A second lipid stain, Dragendorff stain (Beiss, 1964; Wagner and Hoerhammer, 1961) (solution I: 2.7 g bismuth nitrate in 100 ml 20% acetic acid; solution II: 10 g KI in 25 ml water) was used. Before use 20 ml solution I was mixed with 5 ml solution II and diluted with 70 ml water). Dragendorff reagent is used to detect choline-containing lipids such as PC, lyso-PC and sphingomyelin. It identifies these lipids by binding to the quaternary ammonium groups present in their structures giving rise to a yellow/orange spot.

Lipid location was also revealed under UV light. Plates were sprayed with 0.05% (w/v) ANSA in methanol and, when examined under UV light, lipid location was visible. ANSA is a non-destructive reagent and was employed when further analysis of the lipids was required.

A method for charring the lipids present was also undertaken. Plates were removed from the TLC chamber, residual solvent was allowed to evaporate and plates were then sprayed with sulphuric acid (10%, by vol.) in methanol. The plate was placed on a heating block and as the temperature of the plate increased the lipids were charred by the acid and appeared as black spots on the plate. This further enabled the visualisation and identification of individual lipids.

In conjunction with the outlined specific colour reagents, known authentic standards (Sigma, UK; Nu-Check, USA), were used to confirm identification of lipids. The standards were run under identical chromatographic conditions as the sample and compared so that identification was achieved by co-chromatography. Once lipids had been identified, a non-destructive staining method such as iodine vapour or ANSA was used to locate lipids.

2.1.5 One Dimensional Separation – Polar and Non-Polar Lipids

One-dimensional TLC may be considered as efficient as common two-dimensional techniques. A major advantage of employing a one-dimensional system is that more lipid extract may be applied and sufficiently separated thus reducing the number of plates needed for lipid separation. It is also a faster technique. As with the two-dimensional system an appropriate solvent system needs to be used for efficient separation. The solvent systems used are outlined in Table 2.1 (Hamilton and Hamilton, 1992).

Table 2.1: One-dimensional solvent systems used for the routine separation of polar and non-polar lipid classes by thin-layer chromatography (TLC).

POLAR		NON-POLAR	
Solvents	**Volume (ml)**	**Solvents**	**Volume (ml)**
Methyl acetate	25	Hexane	80
Propan-2-ol	25	Diethyl ether	20
Chloroform	25	Acetic acid	2
Methanol	10		
0.25% Potassium chloride	9		

2.3 Preparation of Fatty Acid Methyl Esters

Before lipid samples can be analysed by gas liquid chromatography (GLC) it is necessary to convert them to volatile derivatives, for example methyl esters. This has the added benefit of improving GLC peak shape and resolution. Acid-catalysed esterification and transesterification involves esterifying and transesterifying non-esterified fatty acids and *O*-acyl lipids respectively by heating them with an excess of anhydrous methanol in conjunction with an acid catalyst.

$$RCOOR' + CH_3OH \xleftrightarrow{H^+} RCOOCH_3 + R'OH$$

$$RCOOH + CH_3OH \xleftrightarrow{H^+} RCOOCH_3 + H_2O$$

Choosing the correct derivatization of fatty acids depends on the type of analysis to be performed. For GLC analysis, acid-catalysed esterification and transesterification was chosen. An aliquot of total lipid extract or individual lipids (separated by TLC) was added to 2.5% H_2SO_4 in dry methanol/toluene 2:1 (v/v) (3 ml). A known volume of internal standard, C15:0 in toluene (1 μg/μl), was added. Samples were refluxed at 70 °C

for 2 h. Care was taken not to avoid any possible decomposition of PUFAs by avoiding super heating the samples or storing for inappropriate lengths of time.

To extract the methyl esters, 5% aqueous sodium chloride (3 ml) was added to the sample and the methyl esters were extracted with analytical grade petroleum ether (60 – 80°C fraction) (Fischer, UK) (3 ml x 2) using Pasteur pipettes to separate the layers. The extracted petroleum ether layer was washed with 2% aqueous potassium bicarbonate which was added to neutralise any residual acid that may be present in the sample. The extracted methyl esters were dried with anhydrous sodium sulphate and evaporated under nitrogen. Samples were re-dissolved in a known volume of high performance liquid chromatography HPLC grade hexane (Fisher Scientific, UK) and transferred to glass gas liquid chromatography injection vials (Chromacol Ltd., UK). Samples were injected into a Clarus 500 (Perkin Elmer, Connecticut) gas chromatograph (GC) fitted with a flame ionising detector (FID) (Perkin-Elmer, Connecticut). A polar capillary column (30 m x 0.25 mm) (Elite 225 Perkin Elmer, USA) was used for fatty acid analysis.

2.3 Gas Liquid Chromatography

GLC is an important technique used to analyse the fatty acid derivatives of lipids. The principle is based on vaporising the analyte which is carried by an inert gas known as the mobile phase. On reaching the oven the temperature is gradually increased in order to increase vapour pressures and allow analysis time to be reduced. As the analyte moves through the GLC it passes through the stationary phase, a long cylindrical column with an inert polymer bound to it. Capillary columns are most common today as they allow greater separation and better resolution in the resulting peaks. The different physical and chemical prosperities of the anlayte's components absorb onto the stationary phase to different degrees resulting in different elution times for each component. As the analyte leaves the column it is combusted and the resulting ions bombarding the detector are recorded giving rise to the resulting chromatograms. The signal recorded relates to the proportion of reduced carbon atoms in the flame meaning that the detector is sensitive to mass rather than concentration.

Fatty acid profiles were determined by gas liquid chromatography (GLC). The temperature programmed employed was: 170°C for 3 min, followed by heating at 220°C at 4°C/min, and finally 220°C for 15 min. The advantage of using a temperature programme means that the oven temperature is initially low allowing the elution of the more volatile components. The oven temperature is then gradually increased at a constant rate allowing an increase in vapour pressure and quicker analytical times as the elution of the less volatile components becomes faster with the increased vapour pressure. The carrier was nitrogen at a flow rate of 10 ml/min. A split ratio of 20:1 was employed. Using a split ratio has the advantage of ensuring rapid delivery of the sample into the column. The FAME were identified by comparing retention times (RT) with those obtained from a fish oil standard, menhaden oil, (Sigma, UK) as well as specific fatty acid standards (Nu-Chek Prep. Inc., USA).

CHAPTER 3

Lipid and Fatty Acid Composition of Farmed Fresh-Water Oncorhynchus mykiss

3.0 Introduction

Fish is considered an important global commodity and fish oils, containing PUFAs are considered valuable nutritional components. Since it was reported by Bang *et al.* (1971) and Bang and Dyerberg (1980) that a high intake of n-3 PUFAs in the Greenland Eskimo population reduced cardiovascular mortality, the scientific interest in n-3 PUFAs has developed exponentially. Currently there is an abundance of evidence supporting the theory that n-3 PUFAs can have protective roles a variety of disease states, including coronary artery disease, most notably sudden cardiac death (Leaf *et al.*, 2003; Metcalf *et al.*, 2007), cancer (Chang *et al.*, 1997; Chapkin *et al.*, 2007; Reddy *et al.*, 1991;), neurodevelopmental and neurological disorders (Green *et al.*, 2007; Schaefer *et al.*, 2006), RA (Calder and Zurier, 2001; Leeb *et al.*, 2006) and a host of other chronic inflammatory conditions (Calder and Grimble, 2002). The evidence to support the beneficial effects of n-3 PUFAs is so convincing that the American Heart Association (AHA) recommends at least two servings of oily fish per week in conjunction with foods rich in α-linolenic acid (Kris-Etherton *et al.*, 2002). Also, in the UK, current daily recommendations for adults are 450 mg/d EPA and DHA (Givens and Gibbs, 2008).

Much debate exists over the classification of EFAs (Cunnane, 2003). However, despite this ongoing debate, it is generally accepted that the 18 carbon n-6 and n-3 fatty acids, linoleic and α-linolenic acid, are considered to be EFAs. Both linoleic and α-linolenic can be converted to the longer chain PUFAs, AA, EPA and DHA by a series of elongation and desaturation steps that occur in the endoplasmic reticulum of the liver (Arterburn *et al.*, 2006). However, the rate of conversion from the 18 carbons to the long chain PUFAs is extremely limited in humans (Plourde and Cunnane, 2007) and, because of this, both EPA and DHA are often classified as EFAs also. Due to this they are incorporated into infant formulae to ensure sufficient levels for normal infant development (Crawford, 1993).

The main sources of EPA and DHA in the diet are fish and fish oils. Unlike mammals, fish cellular membranes contain low levels of AA but accumulate rather significant levels of EPA and DHA (Cejas *et al.*, 2004) and are, therefore, a particularly rich source of these long chain PUFAs. However, their quantities are dependent on several parameters including species and can be influenced by various environmental factors including the diet (Bandarra *et al.*, 2006; Gulliou *et al.*, 1995; Turchini *et al.*, 2006; Visentainer *et al.*, 2005), sex (Satue and Lopez, 1996), salinity of the environment, (Cordier *et al.*, 2002; Innis *et al.*, 1995), surrounding water temperature and season (Hazel, 1979; Luzia *et al.*, 2003; Senso *et al.*, 2007).

Another important factor which can affect EPA and DHA levels is whether the fish are wild or farm-reared (Abrami *et al.*, 1992; George and Bhopal, 1995). Presently, wild fish stocks are at historically low levels and it has been estimated that only 10% of big-fish (e.g. tuna, swordfish) stocks and large ground-fish (e.g. cod, halibut, skates) remain in the seas (Myers and Worm, 2003). The International Council for Exploration of the Sea (ICES) have, for some time, being advising governments to implement the closure of several fisheries in order to avoid complete eradication of certain fish species (www.ices.dk). The Canadian fishing industry is a prime example of how dire the situation has become. Over ten years ago several Canadian Atlantic cod populations had been reduced to such critical levels that a moratorium was implemented on the fishing industry (Myers and Worm, 2003). In the years since this implementation it appears the cod stocks together with the fishing industry have yet to recover (Schrank, 2005).

Thus wild fisheries have reached a critical cross-road and for several years the output from the traditional fishing industry has stagnated. In accordance with the problems faced by wild fisheries the aquaculture industry has risen dramatically in previous years (Naylor *et al.*, 2000). One example is that of farm-reared salmon. The output of farm-reared salmon now surpasses the landing of wild salmon (Hannesson, 2003) and it is estimated that the production of aquaculture has more than doubled in the last two decades (Food and Agricultural Organisation, 1999). However, the aquaculture industry is not without problems and one such problem encountered by the aquaculture industry is

the belief that farm-reared fish contained less n-3 PUFAs than their wild counter-parts. However, recent findings have found this perception to be inaccurate (Cahu *et al.*, 2004). In fact it has been reported that, in general, farmed fish have higher total lipid levels than wild fish and that on an equal weight basis farmed fish fillets provided greater levels of n-3 PUFAs (especially EPA and DHA) than wild fish fillets (Cahu *et al.* 2004). In agreement with this idea, it has also been documented that farm-raised salmon and trout contain similar levels of EPA and DHA compared to their wild-type counterparts (Kris-Etherton *et al.*, 2002). Moreover, it has been reported that farmed salmon contained more than twice the fat as their wild counter-part whereas farmed catfish were noted as having five times the fat levels as wild catfish (Nettleton and Exler, 1992). Differences between wild and captive fish may be due to dietary manipulation of captive fish which are fed nutrient controlled diets (often containing fish lipids) compared to the wild counterparts whose diet is predominantly habitat-dependent.

As with marine fisheries, aquaculture produces vast quantities of waste and stringent regulations exist on disposal and treatment of aquaculture generated waste (www.scotland.gov.uk/Publications/2005/03;www.defra.gov.uk). Treatment and disposal have large economical and environmental implications for the industry. Elementary investigation has shown farmed fish waste to be enriched with valuable n-3 PUFAs (Kolakowska *et al.*, 2006) and the logic of disposing of such a rich source of n-3 PUFAs needs to be revised. The objective of this study was to analyse the various tissues of farmed trout (*Oncorhynchus mykiss*) and components of fish waste and determine if the latter would be a suitable source of n-3 PUFAs.

Chapter 3: Materials & Methods

3.1 Collection and Dissection of Rainbow Trout *(Oncorhynchus mykiss)*

Rainbow trout *(Oncorhynchus mykiss)* fed preformed pellets were obtained from Crucorney trout farm, Wales. Fish were killed immediately, covered in ice to prevent any degradation and transported back to the laboratory for immediate dissection. On arrival at the laboratory the fish were weighed before immediately being dissected.

3.2 Removal of Organs for Analysis

Organs selected for analysis included: liver, muscle, heart, spleen, adipose tissue (visceral), brain and eyes. An incision was made behind the pectoral fin and along the full length of the underside of the body to the tail fin. Once removed from the fish all tissues were rinsed or perfused (heart and liver) with physiological saline (0.9%, w/v) and weighed.

3.3 Extraction of Trout Lipids

Trout lipids were extracted using the Garbus method (Garbus *et al.*, 1963) as previously described (section 2.0). Immediately after weighing, the tissues were homogenised in 3 ml chloroform/methanol (1:2, by vol.) using a mortar and pestle and transferred to 10 ml glass tubes. The mortar and pestle were rinsed with 2 ml chloroform/methanol (1:2, by vol.) and this was added to the sample. After allowing extraction at room temperature for at least 15 minutes, the homogenate was filtered through glass wool in order to remove any solid material. Chloroform (2 ml) was added to the extract, followed by 2 ml Garbus solution (2 M KCl in 0.5 M potassium phosphate buffer, pH 7.4) and 2 ml distilled water.

Samples were vortexed and centrifuged on a Baird & Tatlock Auto Bench Centrifuge Mark IV for 5 min at 1000 x g_{av} in order to separate the sample into two phases. The upper aqueous layer was removed and discarded using Pasteur pipettes. The lower lipid-containing layer was transferred to a clean 5 ml glass tube and dried under a stream of nitrogen. Lipids were dissolved in a known volume of chloroform and 0.05% (w/v) butylated hydroxytoluene was added. All samples were stored at -20°C under nitrogen.

3.4 Separation of Individual Lipids using Thin Layer Chromatography and Preparation of Methyl Esters for Gas Chromatographic Analysis

See section 2.1 – 2.3 for standard methods.

3.5 Silver Nitrate Thin Layer Chromatography

Silver nitrate TLC involves the incorporation of silver ions with stationary phase, usually the silica gel. This form of TLC is an effective tool to use when it is necessary to separate lipids according to the number of *cis*-double bonds present in the fatty acids. The purpose of employing silver nitrate-TLC was to aid the identification of particular fatty acids (20:1 and 22:1). When a lipid sample is applied to the silver ion-TLC impregnated plate, complexes between the silver ions and the double bonds of fatty acids are formed. Other parameters which influence the separation of fatty acids are whether the bonds are in the *cis* or *trans* conformation, the position of the double bonds in the aliphatic chain as well as the chain length (Christie, 2003). Lipids samples must be derivatised (e.g. converted to fatty acid methyl esters (FAMEs)) before silver ion TLC.

Silica gel 60 plates (Merck, Germany) were impregnated with silver nitrate by immersing the plates for 1 min into a solution of 4% silver nitrate in methanol/water (9/1, by vol). Plates were drained and dried for 2 min in dim light (to prevent the oxidation of the silver ions) in a well-ventilated fume hood. Plates were stored desiccated in the dark until required.

Two separate solvent systems were used to develop the plates. The first solvent system, hexane/diethyl ether (90:10, by vol.) was used to separate saturated, monounsaturated and di-unsaturated fatty acids. The second system consisting of toluene/ethyl acetate (90:10, by vol.) and was used for appropriate separation of PUFAs. To separate the fatty acids, an aliquot of FAMEs was applied to the pre-activated plate (100°C x 1 h) and the plate was placed in a TLC solvent chamber. When the solvent front had reached the top of the plate, plates were removed and dried in a stream of cold air. To reveal the location of the fatty acids, plates were sprayed with water and the positions of the separated fatty acid bands compared to standards.

To elute the fatty acids from the silica gel, hexane/diethyl ether (1:1, by vol.) (6 ml) was added to the scraped bands and vortexed. Aqueous 20% sodium chloride (1 ml) was added, the tube sealed and the mixture vortexed. It was not necessary to centrifuge the samples as the aqueous and lipid layers separated easily. The top organic layer was removed to a clean tube. The extraction of fatty acids was repeated twice more and the organic layers of each elution combined. Samples were taken to dryness under nitrogen and the lipid components were dissolved in a known volume of hexane before being analysed by GLC (sections 2.3, 2.4).

3.6 Percentage Fatty Acid Composition of Feed for *Oncorhynchus mykiss*

An abundance of evidence has shown that the fatty acid composition of the diet is reflected in the acyl make up of the fish especially when storage TAG is accumulated (Bell *et al.*, 1997; Miller *et al.*, 2007; Torstensen *et al.*, 2005). The aquaculture system used to produce the *Oncorhynchus mykiss* used specifically designed food pellets to fulfil the nutritional requirements of the fish. According to the manufacturer's details these pellets contained approximately 40% crude protein, 15% oil, 6% fibre and 6% ash (Benthan Trout Farming Limited, UK) (we assume the remainder 33% to be carbohydrate). Based on total acyl composition we determined the total lipid content of the feed to be 70 mg fatty acid/g feed. The percentage acyl distribution in the feed is illustrated overleaf (Table 3.1).

Individual fatty acids were identified by co-chromatography with known standards (Nu-Check Prep., USA). Some fatty acids were more difficult to identify (20:1 isomers and 22:1) and were first separated by silver nitrate TLC (section 3.5) to determine the degree of unsaturation. Following this co-chromatography was used. However, we were only able to identify one of the 20:1 isomers (n-9). The literature widely reports the presence of high quantities of 20:1 n-9 and minor levels of 20:1 n-11 in fish oils. However, we were unable to confirm the identity of the second 20:1 isomer as n-11 by co-chromatography or GC-MS although it is highly likely to be the n-11 isomer.

The principle saturated fatty acids included 16:0, 14:0 and 18:0. The monounsaturated fatty acids, by quanity, were 18:1 n-9 > 22:1 > 20:1 n-9 > 16:1 n-7 > 18:1 n-7 > 20:1. The literature states that dietary lipids are reflected in tissue compositions so we assumed that these fatty acids would be commonly found in the organs analysed. EPA was present at approximately 5% (3.6 mg EPA/g wet weight of feed) whilst DHA composed approximately 8% of the acyl composition (5.5 mg DHA/g wet weight of feed).

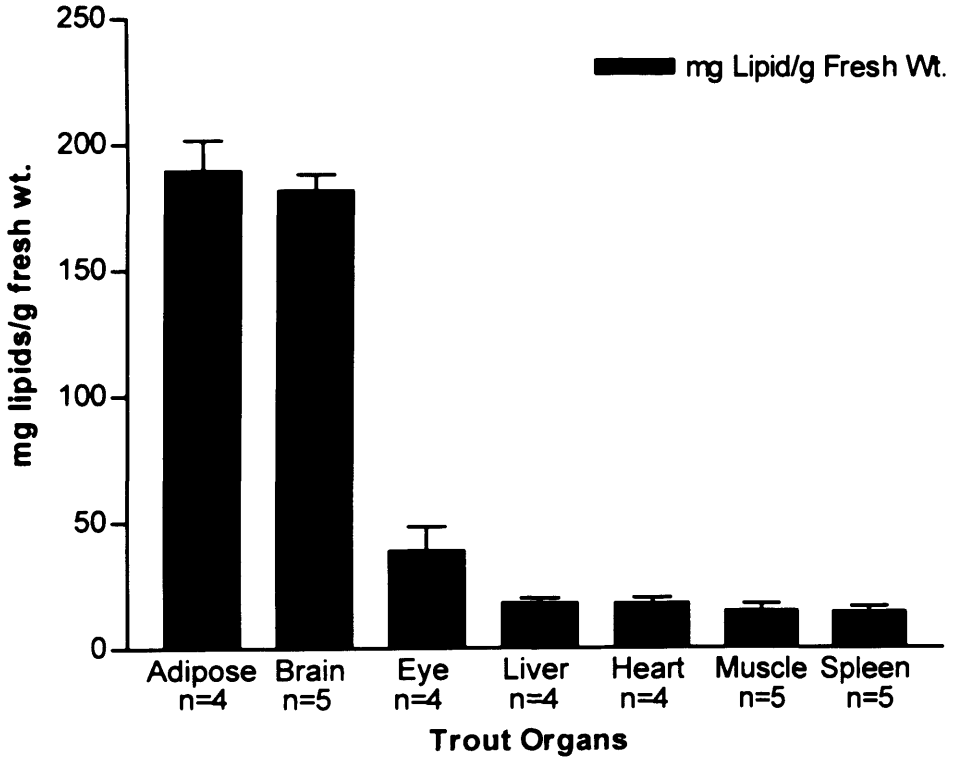
Table 3.1: Fatty acid analysis of food pellets used in the aquaculture of *Oncorhynchus mykiss*. Results as means ± SD. tr., trace (< 0.05).

Fatty Acid	Feed (n=3)
14:0	3.4 ± 0.1
16:0	12.9 ± 0.6
16:1 n-7	4.3 ± 0.1
16:2 n-4	0.2 ± tr.
16:3 n-4	0.1 ± tr.
18:0	2.5 ± 0.1
18:1 n-9	18.0 ± 0.9
18:1 n-7	3.2 ± 0.1
18:2 n-6	0.3 ± tr.
18:3 n-3	14.4 ± 1.0
18:3 n-4	1.5 ± 0.1
18:4 n-3	1.6 ± 0.1
20:1 n-9	8.7 ± 0.4
20:1	0.4 ± tr.
20:4 n-6	0.2 ± tr.
20:4 n-3	3.7 ± 0.2
20:5 n-3	**5.2 ± 0.2**
22:1	9.4 ± 0.5
22:2	0.3 ± tr.
22:5 n-3	1.8 ± 0.1
22:6 n-3	**7.9 ± 0.3**

3.7 Total Lipid Extracted from *Oncorhynchus mykiss* Tissues

The range of total lipid found in individual tissues of *Oncorhynchus mykiss* was from 14 mg/g fresh weight to 190 mg/g fresh weight (Fig. 3.1). As expected, the visceral adipose tissue yielded the highest levels of total lipid (190 mg/g fresh weight). Brain also contained comparable total levels (180 mg/g fresh weight). Total lipids from the eye showed significantly higher levels than the other organs (apart from adipose tissue and brain) with approximately 40 mg/g fresh weight. The liver, heart, muscle and spleen were found to contain rather similar levels of total lipid extract ranging from approximately 18 mg/g fresh weight for liver and heart to about 15 mg/g fresh weight for muscle and spleen.

Fig. 3.1: Total lipid extracted from individual tissues of *Oncorhynchus mykiss* (mg lipid/g fresh weight). Results as means ± SD. Adipose = visceral adipose tissue.



3.8 Individual Lipids Found in *Oncorhynchus mykiss* Tissues

3.8.1 Polar Lipids from *Oncorhynchus mykiss*

The principal polar lipids (for identification method see section 2.1.4) found in all tissues consisted of phosphatidylcholine (PC), phosphatidylethanolamine, (PE), phosphatidylinositol (PI) and phosphatidylserine (PS) with minor levels of sphingomyelin (SPH), diphosphatidylglycerol (DPG) and lyso-phosphatidylcholine (lyso-PC) also detected in some tissues (Table 3.2). PC was the main lipid found in all organs except adipose tissue where it constituted approximately 14% of the total polar lipids detected. In all other organs PC represented approximately 50% of the total polar lipids present. However, in the spleen PC accounted for only 30% of polar lipids. PE was presented in approximately equal distribution, 20%, across all organs analysed. In terms of percentage polar lipids, PI was present in the highest amounts in adipose tissue, 31%, whereas in all other organs examined PI accounted for approximately 11% of the total polar lipids. The spleen did show a slightly elevated level of PI, 2.2 mg/g fresh weight (18%). PS was present in all organs at approximately 8% although there were two exceptions to this rule. Both adipose tissue and spleen displayed almost four times the levels of PS found in the other organs, approximately 32%. In both the brain and adipose tissue SPH consisted of approximately 12% of the total polar lipids whereas in all other organs it composed approximately 2.5% of the polar lipids. The heart was seen to have the highest levels of DPG, consisting of 8% total polar lipids. This was consistent with the high mitochondrial content of heart and the localisation of DPG in the inner mitochondrial membrane. Brain and muscle contained approximately 6% and 7% respectively whilst DPG was found to compose 4% and 2.5% of liver and spleen polar lipids respectively. No DPG was detected in the eye or adipose tissue, two tissues with a very low mitochondrial content. The rather unusual polar lipid content of adipose tissue may have been due to its relative paucity of internal membranes. Lyso-PC was detected in the heart, eye, spleen and liver at the following levels 9%, 6%, 2.5% and 1.6% respectively. No lyso-PC was detected in the adipose tissue, brain or muscle.

Table 3.2: Polar acyl lipids detected in various tissues of *Oncorhynchus mykiss* (mg fatty acid/g fresh weight). Results as means ± SD n=4. Adipose = visceral adipose tissue. Percentages are given in parenthesis. n.d., none detected; tr. < 0.05.

	mg fatty acid/g fresh wt.							
Tissue	**PC**	**PE**	**PI**	**PS**	**SPH**	**DPG**	**Lyso-PC**	**Total**
Adipose	0.6 ± 0.3	0.5 ± 0.2	1.3 ± 0.2	1.3 ± 0.5	0.5 ± 0.1	n.d.	n.d.	4.2
	(14)	(12)	(31)	(31)	(12)			(100)
Brain	1.2 ± 0.3	0.5 ± 0.2	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.2 ± tr.	n.d.	2.8
	(43)	(17)	(11)	(11)	(11)	(7)		(100)
Eye	7.7 ± 2.4	3.3 ± 1.1	1.7 ± 0.5	1.3 ± 0.5	0.3 ± 0.1	n.d.	0.6 ± 0.2	14.9
	(52)	(22)	(11)	(9)	(2)		(4)	(100)
Heart	2.3 ± 0.4	1.2 ± 0.5	0.4 ± 0.1	0.3 ± 0.3	0.2 ± 0.1	0.4 ± 0.1	0.5 ± 0.2	5.3
	(43)	(23)	(8)	(6)	(3)	(8)	(9)	(100)
Liver	6.6 ± 1.4	2.7 ± 0.8	1.3 ± 04	0.7 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.2 ± tr.	12.3
	(54)	(22)	(11)	(5)	(2)	(4)	(2)	(100)
Muscle	1.5 ± 0.4	0.7 ± 0.3	0.4 ± 0.1	0.3 ± 0.1	0.1 ± tr.	0.2 ± 0.1	n.d.	3.2
	(47)	(22)	(13)	(9)	(3)	(6)		(100)
Spleen	3.5 ± 1.3	2.0 ± 0.2	2.2 ± tr.	4.0 ± 1.8	0.2 ± tr.	0.3 ± tr.	0.3 ± 0.1	12.5
	(28)	(16)	(18)	(32)	(1)	(2)	(2)	(100)

*PC, Phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; SPH, sphingomyelin; DPG, diphosphatidylglycerol and lyso-PC, lyso-phosphatidylcholine.

3.8.2 Non-Polar Lipids from *Oncorhynchus mykiss*

In terms of the non-polar lipids identified, as expected, TAG was the major lipid detected representing over 90% of the total non-polar lipids in most tissues (Table 3.3). The level of TAG in heart tissue was the only exception where approximately 75% of non-polar lipids consisted of TAG. The remaining non-polar lipids in heart consisted of 12% 1,2-diacylglycerol (1,2 DAG), 9% 1,3-diacylglycerol (1,3 DAG) and 9% non-esterified fatty acids (NEFAs).

Table 3.3: Non-polar acyl lipids detected in tissues of *Oncorhynchus mykiss* (mg fatty acid/g fresh weight). Results as means ± SD (n=5). Adipose = visceral adipose tissue. Percentages given in parenthesis. n.d., none detected; tr. < 0.05).

	mg fatty acid/g fresh wt.				
Tissue	**TAG**	**1,2 DAG**	**1,3 DAG**	**NEFA**	**Total**
Adipose	131.6 ± 33.7	4.1 ± 2.0	4.1 ± 2.1	n.d.	139.8
	(94)	(3)	(3)		(100)
Brain	122.4 ± 59.8	0.6 ± 0.2	0.5 ± 0.1	1.6 ± 0.7	125.1
	(98)	(0.5)	(0.4)	(1.3)	(100)
Eye	27.1 ± 10.0	0.1 ± tr.	0.2 ± 0.1	0.1 ± tr.	27.5
	(99)	(0.4)	(0.7)	(0.4)	(100)
Heart	2.5 ± 1.2	0.4 ± 0.2	0.3 ± 0.1	0.3 ± 0.1	3.3
	(76)	(12)	(9)	(9)	(100)
Liver	2.0 ± 0.3	0.1 ± tr.	0.1 ± tr.	n.d.	2.2
	(91)	(4.5)	(4.5)		(100)
Muscle	20.2 ± 8.0	0.1 ± tr.	0.1 ± tr.	0.2 ± 0.1	20.6
	(98)	(0.5)	(0.5)	(1.0)	(100)
Spleen	6.6 ± 1.4	0.1 ± tr.	0.2 ± tr.	0.2 ± tr.	7.1
	(93)	(1.4)	(2.8)	(2.8)	(100)

*TAG, Triacylglycerol; 1,2-DAG, 1,2 diacylglycerol; 1,3-DAG, 1.3 diacylglycerol; NEFAs, non-esterified fatty acids.

3.5 Acyl Distribution in Phosphatidylcholine from Individual Tissues of *Oncorhynchus mykiss*

Some notable differences in the fatty acid composition of PC were apparent when the lipid was isolated from individual tissues of *Oncorhynchus mykiss* (Table 3.4). Variable levels of the principle saturated fatty acid, palmitic acid (16:0) were observed. PC from brain, heart and muscle had the greatest level with approximately 40% 16:0. PC from spleen was seen to be composed of 35% palmitic acid whilst the eye and liver demonstrated approximately 28%. The lowest level of 16:0 was detected in adipose tissue. Elevated levels of palmitoleic acid (16:1 n-7) were detected in adipose tissue (6%). Lower levels were observed in the brain and eye (approximately 2.3%) while the liver, muscle and spleen demonstrated approximately 1.4% 16:1 n-7 in PC. Variable levels of stearic acid (18:0) were also found in different tissues. Adipose tissue, brain, eye and liver all contained approximately 8% 18:0. PC from heart and spleen contained half of this amount (4%) while muscle PC only contained approximately 2% 18:0. Oleic acid, 18:1 n-9, was the principle monounsaturated fatty acid in PC from all tissues except adipose (13%). Highest levels of 18:1 n-9 were found in the eye and spleen, 19% and 23% respectively. PC from the remaining tissues was composed of approximately 11% 18:1 n-9. The n-6 fatty acid, 18:2, was present in PC from all tissues at approximately 3% with the exception of the eye (1.7%). PC present in adipose differed significantly from the other tissues in terms of the principle monounsaturated fatty acids. The major monounsaturated fatty acids in adipose tissue consisted of eicosaenoic acid, 20:1 n-9; oleic acid, 18:1; docosaenoic acid, 22:1 and palmitoleic acid, 16:1 n-7. Both 20:1 n-9 and 22:1 were found at significantly elevated levels in PC from adipose tissue (15% and 8% respectively) compared to other tissues. PC from the remaining organs was composed of approximately 2.5% 20:1 although slightly elevated levels were detected in the liver, 5%. 22:1 was only found at approximately 1% in all tissues except liver where it constituted 2.4% of PC. AA (20:4 n-6) was present in particularly low levels in PC from brain, eye and liver (0.4%). In PC from muscle and spleen AA constituted approximately 1% while no AA was detected in PC from adipose tissue. EPA, (20:5 n-3) was also not detected in PC from adipose tissue but was observed at approximately 5% in heart, liver, muscle and spleen. However, in both the brain and eye PC contained only 1.5% EPA.

DHA, 22:6 n-3, was the second most abundant fatty acid detected in all tissues with the exception of adipose. DHA was highest in PC from liver and muscle (30%), whereas heart and eye contained approximately 25%. Lower levels were observed in the brain and spleen, 18% and 13% respectively whilst PC from adipose tissue was contained 8% DHA.

3.6 Acyl Distribution in Phosphatidylethanolamine from Individual Tissues of *Oncorhynchus mykiss*

Differences in the fatty acid profile of PE from individual tissues were also observed (Table 3.5). In contrast to PC, two principal saturated fatty acids in PE were observed. PE from brain contained the highest levels of 16:0 and 18:0 (22% and 18% respectively). Heart and muscle PE contained approximately 17% 16:0 while 18:0 was approximately 14% and 8% respectively. The eye, liver and spleen PE were composed of approximately 11% 16:0 and 9% 18:0. The principal monounsaturated fatty acid was 18:1 n-9 (approximately 10%) in PE from most tissues although 18:1 n-9 constituted approximately 21% of PE in the eye. Adipose tissue was an exception where the principal monounsaturated fatty acids were 20:1 n-9 and 22:1 (21% and 15% respectively). In all other tissues 20:1 n-9 and 22:1 constituted approximately 4% and 1-1.5% although slightly elevated levels of 20:1 n-9 were detected in PE from the liver and spleen.

In terms of the PUFAs, in general the principal PUFAs observed in PE in all tissues were DHA > 18:2 n-6 > EPA = AA. Organs with the greatest levels of AA in PE included the brain, eye and spleen (approximately 3%). Reduced levels were detected in heart and muscle (1.5% and 1.2%) and less than 0.5% AA was detected in PE from liver. Similar to PC no AA was detected in adipose tissue. EPA was similar to AA in that it too was not found in PE from adipose tissue. Approximately 3% EPA was observed in PE from eye, heart, muscle and spleen whereas liver PE contained 1.5% EPA. Minor levels of EPA were detected in the brain (1%). The highest level of DHA, 40%, was present in PE from muscle, followed by approximately 35% DHA in PE from the eye, heart, liver and spleen. About 10% DHA was detected in PE from both adipose tissue and brain.

Table 3.4: Fatty acid analysis of phosphatidylcholine from individual tissues of *Oncorhynchus mykiss*. Results as means of % fatty acids ±SD. tr, trace (< 0.05), n.d., none detected.

Fatty Acid	Adipose tissue n = 5	Brain n = 5	Eye n = 4	Heart n= 3	Liver n = 5	Muscle n=5	Spleen n = 5
14:0	0.6 ± 0.3	2.7 ± 0.5	1.5 ± 0.3	1.4 ± 0.1	1.6 ± 0.2	1.8 ± 0.6	2.1 ± 1.1
16:0	26.6 ± 7.7	39.5 ± 2.1	29.5 ± 1.0	40.4 ± 6.2	27.0 ± 4.3	35.9 ± 5.7	39.3 ± 4.8
16:1 n-7	5.9 ± 0.4	2.6 ± 0.5	2.1 ± 0.5	0.8 ± 0.3	1.5 ± 0.4	1.3 ± 0.2	1.4 ± 0.2
16:2 n-4	2.2 ± 1.7	0.5 ± 0.3	0.6 ± 0.9	0.1 ± tr.	0.4 ± tr.	0.2 ± tr.	0.2 ± tr.
16:3 n-4	2.0 ± 1.0	2.4 ± 0.5	0.6 ± 0.1	0.5 ± 0.1	0.4 ± tr.	0.6 ± tr.	0.4 ± 0.1
18:0	8.1 ± 1.9	8.3 ± 0.7	8.5 ± 0.5	4.4 ± 0.8	7.0 ± 2.0	2.2 ± 0.3	4.1 ± 0.4
18:1 n-9	13.0 ± 5.3	11.7 ± 2.4	18.9 ± 1.2	12.6 ± 1.9	11.5 ± 1.3	9.0 ± 1.4	22.5 ± 1.2
18:1 n-7	n.d.	1.6 ± 0.4	2.4 ± 0.2	1.5 ± tr.	3.1 ± 0.9	2.1 ± 0.2	3.0 ± 1.8
18:2 n-6	4.3 ± 0.8	2.3 ± 0.4	1.7 ± 1.0	2.2 ± 0.3	3.4 ± 0.7	3.2 ± 0.5	2.7 ± 0.5
18:3 n-3	n.d.	0.5 ± 0.2	0.1 ± tr.	0.2 ± tr.	0.1 ± 0.1	0.2 ± tr.	0.2 ± 0.1
18:3 n-4	1.1 ± 0.6	0.6 ± 0.2	0.2 ± tr.	0.4 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.1 ± 0.1
18:4 n-3	n.d.	0.1 ± tr.	0.2 ± tr.	0.2 ± 0.1	0.1 ± tr.	0.4 ± 0.1	0.1 ± tr.
20:1 n-9	15.2 ± 3.8	2.4 ± 0.4	2.0 ± 0.3	1.9 ± 0.4	5.2 ± 1.8	1.7 ± 0.2	2.4 ± 0.4
20:1	n.d.	1.4 ± 0.3	0.8 ± 0.2	n.d.	n.d.	n.d.	n.d.
20:4 n-6	n.d.	0.4 ± 0.1	0.3 ± 0.1	1.6 ± 0.2	0.4 ± 0.2	1.0 ± 0.2	1.1 ± 0.6
20:4 n-3	0.9 ± 0.4	0.5 ± 0.2	3.6 ± 0.2	0.5 ± 0.1	0.7 ± 0.3	0.7 ± 0.1	0.4 ± 0.2
20:5 n-3	**n.d.**	**2.2 ± 0.4**	**1.1 ± 0.5**	**5.6 ± 0.8**	**4.4 ± 2.2**	**6.5 ± 1.4**	**3.9 ± 1.1**
22:1	7.5 ± 1.3	1.3 ± 0.3	0.6 ± 0.1	0.3 ± 0.1	2.4 ± 1.1	0.7 ± 0.2	0.7 ± 0.2
22:2	5.7 ± 2.1	1.1 ± 0.2	0.8 ± 0.1	0.7 ± 0.3	0.4 ± 0.1	0.5 ± 0.1	1.0 ± 0.2
22:5 n-3	n.d.	n.d.	n.d.	0.7 ± 0.1	1.5 ± 0.4	1.0 ± 0.3	0.5 ± 0.2
22:6 n-3	**8.1 ± 2.7**	**18.1 ± 2.0**	**25.3 ± 1.4**	**23.9 ± 3.6**	**28.7 ± 3.2**	**30.5 ± 6.7**	**13.4 ± 4.0**

Table 3.5: Fatty acid analysis of phosphatidylethanolamine from individual tissues of *Oncorhynchus mykiss*. Results as means of % fatty acids ± SD. tr, trace (< 0.05), n.d., none detected.

Fatty Acid	Adipose tissue n = 5	Brain n = 5	Eye n = 4	Heart n = 3	Liver n = 5	Muscle n=5	Spleen n = 3
14:0	0.8 ± 0.2	2.6 ± 1.0	0.5 ± 0.2	0.5 ± 0.2	1.7 ± 0.2	0.4 ± 0.2	0.3 ± 0.1
16:0	12.8 ± 2.2	21.5 ± 2.9	9.9 ± 1.6	16.7 ± 2.9	11.4 ± 1.4	16.6 ± 3.7	10.4 ± 1.6
16:1 n-7	7.3 ± 2.1	5.4 ± 1.4	0.9 ± 0.4	1.3 ± 0.4	0.7 ± 0.3	1.5 ± 0.5	0.9 ± 0.3
16:2 n-4	3.2 ± 0.8	1.6 ± 0.5	0.3 ± tr.	0.3 ± 0.1	0.3 ± tr.	0.2 ± 0.2	0.3 ± 0.1
16:3 n-4	1.7 ± 0.5	3.5 ± 1.3	1.0 ± 0.2	1.2 ± 0.2	0.5 ± tr.	1.1 ± 0.2	1.0 ± 0.4
18:0	12.8 ± 2.2	17.7 ± 1.6	8.8 ± 1.1	13.7 ± 1.9	9.3 ± 2.8	7.9 ± 1.1	8.2 ± 0.6
18:1 n-9	9.3 ± 5.4	10.9 ± 5.1	20.7 ± 2.5	9.2 ± 1.0	11.8 ± 2.9	8.8 ± 1.2	12.4 ± 1.8
18:1 n-7	9.3 ± 3.5	2.7 ± 0.7	3.1 ± 1.7	4.7 ± 0.6	5.0 ± 0.9	2.7 ± 0.5	6.1 ± 0.9
18:2 n-6	4.9 ± 1.8	3.4 ± 0.3	3.6 ± 0.8	4.2 ± 0.5	4.7 ± 1.1	4.2 ± 0.6	5.4 ± 0.9
18:3 n-3	n.d.	1.8 ± 0.6	0.5 ± 0.2	0.6 ± 0.1	0.0 ± tr.	0.6 ± 0.1	0.8 ± 0.4
18:3 n-4	n.d.	1.4 ± 0.3	0.4 ± 0.1	0.6 ± 0.1	0.3 ± 0.1	1.4 ± 1.1	0.5 ± 0.4
18:4 n-3	n.d.	n.d.	0.2 ± tr.	0.4 ± 0.1	0.3 ± tr.	0.4 ± 0.2	0.4 ± 0.1
20:1 n-9	21.3 ± 6.8	4.0 ± 0.5	3.8 ± 0.7	4.6 ± 0.2	8.8 ± 0.6	3.6 ± 2.1	6.5 ± 0.9
20:1	n.d.	1.1 ± 0.3	n.d.	0.3 ± tr.	n.d.	n.d.	n.d.
20:4 n-6	n.d.	3.0 ± 0.6	2.4 ± 1.2	1.5 ± 0.3	0.4 ± 0.1	1.2 ± 0.2	3.3 ± 0.7
20:4 n-3	2.1 ± 0.7	1.8 ± 0.5	0.7 ± 0.1	0.8 ± 0.4	4.1 ± 1.9	1.2 ± 0.5	0.3 ± tr.
20:5 n-3	**n.d.**	**1.0 ± 0.4**	**3.6 ± 0.4**	**2.5 ± 0.3**	**1.6 ± 1.9**	**3.2 ± 0.4**	**3.2 ± 0.2**
22:1	14.5 ± 5.2	1.0 ± 0.3	0.8 ± 0.2	2.1 ± 0.4	3.3 ± 1.5	1.7 ± 0.8	1.6 ± 0.6
22:2	2.1 ± 0.4	1.4 ± 0.4	1.1 ± 0.6	2.2 ± 1.4	0.7 ± 0.1	0.7 ± 0.2	1.3 ± 0.3
22:5 n-3	n.d.	1.5 ± 0.7	1.7 ± 0.2	1.9 ± 0.6	0.9 ± 0.2	1.7 ± 0.5	0.9 ± 0.2
22:6 n-3	**9.9 ± 4.8**	**12.7 ± 3.0**	**36.3 ± 2.2**	**32.5 ± 2.7**	**35.2 ± 2.2**	**40.6 ± 6.8**	**35.7 ± 3.9**

3.7 Acyl Distribution in Phosphatidylinositol from Individual Tissues of *Oncorhynchus mykiss*

In terms of the saturated fatty acids detected in PI from all tissues 18:0 was consistently greater than 16:0, often strikingly so (Table 3.6). This observation is a stark contrast to PC (Table 3.4) where 16:0 was the principal saturated fatty acid. Even though 18:0 was the principal saturated fatty acid in PE (Table 3.5) the levels of 18:0 observed in PI were much greater than found for PE. Adipose tissue had the lowest amounts of saturated fatty acids in PI with only 7% 16:0 and 10% 18:0. Moreover, this tissue also had the lowest ratio of 18:0 to 16:0. PI from the spleen contained approximately seven times more 18:0 than 16:0 while there was five times more 18:0 than 16:0 detected in PI from the muscle. The heart contained quadruple the amount of 18:0 compared to 16:0. PI from the eye contained treble the amount of 18:0 than 16:0 while PI both the liver and brain contained twice as much 18:0 than 16:0. The principal monounsaturated fatty acid in PI from all tissues except adipose was 18:1 n-9. Adipose tissue together with the eye, heart and liver were composed of approximately 8% 18:1 n-9. Marginally lower levels were observed in the brain, muscle and spleen, approximately 6%. As for the other phosphoglycerides (section 3.5, 3.6) elevated levels of 20:1 n-9 and 22:1 were detected in adipose tissue compared to the other tissues examined. 20:1 n-9 accounted for 22% of the fatty acid profile of PI in adipose tissue compared to the 3% found in other tissues. 22:1 was observed at a level of 27% in adipose tissue which was in stark contrast to the approximate 2% found in the other tissues.

In terms of the PUFA content of PI the principal fatty acids were the same in PE and PI (Table 3.5, 3.6) although the levels varied. Generally speaking, DHA > 20:4 n-6 > EPA > 18:2 n-6. PI demonstrated dramatic differences in AA in comparison to the levels detected in both PC and PE. Minor levels of AA were observed in both PC and PE in all tissues (Tables 3.4, 3.5). However, in PI from liver and spleen the levels of AA were substantial. The liver and spleen contained approximately 22.5% AA, dramatically more than that for PC or PE (Table 3.4, 3.5) and was twice the level detected in eye and heart and more than six times the levels noted in brain and muscle. However, elevated levels of AA compared to PC and PE were also detected in PI from eye and heart (11% and 9% respectively). PI from the

brain and the muscle contained approximately 3.5% AA. Similar to the PC and PE (Tables 3.4, 3.5) AA was not detected in PI from adipose tissue. The absence of EPA from adipose tissue was also observed together with the low levels detected in brain. However, for the remaining tissues EPA was detected at approximately 2.5%. DHA was a principal component of PI in all tissues, though particular tissues displayed elevated levels. PI from muscle was composed of 24% DHA whereas in all remaining tissues DHA accounted for approximately 10% of the fatty acid composition of PI, the only exception being adipose tissue where DHA constituted 2% of the fatty acids in PI. Overall, PI (Table 3.6) contained less DHA than PC and PE (Tables 3.4, 3.5).

Table 3.6: Fatty acid analysis of phosphatidylinositol from individual tissues of *Oncorhynchus mykiss*. Results as means of % fatty acids ± SD. tr., trace (< 0.05); n.d., none detected.

Fatty Acid	Adipose tissue	Brain	Eye	Heart	Liver	Muscle	Spleen
	n = 3	n = 4	n = 4	n = 3	n = 4	n=4	n = 3
14:0	0.5 ± 0.2	2.0 ± 0.5	0.7 ± 0.2	0.6 ± 0.2	1.9 ± 0.3	0.7 ± 0.5	0.3 ± 0.2
16:0	6.8 ± 2.8	17.9 ± 0.5	13.0 ± 6.2	10.7 ± 1.5	7.9 ± 0.4	7.7 ± 1.1	5.0 ± 0.2
16:1 n-7	5.0 ± 1.8	6.4 ± 1.2	3.5 ± 1.3	2.0 ± 0.2	0.5 ± 0.1	1.6 ± 0.1	2.2 ± 0.7
16:2 n-4	1.6 ± 0.6	0.6 ± tr.	1.3 ± 1.2	0.2 ± tr.	0.8 ± 0.4	0.2 ± 0.2	0.1 ± tr.
16:3 n-4	1.0 ± 0.5	2.4 ± 0.4	1.2 ± 0.1	1.1 ± 0.1	0.7 ± tr.	1.1 ± 0.3	1.0 ± 0.1
18:0	9.8 ± 3.8	32.0 ± 3.4	35.0 ± 2.5	40.7 ± 5.0	34.4 ± 8.9	40.4 ± 2.3	34.0 ± 1.8
18:1 n-9	9.7 ± 0.3	5.9 ± 0.9	8.1 ± 1.5	7.2 ± 0.5	7.1 ± 0.9	5.8 ± 0.9	6.2 ± 0.7
18:1 n-7	nd	1.7 ± 0.5	3.1 ± 0.1	2.3 ± 0.1	1.5 ± 0.3	1.7 ± 0.2	1.7 ± 0.5
18:2 n-6	4.8 ± 1.9	3.1 ± 0.5	2.7 ± 0.9	2.2 ± 0.6	0.7 ± 0.4	1.5 ± 0.3	1.9 ± 0.2
18:3 n-3	nd	1.6 ± 0.2	1.0 ± 0.7	0.6 ± 0.3	0.7 ± 0.1	0.6 ± 0.2	0.5 ± 0.1
18:3 n-4	1.4 ± 0.9	1.2 ± 0.4	0.7 ± 0.2	0.6 ± 0.2	0.4 ± 0.2	0.7 ± 0.3	0.4 ± 0.4
18:4 n-3	nd	nd	nd	0.8 ± 0.4	0.3 ± 0.1	0.4 ± 0.4	0.1 ± tr.
20:1 n-9	22.0 ± 11.9	2.6 ± 0.6	3.8 ± 0.6	3.4 ± 0.9	3.4 ± 0.7	2.5 ± 1.4	4.0 ± 0.1
20:1	nd	4.1 ± 0.5	nd	0.3 ± tr.	nd	0.4 ± 0.3	nd
20:4 n-6	nd	2.6 ± 0.8	10.7 ± 5.4	9.0 ± 0.7	22.9 ± 4.5	3.9 ± 0.7	21.6 ± 3.1
20:4 n-3	1.5 ± 0.9	1.5 ± 0.8	0.9 ± 0.2	1.8 ± 0.8	3.5 ± 0.7	1.1 ± 0.1	0.5 ± 0.4
20:5 n-3	**nd**	**0.6 ± 0.5**	**3.2 ± 0.8**	**1.5 ± 0.3**	**1.4 ± 0.6**	**3.0 ± 0.2**	**2.6 ± 0.7**
22:1	27.2 ± 5.2	1.0 ± 0.7	nd	2.6 ± 0.8	3.2 ± 2.2	1.2 ± 0.6	0.9 ± 0.2
22:2	6.8 ± 1.3	6.4 ± 1.0	2.8 ± 0.3	2.6 ± 1.5	0.8 ± 0.2	0.6 ± 0.2	2.3 ± 1.5
22:5 n-3	nd	nd	0.6 ± 0.5	0.4 ± 0.1	0.7 ± tr.	1.2 ± 0.3	2.7 ± 1.8
22:6 n-3	**1.8 ± 1.2**	**6.5 ± 1.39**	**10.4 ± 3.3**	**9.3 ± 2.9**	**9.5 ± 2.1**	**24.3 ± 5.5**	**12.2 ± 0.5**

3.8 Acyl Distribution in Phosphatidylserine from Individual Tissues of *Oncorhynchus mykiss*

In terms of the acyl composition of PS, the main saturated fatty acids included 18:0 and 16:0 (Table 3.6). In all tissues 18:0 was significantly greater than the levels of 16:0. The greatest 18:0 to 16:0 ratio was observed in spleen where there was four times as much 18:0 as 16:0. Liver and muscle contained similar levels of 16:0 and 18:0 (approximately 12% and 28%, respectively). Similar levels of 16:0 were reported in PS from the heart compared to liver and muscle but PS from the heart contained approximately 37% 18:0, the highest level of 18:0 observed in any of the organs examined. The principal monounsaturated fatty acid detected in PS from all tissues (with the exception of adipose tissue) was 18:1 n-9. The highest level of 18:1 n-9 was observed in PS from the eye (11%) and was seen to be almost four times greater than the other principal monounsaturated fatty acid, 16:1. PS from the heart, liver and spleen contained approximately 4% 18:1 n-9. Elevated levels of 18:1 n-9 were observed in PS from muscle (8%) and the ratio of 18:1 n-9 and 16:1 was approximately 2.7. As previously seen with the other phosphoglycerides (Tables 3.4 – 3.6) 20:1 n-9 and 22:1 were the dominant monounsaturated fatty acids in PS from adipose tissue (23% and 19%, respectively).

With regard to the PUFAs, as previously seen (Tables 3.4 – 3.6), DHA was the principal PUFA in all tissues apart from adipose tissue. For most tissues the PUFA ranking was DHA > AA = 18:2 n-6 = EPA (Table 3.7). AA was highest in PS from brain (6%). PS from the heart and muscle contained comparable levels of AA (3%) while lower levels were observed in spleen and liver. As seen for PC, PE and PI (Table 3.4 – 3.6) no AA was detected in PS from adipose tissue. Also, no AA was observed in PS from the eye. EPA was rather low in PS from the muscle, liver, spleen and eye. Slightly higher and comparable levels were found in PS in brain and heart (1.2%). As observed for the aforementioned phosphoglycerides (Table 3.4 – 3.6), no EPA was detected in PS from adipose tissue. DHA was highest in PS from the spleen, eye and liver (approximately 35%). Muscle PS contained 25% DHA while the heart contained 16% DHA. The lowest levels of DHA were found in PS from the brain and adipose tissue (7% and 2% respectively).

