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FATTY ACIDS COMPOSITION IN SOUTH AFRICAN FRESH WATER FISH AS INDICATORS OF FOOD QUALITY FOR HUMAN CONSUMPTION

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A research report submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Science

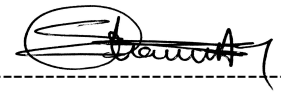
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WITS University, Johannesburg, 2007

DECLARATION

I declare that this research report is my own, unaided work. It is being submitted for the Degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.



(Signature of Candidate)

----------**30**--------Day of------**May**---------2007

ABSTRACT

Lipid classes and fatty acid composition (nutritive quality) of three commercially important fresh water fish species *Oreochromis Mossambicus* (Mozambique Tilapia), *Clarias Gariepinus* (Sharptooth Catfish) and *Cyprinus Carpio* (Carp) obtained from an aquaculture, different river systems and fish markets in South Africa were investigated. Fish fillets were prepared in the laboratory and used as representative samples for extraction of lipids through the Folch extraction method (using chloroform methanol at the ratio of 2:1). The structural separation of esterified fatty acids from fish lipids was conducted using gas chromatograph. Identification of fatty acids (FAs) composition was done by comparing the retention times of samples with the ones for all FAs standards and by spiking with commercially available fatty acids standards. Total lipid content of Tilapia fish was higher than that of Cat and Carp fish. Palmitic acid (16:0) was found to be the most abundant fatty acid (18.24 to 21.84 %) in all analysed fish species. Appreciable quantities of essential polyunsaturated fatty acids such as docosahexaenoic acid DHA (22:6 n-3, 3.92 to 6.16%), eicosapentaenoic acid EPA (20:5 n-3, 1.91 to 2.92 %) and Arachidonic acid (20:4 n-6, 7.19 to 8.50 %) were also found. EPA + DHA values were much higher on Tilapia fish lipid fractions in comparison with other fish lipid fractions. Observations showed that fish species from Gauteng province were the richest in lipid fractions as compared to those from other provinces. Of all fish species, cultured fish were found to be highly characterised by high levels of FAs as compared to the fish species collected from the river systems. This could be attributed to the FAs composition of their diet. The study points out that all fish species under study contain appreciable

levels of n-3 polyunsaturated fatty acids and would therefore be suitable for highly unsaturated low-fat diets.

In loving memory of my granny and sister

Shalati Sithole and Charllote Chauke

"There was only one catch……..."

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LIST OF ABBREVIATIONS

FAs	**Fatty Acids**
PUFA	**Poly unsaturated Fatty Acid**
FAMEs	**Fatty Acid Methyl Esters**
FFA	**Free Fatty Acid**
EFA	**Essential Fatty Acids**
BHT	**Butylated Hydroxytoluene**
GC	**Gas Chromatography**
FID	**Flame Ionization Detector**
DHA	**Docosahexaenoic Acid**
EPA	**Eicosapentaenoic Acid**
AA	**Arachidonic Acid**
n-3	**Omega-3**
n-6	**Omega-6**
RSD	**Relative Standard Deviation**
LA	**Linoleic Acid**
LLE	**Liquid-Liquid Extraction**
MAE	**Microwave Assissted Extraction**

CHAPTER 1 : INTRODUCTION

1.1. GENERAL INTRODUCTION

The discovery of chromatography by Tswett is usually cited as the beginning of chromatography (Tswett, 1903 as cited by Brondz, 2002). However, Tswett's work was predated by the application of separation techniques by Day who first applied separation science on petroleum analysis (complex mixture of lipids) by passing sample solution through a glass column packed with solid calcium carbonate (Day, 1897 as cited by Brondz, 2002). Many literatures indicate that the first satisfactory separation of C6-C18 free fatty acids, with least tailing and good separation between critical C16:1 and C16 pair was presented by Brondz *et al*., (Brondz *et al.,* 1983).

In nature, fatty acids (FAs) exist as pure substances or as parts of more complex molecules known as lipids. These complex molecules are soluble in organic solvents such as ether, chloroform-methanol mixture, hexane, benzene and petroleum ether (Brondz, 2002).

Fatty acids (both saturated and unsaturated) are straight chain carboxylic acids (e.g. palmitic acids, stearic acid, etc) with a number of carbon atoms in their molecules. This is true for the greater majority of structures and for the more abundant acids. Chain lengths range from 2 to over 80 carbon atoms. However, the most common are between C_{12} and C_{22} (Brondz, 2002; Gunstone, 1996).

The effects of dietary fatty acid composition on fatty acid metabolism especially in connection with the essential fatty acid requirements of several fish species have been

widely studied (Steffens, 1997). Research indicate that there is no doubt that the composition of fatty acids reflects to a greater extent that of the fish's diet (determined by what the fish eats) (Steffens, 1997). Thus there is a huge variation in fatty acid composition of different individual fish of the same species, mainly owing to differences in the food they feed on (Steffens, 1997).

Several fresh water fish species are rich in Omega-6 (n-6) polyunsaturated fatty acids (PUFA) such as arachidonic acid and linoleic acid (LA). This is attributed to the lipid composition of the plankton they feed on. However, fresh water fish are a main source of Omega-3 (n-3) PUFA especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Gunstone, 1996; Steffens, 1997).

Unlike their fresh water counterparts, marine fish oils/ or lipids are highly characterized by low levels of linoleic acid (18:2 n-6) and linolenic acid (18:3 n-3), with high levels of long-chain n-3 polyunsaturated fatty acids. Eicosapentaenoic acid (20:5 n-3) and docosahexaenoic acid (22:6 n-3) are the predominant n-3 fatty acids (Mendez *et al.,* 1996). A study conducted by Stansby (1967) found that river fingerlings of Coho salmon had a very high level of linoleic acid, while ocean matured salmon contained a much lower percentage of this type of fatty acid.

Scientific studies have shown that consumption of fish containing high levels of fatty acids (especially DHA and EPA) has positive benefits to human health particularly in preventing cardiovascular diseases and other related diseases (Steffens, 1997; British nutrition foundation, 1992). Fatty acids serve as energy reserves, structural and functional components in the living organisms including human beings. The quality of the fish is

therefore linked to the type and quantity of their fatty acids (Gunstone, 1996; Steffens, 1997). Malnutrition and shortage of foods containing high levels of this type of fatty acid (especially fish) in rural areas of South Africa might be the underlying reason for the higher incidence of cardiovascular diseases among the population (Menong as cited by Dlale, 2005).

EPA and DHA are known to play a major role in modulating the biosynthesis of eicosanoids (Varljen *et al.,* 2003). Moreover, DHA is found in high concentrations in membranes of important organs, possibly influencing membrane-lipid dependent functions, especially in retina and brain (Varljen *et al.,* 2003; Hoffman *et al.,* 1993). Therefore, the nutritional importance of fish consumption is associated largely with the n-3 PUFA content (Ackman, 1989). These findings have created a new market for fish oil as a food and dietary supplement. Several other products with technical and cosmetic applications based on fish oil fatty acids have also been developed and produced commercially (Ackman, 1989).

Many studies have been conducted to determine fatty acids composition of lipids on different kinds of living organisms in many countries (Mendez *et al.,* 1996). However, few of this kind have been carried out for many of South African fish species. Shortage of knowledge on the fatty acids compositions on South African fish species might be the underlying reason for low consumption of fish among the South African population.