3.9 Acyl Distribution in Sphingomyelin from Individual Tissues of *Oncorhynchus mykiss*

Sphingomyelin (SPH) was found in all tissues examined (Table 3.8). The main saturated fatty acids detected in SPH from all tissues were 16:0 and 18:0. Equal levels of 16:0 and 18:0 were observed in SPH in adipose tissue, brain, eye and heart. However, lower absolute levels of both fatty acids were observed in adipose tissue compared to the other tissues. Spleen contained the highest level of 16:0 (33%) while the eye, liver and heart contained comparable levels (approximately 24%). Muscle and brain contained approximately 18% 16:0 in SPH, significantly lower than the spleen, eye, liver and heart (approximately 18%). In terms of 18:0, the eye contained the greatest levels (22%). In SPH from the brain, heart, liver and spleen 18:0 constituted approximately 18%. Equal proportions of 16:0 and 18:0 were observed in SPH from adipose tissue (approximately 9%). In terms of the monounsaturated fatty acids 18:1 n-9 and 16:1 n-7 were the principal fatty acids. 18:1 n-9 was present in excess of 16:1 n-7 in SPH from the heart, liver and spleen while comparable levels of 18:1 n-9 and 16:1 n-7 were observed in adipose tissue, brain, eye and muscle. Adipose tissue was again the exception with 20:1 n-9 and 22:1 being the principal monounsaturated fatty acids (28% and 13% respectively).

In terms of the PUFA content of SPH, the main PUFAs in most tissues included DHA > 20:4 n-3 > 18:2 n-6 > EPA. The highest level of AA were detected in brain and muscle (approximately 4%), followed by SPH from the eye (2.4%) with minor levels detected in spleen and heart (approximately 1%). No AA was found in SPH from either adipose tissue or liver. EPA in SPH from brain, liver and muscle was just over 2%, with very low levels detected in the eye, spleen and heart. No EPA was evident in SPH from adipose tissue. DHA was found at the greatest level in SPH from the liver and muscle (approximately 13%). The spleen, heart and eye contained approximately 6% DHA while the lowest levels were present in the eye and adipose tissue (3% and 1% respectively).

Table 3.7: Fatty acid analysis from phosphatidylserine in individual tissues of *Oncorhynchus mykiss*. Results as means of % fatty acids ± SD. tr., trace (< 0.05); n.d., none detected.

Fatty Acid	Adipose tissue	Brain	Eye	Heart	Liver	Muscle	Spleen
	n = 5	n = 5	n = 3	n = 4	n = 4	n = 5	n = 3
14:0	0.7 ± 0.1	2.6 ± 0.5	0.5 ± 0.1	0.8 ± 0.3	0.4 ± 0.2	2.4 ± 0.1	0.2 ± 0.1
16:0	7.9 ± 3.7	18.6 ± 2.1	9.4 ± 0.9	13.5 ± 1.6	11.1 ± 2.1	13.1 ± 3.5	7.3 ± 1.3
16:1 n-7	8.2 ± 2.2	7.8 ± 1.5	2.9 ± 0.9	2.6 ± 0.8	0.6 ± 0.3	3.3 ± 1.0	1.2 ± 0.4
16:2 n-4	1.4 ± 0.3	0.7 ± 0.1	0.5 ± 0.1	1.2 ± 0.7	0.7 ± 0.3	0.4 ± 0.4	0.3 ± 0.1
16:3 n-4	1.4 ± 0.3	2.3 ± 0.3	1.0 ± 0.2	0.7 ± 0.4	1.2 ± 0.2	1.5 ± 0.4	1.1 ± 0.1
18:0	17.8 ± 9.6	24.5 ± 2.1	27.4 ± 1.6	36.5 ± 3.8	29.0 ± 2.7	27.8 ± 3.6	29.7 ± 0.7
18:1 n-9	7.7 ± 1.9	6.6 ± 0.7	10.8 ± 1.3	4.9 ± 1.6	3.5 ± 0.4	7.8 ± 3.6	4.5 ± 1.2
18:1 n-7	n.d.	2.4 ± 1.1	2.9 ± 0.3	2.4 ± 0.2	2.0 ± 0.4	2.1 ± 0.8	3.9 ± 0.3
18:2 n-6	4.4 ± 0.8	3.2 ± 0.6	1.8 ± 0.1	1.9 ± 0.6	0.7 ± 0.1	1.8 ± 0.3	1.3 ± 0.4
18:3 n-3	1.7 ± 0.6	2.2 ± 0.8	0.5 ± 0.1	0.8 ± 0.2	0.4 ± 0.2	2.0 ± 1.1	0.5 ± 0.3
18:3 n-4	n.d.	1.6 ± 1.2	1.0 ± 0.5	1.1 ± 0.2	0.4 ± 0.1	1.1 ± 0.3	0.6 ± 0.3
18:4 n-3	n.d.	1.2 ± 0.5	0.5 ± 0.1	0.7 ± 0.3	0.7 ± 0.1	0.8 ± 0.8	0.4 ± tr.
20:1 n-9	22.6 ± 9.2	3.8 ± 0.7	3.8 ± 0.3	3.7 ± 0.4	3.7 ± 0.7	3.8 ± 0.6	6.8 ± 1.5
20:1	n.d.	0.7 ± 0.6	0.7 ± 0.2	n.d.	n.d.	0.9 ± 0.1	n.d.
20:4 n-6	n.d.	5.5 ± 0.9	n.d.	2.7 ± 0.1	1.2 ± 0.6	2.7 ± 0.1	1.0 ± 0.6
20:4 n-3	1.3 ± 0.7	1.1 ± 0.5	0.9 ± 0.8	0.6 ± 0.7	3.3 ± 1.2	0.5 ± 0.2	0.6 ± 0.3
20:5 n-3	**n.d.**	**1.2 ± 1.0**	**0.9 ± 0.2**	**1.2 ± 0.8**	**0.7 ± 0.5**	**0.8 ± 0.4**	**0.6 ± 0.2**
22:1	18.5 ± 7.6	2.5 ± 1.8	1.9 ± 0.3	2.7 ± 0.5	8.0 ± 3.1	1.2 ± 0.5	n.d.
22:2	4.1 ± 3.1	n.d.	n.d.	5.1 ± 2.4	0.8 ± 0.2	1.0 ± 0.4	n.d.
22:5 n-3	n.d.	4.2 ± 2.3	n.d.	0.9 ± 0.7	0.8 ± 0.2	1.8 ± 0.7	1.2 ± 0.2
22:6 n-3	**1.7 ± 1.5**	**7.2 ± 3.7**	**32.6 ± 4.4**	**15.9 ± 2.8**	**32.1 ± 1.1**	**24.6 ± 5.8**	**38.7 ± 3.8**

Table 3.8: Fatty acid analysis of sphingomyelin from individual tissues of *Oncorhynchus mykiss*. Results as means of % fatty acids ± SD. tr., trace (< 0.05); n.d., none detected.

Fatty Acid	Adipose tissue	Brain	Eye	Heart	Liver	Muscle	Spleen
	n = 4	n = 5	n = 4	n = 4	n = 3	n = 3	n = 3
14:0	0.9 ± 0.1	2.7 ± 0.6	2.6 ± 0.9	2.8 ± 1.5	3.5 ± 1.1	1.5 ± 0.2	4.4 ± 2.9
16:0	9.0 ± 3.3	18.9 ± 4.9	22.6 ± 7.1	23.6 ± 7.9	26.7 ± 2.2	17.4 ± 5.8	32.5 ± 2.1
16:1 n-7	7.9 ± 1.5	7.4 ± 1.6	8.7 ± 0.7	3.4 ± 1.4	1.8 ± 0.2	5.3 ± 2.4	3.2 ± 1.0
16:2 n-4	1.2 ± 0.2	1.0 ± 0.6	0.9 ± tr.	4.2 ± 0.4	2.4 ± 0.4	0.5 ± 0.5	0.3 ± 0.3
16:3 n-4	2.3 ± 1.5	1.9 ± 0.3	2.4 ± 0.4	1.2 ± 0.3	2.7 ± 0.8	1.4 ± 0.3	1.3 ± 0.2
18:0	10.0 ± 4.3	16.7 ± 3.4	22.1 ± 6.0	18.7 ± 3.8	17.5 ± 2.5	10.5 ± 6.3	18.4 ± 1.6
18:1 n-9	5.9 ± 2.6	6.4 ± 1.9	8.1 ± 2.8	7.7 ± 5.7	11.9 ± 3.1	6.0 ± 1.4	9.2 ± 2.3
18:1 n-7	n.d.	2.2 ± 0.9	1.6 ± 0.8	1.4 ± 0.5	2.7 ± 0.2	3.5 ± 2.3	3.3 ± 1.2
18:2 n-6	3.3 ± 1.9	5.2 ± 4.5	5.2 ± 0.8	3.8 ± 0.9	1.7 ± 0.9	7.5 ± 4.9	3.6 ± 0.8
18:3 n-3	n.d.	2.7 ± 1.1	1.8 ± 0.4	1.3 ± 0.4	1.3 ± 0.2	5.4 ± 4.2	0.9 ± 0.3
18:3 n-4	n.d.	2.3 ± 1.2	1.3 ± 1.1	1.2 ± 0.9	n.d.	2.1 ± 0.4	0.8 ± 0.3
18:4 n-3	3.4 ± 2.7	2.7 ± 0.9	n.d.	0.9 ± 0.4	n.d.	2.7 ± 1.8	1.0 ± 0.6
20:1 n-9	31.3 ± 10.8	4.8 ± 0.7	1.5 ± 0.5	2.5 ± 1.3	7.4 ± 0.2	1.6 ± 0.2	3.9 ± 1.4
20:1	n.d.	5.2 ± 1.4	7.7 ± 0.7	3.3 ± 0.7	n.d.	4.6 ± 1.0	n.d.
20:4 n-6	n.d.	3.9 ± 2.1	2.4 ± 0.6	1.1 ± 0.7	n.d.	3.6 ± 1.7	0.9 ± 0.2
20:4 n-3	3.8 ± 1.7	2.7 ±1.4	4.2 ± 5.7	2.9 ± 1.2	2.1 ± 0.3	3.5 ± 2.4	0.3 ± 0.3
20:5 n-3	**n.d.**	**2.2 ± 1.8**	**0.4 ± 0.1**	**0.3 ± 0.2**	**3.5 ± 1.9**	**1.3 ± 0.5**	**0.6 ± 0.2**
22:1	13.3 ± 5..3	5.9 ± 2.3	n.d.	4.5 ± 2.4	4.3 ± 0.5	3.4 ± 1.5	4.9 ± 1.6
22:2	6.5 ± 3.7	4.3 ± 2.7	n.d.	5.2 ± 3.5	n.d.	2.4 ± 0.8	3.1 ± 1.8
22:5 n-3	n.d.	n.d.	n.d.	4.2 ± 3.8	n.d.	0.9 ± 0.0	0.5 ± 0.9
22:6 n-3	**1.5 ± 0.5**	**3.3 ± 1.0**	**7.5 ± 1.3**	**5.7 ± 3.3**	**12.3 ± 4.1**	**14.2 ± 8.2**	**6.7 ± 4.1**

3.10 Acyl Distribution in Diphosphatidylglycerol from Individual Tissues of *Oncorhynchus mykiss*

Diphosphatidylglycerol (DPG) was detected in the brain, heart, liver, muscle and spleen (Table 3.9). As with the other phoshoglycerides analysed (Tables 3.4 – 3.8) 16:0 and 18:0 were the principal saturated fatty acids. Both fatty acids occurred in approximately equal percentages in all tissues but the total saturated fatty acid content varied from 47% in brain to 20% in liver. In terms of the monounsaturated acyl composition of DPG 18:1 n-9 was the dominant fatty acid ranging from 8% in muscle to 15% in spleen. However, 18:1 n-7, 16:1 n-7, 20:1 and 22:1 were also significant components of DPG.

The PUFA content of DPG in all tissues consisted of DHA > 18:2 n-6 > EPA = AA = 20:4 n-3. In contrast to the other phosphoglycerides (Table 3.4 – 3.8) 18:2 n-6 was found in appreciable amounts in DPG from most tissues constituting approximately 13% in DPG from the heart, liver, muscle and spleen. The levels of AA and EPA were equal in DPG from all tissues (approximately 1.5%). DHA was the main PUFA in most tissues. Liver DPG was composed of 35% DHA whereas the spleen, muscle and heart contained approximately 23% DHA while brain was composed of only 5% DHA. Linoleic acid (18:2 n-6) was quite high in most tissues and was the major PUFA in DPG from brain.

Table 3.9: Fatty acid analysis of diphosphatidylglycerol (cardiolipin) from individual tissues of *Oncorhynchus mykiss*. Results as means of % fatty acids ± SD. tr., trace (< 0.05); n.d., none detected. No diphosphatidylglycerol was detected in the lipid extracts from the adipose tissue and eye.

Fatty Acid	Brain	Heart	Liver	Muscle	Spleen
	n = 5	n = 3	n = 4	n = 5	n = 5
14:0	2.8 ± 1.0	1.7 ± 0.3	2.4 ± 0.9	0.9 ± 0.5	1.7 ± 0.8
16:0	22.7 ± 2.4	13.2 ± 5.6	10.6 ± 1.8	13.8 ± 3.3	13.2 ± 4.1
16:1 n-7	6.2 ± 1.7	2.2 ± 0.6	1.9 ± 0.6	2.9 ± 1.2	4.3 ± 1.2
16:2 n-4	1.0 ± 0.4	0.4 ± 0.2	1.5 ± 0.5	0.3 ± 0.3	0.6 ± 0.3
16:3 n-4	2.5 ± 0.3	0.6 ± 0.3	1.8 ± 0.7	1.1 ± 0.5	1.1 ± 0.5
18:0	21.7 ± 1.9	9.9 ± 6.9	6.9 ± 1.4	7.0 ± 1.9	10.0 ± 1.4
18:1 n-9	6.7 ± 1.5	11.3 ± 2.4	9.3 ± 1.4	7.7 ± 1.7	14.5 ± 1.6
18:1 n-7	3.7 ± 0.5	4.1 ± 0.8	3.0 ± 0.2	6.5 ± 0.5	6.5 ± 0.4
18:2 n-6	5.7 ± 0.9	15.0 ± 2.3	12.0 ± 1.6	14.8 ± 3.6	9.9 ± 2.3
18:3 n-3	2.0 ± 0.4	0.7 ± 0.1	0.7 ± 0.2	1.3 ± 0.5	1.1 ± 0.4
18:3 n-4	2.0 ± 1.2	2.8 ± 0.3	1.0 ± 0.4	2.1 ± 0.3	0.8 ± 0.3
18:4 n-3	1.7 ± 0.7	0.9 ± 0.3	0.4 ± tr.	0.9 ± 0.6	0.6 ± 0.3
20:1 n-9	3.4 ± 0.6	3.1 ± 0.8	3.8 ± 0.5	4.5 ± 1.0	5.2 ± 1.1
20:1	5.4 ± 0.8	0.3 ± 0.2	n.d.	0.9 ± 0.6	n.d.
20:4 n-6	1.4 ± 0.7	1.5 ± 0.7	1.7 ± 0.5	1.4 ± 0.9	1.7 ± 0.8
20:4 n-3	1.3 ± 0.8	1.3 ± 0.3	1.3 ± 0.2	1.2 ± 0.3	0.7 ± 0.6
20:5 n-3	**1.8 ± 0.6**	**1.0 ± 0.2**	**1.2 ± 0.6**	**1.0 ± 0.2**	**1.3 ± 0.5**
22:1	1.4 ± 0.3	3.3 ± 0.9	5.9 ± 1.4	4.4 ± 1.1	1.6 ± 0.5
22:2	1.9 ± 1.2	1.5 ± 1.2	n.d.	1.1 ± 0.5	4.7 ± 3.0
22:5 n-3	n.d.	1.0 ± 0.9	2.0 ± 0.5	0.9 ± 0.3	0.2 ± 0.5
22:6 n-3	**4.9 ± 2.0**	**24.4 ± 7.5**	**35.0 ± 4.5**	**24.5 ± 5.8**	**20.1 ± 3.9**

3.11 Acyl Distribution in Lyso-Phosphatidylcholine from Individual Tissues of *Oncorhynchus mykiss*

Lyso-phosphatidylcholine (lyso-PC) was only detected in eye, heart, liver and spleen (Table 3.10). 16:0 and 18:0 were the main saturated fatty acids and the total content of saturates in lyso-PC varied from about 30% in the eye to 50% in the liver. 18:0 was the principal saturated fatty acid found in the eye and heart (over 20%) while in the liver 16:0 and 18:0 were found at equal levels. In lyso-PC from the spleen the levels of 16:0 were more than twice the level of 18:0 (30% and 12% respectively). The principal monounsaturated fatty acids included 16:1 n-7 (except for heart), 18:1 n-9 and n-7 and 20:1 n-9.

The PUFA content of lyso-PC from the eye was primarily composed of DHA (25%), 18:2 n-6 (7%), EPA, AA and 16:3 n-4 (approximately 2%). DHA was also the main PUFA found in heart, liver and spleen with both AA and EPA constituting approximately 2% of the lyso-PC composition.

Differences between PC (Table 3.4) and lyso-PC (Table 3.10) from the same tissues will likely reflect the positional distribution of fatty acids and, hence, the selective loss of acids from the *sn*-2 position as lyso-PC is formed.

Table 3.10: Fatty acid analysis of lyso-phosphatidylcholine from individual organs of *Oncorhynchus mykiss*. Results as means of % fatty acids ± SD. tr., trace (< 0.05); n.d., none detected. No lyso-phosphatidylcholine was detected in adipose tissue, brain and muscle.

Fatty Acid	Eye	Heart	Liver	Spleen
	n = 4	n = 3	n = 3	n = 3
14:0	1.6 ± 0.2	1.6 ± 0.9	1.8 ± 0.8	1.3 ± 0.6
16:0	13.6 ± 2.2	17.2 ± 8.6	26.2 ± 6.2	30.2 ± 12.2
16:1 n-7	11.0 ± 3.0	6.9 ± 2.8	0.1 ± tr.	4.5 ± 1.0
16:2 n-4	1.3 ± 0.4	1.5 ± 0.9	3.8 ± 0.8	0.4 ± 0.4
16:3 n-4	2.5 ± 0.6	1.9 ± 0.1	2.8 ± 0.6	1.3 ± 2.4
18:0	21.6 ± 7.2	24.7 ± 5.2	22.2 ± 10.0	12.1 ± 7.7
18:1 n-9	9.0 ± 2.9	6.4 ± 0.5	7.9 ± 2.0	3.8 ± 2.3
18:1 n-7	2.2 ± 1.6	6.0 ± 0.9	2.9 ± 0.6	3.1 ± 0.1
18:2 n-6	6.5 ± 2.3	4.1 ± 0.4	2.6 ± 0.6	1.7 ± 1.0
18:3 n-3	1.9 ± 1.0	1.6 ± 0.2	1.1 ± 0.2	0.9 ± 0.6
18:3 n-4	1.7 ± 0.4	1.0 ± 0.2	1.1 ± tr.	0.6 ± 0.5
18:4 n-3	n.d.	1.9 ± 0.8	n.d.	n.d.
20:1 n-9	3.9 ± 1.1	4.3 ± 0.4	7.1 ± 3.6	5.9 ± 0.4
20:1	n.d.	1.9 ± 04	n.d.	n.d.
20:4 n-6	1.6 ± 0.2	2.4 ± 1.5	2.2 ± 1.7	1.1 ± 0.2
20:4 n-3	1.0 ± 0.9	2.7 ± 1.0	2.7 ± 0.6	0.7 ± 0.9
20:5 n-3	**2.1 ± 0.9**	**3.6 ± 1.1**	**2.2 ± 0.8**	**1.5 ± 0.9**
22:1	n.d.	4.6 ± 0.9	n.d.	n.d.
22:2	n.d.	n.d.	n.d.	n.d.
22:5 n-3	n.d.	n.d.	n.d.	n.d.
22:6 n-3	**24.8 ± 8.2**	**10.3 ± 3.7**	**9.5 ± 1.5**	**11.0 ± 1.0**

3.12 Acyl Distribution in Triacylglycerol from Individual Tissues of *Oncorhynchus mykiss*

The fatty acid composition of the major storage lipid, TAG, is shown in Table 3.11. The percentages of acyl components of TAG were similiar between tissues apart from some minor discrepancies. The principal saturated fatty acid in each organ was 16:0 which was present at approximately 15%. Both 14:0 and 18:0 were the other remaining saturated fatty acids and the composition was consistent between individual organs (approximately 3% and 3.5% respectively). The major monounsaturated fatty acid in TAG from all organs was 18:1 n-7 (approximately 26%). Both 20:1 n-9 and 22:1 were also prominent components of TAG (12% and 10% respectively) and 16:1 n-7 was present at approximately 5% in all tissues. In terms of the PUFAs, 18:2 n-6 was the principal PUFA consisting of 10 – 13% in each tissue apart from two exceptions. TAG from both the heart and liver contained of 2.7% and 5.1% linolenic acid respectively, significantly less than that found in the remaining tissues. EPA was present at approximately 1.5% in all tissues although elevated levels were detected in the TAG from the liver (7.5%). DHA composed 5% of the TAG fraction in the brain, eye, liver, muscle and eye. TAG from adipose tissue and the heart contained only 3% and 2% DHA respectively (Table 3.11).

Table 3.11: Fatty acid analysis of triacylglycerols from individual tissues of *Oncorhynchus mykiss*. Results as means of % fatty acids ± SD. tr., trace (< 0.05), n.d., none detected.

Fatty Acid	Adipose tissue n = 4	Brain n = 5	Eye n = 5	Heart n= 3	Liver n = 3	Muscle n = 5	Spleen n = 4
14:0	2.7 ± 0.2	3.5 ± 0.2	3.1 ± 0.2	2.3 ± 0.9	3.0 ± 1.6	2.9 ± 0.3	2.2 ± 0.5
16:0	15.2 ± 0.8	15.4 ± 1.1	14.4 ± 1.1	19.1 ± 0.8	18.0 ± 4.6	16.1 ± 1.2	14.2 ± 0.8
16:1 n-7	4.0 ± 2.3	6.4 ± 0.5	5.4 ± 0.3	5.4 ± 0.6	4.9 ± 0.8	6.3 ± 0.3	4.4 ± 0.4
16:2 n-4	0.2 ± tr.	0.3 ± tr.	0.3 ± tr.	0.3 ± 0.2	0.3 ± 0.1	0.3 ± tr.	0.2 ± tr.
16:3 n-4	0.5 ± 0.1	0.5 ± tr.	0.5 ± 0.1	0.7 ± 0.2	0.5 ± 0.2	0.6 ± tr.	0.6 ± tr.
18:0	3.2 ± 0.3	2.9 ± 0.2	2.8 ± 0.3	4.9 ± 0.9	3.7 ± 0.6	3.0 ± 0.3	3.5 ± 0.3
18:1 n-9	26.2 ± 2.5	27.7 ± 2.4	26.2 ± 0.6	27.2 ± 0.9	29.5 ± 1.9	26.9 ± 0.8	24.5 ± 2.2
18:1 n-7	5.6 ± 2.9	0.4 ± 0.1	5.0 ± 2.4	5.7 ± 1.7	4.0 ± 0.3	0.3 ± tr.	5.1 ± 2.6
18:2 n-6	9.9 ± 0.3	12.2 ± 0.6	12.4 ± 0.9	2.7 ± 1.2	5.1 ± 3.8	12.7 ± 0.7	10.0 ± 0.9
18:3 n-3	0.7 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	0.3 ± 0.1	2.7 ± 2.2	0.9 ± 0.5	0.8 ± 0.2
18:3 n-4	0.7 ± 0.1	1.0 ± 0.1	0.9 ± 0.5	0.5 ± 0.6	0.3 ± 0.2	0.6 ± 0.5	0.7 ± 0.1
18:4 n-3	0.1 ± 0.1	0.3 ± 0.1	0.3 ± 0.3	1.4 ± 0.3	0.6 ± 0.1	0.1 ± tr.	0.2 ± 0.1
20:1 n-9	12.7 ± 0.7	10.4 ± 1.1	9.7 ± 0.8	12.7 ± 1.7	11.8 ± 1.5	10.9 ±0.3	13.1 ± 1.4
20:1	0.1 ± 0.1	n.d.	n.d.	n.d.	n.d.	0.1 ± tr.	n.d.
20:4 n-6	0.8 ± 0.1	0.9 ± 0.2	0.8 ± 0.1	0.2 ± 0.2	0.4 ± 0.2	0.5 ± 0.4	0.4 ± 0.2
20:4 n-3	0.5 ± 0.2	0.8 ± 0.1	0.8 ± 0.1	0.5 ± 0.3	1.2 ± 0.7	0.9 ± 0.3	1.0 ± 0.3
20:5 n-3	**1.0 ± 0.2**	**1.7 ± 0.1**	**1.9 ± 0.3**	**1.1 ± 0.1**	**7.3 ± 2.1**	**1.8 ± 0.2**	**1.7 ± 0.2**
22:1	11.8 ± 1.5	8.6 ± 0.8	8.1 ± 1.2	12.4 ± 1.5	0.5 ± 0.5	8.9 ± 0.5	12.1 ± 1.7
22:2	0.5 ± 0.1	n.d.	n.d.	0.9 ± 0.6	0.9 ± 0.3	0.2 ± tr.	0.6 ± 0.4
22:5 n-3	0.5 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.6 ± 0.5	1.0 ± 0.4	0.8 ± 0.1	0.6 ± 0.1
22:6 n-3	**2.9 ± 0.6**	**5.3 ± 0.8**	**5.5 ± 5.0**	**2.0 ± tr.**	**5.8 ± 0.9**	**5.5 ± 0.8**	**4.2 ± 0.9**

3.14 Acyl Distribution in 1,2-Diacylglycerol from Individual Tissues of *Oncorhynchus mykiss*

The acyl composition of 1,2 diacylglycerol (DAG) was less consistent than what was observed for the TAG fraction in each organ (Table 3.12). For 1,2-DAG from most tissues the levels of 16:0 and 18:0 were about equal although the specific levels between tissues varied quite significantly. The highest levels of 16:0 and 18:0 were found in muscle (approximately 27%) whilst the lowest levels were present in heart (9% 16:0 and 18% 18:0). In terms of the monounsaturated fatty acids, adipose tissue and the eye contained approximately equal proportions of 18:1, 20:1 n-9 and 22:1. Brain tissue had rather high levels of 22:1 and also contained approximately 9% and 5% of 18:1 and 20:1 n-9, respectively. The liver, muscle and spleen were composed of equal levels of 18:1 and 20:1 n-9 and little or no 22:1 was detected. The major PUFA was usually 18:2 n-6 which comprised approximately 6% of the acyl composition of 1,2-DAG in all tissues. Unusually, EPA was highest in 1,2-DAG from adipose tissue (4%) while 1,2-DAG from the heart contained approximately 3% EPA. DHA was greatest in the spleen (6%) while the liver, heart, eye and adipose tissue contained approximately 4%. 1,2-DAG from both muscle and brain had the lowest proportions of 1,2-DAG. AA was found at high levels in 1,2-DAG from liver (14%).

3.15 Acyl Distribution in 1,3-Diacylglycerol from Individual Tissues of *Oncorhynchus mykiss*

Table 3.13 outlines the acyl composition of 1,3-DAG. Similar to 1,2-DAG (Table 3.12) variations in fatty acyl compositions were detected between tissues. 16:0 comprised approximately 17% of 1,3-DAG in most tissues (adipose, 8%) whilst 18:0 varied from 22% and 20% in muscle and heart to 12% in both the spleen and brain. As seen with 1,2-DAG 22:1 n-9 was the dominant monounsaturated fatty acid present in 1,3-DAG from the brain (30%) followed by 8% 18:1 and 4% 20:1. Adipose tissue 1,3-DAG contained 19% 18:1 n-9 and equal proportions of 20:1 n-9 and 22:1. In the eye equal levels of 18:1 and 22:1 were present (approximately 10%) while 4% was made up of 20:1 n-9. No 22:1

was detected in 1,3-DAG from the heart and the monounsaturated fatty acids comprised 16:1 n-7, 18:1 n-9 and 20:1 n-9. The liver 1,3-DAG displayed a significantly high level of 20:1 n-9 (12.8%). Muscle and spleen were composed of predominantly equal proportions of 16:1, 18:1 and 20:1. Of the PUFA content of 1,3-DAG 18:2 n-6 comprised approximately 5% in all tissues although a lower proportion was reported for 1,3-DAG from brain (2.6%). EPA was a minor component of 1,3-DAG from all tissues with the exception of the liver where it constituted approximately 9% of the acyl composition. DHA was significantly elevated in 1,3-DAG from the eye (28%) while in the spleen and liver it composed 10% and 6% respectively. 1,3-DAG from adipose tissue contained approximately 5%, muscle 4%, heart 3% and 1.2% was found in the brain.

Table 3.12: Fatty acid analysis of 1,2-diacylglycerol from individual tissues of *Oncorhynchus mykiss*. Results as means of % fatty acids ± SD. tr, trace (< 0.05), n.d., none detected.

Fatty Acid	Adipose tissue n = 4	Brain n = 5	Eye n = 5	Heart n = 4	Liver n = 4	Muscle n=5	Spleen n = 5
14:0	0.2 ± 0.4	4.1 ± 2.0	1.9 ± 0.4	1.3 ± 0.4	8.6 ± 2.1	3.8 ± 1.2	1.8 ± 0.2
16:0	11.0 ± 7.5	18.8 ± 0.7	19.3 ± 3.3	9.2 ± 6.5	20.1 ± 2.7	26.2 ± 4.4	17.0 ± 1.8
16:1 n-7	10.2 ± 1.9	2.0 ± 0.7	6.5 ± 2.1	11.7 ± 4.3	8.5 ± 1.3	7.2 ± 2.8	10.5 ± 1.6
16:2 n-4	0.2 ± 0.2	1.7 ± 0.5	2.4 ± 1.7	3.2 ± 0.3	2.6 ± 0.3	2.6 ± 2.3	2.3 ± 0.8
16:3 n-4	1.5 ± 0.2	1.5 ± 0.5	2.2 ± 0.6	2.6 ± 0.6	6.4 ± 0.6	2.2 ± 1.5	2.1 ± 0.6
18:0	16.2 ± 2.4	18.5 ± 1.9	19.9 ± 6.2	17.8 ± 4.7	16.6 ± 3.2	27.2 ± 8.9	17.6 ± 4.5
18:1 n-9	6.8 ± 2.9	9.0 ± 1.8	10.2 ± 5.2	4.4 ± 0.3	5.6 ± 2.7	7.5 ± 2.4	6.4 ± 3.5
18:1 n-7	2.6 ±1.8	n.d.	1.9 ± 1.3	1.1 ± 2.3	n.d.	n.d.	2.7 ± 0.6
18:2 n-6	7.4 ± 1.1	4.0 ± 0.2	7.3 ± 1.4	6.4 ± 2.0	5.2 ± 1.4	4.1 ± 0.8	6.7 ± 2.3
18:3 n-3	2.5 ± 0.9	1.2 ± 0.5	1.5 ± 0.4	5.1 ± 4.6	3.9 ± 0.6	2.5 ± 1.4	3.4 ± 2.0
18:3 n-4	3.8 ± 0.8	1.2 ± 0.2	2.4 ± 1.9	3.2 ± 1.6	n.d.	1.7 ± 1.1	2.5 ± 0.7
18:4 n-3	3.8 ± 2.6	2.6 ± 1.7	2.0 ± 0.7	2.8 ± 0.7	0.9 ± 0.1	1.6 ± 0.9	1.7 ± 0.7
20:1 n-9	9.1 ± 1.4	5.0 ± 3.2	6.3 ± 2.2	8.4 ± 4.8	6.6 ± 1.7	5.1 ± 2.3	5.9 ± 0.8
20:1	3.6 ± 1.3	3.6 ± 0.6	n.d.	n.d.	n.d.	n.d.	n.d.
20:4 n-6	4.1 ± 2.0	1.2 ± 0.4	2.4 ± 1.6	2.2 ± 1.8	14.4 ± 4.5	1.9 ± 0.7	3.3 ± 2.7
20:4 n-3	0.1 ± tr.	0.9 ± 0.2	1.2 ± 0.3	2.5 ± 0.3	n.d.	0.5 ± tr.	3.7 ± 0.5
20:5 n-3	**4.4 ± 1.7**	**0.6 ± 0.3**	**0.6 ± 0.2**	**2.9 ± 1.4**	**n.d.**	**0.9 ± 0.3**	**0.2 ± 0.4**
22:1	10.9 ± 4.3	22.6 ± 5.2	9.5 ± 5.6	1.0 ± 0.3	n.d.	1.9 ± 1.3	n.d.
22:2	0.1 ± 0.1	0.9 ± 0.2	n.d.	6.9 ± 4.2	n.d.	3.6 ± 2.2	n.d.
22:5 n-3	0.5 ± 0.1	n.d.	n.d.	4.9 ± 1.0	n.d.	1.7 ± 1.2	7.4 ± 0.8
22:6 n-3	**3.1 ± 1.1**	**1.4 ± 0.5**	**4.0 ± 1.**	**3.8 ± 1.9**	**4.2 ± 0.6**	**1.3 ± 1.2**	**5.9 ± 1.0**

Table 3.13: Fatty acid analysis of 1,3-diacylglycerol from individual tissues of *Oncorhynchus mykiss*. Results as means of % fatty acids ± SD. tr., trace (< 0.05), n.d., none detected.

Fatty Acid	Adipose tissue n = 4	Brain n = 4	Eye n = 4	Heart n = 4	Liver n = 4	Muscle n = 4	Spleen n = 3
14:0	0.4 ± 0.4	4.1 ± 2.0	1.2 ± 0.4	1.6 ± 0.7	4.7 ± 1.4	5.1 ± 1.5	1.4 ± 0.5
16:0	7.7 ± 3.4	18.4 ± 4.6	14.4 ± 4.8	17.8 ± 3.9	18.0 ± 4.0	19.9 ± 2.1	11.2 ± 4.1
16:1 n-7	10.1 ± 3.1	2.5 ± 0.4	5.1 ± 1.9	6.0 ± 2.9	7.1 ± 3.5	9.5 ± 1.5	5.5 ± 1.5
16:2 n-4	0.6 ± 0.8	1.4 ± 0.6	1.0 ± 0.5	2.8 ± 1.0	1.0 ± 0.7	1.7 ± 0.0	2.2 ± 0.2
16:3 n-4	2.3 ± 0.7	1.8 ± 0.7	1.4 ± 0.3	2.7 ± 1.2	3.9 ± 1.3	2.1 ± 0.3	2.0 ± 1.1
18:0	18.9 ± 4.4	12.7 ± 3.4	18.0 ± 6.3	20.7 ± 3.3	16.7 ± 4.5	22.1 ± 6.4	11.7 ± 4.9
18:1 n-9	10.8 ± 5.7	8.0 ± 4.4	8.3 ± 3.9	5.8 ± 1.1	7.1 ± 3.8	7.3 ± 2.6	6.7 ± 3.7
18:1 n-7	4.8 ± 1.1	n.d.	n.d.	1.7 ± 2.2	n.d.	n.d.	2.4 ± 0.9
18:2 n-6	5.6 ± 0.4	2.6 ± 1.8	4.5 ± 2.5	5.3 ± 1.8	4.8 ± 1.8	4.5 ± 1.0	3.2 ± 1.4
18:3 n-3	2.6 ± 2.5	1.1 ± 0.2	1.2 ± 0.8	2.4 ± 1.3	3.1 ± 1.7	3.6 ± 1.9	2.3 ± 0.3
18:3 n-4	3.0 ± 1.3	1.1 ± 0.3	0.9 ± 0.5	1.8 ± 0.8	0.7 ± tr.	2.1 ± 0.7	1.1 ± 0.7
18:4 n-3	3.5 ± 2.1	1.8 ± 0.3	1.6 ± 1.0	1.7 ± 0.6	n.d.	2.8 ± 1.2	0.6 ± 0.2
20:1 n-9	9.0 ± 3.5	3.6 ± 1.5	4.0 ± 0.5	2.9 ± 1.0	12.8 ± 5.0	6.7 ± 0.2	5.3 ± 2.3
20:1	n.d.	3.3 ± 0.7	n.d.	5.2 ± 1.9	n.d.	n.d.	n.d.
20:4 n-6	1.6 ± 1.0	1.5 ± 1.3	0.9 ± 0.0	5.6 ± 2.7	8.0 ± 0.4	1.1 ± 0.0	2.0 ± 0.4
20:4 n-3	3.0 ± 0.7	2.3 ± 1.5	2.2 ± 0.1	3.0 ± 1.4	1.1 ± 0.1	1.1 ± 1.5	2.0 ± 0.6
20:5 n-3	**0.3 ± 0.5**	**2.4 ± 1.3**	**0.6 ± 0.1**	**1.0 ± tr.**	**8.7 ± 0.1**	**0.9 ± 0.7**	**1.8 ± 0.5**
22:1	7.4 ± 2.4	29.7 ± 5.2	11.0 ± 2.4	n.d.	n.d.	1.7 ± tr.	1.7 ± 0.9
22:2	3.2 ± 2.6	1.6 ± 0.5	n.d.	n.d.	n.d.	6.5 ± 2.0	24.2 ± 5.4
22:5 n-3	n.d.	n.d.	n.d.	10.3 ± 1.4	4.0 ± 1.0	1.4 ± 2.0	4.1 ± 2.0
22:6 n-3	**5.2 ± 3.1**	**1.2 ± 0.7**	**27.5 ± 9.2**	**2.9 ± 0.7**	**6.1 ± 0.5**	**3.7 ± 1.7**	**10.3 ± 4.2**

3.16 Discussion

The dietary intake of fish and fish-derived products has increased dramatically in recent years (www.fao.org; www.who.int) due to the acceptance that EPA and DHA have beneficial effects on human health and disease (Kris-Etherton *et al.*, 2001; Kromann and Green, 1980; Siscovick *et al.*, 2003). However, as the world's fishing stocks are limited and have come under enormous pressure from over-fishing, mis-management and environmental factors (Schrank, 2005) aquaculture has developed into an ever-expanding and profitable industry (Food and Agricultural Organisation, 1999; Naylor *et al.*, 2000). As the industry continues to develop, the issue of waste disposal becomes increasingly more relevant. In this chapter we set out to categorise the levels of total and individual lipids (together with their acyl components) in the various tissues of farm-reared *Oncorhynchus mykiss*, many of which could contribute to waste.

The impact of diet on the lipid and fatty acid composition of fish has been extensively reviewed and it is now accepted that tissue lipid and fatty acid composition are reflective of the type of diet consumed (Jeong *et al.*, 2002; Karapanagiotidis *et al.*, 2007; Miller *et al.*, 2007; Stubhaug *et al.*, 2005). We determined that the principal fatty acids found in the diet of the trout used in these experiments included 18:1 n-9 (18%), 18:3 n-3 (14%), 16:0 (13%), 22:1 (9%), 20:1 n-9 (9%), DHA (8%) and EPA (5%). It was therefore not surprising that the acyl composition of the tissues examined were predominantly composed of the aforementioned fatty acids (Tables 3.4 – 3.10). Kennedy *et al.* (2005) observed that fish reared on a diet which contained elevated levels of conjugated linolenic acid (CLA) incorporated CLA into tissue lipids with a two-fold increase in CLA observed in the liver compared to the flesh. Further evidence suggests that replacement of EPA and DHA rich diets with vegetable oil diets results in lower tissue concentrations of EPA and DHA, despite the biosynthetic activity that converts 18:3 n-3 to EPA and DHA (Karapanagiotidis *et al.*, 2007). Another study which investigated the effect of feeding diets rich in linseed oil altered the fatty acid composition of the muscle and liver resulting in a decrease in the concentration of highly unsaturated n-3 PUFAs (EPA, DHA) in the non-polar fraction of fish flesh, whilst no effect was observed in the polar fraction (Menoyo *et al.*, 2005). Menoyo *et al.* (2005) did however notice an increase in 18:3 n-3 and an accumulation

of desaturation and elongation products in the liver. Bell *et al.* (2003) concluded that a significant loss in tissue n-3 PUFAs were observed in Atlantic salmon when dietary fish oil was replaced with > 66% vegetable oils.

In order for offal from farmed fish to be considered as a potential source of n-3 PUFAs it was necessary to determine the tissue levels of such fatty acids. In terms of total lipid (Fig. 3.1), as expected, the visceral adipose tissue contained the highest levels of lipids (19g/100g fresh weight). We found that brain tissue was composed of approximately 18 g of total lipid/100 g fresh weight. Previous research has reported approximately 7% of total lipid in the brain of *Oncorhynchus mykiss,* (Stoknes *et al.*, 2004). Stoknes *et al.* (2004) also reported significant differences in the total lipid found in brain between species. They reported the lowest levels of total lipid in Portuguese and black dogfish brains (3.8% and 3.2%) whereas for salmon they reported 7%. It is known that the total lipid content in brain is elevated during development and discrepancies may be due to variations in fish development (Mourente and Tocher, 1992). Differences in water temperature have also been found to affect brain lipid composition (Johnston and Roots, 1964). Differences between what we determined as the total lipid content in *Oncorhynchus mykiss* and what was reported by Stoknes *et al.* (2004) may be explained by the age of the fish and/or temperature acclimation. Also, it is widely known that season and farming conditions affect levels of lipids (Ackman and Takeuchi, 1986; Argen *et al.*, 1987; Senso *et al.*, 2007), therefore this may account for differences observed between our data and those of Stoknes *et al.* (2004).

Total lipid in the eye of *Oncorhynchus mykiss* was determined to be approximately 3.9 g/100 g fresh weight (4%) (Fig. 3.1). Previous research (Stoknes *et al.*, 2004) provided evidence that the eyes of salmon and trout contained particularly high amount of lipids compared to several other species (cod, saithe, redfish, Portuguese and black dogfish and the leafscale gluper shark). However, we determined the levels of total lipid in the eye (4%) of *Oncorhynchus mykiss* to be much less than the values reported by Stoknes *et al.* (2004) for both trout and salmon (48% and 36% respectively). Moreover, our results were more similar to the levels reported for Portuguese dogfish.

The levels of total lipid determined in the liver, heart, muscle and spleen were all comparable to each other (18 – 14 mg/g fresh weight) (Fig. 3.1). The levels of total lipid reported by Bandarra *et al.* (2006) in the liver and muscle for *Oncorhynchus mykiss* were 2.8% and 4.1% respectively. The fish used by Bandarra *et al.* (2006) were classified as juveniles whereas the fish used in our experiments were considered adult (~2.5 yrs) and differences in age may explain these variations in total lipid content in the liver and muscle. Also, another factor that should be taken into account is season. Bandarra *et al.* (2006) do not specify in which season the fish were reared and the literature suggests that season has a large effect on lipid accumulation and composition in fish (Agren *et al.*, 1987; Senso *et al.*, 2007). Miller *et al.* (2007) found similar levels of total lipid in the muscle of Atlantic salmon, 17.8 mg/g fresh weight to our levels for trout. They also reported that the levels of total lipid were dependent on the type of diet consumed. When the fish were subjected to a high fish oil diet (24% EPA and 15% DHA) the levels of total lipid in muscle increased significantly. However, the use of such EPA- and DHA-saturated diets have implications in terms of the organoleptic qualities of the flesh for human consumption. Therefore, the levels of EPA and DHA are normally lower (the diet used in our analysis consisted of approximately 5% EPA and 8% DHA). Our results for total lipid in muscle (1.5%) are also consistent with the levels of total lipid reported in the muscle of the carnivorous, fresh-water fish, the zander *(Sander lucioperca)* analysed during the winter months (1.3%) but not during the spring (0.6%), summer (0.8%) or autumn (0.6%) (Guler *et al.*, 2007). Differences between total lipid in the muscle of wild and farm-reared fish have also been recorded. Rueda *et al.* (2001) reported that the total fat content of sea bream was elevated in the white muscle and liver of reared sea bream compared to its wild counterpart. Interestingly, no differences between total lipid in muscle and liver of wild and captive sea bream were observed by Cejas *et al.* (2004). As only farm-reared trout were analysed in our experiments it may be interesting to compare the levels of total liver and muscle lipid to wild trout of similar age. Another factor that may affect total liver lipid is water temperature. Hazel (1979) observed that cold-acclimated (5°C) trout *(Salmon gairdneri)* possessed significantly more liver tissue per unit of body weight, and significantly less non-polar lipid per gram of liver than warm-acclimated (20°C) individuals.

Total lipid in the heart muscle was determined to be approximately 17.7 mg/g fresh weight (1.8%) (Fig. 3.1). Similar levels were reported by Castell *et al.*, (1972). They found approximately 2.1% lipid in rainbow trout hearts *(Salmo gairdneri)* although this was dependent on the diet and varied from 2.1% - 3.5%.

Individual lipids play a variety of specific roles in fish biology and oftentimes the lipid content of fish can markedly exceed the protein content, indicating the major role lipids and their specific acyl components play as sources of metabolic energy in fish, growth, reproduction and migration (Kennedy *et al.*, 2005). Specific lipids can either be incorporated into cellular membranes of tissues, catabolised to provide energy or deposited in adipose tissue as an energy reserve (Tocher, 2003). We separated the individual lipid classes and determined that the main non-polar lipid in all tissues was TAG (Table 3.2). Of the phosphoglycerides, PC, PE, PI and PS were the predominant polar lipids in all tissues. Minor levels of SPH, DPG and lyso-PC were also identified in certain tissues (Table 3.1). These particular phospholipids were previously reported as the major polar lipids in the total body lipids of rainbow trout, *Salmo gairdneri* (Castledine and Buckley, 1982). As previously discussed, (section 3.8.1), significant variations in the percentages of individual phospholipids were observed between tissues whereas the TAG was consistently the predominant non-polar lipid in all tissues (section 3.8.2).

Similar to the results reported by Castledine and Buckley (1982), we observed significant heterogeneity in the acyl constituent and their percentages for the individual phosphoglycerides. However, the TAG fraction displayed relatively similar fatty acid patterns in each tissue apart from some minor details in liver-derived TAG (Table 3.10). The TAG fraction of muscle from one-year old black seabream (Rodriguez *et al.*, 2004) was relatively similar to our observations although we observed higher levels of 18:1 n-9 in *Oncorhynchus mykiss*. As previously mentioned, fish lipid and acyl composition can be influenced by numerous conditions including season, water temperature and habitat. Cejas *et al.* (2004) determined significant differences between the acyl composition of TAG from liver and muscle in wild and captive white seabream. In terms of the PUFA content of TAG from the liver our findings compare well with the levels of 18:2 n-6 and EPA recorded for captive seabream although the the DHA levels were more closely related to the levels

observed from the wild seabream (Cejas *et al.*, 2004). Some possible explanations for this may be due to the age differences, genetics, rearing methods and habitat variations between the seabream and *Oncorhynchus mykiss.*

Overall, as expected, the membrane lipids of *Oncorhynchus mykiss* demonstrated greater levels of EPA and DHA compared to the non-polar lipid fractions. In contrast to mammalian-derived membrane lipids, AA was a minor component of membrane lipids in most tissues which was consistent with the literature (Cejas *et al.*, 2004; Tocher and Sargent, 1987). In comparison to marine fish, fresh-water fish such as *Oncorhynchus mykiss* have the capacity to synthesize the longer chain PUFAs from their 18 carbon precursors (Henderson and Tocher, 1987). However, it has been reported that the efficiency of this pathway is not very high and it has been established that feeding preformed EPA and DHA results in higher tissue levels of EPA and DHA. It was interesting to observe that, despite the diet being composed of 5% EPA, tissue levels of EPA remained quite low whereas tissue DHA was much greater than the 8% DHA that the diet provided. As fish lipid and fatty acid composition can be manipulated by dietary intervention as well as seasonal, habitat and rearing methods it would be interesting to repeat the analyses to determine the effects of each of these parameters. However, the relative enrichement of DHA over EPA in trout tissue lipids, compared to diet, suggests that trout have a significant capacity to biosynthesise DHA.

To conclude we determined that farmed *Oncorhynchus mykiss* tissues were a rich source of the valuable n-3 PUFAs and, thus, fish waste could provide these acids, especially EPA and DHA for nutraceutical development whilst reducing disposal costs and environmental implications.

CHAPTER 4

Effect on Storage Time and Processing Method on the n-3 Polyunsaturated Fatty Acid Content of Farmed Oncorhynchus mykiss Waste Tissues

4.0 Introduction

Rendering has long been employed in the extraction of lipids from a variety of animal products including fish. In brief, rendering means to 'split open by the process of heating'. There are two main forms of rendering, wet and dry systems. Dry rendering involves the removal of vapour once the tissue has been disrupted by steam, whereas with wet rendering, heat is applied to melt the fat and the resulting meal and fat still contain water after the decanting phase (Woodgate and van der Veen, 2004).

Throughout the fish oil industry wet rendering is commonly employed as the principal method for the extraction of fish lipids (Aidos *et al.*, 2003). This involves mincing the starting material which is then conveyed through a steam heated continuous cooker where it is heated to 100 °C. Such conditions sterilise the fish whilst coagulating proteins and disrupting cell membranes facilitates the separation of oil from the dry matter. Once rendered, the material moves to a continuous screw-press system where most of the liquid is squeezed out to form a press-cake which is removed and sent for drying thus separating the solid and liquid phases. The liquid phase (oil, water and some dissolved proteins and salts) is passed through a decanter which removes dissolved proteins and salts. Such proteins and salts are transported to the drier and combined with the press-cake. Liquid from the decanter is fed to separators where the oil is removed and enters the refining process. Refining involves several or all of the following steps: removal of free fatty acids and some metal contaminants, bleaching, winterization (removal of saturated fats by a cold press or filter-type system) and de-odourisation. At this stage vitamins which may have been removed during de-odourisation may be added back to the oil depending on its final use (Breivik, 2007; Gunstone *et al.*, 2007).

In order to compare wet rendering to other methods of lipid extraction, a laboratory scale model of wet rendering was employed. For comparison, we used Garbus (laboratory-based) method for lipid extraction. The third method used was a single solvent and was based on an alternative procedures used in some industrial processes (Gunstone *et al.*, 2007).

The Bligh and Dyer method of which that of Garbus *et al.* (1963) is a variant, is commonly used with natural tissues and is widely regarded as one of the most effective methods for extracting lipids. However, employing this method on an industrial scale basis would not prove cost-effective and several problems would be encountered. Not only would this method give rise to huge solvent disposal costs it would also demand a longer analysis time, specialised equipment, skilled personnel and extensive health and safety measures (Johnson and Barnett, 2003). Moreover, chloroform is now considered a possible carcinogen and its use in the production of food and foodstuff for human and animal consumption is strictly prohibited (Fang *et al.*, 2007; van Vleet and Schnellmann, 2003).

The final system investigated was a single-solvent based method. Petroleum ether was the solvent used as it is commonly used in the Soxhlet method for lipid extraction. In brief, the Soxhlet method involves the starting material being suspended in a porous thimble of a Soxhlet apparatus, for example the Büchi Universal Extraction System, (Flawil, Switzerland) where the lipids are extracted by petroleum ether or its vapour (Manirakiza *et al.*, 2001). Disadvantages of this method include the lengthy analysis time required, poor extraction of polar lipids, large volumes of solvents and in some cases, boiling of hazardous solvents (Johnson and Barnett, 2003). Due to such complications this method is primarily used as a reference method and, nowadays, is less used for the commercial production of oil. The petroleum ether method described in this chapter is a modification of the cold Soxhlet method.

Chapter 4: Materials & Methods

4.1 Collection and Dissection of *Oncorhynchus mykiss*

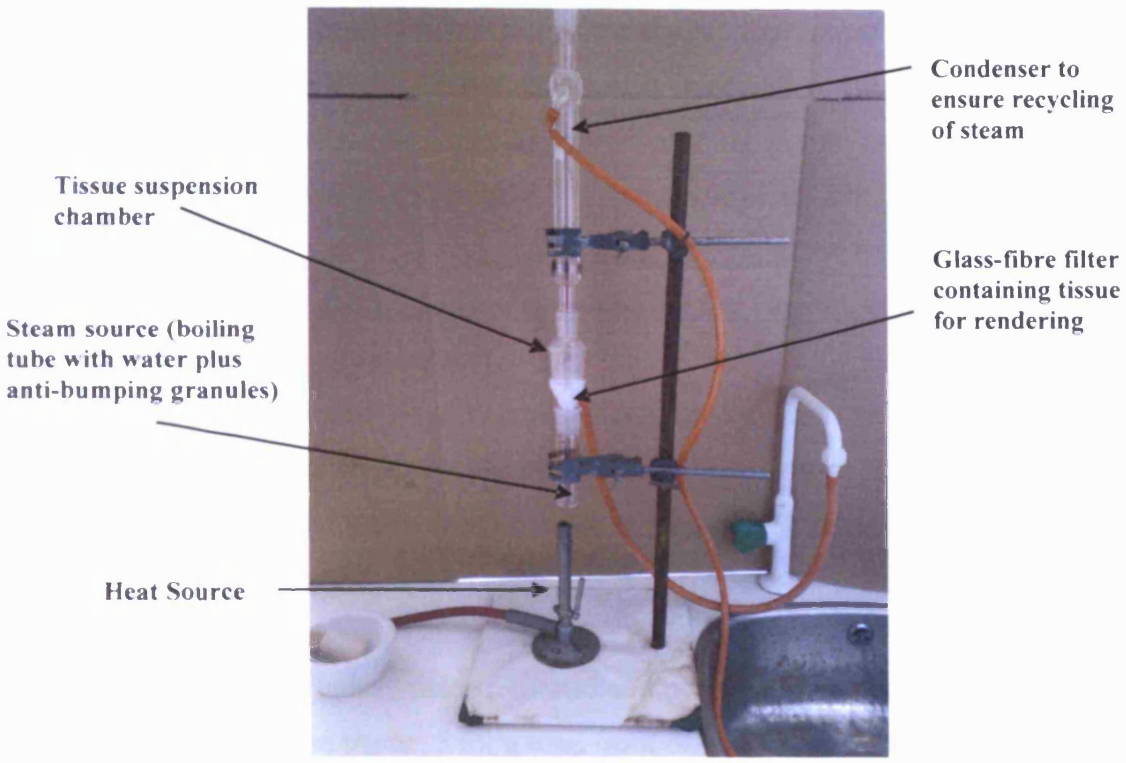
Rainbow trout were collected fresh from the trout farm on the morning of the experiment and transported back to the laboratory stored on ice. For the experiments using visceral adipose tissue, the tissue was dissected from the intestinal cavity and rinsed in physiological saline. For the experiments using total trout offal as the lipid source liver, spleen, eyes, heart, brain and eggs were dissected from the fish, rinsed in physiological saline and blended together to ensure complete homogenisation of the tissue. The resulting paste was weighed (Extend, Sartorius, USA) and allocated to the various lipid extraction methods and time points (0 h, 24 h and 48 h).