1.2. RESEARCH OBJECTIVES

1.2.1. General Objective

This study is aimed at determining the fatty acid composition of lipids of the main important fresh water fish species in South Africa and relates their importance to human nutrition.

1.2.2. Specific Objectives

- To identify and quantify fatty acids composition of lipids of the main important (highly consumed) fresh water fish species found in South Africa.
- To determine whether the regional location has an influence on fatty acids composition of fresh water fish
- To find out human nutritional value of different fish species based on their fatty acids composition.
- To come up with recommendations on types of fish organisms suitable for people with specific deficiencies and those that can be used in aquaculture projects.

1.3. JUSTIFICATION OF THE RESEARCH PROJECT

Poverty and Poor nutrition in rural areas of South Africa have accelerated the spread of some diseases in such areas. Implementation of aquaculture (fish farming) projects for community benefit can play a major role in supplementing the nutritional level of food they consume.

The main protein source in South Africa is chicken and beef meat. The fatty acids composition of these types of food consist mainly of stearic and palmitic acids, with very low levels of unsaturated fatty acids. This might be the leading reason for high incidence of cardiovascular diseases among the population. South Africa has enough fresh water ecosystems which can provide fish for consumption. However, consumption among the population is very low. This might be due to lack of information available on the fatty acids composition of South African fish species. The quality of the fish in terms of fatty acid composition from various parts of South Africa is also not fully known.

Fish is a major source of food for many human populations, which has stimulated many studies to collect information about the quality of fish species all over the world. Fatty acid composition data for different marine, freshwater and farmed fish species, especially originating from Hungary, Malaysia, United States, Canada and Japan are available in the literature (Steffens *et al.*, 1993; Zuraini *et al.,* 2005). However, published information about the composition of fatty acids in South African fresh water fish species is scarce. The aim of the present study is to collect information on fatty acid composition of three commercially important fish species in South African fresh water ecosystems. They occupy an important place in the fishing activity of the country because they represent an important food source.

The study generated data on the level of lipids content on South African fish, which can be applicable in human nutrition and thus can contribute to reduced risk and prevention of various human diseases and to the improvement of the health status of South African citizens. Traditionally liver oil of the Winghead and Sandbar fish has been used to relieve

muscular pain as well as arthritis in Pakistan (Gopakumar and Nair, 1972). Various reports on the study and identification of fish liver oil having high pharmacological activity potential as a hypolipidemic agent (Zuraini *et al.,* 2005), an antiarthritic agent and preventing agent for renal damage (Stahl *et al.*, 1988) inspired this research to be undertaken. Studying the fish fatty acid composition from various parts of South Africa will give an indication on the quality of the fresh water fish in the country.

CHAPTER 2: LITERATURE REVIEW

2.1. LIPIDS AND FATTY ACIDS COMPOSITION

Due to their fatty acids importance on human health, essential oils have gained more application in pharmaceutical, food and fragrance preparations (Stahl *et al.*, 1988). It is not surprising that many techniques have been developed to harness these essential oils for use in various applications. Freshwater fish species serve as a valuable source of essential fatty acids. They therefore form an important component of diet for many peoples of the world, mainly as a general source of nutritional components, as low-fat, high protein food and as a source of polyunsaturated fatty acids.

Lipids refer to compounds based on fatty acids or closely related compounds such as the corresponding alcohols (glycerol) or the sphingosine bases (Gunstone, 1996). They are soluble in non-polar solvents such as chloroform, hydrocarbons, and they serve to insulate the nervous system where the white substance consists of cerebrosides and other lipids (Gunstone, 1996). Fatty acids form components of this complex mixture. They constitute components of the fixed oils, fats, waxes, lipopolysaccharides, glycerophospholipids, glycoglycerolipids and spingolipids (Brondz, 2002; Mensink *et al*., 1988). Fatty acids have attracted more scrutiny as precursors for prostaglandin synthesis and possible genetic regulators (Gunstone, 1996).

Both saturated and unsaturated fatty acids refer to straight chain compounds with an even number of carbon atoms in their molecules. This is true for many types of fatty acids (British Nutrition Foundation, 1992). However, acids with an odd number of carbon

atoms are also present at a very low scale, and mainly occur as do those with branched chains or with carboxylic units (Gunstone, 1996). The chain length ranges from 2 to 80 carbon atoms, but they are mainly found to be highly dominant with carbon atoms ranging from C12- C22 (British nutrition foundation, 1992).

Table 1. Most common fatty acids nomenclature (both saturated and unsaturated) found between the carbon range of C12 to C22

Systematic name	**Trivial name/abbreviation**	**Shorthand notation**
Saturated		
Dodecanoic	Lauric	(12:0)
Tetradecanoic	myristic	(14:0)
Hexadecanoic	palmitic	(16:0)
Octadecanoic	stearic	(18:0)
Unsaturated		
Cis-9-hexadecenoic	palmitoleic	(16:1 n-7)
Cis-9-octadecenoic	oleic	(18:1 n-9)
Trans-9-octadecenoic	elaidic	(18:1 n-9)
Cis-11-eicosaenoic	gadoleic	(20:1 n-9)
Cis-13-docasaenoic	erucic	(22:1 n-9)
Cis-11-docasaenoic	cetoleic	(22:1 n-11)
Cis-15-tetracosaenoic	nervonic	(24:1 n-9)
Cis,cis,9,12-octadecadienoic	Linoleic	(18:2 n-6)
Trans-5,cis-9,cis-12-octadecatrienoic	columbinic	(18:3 n-6)
All cis,9,12,15-octadecatrienoic	alpha linolenic	(18:3 n-3)
All cis,6,9,12-octadecatrienoic	gamma linolenic	(18:3 n-6)
All cis,6,9,12,15-octadecatetraenoic	stearidonic	(18:4 n-3)
All cis,11,14,17-eicosatrienoic	Mead	(20:3 n-9)
All cis,8,11,14,-eicosatrienoic	Dihomo gamma linolenic	(20:3 n-6)
All cis,8,11,14,17-eicosatetraenoic	ETA	(20:4 n-3)
All cis,5,8,11,14,-eicosatetraenoic	arachidonic	(20:4 n-6)
All cis,5,8,11,14,17-eicosapentaenoic	EPA	(20:5 n-3)
All cis,7,10,13,16-docosatetraenoic	adrenic	(22:4 n-6)
All cis,7,10,13,16,19-docosapentaenoic	----------	(22:5 n-3)
All cis,4,7,10,13,16-docosapentaenoic	DPA	(22:5 n-6)
All cis,4,7,10,13,16,19-docosahexaenoic	DHA	(22:6 n-3)

Source: British Nutrition Foundation, (1992).

Fatty acids with each of the carbon atoms in the chain except the two terminal ones, is bonded to two hydrogen atoms, they are said to be saturated. While the one with a carbon atom bonded to only one hydrogen is said to be unsaturated (Gunstone, 1996; British Nutrition Foundation, 1992). Polyunsaturated fatty acids occur in biosynthetically related families, with n-6 acids based on linoleic and ω-3 acids based on α-linolenic as the most important one (Pepping, 1999).