For both starting materials, time zero samples were extracted immediately at room temperature by each method under investigation whereas samples for the 24 and 48 h time points were exposed to the atmosphere and sunlight at room temperature (25 °C) in order to mimic the probable offal storage conditions on farms before they were extracted.

4.2 Trout Oil Lipid Extraction using a Wet Rendering Method

In order to mimic the conditions employed by industry for the wet rendering extraction of fish oils a bench-top apparatus was developed. This consisted of a condenser fitted to a glass chamber where the tissue was suspended on glass-fibre membrane (Whatman, UK). The role of the condenser was to prevent evaporation of the steam during the rendering process so that the steam was recycled throughout the process. Attached to the suspension chamber was the steam source, a glass boiling tube containing distilled water (15 ml) and anti-bumping granules (BDH, UK) (Fig. 4.1). Steam was generated by heating the water using a Bunsen burner. The presence of condensed water droplets above the tissue indicated that the steam was in fact passing through the glass-fibre membrane and, thus, rendering the tissue.

Fig. 4.1: Laboratory scale apparatus used for the wet rendering of trout tissues.



Starting material was placed on the moistened glass-fibre filter which was positioned inside the suspension chamber. The suspension chamber was located between the condenser and the boiling tube and held together using a clamp.

To initiate the rendering process water (15 ml) in the boiling tube was heated using a Bunsen burner until a steady flow of steam was moving up through the boiling tube and suspension chamber. To prevent evaporation of the steam, cold water was continuously passed though the condenser. This process was undertaken for 15 min before the apparatus was allowed to cool and the tissue was transferred to a tissue separating cloth placed inside a mortar and pestle. Here the tissue was pressed and the resulting liquid was transferred to a glass test tube. The water (15 ml) utilised to render the material was used to rinse the mortar and pestle and added to the test tube. As a final wash, distilled water (2 ml) was used to rinse the mortar and pestle and this was combined with the initial water and sample.

In order to determine that the liquid phase had not become acidic due to the generation of free fatty acids during the rendering process the pH was tested with full-range pH paper (Whatman, UK). To separate the oil, the sample was centrifuged on a Baird & Tatlock Auto Bench Centrifuge Mark IV for 5 min at 1000 x g_{av} (x2). The top lipid layer was removed using a glass pipette and transferred to a glass vial, flushed with nitrogen, dissolved in chloroform (1 ml) and stored at -20°C.

4.3 Trout Oil Lipid Extraction using the Garbus Method

Once removed and rinsed in physiological saline, the tissue (adipose tissue or total trout offal) was extracted by the Garbus method (Garbus *et al.*, 1963). Depending on the amount and type of tissue used as the starting material for lipid extraction different amounts of solvents were used.

Samples were homogenised using a mortar and pestle in chloroform/methanol (1:2, by vol.) (2.5 ml/g adipose tissue, 5 ml/g total trout offal homogenate) and transferred to a glass 10 ml tube via a glass wool filter to remove any residual particles. Mortar and pestle were rinsed with chloroform/methanol (1:2, by vol.) and this was combined with the sample. Chloroform (2 ml/g adipose tissue, 4 ml/g total trout offal homogenate) was then added to sample together with Garbus solution (2 ml/g adipose tissue, 4 ml/g total trout offal homogenate) (2 M KCl in 0.5 M potassium phosphate buffer, pH 7.4) and distilled water (1 ml/g adipose tissue, 2 ml/g total trout offal homogenate). After thorough mixing, the solutions were allowed to stand for 15 min at room temperature to allow the separation of the two phases. The mixture was vortexed again and centrifuged on a Baird & Tatlock Auto Bench Centrifuge Mark IV for 5 min at 1000 x g_{av}. The upper aqueous layer was removed and discarded using Pasteur pipettes. The lower lipid-containing layer was transferred to a clean 10 ml glass tube and dried under a stream of nitrogen. Lipids were dissolved in a known volume of chloroform and stored at -20°C. Preliminary data from our laboratory indicated that qualtitative extraction was achieved using the volumes outlined.

4.4 Trout Oil Lipid Extraction using an Industrial Solvent Method

A solvent system based on frequently-used industrial methods for lipid extraction was the third method compared in these experiments. Tissue was homogenised in mortar and pestle with petroleum ether (3 ml/g adipose tissue, 4 ml/g total trout offal homogenate) before being filtered through glass wool into a 10 ml tube. The mortar and pestle were rinsed with 2 ml petroleum ether and this was added to the homogenate. Sodium chloride (5%) (3 ml/g adipose tissue, 4 ml/g total trout offal homogenate) was added to the homogenate and the mixture vortexed followed by centrifugation (x2) on a Baird & Tatlock Auto Bench Centrifuge Mark IV for 5 min at 1000 x g_{av}. The upper lipid layer was removed and brought to dryness under nitrogen before being dissolved in chloroform and stored at -20°C.

4.5 Effect of Storage Time and Extraction Method on Total Lipid Extracted from Total Trout Offal

In terms of total lipid extracted the Garbus extraction proved to be the most efficient method employed yielding approximately 38 mg lipid/g tissue homogenate at each time point. As for the petroleum ether and rendering methods, both yielded approximately 15 mg lipid/g tissue homogenate on initial extraction. With the petroleum ether method, the yield increased significantly ($p < 0.001$) with time yielding approximately 32 mg/g tissue homogenate at time 48 h (Fig. 4.2A).

The rendering method also demonstrated significantly increased ($p < 0.05$) lipid yields with storage time – increasing from 16 mg/g at time zero to 21 mg/g at time 24 h with a further increase to 25 mg/g at time 48 h (Fig. 4.2A).

At time zero the Garbus method was significantly more effective than both the petroleum ether and rendering ($p < 0.001$) methods. At time 24 h no statistical differences were observed in total lipid yield between the Garbus and petroleum ether methods but both these methods were significantly different to the rendering method ($p < 0.001$) (Fig. 4.1). The rendering method saw an increase in total lipid yield at 48 h and due to this no difference was observed with the petroleum ether method but the yield was still significantly less that that observed for the Garbus method at 48 h ($p < 0.05$). Overall, the Garbus method was consistently more effective at extracting total lipid at each time point compared to the petroleum ether and rendering methods. Moreover, it gave maximum yield at time zero, in keeping with its efficiency as a laboratory method for lipid extraction (Fig. 4.2A).

4.6 Effect of Storage Time and Extraction Method on Triacylglycerol Yields from Total Trout Offal

The greatest amount of TAG extracted at time zero was 32 mg/g tissue homogenate and was achieved by the Garbus method. Both the petroleum ether and rendering methods produced approximately one third of this yield (11 mg/g tissue homogenate) (Fig. 4.2B).

No statistically significant changes were observed in TAG yield with the Garbus method at each time point. However, the TAG yield produced by the petroleum ether method at 24 h and 48 h was significantly different to time zero ($p < 0.001$). However, no differences were observed between 24 and 48 h. The rendering method also demonstrated an increase in TAG yield with time. The increase in the TAG fraction was significantly different at 48 h compared to time zero ($p < 0.05$). For both the petroleum ether and rendering methods the apparent increase in extraction efficiency with time may be attributed to degradation of the tissue structure by phospholipases and proteinases during storage.

Overall, the levels of TAG produced by the Garbus method were shown to be the highest and most consistent over time (Fig. 4.2B). Generally speaking, the Garbus method was significantly more effective at extracting the TAG fraction compared to both the petroleum ether and rendering methods ($p < 0.001$) at time zero. As seen with the extraction of total lipid, (Fig. 4.2A) Garbus and petroleum ether methods were significantly more effective at time 24 h compared to the rendering method ($p < 0.001$) but not different from each other. By time 48 h the rendering method was only significantly different from the Garbus method ($p < 0.05$) but had become comparable to the petroleum ether method (Fig. 4.2B).

Fig. 4.2A: Comparison of the efficiency of extraction by three lipid extraction methods used to extract total lipid from total trout offal after different storage times. Results as means ± SD (n=5). * denotes significance of storage time within method. † denotes significance between methods.

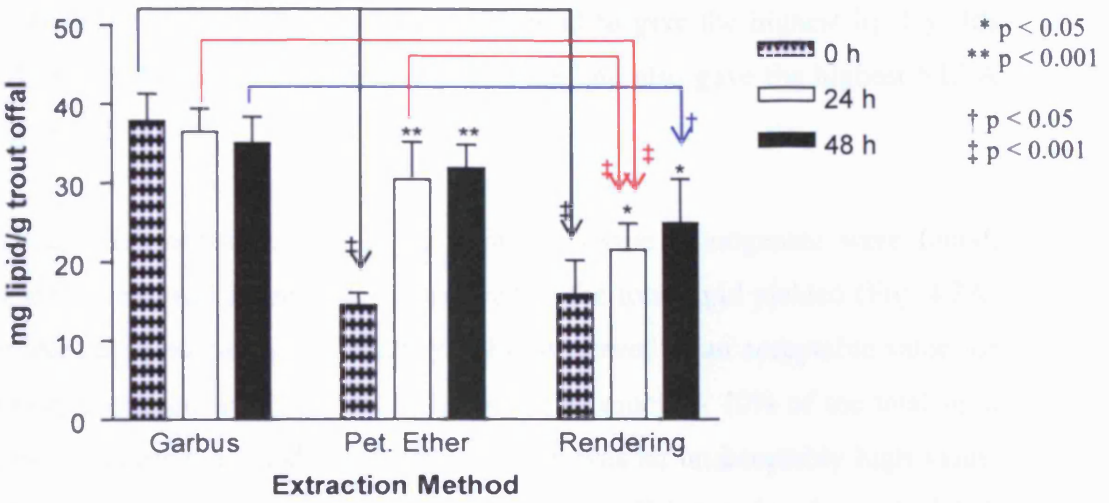
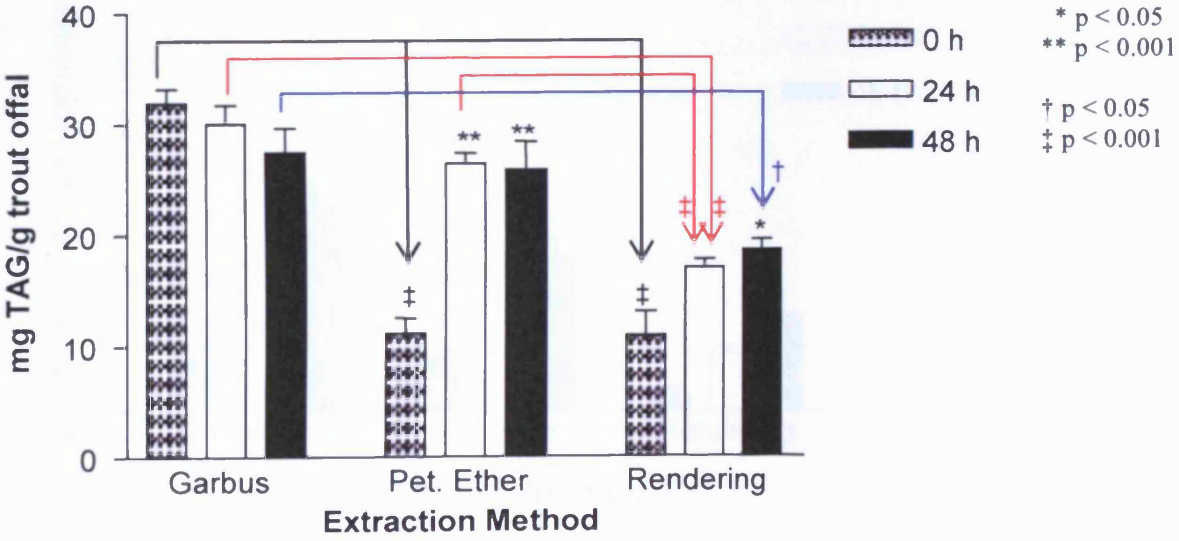


Fig. 4.2B: Comparison of the efficiency of extraction by three lipid extraction methods used to extract triacylglycerols from total trout offal after different storage times. Results as means ± SD (n=4). * denotes significance of storage time within method. † denotes significance between methods.

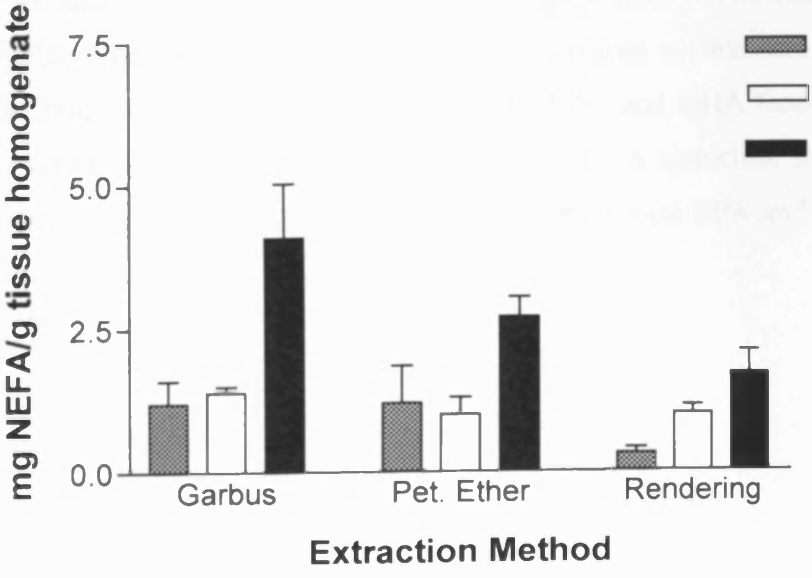


4.7 Effect of Storage Time and Extraction Method on Non- Esterified Fatty Acid Yields from Total Trout Offal

Since NEFAs are most likely released by tissue lipases or phospholipases, then higher amounts found after storage of the offal at room temperature are to be expected. Because the Garbus method of extraction was found to give the highest lipid yields (Figs. 4.2A, 4.2B) it was not surprising that the method also gave the highest NEFA values (Fig. 4.2C).

After 24 h approximately 0.5 – 1.2 mg NEFA/g tissue homogenate were found, depending on the extraction method. Compared to the total lipid yielded (Fig. 4.2A) this represented approximately 2% which might be viewed as an acceptable value for an industrially-produced lipid. By 48 h, however, as much as 10% of the total lipid extracted by the Garbus method was NEFA, which was an unacceptably high value. If these amounts reflect the deterioration in quality of offal stored under typical fish farm conditions, then the storage conditions would need modifying.

Fig. 4.2C: Yield of non-esterified fatty acids (mg FFA/g tissue homogenate) over time in total trout offal evaluated by different extraction methods. Results as means ± SD (n=5).



4.8 Effect of Storage Time and Extraction Method on the Fatty Acid Profiles of Individual Lipids from Total Trout Offal

The effects of storage time and extraction method on the percentage changes of the fatty acid profiles of individual lipids from total trout offal are outlined below (Tables 4.1 – 4.16). EPA and DHA are the most important fatty acids found in fish oil due to their biological properties and their levels need to be conserved during processing of the oil. Figs 4.3-4.8 represent the effect of tissue storage time on the levels of these fatty acids in the total polar lipid fraction as well as the triacylglycerol and the non-esterified fatty acid (NEFA) fractions extracted using the different methods.

The major fatty acids found in the total polar lipid fraction extracted using the Garbus method at time zero included DHA > 16:0 > 18:1 n-9 > EPA > 18:2 n-6. Over storage time there was a decrease in the percentage of DHA to 21.6% and of EPA to 2.9% which was coincident with a relative increase in the main monounsaturated fatty acids, 16:1, 18:1 n-9, 20:1 n-9, 22:1 and 24:1. These effects were not unexpected as the levels of PUFAs are believed to decrease with time, due to deterioration of the tissue, resulting in a relative increase in monounsaturated fatty acids (Table 4.2). As EPA and DHA are found in greater amounts in the total polar lipids it was necessary to look at the relative changes in lipid classes at different times in order to explain the decrease in EPA and DHA over time. Table 4.1 outlines the major lipids recovered by the three different extraction methods at the three time points examined. For the Garbus method the yield of total polar lipids was greatest at time zero. Moreover, the total polar fraction was shown to contain more EPA and DHA than any other lipid fraction examined (Tables 4.2, 4.5, 4.8, 4.11 4.14). A reduction in the total polar fraction with time partly accounts for the reduction of total EPA and DHA seen with the Garbus method (Figs. 4.3, 4.4).

Table 4.1 Effect of tissue storage time and extraction method on total trout offal lipids (mg/g tissue homogenate) based on total fatty acid analysis. Percentage of total lipid values are given in parenthesis. Results as means ± SD (n=4).

	GARBUS				
	TAG	**MAG**	**DAG**	**NEFA**	**TP**
0 h	32.0 ± 2.5	0.8 ± 0.6	0.3 ± 0.1	1.2 ± 0.8	3.0 ± 1.7
	(92)	(1.2)	(0.4)	(1.9)	(4.9)
24 h	30.1 ± 3.4	1.0 ± 0.3	0.5 ± 0.2	1.4 ± 0.2	1.9 ± 0.4
	(88.6)	(2.8)	(1.5)	(4.3)	(5.3)
48 h	27.4 ± 4.5	1.2 ± 0.4	1.3 ± 0.6	4.1 ± 1.9	1.3 ± 0.3
	(78.3)	(3.2)	(3.6)	(11.3)	(3.7)
	PETROLEUM ETHER				
	TAG	**MAG**	**DAG**	**NEFA**	**TP**
0 h	11.2 ± 2.7	0.6 ± 0.6	0.2 ± 0.2	1.2 ± 1.3	0.2 ± 0.1
	(95.4)	(1.7)	(0.4)	(2.2)	(0.3)
24 h	26.5 ± 1.8	1.0 ± 0.6	0.7 ± 0.6	1.0 ± 0.6	0.5 ± 0.2
	(90.3)	(3.3)	(2.1)	(3.2)	(1.7)
48 h	25.9 ± 4.5	0.6 ± 0.3	0.7 ± 0.1	2.7 ± 0.7	0.7 ± 0.3
	(84.7)	(2.0)	(2.4)	(8.7)	(2.2)
	RENDERING				
	TAG	**MAG**	**DAG**	**NEFA**	**TP**
0 h	10.9 ± 3.8	0.3 ± 0.1	0.1 ± 0.0	0.3 ± 0.2	0.3 ± 0.2
	(91.9)	(2.5)	(0.8)	(2.0)	(2.8)
24 h	17.0 ± 1.3	0.4 ± 0.1	0.4 ± 0.2	1.0 ± 0.3	0.3 ± 0.1
	(89.4)	(1.9)	(2.0)	(5.1)	(1.7)
48 h	18.6 ± 1.7	0.5 ± 0.1	0.3 ± 0.1	1.7 ± 0.8	0.2 ± 0.1
	(87.5)	(2.2)	(1.2)	(8.1)	(0.3)

* TAG, triacylglycerol; DAG, diacylglycerol (1,2-DAG and 1,3-DAG combined); MAG, monoacylglycerol; NEFA, non-esterified fatty acids; TP, total polar lipid fraction.

The major fatty acids observed in the total polar lipids extracted by the petroleum ether method included 18:1 n-9 > DHA > 16:0 > 18:2 n-6 > 18:0 > 16:1 n-7 > 22:1 > 20:1 n-9 > EPA (Table 4.3). The levels of the monounsaturated fatty acids 20:1 n-9 and 24:1 extracted with the petroleum ether method increased with time whilst there was a decrease in the level of 16:1 n-7. No significant changes in the levels of EPA and DHA were detected (Fig. 4.3-4.4).

In order to explain changes in the acyl composition of the total polar fraction extracted by the petroleum ether method it is important to consider changes in the different lipid fractions (Table 4.1). The total polar lipid fraction was lowest at time zero and increased over time (0.3% at time zero to 2.2% at time 48 h). Petroleum ether as a non-polar solvent is not considered to be an effective method at extracting the more polar lipids. Such an increase can be explained by the effect of storage time and tissue degradation. Degradation of the tissue with time would be expected to result in a loss of membrane integrity which in turn could cause the release of membrane lipids and fatty acids. Due to this release, the lipids and fatty acids may become easier to extract with petroleum ether resulting in an increase of such components.

In the total polar lipid fraction extracted by the rendering method from total trout offal the major fatty acids consisted of 18:1 n-9 > 16:0 = DHA > 18:2 n-6 > 20:1 n-9 > 22:1 > EPA (Table 4.4). No statistically significant changes in percentage fatty acids were observed with time. Overall, the pattern of fatty acids in the polar lipid fraction was similar to that yielded by the petroleum ether method (Table 4.4). Thus, unlike the Garbus method, no significant differences were observed in the percentages of EPA and DHA with time (Table 4.4, Figs. 4.3 and 4.4). In general, no major differences in the percentage acyl composition of total polar lipids extracted using the rendering method were recorded with storage time (Table 4.4). Moreover, the percentage of total polar lipids (Table 4.1) did not change from zero h to 48 h (and remained low throughout) and the lack of changes for EPA and DHA may be attributed to this.

Comparison of the three methods used to extract total polar lipids showed that the Garbus method was best for extracting PUFAs. Petroleum ether is not considered a

good method for extraction of total polar lipids and because total polar lipids are enriched with PUFAs, the lower levels of DHA and EPA in petroleum ether extracts may be due to reduced extraction efficiency.

In terms of the acyl composition of the TAG fraction, there was very little variation between extraction methods. At time zero for all three extraction methods, the principal fatty acids in TAG included 18:1 n-9 > 18:2 n-6 > 16:0 > DHA > 20:1 n-9 = 22:1 > 18:0 > EPA (Tables 4.5 – 4.7). No apparent changes in the acyl composition of the TAG fraction were observed over time with any of the three methods examined. This included the long chain PUFAs, EPA and DHA (Figs. 4.5 and 4.6) for which no significant changes were detected either with extraction method or with storage time. Each method yielded approximately 2% EPA and 8% DHA in the TAG fraction at each time point (Tables 4.5 – 4.7).

The major fatty acids in the diacylglycerol (DAG) fraction extracted by the Garbus method at time zero were 18:1 n-9 > 16:0 = 18:2 n-6 > 20:1 n-9 = 22:1 > DHA > 18:0 > EPA > 16:1 n-7. Surprisingly, the levels of EPA and DHA appeared to increase with time. This may reflect selective hydrolysis of TAG which may enrich the DAG fraction with EPA and DHA. Due to the low levels of DAG present in total trout offal it is not unexpected that some of the minor fatty acids were not detected after lipid extraction (Table 4.8).

Very few of the minor fatty acids were detected in the DAG fraction extracted by the petroleum ether method at time zero (Table 4.9). However, most of these minor acyl components were detected for tissue stored for 24 or 48 h. This effect may be due to degradation of the tissue over time resulting in a subsequent increase in the DAG fraction (Table 4.1). The major fatty acids present in DAG extracted at time zero included 18:1 n-9 > 16:0 = 18:2 n-6 > 18: 0 > 22:1 = DHA = 20:1 n-9 > EPA > 16:1 n-7. There were no obvious and consistent changes in the fatty acid composition of the DAG fraction with tissue storage time (Table 4.9).

The DAG fraction extracted by the rendering method (Table 4.10) at time zero was similar to that extracted by the petroleum ether method (Table 4.9) in that many minor fatty acids were not detected. With time, these components became detectable

perhaps due to tissue degradation and generation of more DAG. The principal fatty acids included 18:1 n-9 > 16:0 = DHA = 22:1 = 20:1 n-9 = 18:2 n-6 > 18:0 > EPA. After 48 h of tissue storage, there seemed to be a decrease in the relative proportion of EPA and DHA (Table 4.10).

For each extraction method the levels of DAG increased as a function of storage time (Table 4.1). DAG is a product of TAG hydrolysis by lipases or of phospholipid catabolism from phospholipase C. Activity of either of these enzymes might be expected to increase DAG levels during storage.

The detection of minor fatty acids in the monoacylglycerol (MAG) fraction extracted using the three methods under investigation was noticeably better than for DAG (Tables 4.11 – 4.13 and 4.8 – 4.10). As seen with previous non-polar lipid fractions the major fatty acid found in MAG extracted using three methods was the monounsaturated fatty acid, 18:1 n-9. The remaining fatty acids that constituted the MAG fraction included 16:0, 18:0, 16:1 n-7, 20:1 n-9, 22:1, 18:2 n-6, EPA and DHA. For each method examined, no significant changes were observed in the fatty acid profiles over tissue storage time. However, the levels of EPA and DHA were observed to be somewhat variable over time, although these changes were not significantly different for any of the three methods (Tables 4.11 – 4.13). Table 4.1 indicates that, although the MAG fraction tended to increase as a function of storage time for each extraction method, any increases were not statistically significant.

No variation due to the extraction method used was detected in the percentage acyl composition of NEFAs generated with time (Tables 4.14 – 4.16, Figs. 4.7 and 4.8). The major fatty acids present in the NEFA fraction were consistent and also independent of tissue storage time. The approximate levels of the principle fatty acids detected at each time point for each method were 18:1 n-9 > DHA > 18:2 n-6 > 16:0 > 20:1 n-9 > 22:1 > EPA > 18:0 > 16:1 n-7 (Tables 4.14 – 4.16). In terms of percentage lipids (based on total fatty acid analysis) the NEFA fraction increased as a function of tissue storage time for each extraction method investigated (Table 4.1). However, the levels of NEFAs generated were different between extraction methods. The Garbus method recovered the greatest amount of NEFAs with time (Table 4.1). However, the Garbus method was the most effective method of lipid extraction (Fig. 4.2A, 4.2B)

and, due to this, greater levels of NEFAs were expected. The petroleum ether and rendering methods also saw an increase in NEFA levels with time. After 48 h these represented 8-9% of the total lipid (Table 4.1). The rise in percentage levels of NEFAs is consistent with tissue degradation and release of fatty acids from complex lipids.

Fig. 4.3: EPA values as percentage of total fatty acids in the total polar lipid fraction of trout offal recovered using three methods of lipid extraction at different tissue storage times. Results as means ±SD (n=3). * denotes significance of storage time within method. † denotes significance between methods.

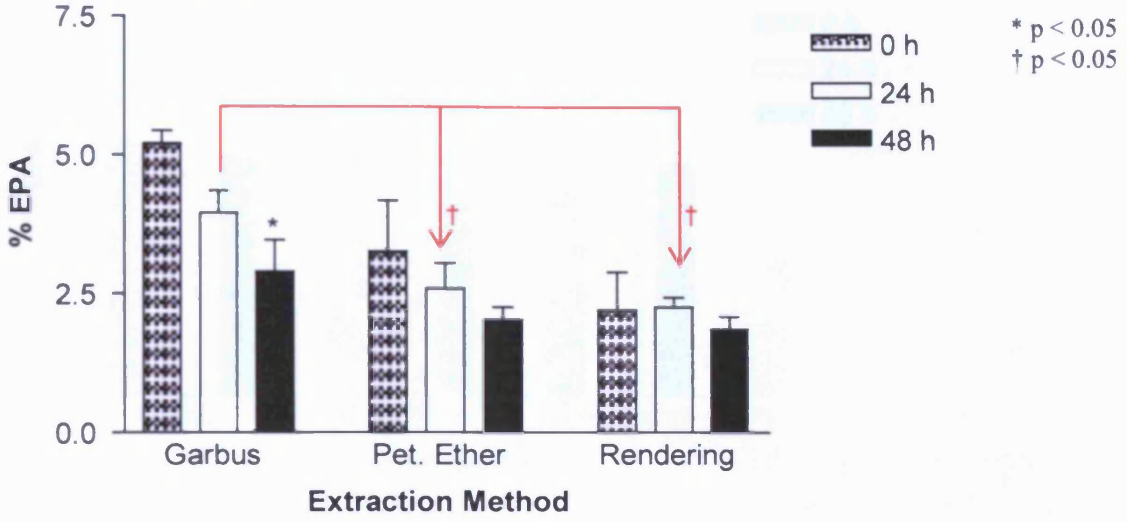


Fig. 4.4: DHA values as percentage of total fatty acids in the total polar lipid fraction of trout offal extracted using three methods of lipid extraction at different tissue storage times. Results as means ±SD (n=3). * denotes significance of storage time within method. † denotes significance between methods.

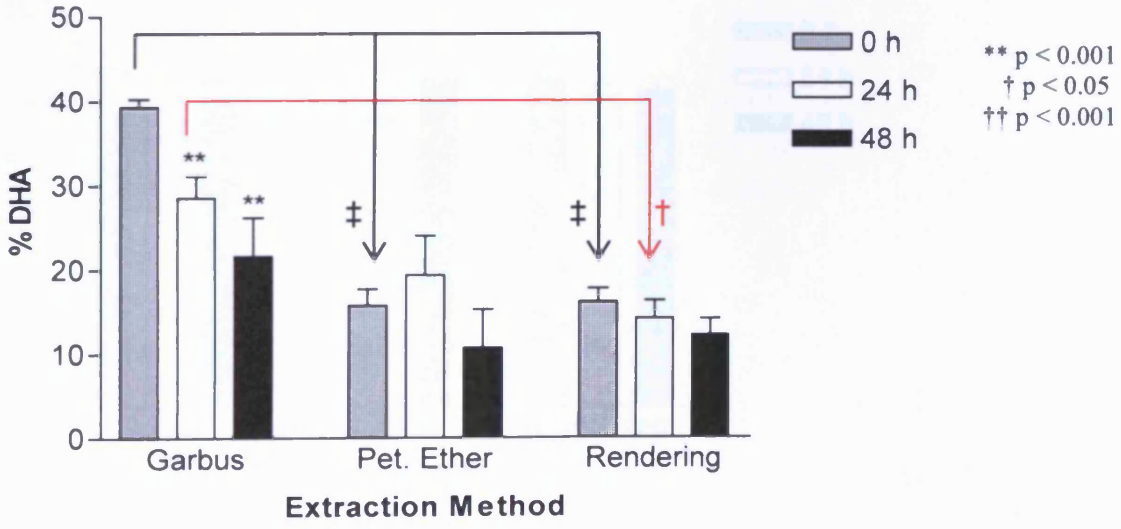


Fig. 4.5: EPA values as percentage of total fatty acids in the triacylglycerol fraction of trout offal extracted using three methods of lipid extraction at different tissue storage times. Results as means ±SD (n=3).

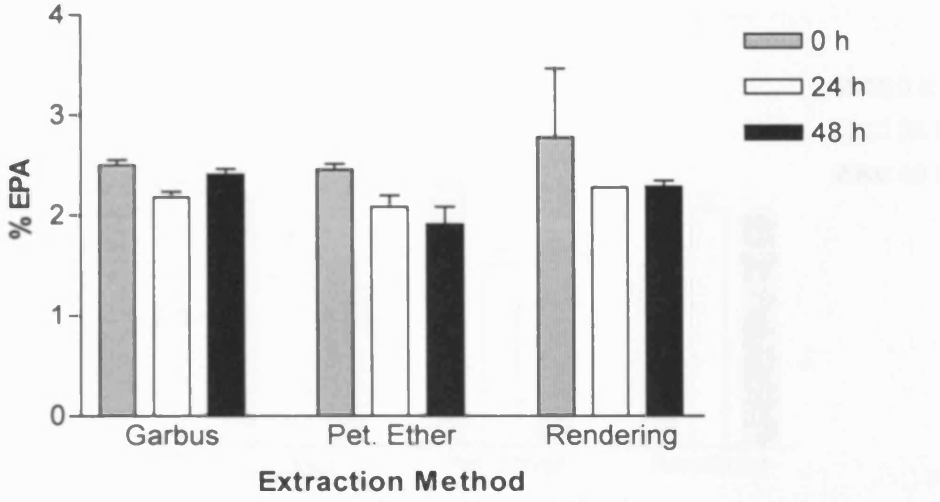


Fig. 4.6: DHA values as percentage of total fatty acids in the triacylglycerol fraction of trout offal extracted using three methods of lipid extraction at different tissue storage times. Results as means ±SD (n=3).

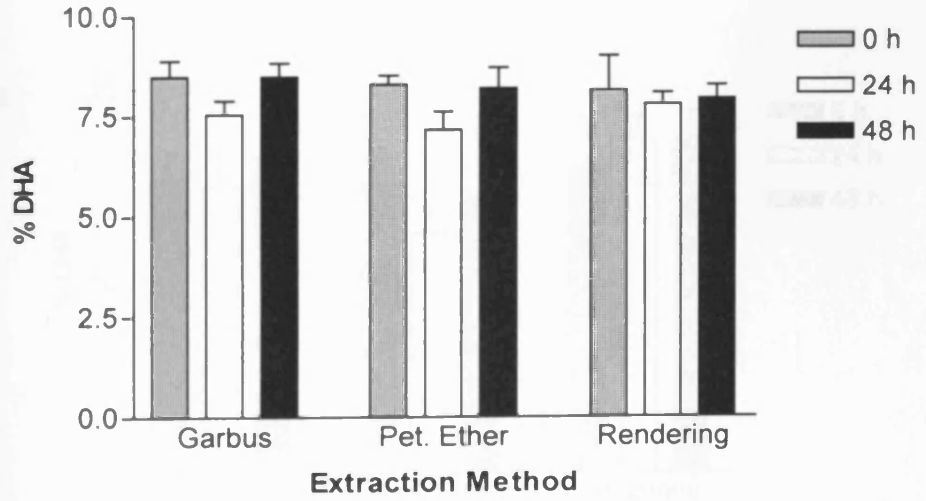


Fig. 4.7: EPA values as percentage of total fatty acids in the non-esterified fatty acid fraction (NEFA) in trout offal extracted using three methods of lipid extraction at different tissue storage times. Results as means ±SD (n=3).

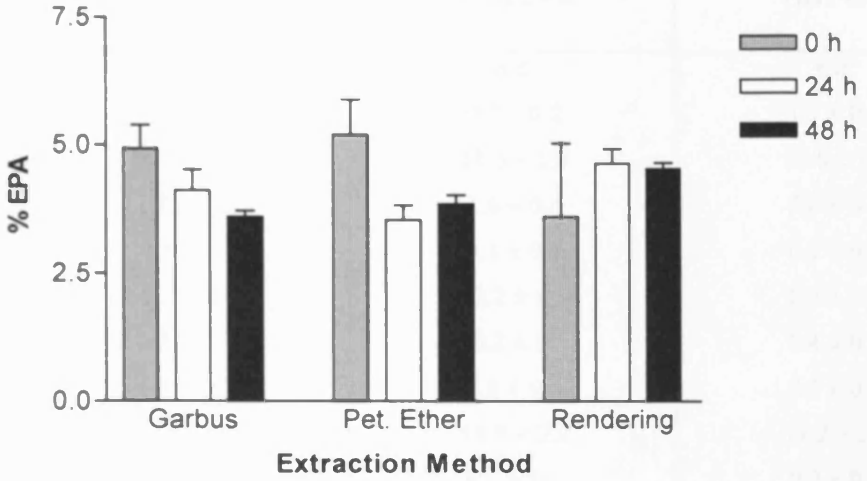


Fig. 4.8: DHA values as percentage of total fatty acids in the non-esterified free fatty acid fraction (NEFA) of trout offal extracted using three methods of lipid extraction at different tissue storage times. Results as means ±SD (n=3).

Table 4.2: Percentage fatty acid composition of the total polar lipids from total trout offal extracted using the Garbus method after different tissue storage times. Results as means ± SD (n=3). n.d. = none detected, tr. = <0.05.

Fatty Acids	Time zero	Time 24 h	Time 48 h
12:0	0.1 ± 0.1	n.d.	n.d.
14:0	0.8 ± 0.2	0.7 ± 0.2	1.3 ± 0.6
16:0	18.7 ± 1.0	18.5 ± 1.5	16.6 ± 1.7
16:1 n-7	1.2 ± 0.2	1.6 ± 0.4	2.0 ± 0.4
16:1	n.d.	0.1 ± 0.1	0.1 ± 0.2
17:0	0.2 ± tr.	0.2 ± tr.	0.2 ± 0.1
17:1	0.2 ± 0.1	0.2 ± tr.	0.4 ± 0.1
18:0	4.9 ± 0.3	5.8 ± 0.5	5.5 ± 0.5
18:1 n-9	12.1 ± 0.9	16.9 ± 2.6	19.2 ± 3.6
18:1 n-7	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
18:2 n-6	4.7 ± 0.3	6.4 ± 1.0	7.6 ± 1.2
18:2 n-3	0.1 ± tr.	0.2 ± tr.	0.2 ± tr.
18:3 n-6	0.6 ± 0.1	0.8 ± tr.	0.8 ± 0.1
18:3 n-3	0.4 ± 0.1	0.5 ± 0.1	0.5 ± tr.
20:0	n.d.	0.2 ± 0.2	0.2 ± tr.
20:1 n-9	2.3 ± 0.2	4.2 ± 1.1	6.1 ± 1.9
20:2	0.8 ± 0.1	1.0 ± 0.2	0.9 ± 0.1
20:3 n-9	0.5 ± 0.1	0.3 ± 0.2	0.6 ± 0.5
20:4 n-6	1.8 ± 0.1	1.2 ± 0.8	0.9 ± 0.6
20:4 n-3	0.7 ± 0.1	0.5 ± 0.1	0.9 ± 0.1
20:5 n-3	**5.2 ± 0.4**	**3.9 ± 0.7**	**2.9 ± 1.0**
22:1	1.3 ± 0.2	3.5 ± 1.3	6.2 ± 2.3
22:2	n.d.	n.d.	n.d.
22:3	tr.	n.d.	0.3 ± 0.1
23:0	0.4 ± 0.4	0.3 ± 0.2	n.d.
22:5 n-3	1.3 ± 0.2	1.1 ± 0.3	0.8 ± 0.2
22:6 n-3	**39.4 ± 1.6**	**28.5 ± 4.5**	**21.6 ± 7.9**
24:0	n.d.	tr. ± 0.1	n.d.
24:1	2.3 ± 0.1	3.0 ± 0.6	3.9 ± 0.3

Table 4.3: Percentage fatty acid composition of the total polar lipid fraction from total trout offal extracted using the petroleum ether method after different tissue storage times. Results as means ± SD (n=3). n.d. = none detected, tr. = <0.05.

Fatty Acids	Time zero	Time 24 h	Time 48 h
12:0	n.d.	n.d.	0.1 ± 0.1
14:0	n.d.	1.1 ± 2.3	2.0 ± 0.1
16:0	15.7 ± 2.4	15.2 ± 5.3	16.8 ± 1.5
16:1 n-7	9.2 ± 3.2	2.1 ± 0.6	2.6 ± 0.7
16:1	n.d.	n.d.	n.d.
17:0	n.d.	0.2 ± 0.2	0.3 ± 0.1
17:1	n.d.	0.3 ± 0.4	0.3 ± 0.1
18:0	9.9 ± 3.8	6.0 ± 3.5	5.4 ± 0.3
18:1 n-9	24.8 ± 2.4	18.3 ± 5.4	22.3 ± 1.3
18:1 n-7	n.d.	n.d.	0.1 ± 0.1
18:2 n-6	10.9 ± 1.1	8.1 ± 1.9	9.1 ± 0.2
18:2 n-3	n.d.	n.d.	0.1 ± tr.
18:3 n-6	0.6 ± tr.	1.1 ± 0.2	1.2 ± 0.2
18:3 n-3	n.d.	0.3 ± 0.2	0.5 ± 0.2
20:0	n.d.	n.d.	n.d.
20:1 n-9	4.4 ± 0.9	8.1 ± 2.2	8.0 ± 1.5
20:2	n.d.	1.0 ± 0.1	0.7 ± 0.5
20:3 n-9	n.d.	0.4 ± 0.6	0.5 ± 0.2
20:4 n-6	n.d.	0.9 ± 0.6	0.9 ± 0.1
20:4 n-3	n.d.	1.4 ± 1.0	0.8 ± 0.2
20:5 n-3	**3.3 ± 1.6**	**3.0 ± 0.8**	**2.0 ± 0.4**
22:1	7.5 ± 1.2	9.3 ± 2.9	8.3 ± 1.4
22:2	n.d.	n.d.	n.d.
22:3	n.d.	n.d.	n.d.
23:0	n.d.	n.d.	n.d.
22:5 n-3	n.d.	n.d.	3.1 ± 5.3
22:6 n-3	**15.7 ± 3.4**	**19.3 ± 8.2**	**10.7 ± 7.9**
24:0	n.d.	n.d.	n.d.
24:1	n.d.	4.3 ± 1.3	3.2 ± 0.4

Table 4.4 Percentage fatty acid composition of the total polar lipid fraction from total trout offal extracted using the rendering method after different tissue storage times. Results as means ± SD (n=3). n.d. = none detected, tr. = <0.05.

Fatty Acids	Time zero	Time 24 h	Time 48 h
12:0	n.d.	0.1 ± 0.2	n.d.
14:0	1.3 ± 0.4	0.8 ± 0.5	1.8 ± 0.4
16:0	15.8 ± 7.3	13.5 ± 1.4	18.6 ± 4.2
16:1 n-7	2.0 ± 0.2	2.2 ± 0.4	3.4 ± 0.7
16:1	n.d.	n.d.	n.d.
17:0	0.2 ± 0.3	0.2 ± 0.1	0.4 ± 0.1
17:1	0.2 ± 0.2	0.3 ± tr.	0.9 ± 0.9
18:0	7.1 ± 1.7	5.6 ± 0.4	5.5 ± 0.7
18:1 n-9	23.5 ± 1.5	23.0 ± 1.4	20.4 ± 4.2
18:1 n-7	n.d.	n.d.	n.d.
18:2 n-6	10.9 ± 5.6	10.7 ± 0.7	9.6 ± 0.5
18:2 n-3	n.d.	0.1 ± tr.	0.3 ± 0.1
18:3 n-6	1.6 ± 0.8	1.1 ± 0.1	1.1 ± 0.1
18:3 n-3	1.0 ± 0.7	0.8 ± 0.2	0.9 ± 0.3
20:0	n.d.	0.1 ± 0.1	0.2 ± 0.3
20:1 n-9	9.0 ± 2.9	8.5 ± 1.2	8.7 ± 2.0
20:2	0.5 ± 0.5	1.2 ± 0.2	0.5 ± 0.4
20:3 n-9	0.2 ± 0.3	0.3 ± 0.2	0.3 ± 0.2
20:4 n-6	0.5 ± 0.6	0.8 ± 0.2	0.6 ± 0.5
20:4 n-3	0.5 ± 0.6	1.3 ± 0.3	0.7 ± 0.5
20:5 n-3	**2.2 ± 1.2**	**2.3 ± 0.3**	**1.9 ± 0.4**
22:1	8.1 ± 2.4	9.6 ± 1.7	8.6 ± 1.9
22:2	n.d.	n.d.	n.d.
22:3	n.d.	n.d.	n.d.
23:0	n.d.	n.d.	n.d.
22:5 n-3	0.5 ± 0.6	0.8 ± 0.6	n.d.
22:6 n-3	**16.2 ± 2.7**	**14.2 ± 3.7**	**12.2 ± 3.4**
24:0	0.4 ± 0.8	0.1 ± 0.2	0.1 ± 0.2
24:1	1.9 ± 1.5	2.6 ± 0.5	3.6 ± 0.9

Table 4.5: Percentage fatty acid composition of the triacylglycerol fraction from total trout offal extracted using the Garbus method after different tissue storage times. Results as means ± SD (n=3). n.d. = none detected, tr. = <0.05.

Fatty Acids	Time zero	Time 24 h	Time 48 h
12:0	0.1 ± tr.	0.1 ± 0.1	tr.
14:0	3.2 ± 0.1	3.1 ± 0.1	3.0 ± 0.1
16:0	13.1 ± 0.8	13.2 ± 0.9	12.9 ± 0.9
16:1 n-7	4.4 ± 0.7	4.4 ± 0.6	4.3 ± 0.6
16:1	0.1 ± tr.	0.1 ± tr.	0.2 ± 0.1
17:0	0.2 ± tr.	0.2 ± tr.	0.3 ± 0.1
17:1	0.4 ± tr.	0.4 ± tr.	0.3 ± 0.2
18:0	2.7 ± 0.3	2.8 ± 0.4	2.7 ± 0.4
18:1 n-9	24.8 ± 1.0	25.8 ± 1.0	25.5 ± 1.1
18:1 n-7	0.3 ± tr.	0.3 ± tr.	0.3 ± tr.
18:2 n-6	15.7 ± 0.6	15.5 ± 0.7	15.5 ± 0.6
18:2 n-3	0.3 ± tr.	0.3 ± tr.	0.3 ± tr.
18:3 n-6	1.7 ± 0.1	1.6 ± 0.1	1.4 ± 0.1
18:3 n-3	1.5 ± 0.1	1.3 ± 0.1	1.4 ± 0.1
20:0	0.1 ± tr.	0.1 ± tr.	0.1 ± tr.
20:1 n-9	7.7 ± 0.6	8.2 ± 0.6	8.0 ± 0.6
20:2	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
20:3 n-9	0.3 ± tr.	0.3 ± tr.	0.3 ± 0.1
20:4 n-6	0.4 ± tr.	0.3 ± 0.1	0.3 ± 0.1
20:4 n-3	1.1 ± 0.1	1.1 ± 0.1	0.9 ± 0.5
20:5 n-3	**2.5 ± 0.1**	**2.2 ± 0.1**	**2.4 ± 0.1**
22:1	7.7 ± 0.6	8.2 ± 0.6	8.1 ± 0.6
22:2	0.1 ± tr.	0.1 ± tr.	0.1 ± tr.
22:3	n.d.	n.d.	0.1 ± 0.1
23:0	0.1 ± tr.	0.2 ± tr.	0.1 ± 0.1
22:5 n-3	1.0 ± 0.1	0.8 ± 0.1	0.9 ± 0.1
22:6 n-3	**8.5 ± 0.7**	**7.6 ± 0.6**	**8.5 ± 0.6**
24:0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± tr.
24:1	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.1

Table 4.6: Percentage fatty acid composition of the triacylglycerol fraction from the total trout offal extracted using the petroleum ether method after different tissue storage times. Results as means ± SD (n=3). n.d. = none detected, tr. = <0.05.

Fatty Acids	Time zero	Time 24 h	Time 48 h
12:0	0.1 ± tr.	0.1 ± tr.	0.1 ± tr.
14:0	3.1± 0.2	3.0 ± 0.3	3.1 ± 0.1
16:0	13.1± 0.8	13.4 ± 0.9	13.0 ± 1.0
16:1 n-7	4.5± 0.6	4.4 ± 0.6	4.2 ± 0.7
16:1	0.1± tr.	0.1 ± tr.	0.1 ± tr.
17:0	0.2± 0.1	0.2 ± tr.	0.2 ± tr.
17:1	0.4± 0.1	0.4 ± tr.	0.4 ± tr.
18:0	2.7± 0.3	2.9 ± 0.4	2.7 ± 0.3
18:1 n-9	25.4± 1.0	26.2 ± 1.2	25.3 ± 0.8
18:1 n-7	0.3± tr.	0.3 ± tr.	0.3 ± tr.
18:2 n-6	15.7± 0.6	15.2 ± 0.6	15.6 ± 0.7
18:2 n-3	0.3± tr.	0.3 ± tr.	0.3 ± tr.
18:3 n-6	1.7± 0.1	1.5 ± 0.1	1.7 ± 0.1
18:3 n-3	1.4± 0.1	1.3 ± 0.1	1.4 ± 0.1
20:0	0.1± tr.	0.1 ± tr.	0.1 ± tr.
20:1 n-9	7.9± 0.6	8.3 ± 0.6	8.3 ± 0.4
20:2	0.9± 0.1	0.9 ± 0.1	0.9 ± 0.1
20:3 n-9	0.3± tr.	0.3 ± 0.1	0.3 ± tr.
20:4 n-6	0.3± tr.	0.3 ± 0.1	0.3 ± tr.
20:4 n-3	1.1± 0.1	1.0 ± 0.1	1.1 ± 0.1
20:5 n-3	**2.5± 0.1**	**2.1 ± 0.2**	**1.9 ± 0.6**
22:1	7.8± 0.6	8.9 ± 0.5	8.3 ± 0.5
22:2	0.1± tr.	0.1 ± tr.	0.1 ± tr.
22:3	tr.	tr.	0.2 ± tr.
23:0	0.1± tr.	0.2 ± tr.	tr.
22:5 n-3	1.0 ± 0.1	0.8 ± 0.1	1.0 ± tr.
22:6 n-3	**8.3± 0.4**	**7.2 ± 0.8**	**8.2 ± 0.9**
24:0	0.1± tr.	0.2 ± tr.	0.2 ± tr.
24:1	0.7± 0.1	0.6 ± 0.2	0.7 ± tr.

Table 4.7: Percentage fatty acid composition of the triacylglycerol fraction from total trout offal extracted using the rendering method at different tissue storage times. Results as means ± SD (n=3). n.d. = none detected, tr. = <0.05.

Fatty Acids	Time zero	Time 24 h	Time 48 h
12:0	tr.	tr.	0.1 ± tr.
14:0	2.7 ± 0.7	2.9 ± 0.2	3.1 ± 0.1
16:0	13.4 ± 0.9	13.0 ± 0.9	13.1 ± 0.8
16:1 n-7	4.2 ± 0.8	4.3 ± 0.6	4.2 ± 0.7
16:1	0.1 ± 0.1	0.1 ± tr.	0.1 ± 0.1
17:0	0.2 ± tr.	0.2 ± tr.	0.2 ± tr.
17:1	0.4 ± 0.1	0.4 ± tr.	0.4 ± tr.
18:0	2.9 ± 0.5	2.8 ± 0.3	2.8 ± 0.3
18:1 n-9	26.0 ± 0.9	25.7 ± 0.9	25.7 ± 0.8
18:1 n-7	0.3 ± tr.	0.3 ± tr.	0.3 ± tr.
18:2 n-6	16.2 ± 1.4	15.5 ± 0.6	15.4 ± 0.6
18:2 n-3	0.4 ± 0.1	0.3 ± tr.	0.3 ± tr.
18:3 n-6	1.6 ± 0.1	1.7 ± 0.1	1.7 ± 0.1
18:3 n-3	1.3 ± 0.2	1.4 ± 0.1	1.4 ± 0.1
20:0	0.2 ± 0.1	0.1 ± tr.	0.1 ± tr.
20:1 n-9	7.4 ± 1.8	8.2 ± 0.6	8.1 ± 0.6
20:2	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
20:3 n-9	0.4 ± 0.2	0.3 ± 0.1	0.3 ± tr.
20:4 n-6	0.6 ± 0.5	0.3 ± 0.1	0.3 ± tr.
20:4 n-3	1.1 ± 0.2	1.4 ± 0.6	1.1 ± tr.
20:5 n-3	**2.8 ± 1.2**	**2.3 ± tr.**	**2.3 ± 0.1**
22:1	6.8 ± 2.5	8.2 ± 0.6	8.1 ± 0.6
22:2	0.1 ± tr.	0.1 ± tr.	0.1 ± tr.
22:3	0.1 ± 0.2	tr.	0.1 ± tr.
23:0	0.1 ± 0.1	0.2 ± tr.	tr.
22:5 n-3	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
22:6 n-3	**8.2 ± 1.5**	**7.8 ± 0.5**	**7.9 ± 0.6**
24:0	0.1 ± 0.1	0.2 ± tr.	0.2 ± tr.
24:1	0.6 ± 0.2	0.7 ± 0.1	0.6 ± 0.1

Table 4.8: Percentage fatty acid composition of the diacylglycerol (1,2 and 1,3 diacylglycerol) fraction from total trout offal extracted using the Garbus method after different tissue storage times. Results as means ± SD (n=3). n.d. = none detected, tr. = <0.05.

Fatty Acids	Time zero	Time 24 h	Time 48 h
12:0	n.d.	n.d.	0.2 ± tr.
14:0	1.7 ± 0.2	0.9 ± 0.2	1.8 ± 0.6
16:0	14.7 ± 2.2	11.4 ± 1.8	13.1 ± 1.6
16:1 n-7	2.4 ± 0.8	2.1 ± 0.1	3.0 ± 0.2
16:1	n.d.	n.d.	n.d.
17:0	n.d.	0.2 ± 0.1	0.2 ± tr.
17:1	n.d.	0.4 ± 0.2	0.4 ± 0.1
18:0	6.1 ± 0.9	4.7 ± 1.1	3.7 ± 0.3
18:1 n-9	26.9 ± 2.2	21.1 ± 4.8	22.4 ± 2.3
18:1 n-7	n.d.	n.d.	2.7 ± 2.4
18:2 n-6	13.8 ± 0.8	11.1 ± 1.7	12.7 ± 1.3
18:2 n-3	n.d.	0.2 ± tr.	0.3 ± tr.
18:3 n-6	1.3 ± 0.9	1.7 ± 0.3	1.4 ± 0.2
18:3 n-3	1.1 ± 0.7	2.4 ± 0.7	1.2 ± 0.1
20:0	n.d.	0.2 ± 0.4	n.d.
20:1 n-9	10.3 ± 1.2	9.0 ± 2.1	7.4 ± 0.4
20:2	0.2 ± 0.4	1.2 ± 0.3	0.9 ± 01
20:3 n-9	n.d.	0.1 ± 0.2	0.4 ± 0.1
20:4 n-6	n.d.	1.1 ± 0.3	0.7 ± 0.1
20:4 n-3	0.7 ± 0.9	2.7 ± 1.3	1.6 ± 0.3
20:5 n-3	**2.6 ± 0.4**	**5.3 ± 1.7**	**3.1 ± 0.2**
22:1	10.8 ± 0.9	9.6 ± 3.1	8.0 ± 0.5
22:2	n.d.	n.d.	n.d.
22:3	n.d.	n.d.	n.d.
23:0	n.d.	n.d.	n.d.
22:5 n-3	n.d.	1.9 ± 0.6	1.0 ± 0.1
22:6 n-3	**7.5 ± 1.8**	**12.4 ± 8.6**	**13.3 ± 1.9**
24:0	n.d.	n.d.	n.d.
24:1	n.d.	0.3 ± 0.5	0.6 ± 0.0

Table 4.9: Percentage fatty acid composition of the diacylglycerol (1,2 and 1,3 diacylglycerol) fraction from total trout offal extracted using the petroleum ether method after different tissue storage times. Results as means ± SD (n=3). n.d. = none detected, tr. = <0.05.

Fatty Acids	Time zero	Time 24 h	Time 48 h
12:0	n.d.	0.2 ± tr.	0.1 ± 0.2
14:0	n.d.	1.3 ± 0.9	2.4 ± 0.3
16:0	14.8 ± 1.5	11.0 ± 4.3	13.8 ± 0.8
16:1 n-7	4.4 ± 2.1	2.0 ± 0.5	3.4 ± 1.1
16:1	n.d.	n.d.	n.d.
17:0	n.d.	n.d.	n.d.
17:1	1.4 ± 0.7	0.1 ± 0.2	0.3 ± tr.
18:0	11.8 ± 2.6	4.5 ± 1.5	4.6 ± 0.9
18:1 n-9	19.7 ± 5.0	18.2 ± 4.1	23.8 ± 0.8
18:1 n-7	n.d.	n.d.	0.1 ± 0.1
18:2 n-6	12.8 ± 2.6	11.3 ± 1.9	13.5 ± 0.4
18:2 n-3	n.d.	0.2 ± tr.	0.3 ± 0.1
18:3 n-6	n.d.	1.9 ± 0.6	1.5 ± 0.4
18:3 n-3	1.9 ± 0.4	2.1 ± 0.2	1.2 ± 0.2
20:0	n.d.	0.2 ± 0.4	0.2 ± 0.2
20:1 n-9	6.8 ± 1.6	7.3 ± 2.8	8.1 ± 1.0
20:2	n.d.	1.0 ± 0.3	0.7 ± 0.5
20:3 n-9	n.d.	0.2 ± 0.3	0.4 ± 0.1
20:4 n-6	n.d.	0.9 ± 0.3	0.8 ± 0.1
20:4 n-3	n.d.	3.1 ± 0.4	1.4 ± 0.2
20:5 n-3	**5.9 ± 0.8**	**5.8 ± 3.1**	**3.0 ± 0.4**
22:1	10.4 ± 2.9	8.5 ± 3.4	7.8 ± 0.9
22:2	n.d.	n.d.	n.d.
22:3	n.d.	n.d.	n.d.
23:0	n.d.	0.2 ± 0.4	0.2 ± 0.1
22:5 n-3	n.d.	1.5 ± 1.3	1.0 ± 0.1
22:6 n-3	**10.1 ± 4.3**	**18.5 ± 9.8**	**11.0 ± 1.5**
24:0	n.d.	n.d.	tr.
24:1	n.d.	n.d.	0.5 ± 0.1

Table 4.10: Percentage fatty acid composition of the diacylglycerol (1,2 and 1,3 diacylglycerol) fraction from total trout offal extracted using the rendering method after different tissue storage times. Results as means ± SD (n=3). n.d. = none detected, tr. = <0.05.

Fatty Acids	Time zero	Time 24 h	Time 48 h
12:0	n.d.	n.d.	n.d.
14:0	1.0 ± 0.8	1.0 ± 0.5	2.2 ± 0.6
16:0	14.1 ± 2.3	13.7 ± 2.8	16.7 ± 2.4
16:1 n-7	0.5 ± 0.8	2.5 ± 1.5	3.2 ± 1.0
16:1	n.d.	n.d.	n.d.
17:0	n.d.	0.2 ± 0.1	1.0 ± 0.3
17:1	1.0 ± 0.7	0.3 ± 0.2	0.8 ± 0.7
18:0	10.6 ± 2.6	6.1 ± 2.3	5.0 ± 0.8
18:1 n-9	24.0 ± 7.1	23.2 ± 2.6	25.0 ± 2.5
18:1 n-7	n.d.	n.d.	n.d.
18:2 n-6	10.2 ± 1.6	12.9 ± 2.2	11.6 ± 1.7
18:2 n-3	n.d.	0.3 ± 0.2	0.4 ± 0.4
18:3 n-6	0.3 ± 0.5	1.6 ± 0.2	1.3 ± tr.
18:3 n-3	n.d.	1.7 ± 0.3	0.7 ± 0.5
20:0	n.d.	0.1 ± 0.1	0.1 ± 0.2
20:1 n-9	9.2 ± 4.3	7.8 ± 2.2	10.3 ± 1.3
20:2	0.3 ± 0.6	1.2 ± 0.3	0.5 ± 0.3
20:3 n-9	n.d.	0.3 ± 0.2	0.4 ± 0.1
20:4 n-6	n.d.	0.8 ± 0.2	0.4 ± 0.3
20:4 n-3	n.d.	1.7 ± 0.7	1.3 ± 0.6
20:5 n-3	**3.0 ± 1.4**	**3.4 ± 0.8**	**1.3 ± 0.9**
22:1	12.7 ± 2.3	9.0 ± 2.2	10.5 ± 1.0
22:2	n.d.	n.d.	n.d.
22:3	n.d.	n.d.	n.d.
23:0	n.d.	n.d.	n.d.
22:5 n-3	n.d.	1.3 ± 0.2	1.6 ± 0.3
22:6 n-3	**13.2 ± 1.8**	**12.6 ± 2.6**	**5.8 ± 2.9**
24:0	n.d.	n.d.	n.d.
24:1	n.d.	n.d.	n.d.

Table 4.11: Percentage fatty acid composition of the monoacylglycerol fraction from total trout offal extracted using the Garbus method after different tissue storage times. Results as means ± SD (n=3). n.d. = none detected, tr. = <0.05.

Fatty Acids	Time zero	Time 24 h	Time 48 h
12:0	n.d.	n.d.	n.d.
14:0	2.3 ± 1.5	0.7 ± 0.6	1.5 ± 0.7
16:0	16.3 ± 7.5	11.0 ± 1.8	12.2 ± 1.7
16:1 n-7	2.8 ± 1.0	2.9 ± 1.4	3.5 ± 1.3
16:1	n.d.	n.d.	n.d.
17:0	0.3 ± 0.2	0.2 ± 0.1	0.2 ± tr.
17:1	0.1 ± tr.	0.2 ± tr.	0.4 ± tr.
18:0	5.4 ± 1.2	5.0 ± 1.1	4.0 ± 0.5
18:1 n-9	22.5± 2.1	25.7 ± 2.3	25.0 ± 1.7
18:1 n-7	n.d.	n.d.	n.d.
18:2 n-6	13.4 ± 2.9	14.4 ± 1.7	15.0 ± 0.2
18:2 n-3	0.1 ± 0.1	0.1 ± 0.2	0.3 ± 0.1
18:3 n-6	1.7 ± 0.3	1.8 ± 0.1	1.8 ± 0.1
18:3 n-3	1.2 ± 0.4	1.1 ± 0.2	1.3 ± 0.1
20:0	0.5 ± 0.1	0.4 ± 0.3	tr.
20:1 n-9	7.6 ± 1.1	9.1 ± 1.5	7.7 ± 0.7
20:2	0.9 ± 0.3	1.1 ± 0.1	1.0 ± 0.1
20:3 n-9	1.1 ± 0.7	0.2 ± 0.2	0.4 ± 0.1
20:4 n-6	0.9 ± 0.5	0.6 ± 0.2	0.8 ± 0.3
20:4 n-3	1.3 ± 0.6	1.6 ± 0.7	1.2 ± 0.1
20:5 n-3	**2.4 ± 0.8**	**2.2 ± 0.4**	**2.4 ± 0.4**
22:1	6.5 ± 1.0	9.1 ± 2.4	7.3 ± 0.8
22:2	n.d.	0.1 ± 0.2	0.1 ± 0.1
22:3	n.d.	n.d.	0.3 ± 0.1
23:0	n.d.	n.d.	n.d.
22:5 n-3	0.4 ± 0.4	0.8 ± 0.6	1.0 ± 0.1
22:6 n-3	**11.5 ± 5.3**	**9.9 ± 1.5**	**12.3 ± 2.4**
24:0	0.5 ± tr.	0.2 ± 0.5	0.3 ± 0.1
24:1	0.7 ± 0.1	1.5 ± 0.7	0.6 ± 0.1

Table 4.12: Percentage fatty acid composition of the monoacylglycerol fraction from total trout offal extracted using the petroleum ether method after different tissue storage times. Results as means ± SD (n=3). n.d. = none detected, tr. = <0.05.

Fatty Acids	Time zero	Time 24 h	Time 48 h
12:0	n.d.	n.d.	tr.
14:0	0.3 ± 0.5	1.0 ± 0.2	2.2 ± 0.3
16:0	13.9 ± 3.7	12.9 ± 2.3	13.5 ± 0.1
16:1 n-7	5.9 ± 3.4	2.7 ± 1.3	3.8 ± 1.4
16:1	n.d.	n.d.	n.d.
17:0	n.d.	0.2 ± 0.4	0.2 ± 0.1
17:1	n.d.	0.2 ± 0.2	0.3 ± 0.1
18:0	8.2 ± 3.7	4.6 ± 0.6	4.7 ± 1.2
18:1 n-9	25.9 ± 3.1	26.9 ± 1.9	25.3 ± 1.3
18:1 n-7	n.d.	n.d.	n.d.
18:2 n-6	15.4 ± 1.7	15.5 ± 0.1	14.8 ± 0.3
18:2 n-3	n.d.	0.2 ± tr.	0.3 ± 0.1
18:3 n-6	1.5 ± 1.3	1.9 ± 0.1	1.7 ± 0.1
18:3 n-3	0.5 ± 0.1	1.2 ± 0.2	1.2 ± 0.2
20:0	n.d.	n.d.	n.d.
20:1 n-9	6.3 ± 2.1	8.3 ± 0.5	7.1 ± 0.6
20:2	0.3 ± 0.1	1.2 ± 0.1	0.9 ± 0.1
20:3 n-9	n.d.	0.5 ± 0.1	0.4 ± 0.1
20:4 n-6	n.d.	0.5 ± 0.1	0.7 ± 0.4
20:4 n-3	0.5 ± 0.1	1.7 ± 0.3	1.1 ± 0.1
20:5 n-3	**1.5 ± 0.4**	**2.1 ± 0.3**	**2.2 ± 0.2**
22:1	7.7 ± 2.6	7.9 ± 0.9	7.6 ± 0.6
22:2	n.d.	n.d.	n.d.
22:3	n.d.	n.d.	n.d.
23:0	n.d.	n.d.	n.d.
22:5 n-3	0.4 ± 0.8	1.2 ± 0.2	1.2 ± 0.1
22:6 n-3	**11.0 ± 2.9**	**9.3 ± 1.9**	**10.8 ± 1.7**
24:0	n.d.	n.d.	n.d.
24:1	n.d.	n.d.	n.d.

Table 4.13: Percentage fatty acid composition of the monoacylglycerol fraction from total trout offal extracted using the rendering method after different tissue storage times. Results as means ± SD (n=3). n.d. = none detected, tr. = <0.05.

Fatty Acids	Time zero	Time 24 h	Time 48 h
12:0	tr.	n.d.	n.d.
14:0	1.8 ± 0.6	0.6 ± 0.5	2.2 ± 0.3
16:0	13.1± 1.4	11.1 ± 1.6	14.6 ± 1.9
16:1 n-7	4.1 ± 1.4	2.4 ± 1.2	4.2 ± 1.1
16:1	n.d.	n.d.	n.d.
17:0	0.2 ± 0.2	0.1 ± 0.1	0.8 ± 0.1
17:1	0.3 ± 0.2	0.3 ± 0.3	0.9 ± 0.8
18:0	4.3 ± 1.0	4.7 ± 0.8	4.3 ± 0.7
18:1 n-9	25.9 ± 3.8	25.8 ± 1.3	25.6 ± 1.6
18:1 n-7	0.1 ± 0.1	n.d.	n.d.
18:2 n-6	15.9 ± 1.2	14.6 ± 1.6	14.5 ± 1.4
18:2 n-3	0.2 ± 0.1	0.2 ± 0.2	0.2 ± 0.2
18:3 n-6	1.7 ± 0.2	1.8 ± 0.2	1.8 ± 0.1
18:3 n-3	1.4 ± 0.3	1.3 ± 0.4	1.3 ± 0.1
20:0	n.d.	n.d.	n.d.
20:1 n-9	8.7 ± 1.1	9.3 ± 2.2	7.5 ± 0.4
20:2	0.7 ± 0.5	1.2 ± tr.	0.6 ± 0.4
20:3 n-9	0.3 ± 0.2	0.3 ± 0.3	0.5 ± 0.2
20:4 n-6	0.4 ± 0.3	0.8 ± 0.2	0.3 ± 0.3
20:4 n-3	0.7 ± 0.5	2.2 ± 1.2	1.3 ± 0.6
20:5 n-3	**2.1 ± 0.2**	**2.9 ± 0.9**	**1.9 ± 0.2**
22:1	8.1 ± 0.7	9.0 ± 2.7	6.5 ± 0.3
22:2	n.d.	n.d.	n.d.
22:3	n.d.	n.d.	n.d.
23:0	n.d.	n.d.	n.d.
22:5 n-3	0.6 ± 0.4	1.4 ± 0.3	0.5 ± 0.4
22:6 n-3	**8.7 ± 1.2**	**11.5 ± 0.8**	**10.1 ± 1.5**
24:0	n.d.	n.d.	n.d.
24:1	0.6 ± 0.4	1.0 ± 0.2	0.5 ± 0.3

Table 4.14: Percentage fatty acid composition of the non-esterified fatty acid fraction from total trout offal extracted using the Garbus method after different tissue storage times. Results as means ± SD (n=3). n.d. = none detected, tr. = <0.05.

Fatty Acids	Time zero	Time 24 hr	Time 48 h
12:0	0.2 ± 0.4	n.d.	n.d.
14:0	1.5 ± 0.2	1.2 ± 0.4	1.8 ± 0.4
16:0	14.0 ± 1.9	14.7 ± 1.5	14.4 ± 0.8
16:1 n-7	2.4 ± 0.2	2.5 ± 0.7	3.3 ± 0.7
16:1	n.d.	n.d.	0.1 ± 0.1
17:0	0.2 ± 0.1	0.3 ± 0.1	0.2 ± tr.
17:1	0.2 ± 0.2	0.3 ± 0.1	0.5 ± tr.
18:0	4.2 ± 0.9	4.7 ± 1.2	3.6 ± 0.3
18:1 n-9	22.0 ± 1.5	22.5 ± 2.6	22.2 ± 2.3
18:1 n-7	n.d.	0.4 ± tr.	0.3 ± tr.
18:2 n-6	15.6 ± 1.4	14.4 ± 1.9	13.6 ± 1.1
18:2 n-3	0.3 ± 0.2	0.4 ± 0.1	0.3 ± tr.
18:3 n-6	1.5 ± 0.9	1.1 ± 0.7	1.1 ± 0.7
18:3 n-3	1.5 ± 0.4	1.1 ± 0.4	1.3 ± 0.2
20:0	n.d.	0.2 ± 0.1	0.1 ± tr.
20:1 n-9	4.7 ± 0.4	5.3 ± 0.7	5.8 ± 0.3
20:2	0.8 ± 0.1	1.1 ± 0.2	0.9 ± 0.1
20:3 n-9	0.5 ± 0.1	0.5 ± 0.1	0.5 ± tr.
20:4 n-6	1.4 ± 0.2	1.3 ± 0.3	1.0 ± 0.1
20:4 n-3	1.4 ± 0.2	1.1 ± 0.6	1.2 ± 0.1
20:5 n-3	**4.9 ± 0.8**	**4.1 ± 0.7**	**3.9 ± 0.2**
22:1	4.4 ± 0.9	5.3 ± 1.4	5.2 ± 0.5
22:2	n.d.	tr. ± 0.1	0.1 ± tr.
22:3	n.d.	n.d.	n.d.
23:0	0.2 ± 0.5	0.8 ± 0.3	0.4 ± tr.
22:5 n-3	1.3 ± 0.2	1.2 ± 0.2	1.1 ± 0.2
22:6 n-3	**15.4 ± 2.5**	**15.0 ± 3.4**	**16.5 ±2.8**
24:0	n.d.	n.d.	0.1 ± 0.1
24:1	1.4 ± 1.0	0.5 ± 0.1	0.7 ± 0.2

Table 4.15: Percentage fatty acid composition of the non-esterified fatty acid fraction from total trout offal extracted using the petroleum ether method after different tissue storage times. Results as means ± SD (n=3). n.d. = none detected, tr. = <0.05.

Fatty Acids	Time zero	Time 24 hr	Time 48 h
12:0	n.d.	tr.	tr.
14:0	1.4 ± 0.8	1.3 ± 1.1	2.8 ± 0.2
16:0	13.0 ± 2.6	13.8 ± 4.4	13.4 ± 0.5
16:1 n-7	2.5 ± 0.5	3.0 ± 0.9	3.5 ± 0.9
16:1	n.d.	0.1 ± 0.2	tr.
17:0	n.d.	0.2 ± 0.1	0.2 ± tr.
17:1	0.6 ± 0.7	0.2 ± 0.1	0.4 ± 0.1
18:0	5.0 ± 0.8	5.0 ± 1.1	3.2 ± 0.6
18:1 n-9	22.3 ± 3.0	23.1 ± 3.9	23.2 ± 0.5
18:1 n-7	0.7 ± 0.5	0.2 ± 0.2	0.3 ± tr.
18:2 n-6	16.4 ± 2.4	13.4 ± 3.3	14.6 ± 0.9
18:2 n-3	0.2 ± tr.	0.2 ± 0.2	0.4 ± tr.
18:3 n-6	1.9 ± 0.3	1.5 ± 0.2	1.6 ± 0.1
18:3 n-3	1.4 ± 0.3	1.1 ± 0.3	1.4 ± 0.2
20:0	n.d.	0.1 ± 0.1	1.6 ± 0.9
20:1 n-9	4.7 ± 0.7	7.2 ± 2.5	4.7 ± 3.2
20:2	1.4 ± 0.7	1.2 ± 0.2	0.9 ± 0.1
20:3 n-9	0.3 ± 0.3	0.4 ± 0.3	0.6 ± 0.3
20:4 n-6	1.4 ± 0.2	0.9 ± 0.6	0.8 ± 0.4
20:4 n-3	1.5 ± 0.3	1.9 ± 1.5	1.2 ± tr.
20:5 n-3	**5.2 ± 1.2**	**3.5 ± 0.5**	**3.9 ± 0.3**
22:1	4.1 ± 0.9	8.5 ± 3.5	5.4 ± 0.7
22:2	n.d.	n.d.	n.d.
22:3	n.d.	n.d.	n.d.
23:0	n.d.	n.d.	n.d.
22:5 n-3	0.8 ± 0.1	0.9 ± 0.6	1.2 ± 0.1
22:6 n-3	**15.2 ± 3.1**	**12.5 ± 2.4**	**14.8 ± 1.7**
24:0	n.d.	n.d.	n.d.
24:1	n.d.	n.d.	n.d.

Table 4.16: Percentage fatty acid composition of the non-esterified fatty acid fraction from total trout offal extracted using the rendering method after different tissue storage times. Results as means ± SD (n=3). n.d. = none detected, tr. = <0.05.

Fatty Acids	Time zero	Time 24 hr	Time 48 h
12:0	n.d.	n.d.	n.d.
14:0	0.9 ± 0.6	0.9 ± 0.6	1.6 ± 0.1
16:0	15.6 ± 4.5	13.1 ± 1.1	12.6 ± 1.5
16:1 n-7	1.9 ± 1.1	2.6 ± 1.0	3.1 ± 0.8
16:1	n.d.	n.d.	n.d.
17:0	0.1 ± 0.1	0.2 ± tr.	0.3 ± 0.2
17:1	0.2 ± 0.2	0.3 ± 0.2	0.4 ± 0.1
18:0	6.8 ± 1.8	4.5 ± 1.4	2.9 ± 0.5
18:1 n-9	23.1 ± 3.5	20.5 ± 8.5	23.6 ± 2.4
18:1 n-7	0.17 ± 0.1	0.2 ± 0.2	0.3 ± 0.2
18:2 n-6	13.8 ± 2.2	11.3 ± 6.3	14.3 ± 1.4
18:2 n-3	0.1 ± 0.2	0.4 ± tr.	0.4 ± 0.1
18:3 n-6	1.2 ± 0.8	1.7 ± 0.2	1.6 ± 0.2
18:3 n-3	0.9 ± 0.7	1.3 ± 0.3	1.3 ± 0.2
20:0	n.d.	0.1 ± tr.	0.1 ± 0.1
20:1 n-9	6.0 ± 1.2	6.4 ± 1.5	5.3 ± 0.6
20:2	0.7 ± 0.5	1.3 ± 0.4	0.9 ± 0.1
20:3 n-9	0.3 ± 0.3	0.9 ± 0.4	0.5 ± 0.1
20:4 n-6	1.0 ± 0.7	1.5 ± 0.3	1.3 ± 0.1
20:4 n-3	1.1 ± 0.8	1.7 ± 0.5	1.3 ± 0.1
20:5 n-3	**3.6 ± 2.5**	**4.7 ± 0.5**	**4.6 ± 0.2**
22:1	6.0 ± 2.7	6.7 ± 2.8	4.3 ± 0.3
22:2	n.d.	0.1 ± 0.1	tr.
22:3	n.d.	n.d.	n.d.
23:0	0.2 ± 0.4	1.0 ± 0.3	0.6 ± 0.2
22:5 n-3	0.8 ± 1.0	1.5 ± 0.3	1.3 ± 0.2
22:6 n-3	**16.0 ± 4.6**	**17.4 ± 2.2**	**17.5 ± 2.4**
24:0	n.d.	n.d.	n.d.
24:1	n.d.	n.d.	n.d.

4.9 Effect of Storage Time and Extraction Method on Total Lipid Extracted from Visceral Adipose Tissue

In order to compare starting materials, visceral adipose tissue was also used as a lipid source. It was very uncommon to find subcutaneous adipose tissue whereas visceral adipose tissue was present in all fish analysed. In contrast to the trends seen with total trout offal (Fig. 4.2B) all three methods used for lipid extraction produced almost equal yields of total lipids at each time point (Fig. 4.9). The only exception to this trend was seen with the rendering method at which the levels dropped from approximately 165 mg lipid/g fresh weight at time zero to 98 mg lipid/g fresh weight at 48 h. This decrease in the total lipid extracted was not seen with the other two methods where the levels remained stable at about 200 mg/g at each time point (Fig. 4.9).

4.10 Effect of Storage Time and Extraction Method on Triacylglycerol Yields from Visceral Adipose Tissue

For the Garbus method no difference in extraction efficiency was noted between TAG yield at 24 h but by 48 h TAG yield had significantly decreased ($p < 0.05$) compared to time zero (Fig. 4.10). No significant differences in TAG yield for the petroleum ether method were detected at any of the three time points examined. The Garbus and petroleum ether method produced similar yields of TAGs at each time point. However, the rendering method was observed to yield significantly less ($p < 0.05$) TAG than either the Garbus or petroleum ether methods at zero and 48 h. Within the rendering experiment the TAG yields were comparable at zero and 24 h but by 48 h a significant decrease ($p < 0.05$) was observed (Fig. 4.10).

Fig. 4.9: Comparison of the yield of three methods used to extract total lipid from visceral adipose tissue after different tissue storage times. Results as means ± SD (n=3). * denotes significance of storage time within method. † denotes significance between methods.

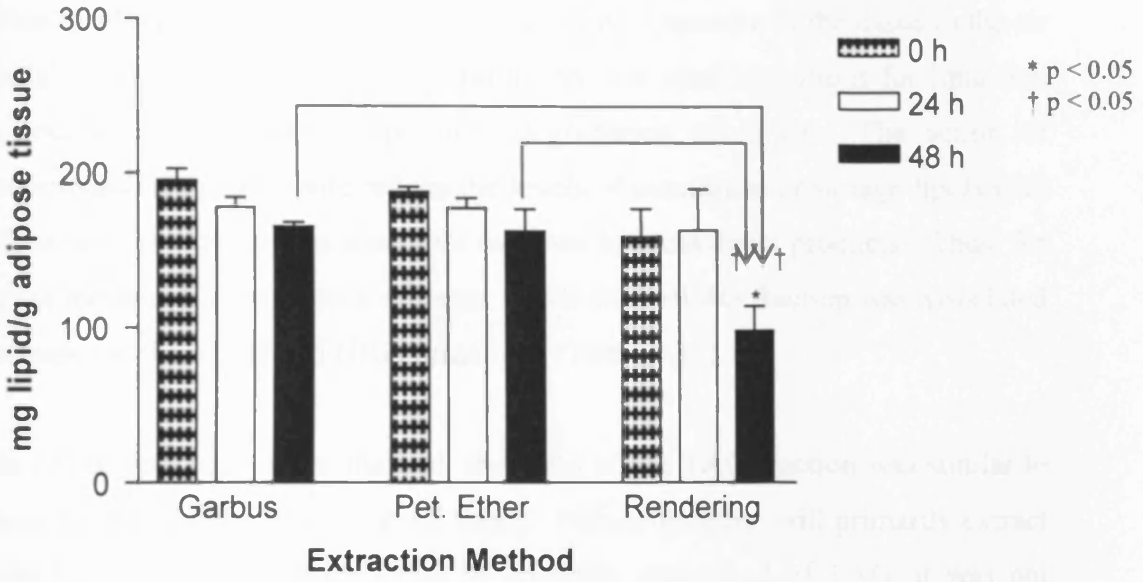
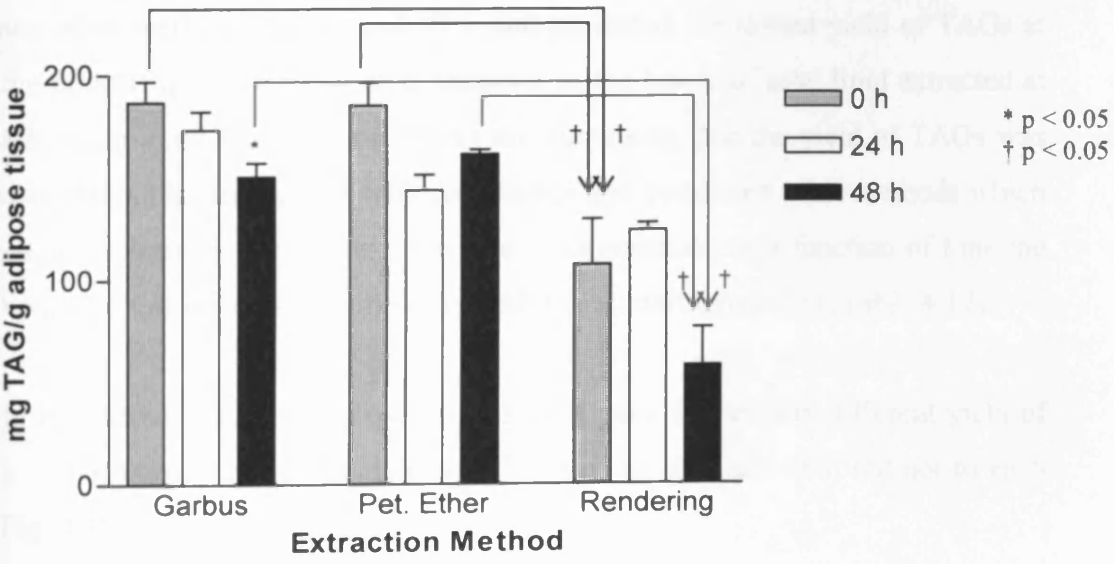


Fig. 4.10: Comparison of the yield of three methods used to extract triacylglycerols from visceral adipose tissue after different tissue storage times. Results as means ± SD (n=3). * denotes significance of storage time within method. † denotes significance between methods.



In order to explain the effect of extraction method and tissue storage time on total lipid and TAGs extracted from visceral adipose tissue it was important to consider the distribution of lipid classes at each time point (Table 4.17). As mentioned previously, tissue degradation increases in accordance with increasing storage time. Storage conditions employed for the visceral adipose tissue were modelled on the expected conditions in which the offal may be stored on farms. Exposure of the tissue to the air and sunlight and storage at room temperature are not ideal conditions for lipid-rich tissues and would be likely to promote degradation of lipids. The action of phospholipases and lipases could reduce the levels of membrane or storage lipids with time. In some cases there was a notable increase in breakdown products. Thus, for the Garbus method of extraction a decrease of 5% in the TAG fraction was associated with increases in the MAG and NEFA fractions (Table 4.17).

In terms of the petroleum ether method, the yield of the TAG fraction was similar to that found for the Garbus method at all times. Petroleum ether will primarily extract non-polar lipids and, as adipose tissue is primarily composed of TAG, it was not surprising that the high levels of TAG were extracted using the petroleum ether method (Fig. 4.10). The decrease in TAG observed at 48 h may be associated with the increase in degradation products observed over time (Table 4.17) due to the action of lipases during storage over time.

The rendering method was significantly different ($p < 0.05$) from both the Garbus and petroleum ether methods at zero and 48 h and produced the lowest yield of TAGs at each time point (Fig. 4.10). Due to a decrease in the levels of total lipid extracted at time 48 h by the rendering method it was not surprising that the yield of TAGs was reduced at time 48 h. In contrast with the Garbus and petroleum ether methods which both saw an increase in the minor lipids due to degradation as a function of time the minor lipids did not increase significantly with the rendering method (Table 4.17).

Overall, the Garbus and petroleum ether methods gave statistically different yield of TAG ($p < 0.05$) compared to the rendering method at zero and 48 h but not to each other (Fig. 4.10)

Table 4.17 Effect of tissue storage time and extraction method on visceral adipose tissue lipids (mg/g tissue homogenate) based on total fatty acid analysis. Percentage lipids are given in parenthesis. Results as means ± SD (n=3). n.d. = none detected.

	GARBUS				
	TAG	**MAG**	**DAG**	**NEFA**	**TP**
0 h	176.9 ± 45.3	n.d.	2.2 ± 0.7	n.d.	3.0 ± 1.7
	(97.2)		(1.2)		(1.9)
24 h	173.2 ± 15.7	4.3 ± 1.1	1.7 ± 0.9	6.4 ± 0.2	4.6 ± 1.6
	(91)	(2.3)	(0.9)	(3.4)	(2.5)
48 h	150.6 ± 11.6	3.1 ± 0.9	1.9 ± 0.3	5.1 ± 1.5	2.1 ± 1.1
	(92.5)	(1.9)	(1.2)	(3.1)	(1.3)
	PETROLEUM ETHER				
	TAG	**MAG**	**DAG**	**NEFA**	**TP**
0 h	199.5 ± 16.0	3.4 ± 1.4	2.2 ± 0.9	0.8 ± 0.3	1.4 ± 0.1
	(96.5)	(1.7)	(1.0)	(0.3)	(0.7)
24 h	144.0 ± 12.9	1.3 ± 0.3	0.9 ± 0.5	4.2 ± 2.1	1.4 ± 0.9
	(94.8)	(0.8)	(0.6)	(2.8)	(1.0)
48 h	161.5 ± 4.0	3.9 ± 0.3	1.8 ± 0.7	4.6 ± 0.6	2.3 ± 0.2
	(94.2)	(1.9)	(0.8)	(1.8)	(1.9)
	RENDERING				
	TAG	**MAG**	**DAG**	**NEFA**	**TP**
0 h	107.5 ± 38.6	0.4 ± 0.2	1.6 ± 0.5	1.8 ± 0.2	0.9 ± 0.1
	(96)	(0.4)	(1.2)	(1.6)	(0.8)
24 h	124.4 ± 5.9	0.7 ± 0.3	0.5 ± 0.1	1.6 ± 0.8	0.6 ± 0.2
	(97.4)	(0.5)	(0.4)	(1.2)	(1.0)
48 h	57.9 ± 22.2	0.5 ± 0.3	0.5 ± 0.1	1.1 ± 0.7	0.4 ± 0.1
	(96.3)	(0.8)	(0.1)	(1.7)	(0.7)

* TAG, triacylglycerol; DAG, diacylglycerol (1,2-DAG and 1,3-DAG combined); MAG, monoacylglycerol; NEFA, non-esterified fatty acids; TP, total polar lipid fraction.

Comparing both types of starting material, total trout offal and adipose tissue, it is quite apparent that, as expected, the levels of both total lipid and TAGs vary significantly. In terms of total trout offal the levels of total lipid extracted (Fig. 4.2A, 4.9) were approximately four and a half times lower than those produced from the adipose tissue for the Garbus method. This was also true for the petroleum ether and rendering methods although the reduction was even more pronounced, six and seven times, respectively (Fig. 4.2A, 4.10).

The levels of TAG produced from the adipose tissue were also far greater than the levels produced from total trout offal (Fig. 4.2B, 4.10). Across each method the levels of TAGs yielded from adipose tissue were almost five times greater from those from total trout offal on a gram fresh weight basis. At time zero for both the petroleum ether and rendering methods the differences in TAG levels produced from both starting materials were even greater. TAG extracted from total offal using the Garbus method at time zero produced 32 mg/g tissue homogenate whereas when extracted from adipose tissue the TAG fraction was almost six times this yield (approximately 200 mg/g fresh weight). A similar trend was seen for petroleum ether extraction where there was a twenty-fold increase in total offal TAG at time zero and an eleven-fold increase was observed with the rendering method when adipose tissue was used (Fig. 4.2B, 4.10). Clearly, adipose tissue is an excellent source of TAG and can be extracted well by a petroleum ether method.

4.11 Effect of Storage Time and Extraction Method on Non-Esterified Fatty Acids from Visceral Adipose Tissue

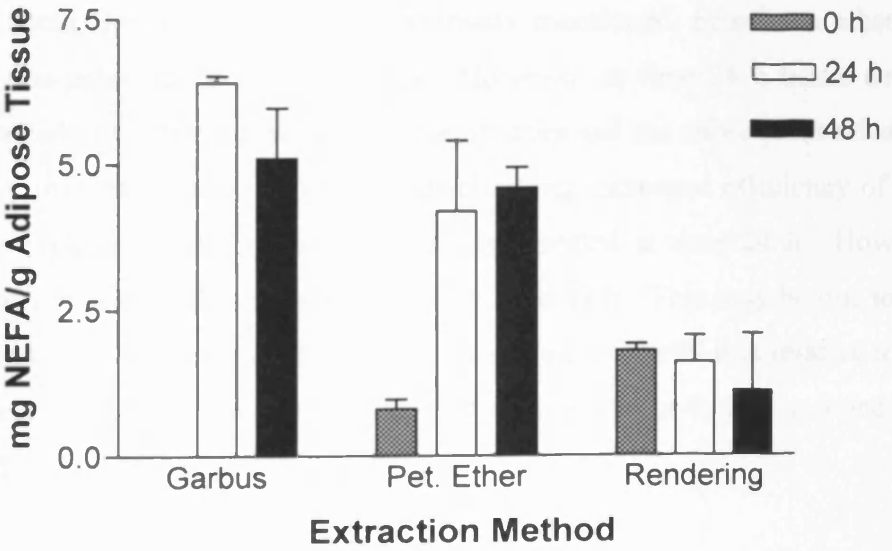
NEFAs generated during the storage and processing of adipose tissue revealed some unexpected results (Fig. 4.11). When fresh visceral adipose tissue was extracted with the Garbus method no NEFAs were detected. This was quite unusual as NEFAs were detected with both the petroleum ether and rendering methods and were also detected with the Garbus method when total trout offal was used (Fig. 4.2C). It would be necessary to repeat this experiment to determine NEFAs at time zero as the current result may be due to an experimental error such as a problem with the extraction procedure or separation of the lipids by TLC. At time 24 and 48 h NEFAs were detected with the Garbus method at approximately 5 mg/g fresh weight.

NEFAs were detected with both the petroleum ether and rendering methods at each time point analysed. There was a steady increase in the NEFA fraction with the petroleum ether method over time increasing from 0.6 mg/g fresh weight to 4.3 mg/g fresh weight by time 48 h.

These trends were not consistent with the changes in NEFAs observed from total trout offal. When total trout offal was used as the starting material NEFAs were detected at time zero, increased over 24 h with a further, more pronounced increase observed by time 48 h. There was no apparent plateau effect observed as seen in the adipose tissue experiments (Figs. 4.2C, 4.11).

Appreciable amounts of NEFAs were found at time zero for the rendering method and did not change significantly at 24 h or 48 h. This pattern was also seen in the NEFA fraction from total trout offal (Figs. 4.2C, 4.11).

Fig. 4.11: Yield of non-esterified fatty acids (mg NEFA/g tissue homogenate) over storage time in visceral adipose tissue evaluated by different extraction methods. Results as means ± SD (n=3).



4.12 Effect of Storage Time and Extraction Method on the Fatty Acid Profiles of Individual Lipids from Visceral Adipose Tissue

Percentage changes in fatty acid profiles for individual lipids from adipose tissue extracted by the three methods employed are outlined in Tables 4.18 – 4.33.

Total polar lipids appeared to demonstrate the greatest changes in fatty acid profiles on tissue storage. For the total polar lipids extracted using the Garbus method the major fatty acids included: 18:1 n-9 > 16:0 = DHA > 22:1 = 18:2 n-6 = 20:1 n-9 > 18:0 > 16:1 n-7 = EPA (Table 4.18). A decrease in the percentages of EPA and DHA was observed over time and this correlated to a corresponding increase in the major monounsaturated fatty acids 16:1, 18:1 n-9, 20:1 n-9 and 22:1 (Table 4.18). This effect had also been observed in the total polar lipid fraction extracted from total trout offal using the Garbus method (Table 4.2).

For the total polar lipids extracted using the petroleum ether method the major fatty acids detected were 18:0 > 18:1 n-9 > 16:0 > 20:1 n-9 > DHA > 22:1 > 18:2 n-6 and 16:1 n-7 (Table 4.19). The levels of DHA and EPA present in the total polar lipids extracted by the petroleum ether method at time zero were found at approximately half the yield as seen with the Garbus extraction. There was a noticeable increase in both EPA and DHA at time 24 h and this may be due to tissue degradation which allowed better lipid extraction. As previously mentioned, petroleum ether primarily extracts non-polar lipids such as TAGs. However, at time 24 h tissue deterioration may have led to the breakdown of cell membranes and the subsequent release of EPA and DHA from membrane phospholipids allowing increased efficiency of extraction and may explain the slightly elevated levels reported at time 24 h. However, both these fatty acids are reduced even further by time 48 h. This may be due to oxidation and degradation over time (Table 4.19). In accordance with this relative increases in the percentages of the monounsaturated fatty acids, 18:1 n-9, 20:1 n-9 and 22:1 were observed at time 48 h (Table 4.19).

The efficiency of extraction of the total polar lipid fraction extracted using the rendering method was less than both the Garbus and petroleum methods (Table 4.20). This meant that minor fatty acids in the total polar lipids could not be detected at time

zero. However, over time many of these acids became detectable, an effect which may be due to tissue breakdown thus allowing better penetration of the cells and, henceforth, their membrane lipids. The major fatty acids detected included: 18:1 n-9 > 20:1 n-9 > 16:1 n-7 > 16:0 > 18:1 n-7 > 22:1 > 18:2 n-6 > EPA and DHA. One obvious difference between this method and the Garbus and petroleum ether method is the poor extraction of DHA and the large percentage of monoenoic fatty acids such as 16:1, 20:1 n-9 and 22:1. Over time the levels of saturated fatty acids were seen to increase, particularly 12:0, 14:0, 16:0, 18:0 and 20:0. Some of the monounsaturated fatty acids actually decreased with time, 16:1, 18:1 n-9 and 20:1 n-9 (Table 4.20).

Overall, in terms of DHA percentage in the total polar lipid fraction the Garbus method was significantly more effective than either the petroleum ether or the rendering methods ($p < 0.05$ and $p < 0.001$ respectively). The petroleum ether method was also significantly more effective ($p < 0.05$) compared to the rendering method (Fig. 4.12).

Fig. 4.12: EPA values as percentage of the total fatty acids in the total polar lipids of visceral adipose tissue extracted using three methods of lipid extraction after different tissue storage times. Results as means ±SD (n=3). * denotes significance of storage time within method. † denotes significance between methods.

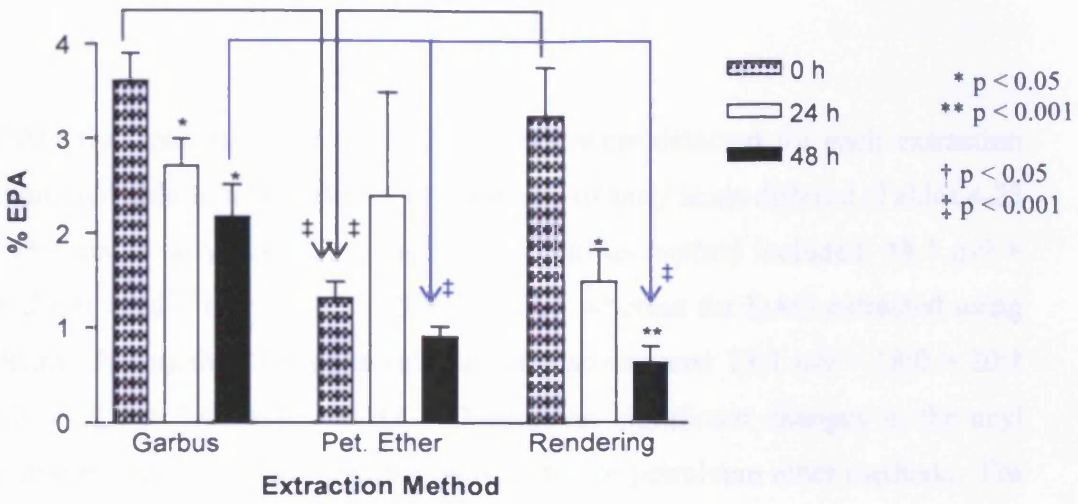
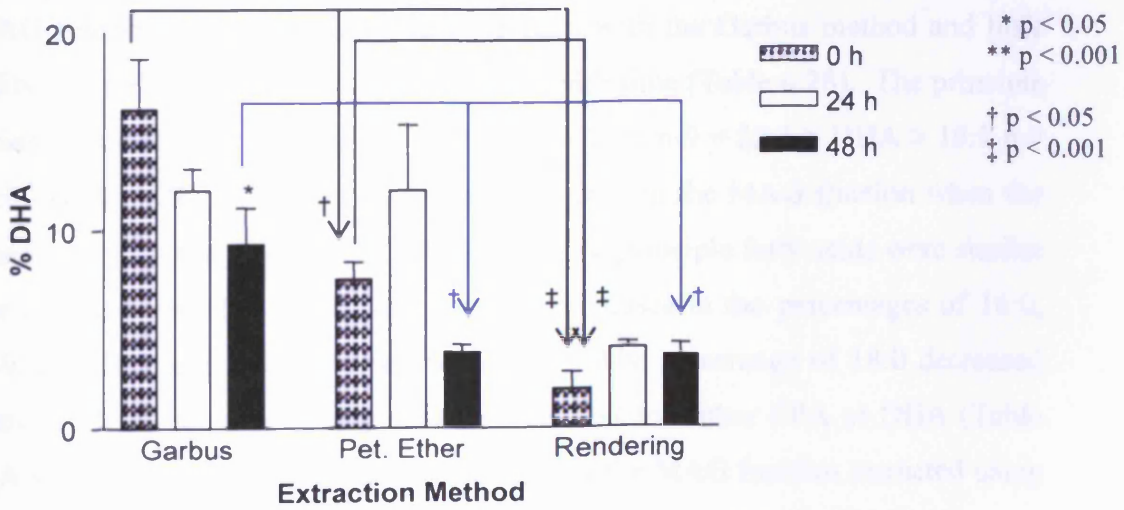


Fig. 4.13: DHA values as percentage of total fatty acids the total polar lipids of visceral adipose tissue extracted using the three methods of lipid extraction after different tissue storage times. Results as means ±SD (n=3). * denotes significance of storage time within method. † denotes significance between methods.



In terms of the effect of storage time and extraction method on the acyl composition of the TAG fraction no obvious differences were noted (Tables 4.22 – 4.24). The major fatty acids detected with each method were: 18:1 n-9 > 16:0 = 18:2 n-6 > 22:1 = 20:1 n-9 = DHA > 16:1 n-7 > 14:0 = EPA (Tables 4.22 – 4.24). Despite EPA and DHA being highly unsaturated and, therefore, more prone to degradation the levels of both these fatty acids remained constant over time. Independent of the method used approximately 2% EPA and 8% DHA was detected at each time point (Figs. 4.14, 4.15).

For the DAG fraction, the same main fatty acids were detected for each extraction method examined although the relative proportions of fatty acids differed (Tables 4.25 – 4.27). The major fatty acids detected by the Garbus method included: 18:1 n-9 > 16:0 > 18:2 n-6 > 20:1 n-9 = 22:1 = DHA = 18:0 whereas for DAG extracted using the petroleum ether method the predominant fatty acids were: 18:1 n-9 > 18:0 > 20:1 n-9 = 16:0 > 22:1 > 18:2 n-6 > DHA. Overall no significant changes in the acyl composition were detected with time for the Garbus or petroleum ether methods. For the rendering method many of the minor fatty acids were not detected at time zero but this improved with time (Table 4.27). This may be due to the degradation of the tissue over time allowing for easier extraction of lipids including catabolic intermediates such as DAG. Interestingly the percentages of 12:0, 14:0, 16:0, 18:0 increased with time while 16:1 n-7 decreased. An effect of storage time on the acyl composition was not found in the DAG fraction extracted by the Garbus or petroleum ether methods (Tables 4.25, 4.26).

The MAG fraction was not detected at time zero with the Garbus method and little variability in its acyl composition was recorded with time (Table 4.28). The principle fatty acids included: 18:1 n-9 > 16:0 = 18:2 n-6 > 20:1 n-9 = 22:1 > DHA > 16:1 n-7 and 18:0 (Table 4.28). Some changes were detected in the MAG fraction when the petroleum ether method was used (Table 4.29). The principle fatty acids were similar to those detected with the Garbus method but increases in the percentages of 16:0, 18:1 n-9, and 18:2 n-6 were detected with time. The percentage of 18:0 decreased with time while no apparent changes were observed for either EPA or DHA (Table 4.29). A similar decrease was observed for 18:0 in the MAG fraction extracted using the rendering method (Table 4.30). Similar to the effect seen in DAG when the

rendering method was used the levels of the saturated fatty acids, 12:0 14:0 and 16:0 increased with time. In terms of EPA and DHA, no significant changes were observed over time (Table 4.30).

NEFAs were not detected at time zero with the Garbus method. However, as previously mentioned this was quite unexpected (Fig. 4.11). NEFAs were detected at time zero for both the petroleum ether and rendering methods and also with the Garbus method when total trout offal was used (Fis. 4.11). It would be advisable to repeat the Garbus extraction of adipose tissue at time zero and it is likely that NEFAs were not detected due to a problem with the extraction method or separation of lipids by TLC.

The major fatty acids found in the NEFA fraction derived using all three extraction methods included: 18:1 n-9 > 18:2 n-6 > 16:0 > 20:1 n-9 > DHA > 22:1 > 18:0 > 16:1 n-7 and EPA. There was no significant effect of time on the fatty acid composition of the NEFA fraction derived with all three methods (Tables 4.31 – 4.33). In terms of EPA and DHA some minor differences were noted between methods. No changes in EPA and DHA were observed between 24 and 48 h whilst with the petroleum ether method both EPA and DHA had increased by 24 h with no further increases observed at 48 h. The rendering method saw an increase in EPA at 24 h but DHA was seen to increase at 48 h (Table 4.31 – 4.33 and Figs. 4.16, 4.17).

Comparing the results obtained with adipose tissue to those produced from total trout offal some similarities are observed. With both starting materials, the total polar lipids displayed the greatest changes in terms of fatty acid profiles. The levels of EPA and DHA in total polar lipids decreased in a time-dependent fashion for both starting materials (Tables 4.2 – 4.4 and 4.18 – 4.20). The method that produced the greatest levels of EPA and DHA from both tissue sources at all times was the Garbus method (Tables 4.2 – 4.4 and 4.18 – 4.20).

For the major lipid class, TAG, the consistency of the fatty acid profiles as a function of time was maintained independent of the starting material used (Tables 4.5 – 4.7 and 4.22 – 4.24). Also, the percentage levels of both EPA and DHA from the TAG fraction, about 2% and 8% respectively was independent of lipid source (Tables 4.5 – 4.7 and 4.22 – 4.24).

Overall, as expected, the yield of both total lipid and TAG was far greater from adipose tissue compared to the total trout offal (Fig. 4.2A, 4.2B, 4.12, 4.13). However, despite higher yields of both total lipid and TAG the levels of one of the most important fatty acids, DHA, was significantly and more consistently greater in all lipids derived from total trout offal (Tables 4.1 – 4.16, 4.18 – 4.25). EPA levels were detected at approximately 2% in all lipids from both sources (Tables 4.1 – 4.16, 4.18 – 4.25). In terms of method, the Garbus method proved to be significantly more effective in yielding both total lipid and TAG from total trout offal (Fig. 4.2A, 4.2B). It also demonstrated increased efficiency in the extraction of the minor fatty acids at time zero compared to the other two methods investigated (Tables 4.1 – 4.16). When adipose tissue was used as the starting material all three methods demonstrated equal efficiency of extraction at zero and 48 h although a significant decrease at 48 h for the rendering method was observed for total polar and TAG extraction (Figs. 4.12, 4.13). Generation of oxidation products (Figs. 4.3 – 4.11) associated with storage time were noted to increase with time when both total trout offal and adipose tissue were used although, as previously mentioned, (section 4.11), some discrepancies were observed in terms of NEFAs from adipose tissue at time zero for the Garbus method.

Fig. 4.14: EPA values as percentage of total fatty acids in the triacylglycerol fraction of visceral adipose tissue using three methods of lipid extraction after different tissue storage times. Results as means ± SD (n=3).

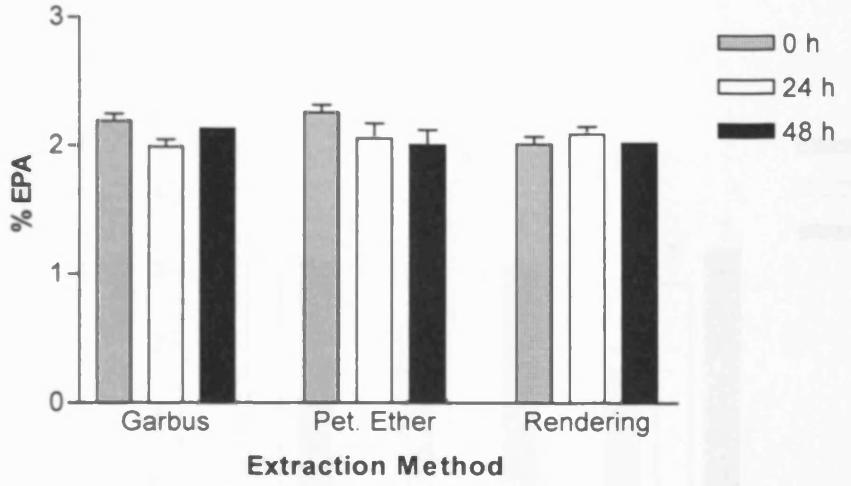


Fig. 4.15: DHA values as percentage values of total fatty acids in the triacylglycerol fraction of visceral adipose tissue using three methods of lipid extraction after different tissue storage times. Results as means ± SD (n=3).