2.1.1. Nomenclature and Distribution of Fatty Acids in Nature

2.1.1.1. Saturated fatty acids

Saturated fat refers to fats that contain mainly saturated fatty acids. These are mainly found in animal fatty tissues (Collins, 2005). Saturated fatty acids have the formula H(CH2)n-COOH where n ranges from 0 to 40 or more. Formic acid is the first member in the homology series of saturated carboxylic acids C1-C16 (Beyer, 2001). They do not contain any double bonds or other functional groups along the chain. Saturated fatty acids form straight chains and, as a result, can be packed together very tightly, allowing living organisms to store chemical energy very densely. The fatty tissues of animals contain large amounts of long-chain saturated fatty acids (Collins, 2005; Wilkipedia, 2005).

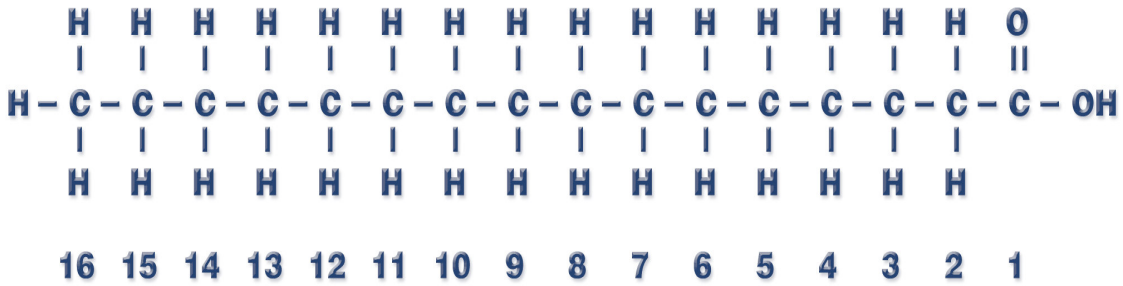


Figure 1. Straight chain Palmitic fatty acid with its carbon atoms saturated with hydrogen atoms (source: Microbial ID, 2000).

Lipid-bound C_4 to C_{12} fatty acids are in essence mainly found in milk fats and animal tissues, while the medium-chain compounds occur in seed oils, such as coconut oil. Palmitic acid is one of the most abundant fatty acids in nature and is found in the lipids of all organisms. Stearic acid is also relatively common (Microbial ID, 2000). The higher saturated fatty acids are solid at room temperature and are comparatively inert chemically (Christie, 1989).

2.1.1.2. Monoenoic fatty acids

Monoenoic fatty acids (or monounsaturated fatty acids) are straight-chain even-numbered fatty acids with 10 to more than 30 carbon atoms and containing one cis-double bond. The double bond can be in a variety of different positions, and this is specified in the systematic nomenclature in relation to the carboxyl group. Thus, the most abundant monoenoic fatty acid in tissues is probably *cis*-9-octadecenoic acid, also termed oleic acid (18:1) (Christie, 1989).

Animal and plant lipids frequently contain families of monoenoic fatty acids with similar terminal structures, but with different chain-lengths, that may arise from a common precursor either by chain-elongation or by *beta*-oxidation (Christie, 1989;). *cis*-Monoenoic fatty acids with 18 carbons or less melt below room temperature (*trans*-isomers have somewhat higher melting points). Because of the presence of the double bond, they are more susceptible to oxidation than are the saturated fatty acids (Collins, 2005).

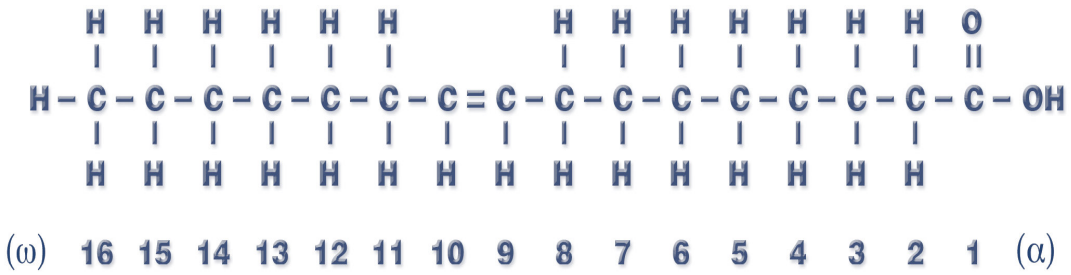


Figure 2. Cis bonded monoenoic fatty acid (palmitoleic acid) with a single double bond (source: Microbial ID, 2000).

2.1.1.3. Polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFA) refer to the type of fatty acids consisting of more than one double bond on their structures. They are liquid at room temperature and in the refrigerator (American Heart Association, 2006). PUFA are categorized according to their chemical structures, from which the most important are those of n-3 and n-6 fatty acids. This is because these two categories of PUFA are not synthesized by an animal's body, therefore they should be provided from the diet (American Heart Association, 2006). The n-6 series are derived from linoleic acid (LA) while the n-3 series from α-linolenic acid (ALA) (Steffens, 1997).

Of all polyunsaturated fatty acids, docosahexaenoic and eicosapentaenoic acids are the main important components of the Omega-3 fatty acids. Studies show that marine fish are the major source of Omega-3 fatty acids in nature, including docosahexaenoic and eicosapentaenoic acid (Steffens, 1997; Mendez *et al.,* 1996; Christie, 1989; Dayhuff and Wells, 2005). Linoleic acid (*cis*-9,*cis*-12-octadecadienoic acid) is the most wide-spread polyunsaturated fatty acid, and it is found in most animal and plant tissues. It serves as

the precursor of a family of fatty acids that is formed by desaturation and chain elongation (e.g arachidonic acid (20:4 n-6)), in which the terminal (n-6) structure is retained (Christie, 1989).

For all unsaturated fatty acids, the hydrocarbon tail around C=C double bond can be arranged in two ways. For *trans bonds*, hydrogen atoms are on the opposite sides of the double bonds such that one is "up" and the other one is located "down" across from each other. In *cis bonds,* hydrogen atoms attached to the double bond are either both "up" or both "down" such that both are on the same side of the molecule (American heart association, 2006; Carter, 2000; Christie, 1989). Trans fatty acid have a negative influence on the quality of fatty acids, by increasing the level of low-density lipoprotein LDL ("bad") cholesterol while decreasing high density lipoprotein HDL ("good) cholesterol. Polyunsaturated fatty acids can be dienoic, trienoic, tetrapentaenoic and hexaenoic acids (Brondz, 2002).

2.1.2. Omega-3 and Omega-6 Fatty Acids (Essential Fatty Acids)

The health effects of Omega-3 fatty acids describe the remarkable developments in fish oil research, in the past decades that have turned traditional thinking about fatty acids upside down. Starting with the striking observation that the native Inuit (raw flesh) high fat diet is associated with the absence of heart disease, this has led to a number careful studies demonstrating the involvement of Omega-3 fatty acids in human growth and development. Omega-3 fatty acids and health leads to the threshold of understanding on how these fatty acids function in the immune system and in cancer (British nutrition foundation, 1992).