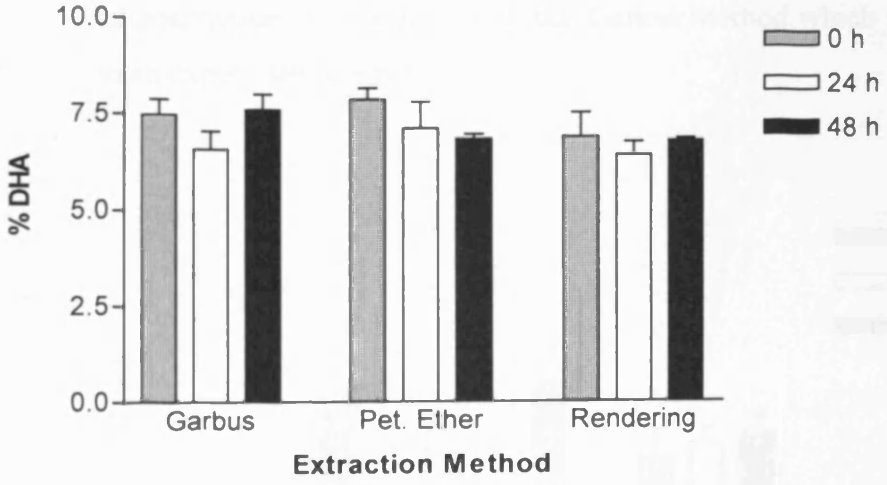


Fig. 4.16: EPA values as percentage of total fatty acids in the non-esterified fatty acid (NEFA) fraction of visceral adipose tissue using three methods of lipid extraction after different tissue storage times. Results as means ± SD (n=3). Unexpectedly, NEFAs were not detected in adipose tissue at time zero with the Garbus method which may be due to an experimental error.

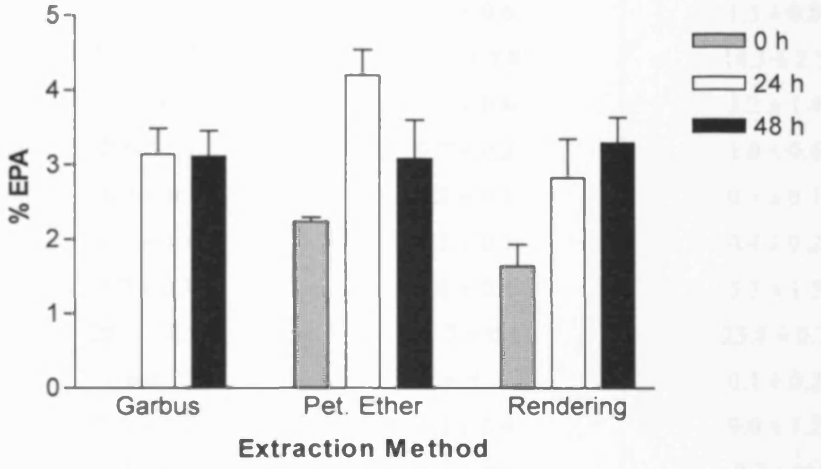


Fig. 4.17: DHA values as percentage of total fatty acids in the non-esterified fatty acid (NEFA) fraction of visceral adipose tissue extracted using three methods of lipid extraction after different tissue storage times. Results as means ± SD (n=3). Unexpectedly, NEFAs were not detected in adipose tissue at time zero with the Garbus method which may be due to an experimental error.

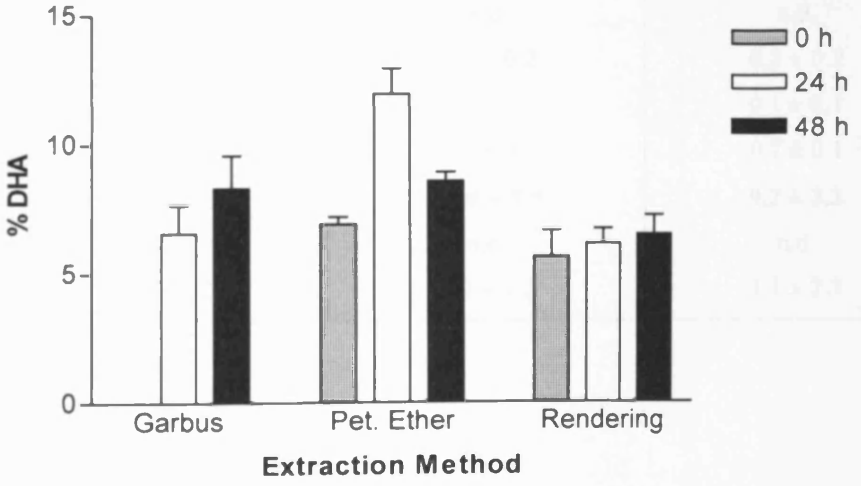


Table 4.18: Percentage fatty acid composition of the total polar lipids from visceral adipose tissue extracted using the Garbus method after different tissue storage times. Results as means ± SD (n=3). n.d. = none detected, tr. = <0.05.

Fatty Acids	Time zero	Time 24 h	Time 48 h
12:0	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.2
14:0	1.3 ±0.1	0.6 ± 0.6	1.3 ± 0.8
16:0	16.7 ± 0.9	14.0 ± 3.8	14.3 ± 2.3
16:1 n-7	2.3 ± 0.2	2.0 ± 0.6	3.2 ± 1.4
16:1	0.5 ± 0.3	0.7 ± 0.2	1.0 ± 0.6
17:0	0.3 ± tr.	0.3 ± 0.1	0.3 ± 0.1
17:1	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.2
18:0	5.7 ± 0.3	6.8 ± 0.1	5.3 ± 1.5
18:1 n-9	20.5 ± 0.5	21.7 ± 0.1	23.8 ± 0.7
18:1 n-7	n.d.	n.d.	0.1 ± 0.2
18:2 n-6	7.3 ± 1.3	7.3 ± 0.4	9.0 ± 1.2
18:2 n-3	0.2 ± 0.1	0.2 ± tr.	0.2 ± tr.
18:3 n-6	0.6 ± 0.2	0.7 ± 0.2	0.9 ± 0.2
18:3 n-3	0.7 ± 0.2	0.6 ± 0.2	1.1 ± 0.5
20:0	0.4 ± 0.3	0.3 ± 0.1	0.2 ± 0.2
20:1 n-9	6.2 ± 1.2	8.8 ± 1.6	9.5 ± 0.5
20:2	0.7 ± 0.2	0.9 ± 0.2	0.8 ± 0.1
20:3 n-9	1.1 ± 0.2	0.7 ± 0.1	0.4 ± 0.2
20:4 n-6	1.7 ± 0.2	1.7 ± 0.3	1.1 ± 0.6
20:4 n-3	0.8 ± tr.	0.7 ± 0.3	0.6 ± 0.1
20:5 n-3	**3.6 ± 0.5**	**2.7 ± 0.5**	**2.2 ± 0.6**
22:1	7.8 ± 1.6	10.5 ± 2.4	10.9 ± 0.7
22:2	n.d.	n.d.	n.d.
22:3	0.1 ± tr.	0.2 ± 0.2	0.2 ± 0.2
23:0	0.4 ± tr.	0.2 ± 0.1	0.1 ± 0.1
22:5 n-3	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
22:6 n-3	**16.1 ± 4.5**	**12.0 ± 1.9**	**9.2 ± 3.2**
24:0	n.d.	n.d.	n.d.
24:1	4.0 ± 0.2	5.5 ± 1.2	3.1 ± 2.3

Table 4.19: Percentage fatty acid composition of the total polar lipids from visceral adipose tissue extracted using the petroleum ether method after different tissue storage times. Results as means ± SD (n=3). n.d. = none detected, tr. = <0.05.

Fatty Acids	Time zero	Time 24 h	Time 48 h
12:0	0.2 ± 0.3	0.5 ± 0.2	n.d.
14:0	0.5 ± 0.4	1.0 ± 0.5	0.6 ± 0.4
16:0	12.0 ± 5.5	16.2 ± 1.5	13.9 ± 1.5
16:1 n-7	5.2 ± 2.9	2.2 ± 0.6	1.6 ± 0.5
16:1	n.d.	1.7 ± 1.5	1.9 ± 1.4
17:0	0.8 ± 0.4	0.4 ± 0.1	0.5 ± 0.1
17:1	0.1 ± 0.2	0.3 ± 0.1	0.2 ± 0.2
18:0	17.6 ± 3.2	10.6 ± 2.8	12.7 ± 2.2
18:1 n-9	23.7 ± 8.6	20.0 ± 1.9	25.0 ± 0.6
18:1 n-7	n.d.	0.1 ± 0.1	n.d.
18:2 n-6	5.5 ± 1.0	6.5 ± 1.4	8.3 ± 0.7
18:2 n-3	n.d.	0.2 ± 0.2	0.1 ± 0.1
18:3 n-6	0.7 ± 0.6	0.6 ± 0.1	0.9 ± 0.1
18:3 n-3	1.8 ± 0.4	1.1 ± 0.6	1.3 ± 0.2
20:0	0.1 ± 0.2	1.3 ± 1.6	n.d.
20:1 n-9	10.4 ± 1.9	7.9 ± 3.0	12.3 ± 0.2
20:2	0.3 ± 0.0	0.8 ± 0.1	0.7 ± 0.1
20:3 n-9	0.3 ± 0.0	0.7 ± 0.2	0.4 ± 0.1
20:4 n-6	2.2 ± 0.4	1.8 ± 0.2	0.5 ± 0.2
20:4 n-3	0.3 ± 0.5	0.7 ± 0.1	0.5 ± 0.5
20:5 n-3	**1.3 ± 0.3**	**2.4 ± 1.9**	**0.9 ± 0.2**
22:1	7.3 ± 1.5	6.5 ± 2.5	11.4 ± 1.0
22:2	0.2 ± 0.4	0.2 ± 0.2	n.d.
22:3	n.d.	n.d.	n.d.
23:0	0.5 ± 0.8	0.1 ± tr.	n.d.
22:5 n-3	n.d.	0.4 ± 0.5	0.3 ± 0.2
22:6 n-3	**7.5 ± 1.5**	**12.0 ± 5.7**	**3.8 ± 0.7**
24:0	n.d.	0.2 ± 0.3	n.d.
24:1	1.9 ± 1.8	3.7 ± 0.6	2.2 ± 0.4

Table 4.20: Percentage fatty acid composition of the total polar lipids from visceral adipose tissue extracted using the rendering method after different tissue storage times. Results as means ± SD (n=3). n.d. = none detected, tr. = <0.05.

Fatty Acids	Time zero	Time 24 h	Time 48 h
12:0	n.d.	1.9 ± 0.3	6.0 ± 1.8
14:0	0.6 ± 0.9	4.2 ± 2.4	4.2 ± 0.9
16:0	10.4 ± 1.5	17.3 ± 1.9	17.8 ± 2.1
16:1 n-7	10.9 ± 1.4	7.7 ± 1.7	2.2 ± 0.5
16:1	n.d.	0.7 ± 0.8	0.1 ± 0.2
17:0	1.2 ± 0.3	0.7 ± 0.8	0.6 ± 0.2
17:1	n.d.	0.6 ± 0.5	0.5 ± 0.8
18:0	1.7 ± 0.8	5.5 ± 2.7	11.8 ± 1.6
18:1 n-9	29.4 ± 4.6	19.2 ± 6.3	21.0 ± 3.7
18:1 n-7	9.8 ± 4.5	1.2 ± 0.1	n.d.
18:2 n-6	4.4 ± 1.3	8.0 ± 4.0	7.7 ± 2.5
18:2 n-3	n.d.	0.1 ± 0.1	0.2 ± 0.3
18:3 n-6	0.4 ± 0.6	0.7 ± 0.6	0.8 ± 0.7
18:3 n-3	4.0 ± 0.7	1.3 ± 0.3	1.9 ± 0.7
20:0	n.d.	n.d.	3.3 ± 0.4
20:1 n-9	15.4 ± 1.3	13.2 ± 2.7	9.6 ± 0.9
20:2	n.d.	n.d.	0.5 ± 0.2
20:3 n-9	n.d.	n.d.	0.7 ± 0.7
20:4 n-6	0.2 ± 0.8	0.7 ± 0.6	0.1 ± 0.7
20:4 n-3	tr.	1.1 ± 0.5	0.5 ± 0.2
20:5 n-3	**3.2 ± 0.9**	**1.5 ± 0.6**	**0.6 ± 0.3**
22:1	5.4 ± 1.5	8.2 ± 2.5	7.7 ± 1.4
22:2	n.d.	0.9 ± 0.6	0.3 ± 0.2
22:3	n.d.	n.d.	n.d.
23:0	n.d.	n.d.	n.d.
22:5 n-3	n.d.	0.2 ± 0.3	0.2 ± 0.3
22:6 n-3	**1.9 ± 1.5**	**4.0 ± 0.5**	**3.6 ± 1.1**
24:0	n.d.	0.8 ± 0.5	n.d.
24:1	n.d.	0.5 ± 0.9	0.5 ± 0.3

NB; there is no Table 4.21.

Table 4.22: Percentage fatty acid composition of the triacylglycerol fraction from visceral adipose tissue extracted using the Garbus method after different tissue storage times. Results as means ± SD (n=3). n.d. = none detected, tr. = <0.05.

Fatty Acids	Time zero	Time 24 h	Time 48 h
12:0	tr.	0.1 ± tr.	0.1 tr.
14:0	3.5 ± tr.	3.4 ± 0.2	3.3 ± 0.1
16:0	14.0 ± 0.4	13.7 ± 0.4	13.5 ± 0.4
16:1 n-7	5.0 ± 0.1	5.2 ± 0.2	5.3 ± 0.2
16:1	0.1 ± tr.	0.1 ± tr.	0.1 ± tr.
17:0	0.2 ± tr.	0.2 ± 0.1	0.2 ± tr.
17:1	0.5 ± tr.	0.5 ± tr.	0.4 ± tr.
18:0	2.5 ± 0.6	2.8 ± 0.3	2.7 ± 0.4
18:1 n-9	26.3 ± 0.7	26.4 ± 0.5	26.0 ± 1.4
18:1 n-7	0.5 ± 0.9	0.5 ± 0.9	0.8 ± 0.8
18:2 n-6	9.5 ± 3.8	13.9 ± 0.1	14.1 ± 0.1
18:2 n-3	4.9 ± 2.8	0.4 ± 0.4	0.4 ± tr.
18:3 n-6	1.4 ± tr.	0.9 ± 0.7	1.0 ± 0.8
18:3 n-3	1.1 ± 0.9	1.3 ± 0.2	1.5 ± 0.1
20:0	0.4 ± 0.1	0.3 ± 0.1	0.2 ± tr.
20:1 n-9	8.1 ± 0.1	8.5 ± 0.2	8.2 ± 0.2
20:2	0.8 ± tr.	0.8 ± tr.	0.8 ± tr.
20:3 n-9	0.4 ± tr.	0.4 ± tr.	0.4 ± tr.
20:4 n-6	0.4 ± tr.	0.4 ± tr.	0.4 ± tr.
20:4 n-3	1.0 ± 0.1	0.9 ± tr.	0.9 ± 0.1
20:5 n-3	**2.2 ± 0.1**	**2.0 ± 0.1**	**2.1 ± tr.**
22:1	8.2 ± 0.3	8.6 ± 0.3	8.0 ± 0.1
22:2	0.1 ± tr.	0.5 ± 0.6	0.1 ± tr.
22:3	0.1 ± tr.	n.d.	0.1 ± tr.
23:0	0.2 ± tr.	0.2 ± tr.	0.2 ± tr.
22:5 n-3	0.6 ± 0.4	0.7 ± 0.4	0.9 ± tr.
22:6 n-3	**7.5 ± 0.7**	**6.6 ± 0.8**	**7.6 ± 0.7**
24:0	0.1 ± tr.	0.1 ± tr.	0.1 ± 0.1
24:1	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.1

NB; there is no Table 4.21

Table 4.23: Percentage fatty acid composition of the triacylglycerol fraction from visceral adipose tissue extracted using the petroleum ether method after different tissue storage times. Results as means ± SD (n=3). n.d. = none detected, tr. = <0.05.

Fatty Acids	Time zero	Time 24 h	Time 48 h
12:0	tr.	tr.	tr.
14:0	3.1 ± 0.3	3.7 ± 0.3	2.5 ± 1.0
16:0	13.1 ± 0.1	13.9 ± 1.0	13.7 ± 0.5
16:1 n-7	5.6 ± 0.6	5.9 ± 0.2	5.5 ± 0.4
16:1	0.1 ± tr.	0.1 ± tr.	0.1 ± tr.
17:0	0.1 ± tr.	0.2 ± tr.	0.2 ± tr.
17:1	0.2 ± tr.	0.5 ± tr.	0.5 ± tr.
18:0	2.3 ± 0.3	2.1 ± 0.2	1.9 ± 0.8
18:1 n-9	26.4 ± 0.3	24.5 ± 2.9	27.3 ± 0.1
18:1 n-7	0.4 ± tr.	0.6 ± 0.5	1.4 ± 0.9
18:2 n-6	14.0 ± 0.3	14.0 ± 0.9	13.9 ± 0.7
18:2 n-3	0.4 ± tr.	0.4 ± tr.	0.4 ± 0.1
18:3 n-6	1.5 ± tr.	1.0 ± 0.8	1.4 ± 0.1
18:3 n-3	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.2
20:0	0.5 ± tr.	0.3 ± 0.1	0.2 ± tr.
20:1 n-9	8.5 ± 0.2	8.7 ± 0.6	8.6 ± 0.2
20:2	0.8 ± tr.	0.8 ± 0.1	0.8 ± tr.
20:3 n-9	0.4 ± tr.	0.4 ± tr.	0.4 ± tr.
20:4 n-6	0.4 ± tr.	0.4 ± tr.	0.4 ± tr.
20:4 n-3	1.0 ± tr.	1.0 ± 0.1	0.9 ± 0.1
20:5 n-3	**2.3 ± 0.1**	**2.1 ± 0.2**	**2.0 ± 0.2**
22:1	7.7 ± 0.2	8.9 ± 1.1	8.0 ± 0.3
22:2	0.1 ± tr.	0.1 ± tr.	0.1 ± tr.
22:3	0.1 ± tr.	0.1 ± tr.	0. 1± tr.
23:0	0.2 ± tr.	0.2 ± tr.	0.1 ± tr.
22:5 n-3	0.7 ± 0.5	0.9 ± 0.1	0.8 ± 0.1
22:6 n-3	**7.8 ± 0.5**	**7.1 ± 1.2**	**6.8 ± 0.2**
24:0	0.1 ± tr.	0.1 ± tr.	0.1 ± tr.
24:1	0.7 ± tr.	0.7 ± 0.2	0.7 ± 0.1

Table 4.24: Percentage fatty acid composition of the triacylglycerol fraction from visceral adipose tissue extracted using the rendering method at different tissue storage times. Results as means ± SD (n=3). n.d. = none detected, tr. = <0.05.

Fatty Acids	Time zero	Time 24 h	Time 48 h
12:0	tr.	0.1 ± tr.	0.1 ± tr.
14:0	3.2 ± 0.3	3.3 ± 0.2	3.1 ± 0.4
16:0	14.0 ± 0.9	13.6 ± 0.2	13.9 ± 0.2
16:1 n-7	5.6 ± 0.6	5.4 ± 0.1	5.0 ± 0.2
16:1	0.1 ± tr.	0.2 ± 0.2	0.1 ± tr.
17:0	0.2 ± tr.	0.2 ± tr.	0.2 ± tr.
17:1	0.5 ± tr.	0.2 ± tr.	0.3 ± 0.2
18:0	2.6 ± 0.5	2.7 ± 0.3	3.0 ± 0.1
18:1 n-9	27.0 ± 0.5	27.9 ± 0.1	27.1 ± 0.3
18:1 n-7	0.3 ± tr.	0.3 ± tr.	0.2 ± 0.2
18:2 n-6	13.7 ± 0.3	14.0 ± 0.21	14.1 ± 0.1
18:2 n-3	0.3 ± tr.	0.4 ± tr.	0.4 ± tr.
18:3 n-6	1.4 ± tr.	0.9 ± 0.7	0.9 ± 0.7
18:3 n-3	1.4 ± 0.1	1.4 ± 0.2	0.9 ± 0.7
20:0	tr.	0.2 ± tr.	0.2 ± tr.
20:1 n-9	8.5 ± 0.4	8.5 ± 0.3	8.5 ± 0.3
20:2	0.8 ± tr.	0.8 ± tr.	0.9 ± 0.1
20:3 n-9	0.4 ± tr.	0.4 ± 0.1	0.5 ± tr.
20:4 n-6	0.4 ± tr.	0.3 ± 0.1	0.4 ± tr.
20:4 n-3	1.0 ± 0.2	0.9 ± 0.1	0.9 ± 0.1
20:5 n-3	**2.0 ± 0.1**	**2.1 ± 0.1**	**2.0 ± tr.**
22:1	8.0 ± 0.2	7.9 ± 0.3	8.4 ± 0.3
22:2	0.1 ± tr.	0.1 ± tr.	0.1 ± tr.
22:3	tr.	0.1 ± 0.1	0.1 ± 0.1
23:0	0.1 ± 0.1	0.1 ± tr.	0.1 ± 0.1
22:5 n-3	0.8 ± 0.1	0.9 ± tr.	0.9 ± tr.
22:6 n-3	**6.8 ± 1.1**	**6.4 ± 0.6**	**6.8 ± 0.1**
24:0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± tr.
24:1	0.7 ± 0.1	0.6 ± 0.1	0.8 ± 0.1

Table 4.25: Percentage fatty acid composition of the diacylglycerol (1,2- and 1,3 diacylglycerol) fraction from visceral adipose tissue extracted using the Garbus method after different tissue storage times. Results as means ± SD (n=3). n.d. = none detected, tr. = <0.05.

Fatty Acids	Time zero	Time 24 h	Time 48 h
12:0	0.3 ± 0.1	0.4 ± 0.3	0.2 ± 0.1
14:0	2.0 ± 0.4	1.8 ± 0.6	1.2 ± 0.4
16:0	15.5 ± 0.4	13.3 ± 0.2	12.2 ± 1.7
16:1 n-7	3.5 ± 0.4	2.8 ± tr.	2.8 ± 0.3
16:1	0.3 ± 0.1	0.2 ± tr.	0.5 ± 0.5
17:0	0.3 ± tr.	0.3 ± 0.1	0.2 ± tr.
17:1	0.5 ± 0.1	0.6 ± tr.	0.5 ± 0.1
18:0	4.4 ± 0.2	5.0 ± 1.4	4.4 ± 0.9
18:1 n-9	27.3 ± 0.6	24.3 ± 1.9	25.2 ± 2.9
18:1 n-7	n.d.	n.d.	0.1± 0.2
18:2 n-6	12.1 ± 0.3	11.8 ± 0.5	11.6 ± 0.6
18:2 n-3	0.3 ± 0.1	0.4 ± tr.	0.4 ± 0.2
18:3 n-6	0.8 ± 0.5	1.0 ± 0.7	0.8 ± 0.8
18:3 n-3	1.0 ± tr.	1.8 ± 0.5	1.8 ± 0.9
20:0	0.2 ± 0.2	n.d.	n.d.
20:1 n-9	9.5 ± 0.4	8.9 ± 0.7	9.4 ± 1.2
20:2	0.8 ± 0.1	0.6 ± tr.	0.6 ± 0.1
20:3 n-9	0.4 ± 0.1	0.5 ± 0.1	0.4 ± tr.
20:4 n-6	1.4 ± 0.1	1.1 ± 0.2	1.1 ± 0.3
20:4 n-3	0.7 ± 0.2	1.2 ± 0.2	1.1 ± 0.2
20:5 n-3	**2.1 ± tr.**	**3.8 ± 1.0**	**3.4 ± 1.3**
22:1 n-9	8.5 ± 0.5	8.3 ± 0.9	8.7 ± 1.0
22:2	n.d.	0.2 ± 0.3	0.2 ± 0.3
22:3	0.6 ± 0.3	0.6 ± tr.	0.9 ± 0.4
23:0	0.5 ± 0.4	0.5 ± tr.	0.3 ± 0.4
22:5 n-3	0.6 ± tr.	1.1 ± 0.2	1.2 ± 0.4
22:6 n-3	**5.8 ± 1.0**	**8.8 ± 1.1**	**9.9 ± 1.9**
24:0	0.3 ± 0.3	n.d.	0.1 ± 0.1
24:1	0.8 ± 0.4	0.8 ± 0.8	1.0 ± 0.1

Table 4.26: Percentage fatty acid composition of the diacylglycerol (1,2- and 1,3 diacylglycerol) fraction from visceral adipose tissue extracted using the petroleum ether method after different tissue storage times. Results as means ± SD (n=3). n.d. = none detected, tr. = <0.05.

Fatty Acids	Time zero	Time 24 h	Time 48 h
12:0	0.7 ± 0.1	0.8 ± 0.3	0.3 ± 0.3
14:0	1.2 ± 0.7	2.1 ± 0.5	0.7 ± 0.7
16:0	10.7 ± 5.7	14.8 ± 1.0	13.9 ± 0.8
16:1 n-7	1.6 ± 0.3	3.7 ± 1.8	1.6 ± 0.4
16:1	3.3 ± 0.6	0.1 ± 0.1	3.9 ± 1.2
17:0	0.6 ± 0.5	0.4 ± 0.1	0.7 ± 0.2
17:1	0.5 ± 0.5	0.2 ± 0.1	0.7 ± 0.1
18:0	15.0 ± 5.2	9.2 ± 2.2	15.0 ± 3.2
18:1 n-9	21.3 ± 3.2	24.6 ± 0.6	21.2 ± 2.4
18:1 n-7	n.d.	n.d.	n.d.
18:2 n-6	8.8 ± 1.8	10.3 ± 0.8	7.4 ± 1.2
18:2 n-3	n.d.	0.3 ± 0.2	0.1 ± 0.2
18:3 n-6	1.1 ± 0.1	1.0 ± 0.2	1.1 ± 0.5
18:3 n-3	1.6 ± 0.9	2.2 ± 0.5	2.0 ± 0.4
20:0	n.d.	n.d.	n.d.
20:1 n-9	12.0 ± 0.5	10.4 ± 1.3	12.4 ± 1.0
20:2	1.4 ± 1.1	0.8 ± 0.2	0.4 ± 0.4
20:3 n-9	0.9 ± 0.2	0.4 ± 0.1	0.1 ± 0.2
20:4 n-6	0.6 ± tr.	1.3 ± 0.2	1.7 ± 0.6
20:4 n-3	0.6 ± 0.5	0.8 ± 0.4	1.3 ± 0.5
20:5 n-3	**1.2 ± 0.1**	**1.3 ± 0.5**	**0.5 ± 0.5**
22:1	10.9 ± 5.1	9.4 ± 1.2	10.6 ± 1.0
22:2	n.d.	0.2 ± 0.3	n.d.
22:3	n.d.	0.4 ± 0.3	0.3 ± 0.2
23:0	n.d.	n.d.	n.d.
22:5 n-3	0.5 ± 0.4	0.6 ± 0.1	0.1 ± 0.2
22:6 n-3	**5.5 ± 2.1**	**4.8 ± 1.1**	**2.7 ± 0.4**
24:0	n.d.	0.2 ± 0.3	n.d.
24:1	n.d.	0.6 ± 0.7	1.1 ± 0.9

Table 4.27: Percentage fatty acid composition of the diacylglycerol from visceral adipose tissue using the rendering method of extraction after different tissue storage times. Results as means ± SD. n.d. = none detected, tr. = <0.05.

Fatty Acids	Time zero	Time 24 h	Time 48 h
12:0	n.d.	5.5 ± 2.7	4.0 ± 1.3
14:0	n.d.	2.8 ± 0.1	3.1 ± 0.4
16:0	12.6 ± 1.5	15.7 ± 4.6	14.2 ± 1.0
16:1 n-7	9.1 ± 1.3	2.5 ± 0.8	2.4 ± 0.3
16:1	n.d.	1.7 ± 0.7	0.1 ± 0.2
17:0	0.8 ± 0.2	0.7 ± 0.4	0.4 ± 0.4
17:1	n.d.	1.4 ± 1.6	1.8 ± 1.6
18:0	1.5 ± 0.3	4.7 ± 2.0	5.0 ± 1.2
18:1 n-9	23.7 ± 5.3	21.9 ± 5.1	22.0 ± 1.9
18:1 n-7	14.6 ± 8.2	n.d.	n.d.
18:2 n-6	6.0 ± 1.8	9.6 ± 1.8	8.9 ± 0.9
18:2 n-3	n.d.	0.4 ± 0.1	0.1 ± 0.1
18:3 n-6	0.4 ± 0.4	2.1 ± 1.5	1.4 ± 0.1
18:3 n-3	2.9 ± 1.8	1.3 ± 0.9	1.8 ± 1.3
20:0	0.4 ± 0.3	0.1 ± tr.	0.4 ± 0.7
20:1 n-9	14.2 ± 2.9	11.0 ± 3.0	14.3 ± 2.6
20:2	0.3 ± 0.3	0.1 ± 0.2	0.5 ± 0.9
20:3 n-9	n.d.	0.6 ± 0.8	0.3 ± 0.5
20:4 n-6	n.d.	0.4 ± 0.4	0.7 ± 0.1
20:4 n-3	0.5 ± 0.5	0.9 ± 0.3	0.5 ± tr.
20:5 n-3	**2.7 ± 0.9**	**1.3 ± 0.3**	**3.5 ± 1.2**
22:1	7.7 ± 1.9	7.5 ± 2.8	8.2 ± 0.9
22:2	n.d.	2.7 ± 1.8	0.9 ± 0.6
22:3	n.d.	n.d.	0.1 ± tr.
23:0	n.d.	n.d.	n.d.
22:5 n-3	n.d.	0.4 ± 0.3	0.2 ± 0.3
22:6 n-3	**2.4 ± 0.1**	**4.3 ± 0.2**	**3.4 ± 0.4**
24:0	n.d.	0.4 ± 0.4	0.2 ± 0.3
24:1	n.d.	0.2 ± 0.2	2.0 ± 0.7

Table 4.28: Percentage fatty acid composition of the monoacylglycerol fraction from visceral adipose tissue extracted using the Garbus method after different tissue storage times. Results as means ± SD (n=3). n.d. = none detected, tr. = <0.05. No MAG was detected at time zero.

Fatty Acids	Time 24 hr	Time 48 h
12:0	0.3 ± 0.1	0.3 ± 0.1
14:0	1.9 ± 0.2	1.7 ± 0.5
16:0	13.8 ± 0.5	14.3 ± 0.6
16:1 n-7	5.0 ± 0.2	4.9 ± 1.4
16:1	0.2 ± tr.	0.3 ± 0.1
17:0	0.2 ± tr.	0.3 ± 0.1
17:1	0.3 ±0.1	0.6 ± tr.
18:0	4.5 ± 0.9	4.4 ± 0.4
18:1 n-9	27.8 ± 0.3	27.6 ± 1.1
18:1 n-7	n.d.	n.d.
18:2 n-6	13.9 ± 0.3	13.7 ± 0.5
18:2 n-3	0.3 ± 0.1	0.3 ± tr.
18:3 n-6	1.4 ± 0.1	1.0 ± 0.6
18:3 n-3	1.4 ± 0.2	1.3 ± 0.2
20:0	0.1 ± 0.1	n.d.
20:1 n-9	8.8 ± 0.4	8.7 ± 0.2
20:2	0.8 ± 0.2	0.7 ± 0.1
20:3 n-9	0.5 ± 0.1	0.4 ± tr.
20:4 n-6	0.5 ± 0.4	0.5 ± 0.1
20:4 n-3	0.9 ± 0.1	0.7 ± 0.1
20:5 n-3	**1.6 ± 0.2**	**1.5 ± 0.1**
22:1	7.3 ± 0.5	8.1 ± 1.0
22:2	0.1 ± 0.2	n.d.
22:3	0.2 ± 0.2	0.1 ± 0.1
23:0	0.1 ± 0.2	n.d.
22:5 n-3	0.8 ± 0.1	0.7 ± 0.1
22:6 n-3	**6.9 ± 0.4**	**7.3 ± 1.0**
24:0	n.d.	n.d.
24:1	0.6 ± 0.1	0.5 ± 0.1

Table 4.29: Percentage fatty acid composition of the monoacylglycerol fraction from visceral adipose tissue extracted using the petroleum ether method after different tissue storage times. Results as means ± SD (n=3). n.d. = none detected, tr. = <0.05.

Fatty Acids	Time zero	Time 24 h	Time 48 h
12:0	n.d.	0.6 ± 0.3	0.1 ± tr.
14:0	n.d.	2.0 ± 0.8	0.5 ± 0.2
16:0	7.4 ± 1.7	14.3 ± 1.1	12.5 ± 1.0
16:1 n-7	4.2 ± 2.3	5.1 ±0.2	2.4 ± 0.3
16:1	n.d.	0.2 ± 0.1	0.2 ± 0.2
17:0	0.6 ± 0.5	0.4 ± tr.	0.4 ± 0.1
17:1	0.6 ± 0.9	0.5 ± 0.2	0.2 ± 0.1
18:0	19.1 ± 3.6	7.4 ± 1.3	9.3 ± 2.1
18:1 n-9	21.9 ± 2.6	25.1 ± 0.5	27.3 ± 2.0
18:1 n-7	n.d.	n.d.	n.d.
18:2 n-6	9.2 ± 1.9	13.5 ± 0.2	13.4 ± 0.8
18:2 n-3	n.d.	0.3 ± 0.1	0.2 ± 0.2
18:3 n-6	1.0 ± 0.3	1.4 ± tr.	1.4 ± 0.1
18:3 n-3	2.1 ± 1.3	1.5 ± 0.2	1.4 ± 0.3
20:0	n.d.	n.d.	n.d.
20:1 n-9	11.9 ± 0.1	8.4 ± 0.6	10.3 ± 0.3
20:2	0.6 ± 0.6	0.7 ± tr.	0.8 ± 0.1
20:3 n-9	0.2 ± 0.3	0.5 ± tr.	0.4 ± 0.1
20:4 n-6	3.0 ± 2.3	0.9 ± 0.5	0.4 ± 0.2
20:4 n-3	1.0 ± 0.9	1.0 ± 0.1	1.2 ± 0.4
20:5 n-3	**1.3 ± 0.5**	**1.7 ± tr.**	**1.5 ± 0.2**
22:1	8.0 ± 2.0	6.1 ± tr.	7.8 ± 0.6
22:2	n.d.	n.d.	n.d.
22:3	n.d.	n.d.	n.d.
23:0	n.d.	0.1 ± 0.1	0.2 ± 0.3
22:5 n-3	0.3 ± 0.5	0.5 ± 0.4	0.7 ± tr.
22:6 n-3	**6.8 ± 1.3**	**7.8 ± 1.1**	**7.3 ± 0.2**
24:0	n.d.	n.d.	n.d.
24:1	0.8 + 0.1	0.5 ± 0.1	0.2 ± 0.3

Table 4.30: Percentage fatty acid composition of the monoacylglycerol from visceral adipose tissue over time using the rendering method of extraction after different tissue storage times. Results as means ± SD. n.d. = none detected, tr. = <0.05.

Fatty Acids	Time zero	Time 24 h	Time 48 h
12:0	n.d.	2.1 ± 0.5	2.7 ± 0.7
14:0	0.1 ± 0.2	4.2 ± 2.9	2.8 ± 0.7
16:0	9.2 ± 1.3	14.8 ± 2.1	15.8 ± 2.7
16:1 n-7	4.6 ± 1.5	9.1 ± 4.5	3.7 ± 1.4
16:1	0.3 ± 0.5	1.0 ± 1.3	n.d.
17:0	0.5 ± 0.4	0.2 ± 0.2	0.6 ± 0.2
17:1	0.2 ± 0.3	0.3 ± 0.3	0.7 ± 0.3
18:0	16.4 ± 5.5	5.1 ± 3.1	6.6 ± 2.7
18:1 n-9	19.4 ± 1.9	20.6 ± 8.3	21.5 ± 1.4
18:1 n-7	n.d.	n.d.	n.d.
18:2 n-6	8.1 ± 1.0	10.3 ± 3.3	10.7 ± 0.6
18:2 n-3	0.2 ± 0.3	0.3 ± 0.2	0.1 ± 0.2
18:3 n-6	1.1 ± 0.2	1.2 ± 0.2	0.7 ± 0.2
18:3 n-3	2.2 ± 0.7	2.5 ± 0.2	1.6 ± 1.2
20:0	0.5 ± 0.1	0.2 ± tr.	0.7 ± 0.4
20:1 n-9	10.5 ± 0.9	10.7 ± 2.0	11.0 ± 3.2
20:2	0.5 ± 0.5	0.3 ± tr.	0.2 ± 0.3
20:3 n-9	0.3 ± 0.5	0.5 ± 0.6	0.8 ± tr.
20:4 n-6	2.7 ± 0.6	0.4 ± 0.4	1.1 ± 0.8
20:4 n-3	1.5 ± 0.2	1.5 ± 1.1	0.5 ± 0.5
20:5 n-3	**1.7 ± 0.5**	**1.8 ± 0.5**	**3.4 ± 2.2**
22:1	7.9 ± 2.0	6.0 ± 1.1	7.3 ± 1.5
22:2	n.d.	1.3 ± 0.9	n.d.
22:3	n.d.	n.d.	n.d.
23:0	4.2 ± 0.8	n.d.	n.d.
22:5 n-3	0.5 ± 0.5	0.5 ± 0.4	0.2 ± 0.3
22:6 n-3	**5.6 ± 1.8**	**5.2 ± 2.4**	**4.9 ± 0.3**
24:0	n.d.	n.d.	n.d.
24:1	1.1 ± 0.9	0.4 ± 0.4	2.6 ± 0.9

Table 4.31: Percentage fatty acid composition of the non-esterified fatty acid fraction (NEFA) from visceral adipose tissue extracted using the Garbus method after different tissue storage times. Results as means ± SD (n=3). n.d. = none detected, tr. = <0.05. Unexpectedly, no NEFA fraction was detected at time zero possibly due to an experimental error.

Fatty Acids	Time 24 hr	Time 48 h
12:0	0.2 ± tr.	0.2 ± 0.1
14:0	1.0 ± 0.3	1.2 ± 0.2
16:0	12.4 ± 0.2	13.6 ± 0.7
16:1 n-7	3.7 ± 0.3	3.5 ± 1.1
16:1	0.2 ± tr.	0.5 ± 0.3
17:0	0.2 ± tr.	0.2 ± tr.
17:1	0.4 ± 0.1	0.4 ± tr.
18:0	3.8 ± 0.2	4.4 ± 1.1
18:1 n-9	29.5 ± 2.1	27.0 ± 2.5
18:1 n-7	0.7 ± 0.6	0.4 ± tr.
18:2 n-6	14.0 ± 1.1	13.7 ± 0.9
18:2 n-3	0.4 ± 0.2	0.4 ± tr.
18:3 n-6	0.8 ± 0.5	1.1 ± 0.1
18:3 n-3	1.0 ± 0.1	1.0 ± 0.2
20:0	0.2 ± 0.2	0.3 ± 0.1
20:1 n-9	7.4 ± 0.6	7.0 ± 0.5
20:2	1.0 ± 0.3	1.0 ± 0.2
20:3 n-9	0.7 ± 0.2	0.7 ± 0.1
20:4 n-6	1.4 ± 0.3	1.7 ± 0.4
20:4 n-3	1.2 ± tr.	1.0 ± 0.2
20:5 n-3	**3.1 ± 0.6**	**3.1 ± 0.4**
22:1	6.3 ± 0.8	5.5 ± 0.6
22:2	0.1 ± 0.1	0.2 ± 0.3
22:3	0.5 ± 0.6	0.8 ± 0.4
23:0	1.3 ± 1.1	1.0 ± 0.9
22:5 n-3	0.9 ± 0.1	0.8 ± 0.2
22:6 n-3	**6.6 ± 1.9**	**8.3 ± 2.2**
24:0	0.4 ± 0.3	0.3 ± 0.3
24:1	0.8 ± 0.3	0.7 ± 0.2

Table 4.32: Percentage fatty acid composition of the non-esterified fatty acid fraction from visceral adipose tissue extracted using the petroleum ether method after different tissue storage times. Results as means ± SD (n=3). n.d. = none detected, tr. = <0.05.

Fatty Acids	Time zero	Time 24 h	Time 48 h
12:0	0.7 ± 0.5	0.2 ± tr.	0.1 ± 0.2
14:0	1.9 ± 0.4	0.9 ± 0.2	1.0 ± 0.1
16:0	14.0 ± 1.2	11.8 ± 0.8	12.9 ± 1.2
16:1 n-7	2.1 ± 0.3	3.8 ± 0.3	3.4 ± 1.3
16:1	n.d.	0.2 ± tr.	0.3 ± tr.
17:0	0.5 ± 0.1	0.2 ± tr.	0.3 ± 0.1
17:1	0.2 ± 0.0	0.4 ± 0.1	0.4 ± 0.1
18:0	10.6 ± 2.1	4.8 ± 0.6	5.6 ± 1.8
18:1 n-9	22.8 ± 1.5	24.1 ± 1.4	25.4 ± 0.4
18:1 n-7	n.d.	0.2 ± 0.2	0.1 ± 0.1
18:2 n-6	10.9 ± 0.8	12.9 ± 1.5	12.7 ± 1.1
18:2 n-3	0.5 ± tr.	0.4 ± 0.1	0.3 ± 0.1
18:3 n-6	0.7 ± 0.3	0.6 ± 0.5	1.1 ± 0.1
18:3 n-3	1.1 ± 0.9	1.5 ± 0.2	1.3 ± 0.2
20:0	0.3 ± 0.2	0.4 ± tr.	0.2 ± 0.2
20:1 n-9	8.3 ± 0.1	6.8 ± 0.1	8.1 ± 0.9
20:2	0.6 ± tr.	1.0 ± 0.2	0.8 ± 0.1
20:3 n-9	0.4 ± 0.1	0.8 ± 0.1	0.6 ± 0.1
20:4 n-6	0.9 ± 0.4	1.8 ± 0.3	1.4 ± 0.2
20:4 n-3	0.8 ± tr.	1.3 ± 0.2	1.1 ± 0.2
20:5 n-3	**2.2 ± 0.1**	**4.2 ± 0.6**	**3.1 ± 0.9**
22:1	6.5 ± 0.5	5.3 ± 0.6	6.8 ± 0.9
22:2	tr.	tr.	n.d.
22:3	tr.	1.5 ± 1.5	2.2 ± 0.4
23:0	4.6 ± 0.8	0.8 ± 1.3	n.d.
22:5 n-3	0.8 ± 0.2	1.2 ± tr.	1.0 ± 0.2
22:6 n-3	**6.9 ± 0.5**	**12.0 ± 1.7**	**8.6 ± 0.6**
24:0	1.0 ± 0.9	0.6 ± 0.5	0.8 ± 0.2
24:1	0.9 ± 0.3	0.6 ± 0.2	0.7 ± 0.2

Table 4.33: Percentage fatty acid composition of the non-esterified fatty acid fraction from visceral adipose tissue extracted using the rendering method of extraction after different tissue storage times. Results as means ± SD. n.d. = none detected, tr. = <0.05.

Fatty Acids	Time zero	Time 24 h	Time 48 h
12:0	n.d.	1.4 ±0.9	0.8 ± 0.3
14:0	0.1 ± 0.1	2.5 ±1.1	1.4 ± 0.5
16:0	9.2 ± 1.3	14.3 ±2.5	13.2 ± 0.7
16:1 n-7	4.6 ± 1.5	3.2 ±2.8	2.8 ± 1.4
16:1	0.3 ± 0.3	0.2 ±0.2	0.6 ± 0.8
17:0	0.5 ± 0.4	1.0 ±0.2	0.3 ± 0.1
17:1	0.2 ± 0.3	0.3 ±0.3	0.5 ± 0.1
18:0	16.4 ± 5.5	5.1 ±0.2	4.2 ± 0.1
18:1 n-9	19.4 ± 1.9	20.6 ±8.3	24.9 ± 1.2
18:1 n-7	n.d.	2.1 ±0.2	0.1 ± 0.1
18:2 n-6	8.1 ± 0.4	10.6 ±3.2	12.7 ± 0.9
18:2 n-3	0.2 ± 0.3	0.3 ±0.2	0.6 ± 0.1
18:3 n-6	1.1 ± 0.2	2.7 ±2.6	1.0 ± 0.6
18:3 n-3	2.2 ± 0.7	1.7 ±0.9	1.1 ± 0.9
20:0	0.5 ± 0.1	0.3 ±0.3	0.5 ± 0.6
20:1 n-9	10.5 ± 0.9	11.4 ± 5.3	8.4 ± 1.5
20:2	0.5 ± 0.4	0.8 ± 0.7	1.0 ± 0.1
20:3 n-9	0.3 ± 0.4	0.5 ± 0.5	0.7 ± 0.1
20:4 n-6	2.7 ± 0.5	1.5 ± 0.7	1.5 ± 0.3
20:4 n-3	1.5 ± 0.2	0.9 ± 0.8	1.0 ± 0.2
20:5 n-3	**1.7 ± 0.5**	**2.8 ± 0.9**	**3.3 ± 0.6**
22:1	7.9 ± 2.0	6.1 ± 1.8	6.2 ± 0.9
22:2	n.d.	1.0 ± 1.5	n.d.
22:3	n.d.	n.d.	1.5 ± 1.1
23:0	4.2 ± 0.1	0.3 ± 0.5	0.6 ± 0.1
22:5 n-3	0.5 ± 0.5	0.6 ± 0.3	1.0 ± 0.2
22:6 n-3	**5.6 ± 1.8**	**6.1 ± 1.0**	**9.5 ± 1.3**
24:0	0.8 ± 0.3	1.0 ± 0.8	0.3 ± 0.3
24:1	1.1 ± 0.9	0.6 ± 0.6	0.6 ± 0.3

4.13 Discussion

It has been well documented that different methods of lipid extraction give rise to different yield of lipids (Ewald *et al.*, 1998; de Boer, 1998; Randall *et al.*, 1991). However, very few publications exist which report specific details on the most commonly-used commercial method of lipid extraction, rendering. Due to a lack of information on the rendering method, we set out to compare extraction efficiencies between the commercially-used rendering method and what can be regarded as the best method of lipid extraction, the Garbus method, together with a variation of the Soxhlet method.

The Garbus method is an adaptation of the Bligh and Dyer procedure and is a reliable method for lipid extraction and offers the unique advantage of extracting the more difficult-to-extract lipids such as highly charged phospholipids (e.g. higher inositides) (Christie, 2003). In keeping with its reputation we determined that the yields of both total lipid and TAG were significantly and, more consistently, greater with the Garbus method than either of the other two methods of extraction examined (Figs. 4.2A, 4.2B, 4.12, 4.13). However, despite the Garbus method being generally considered a 'gold standard' in lipid extraction it is not used commercially due to toxicity and cost-related issues (Fang *et al.*, 2007). Previous research has been undertaken to determine a method which reports comparable yields to that of the chloroform/methanol-based Garbus method. Gunnlaugsdottir and Ackman (1993) investigated the efficiency of extraction of two chloroform/methanol/water methods, Bligh and Dyer (of which the Garbus is a variant) and Smith-Ambrose-Knobl compared to a hexane/isopropanol method. They determined that the hexane/isopropanol method was a less effective method of lipid extraction compared to the chloroform/methanol/water-based methods. It was also reported that the hexane/isopropanol method was far less efficient in extracting the more polar lipid classes (Gunnlaugsdottir and Ackman, 1993). These conclusions reflect our findings for the Garbus and petroleum ether methods in terms of total lipid, TAG yield and extraction of the acyl components of lipids from total trout offal (Figs. 4.2A, 4.2B, Tables 4.2 – 4.16).

When total trout offal was employed as the starting material, the Garbus method yielded the maximum amount of total lipid (approximately 36.5 mg/g tissue homogenate) at each time point examined whereas the petroleum ether method gave a total lipid yield of 14.5 mg/g tissue homogenate at time zero. However, the yield from the petroleum ether method increased with tissue storage time and reached comparable yields with the Garbus method after 24 h. Yield of TAG as well as yield of the minor acyl constituents improved with time (Figs. 4.2A, 4.2B, Tables 4.3, 4.9, 4.12, 4.15). Similar to Gunnlaugsdottir and Ackamn (1993) we also found that the more polar lipids were poorly extracted with the petroleum ether method and hypothesised that an increase in the total polar fraction was more reflective of tissue degradation and the subsequent release of complex lipids rather than an increase in method effectiveness (Aidos *et al.*, 2003b).

Ewald *et al.* (1998) also investigated the differences between the Bligh and Dyer and the Soxhlet extraction methods on muscle from various fish species including salmon, herring, cod and Northern pike. The solvent system used for the Soxhlet method was acetone/hexane (10:7, by vol.) and they found that the method of Bligh and Dyer resulted in a greater yield of total lipid compared to the Soxhlet method. For salmon they reported significantly higher yields of total lipid with the Bligh and Dyer method (12.8%) compared to the Soxhlet method (11.72%). Our results correspond with the trend observed by Ewald *et al* (1998) (i.e. our Garbus method resulted in a greater yield of total lipid, 3.8%, compared to the petroleum ether method, 1.5%, (a Soxhlet variant) used in our experiments). Manirakiza *et al.* (2001) also compared variations of the Bligh and Dyer method to different solvents used with the Soxhlet method and reported higher total lipid yields with the Bligh and Dyer method and its variants rather than with the Soxhlet. The Bligh and Dyer method and its variants achieved recoveries of approximately 72 mg/g fresh wt. of total lipid from fish flour (dried and powdered fish used for animal/fish food stuffs) whereas the standard Soxhlet method using acetone:hexane (1:4, by vol.) gave approximately 51 mg/g fresh wt. of total lipid. As different starting materials were used in the present study and that of Manirakiza *et al.* (2001), it was to be expected that the levels of total lipid yield would be significantly different. Nevertheless, the trend is similar. We determined that for

the total trout offal at time zero the total lipid yield with the Garbus method was about twice that seen with the petroleum ether method (Fig. 4.2A).

Inter-method differences observed when total trout offal was used were not found when the starting material was visceral adipose tissue (Figs. 4.9, 4.10). At time zero and 24 h no disparities between methods were observed but by 48 h the level of efficiency for the rendering method had decreased and was significantly different from both the Garbus and petroleum ether methods (Figs. 4.9, 4.10). Since adipose tissue is heavily dominated by TAG accumulating in lipid droplets, the comparability of the extraction methods probably reflects this. Very little literature is available on the use of specific tissues such as adipose tissue in terms of monitoring efficiency of different methods of lipid extraction.

Commercial production of fish oil is most commonly achieved by a rendering method which relies on the use of steam to rupture cells and liberate the oil, followed by a series of purification steps (Aidos *et al.*, 2003a; Pigott and Tucker, 1990). Due to this, a model system of rendering was employed to determine yield from this method in terms of total lipid, TAG, acyl composition and EPA and DHA levels. We found that the yield from total lipid and TAG extraction increased with tissue storage time when total trout offal was used (Figs. 4.2A, 4.2B). However, no differences were observed when visceral adipose tissue was used as a starting material (Figs. 4.9, 4.10). Visceral adipose tissue is primarily used as a storage depot for fatty acids catabolised for energy metabolism (Henderson and Tocher, 1987). It is predominantly composed of the non-polar lipid TAG and we observed that TAG-enriched adipose tissues produced greater yields of both total lipid and TAG with rendering compared to the phospholipid-enriched tissues in total trout offal homogenate (Figs. 4.2A, 4.2B, 4.9, 4.10).

Despite the rendering process being predominantly used for commercial production of fish oils, surprisingly very little literature is available on lipid yields or acyl composition of rendering-derived lipids. From our analysis we determined that total lipids yields depended on both starting material and time of analysis when the rendering method was used (Figs. 4.2A, 4.9). When total trout offal was used the total lipid yield increased from 15.9 mg/g fresh weight of homogenate to 25 mg/g by 48 h. We hypothesised that the increase in yield was probably due to degradation of the

tissue over time by proteinases etc. which would break up the tissue structure thus allowing for easier extraction of complex lipids (Aidos *et al.*, 2003b). Despite this increase in total lipid yield at 48 h the rendering method remained significantly less efficient compared to the Garbus method (Fig. 4.2A). However, when visceral adipose tissue was used as the starting material the rendering method demonstrated equal extraction capacity to that of the Garbus and petroleum ether methods at both zero and 24 h. Nevertheless, total lipid and TAG yield decreased at 48 h and this may be due to degradation such as the susceptibility of the TAG fraction for catabolism by lipases (Athenstaedt and Daum, 2006).

In terms of acyl composition of the lipid classes extracted, we observed that for both starting materials (total trout offal and visceral adipose tissue) there was a general trend for a relative increase in the monounsaturated fatty acids in most lipids with tissue storage time (Tables 4.2 – 4.16, 4.18 – 4.33). As previously mentioned (section 4.8, 4.12) the principal monounsaturated fatty acids consisted of 16:1, 18:1, 20:1 and 22:1 in all lipids derived from either starting material. Aidos *et al.* (2003b) also reported these fatty acids to be the principal fatty acids determined in herring byproducts. Total trout offal contained a high amount of DHA whilst EPA was equally distributed between the trout offal-derived and visceral adipose tissue-derived lipids (Figs. 4.3 – 4.6, 4.12 – 4.15, Tables 4.2 – 4.16, 4.18 – 4.33). In terms of acyl composition the TAG fractions demonstrated very little changes with time whilst acyl changes with time were quite distinct for the total polar lipid fractions especially with regard to DHA which generally decreased significantly with increased storage time (Tables 4.2 – 4.16, 4.18 – 4.33).