Omega-3 (n-3) and omega-6 (n-6) fatty acids are unsaturated "Essential Fatty Acids" (EFAs) that need to be included in the diet because the human metabolism cannot synthesise them from other fatty acids (Zamora, 2005). Since these fatty acids are polyunsaturated, the terms n-3 PUFAs and n-6 PUFAs are applied to omega-3 and omega-6 fatty acids, respectively. Omega-6 fatty acids (e.g Linoleic) consist of double bond(s) six carbons away from the "Omega" carbon, while Omega-3 fatty acids (e.g Linolenic) consist of double bond(s) three carbons away from the "omega" carbon (Brondz, 2002).

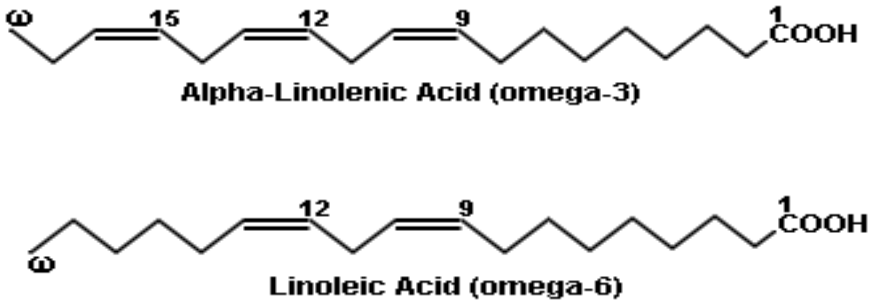


Figure 3. The structural representation of Omega-3 and Omega-6 fatty acids with carboxylic group at the "head" and Omega group at the "tail" (source: Zamora, 2005).

Docosahexaenoic acid (DHA) and arachidonic acid (AA) are both unsaturated fatty acids crucial for the optimal development of the brain and eyes. The importance of DHA and AA in infant nutrition is well established (Robert, 1996; Steffens, 1997; Lloyd and Ackman *et al*., 1980). Both substances are routinely added to infant formulas. Excessive amounts of omega-6 polyunsaturated fatty acids and a very high omega-6/omega-3 ratio have been linked with pathogenesis (origination and development of diseases) (Zamora, 2005).

2.1.3. Differences in Fatty Acids Composition between Marine Fish and Freshwater Fish Oils

The fatty acid composition of freshwater fish is highly characterised by high level of n-6 polyunsaturated fatty acids especially linoleic as well as the arachidonic acid. In comparison with marine fish, freshwater fish generally contains very high levels of C18 PUFA, however, they also contains substantial amount of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Ackman, 1967; Cowey and Sargent, 1972; Bell *et al,* 2001; Steffens, 1997).

In comparison Steffens, (1997) further indicate that high levels of long-chain n-3 polyunsaturated fatty acids such as eicosapentaenoic acid and docosahexaenoic acid predominantly characterizes the lipids of marine fish species, with low levels of linoleic acid and linolenic acid.

2.2. FACTORS INFLUENCING FATTY ACIDS COMPOSITION IN FISH

The level of lipids in fish organisms varies widely, not only between different species, but also within different organisms of the same species depending on feeding grounds, water temperature and salinity, seasonal variations and other factors (Schwalme *et al.*, 1993). In some species, sex, maturity and age contribute to significant difference (Rasoarahona *et al.,* 2005). Because of this, fatty acids composition on fish of the same species tends to differ from one water course to another depending on these factors.

2.2.1. Effects of Diet

Studies indicate that the type of feeds fed to fish population can have a greater influence on the fatty acid composition of the fish (Steffens, 1997). Fresh water fish contains high levels of n-6 PUFA, but however, it also contains substantial amount of EPA and DHA (Ackman, 1967; Cowey and Sargent, 1972; Castell, 1979; Steffens, 1989 as cited by Steffens, 1997), this reflect to large extent that of its diet (Cowey, 1993; Watanabe, 1982). Experimental study by Boggio *et al.,* (1985), found that feeding fish of diet containing high levels of n-3 fatty acids markedly increases the proportion of these acids in the tissues of the fish and thereby making them to contain high level of n-3 PUFA.

Table 2. FAs composition (%) of muscle total lipids of marketable carp (1000g) from natural waters (unfed) and from aquaculture (supplementary feeding with wheat and rye)

Fatty acid	**Carp from natural waters (unfed)**	**Carp from fishpond (suppl. Fed with wheat and rye)**
14:0	0.7	1.0
16:0	12.9	18.6
16:1 n-7	7.3	8.0
18:0	5.1	6.2
18:1 n-9	14.8	43.3
18:2 n-6	7.9	6.0
18:3 n-3	2.9	
18:3 n-3 and 20:1 n-9		3.4
20:1 n-9	2.0	
20:2 n-6	0.9	-
20:3 n-9	-	1.7
20:3 n-6	0.9	0.6
20:4 n-6	8.6	3.2
20:4 n-3	1.0	-
20:5 n-3	8.8	1.4
22:4 n-6	1.8	-
22:5 n-6	1.6	0.9
22:5 n-3	4.4	0.8
22:6 n-3	6.5	3.1

Source: Csengeri and Farkas, (1993).

The table above shows some of the most important observations obtained in a study conducted in Hungary with myristic acid being the acid consisting of high proportion.

The marine weeds and other phytoplanktons are characterised by high level of long-chain n-3 polyunsaturated fatty acids. This turns to reach marine animals (fish) through food chains and food webs, and thus makes them also to be good sources of n-3 PUFA predominantly eicosapentaenoic acid (20:5 n-3) and docosahexaenoic acid (22:6 n-3) (Stansby *et al.*, 1990). Marine fish depend on a dietary supply of polyunsaturated n-3 fatty acids for rapid growth, since in many cases they have only a very limited desaturase activity (Stansby *et al*., 1967; Walker, 1997).

Most studies have come to a similar conclusion that the fatty acids composition of lipids on fish resembles its dietary composition. A study conducted on catfish by Worthington and Lovell, (1973) as cited by Steffens, (1997) found that higher level of concentrations of EPA and DHA were put high up in the carcass of catfish kept on diet containing 7.3% menhaden oil instead of 7.3% soybean oil.

2.2.2. Temperature

Environmental conditions have a greater influence on the fatty acids composition in both freshwater and marine waters. Understanding this has been a matter of concern by many scientists and researchers. The roles of environmental and physiological factors underlying these changes were evaluated by Schwalme *et al.*, (1993). Studies have shown that, fish organisms of the same species found in similar environment but in different temperature ranges tend to have differences in terms of their fatty acids composition and concentrations.

Fish which are found in warm water ecosystems have low concentrations of polyunsaturated fatty acids than those which are found in cold water environments. Fish need high PUFA to provide tolerance to low water temperatures, and this causes fish in cold environments to have a high PUFA content than those in warm waters such as tropical areas (Rasoarahona *et al.*, 2005). There are a number of experiments demonstrating the effect of environmental temperature on fatty acid composition of aquatic animals (Rasoarahona *et al.*, 2005). The general trend toward higher content of long chain PUFA at lower temperatures is quite clear. The Σn-6/ Σn-3 ratio decreases with a decrease in temperature (Meed and Kayama, 1967). If the trends in fatty acid composition can be taken as clues to the essential fatty acids requirements of fish, the Σn-3 requirement would be greater for fish raised at lower temperatures. Fish raised in warmer waters, such as common carp, channel catfish, and tilapia may do better with a mixture of Σn-6 and Σn-3 fatty acids (Meed and Kayama, 1967; Cowey and Sargent, 1977).

2.2.3. Seasonal Variations

Seasonal changes also have an influence on fatty acids composition of fish. Seasonal changes have been observed in total lipid quantity of various aquatic organisms specifically on fish.

A study conducted on three tilapia species (*Oreochromis niloticus*, Oreochromis *macrochir* and *tilapia rendalli)* during two seasons (autumn and winter), shows that the Σn-3/ Σn-6 ratio varies, for each species according to season and species. The results indicate that the Σn-3 turns to be lower in autumn and rising up in winter. This was

explained by a decreasing value of the Σn-3 PUFA total value from 12% to 7.3% for *Oreochromis* niloticus, 13,9% to 7.7% for *tilapia rendalli* and 14.4% to 8.3% for *Oreochromis macrochir* and an increase in Σn-6 PUFA total value from 13.3% to 15.6%, 10.7% to 13.0% and 8.7% to 16.0% respectively (Rasoarahona *et al.*, 2005). On this study, the influence of changes on feeding conditions based on seasonal changes was held constant.

2.2.4. Species Type

Studies show that, the difference in species type have an influence on the fatty acid composition of fish within the same geographical location (even within the same water body). This is highly influenced by the level of fatty acids biosynthesis and metabolism in the fish's body. Moreover, the level of PUFA required by the fish species to tolerate the environmental conditions also has an influence (Halver, 2000; Quillet *et al*., 2005). In the publication by Pratt and Matthews (2004) on super foods, Salmon fish was rated to be one of the richest in fatty acids composition specifically in polyunsaturated ones (Pratt and Matthews, 2004).

2.3. ESSENTIAL FATTY ACIDS AND HUMAN NUTRITION

There is a wide agreement that dietary fat in relation to both quality and quantity can influence early development (i.e. during pregnancy and in the neonate) of coronary heart disease, plasma lipids, atherosclerosis, thrombogenesis, blood pressure, cardiac arrhythmias, cancer, diabates, skin diseases and disorders of the immune system. As a consequence of many investigations dietary recommendations have been made for patients with these conditions and for the general population (Gunstone, 1996).

Overproduction of eicosanoids (prostaglandins, thromboxanes and leukotriens), which are hormone-like compounds produced from arachidonic acid have been linked to high health disorders (Burr and Burr, 1930). Consumption of food (fish) containing high level of omega-3 type of fatty acids especially EPA and DHA can slow down the production of the eicosanoids and thus preventing various health disorders in humans (British Nutrition Foundation, 1992).

The beneficial effects of various essential fatty acids in human nutrition are of considerable interest. Therefore, as a result many long-term studies of the beneficial effects of eating fish with regard to the incident of various disease attacks were conducted (Stansby, 1990).

Studies show that consumption of fish has been associated with a low risk of cardiovascular diseases due to the presence of Omega-3 fatty acid content (Mendez *et al.,* 1996). Inflammation (cause swelling in a part of the body) has emerged as playing a central role in many prevalent diseases not previously believed to involve inflammation, including Alzheimer's disease and cancer. Others include atherosclerosis, arthritis and periodental diseases (Dyer *et al,* 2002; Serhan *et al.*, 2004). Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are precursors for the biosynthesis of eicosanoids and docosanoids, with multiple health benefits (Uauy and Valenzuela, 1992). Danish research on Eskimo food in Greenland suggests that low risk of heart attack in people located in such areas might be linked to the type of food eaten there, which is highly characterised by fish consumption (Bang and Dyerberg, 1972).

Both n-6 and n-3 series FAs have the ability to affect the fluidity, flexibility and permeability of the membranes, they are essential in maintaining impermeability barrier of the skin and are involved in cholesterol transport and metabolism (Steffens, 1997).

2.3.1. Cardiovascular Disease

Ackman (1989) indicate that, Coronary heart disease is the leading cause of death in the United States and in industrialised Western countries. Many reports have appeared since the epidemiological evidence by Bang and Dyerberg (1972) called attention to the low mortality from coronary heart diseases (CHD) among the Greenland Eskimos, which they attributed to potential antiatherosclerotic effects of the diet high in oil of marine fishes. Gutierrez., and de Silva (1993) released a major study dealing with the safety of n-3 and n-6 PUFAs. Based on numerous reports published in the medical literature, Gutierrez., and de Silva (1993) concludes that a high intake of n-3 PUFAs reduces the risk for cardiovascular disease and heart attack and is entirely safe at least up to a level corresponding to 10% of the daily calorie intake. Dietary antioxidants (especially vitamin E) need to be increased if the PUFA intake is increased. Connor, (2000) cited evidence that eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which are found in fish oils are helpful in the prevention and treatment of cardiovascular diseases. DHA is a vital component of the phospholipids in cell membranes throughout the body, but it is particularly abundant in the brain, retina, and sperm.

Fish oils have been found particularly effective in preventing arrhythmias and sudden death from cardiac arrest. Several studies have shown that people who eat fish at least once each week can reduce their risk of sudden cardiac death by 50-70 per cent. EPA has

been found to inhibit blood clotting. While both EPA and DHA contained in fish oils inhibit the development of atherosclerosis (Connor, 2000). Fish oil has important metabolic effects, such as inhibiting platelet aggregation and lowering serum triglyceride levels, which could play a role in the prevention of cardiovascular diseases (Kontogiannea *et al.*, 2000).

2.3.2. Effects on Cancer

Cancer is second only to heart diseases as the leading cause of death in many countries (British Nutrition Foundation, 1988) and South Africa is no exception. The exact cause of most cancers is not known, thus many studies have concentrated on dietary components as initiators or promoters of carcinogenesis (British Nutrition Foundation, 1988).

Many studies have suggested that dietary lipids may be involved in the development of cancer at several sites (British Nutrition Foundation, 1992). Strong evidence by Gunstone *et al*., (1978) and Harris *et al.*, (1997), indicates that high fat diets containing maize oil, sunflower oil, lard or beef tallow increased the incidence of chemically induced colonic tumour (cancer) in populations which consume diets high in such kinds of fats than those who consume less (Gunstone *et al*., 1978; Harris *et al.*, 1997) Low incidence of breast cancer was observed in Greenland and Japan (Katikou *et al.,*2001; Karmeli, 1987), this primarily associated with their traditional diet which contains primarily fish and some seaweed so that the principal components are long chain polyunsaturated fatty acids (PUFA) of the n-3 series. However due to modernisation there has been a change in dietary habits, which had led to an increased consumption of saturated fatty acids and PUFA of n-6 series (British Nutrition Foundation, 1992). In addition, unsaturated fatty

acid and n-6 series particularly linoleic acids have been shown to promote pancreatic carcinogenesis in experimental animals (Jensen *et al.*, 1985; Karmeli, 1987).

Epidemiological, experimental, and mechanistic data implicate n-6 PUFA as an important promotional factor in carcinogenesis. However studies suggest that long chain n-3 PUFAs (specifically fish oil) have an antipromotional effect on the development and progression of wide range of human cancer (Brondz *et al*., 1983). The antitumor effect of EPA is mainly related to its suppression of cell proliferation (de Deckere, 1999). Docosahexaenoic acid (DHA) supplementation in the form of fish oil, has found to have the ability to suppress different types of cancer tumour growth, development and metastasis (Kontogiannea, *et al,* 2000). This clearly indicates that fish oil plays a role in human health by reducing the risk of being affected by cancer and other related diseases.