Overall, the Garbus method of lipid extraction was found to be the most consistent and produced much better yields of total and individual lipids compared to the petroleum ether and rendering methods when the phospolipid-rich tissues were used. Petroleum ether and rendering proved equally as efficient for tissues stored for 24 h, but as mentioned earlier, a decrease in rendering efficiency was observed at 48 h. Some of the literature reports that steam-based methods of lipid extraction method yield lower

levels of saturated fatty acids and higher yields of PUFAs (Aidos *et al.*, 2001; Aidos *et al.*, 2002a) but this finding was not confirmed in our experiments. Our findings demonstrated that, the rendering method is a less efficient method than the Garbus and a petroleum ether-based derivative of the Soxhlet method of lipid extraction. However, despite this, rendering can be employed to produce sufficient levels of lipid yield for commercial production of fish oil with very little toxicity implications. EPA and DHA in total trout offal are present in high enough quantities to make the use of such material a suitable source for fish oil production. However, EPA and DHA are highly unsaturated and, as we have observed, they become degraded over time especially in the phospholipid fractions. In order to preserve the levels some adjustment to farm storage conditions may be advisory. Also, from our analysis we can conclude that a combination of total trout offal as used here (liver, spleen, eyes, heart, brain) and visceral adipose tissue would be a more effective source for the production of trout oils rich in PUFAs.

CHAPTER 5

Effect of Polyunsaturated Fatty Acid Supplementation on Cartilage Degradation in a Model System for Osteoarthritis

5.0 Introduction

An abundance of evidence exists on the ability of long chain n-3 PUFAs, specifically EPA and DHA, to alter the inflammatory response in a multitude of diseases (Calder, 2002, 2005; Kris-Etherton *et al.*, 2001; Simopoulos, 2002). RA, an autoimmune disease, is one such condition where the long chain PUFAs found in fish oil, are thought to ameliorate particular symptoms, both physical and biochemical (Leeb *et al.*, 2003, 2006) associated with the disease. As previously detailed (section 1.5.1.1) the long chain PUFAs, EPA and DHA, are incorporated into the phospholipids of cellular membranes at the expense of the n-6 fatty acid, AA. AA is a precursor for a range of inflammatory mediators such as prostaglandin E_2 and leukotriene B_4 (Fig. 1.8) both of which are considered key mediators in arthritis (Goggs *et al.*, 2005). The n-3 PUFA derived mediators are believed to be less inflammatory than those derived from AA. Replacement of AA with EPA and DHA in the phospholipids of cell membrane of cells results in competition for the COX and LOX enzyme systems and, thereby, gives rise to smaller amounts of the more inflammatory prostanoids (Calder, 2003). An abundance of literature reports on the relationship of n-3 PUFAs with rheumatoid arthritis (Goldberg and Katz, 2007; James *et al.*, 2003; James and Cleland, 1997; Kremer, 2000) but there is relatively little evidence on the effects of n-3 PUFAs in OA.

Development of OA results in a progressive degeneration and erosion of cartilage over time (section 1.3). In brief, catabolism and subsequent loss of cartilage aggrecan, collagen and chondrocyte death results in a compromised ECM coupled with joint inflammation (Sarzi-Puttini *et al.*, 2005). In the early stages of the disease, progressive loss of joint integrity is primarily due to the actions of proteinases, specifically aggrecanases (Sandy *et al.*, 1992). The aggrecanases, of which there are two main specific isoforms, ADAMTS-4 and ADAMTS-5 (Section 1.13), cleave aggrecan within the IGD at the peptide bond Glu^{373}-Ala^{374} resulting in a compromised ECM (Flannery *et al.*, 1992). As joint destruction continues, MMPs become more involved in the erosion of the ECM. Within the IGD of aggrecan the Asn^{341}-Phe^{342} peptide bond is cleaved by MMPs (van Meurs *et al.*, 1999). Two of the major MMPs involved in joint degradation are MMP-3 and MMP-13. MMP-3 is thought to activate collagenases which in turn degrade the collagen matrix of the ECM. MMP-13 is

considered to be involved in destruction of collagen type II which some consider to be the key marker of irreversible joint damage (Cawston, 1998).

The role of lipid metabolism in the pathogenesis and progression of arthritis has been under investigation for some time (James and Cleland, 1997; Lippiello, 1990; Lippiello *et al.*, 1991). Lippiello *et al.* (1991) determined that elevated levels of lipids in synovial fluid could be attributed to arthritic modifications of articular cartilage and the synovial membrane. This study also concluded that patients presenting with osteoarthritis had elevated concentrations of serum fatty acids. Previous studies have suggested that prostaglandin synthesis in chondrocytes is affected by fatty acids and that linoleate may promote reduced collagen type II synthesis in avian chondrocytes (Lippiello, 1990; Watkins *et al.*, 1996). There is also an age-related association between lipid accumulation and osteoarthritic pathogenesis. Evidence indicates an increase in lipid accumulation (and AA in particular) in human articular cartilage which may induce some of the changes observed in chondrocyte metabolism during osteoarthritis (Lippiello, 1990).

Due to the abundance of evidence suggesting a link between n-3 PUFAs in the pathogenesis of rheumatoid arthritis we wanted to elucidate whether such beneficial effects could also be observed in OA. The object of this chapter was to investigate the effects of EPA, DHA and a fish oil preparation, from trout, on protein degradation and mRNA levels of inflammatory and degradative proteins in an *in vitro* model of OA.

Chapter 5: Materials & Methods

5.1 Preparation and Culture of Cartilage Explants

Metacarpophalangeal joints of 7 day-old calves were obtained from the local abattoir, washed thoroughly and sprayed with virusolue (Microsol laboratory disinfectant, Anachem) before the skin was removed from the limb.

The synovial joint was opened in a Class I laminar flow hood. Full depth cartilage was cut away from the bone using sterile conditions and placed in Dulbecco's-modified Eagle's medium (DMEM; Gibco Life Technologies, UK) containing 50 μg/ml gentamicin (Gibco Life Technologies, UK) and 10% foetal calf serum (FCS) (Gibco Life Technologies, UK) for 36 h at 37°C in a humidified atmosphere of 5% CO_2 and 95% O_2. Explants were washed in DMEM (x 3) and fresh DMEM plus 10% FCS was added to explants for a further incubation of 36 h.

5.2 Preparation of Fatty Acid Solutions

Fatty acids (EPA, DHA) (99% minimum purity) (Nu-Check Prep, USA) were dissolved in appropriate volumes of dimethyl sulfoxide (DMSO) (Sigma, UK) to give stock concentration of 20 mg/ml. The fatty acid stock was diluted in de-lipidised FCS (Pan-Biotech, Germany) to yield a working solution of 1 mg/ml. Aliquots of the stock solution and the fatty acid working solutions were derivatised by transesterification and the resulting FAMEs were analysed on GLC in order to confirm the final concentrations (sections 2.3, 2.4). The required fatty acid concentrations (10 - 300 μg/ml) were prepared in DMEM from the working solutions.

5.3 Supplementation of Explants with Polyunsaturated Fatty Acids

Following the stabilisation period (36 h), cultured explants were washed (x3) in serum-free DMEM and one explant was placed in each individual well of a sterile 24-well plate (Greiner, UK) in 1ml DMEM with or without fatty acid-DMSO working solution (10 – 300 μg/ml). Supplemented explants were incubated for 12 h to allow

uptake and incorporation of the fatty acids into the chondrocyte membranes, under aforementioned conditions (section 5.1).

5.4 Inflammatory Stimulation of Explants

Following fatty acid-supplementation (12 h) the media was removed and the explants washed (x3) in serum-free DMEM to remove any residual fatty acids. Interleukin-1 alpha (IL-1α) (10 ng/ml) (Pepro Tech Inc, UK) was added to the explants and incubated in aforementioned conditions for a further 72 h. IL-1α, an inflammatory cytokine, is regularly used to generate inflammation and cartilage degradation *in vitro* (Flannery *et al.*, 1999).

Following incubation with IL-1α, the explants were weighed and stored in RNA*later*® (1 ml) (Sigma, UK) at -20°C until needed for RNA extraction. RNA*later*® is an aqueous, non-toxic tissue and cell storage reagent that stabilizes and protects cellular RNA by rapidly permeating tissues and immediately inactivating RNases, thus maintaining RNA integrity.

5.5 Hydrolysis (Saponification) of Lipids

For some experiments (section 5.3.3) trout oil was used to supplement the explants. In order to allow a comparison with the PUFA supplementation, it was necessary to hydrolyse the trout oil before dissolving it in the DMSO-delipidised FCS complex.

Hydrolysis of lipids can be achieved by heating them in a solution of aqueous ethanolic alkali and allowing them to reflux. The cleaved fatty acids remain water-soluble in alkali conditions and, in order to extract them, acidification of the solution is required. Upon acidification the NEFAs can then be extracted using an organic solvent. If desired, non-saponifiable and water-soluble components can be recovered by using additional steps.

A solution of 0.1 M potassium hydroxide (KOH) in 90% aqueous ethanol was prepared fresh. The lipid sample was placed in a stoppered tube to which the 0.1 M KOH was added (0.25 ml KOH per milligram of lipid sample; up to 6 mg). The lipid was refluxed

at 50°C for 3 h. Acidification of the sample was completed by adding hydrochloric acid (6 M). The pH of the solution was checked using full range pH paper (Whatman, UK). Once acidified, water (2 ml) was added to the mixture and the non-esterified fatty acids were extracted in iso-hexane(2-methylpentane):diethyl ether (1:1, by vol.) (Fischer, UK). The extracted organic layers were passed through short columns of anhydrous sodium sulphate (Sigma, UK) to remove any residual water. The columns of anhydrous sodium sulphate had previously been washed with iso-hexane (2-methylpentane):diethyl either (1:1, by vol). Extraction of the lower aqueous layer was repeated (x2) to ensure complete extraction of NEFAs. The resulting NEFAs were washed thoroughly in potassium hydrogen carbonate ($KHCO_3$) and water to completely remove any residual acid that was present after the extraction procedure. Samples were taken to dryness under N_2, re-dissolved in HPLC hexane (Fischer, UK) and stored at -20°C.

5.6 Preparation of Trout Oil-Derived NEFAs for Supplementation of Explant Cultures

Once the trout oil had been hydrolysed and the NEFAs extracted, an aliquot was derivatised by transesterification and the concentration determined by GLC. Once quantified the trout oil-derived NEFAs were prepared in DMSO-delipidised FCS as previously described (section 5.2) and the final concentrations were confirmed by GLC (section 2.2 and 2.3). Explant cultures were then supplemented with 10 – 300 µg of hydrolysed trout oil-derived NEFAs for 12 h.

5.7 Dismembration of Cartilage Explants for RNA Extraction

This procedure was employed in order to reduce the cartilage explants to a fine powder prior to RNA extraction. The method involved snap freezing the tissue followed by vigorous shaking of the tissues in the presence of a steel ball which resulted in the explant being pulverised to a fine powder.

The cartilage explants were removed from RNA*later*® (Sigma, UK) snap-frozen in liquid nitrogen and placed into a pre-cooled (liquid nitrogen) dismembrator vessel together with a steel ball. The vessel was secured into the dismembrator (Mikro-Dismembrator U, B-Braun Biotech International) and freeze-milled for 1 min at 2000 rpm. Upon completion, Tri reagent (Sigma, UK) (1 ml) was added to the vessel and the Tri reagent-explant powder was transferred to a sterile 1.5 ml micro-centrifuge tube (Corning, USA). Tri-reagent combines phenol and guanidine thiocyanate in a mono-phase solution which facilitates rapid and effective inhibition of RNase activity.

5.8 RNA Isolation

Molecular biology grade chloroform (0.2 ml) was added to milled cartilage samples in Tri-reagent (1 ml). Samples were vortexed and left at room temperature for 15 min. Samples were then centrifuged on a Sanyo Hawk 15/05 refrigerated bench-top centrifuge at 13,200 rpm for 15 min. The upper phase was transferred to a sterile 1.5 ml tube containing 375 µl of 70% molecular biology grade ethanol (Fischer, UK). Total RNA was isolated using RNeasy mini-columns and reagents (Qiagen, UK) according to the manufacture's protocol and eluted in sterile water. Samples were then frozen at -80 °C until needed for reverse transcription of RNA.

The RNeasy columns used in this particular kit separate RNA, based on the binding properties of a silica gel-based membrane. Upon transferring the tissue to the spin column, total RNA binds to the membrane whilst contaminants are removed by a series of buffer washes. RNA is then eluted in RNase-free, sterile water. Using the RNeasy kit allows all RNA molecules greater than 200 nucleotides to be extracted.

To determine RNA purity the ratio of absorbance at 260 and 280 nanometres (nm) was determined. Satisfactory RNA preparations give a ratio between 1.6 and 2.2 for 260/280 nm absorption. Values outside this range indicated significant contamination (by, for example, protein) and such RNA extracts were not used further.

RNA extraction from articular cartilage is known to be more difficult than from many other tissues due complicating factors including low cell content and a high proportion of negatively charged proteoglycans which elute with RNA.

5.9 Oligonucleotide Primer Design

A primer is a short synthetic oligonucleotide designed to have a sequence which is the reverse complement of a region of target DNA to which it can anneal at a specific temperature. Several parameters were taken into account in designing oligonucleotide primers. Each primer was approximately 20 base pairs in length and had a GC content between 40-60%. In addition, the melting temperatures for primers were preferred in the range between 50-65°C. The primers were also assessed for primer-dimer formation. Primer-dimers are the product of non-specific annealing and primer elongation. Possible sites for self-complementarities increase the risk of primer-dimer formation and, therefore, care was taken to ensure the ends of primer sequences were not complementary. Sequences of primers corresponding to the genes of interest were developed using Genbank (the National Institute of Health (NIH) genetic sequence database) and these primer sequences are outlined in Table 5.0. Oligonucleotide primer products were sequenced by the DNA Sequencing Core Unit at Cardiff University.

Table 5.0: Oligonucleotide sequences for primers used for reverse-transcription polymerase chain reaction (RT-PCR). In the sequence for ADAMTS-5, Y denotes a mixture of G and C.

Protein	Sequence of Primers (Forward/Reverse)		Product Size (bp)	Optimal temp (°C)	Cycle Number
GAPDH (Bovine)	5' TGG CAT CGT GGA GGG ACT TAT 3'		299	59.9	28
	5' GAC GCC TGC TTC ACC ACC TTC 3'				
COX-1 (Bovine)	5' GCC CAA CAC TTC ACC CAT CAG 3'		287	59.0	32
	5' CCA GGA AGC AGC CCA AAC ACT 3'				
COX-2 (Bovine)	5' GCT CTT CCT CCT GTG CCT GAT 3'		229	52.3	32
	5' CAT GGT TCT TTC CCT TAG TGA 3'				
ADAMTS-4 (Bovine)	5' TGG ATC CCG AGG AGC CCT GGT 3'		151	54.2	28
	5' TGG CGG TCA GCG TCG TAG TCC 3'				
ADAMTS-5 (Human)	5' GGC CTC TCC CAT GAY GAT TCC 3'		498	62.2	28
	5' TGA GCG AGA ACA CTG GCC CCA 3'				
MMP-3 (Rat)	5' CTT TTG GCG AAA ATC TCT CAG 3'		404	50.0	28
	5' AAA GAA ACC CAA ATG CTT CAA 3'				
MMP-13 (Rat)	5' TTC TGC CAC ACG CTT TTC CTC 3'		273	53.0	35
	5' GGT TGG GGT CTT CAT CTC CTG 3'				
IL-6 (Bovine)	5' TGC AGT CTT CAA ACG AGT GG 3'		378	54.0	28
	5' ACA TTC AAG CCA CAT AGC CA 3'				
TNF-α (Bovine)	5' ACG TCA TTC ATC ACT TTC ATG AGT TC 3'		310	44.0	35
	5' TCT TCT CAA GCC TCA AGT AAC AAG T 3'				
CCOX-I (Bovine)	5' TCC GAT TGA CAT TAT AGC C 3'		204	48.0	21
	5' AAC CTA AAT ACA ACC TCC TTC 3'				
CCOX-II (Bovine)	5' GAG GAG ACT AAC ATT CGG AT 3'		290	48.0	22
	5' GAA GTA GAG ACA ATC TGA ACC 3'				

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; ADAMTS-4, aggrecanases-1; ADAMTS-5, aggrecanases-2; MMP-3, matrix metalloproteinase-3; MMP-13, matrix metalloproteinase-13; IL-6, interleukin-6; TNF-α, tumour necrosis factor-α; CCOX-1, cytochrome c oxidase subunit I; CCOX-2 cytochrome c oxidase subunit II.

5.10 Reverse Transcription Polymerase Chain Reaction

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) was used to investigate the effect of particular fatty acids on the levels of mRNA for various inflammatory genes and ECM proteinases involved in OA.

Complementary DNA (cDNA) was synthesised by reverse transcription using Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Promega, UK). MMLV reverse transcriptase uses single-stranded RNA as a template in the presence of a primer to synthesise a complementary DNA strand. The reaction mixture also contained 5 x RT buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT), RNasin (RNase inhibitor) (Promega, UK), random hexamers , (50 ng/µl) (Abgene, UK) and dNTPs (100 mM) (Promega, UK).

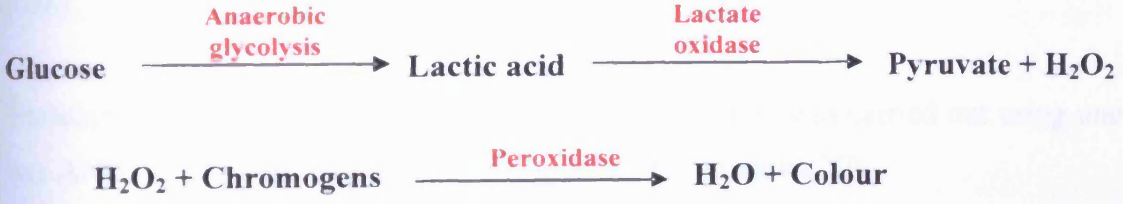
PCR amplification was undertaken using specific oligonucleotide primers (MWG, Germany) which corresponded to the genes of interest (Table 5.0). Initially, the annealing temperatures generated during primer design were used but, in some instances, unspecific bands appeared for some primers. When this problem was encountered, conditions were optimised for each primer set to ensure a single product band of the correct size. $MgCl_2$ concentration gradients (10, 15, 20, 25, 30 mM) as well at temperature gradients were employed to select the correct conditions for primers. $MgCl_2$ is necessary in PCR reactions as Taq polymerase requires a divalent ion as a co-factor and also affects the stringency of binding (lower concentrations provide more stringent conditions thus increasing exact matching of base priming and amplification). In order to maintain the PCR reaction in the logarithmic phase, cycle numbers were optimised for each primer sequence (Table 5.0).

Based on these optimisation experiments, the following conditions for PCR were devised. Following initial denaturation of the cDNA at 95 °C for 5 min, amplification involved 20 – 35 cycles of 1 min at 94 °C, 1 min at the appropriate annealing temperature (Table 5.0), 1 min at 72 °C and a final extension step of 10 min at 72 °C. PCR products were visualised on 2% wt./vol. agarose (Bioline, UK) gels (containing 2% ethidium bromide (Sigma, UK)) under UV light.

5.11 Determination of Explant Metabolism

To assess the metabolic state of cartilage explants a commercially available lactate assay kit (Trinity Biotech, UK) was used to determine the concentration of lactate released into the culture media from the explants during incubation.

The principle behind this assay lies with measuring a glycolysis end product – lactic acid. Lactic acid is metabolised to pyruvate and hydrogen peroxide (H_2O_2) by lactate oxidase. In the presence of the H_2O_2 formed, peroxidase catalyses the oxidative condensation of chromogen precursors to produce a coloured dye which is measured at 540 nm. The increase in absorbance at 540 nm is directly proportional to the lactate concentration of the sample. Changes in the levels of lactate in the media were taken to reflect changes in chrondrocyte metabolism and, thus, were thought to be indicative of chrondrocyte viability.



Lactate standards (0-400 µg/ml) were prepared and 5 µl of these standards or appropriately diluted media samples were added to individual wells of a 96-well plate. Lactate reagent was prepared according to the manufacturer instructions and 250 µl of reagent was added to each well. Samples were left to incubate for 10 min at room temperature before the absorbance at 540 nm was determined using a microplate reader (Opsys MR, Dynex technologies, UK).

5.12 Dimethylmethylene Blue Assay

Determination of proteoglycan degradation was monitored using the dimethylmethylene blue (DMMB) assay which quantifies the levels of sulphated glycosaminoglycans (GAG) released by the cartilage into the culture media (Farndale *et al.*, 1982) . The sulphated GAGs released from cartilage bind to the DMMB dye forming a complex. Generation of this complex gives rise to a metachromatic shift in the absorbance (i.e., colour changes from pink to blue).

Standards of chondroitin sulphate C derived from shark cartilage were prepared (0 – 40 µg/ml) and placed in a 96-well plate together with the appropriately diluted media samples. DMMB solution (200 µl) (32 mg 1,9-DMMB, 20 ml ethanol, 59 ml 1 M sodium hydroxide, 7 ml 98% formic acid and made up to 2 l with water) was added and the absorbance immediately determined at 525 nm due to the instability of the sulphated glycosaminoglycan-DMMB complexes which start to aggregate, and eventually precipitate, as soon as the dye is added to the mixture (Farndale *et al.*, 1986).

Statistical analysis for both GAG and lactate assay results was carried out using one-way ANOVA (post hoc Tukey Test) using SPSS for windows XP.

5.13 Endogenous Acyl Composition of Total Polar Lipids in Bovine Articular Cartilage

To investigate the uptake and incorporation of supplemented fatty acids, typical compositions of explants were determined. The acyl composition of total polar lipids in bovine articular cartilage from the metacarpophalangeal joint was analysed to determine the percentage levels of AA, EPA and DHA (Tables 5.1 – 5.3). The major saturated fatty acids included 18:0 and 16:0 with minor traces of 20:0, 22:0 and 24:0. The principal monounsaturated fatty acid was 18:1 n-9 with minor traces of 18:1 n-7, 16:1 n-7, 20:1 n-9, 22:1 and 24:1. AA constituted approximately 5% of the total acyl composition whilst EPA and DHA were found at 0.8% and 1.5% respectively. Relatively high levels of the unusual n-9 fatty acid, eicosatrienoic acid (20:3), have been previously reported as a major acyl component of total polar lipids from foetal bovine articular cartilage (Adkisson *et al.*, 1991). Here, eicosatrienoic acid was detected at approximately 3% of the total fatty acids (see control values in Tables 5.1 – 5.3).

5.14 Incorporation of α-Linolenic, Eicosapentaenoic and Docosahexaenoic Acids into Total Polar Lipids of Bovine Explant Cultures and the Resulting Effect on the Percentage of Arachidonic Acid

Supplementation of articular cartilage with various doses (10 – 100 μg/ml) of α-linolenic acid, EPA and DHA resulted in dose-dependent increases in the respective fatty acids compared to controls (Fig. 5.1). α-Linolenic acid was found at approximately 0.2% in non-treated cartilage and, following supplementation, the levels increased to 11.5% with 100 μg α-linolenic acid. Supplementation with 50 and 100 μg/ml resulted in a statistically significant increase compared to control levels ($p < 0.001$) (Fig. 5.1).

EPA was detected at approximately 0.6% in control explants but following supplementation with 10 – 100 μg/ml, a dose-dependent increase in EPA ($p < 0.001$) was observed. A final concentration of EPA in polar lipids following 100 μg/ml was approximately 13%. The incorporation of EPA following supplementation resulted in a dose-dependent increase which was statistically significant when compared to control at all doses. DHA was detected at approximately 1.6% in control cultures. Similar to the trends seen with α-linolenic acid and EPA there was a dose-dependent increase observed with DHA supplementation (Fig. 5.1). With 100 μg/ml DHA the final concentration of DHA detected was significantly greater ($p < 0.001$) than controls (approximately 10.8%) (Fig. 5.1). Some variation was observed with 50 μg of DHA (Table 5.3) and it would be advisable to repeat the experiment because of this variation.

Incorporation of the n-3 fatty acids, α-linolenic acid, EPA and DHA into the total polar lipids of chondrocytes was primarily accompanied by a selective decrease in the percentage of 18:1 n-9 (Tables 5.1 – 5.3). With 100 μg/ml ALA there was a 7% decrease in 18:1 n-9 (7%) together with a minor decrease in the minor fatty acids (Table 5.1). EPA supplementation (100 μg/ml) resulted in a 12% increase in EPA accompanied by decreases in 18:1 n-9 (10%) and AA (1.5%). As well as this, a 2.1% increase in DHA was also observed. This increase in DHA following EPA supplementation may have been due to chondrocytes metabolism of EPA to the more unsaturated DHA (Table 5.2). Following 100 μg/ml DHA supplementation there was a 10% increase in DHA of the total polar lipid fraction and a concomitant decrease in 18:1 n-9 (10%). A minor reduction in AA (1.2%) was also observed (Table 5.2).

The uptake of trout oil was not investigated as, in the absence of a specific marker acid or radio-labelled fatty acid, the relative uptake of individual fatty acids from the trout oil mixture would have been very difficult to interpret. Also, the individual levels of EPA and DHA in trout oil (7.5 μg/ml EPA and 31.5 μg/ml DHA) (Table 5.4) were significantly lower than the doses of α-linolenic acid, EPA and DHA used for the uptake experiments.

Fig. 5.1 Increases in the percentage of α-linolenic (ALA), eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) into chondrocyte polar lipids following supplementation of cartilage explants with 10 – 100 µg/ml of the respective fatty acid preparations. Results as means ± SD (n=4).

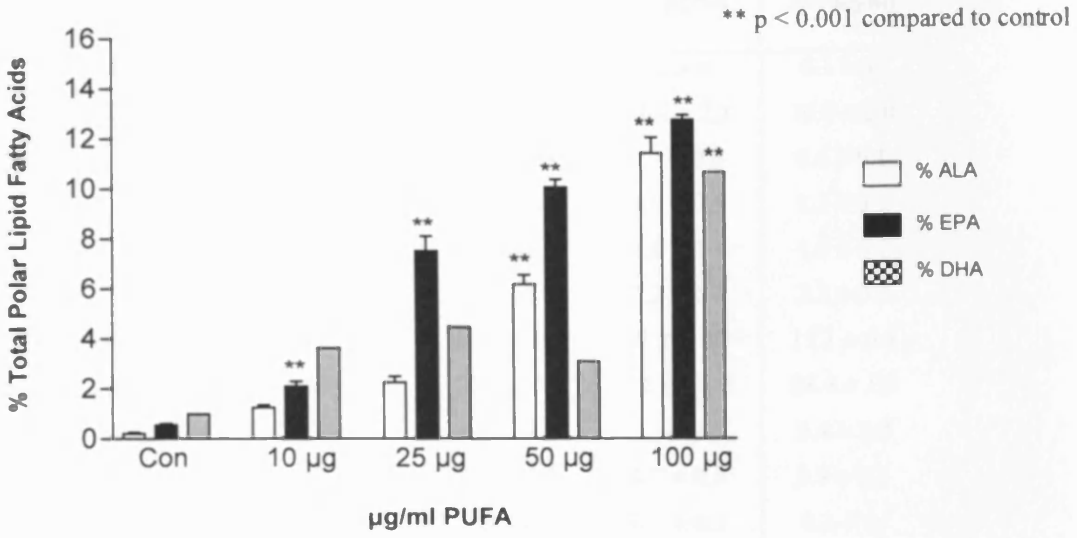


Table 5.1 Percentage acyl composition of total polar lipids extracted from bovine articular cartilage following supplementation with 10 – 100 μg/ml α-linolenic acid. Results as means ± SD (n=4).

Fatty Acids	Control	10 μg/ml	25 μg/ml	50 μg/ml	100 μg/ml
14:0	0.5 ± 0.1	0.3 ± 0.0	0.2 ± tr.	0.2 ± tr.	0.3 ± tr.
16:0	12.4 ± 1.0	7.6 ± 2.2	6.5 ± 1.7	11.1 ± 2.3	10.0 ± 2.0
16:1 n-7	0.9 ± tr.	0.5 ± 0.1	0.4 ± 0.1	0.7 ± 0.3	0.6 ± 0.1
16:1	1.5 ± 0.3	1.0 ± 0.6	1.1 ± 0.9	1.0 ± 0.5	1.1 ± 0.5
17:0	1.6 ± 0.1	0.9 ± 0.7	1.4 ± 0.3	1.8 ± 0.1	1.6 ± 0.1
17:1	2.4 ± 0.3	1.6 ± 0.2	1.7 ± 0.5	2.2 ± 0.3	2.2 ± 0.3
18:0	15.4 ± 0.1	17.7 ± 1.4	16.6 ± 1.3	17.2 ± 0.9	15.1 ± 0.3
18:1 n-9	35.0 ± 1.9	29.4 ± 0.7	28.7 ± 1.3	32.1 ± 4.2	28.4 ± 1.0
18:1 n-7	7.0 ± 0.1	6.3 ± tr.	4.8 ± 3.2	n.d.	3.4 ± 3.0
18:2 n-6	1.4 ± 0.1	1.0 ±0.1	1.0 ± 0.3	0.7 ± 0.1	0.9 ± 0.1
18:2 n-3	0.2 ± 0.3	tr.	0.2 ± 0.2	0.1 ± 0.1	0.3 ± tr.
18:3 n-6	1.8 ± 0.3	1.9 ± 0.3	1.4 ± 0.4	1.6 ± 0.2	1.3 ± tr.
18:3 n-3	**0.2 ± 0.1**	**1.3 ± 0.2**	**2.3 ± 0.5**	**6.2 ± 0.8**	**11.5 ± 1.1**
20:0	0.5 ± 0.2	1.2 ± 0.3	1.1 ± 0.6	0.6 ± 0.1	1.0 ± 0.4
20:1 n-9	0.8 ± tr.	0.6 ± 0.5	1.1 ± 0.1	1.0 ± 0.3	0.8 ± 0.1
20:3 n-9	2.9 ± 1.9	4.1 ± 0.3	4.8 ± 0.3	4.4 ± 0.9	3.7 ± 0.2
20:4 n-6	**4.9 ± 0.4**	**6.5 ± 0.6**	**6.5 ± 0.4**	**5.6 ± 1.1**	**4.8 ± 0.2**
20:4 n-3	0.2 ± tr.	0.5 ± 0.1	0.6 ± 0.3	0.5 ± 0.2	1.0 ± 0.5
20:5 n-3	**0.8 ± tr.**	**0.9 ± 0.1**	**0.7 ± 0.4**	**1.1 ± 0.3**	**0.9 ± 0.1**
22:0	1.3 ± 0.1	1.4 ± 0.3	1.8 ± 0.1	1.7 ± 0.7	1.6 ± 0.3
22:1 n-9	0.4 ± 0.2	0.5 ± 0.3	0.5 ± 0.4	0.7 ± 0.1	0.7 ± 0.1
22:2	1.6 ± 0.1	1.9 ± 0.7	2.3 ± 0.5	1.7 ± 0.3	1.4 ± 0.1
22:5 n-3	2.3 ± 0.1	3.7 ± 0.7	3.8 ± 0.1	2.7 ± 0.5	2.4 ± 0.1
22:6 n-3	**1.5 ± 0.2**	**2.6 ± 0.6**	**2.6 ± 0.1**	**1.7 ± 0.3**	**1.5 ± tr.**
24:0	1.0 ± 0.2	3.3 ± 0.6	3.7 ± 0.6	1.6 ± 0.4	1.6 ± 0.1
24:1	1.6 ± 0.4	3.4 ± 0.2	4.1 ± 0.6	1.8 ± 0.4	1.8 ± 0.1

Table 5.2 Percentage acyl composition of total polar lipids extracted from bovine articular cartilage following supplementation with 10 – 100 μg/ml EPA. Results as means ± SD (n=4).

Fatty Acids	Control	10 μg/ml	25 μg/ml	50 μg/ml	100 μg/ml
14:0	1.0 ± 0.2	0.8 ± 0.3	0.8 ± 0.3	0.7 ± 0.3	0.7 ± 0.3
16:0	18.8 ± 1.4	14.3 ± 3.0	11.1 ± 6.4	14.2 ± 1.4	12.8 ± 2.5
16:1 n-7	1.4 ± 0.1	1.2 ± 0.3	0.9 ± 0.5	1.0 ± 0.3	3.1 ± 4.8
16:1	2.5 ± 0.1	1.9 ± 0.5	1.2 ± 0.5	1.3 ± 0.4	0.9 ± 0.3
17:0	2.0 ± tr.	1.1 ± 0.5	1.8 ± 0.3	1.8 ± 0.1	1.9 ± 0.2
17:1	2.5 ± tr.	2.0 ± 0.4	2.0 ± 0.3	2.1 ± 0.2	1.7 ± 0.2
18:0	17.7 ± 0.4	14.8 ± 1.1	14.9 ± 1.2	15.3 ± 0.8	15.7 ± 1.1
18:1 n-9	31.9 ± 1.2	31.7 ± 1.7	26.0 ± 2.0	24.9 ± 1.7	22.0 ± 2.7
18:1 n-7	n.d.	5.0 ± 3.3	1.3 ± 2.5	6.1 ± 0.9	5.5 ± 1.0
18:2 n-6	0.8 ± 0.1	1.3 ± 0.2	0.7 ± 0.1	0.9 ± 0.2	0.8 ± 0.2
18:2 n-3	0.2 ± tr.	0.4 ± 0.1	0.2 ± 0.2	0.4 ± 0.1	0.3 ± 0.1
18:3 n-6	2.1 ± 0.4	0.9 ± 0.2	1.4 ± 0.7	1.2 ± 0.1	1.1 ± 0.2
18:3 n-3	**0.2 ± tr.**	**0.4 ± 0.2**	**0.3 ± 0.1**	**0.3 ± tr.**	**0.3 ± tr.**
20:0	0.6 ± 0.1	0.6 ± 0.1	1.5 ± 0.3	1.1 ± 0.1	0.8 ± 0.4
20:1 n-9	0.5 ± tr.	1.1 ± 0.2	1.6 ± 0.1	0.9 ± tr.	0.8 ± tr.
20:3 n-9	3.5 ± 0.1	4.3 ± 0.5	3.8 ± 0.6	2.6 ± 0.3	2.3 ± 0.5
20:4 n-6	**6.2 ± tr.**	**4.5 ± 1.0**	**6.7 ± 0.8**	**4.5 ± 0.5**	**4.8 ± 0.5**
20:4 n-3	0.2 ± tr.	0.5 ± 0.2	0.8 ± 0.2	0.4 ± tr.	0.5 ± tr.
20:5 n-3	**0.5 ± 0.1**	**2.1 ± 0.4**	**7.6 ± 1.2**	**10.1 ± 0.6**	**12.9 ± 0.4**
22:0	1.1 ± 0.1	1.4 ± 0.2	1.2 ± 0.4	1.0 ± tr.	1.0 ± 0.1
22:1	0.4 ± tr.	0.7 ± 0.7	1.5 ± 1.2	0.7 ± 0.1	0.7 ± 0.1
22:2	1.4 ± 0.7	1.4 ± 0.3	1.9 ± 0.3	1.8 ± 0.3	1.7 ± 0.1
22:5 n-3	0.2 ± tr.	0.6 ± 0.3	1.5 ± 0.6	0.3 ± tr.	0.4 ± 0.1
22:6 n-3	**1.1 ± 0.1**	**4.1 ± 1.5**	**3.9 ± 1.1**	**2.7 ± 0.3**	**3.2 ± 0.3**
24:0	1.4 ± 0.1	1.4 ± 1.7	2.3 ± 1.4	1.8 ± 0.2	2.0 ± 0.1
24:1	1.0 ± 0.1	1.6 ± 1.3	3.3 ± 2.2	1.9 ± 0.3	2.1 ± 0.3

Table 5.3 Percentage acyl composition of total polar lipids extracted from bovine articular cartilage following supplementation with 10 – 100 μg/ml DHA. Results as means ± SD (n=3).

Fatty Acids	Control	10 μg/ml	25 μg/ml	50 μg/ml	100 μg/ml
14:0	0.7 ± 0.3	1.4 ± 0.3	0.4 ± 0.2	0.7 ± 0.3	0.9 ± 0.2
16:0	15.6 ± 3.7	13.9 ± 3.9	14.8 ± 1.7	12.2 ± 4.4	13.2 ± 2.2
16:1 n-7	1.1 ± 0.3	1.7 ± 0.3	1.4 ± 0.1	0.9 ± 0.2	0.9 ± 0.2
16:1	2.0 ± 0.6	2.4 ± 0.4	2.3 ± 0.4	2.1 ± 0.8	1.9 ± 1.3
17:0	1.8 ± 0.2	1.7 ± 0.6	2.0 ± 0.7	1.5 ± 0.8	1.7 ± 0.6
17:1	2.5 ± 0.2	2.7 ± 0.4	3.1 ± 1.0	2.3 ± 0.4	2.3 ± 0.6
18:0	16.6 ± 1.3	13.6 ± 4.5	15.4 ± 3.5	14.6 ± 4.9	12.8 ± 8.1
18:1 n-9	33.5 ± 2.2	30.4 ± 6.1	30.6 ± 3.3	29.9 ± 3.0	23.6 ± 5.9
18:1 n-7	3.5 ± 0.1	5.2 ± 1.5	0.7 ± 1.5	5.4 ± 1.0	5.2 ± 2.3
18:2 n-6	1.1 ± 0.4	0.9 ± 0.5	0.3 ± 0.2	0.8 ± 0.1	0.6 ± 1.1
18:2 n-3	0.2 ± 0.2	1.6 ± 0.7	1.6 ± 0.7	2.7 ± 0.6	1.9 ± 0.8
18:3 n-6	1.9 ± 0.4	1.0 ± 0.2	0.4 ± 0.1	0.3 ± 0.1	0.6 ± 0.3
18:3 n-3	**0.2 ± 0.1**	**0.5 ± 0.1**	**0.6 ± 0.1**	**0.5 ± 0.2**	**0.3 ± 0.2**
20:0	0.6 ± 0.1	0.9 ± 0.7	1.1 ± 0.2	1.1 ± 0.9	1.0 ± 0.7
20:1 n-9	0.7 ± 0.2	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.5 ± 0.2
20:3 n-9	3.2 ± 1.2	3.4 ± 0.8	3.8 ± 2.2.	3.3 ± 2.1	2.8 ± 1.1
20:4 n-6	**5.6 ± 0.8**	**5.0 ± 1.3**	**5.1 ± 1.7**	**7.0 ± 0.1**	**4.4 ± 1.3**
20:4 n-3	0.2 ± tr.	0.6 ± 0.7	0.2 ± 0.1	0.1 ± tr.	0.2 ± 0.2
20:5 n-3	**0.7 ± 0.1**	**1.0 ± 0.3**	**1.0 ± 0.1**	**1.1 ± 0.5**	**0.7 ± 0.3**
22:0	1.2 ± 0.2	0.8 ± 0.4	0.9 ± 0.3	0.9 ± 0.3	1.1 ± 0.4
22:1	0.4 ± 0.1	0.1 ± 0.2	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
22:2	1.5 ± 0.4	0.1 ± 0.4	1.6 ± 0.2	2.3 ± 0.3	1.7 ± 0.7
22:5 n-3	1.3 ± 1.1	1.5 ± tr.	2.5 ± 0.2	3.1 ± 1.2	1.4 ± 0.4
22:6 n-3	**1.3 ± 0.2**	**2.4 ± 0.1**	**4.5 ± 1.1**	**2.6 ± 2.1**	**10.8 ± 3.4**
24:0	1.2 ± 0.3	3.6 ± 1.4	1.7 ± 0.1	1.5 ± 0.8	2.0 ± 1.2
24:1	1.3 ± 0.4	1.5 ± 0.6	1.8 ± 0.8	1.9 ± 0.9	2.5 ± 0.3

5.15 Glycosaminoglycan Release from PUFA-Supplemented and Interleukin-1α Stimulated Explants

The DMMB assay (section 5.12) for sulphated glycosaminoglycans (GAGs) has been accepted as a quick and effective method for measuring the release of sulphated GAGs from articular cartilage (Farndale *et al.*, 1986). Media collected from bovine explant cultures supplemented with increasing doses of EPA (Fig. 5.3, 5.4), DHA (Fig. 5.5) and trout oil (Fig. 5.6) prior to stimulation with IL-1α were analysed. The effect of the 18 carbon α-linolenic acid was not investigated for two specific reasons, (a) it is not a major component of trout oil and (b) previous research from our laboratory demonstrated that it has little, if any, effect on GAG release (Zainal, 2005).

5.15.1 Effect of EPA Supplementation on Glycosaminoglycan Release

The addition of IL-1α (10 ng/ml) resulted in a significant increase (approximately 6 fold) in the levels of GAG released from articular cartilage compared to the controls minus IL-1α (Figs. 5.3, 5.4). In contrast, the addition of DMSO (1.5%) did not result in any significant change in GAG release compared samples without DMSO. In addition, as expected, no alterations were observed following supplementation with EPA in controls (minus IL-1α).

Supplementation of EPA 10-100 μg/ml (Fig 5.3 A-C) for 12 h resulted in some reductions of GAG release stimulated by the presence of IL-1α. Compared to DMSO + IL-1α, explants supplemented with 10 – 100 μg/ml EPA gave significant effects. In Fig. 5.3A the results for GAG release showed reductions at 100 and 50 μg/ml ($p < 0.001$) and also at 25 μg/ml ($p < 0.05$) compared to DMSO + IL-1α. Significant reductions in GAG release in Fig. 5.3B were observed with only 100 μg/ml ($p < 0.05$). Significant reductions in GAG release was observed at all doses in Fig. 5.3C (25 – 100 μg/ml $p < 0.001$ and 10 μg/ml $p < 0.05$). To investigate whether a further reduction in GAG release could be elicited, increased doses of EPA were employed (Fig. 5.4A-C).

Supplementation of bovine explant cultures with 200-300 μg/ml EPA (Fig. 5.4) for 12 h demonstrated more pronounced effects compared to cultures supplemented with 10-100 μg/ml (Fig. 5.3). Significant reduction of GAG compared to DMSO + IL-1α was observed with 25 μg/ml ($p < 0.05$) and 50-300 μg/ml ($p < 0.001$) in Fig. 5.4A. The experiment shown in Fig. 5.4B showed progressive reduction in GAG release at 50 μg/ml and above. In the experiment shown in Fig. 5.4C GAG reduction was only significant at 200 μg/ml and 300 μg/ml ($p < 0.001$) but not at the lower doses.

Though differences exist between individual experiments there appeared to be a dose-dependent trend in the reduction of IL-1α stimulated GAG release from bovine articular cartilage following supplementation with 10-100 μg/ml EPA (Fig. 5.3). Moreover, the protection offered by 200-300 μg/ml EPA further reduced the levels of GAG release to those seen in control cultures (Fig. 5.4A-C). These effects compare well with previous work from our laboratory which also reported a decrease of GAG release from both bovine explant and monolayer cultures following supplementation with EPA (Hurst 2004, Zainal, 2005). However, it should be noted that both Hurst (2004) and Zainal (2005) reported significant reductions in GAG release at lower concentrations (10 – 25 μg/ml) of EPA.

Fig. 5.3: GAG analysis of medium from three independent experiments using bovine explant cultures, supplemented with or without 10-100 µg/ml EPA for 12 h, ± 10 ng/ml interleukin-1α (IL-1α) for 72 h. (Means ± SD; n=12). The significance of changes was tested using ANOVA post hoc Tukey test compared to DMSO + IL-1α.

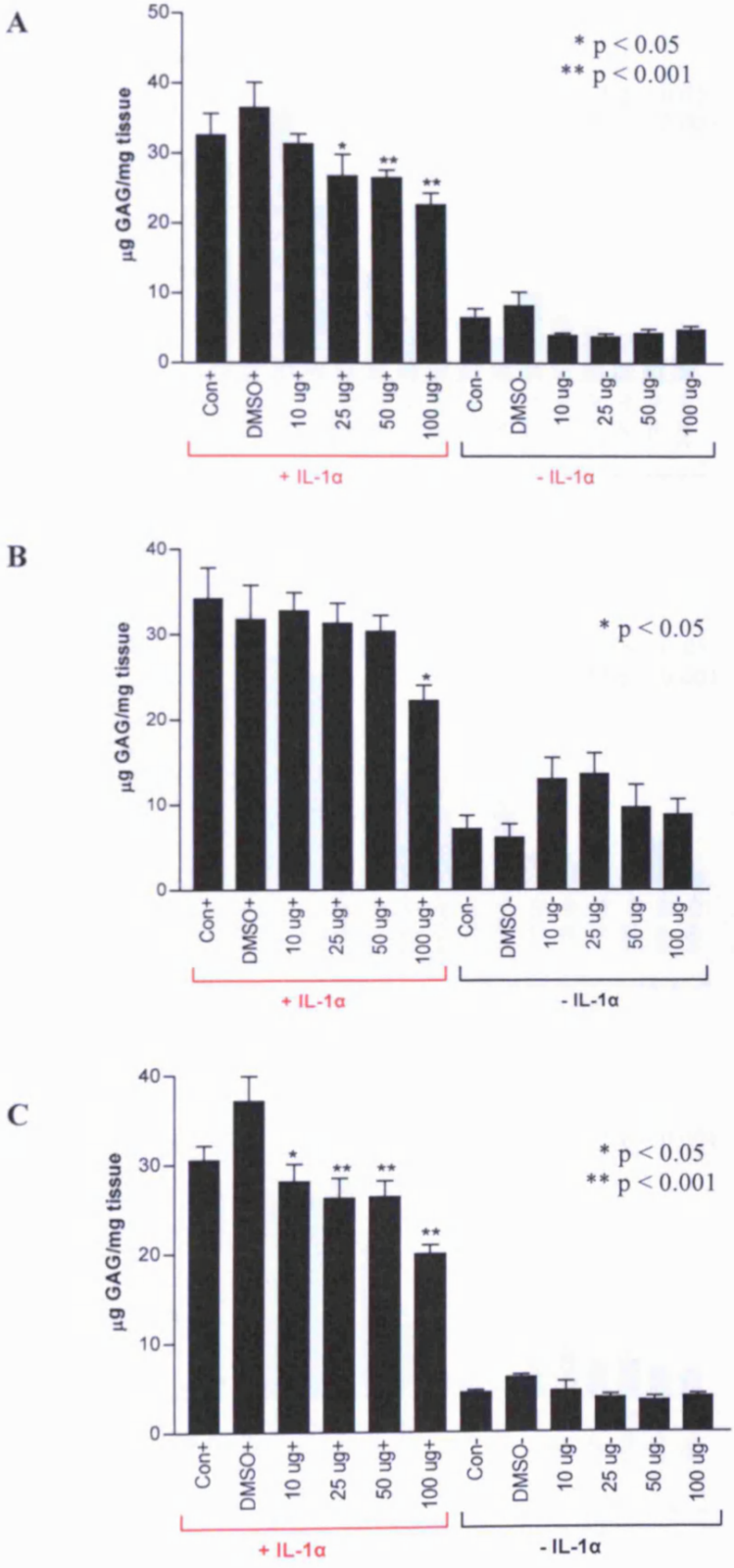
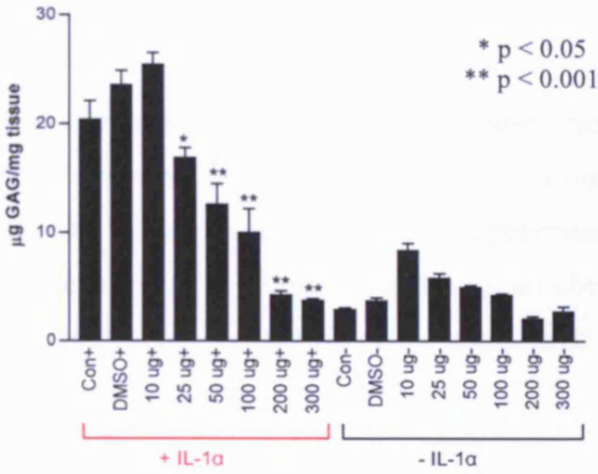


Fig. 5.4: GAG analysis of medium from three independent experiments using bovine explant cultures, supplemented with or without 10-300 µg/ml EPA for 12 h, ± 10 ng/ml interleukin-1α (IL-1α) for 72 h. (Means ± SD; A n=12; B, C n=6). The significance of changes was tested using ANOVA post hoc Tukey test compared to DMSO + IL-1α.

A



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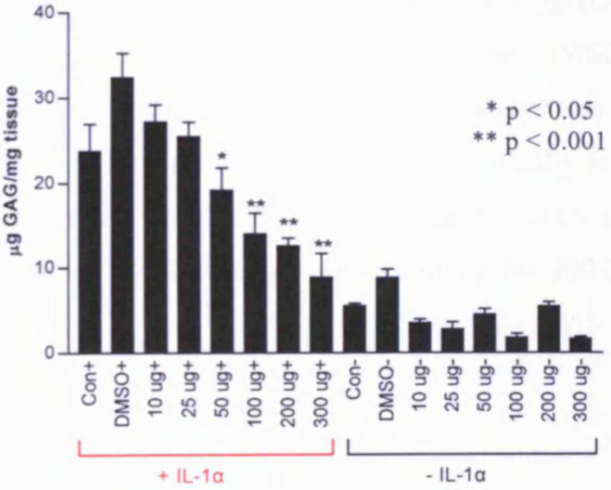

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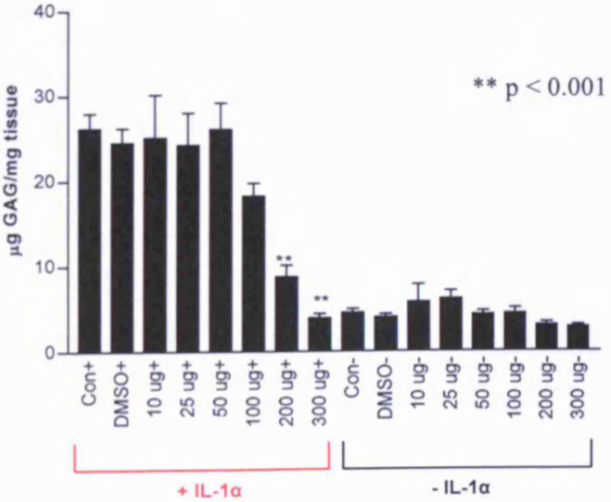


5.15.2 Effect of DHA Supplementation on Glycosaminoglycan Release

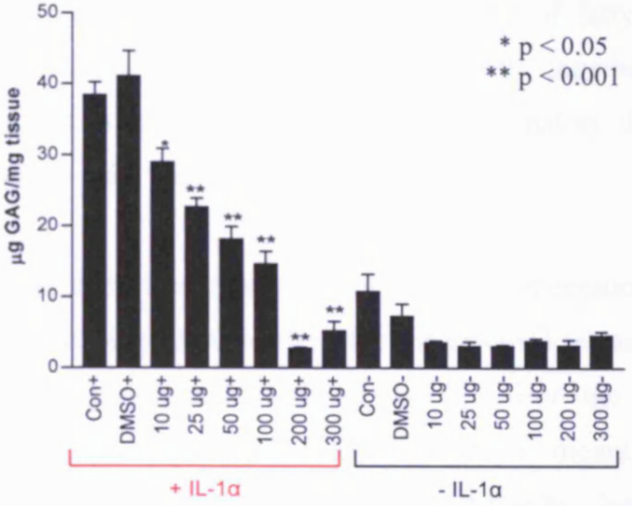
Since EPA and DHA are the two most important long chain PUFAs in fish oil and are both thought to possess 'anti'-inflammatory properties (Serhan and Chiang, 2007; Tziomalos *et al.*, 2007), the effect of DHA supplementation on bovine explant cultures was also examined (Fig. 5.5).

Again, following stimulation with IL-1α, GAG release increased significantly (approximately 5-fold) compared to controls (Fig. 5.5). Control levels of GAG release were comparable to the levels seen with EPA supplementation (Figs. 5.3 and 5.4) and, as previously seen for EPA (Figs. 5.3 and 5.4), no obvious effect on GAG release was noted with DHA supplementation or the addition of DMSO (1.5%) (Fig. 5.5). DHA supplementation was carried out for 12 h.

A statistically significant decrease in GAG release following IL-1α stimulation was found for 10-300 μg/ml DHA ($p < 0.001$) compared to DMSO alone in the first experiment (Fig. 5.5A). For the second experiment, DHA was effective at 50-300 μg/ml (Fig. 5.5B) whereas in the third experiment statistically significant reductions in GAG release were found with 200 and 300 μg/ml DHA (Fig. 5.5C). In all experiments, the levels of GAG release were reduced by 200-300 μg/ml DHA to control (no IL-1α) levels.

Fig. 5.5: GAG analysis of medium from three independent experiments using bovine explant cultures, supplemented with or without 10-300 µg/ml DHA for 24 h, ± 10 ng/ml interleukin-1α (IL-1α) for 72 h. (Means ± SD; n=6). The significance of changes was tested using ANOVA post hoc Tukey test compared to DMSO + IL-1α.