2.3.3. Immune System/ Nervous System Functioning

Animal studies conducted at Oxford University (1996) showed that an increase in fat intake can decrease the number of natural killer (NK) cells found in the blood spleen, which are an integral part of the natural immune response to virus infections. It further indicates that fish oil significantly reduces the NK cells activity in healthy humans. NK cells lowers the ability of the animal's (human) immune and nervous system to function effectively, and as a result they become easily prone to greater risk of diseases (Thies *et al.*, 2001; Thais and Stahl, 1987). British study on natural killer cells conducted on 48 men and women aged between 55 to 75 years, fed with supplements of different types of food rich in different types of fatty acids found that, there was no change in the NK cells activity except in the group taking fish oil (Thies *et al.*, 2001; Popeia *et al*., 2002).

Diets that are low in n-3 fatty acids lead to low brain DHA and thus lead to losses in nervous system function (Salem, *et al*, 1999). In summary, balance in omega-6 by omega-3 fatty acids and in particular, DHA and EPA from fish oils, is essential for human development and in the prevention and amelioration of many common disorders.

2.3.4. Fish Oils and Blood Pressure

Numerous studies have investigated the relationship between dietary fats and blood pressure. Two large European studies conducted by Tocher and Sargent, (1990) as cited by Coulston *et al.,* (2001) show a positive relationship between saturated fatty acids and blood pressure. Clinical intervention trials the results shown that lowering total fat intake from 38-40% to 20-25% of energy and increasing the polyunsaturated fatty acids found in fish and fish oils reduce blood pressure (Williams, 1997; Mensink, *et al.*, 1988). Several studies have been undertaken to determine if supplementation of either fish or fish oils lowers blood pressure. Findings of these studies suggest that blood pressure lowering affect of fish oils may be strongest in individuals with hypertension and in those with clinical atherosclerotic disease or hypercholesterolemia (Morris *et al.,* 1993; Higgs, 1986).

2.3.5. Inflammatory Diseases

The amount and nature of the fatty acid consumed in the diet may play a role in the management of several inflammatory diseases, including inflammatory bowel (Kris-therton, 2000). Consumption of marine foods consisting of precursors n-3 fatty acids is now receiving more attention because of their potential to impact several forms of inflammatory and chronic diseases (Coulston *et al.*, 2001; Stansby, 1990).

Because fish and fish oils contains high level of n-6 fatty acids, their consumption will result in adipose lipid and cell membrane phospholipids that are high in n-6 fatty acyl groups. This tends to activate macrophages, neutrophils, lymphocytes, and other cells, which release membrane lipids that are precursors of potent regulators of the inflammatory response (Coulston *et al.*, 2001; Stansby, 1990). Their release and synthesis lead to recruitment of leukocytes and fibroblasts, which alter permeability of tissues, and increase adherence of leukocytes and platelets to increase generation of harmful oxygen metabolites, and increase synthesis of pro-inflammatory eicosanoids which are products of metabolism of polyunsaturated fatty acids of n-6 series (Coulston *et al.*, 2001).

2.4. OTHER FINDINGS ON RELATIONS BETWEEN POLYUNSATURATED FATTY ACID AND VARIOUS DISEASES

In relation to dietary effects of fish and fish oils to human nutrition, current research has begun to clarify these eicosanoids effects and their relationship to health and diseases, and to genetic variations. On the basis of results thus far, successful applications to major diseases of our times affecting vascular function, inflammatory reactions and immune response are being developed. Such results are definitely known to be important to health professionals and the public alike (Christie, 1997).

Studies indicate that in South Africa one in three men and one in four women usually suffer a stroke or heart attack. One in six men and one in seven women will develop cancer, wherein the most frequent types are breast, lung and prostate cancer. It further indicates that about one in twelve South Africans is diabetic, with 40% affected by high blood pressure (Menong as cited by Dlale, 2005).

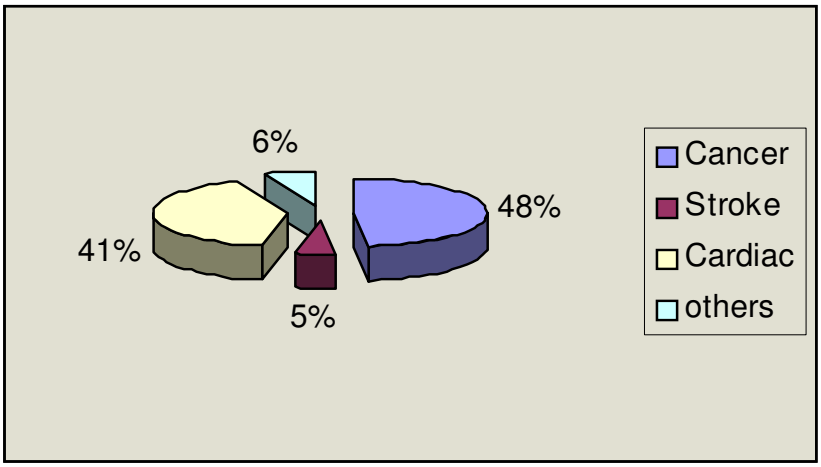


Figure 4. Estimated statistical representations of different diseases in South Africa (Menong as cited by Dlale, 2005).

The statistics above clearly indicates that there is a great need for supplementation of fish and/ or fish oils (specifically Omega-3 PUFA) in dietary composition of South African population in their daily diet, so as to reduce the level of various diseases as indicated by number of researchers on lipid/ or fatty acid chemistry, nutrition and diet therapy.

2.5. SAMPLE PREPARATION AND ANALYSIS

2.5.1. Sample Extraction Methods

Complex biological samples such as those of plants and animal fatty tissues are comprised of complicated matrices of high organic load, and this can not be directly injected into a separation system. To overcome this problem, a series of off-line sample

extraction and treatment is required prior to derivatization or analysis of the fatty acids composition of the animal fatty tissues (Theodoridis *et al.*, 2006).

Quantitative isolation of lipids from tissues in their native state and free of non-lipid contaminants must be accomplished before analysis is attempted (Christie, 1993). Sample extraction is aimed at isolating and concentrating compounds of interest from a sample matrix and transfers them into liquid solvent before analysis (Eskilsson, 2003). In lipid chemistry, this is aimed at the improvement of lipid recovery from any kind of organisms, tissues or cell types, to remove potential interferences from the matrix (Smith, 2003). Since the greatest improvement of the extraction of polar lipids from animal tissues was made by Folch in 1957, many extraction methods which are faster, less man-power demanding, and provide more efficient, reliable and more simpler analyses have been developed (Brondz, 2002; Turner, 2006).

2.5.1.1. Conventional solvent extraction

Solvent extraction is performed by vigorous homogenization or shaking of sample and the extracting organic solvent(s) so as to create the possibility for a large contact area between the solvent and the sample. During the process the analytes are transferred from either the aqueous or solid phase to the organic phase. Efficient phase separation can be achieved by allowing the extraction system to stand before collecting the organic phase. During extraction, the distribution coefficient can be increased by addition of salt to the aqueous or solid phase, which will eliminate analytes from this phase to the organic solvent(s).

Many different solvents or solvent mixtures have been tried for extraction of fatty acids from both bacterial, plants and animal cells. For the solvent(s) to be effective, they should be able to overcome the strong forces of association between tissue lipids and other cellular constituents such as protein and polysaccharides (Christie, 1993). Lipids lacking polar groups such as cholesterol esters or triacylglycerols, are very soluble in hydrocarbons such as hexane, toluene, and also in moderately polar solvents such as diethyl ether or chloroform, and this makes them to be easily extracted (Zahler and Niggli, 1977).

2.5.1.1.1. Folch extraction

Although there have been developments of several modern techniques for extraction process, folch extraction method have remained the method of choice on many lipids extraction and analysis (Kang and Wang, 2005). It is the widely used procedure on extraction of fatty acids from animal origin (Christie, 1993). Folch extraction method involves homogenization of the tissue sample in chloroform/methanol (ratio 2:1) as the extraction solution, to a final volume 20 times the volume of the sample (1 g in 20 ml of solvent mixture) (Folch *et al*., 1957). Christie, (1993) indicates that most lipids scientists have accepted that a mixture of chloroform: methanol as an extracting solvent will thoroughly extract lipids from animal, plants and bacterial tissue than any other solvent or solvent mixtures.

The extracts are filtered after homogenization and washed with a five fold volume of water. This is critical to remove non-lipid components of the extracts, with a concomitant loss of about 0.3% of the tissue lipids in the case of white matter and about 0.6% in the

case of grey matter (Folch *et al.,* 1957). This loss can be reduced by addition of a certain amount of mineral salts. This salt alters the distribution of the lipids and practically eliminates them from the upper phase (Gunstone, 1996; Varljen et al., 2003).

The mixture is allowed to separate; the upper phase contains all of the non-lipid substances, most of the stradin, and only negligible amount of the other lipids. The lower part contains all the tissue lipids except stradin. The volume of the upper and lower phases is 40 and 60% (Folch, *et al.*, 1957). As a result of washing, almost all gangliosides that may have been present also partition to the upper phase and therefore can be lost from analysis. However, these are minor compounds of no critical value to fatty acids analysis since their analysis is specialized. If there is a need to analyse this type of lipids component gangliosides can be recovered from the Folch upper phase by dialysis followed by lyophilization (Christie, 1993).

The main advantage of this method is that it can be run on any scale that is technically feasible. Chloroform has the ability to associate with water molecules, this is presumably due to its weak hydrogen bonds and this is one of its key properties. The Folch extraction method has become a standard method against which other methods are judged.

Although the Folch extraction method (chloroform: methanol) has become the method of choice on fatty acids extraction and analysis. Its toxicity and character as a potent irritant to skin have inspired scientists to look for other methods with the use of different solvents or solvent mixtures. Carlson, (1985) and Ackman, (1980), used dichloromethane-methanol mixture (2:1, v/v) in extraction of plasma and liver in one study, this was found to give results identical to chloroform: methanol and low toxicity.

Propan-2-ol: hexane (3:2, v/v) have also bee used on extraction of fatty acids from animal tissues, this was used mainly due to its low toxicity and good extractive properties. Better recoveries of prostaglandins were reported through the use of this method than in chloroform: methanol (Christie, 1993). Aras *et al.,* (2003), used 1.2M NAOH in 50% of aqueous methanol for the extraction of fatty acids from different tissues of mature trout. Good recovery of fatty acids was observed. although this was not enough when compared with other similar studies with the use of chloroform: methanol (Folch extraction method).

2.5.1.1.2. The Blight and Dyer method

This is the second most commonly used procedure for lipids extraction for plants, animal and bacterial tissues (Christie, 1993). In this method, both extraction and partitioning are simultaneous. The lipids are isolated between two liquids. It was developed as an economic method for extraction of fatty acids from large volume of wet tissue, specifically from frozen fish with the minimum volume of solvent (Blight and Dyer, 1959).

Just like the Folch extraction method, the Blight and Dyer method involves the use of chloroform: methanol (1:2, v/v) with the homogenization of the sample tissue with the extraction solvent or solvent mixture. It is an improvement of the Folch extraction method an important improvement being the replacement of water by 1M NaCL so as to block the binding of some acidic lipids to denatured lipids by producing a biphasic mixture (Christie, 1993).

If applied correctly, this method gives good recoveries of the more important fatty acids. In their study on lipid content and fatty acid composition of fillets on fish, Méndez *et al.* (1996), employed the Blight and Dyer method for extraction. The findings have shown a greater recovery and identification of high concentrations of most important fatty acids of both medium and long chain (Méndez *et al.*, 1996).

2.5.1.2. Microwave-assisted extraction

Microwave assisted extraction (MAE) results from an improvement of microwave digestion method (Eskilsson, 2003). It is an extraction technique which is based on heating of an organic solvent. On this extraction method, a sample and an appropriate solvent mixture are put in the vessel which is then heated by microwave (Turner, 2006).

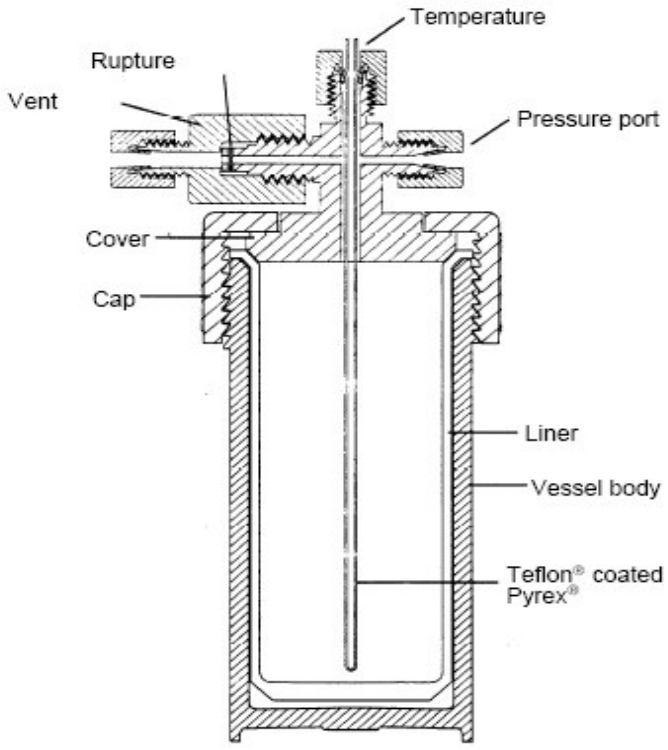


Figure 5. Schematic diagram of a microwave assisted extraction vessel (adopted: Wang, 1997).

MAE provides a technique whereby intact organic compounds can be extracted more rapidly with similar or better recoveries when compared to the conventional extraction process. The ability of solvents to transform electromagnetic energy into heat allows the selection of target specific molecules and deposition of the energy into the whole of the sample, without the usual limitations of heat conduction and convection. The solvent used on extraction is very important since it determines the speed of heating and the selectivity of extraction to obtain an optimal extraction process (Wang, 1997). The heating speed is proportional to the dipole or dielectric constant of the solvent. Many organic solvents are characterised by low dielectric constant which therefore leads to long heating and extraction times. Due to a high dielectric constant, water is easily heated by the microwave assisted extraction technique (Wang, 1997).