A



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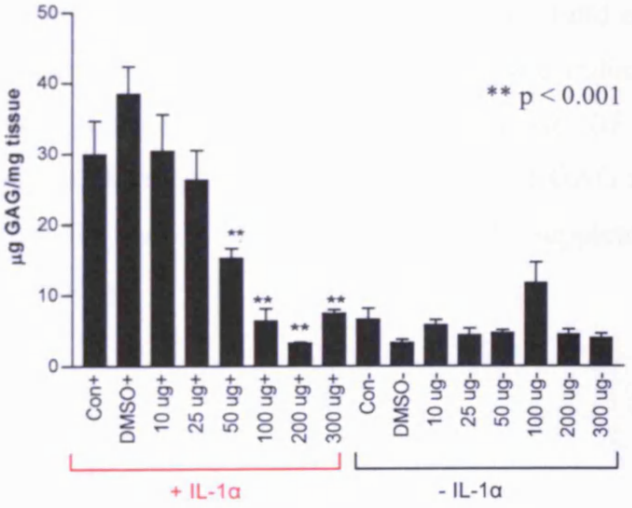

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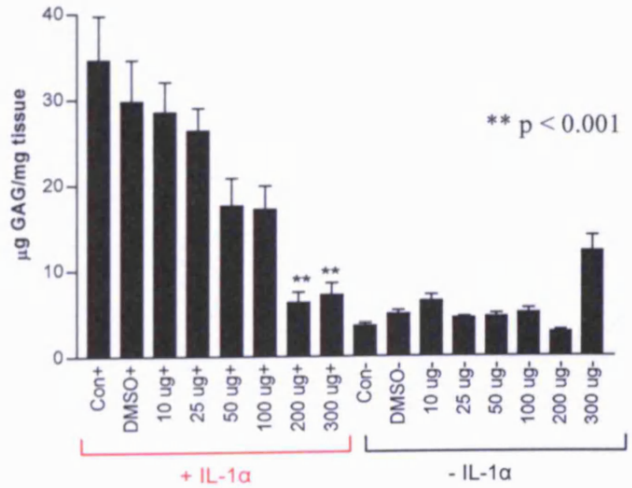


5.15.3 Effect of Trout Oil Supplementation on Glycosaminoglycan Release

Having established the effect of purified n-3 PUFAs on GAG release in the explants following stimulation with IL-1α, we needed to investigate how trout oil preparations would perform. The trout oil used contained a range of fatty acids (Table 5.4) including both EPA (7.5 μg/ml) and DHA (31.5 μg/ml), together with several n-6 PUFAs which would be expected to be more pro-inflammatory than the n-3 PUFAs (Calder, 2005). Linoleic acid was the main n-6 PUFA.

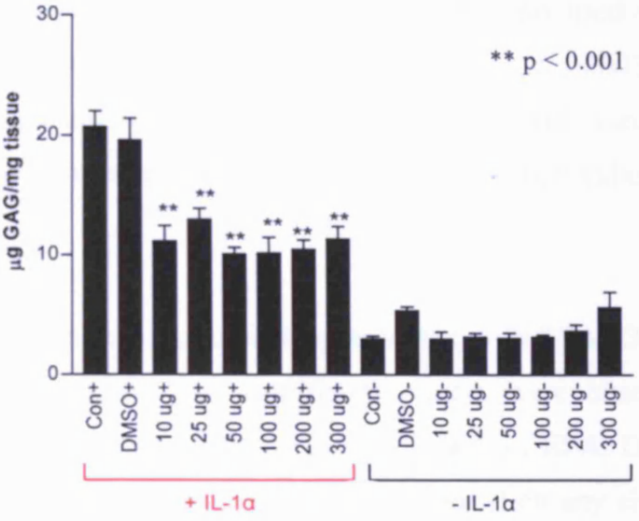
The results for the three experiments using trout oil supplementation are shown in Fig. 5.6. In all cases, a statistically significant reduction in GAG release was noted for the IL-1α treated explants pre-treated with trout oil. For the second and third experiments, variability in the DMSO alone controls meant that only higher concentrations of trout oil gave statistically significant results. Nevertheless, the data for the three experiments were consistent in that (a) trout oil did not affect the no IL-1α treated controls and (b) lowering of the GAG release induced by IL-1 α was achieved at low levels of trout oil and was not much increased (if at all) by the higher doses of this preparation. Clearly, the overall reduction of GAG release seen at high concentrations was less for trout oil than for EPA or DHA supplementation (Figs. 5.3, 5.4 and Fig. 5.5 respectively).

Table 5.4 Percentage acyl composition of the trout oil preparation used to supplement bovine explant cultures. Results as means ± SD (n=3), tr. <0.05.

Fatty Acids	% acyl composition
12:0	0.1 ± tr.
14:0	1.7 ± tr.
16:0	11.3 ± 0.1
16:1 n-7	3.3 ± 0.3
16:1	0.2 ± tr.
17:0	0.1 ± tr
17:1	0.1 ± tr.
18:0	3.1 ± 0.1
18:1 n-9	22.0 ± 1.5
18:1 n-7	2.7 ± 0.1
18:2 n-6	15.0 ± 0.1
18:2 n-3	0.4 ± 0.1
18:3 n-6	1.6 ± 0.2
18:3 n-3	0.7 ± 0.5
20:0	0.2 ± tr.
20:1 n-9	8.8 ± 0.1
20:2	0.2 ± tr.
20:3 n-9	1.1 ± tr.
20:4 n-6	0.5 ± tr.
20:4 n-3	1.1 ± tr.
20:5 n-3	**2.5 ± 0.1**
22:1	10.2 ± 0.2
22:2	0.3 ± tr.
22:3	0.2 ± tr.
23:0	0.1 ± tr.
22:5 n-3	1.1 ± tr.
22:6 n-3	**10.5 ± 0.2**
24:0	0.2 ± tr.
24:1	0.8 ± tr.

Fig. 5.6: GAG analysis of medium from three independent experiments using bovine explant cultures, supplemented with or without 10-300 µg/ml trout oil for 24 h, ± 10 ng/ml interleukin-1α (IL-1α) for 72 h. (Means ± SD; n=6). The significance of changes was tested using ANOVA post hoc Tukey test compared to DMSO + IL-1α.

A



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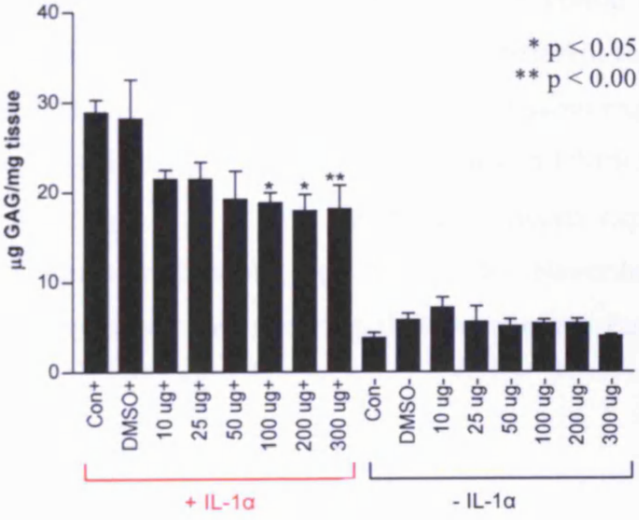

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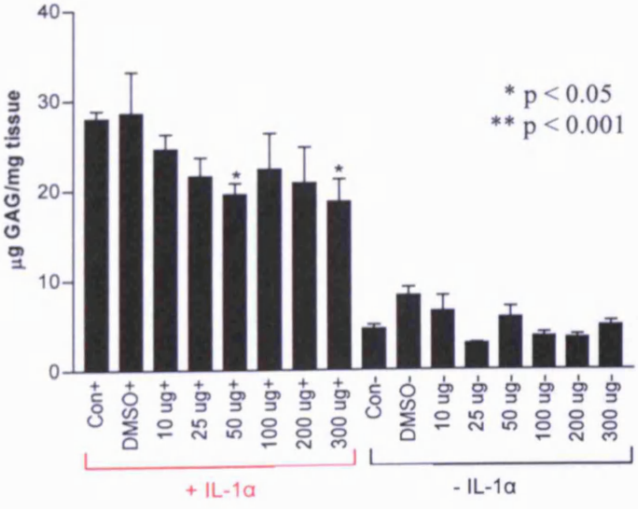

5.16 Effect of Fatty Acid Supplementation on the Metabolic State of Explant Cultures

Respiration in chondrocytes located in articular cartilage is predominantly anaerobic and rates of lactate production (and secretion) are commonly used as a measure of cell or explant metabolic state (Beecher *et al.*, 2007; Ilic *et al.*, 2003; Munteanu *et al.*, 2000). Lactate levels in explant cultures supplemented with various doses of EPA, DHA or trout oil were measured by the lactate oxidase/peroxidase method (Section 5.12 methods).

Lactate production in explant cultures supplemented with EPA, DHA or trout oil are shown in Figs. 5.7 – 5.10. No significant effects were observed between test conditions (+ IL-1α) and control (minus IL-1α) for any of EPA, DHA or the trout oil preparation. DMSO, EPA, DHA and trout oil did not elicit any significant effects on lactate production either (Figs. 5.7 – 5.10). The only exception to this was seen in Fig. 5.7C where 25 μg EPA + IL-1α was significantly different to the test conditions control + IL-1α and DMSO + IL-1α ($p < 0.05$). For the same experiment 10 and 25 μg EPA - IL-1α were significantly different to control and DMSO – IL-1α. As seen for the GAG analysis (Figs. 5.3 – 5.6) variability between experiments was also observed in terms of lactate production (Figs. 5.7 – 5.10). Nevertheless, there were no consistent effects of any treatment, showing that metabolism (as judged by lactate release) was generally not affected.

Fig. 5.7: Lactate analysis of medium from three independent experiment using bovine explant cultures, supplemented with or without 10-100 µg/ml EPA for 24 h, ±10 ng/ml interleukin-1α (IL-1α) for 72 h. (Means ± SD; n=6). The significance of changes was tested using ANOVA post hoc Tukey test compared to DMSO + IL-1α.

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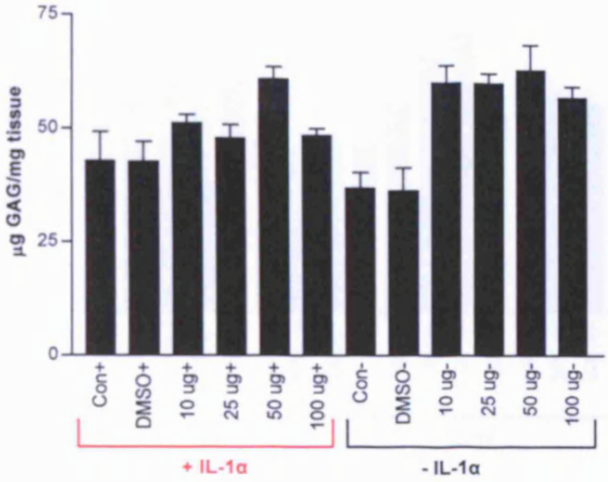

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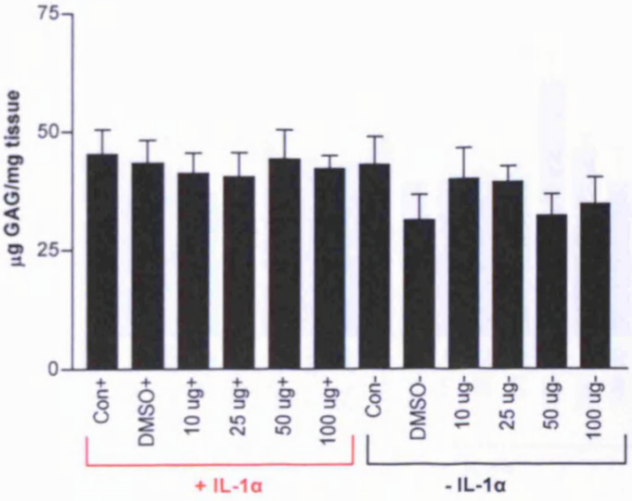

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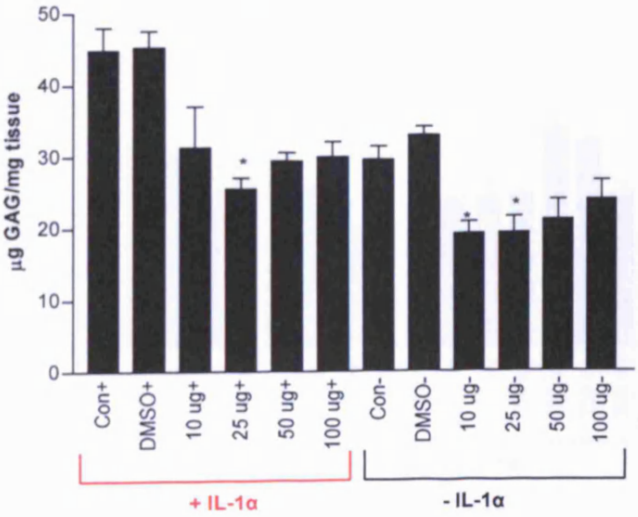

Fig. 5.8: Lactate analysis of medium from three independent experiments using bovine explant cultures, supplemented with or without 10-300 µg/ml EPA for 24 h, ± 10 ng/ml interleukin-1α (IL-1α) for 72 h. (Means ± SD; n=6). The significance of changes was tested using ANOVA post hoc Tukey test compared to DMSO + IL-1α.

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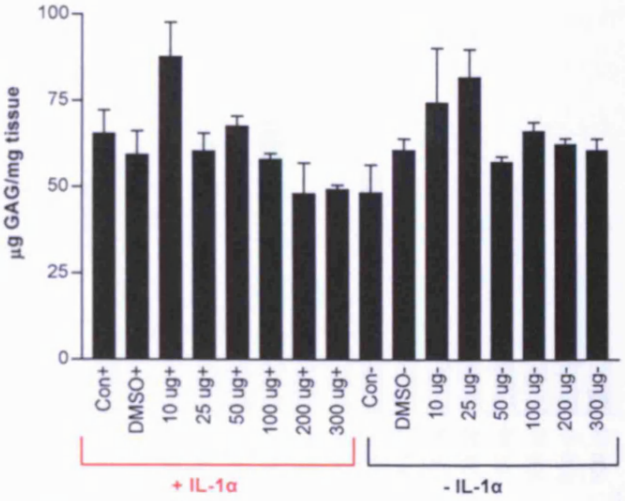

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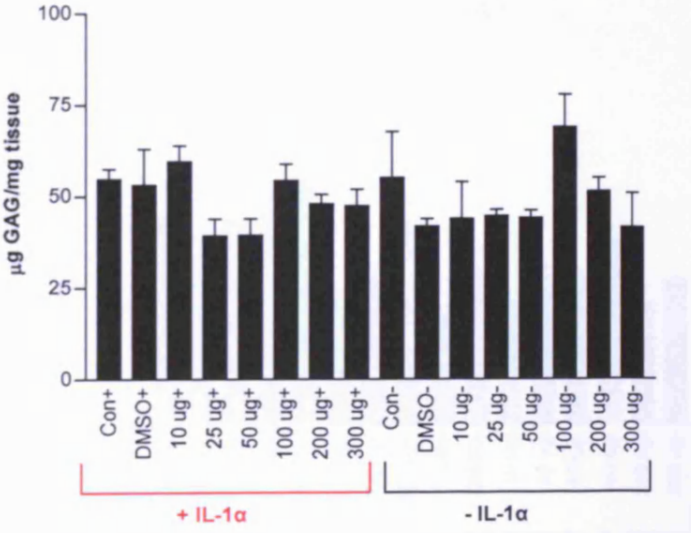

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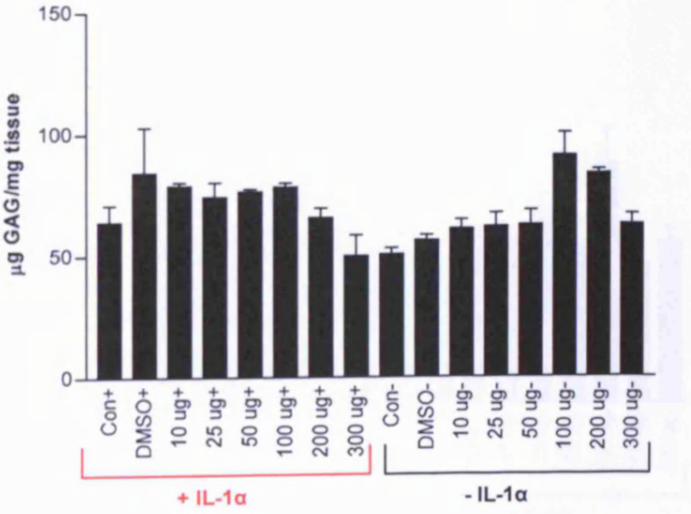
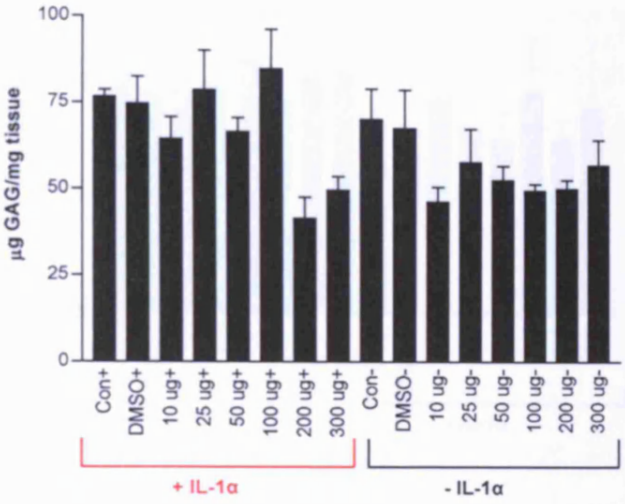


Fig. 5.9: Lactate analysis of medium from three independent experiment using bovine explant cultures, supplemented with or without 10-300 µg/ml DHA for 24 h, ± 10 ng/ml interleukin-1α (IL-1α) for 72 h. (Means ± SD; n=6). The significance of changes was tested using ANOVA post hoc Tukey test compared to DMSO + IL-1α.

A



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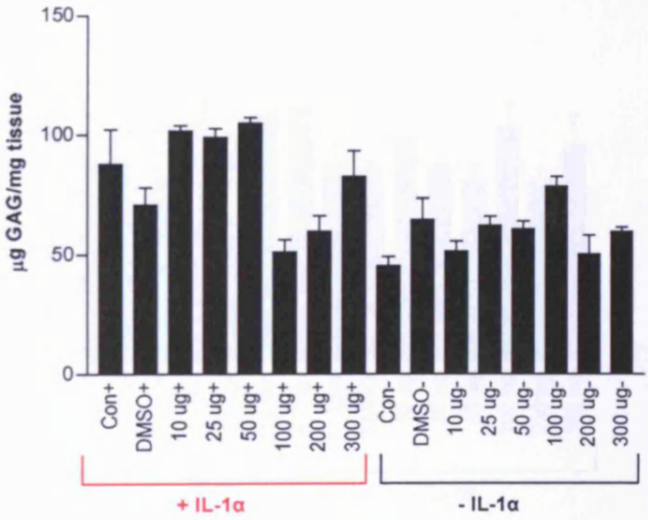

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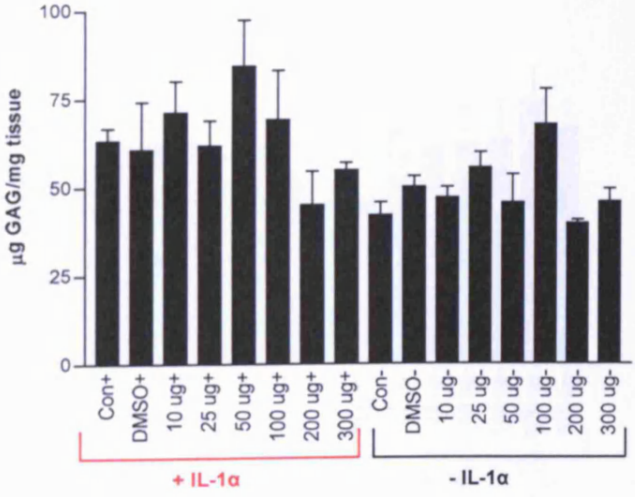
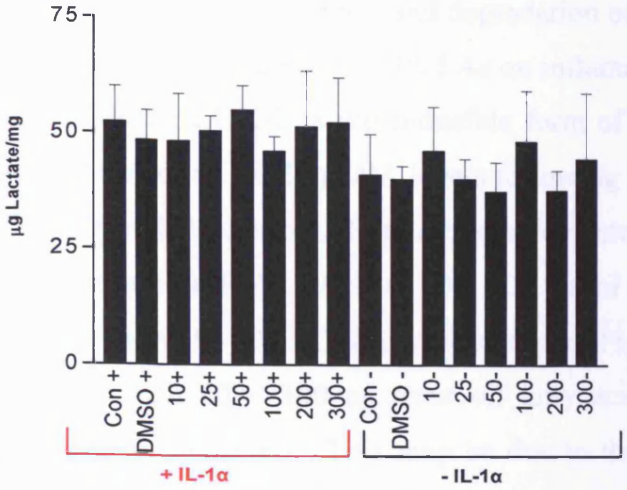


Fig. 5.10: Lactate analysis of medium from bovine explant cultures, supplemented with or without 10-300 µg/ml trout oil for 24 hs, ±10 ng/ml interleukin-1α (IL-1α) for 72 hs. (Means ± SD; n=6). The significance of changes was tested using ANOVA post hoc Tukey test compared to DMSO + IL-1α.

A



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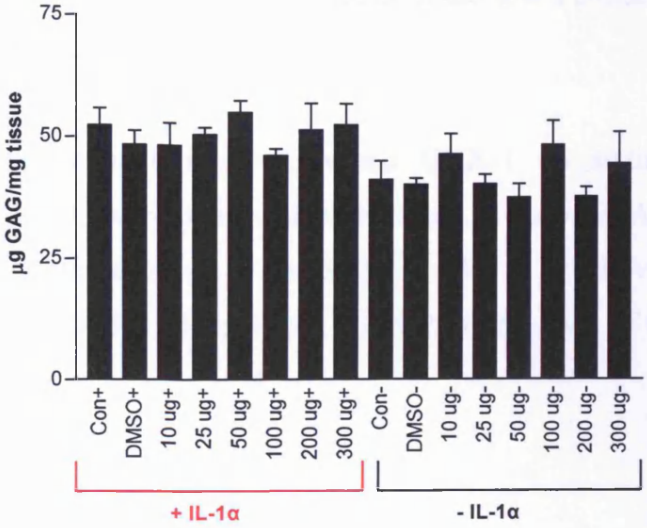

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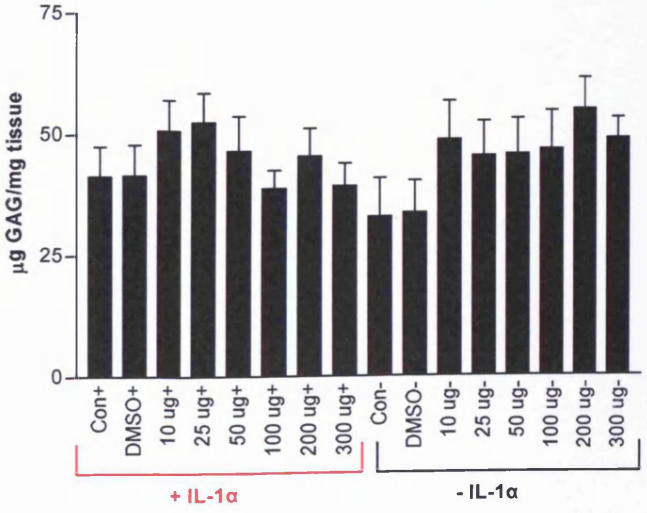


5.17 Effect of Fatty Acid Supplementation on mRNA Levels of Inflammatory and Degradative Proteins Involved in Osteoarthritis

As previously mentioned, (section 5.9), oligonucleotide primers were designed for a number of genes involved in both inflammation and degradation of articular cartilage. One of the most well documented effects of n-3 PUFAs on inflammatory processes is the down-regulation of COX-2. COX-2 is the inducible form of the COX isoforms and we observed up-regulation of COX-2 mRNA levels following incubation with IL-1α (Figs. 5.11 – 5.13) compared to controls. Explants supplemented with 10 – 300 μg EPA demonstrated a reduction in mRNA levels at 50 – 300 μg/ml whereas with DHA reductions were only observed with 200 – 300 μg/ml (compare Figs. 5.11 with 5.12). Following supplementation with 10 – 300 μg trout oil fatty acids no reduction in mRNA levels for COX-2 were observed. This may be due to the presence of other fatty acids in the trout oil mixture. Also, the levels of EPA and DHA (7.5 μg/ml and 31.5 μg/ml respectively) may have been present in too low a concentration to elicit an effect on COX-2 mRNA levels.

With regards to the constitutively-expressed COX-1 no statistically significant changes in mRNA levels were observed with either IL-1α or EPA, DHA or trout oil derived fatty acid supplementation (Figs. 5.11 – 1.13). It is believed that n-3 PUFAs elicit their effect on the inducible isoform, COX-2, at the level of transcription rather than at the protein level.

Fig 5.11: mRNA levels for cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in bovine articular cartilage. RNA was isolated from explants supplemented with 10-300 µg/ml EPA for 12 h. Quantification by densitometry was carried out and gene expression levels were corrected for GAPDH loading (Means ± SEM; n=3). The gels shown are from a representative experiment.

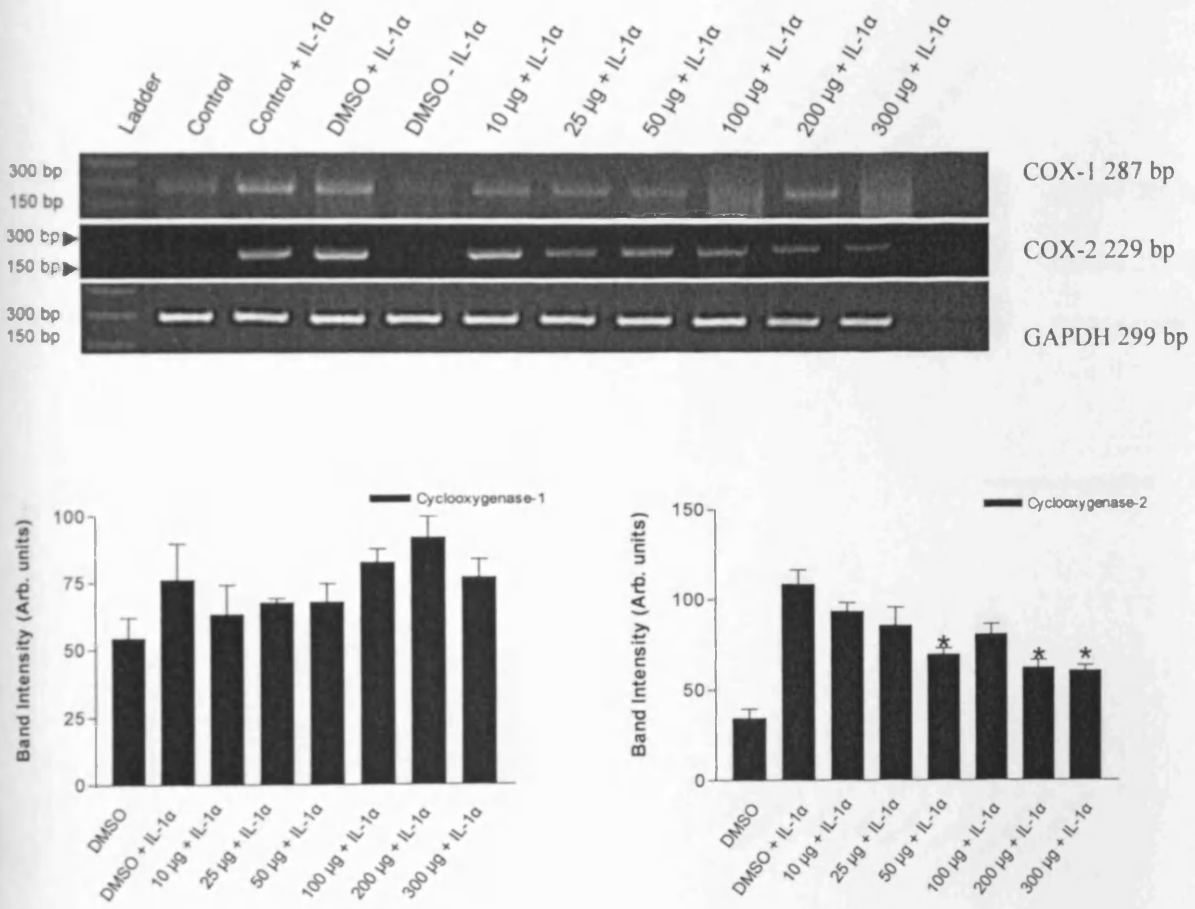


Fig 5.12: mRNA levels for cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in bovine articular cartilage. RNA was isolated from explants supplemented with 10-300 µg/ml DHA for 12 h. Quantification by densitometry was carried out and gene expression levels were corrected for GAPDH loading (Means ± SEM; n=4 COX-2, n=3 COX-1). The gels shown are from a representative experiment.

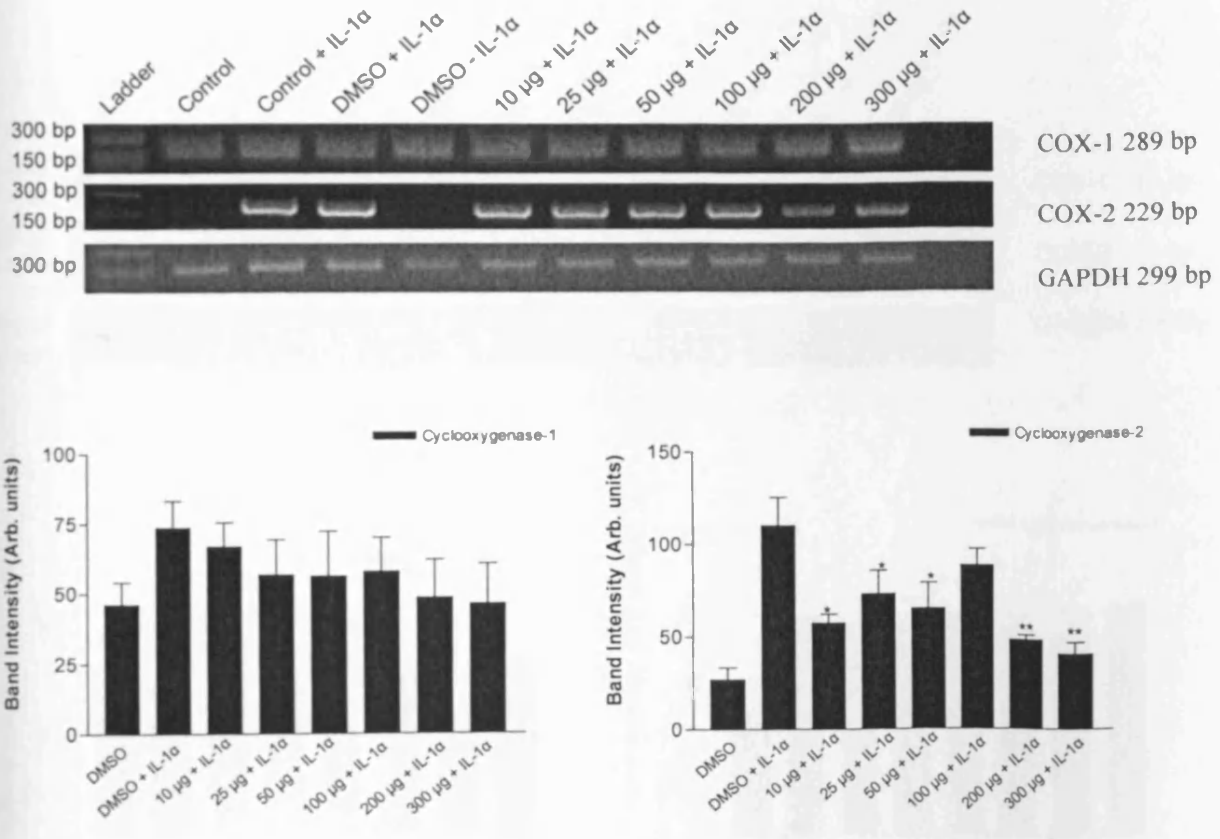
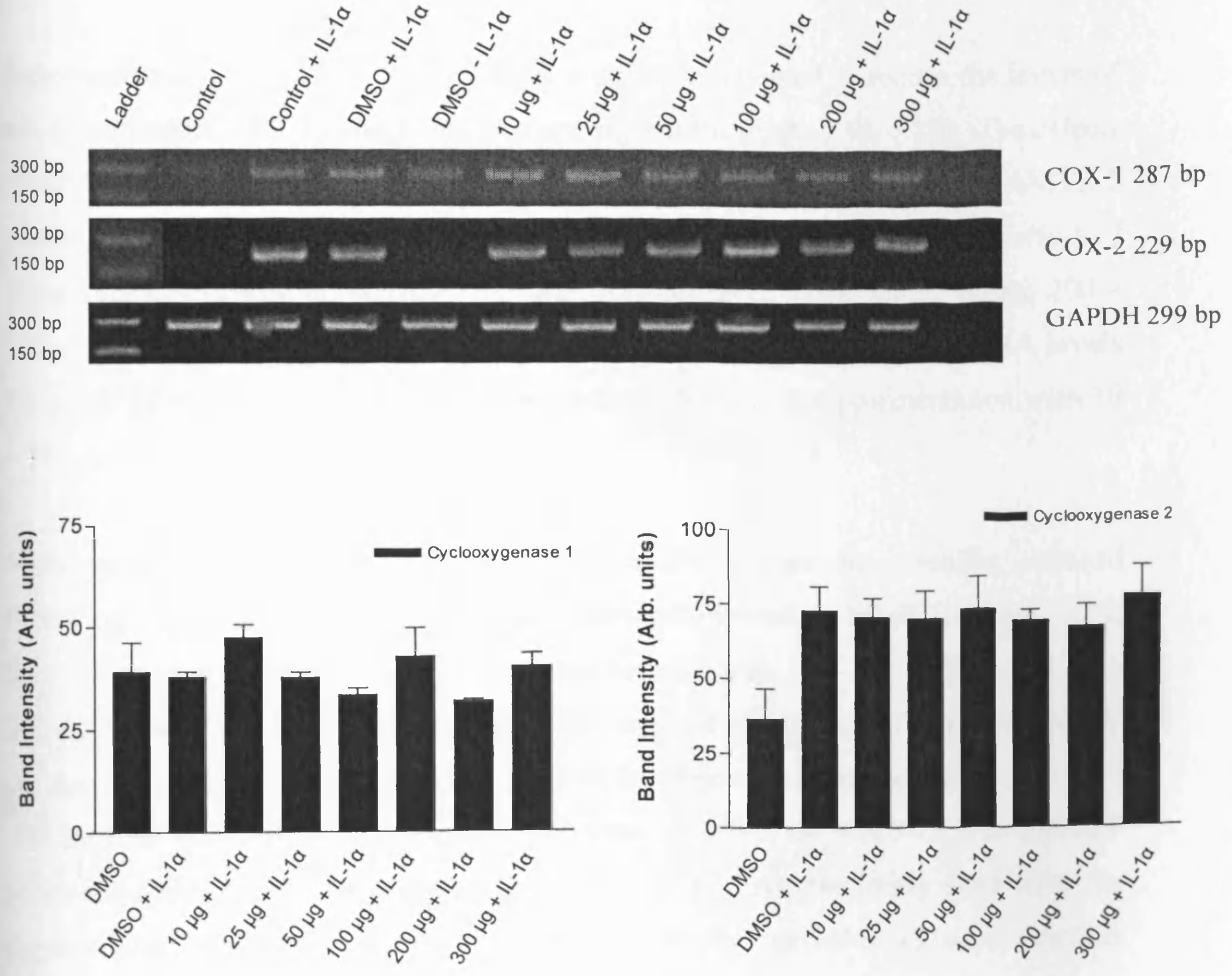


Fig. 5.13: mRNA levels for cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in bovine articular cartilage. RNA was isolated from explants supplemented with 10-300 µg/ml trout oil fatty acids (containing 7.5 µg/ml EPA and 31.5 µg DHA) for 12 h. Quantification by densitometry was carried out and gene expression levels were corrected for GAPDH loading (Means ± SEM; n=4). The gels shown are from a representative experiment.

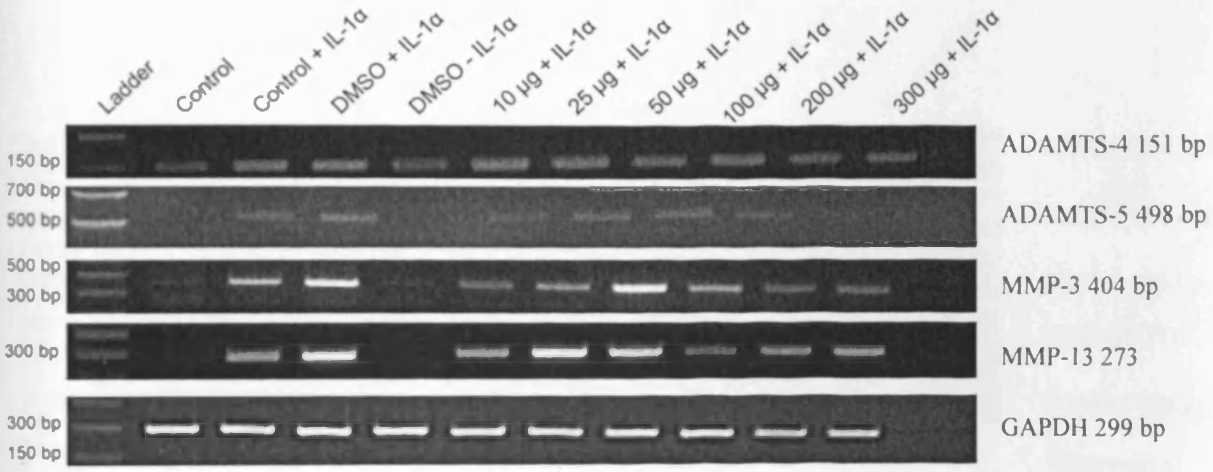


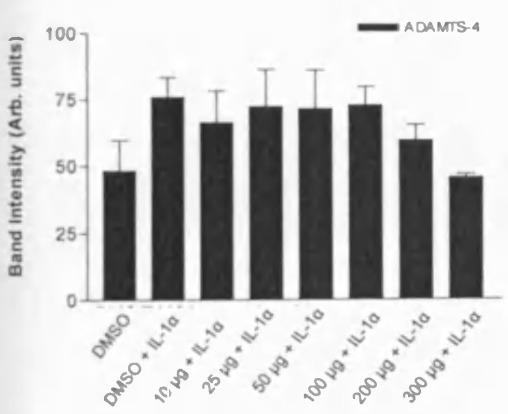
As previously detailed (sections 1.6, 5.0), both aggrecanases and MMPs are involved in the destruction of articular cartilage. We investigated the effects that supplementation of EPA, DHA and trout oil fatty acids may have on expression of the two main aggrecanases, ADAMTS-4 and ADAMTS-5, and two specific MMPs, MMP-3 and MMP-13 (Figs. 5.14 – 5.16) which are thought to be important in cartilage degradation. Basal levels of both the aggrecanases and MMPs were observed in control tissue. Following incubation with IL-1α mRNA levels for these genes were increased.

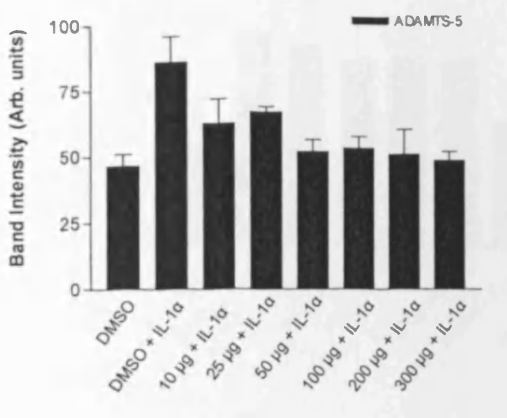
Supplementation with 200 – 300 μg EPA and DHA appeared to reduce the levels of mRNA for ADAMTS-4 though this was not significant (Figs. 5.14, 5.15). The effects of EPA appeared to be more pronounced (though not significant) with ADAMTS-5 where downregulation was observed with 50 – 300 μg EPA (Fig. 5.14). An effect of DHA supplementation on ADAMTS-5 was only observed at the higher doses, 200 – 300 μg DHA (Fig. 5.15) but again this was not significant. No effect on mRNA levels for either ADAMTS-4 or ADAMTS-5 were found following supplementation with 10 – 300 μg trout oil fatty acids (Fig. 5.16).

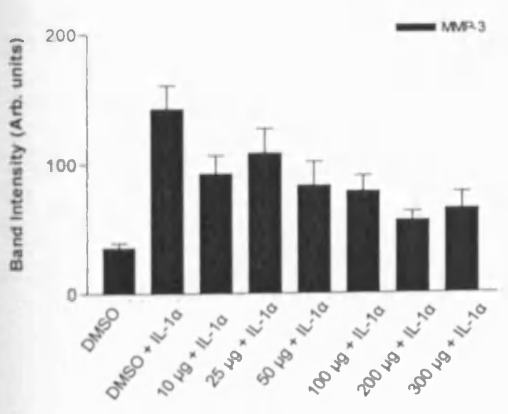
With regards to the MMPs, MMP-3 mRNA levels were more readily reduced following supplementation with EPA and DHA compared to MMP-13 (Figs. 5.14, 5.15). The level of MMP-3 mRNA appeared reduced with 10 – 300 μg EPA and 50 – 300 μg DHA (Figs. 5.14, 5.15) although this was not significant. The mRNA levels for the principal collagenolytic MMP, MMP-13, appeared to be reduced with 100 – 300 μg EPA (though not significantly) whereas no effect on MMP-13 was detected following DHA supplementation (Figs. 5.14 – 5.15). As previously seen with the aggrecanases, no effects on mRNA levels for MMP-3 or MMP-13 were detected following trout oil fatty acid supplementation (Fig. 5.16).

Fig. 5.14: mRNA levels for aggrecanase-1 (ADAMTS-4) and aggrecanases-2 (ADAMTS-5), matrix metalloproteinase 3 and 13 (MMP-3 and MMP-13) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in bovine articular cartilage. RNA was isolated from explants supplemented with 10-300 µg/ml EPA for 12 h. Quantification by densitometry was carried out and gene expression levels were corrected for GAPDH loading (Means ± SEM; n=3). The gels shown are from a representative experiment.









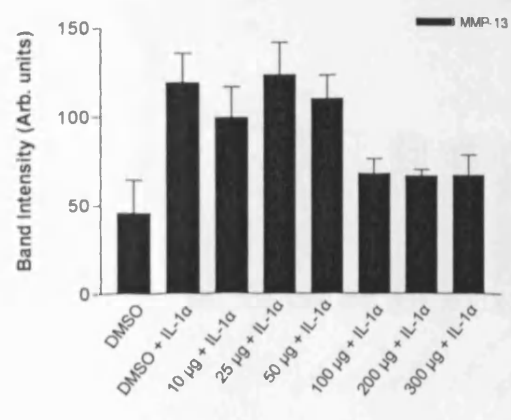


Fig. 5.15: mRNA levels for aggrecanase-1 (ADAMTS-4) and aggrecanases-2 (ADAMTS-5), matrix metalloproteinase 3 and 13 (MMP-3 and MMP-13) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in bovine articular cartilage. RNA was isolated from explants supplemented with 10-300 µg/ml DHA for 12 h. Quantification by densitometry was carried out and gene expression levels were corrected for GAPDH loading (Means ± SEM; n=3). The gels shown are from a representative experiment.

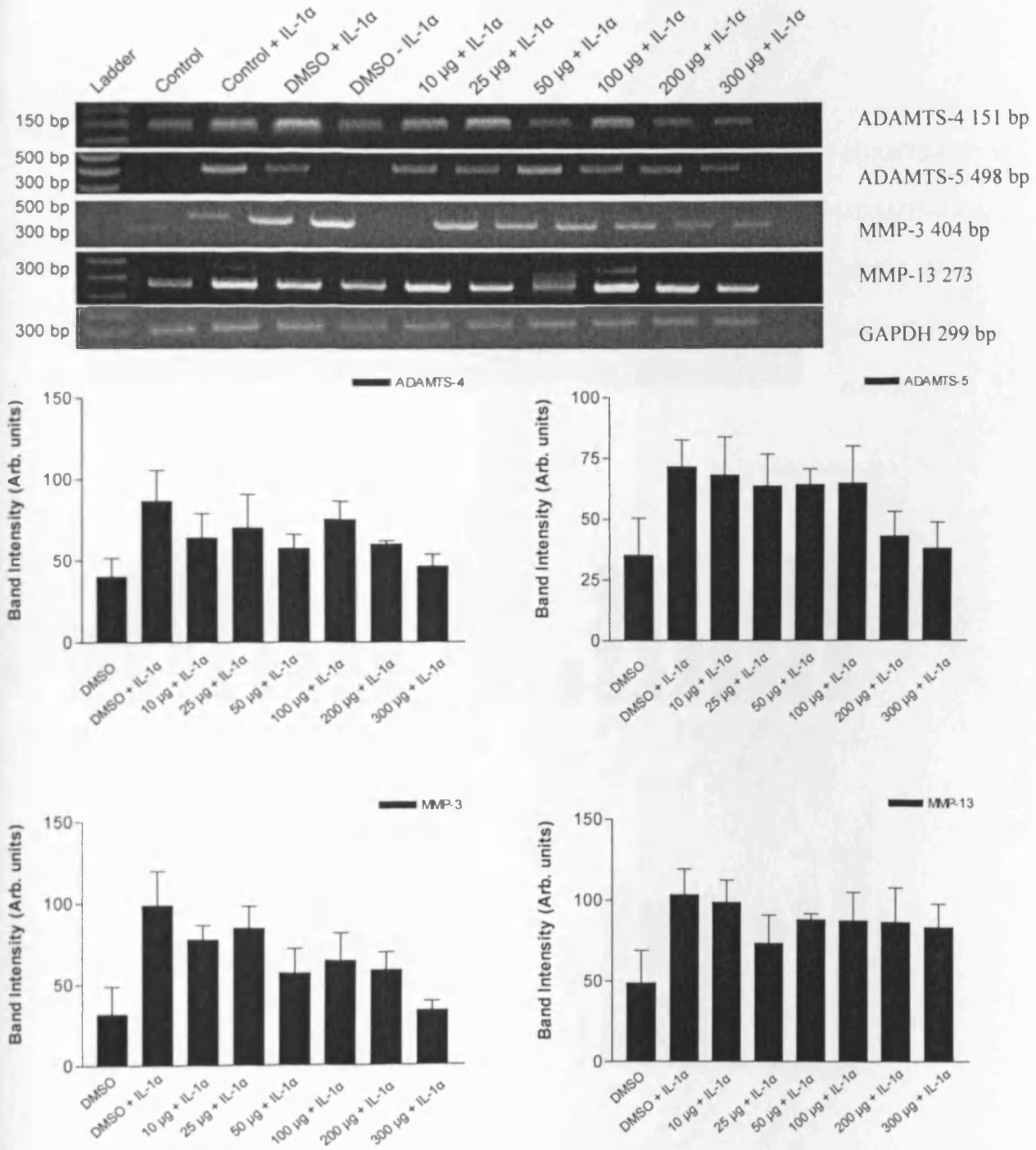
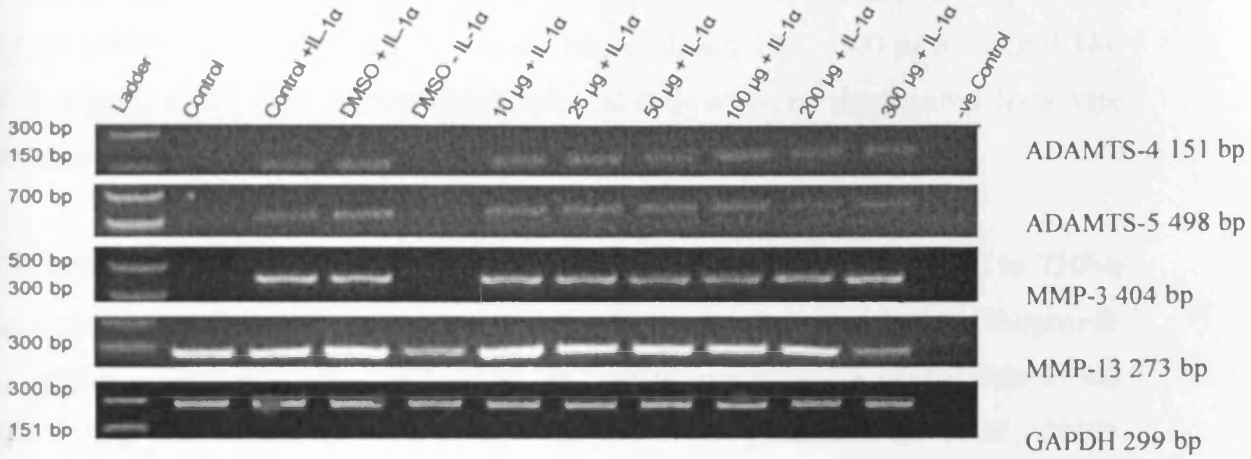
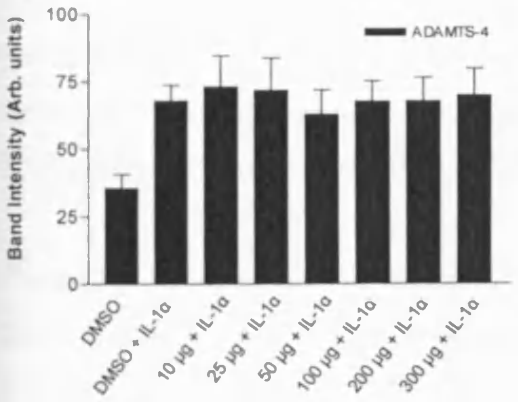
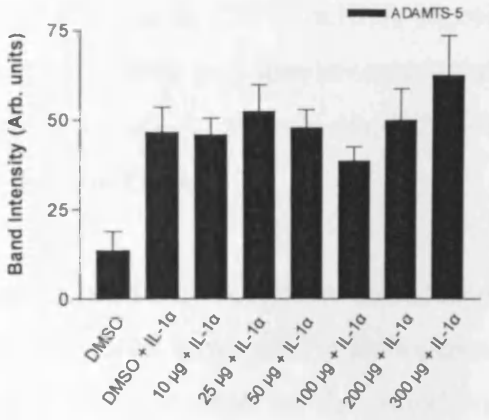
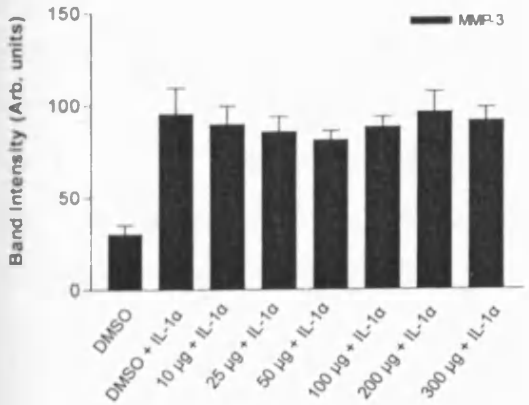


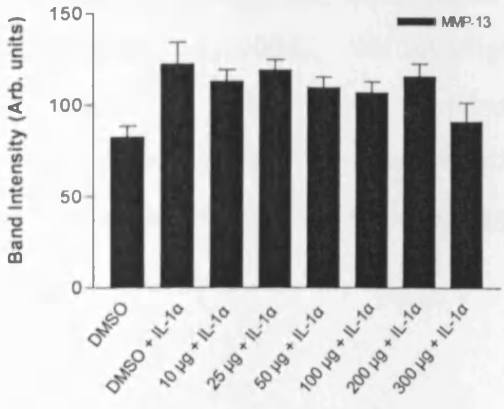
Fig. 5.16: mRNA levels for aggrecanase-1 (ADAMTS-4) and aggrecanases-2 (ADAMTS-5), matrix metalloproteinase 3 and 13 (MMP-3 and MMP-13) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in bovine articular cartilage. RNA was isolated from explants supplemented with 10-300 μg/ml trout oil fatty acids (containing 7.5 μg/ml EPA and 31.5 μg DHA) for 12 h. Quantification by densitometry was carried out and gene expression levels were corrected for GAPDH loading (Means ± SEM; n=4). The gels shown are from a representative experiment.











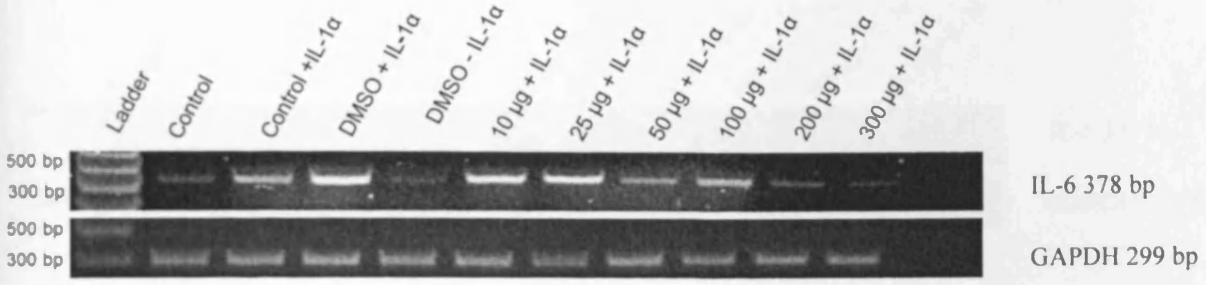
IL-6 is an inducible cytokine and its role in arthritis has been under investigation for some time. However, the results of previous studies detailing the effects on n-3 PUFAs on IL-6 are divided (for review see Sijben and Calder, 2007). We observed an upregulation of mRNA levels for IL-6 following IL-1α treatment compared to controls (Figs. 5.17 – 5.19).

Supplementation of EPA resulted in a dose-dependent decrease in IL-6 mRNA levels with 10 – 300 μg/ml. Even though the effects were more pronounced at the higher doses this was not significant (Fig. 5.17). DHA treatment of explants also appeared to reduce mRNA levels although only at the higher doses, 200 – 300 μg/ml (Fig. 5.18). With regards to the supplementation of trout oil fatty acids, no significant effects were observed (Fig. 5.19).

The effect of trout oil supplementation was also investigated with regard to TNF-α and cytochrome C oxidase subunits I and II. TNF-α is implicated in the pathogenesis of arthritis (Farahat *et al.*, 1993) and the literature reports a potential role of n-3 PUFAs on the reduction of TNF-α mRNA levels (Moghaddami *et al.*, 2007). However, as with IL-6, there is some debate on the relationship of n-3 PUFAs and TNF-α. Incubation with IL-1α resulted in an increase in TNF-α mRNA expression but no consistent effect was observed with trout oil fatty acid supplementation at any dose (Fig. 5.19). It may be of interest to further investigate the role of n-3 PUFAs on TNF-α following supplementation with pure EPA or DHA.

The role of mitochondria in degenerative disease is widely recognized (Maneiro *et al.*, 2003) and the cytochrome c oxidase complex has been implicated in pathogenesis of RA (Henderson *et al.*, 1978). It is thought that the level of the mitochondrial respiratory chain, of which cytochrome c oxidase is a component, decreases in OA although this is still a controversial area (Maneiro *et al.*, 2003). We investigated whether supplementation of trout oil had any effect on the mRNA levels for subunit I and II of the cytochrome c oxidase complex. We observed no effect on either subunit I or II at any dose of trout oil (Fig. 5.20). Moreover, addition of IL-1α did not appear to change levels of these mRNAs.

Fig. 5.17: mRNA levels for interleukin-6 (IL-6) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in bovine articular cartilage. RNA was isolated from explants supplemented with 10-300 µg/ml EPA for 12 h. Quantification by densitometry was carried out and gene expression levels were corrected for GAPDH loading (Means ± SEM; n=3). The gels shown are from a representative experiment.



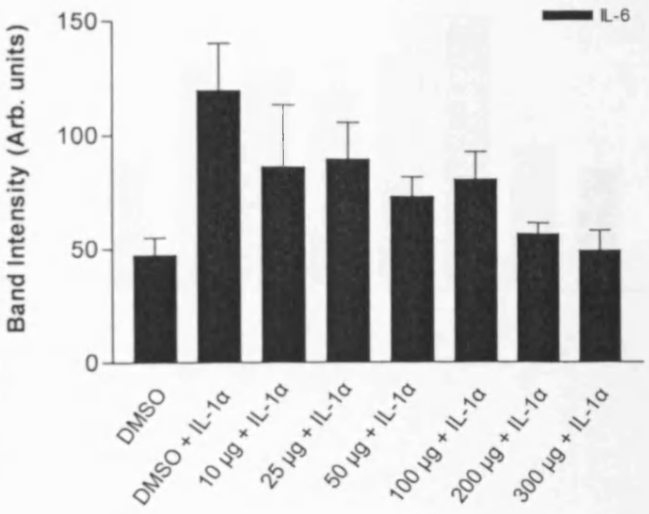


Fig. 5.18: mRNA levels for interleukin-6 (IL-6) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in bovine articular cartilage. RNA was isolated from explants supplemented with 10-300 µg/ml DHA for 12 h. Quantification by densitometry was carried out and gene expression levels were corrected for GAPDH loading (Means ± SEM; n=3). The gels shown are from a representative experiment.

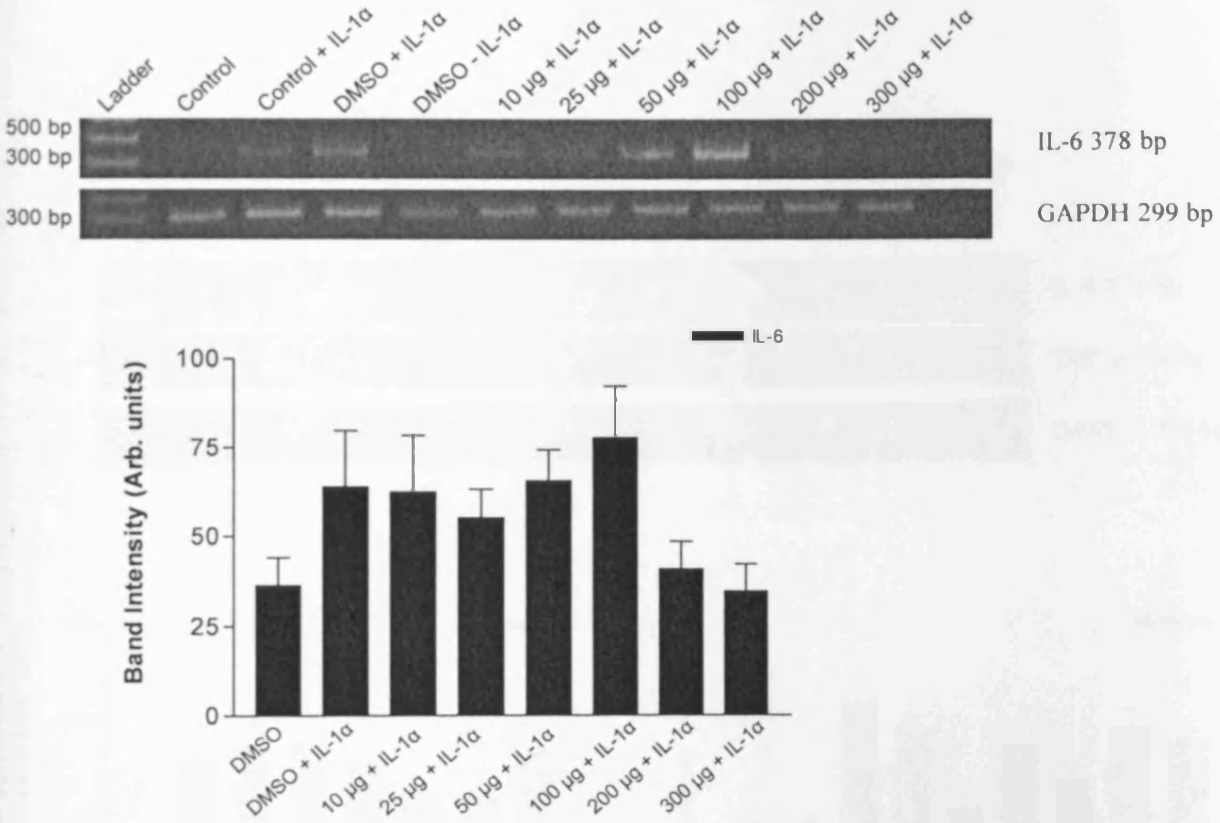


Fig. 5.19: mRNA levels for interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in bovine articular cartilage. RNA was isolated from explants supplemented with 10-300 μg/ml trout oil fatty acids (containing 7.5 μg/ml EPA and 31.5 μg/ml DHA) for 12 h. Quantification by densitometry was carried out and gene expression levels were corrected for GAPDH loading (means ± SEM; n=4). The gels shown are from a representative experiment.

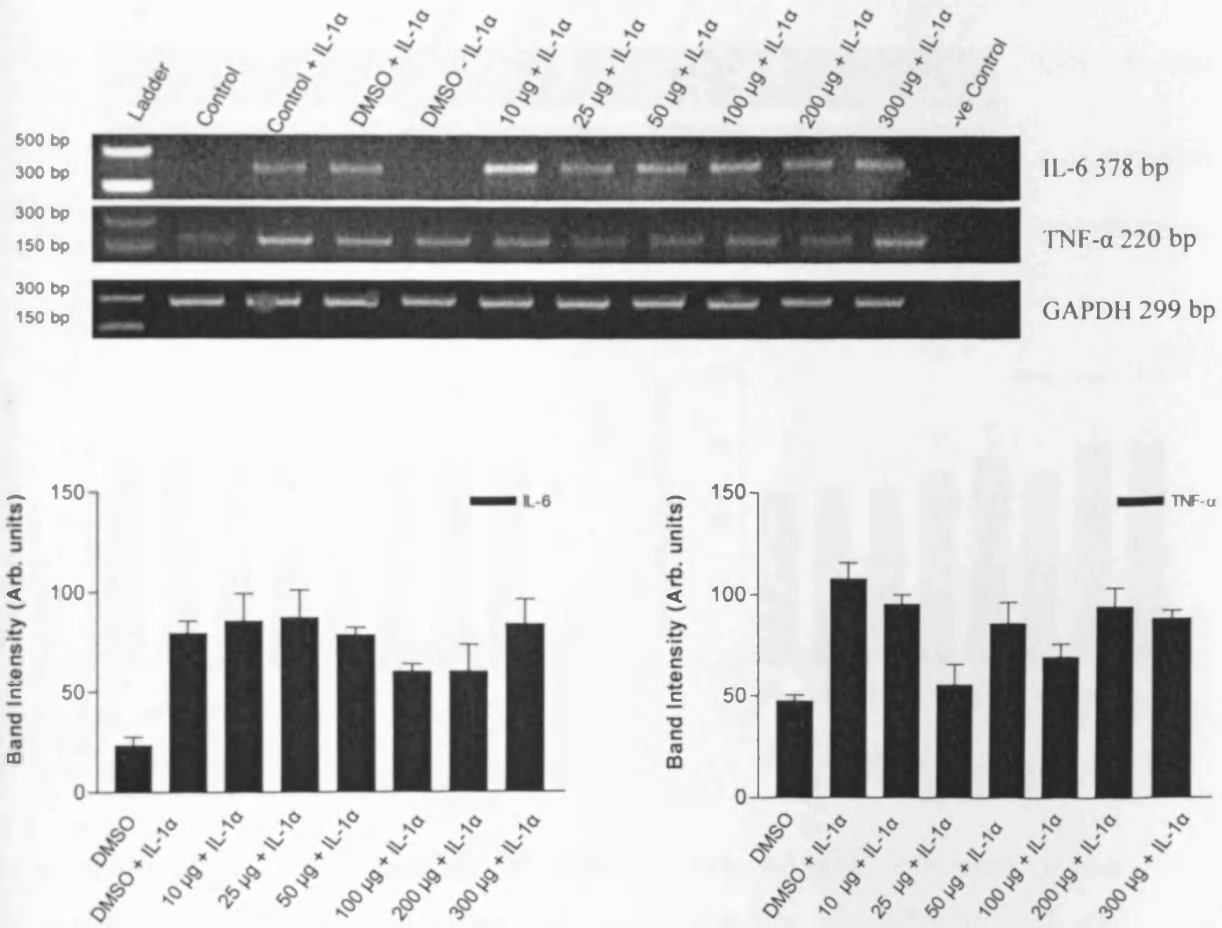
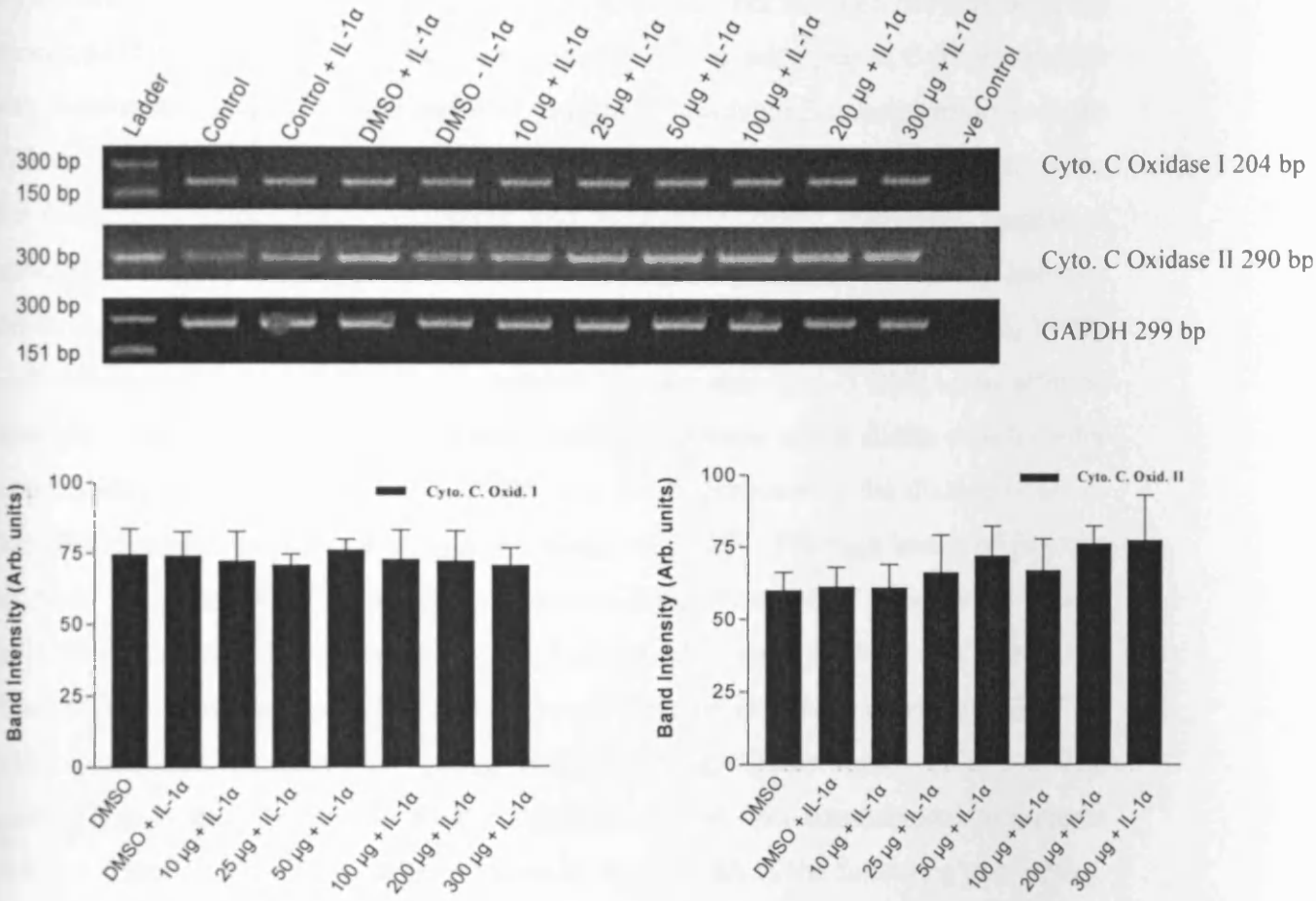


Fig. 5.20: mRNA levels for cytochrome c oxidase subunit I and subunit II and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in bovine articular cartilage. RNA was isolated from explants supplemented with 10-300 μg/ml trout oil fatty acids containing (7.5 μg/ml EPA and 31.5 μg/ml DHA) for 12 h. Quantification by densitometry was carried out and gene expression levels were corrected for GAPDH loading (means ± SEM; n=4). The gels shown are from a representative experiment.



From the results of our analysis of mRNA levels of both inflammatory and degradative proteinases it is apparent that supplementation with EPA or DHA can have potential benefits. However, supplementation with fish oil, containing a mixture of fatty acids, had no apparent effect of on mRNA levels of the genes studied despite a significant reduction in the levels of GAG released. It is possible that n-3 PUFAs supplied by the fish oil may be inducing a protective effect through genes not examined here or by a different mechanism other than gene transcription. Also, the dose of n-3 PUFAs may be too low to elicit an effect of gene expression.

5.18 Discussion

The association between the age and deposition of lipids in articular cartilage has been accepted for quite some time (Bonner *et al.*, 1975) and the accumulation of both intra- and extracellular lipid is considered a normal occurrence due to the presence of such lipids in disease-free tissue (Collins *et al.*, 1965). However, some studies suggest that excessive lipid accumulation may be linked to certain pathological conditions such as degenerative or traumatic arthritis (Lippiello, 1990). Other research has examined the beneficial role of dietary lipids in articular cartilage, for example, in C-57 mice a diet rich in saturated fatty acids was reported to induce age-dependent osteoarthrosis in the animals which was subsequently alleviated by the inclusion of 3% linoleic acid in the diet (Silberberg *et al.*, 1965; Silberberg and Silberberg, 1950). However, despite an association between lipids, fatty acids and cartilage, a possible relationship between initiation and progression of disease has yet to be fully investigated (Lippiello, 1990). Epidemiological studies also confer support for the role of n-3 PUFAs in arthritic diseases. The Japanese population have a high prevalence of the alleles which confer susceptibility for RA (Iikuni *et al.*, 2006) but the occurrence of the disease is lower than Western populations (Rayman and Callaghan, 2006). The high intake of fish oils has been suggested to offer protection against RA (Calder and Zurier, 2001). Also, the Inuit population is renowned for its high fat diet and low rate of CVD. It is believed that, despite the high fat diet consumed, its enrichment with n-3 PUFAs offers protection again CVD (Bang and Dyerberg, 1980; James *et al.*, 2003). Intervention studies where n-3 PUFA supplementation was administered to patients with RA reported significant improvement in some or all of the following parameters, morning stiffness, tender and/or swollen joints, pain, grip strength and clinical assessment of disease activity (Kremer, 2000; Kremer *et al.*, 1995; MacLean *et al.*, 2004). Therefore, due to an abundance of evidence documenting the effect of n-3 PUFAs on inflammation (Sijben and Calder, 2007) and arthritis, we investigated the possible protective effects of n-3 PUFA supplementation on a bovine *in vitro* model of OA.

In order to establish the effect of n-3 PUFAs on the degradative mechanisms involved in OA (GAG release and mRNA gene expression levels, sections 5.15 – 5.17) we first investigated the acyl composition of total polar lipids from articular cartilage explants

(section 5.13). Due to the metabolism of AA, EPA and DHA by the COX and LOX pathways and the generation of eicosanoids, resolvins and their specific role in inflammation we were specifically interested in determining the levels of these fatty acids in the total polar lipid fractions (Table 5.1). In control cultures, we determined AA at approximately 5%, whilst EPA and DHA were determined at approximately 0.8% and 1.5% respectively. A previous study also investigated the acyl composition of total polar lipids in articular cartilage from a number of species (Adkisson *et al.*, 1991). Adkisson *et al.* (1991) determined AA, EPA and DHA composed approximately 5.1%, 0.4% and 1.7% of the acyl composition of total polar lipids in articular cartilage from foetal calf. Our results support these findings. The levels of these fatty acids detected in articular cartilage from newborn pig were less than those observed in foetal calf although a similar trend was reported (i.e., AA was present at significantly higher levels than EPA and DHA). An interesting point was observed by Adkisson *et al.* (1991) with regard to the abundance of an unusual n-9 fatty acid, 20:3. 20:3 n-9 is often seen in animals that have been subjected to EFA deficiency but is normally present in minor amounts, if at all, in most tissues (Holman, 1960). Adkisson *et al.* (1991) reported 5.12% 20:3 n-9 in foetal calf and 6.35% in newborn pig cartilage. Our analysis reported approximately 3% 20:3 n-9 in the total polar lipids of bovine articular cartilage (Table 5.1). Other tissue levels of AA are considerably greater than those recorded in articular cartilage (approximately 8% in muscle, 13% in liver, 14% in kidney and 8% in serum) and several theories to explain the low levels of AA and elevated levels of 20:3 n-9 have been proposed (Adkisson *et al.*, 1991).

Previous studies, both *in vivo* and *in vitro*, have demonstrated that it is possible to enrich cells in n-3 PUFAs (Kew *et al.*, 2004; Kishida *et al.*, 2006; Martin-Chouly *et al.*, 2000; Yaqoob *et al.*, 2000). From the analysis of the total polar lipids from control cartilage we determined the principal n-3 PUFAs, α-linolenic acid, EPA and DHA to be minor components (Table 5.1). Due to the avascular nature of cartilage there was a concern that perhaps the uptake and incorporation of supplemented PUFA may be compromised. However, following supplementation with various concentrations of these PUFAs the polar lipid levels of all three fatty acids increased in a dose-dependent fashion (Fig. 5.1). Previous research has demonstrated the uptake and incorporation of PUFAs into cellular lipids. Incubation of L929 murine

fibrosarcoma cells supplemented with 0 – 50 μM DHA showed a dose-dependent increase in DHA from 2.2% (percentage of total fatty acids) in control cultures to 21.6% DHA with 50 μM (Kishida *et al.*, 2006). Kishida *et al.* (2006) also reported a significant increase in EPA (as a percentage of total fatty acids) following 24 h supplementation with 25 and 50 μM DHA. As no α-linolenic acid was present this increase in EPA may have been due to retro-conversion of DHA. As expected, there was a concomitant reduction in the percentage of 16:1 n-7, 18:1 n-9, 22:5 n-3 and AA following DHA supplementation. As previously mentioned, although the effect of α-linolenic acid was not investigated on GAG and mRNA expression levels (section 5.15, 5.17), we did investigate the uptake and incorporation of α-linolenic acid into total polar lipids (section 5.14.). α-Linolenic acid can be elongated and desaturated to yield the longer chain n-3 PUFAs, EPA and DHA, although the efficiency of this pathway is thought to be extremely low with only approximately 5% of α-linolenic acid being converted to EPA and < 0.5% α-linolenic acid converted to DHA in most tissues (Plourde and Cunnane, 2007). Despite this low conversion rate we were interested to see if increasing the levels of α-linolenic acid might lead to a significant increase in EPA or DHA. However, no increase in the longer chain PUFAs was found (Table 5.1).

Both EPA and DHA supplementation increased significantly their percentages in the total polar lipid fraction. However, some variation was observed with 50 μg of DHA (Table 5.3) and it would be advisable to repeat the experiment because of this variation. It is believed that incorporation of n-3 EPA and DHA would diminish the levels of AA present (Babcock *et al.*, 2000) but we found no reduction in AA following supplementation with α-linolenic acid, EPA or DHA (Tables 5.1 – 5.3). Despite AA being the preferred substrate for COX metabolism (Smith, 2005), EPA competes with AA for the active site of the COX enzyme (Calder, 2001) and the resulting prostanoids are known to be less inflammatory compared to the n-6 derived prostanoids (Calder, 2002). Due to the significant increase of both EPA and DHA in the total polar fraction of articular cartilage chondrocytes we hoped that a reduction in degradative and inflammatory mediators would be observed following incubation with IL-1α (sections 5.15, 5.17).

Culturing cartilage explants is a well-established method for investigating the inflammatory processes involved in arthritis and had been used extensively to test anti-arthritic treatments (Caterson *et al.*, 2000). Destruction of GAG chains that form the ECM is considered to be a useful marker of disease progression (Farndale *et al.*, 1986). An osteoarthritic state can be mimicked *in vitro* by subjecting explants to a catabolic agent such as IL-1α (IL-1β is used for human cartilage), TNF-α, retinoic acid or oncostatin-M (Caterson *et al.*, 2000; Kozaci *et al.*, 2005). Following supplementation with IL-1α for 72 h we observed a significant increase in aggrecan catabolism and a subsequent and significant release in GAG compared to the controls (Figs. 5.3 – 5.6). However, explants supplemented with 10 – 300 μg EPA/DHA or trout oil demonstrated significant reductions in GAG release compared to controls + IL-1α (Figs. 5.3 – 5.6). Our findings are supported by previous studies on both bovine explant and monolayer cultures where supplementation with EPA and, to a lesser extent, DHA also demonstrated a significant reduction in GAG release following treatment with IL-1α (Hurst, 2004; Zainal, 2005). However, in contrast to Hurst (2004) and Zainal (2005), greater concentrations of PUFAs or trout oil were required to elicit a similar effect on GAG reduction. Hurst (2004) observed significant reduction in GAG release with approximately 10 μg EPA but little, if any, reduction was observed at this dose in our experiments (Figs. 5.3 – 5.6). However, the methods used by Hurst (2004) and Zainal (2005) were different from those used in our experiments. Hurst and Zainal used fatty acid-free bovine serum albumin and Tyrode HEPES buffer to solubilise the PUFA whereas in our experiments each PUFA or trout oil-derived fatty acids were dissolved in DMSO and delipidised FBS. As bovine serum albumin is a large protein of approximately 60,000 kDa and due to the avascular nature of cartilage we were concerned that delivery of the fatty acid (which occurs by diffusion through the ECM) may be hindered due to the large size of the protein-fatty acid complex. With regard to the DMSO method, previous research has reported that DMSO may possess some anti-microbial, analgesic and anti-inflammatory effects (Smith *et al.*, 2000). Prior to implementing the DMSO method, preliminary experiments were conducted to determine any possible effects DMSO alone may have on the explants. We observed no effects of DMSO alone (Figs. 5.3 – 5.6). Also, the level of DMSO (1.5%) that the explants were exposed to was low and well within the range normally used for mammalian tissues.

With regard to the effects of PUFA or trout oil supplementation on the IL-1α stimulated release of GAG from explants, significant variations were recorded (Figs. 5.3 – 5.6). A dose-dependent decrease in GAG reduction was recorded although biological variation was also observed. A specific example was supplementation with the trout oil preparation (Figs. 5.6 A-C). These three experiments were carried out simultaneously under the same culture conditions. The trout oil fatty acid preparation (Table 5.4) was the same in all three independent experiments. Despite similar culture conditions and the same fatty acid preparations the results varied significantly between animals. Fig. 5.6A demonstrated significant reduction of GAG release at each concentration of trout oil (10 – 300 μg/ml) whereas significant reductions in GAG release were only observed at 100 – 300 μg/ml in Fig. 5.6B and in Fig. 5.6C at 50 and 300 μg/ml. We hypothesised that such differences were due to biological variation and may suggest that some animals/persons may respond better to n-3 supplementation than others. Given that the levels of EPA and DHA (7.5 and 31.5 μg/ml respectively) were much less in the trout oil fatty acid preparation and taking into account the levels of n-6 PUFAs (together with other classes of fatty acids) such significant reductions in GAG release were surprising (Fig. 5.6).

As previously mentioned, (section 5.16), the metabolic state of the explants was determined by measuring the amount of lactate produced. This assay has been widely used to determine the metabolic state of explants (Ilic *et al.*, 2003; Kozaci *et al.*, 2005; Smith *et al.*, 2000). Using the lactate assay we determined no adverse effects of either IL-1α, PUFA or trout oil supplementation on explant metabolic state. However, it should be noted that a threshold level of lactate production below which explants are deemed non-viable is not reported in the literature and hence, this method is only a crude measure for measuring explant metabolic state, despite its widespread use.

At the time these experiments began no specific assay for determining explant viability was available. Some researchers have investigated certain methods for determining explant viability such as dye-exclusion assays and the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method. Due to the nature of the explants, (thickness of tissues, network of the ECM, low cell content) complications for both these assays deemed them inappropriate. However, a recent breakthrough (Caterson and Hughes Laboratory, Cardiff University) has permitted a

variant of the MTT assay to be used on explants in culture. The method is currently being optimised but such a technique to determine explant viability, rather than metabolic state, would be a much more effective tool for this research. Measuring cell metabolic state is a rather rudimentary method and effects on cell senescence may lead to a mis-interpretation of results. Therefore, it would be advisable to repeat the experiments mentioned in section 5.15 and determine cell viability using the MTT assay.

The role of n-3 PUFAs in the autoimmune disease, RA, had been reviewed extensively in the literature and most conclude that sufferers of RA can benefit from fish oil supplementation (James and Cleland, 1997; Volker and Garg, 1996). However, little evidence is available on the potential role of n-3 PUFAs in OA despite the role of inflammation in the pathogenesis of the disease. The role of cytokines, particularly IL-1α/β, IL-6, IL-10, TNF-α, in the inflammatory process is well documented (Wood, 2006) and inflammation is known to be a key mediator of aggrecan destruction and, hence, cartilage degradation. MMPs and aggrecanases have been identified as the key catabolic enzymes involved in cartilage destruction (Caterson *et al.*, 2000). As previously mentioned (section 1.3, 1.3.1) the MMP and aggrecanase families are composed of multiple enzymes and during our research we focused on MMP-3, MMP-13, ADAMTS-4 and ADAMTS-5 as these are thought to be the main, (or some of the main) enzymes involved in OA (Flannery, 2006).

Supplementation with EPA and DHA was observed to reduce the levels of mRNA for MMP-3, ADAMTS-4 and ADAMTS-5 in a dose-dependent fashion (section 5.17). However, these reductions can only be interpretated as a trend as significance was not reached and we would recommend the use of quantitative PCR for future investigations. Despite MMP-13 being the principle collagenolytic MMP responsible for cleavage of collagen type II in cartilage no effect on MMP-13 was observed following supplementation with n-3 PUFAs (Fig. 5.14, 5.15). The trends observed in our data support those of Zainal (2005) and Hurst (2004) who also observed a decrease in mRNA for these enzymes with increasing doses of EPA, and to a lesser extent DHA. However, with regard to trout oil fatty acid supplementation which had not been investigated previous to this study, we observed no reduction in the expression of mRNA for the aforementioned proteinases (Figs. 5.16) despite the

decrease in the release of GAGs. This indicated that other MMPs and/or aggrecanases may also be involved in the breakdown of cartilage in bovine explant cultures. It is known that MMP-28 and ADAMTS-16 are both upregulated in OA together with MMP-2, -9, -16, ADAMTS-2, -14, -12 (Davidson *et al.*, 2006). Additional research to elucidate whether such enzymes are affected by n-3 PUFA supplementation would be useful.

With regard to mRNA levels of the inflammatory genes, COX-2, IL-6 and TNF-α, some interesting results were observed. COX-2 is often referred to as the inducible isoform of the COXs and the expression of COX-2 is often induced by the action of certain cytokines such as IL-1α as observed here. EPA and DHA both reduced the levels of mRNA for COX-2 (Figs. 5.11, 5.12). Previous studies have also demonstrated a reduction in COX-2 following incubation with long chain n-3 PUFAs. When HT-29 colon cancer cells were incubated with n-3 PUFAs the protein levels of COX-2 were reduced (Calviello *et al.*, 2007). Singh *et al.* (1997) reported that the feeding of high levels of fish oil exerted anti-neoplastic effects on an animal model of carcinogenesis by down-regulating COX-2 expression whereas a diet rich in n-6 PUFAs promoted colon tumourgenesis. Another study investigating the effect of fish oil on an *in vitro* model of colorectal cancer also observed a down-regulation of COX-2 (Llor *et al.*, 2003). Also, a decrease in COX-2 mRNA expression following supplementation with EPA and DHA on bovine monolayer chondrocytes was recorded by Hurst (2004) and Zainal (2005) although the concentrations which elicited these effects were lower than reported here (Figs. 5.5, 5.14) (see previous paragraph).

COX-1 expression is increased during development and is thought to be constitutively expressed in most tissues (Spencer *et al.*, 1999). Such constitutive expression is thought to be due, at least in part, to the relative stability of both COX-1 mRNA and its protein levels (Smith, 2008). From our experiments we observed constitutive expression of COX-1 although the addition of IL-1α appeared to moderately upregulate its mRNA expression compared to controls (Figs. 5.11 – 5.13). Previous research has demonstrated no effect of n-3 PUFAs on COX-1 mRNA levels. When Sprague-Dawley rats were fed diets rich in either n-6 PUFAs (safflower oil) or n-3 PUFAs (menhaden oil) for three weeks no effects on COX-1 mRNA were observed whereas the mRNA for COX-2 was induced in rats fed the high n-6 diet but not the n-

3 rich diet (Badawi *et al.*, 1998). Moreover, Zainal (2005) also found no effect of n-3 PUFA supplementation on COX-1 mRNA levels in bovine monolayer cultures.

IL-6 is a multi-factorial cytokine involved in the regulation of inflammatory and immunoregulatory responses and is inducible by numerous inflammatory agents such as IL-1, TNF-α and interferon gamma (IFN-γ) (Guerne *et al.*, 1990). IL-6 is implicated in both OA and RA and has been detected in the synovial fluid of such patients. Previous research has determined that chondrocytes possess the ability to synthesise IL-6 in response to both physiological and inflammatory stimuli (Bender *et al.*, 1990). We observed upregulation of IL-6 mRNA following incubation with IL-1α (Figs. 5.17 – 5.19). Pre-treatment with n-3 EPA and DHA reduced the levels of mRNA (though significance was not reached) although no effects were observed following treatment with trout oil (Figs. 5.17 – 5.19). A reduction in IL-6 has been previously reported with dietary n-3 PUFAs. In a community-based study it was found that circulating n-3 PUFAs independently associated with lower levels of IL-6 and increased levels of its soluble receptor (IL-6r) (Ferrucci *et al.*, 2006). An abundance of literature describes the relationship n-3 PUFAs and IL-6, however, although some studies report a decrease in IL-6 production, overall the results are quite disparate and further investigation is needed (for review see Sijben and Calder, 2007).

When explants were supplemented with trout oil-derived fatty acids the effect of supplementation on TNF-α was also investigated (Fig. 5.19). TNF-α is an inducible mediator of inflammation, produced by numerous cells including neutrophils, macrophages and lymphocytes and has been identified as a key component of both OA and RA (Farahat *et al.*, 1993). Following incubation with IL-1α we observed an increase in mRNA levels of TNF-α but no significant reduction was found with trout oil-derived fatty acid supplementation (Fig. 5.19). We hypothesised that this may be due to the presence of other classes of fatty acids (saturated, n-6 PUFAs etc) or may indeed indicate that the n-3 PUFAs do not decrease mRNA levels for TNF-α. Similar to previous research on IL-6, the literature is split over the relationship between n-3 PUFAs and TNF-α. Some authors report an increase in TNF-α production (*in vitro)* following feeding of n-3 PUFA-rich diets (Hardardottir and Kinsella, 1991, 1992). Also, when peritoneal macrophages were incubated with n-3 PUFAs *in vitro* an

increase in the secretion of TNF-α was reported. However, when n-3 PUFAs were incubated with RAW 264.7 macrophages a reduction in TNF-α was observed (Skuladottir *et al.*, 2007). Such discrepancies in the literature indicate that further research is necessary to elucidate the effect of n-3 PUFAs on TNF-α.

There is no question of the beneficial role of n-3 PUFA in inflammation, although a clear understanding of the precise mechanisms by which their 'anti'-inflammatory effects are produced is still being investigated. From our research we conclude that significant uptake of n-3 PUFAs is achievable in explant cultures and that such uptake and incorporation can have beneficial effects on the pathogenesis of OA. Pure EPA and DHA supplementation compared to trout oil fatty acids have greater benefits both on GAG destruction and mRNA levels. Due to this, it may be more advantageous to purify EPA and DHA from trout oil rather than using mixtures of the full range of fatty acids extracted from trout tissues.

CHAPTER 6

General Discussion

Chapter 6: General Discussion

The possible relationship between fish oil derived-n-3 PUFAs, EPA and DHA, and human health and disease was first mentioned as far back as the 18th century when it was reported in the London Medical Journal that administering 1–3 tablespoons of cod liver oil 2–4 times daily had beneficial effects in cases of "obstinate chronic rheumatisms, sciaticas of long standing, and in those cases of premature decrepitude" (Percival, 1783). Since then, there has been a significant progress made in understanding the biology of n-3 PUFAs and their protective effects in both health and disease.

Fish are the major source for the long chain PUFAs, EPA and DHA. However, as previously mentioned, the supply of n-3 PUFAs from marine fishing industries is in rapid decline for several reasons (e.g. mis-management of stocks, inaccurate predictions for stock replenishment) which has led to a significant decline in certain species of fish (Schrank, 2005). Cod stocks are one such example, although many other fish species are currently in danger of eradication (Pauly *et al.*, 2002). In contrast to the decline of marine fish species, over the past two decades there has been a significant increase in the production of farmed fish. Currently, the aquaculture industry is playing, and is forecast to continue to play, a significant role in global fish production. A meeting of the Food and Agriculture Organisation Committee on Fisheries (FAO COFI) underscored the importance of the aquaculture industry in complementing the traditional fishing industry, by reducing the pressure on marine fish species and providing a sustainable source of fish, and, subsequently, providing n-3 PUFAs, for human consumption (www.fao.org/focus/e/fisheries/sustaq.htm; Naylor *et al.*, 2000).

Fish farming in the UK is governed by strict regulations with regard to treatment and disposal of waste products (www.defra.gov.uk/fish/fishfarm/waste.htm). The objective of this work was to determine if waste products from one of the most commonly reared fish species, *Oncorhynchus mykiss*, could be a potential source of n-3 PUFAs for the nutraceutical industry.

In chapter three we set out to investigate the lipid and acyl composition of the waste products from *Oncorhynchus mykiss* reared on a controlled-fed, fresh-water fish farm. Current regulations for disposal of fish waste are particularly stringent and incur significant costs to the industry (www.defra.gov.uk/fish/fishfarm/waste.htm). Therefore turning the waste products into a sustainable source of n-3 PUFAs would not only have beneficial effects in reducing costs to the aquaculture industry, it would also lead to a reliable and plentiful source of n-3 PUFAs for nutraceutical development. However, in order for the offal to be considered for this role, it is essential that it contains adequate levels of n-3 PUFAs. As discussed previously, (section 3.6), the farm-reared *Oncorhynchus mykiss* used in these experiments were fed controlled diets containing all the necessary nutrients for growth and development. Initially, we examined the levels of fatty acids contained in the diet. This was important to determine, as it is now accepted that tissue levels of lipids and fatty acids are reflective of those consumed in the diet (Bell *et al.*, 1997; Jeong *et al.*, 2002; Miller *et al.*, 2007). According to our analysis EPA and DHA comprise 5% and 8% of the fatty acyl groups in the lipids in the preformed pellets (Table 3.1). However, we found that the actual tissues levels of both these n-3 PUFAs varied quite significantly depending on tissue and lipid species. The main non-polar lipid, TAG, contained a generally similar ratio of EPA and DHA in all tissues, 2% and 5% respectively (although EPA in the liver was 7% and DHA in the heart and adipose tissue was slightly lower that the other tissues). We reported that, as expected for membrane constituents, the polar lipids contained significantly greater levels of the n-3 PUFAs compared to the non-polar lipids, particularly with respect to DHA. From our analysis we determined that collectively, the waste products from farm-reared *Oncorhynchus mykiss* contain sufficiently high levels of n-3 PUFAs, particularly DHA, for them to be used potentially in nutraceutical development.

However, despite *Oncorhynchus mykiss* waste containing high levels of the n-3 PUFAs, EPA and DHA, the storage and processing of this waste material needed to be investigated to determine if these processes had adverse effects on the levels of these PUFAs. During processing of the farmed fish, the waste by-products are stored on site for quite some time. Due to the fact that this waste is currently disposed of and not processed further, it is often stored under adverse conditions (e.g. exposure to sunlight, unregulated temperatures and lengthy storage time). These conditions are not ideal for

the storage of tissues containing high levels of lipids, especially highly unsaturated molecules. In chapter three, we outlined the lipid and acyl composition of waste tissues from *Oncorhynchus mykiss* but it should be noted that this investigation was carried out on fresh tissues. In chapter four we investigated the effect of storage time and processing method on two different starting materials (total waste and adipose tissue) in order to determine if (a) storage conditions resulted in excessive tissue degradation and subsequent loss of n-3 PUFAs and (b) the effect different processing procedures on lipid and fatty acid yields.

In terms of the two different starting materials used, the total trout offal fraction yielded significantly more polar lipids compared to the adipose tissue fraction which was enriched in TAG. This was not unexpected, as from our initial studies (chapter three), adipose tissue contained low levels of total polar lipids. If total waste from farm-reared *Oncorhynchus mykiss* was to be used as a source of n-3 PUFAs we would recommend that all waste products should be combined to increase the yield of EPA and DHA.

In terms of storage of the waste, (chapter four), it seemed that increased storage time resulted in tissue degradation probably due to the loss of membrane integrity and the action of phospholipases and other catabolising enzymes. In certain regards this effect was advantageous. For example, both the lipid and acyl yields from the petroleum ether and rendering methods increased with time, resulting in more successful extraction from the tissues. However, a disadvantage of increased storage time was the increased generation of NEFAs. As EPA and DHA are the essential PUFAs found in fish tissues their concentration needed to be maintained in order for the tissues to have a use for nutraceutical development. These PUFAs are highly unsaturated and increased storage times, which resulted in increased NEFAs and also reduced the proportions of EPA and DHA in tissues due to the latters' susceptibility for oxidation. Therefore, if farm-reared fish offal was to be employed as source of n-3 PUFAs, its storage conditions need to be reconsidered: for example, on-site storage of offal should be stored under cool (refrigerated) conditions and out of direct sunlight.

With regard to processing of fish waste, the Garbus method was determined to be the most effective method at each time point (section 4.5, 4.6). As discussed previously, (section 4.8), petroleum ether is a non-polar solvent and, therefore, predominantly extracts TAGs whilst the extraction of non-polar lipids was quite inefficient. The rendering method is commonly used for large scale industrial extraction of fish lipids (Gunstone *et al.*, 2007). Although we observed that the rendering method was not as efficient as either the Garbus or the petroleum ether method, reasonable levels of EPA and DHA were extracted. A major advantage that the rendering method offers over the Garbus and petroleum ether methods is the reduction of toxicity issues (through the use of solvents) which makes the rendering method ideal for the production of products for human and animal consumption. With regard to the effect of storage time and rendering we found that increased storage time increased the efficiency of lipid extraction. However, in accordance with increased storage time the production of NEFAs also increased, although the level of NEFAs did not reach the levels seen with either the Garbus or petroleum ether method. We hypothesised that this was primarily due to the greater levels of total lipid extracted with the Garbus and petroleum ether methods rather than a 'protective' effect of the rendering method against NEFA generation (Figs. 4.2A, 4.2C, 4.9, 4.11).

With regard to the acyl composition of individual lipids from the trout waste, there were apparent differences between the results reported in chapter three and chapter four (Tables 3.4 – 3.13, 4.2 – 4.26 and 4.18 – 4.33). The acyl composition for individual lipids outlined in chapter four are for multiple tissues pooled together, whilst in chapter three the lipid and acyl composition are given for specific lipids from individual organs. However, another important factor which may contribute to differences in the fatty acid profiles is season. As previously discussed, (section 3.16), season has a large bearing on both the lipid and fatty acid composition of fish (Ackman and Takeucki, 1986; Agren *et al.*, 1987; Senso *et al.*, 2007). The fish used in chapter three were caught in late November whilst those used in chapter four were caught in April. One example of the effect of season is the level of unsaturated fatty acids found in membrane lipids. In the colder months, membrane polar lipids are often more highly saturated than in the warmer months in order to maintain 'fluidity' of the membranes to compensate for the decrease in water temperature (Johnston and Roots, 1964) (Tables 3.4 – 3.13, 4.3 – 4.26, and 4.16 – 4.33).

The results from both chapter three and four permitted us to conclude that farm-reared *Oncorhynchus mykiss* waste is a rich source of n-3 PUFAs. However, we advise that if minor adjustments were made with regard to storage of the waste tissues, (e.g. refrigeration), the levels of n-3 PUFAs could be maintained for a possible 48 h. We can advise this as, from our analysis, offal stored at room temperature and exposed to direct sunlight was stable for at least 24 h (after 48 h elevated levels of NEFAs were detected. With regard to the processing of such waste tissues, the rendering method, albeit not as efficient as laboratory-scale lipid extraction procedures, could be employed as an effective method of lipid extraction. Bearing in mind that our model rendering system was a small scale, laboratory-based system which was not an exact model of the industrial process, we still achieved adequate yields of total lipids, total TAG, individual lipid separations and adequate recovery of EPA and DHA. Given that an industrial scale rendering system could be more reproducible (e.g. a steamed, twin screw presses are commonly employed to ensure complete disruption, homogenisation and rendering of tissues (Gunstone *et al.*, 2007)), we hypothesise that in a large scale system, the yield of EPA and DHA would be even greater than what we reported here.

Following on from the previous chapters, we wanted to determine if processed trout oil from *Oncorhynchus mykiss* waste could elicit beneficial effects on a model tissue culture system for OA. The bovine tissue culture system employed as a model for OA is widely used as a well-established method and stimulation of the tissue with an inflammatory cytokine (e.g. IL-1α) is regarded as a good model to mimic the inflammatory state seen in osteoarthritic tissues (Caterson et al, 2000; Hughes *et al.*, 1998). As mentioned earlier in this chapter, fish oil has long been implicated with alleviating the symptoms of arthritic disease. Arthritis is a major problem in developed countries and places an enormous burden on national health care systems as well as the devastating effects on quality of life for sufferers of the disease (Lawrence *et al.*, 2007; Helmick *et al.*, 2007; Reginster, 2002). Pharmacological treatments for OA focuses on alleviating the pain and discomfort associated with the disease but no treatment that addresses the pathology currently exits. The pharmacological treatments for OA often result in unwanted side effects (e.g. classical NSAIDS may result in gastric discomfort or, in more extreme cases, ulceration of the gastric lining (section 1.5.1)) (DeWitt, 1999). Dietary intervention has been reported to have

beneficial effects in reducing the inflammation found in patients with rheumatoid arthritis (Rayman and Callaghan, 2006) and, more recently, evidence is emerging that sufferers of OA may also benefit from dietary intervention (Hurst, 2004; Zainal, 2005).

In chapter five we investigated the effect of EPA, DHA and trout oil-derived fatty acid supplementation on a tissue culture model of OA. In terms of EPA, DHA (and α-linolenic acid) supplementation (section 5.14) we found that the levels of these n-3 PUFA were dose-dependently increased in membrane lipids from bovine articular cartilage during incubation. Uptake and incorporation of these PUFAs, did not however, result in a specific decrease in the levels of AA but did alter the percentages of oleic acid, 18:1 n-9. This result indicated that, during the time of the experiment, n-3 PUFA supplementation did not significantly affect the metabolism of AA. We then investigated whether the increase in n-3 PUFAs was enough to induce a protective effect on IL-1α-stimulated GAG degradation. As previously detailed, (section 1.3) aggrecanase cleavage of GAG side chains is induced by IL-1α (Hughes *et al.*, 1998). The release of these GAGs was measured by a colorimetric assay which determined that incubation with IL-1α significantly up-regulated GAG release compared to controls. Pre-incubation with varying doses of EPA and DHA (section 5.15), particularly at the higher doses, was seen to significantly reduce IL-1α stimulated GAG release, therefore, offering protection against cartilage degradation which agreed with previous work from Hurst (2004) and Zainal (2005). However, it should be noted that we used a DMSO method to add PUFAs, in contrast to the technique used by Hurst (2004) and Zainal (2005).

Having determined that supplementation of both EPA and DHA offered articular cartilage protection against aggrecan destruction we decided to examine the effects that n-3 PUFAs may exert on mRNA levels for specific proteinases or 'inflammatory' proteins thought to be involved in the degradation of cartilage and the associated inflammation seen in the pathogenesis of OA. The higher doses of EPA and DHA used in the supplementation resulted in a decrease in COX-2 mRNA, a key enzyme involved in inflammation (section 1.5.1). As discussed earlier, (section 5.18), other investigators have also reported a down regulation of COX-2 mRNA following treatment with EPA and/or DHA (Calviello *et al.*, 2007; Llor *et al.*, 1994; Singh *et al.*,

1997). A reduction in COX-2 mRNA expression and the generation of n-3 PUFA-derived eicosanoids would have the advantage of shifting the inflammatory response from one that was pro-inflammatory (n-6 PUFA-derived eicosanoids) to less inflammatory (section 1.5.1.1).

In addition to investigating the effects of n-3 PUFA supplementation on inflammatory markers such as COX-2, we also looked at mRNA levels for proteinases involved in the loss of ECM integrity. As detailed in section 1.3 and 1.3.1, aggrecanases and MMPs are extensively involved in cartilage degradation (Caterson *et al.*, 2000). Pre-incubation of articular cartilage prior to IL-1α stimulation, led to a decrease in mRNA expression for two of the principle aggrecanases, ADAMTS-4 and -5, as well as MMP-3. However, mRNA for the principle collagenolytic enzyme, MMP-13 was not affected by supplementation with either EPA and/or DHA. We hypothesised that a reduction in the mRNA levels of ADAMTS-4 and -5 could be responsible, at least in part, for the protection seen with regard to GAG release (section 5.15 and 5.17).

Once we had determined that pre-incubation of EPA and DHA in an *in vitro* model of OA resulted in protective effects with regard to protein release and gene expression, we employed the same *in vitro* tissue culture model to test the trout-derived fatty acids. As outlined in Table 5.4, the trout oil preparation contained approximately 2.5% EPA and 10.5% DHA as well as a range of other fatty acids. Interestingly, we also observed a significant decrease in GAG release with various doses of the trout oil preparation (Figs. 5.6A-C). Biological variation was quite apparent as all cultures shown in the aforementioned figures were treated with the same culture materials and the same trout oil fatty acid preparation. The only difference between experiments was the tissue source (individual animals were used per experiment). This in turn suggested that perhaps, more control over the tissue used (e.g. specific cow breed, dietary history) might give more consistent and, perhaps, higher responses to fatty acid supplementation than others. This hypothesis has been previously reported in the literature. Hagfors *et al.* (2005) reported responders and non-responders in a population of Swedish rheumatoid arthritic patients with regards to dietary intervention and clinical improvements. Also, there are reports that some persons respond better to pharmacological treatment than others (e.g. chemotherapy and breast cancer patients). Hence, it is plausible that tissues from some individuals may also

respond differently to supplementation also. Indeed, one can see this result in the data shown in Figs. 5.6A-C.

With regard to supplementation with trout-derived fatty acids and mRNA expression, we did not find any significant changes in relation to mRNA levels for inflammatory genes (e.g. COX-2) or the matrix proteinases (e.g. ADAMTS-5) (see Figs. 5.13, 5.16, 5.19 and 5.20) despite a clearly significant reduction in GAG release (Fig. 5.6 A-C). However, mRNA was the only parameter we measured with respect to gene expression. Enzyme activity levels were not measured and, moreover, the balance of fatty acids would be affected by the presence of a range of n-6 and saturated fatty acids also being present in the trout oil preparation. In comparison with the high levels of EPA and DHA, 300 μg/ml of the trout oil preparation only contained 7.5 EPA and 31.5 μg/ml DHA which, together with the other fatty acids present, may not have been sufficient to elicit a reduction of the mRNA levels. Furthermore, Zainal (2005) noted that EPA was the most effective n-3 PUFA in tissue culture systems to test for OA and this component was rather low in the trout oil mixture. Moreover, as outlined in section 1.5.4.1, n-3 PUFA are known to elicit protective effects by mechanisms other than gene expression (Jump, 2002). Therefore, it may be possible that the trout oil-derived mixture may be exerting protective effects, such as on GAG release, via a different mechanism rather than solely regulating gene expression. Needless to say, these possibilities need to be investigated further as the mechanisms involved are still far from understood. The nature of OA also brings into question the role of other members of the aggrecanase and MMP families. Currently research is ongoing to determine which members of these families may be involved in the pathogenesis and progression of OA or may offer protection from this debilitating disease (Davidson *et al.*, 2006).

From our investigation, we can recommend farmed *Oncorhynchus mykiss* waste to be a rich source of n-3 PUFAs and hypothesise that other varieties of farmed fish (e.g. salmon, which are particularly rich in n-3 PUFAs) may be used as an alternative source of n-3 PUFAs. Given the financial costs for the treatment and disposal of farmed fish waste, combined with the environmental issues associated with waste disposal, we recommend that the use of farmed fish by-products should be readdressed. Given the estimated value of salmon waste alone (£5 million annually in

the UK) and the abundance of n-3 PUFAs in *Oncorhynchus mykiss* we predict that utilising waste products for the generation of n-3 PUFAs would be a profitable exercise which would reduce disposal costs for the aquaculture industry and the associated environmental risks, whilst generating profit from a current waste product. From our research, we can recommend that minor modifications for the on-site storage of waste which would maintain the levels of n-3 PUFAs should be investigated. Not only would the aquaculture industry profit from this exercise but the nutraceutical industry would also reap benefit. As outlined earlier, the aquaculture industry is rapidly expanding and this would guarantee a plentiful supply of waste products with high n-3 PUFA content. Not only this, but the current method for lipid extraction, rendering, is an appropriate method to guarantee adequate yield of lipid as well as n-3 PUFAs, EPA and DHA.

Due to the abundance of research on the beneficial effects of n-3 PUFAs, not solely in terms of arthritis (evidence now suggests the importance of n-3 PUFAs in foetal development, neurological and psychiatric disorders, cancer, CVD and other inflammatory conditions) (Calder, 2004; de Vriese *et al.*, 2003; Koletzko *et al.*, 2001 Tokudome *et al.*, 2006), the demand for n-3 PUFA nutraceuticals will continue to rise and, therefore, transforming a current waste product into a reliable source of n-3 PUFAs would have multi-factorial advantages.

CHAPTER 7

Bibliography

Chapter 7: Bibliography

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