Table 3. Dielectric constant of commonly used solvents

SOLVENTS	**DIELECTRIC CONSTANT(S)**
Water	80.1
Ethanol	25.3
Acetone	21.01
Methylene chloride	8.93
Benzene	2.2825
Chloroform	2.2379
Hexane	1.8865

Source: Wang, 1997

The use of organic solvents in microwave-assisted applications like solvent extraction or synthesis requires some knowledge about the properties of the solvents applied (AntonPaar, 2006). When selecting a solvent, consideration should be given to the microwave-absorbing properties of the solvent, the interaction of the solvent with the matrix and the analyte solubility in the solvent. In highly microwave absorbing samples, efficient extraction can be performed by using pure, microwave transparent solvents (e.g. hexane) (Eskilsson, 2003). The matrix moisture content in the sample improves extraction recoveries, the effect of this depends on the type of extraction solvent used. The available moisture also affects the microwave absorbing ability of a sample and hence contributes to the heating process (Kingston and Jassie, 1988).

Extractions are very different to acid decompositions; this is mainly because different measuring methods require different reagents. On acid decomposition the optical judgement of the degree of extraction is nearly impossible. Therefore, complete extraction of the analyte is only possible via several steps or extraction over a long period of time. To accelerate extraction through the microwave, the temperature is increased as much as possible. As a result this approach enables a considerably improved extraction of analytes in a closed system, e.g. compared to Soxhlet (AntonPaar, 2006).

Reports show that MAE has been utilized in digestion of food and agricultural samples. For other applications this has also been reported to be effective on extraction of organic compounds such as fatty tissues and other organic matter. Over the past decade, interest on the use of MAE has significantly increased. However, utilization of this technique in

food and feed applications is still very low. This might be due to the reason that MAE requires an extensive cleaning-up before any analysis (Eskilsson, 2003; Wang, 1997).

2.5.1.3. Pressurized fluid extraction (PFE)

Since the first article on PFE appeared in 1995, several environmental and biological applications were published (Richter *et al.,* 1995; Eskilsson, 2003). Pressurised fluid extraction is very similar to the Soxhlet extraction; the difference is that in PFE, solvents are used near their super critical region where they have high extraction properties. In that region, the high temperature enables high solubility and high diffusion rate of analytes in the solvent (Fawkes *et al.,* 1982; Turner *et al.,* 2001). Because of its selectivity, short extraction period and environmentally friendly, pressurised fluid extraction is regarded as a promising alternative technique to conventional solvent extraction methods (Hyötyläinen, 2006). This method permits high extraction efficiency with a low solvent volume (15-40 ml) and a short extraction time (15-20 min) with the same solvent employed in the Folch extraction.

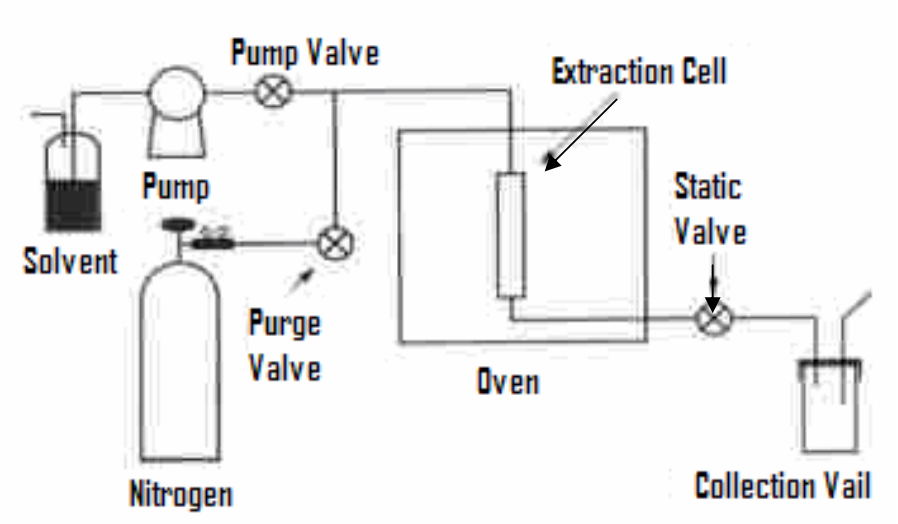


Figure 6. Pressurised fluid extraction technique (Turner, 2006)

The PFE device includes an extraction cell (1 up to 100 ml) maintained at a temperature between 80 and 200°C into which a solvent is pumped and maintained at 10-20 MPa for some minutes. Then, the extract is pushed into a collection vial by a second volume of solvent and finally the whole solvent is pushed with an inert gas flow (Turner, 2006).

For polar substrates, the presence of water can be beneficial since this might have a positive influence on recovery rate. However, for non polar substrate this is not the case. The presence of high water content on the sample might have adverse results in the extracting process thereby leading to a reduced yield due to the co-extraction of water (Francisco, 2002).

Since the invention of this extraction technique, many studies on the application of this techniques on extraction of various compounds including fatty acids in foods, lipids in corn, oats and various fatty tissues from plants and animal cells have been conducted (Ibańez *et al.,* 2006). Although the development of this technique has attracted many scientists for use on analysis of various compounds, its application on animal fatty tissues extraction is still very low.

2.5.1.4. Supercritical fluid extraction (SFE)

Supercritical fluid extraction and fractionation of natural matter is one of the early and most studied applications in the field of sample extraction and purification (Fifield, 1993). In the last 10 years, studies in the extraction of classical compounds like essential animal and seed oils from various sources with or without the addition of a co-solvent have been published. Supercritical extraction of antioxidants, pharmaceuticals, and pesticides has also been studied. The separation of liquid mixtures and the anti-solvent

extraction are other processes that can perform very interesting separations (Fifield, 1993; Reverchon and De Marco, 2006).

Several compounds have been examined as SFE solvents. For example, hydrocarbons such as hexane, pentane and butane, nitrous oxide, sulphur hexafluoride and fluorinated hydrocarbons (Smith, 1999). However, carbon dioxide (CO_2) is the most popular SFE solvent because it is safe, readily available and has a low cost. It allows supercritical operations at relatively low pressures and at near-room temperatures (Reverchon and De Marco, 2006). It is attractive also due to its high diffusivity combined with its high solvent strength (Turner, 2006; Rovessac and Rovessac, 1992).

During extraction, not only target organic compounds will be extracted, but also other undesired compounds have to be taken into consideration. In most cases it is not possible to avoid co-extraction of some compounds belonging to the same family. In order to reduce extraction of other unwanted compounds, fractional separation of the extracts is one of the major ways which can be used to improve the supercritical fluid extraction process selectivity (Reverchon and De Marco, 2006; Turner *et al.,* 2001).

For the supercritical extraction process, the selection of operating conditions depends on the sample matrix to be extracted. The operating temperature should be fixed in the vicinity of the critical point between 35°C and 60°C (Reverchon and De Marco, 2006). This is essential so as to prevent degradation of the analytes (Tissue, 1996). CO_2 flow, particle size of the matrix and duration of the extraction process also have a critical influence on the recovery rate (Smith, 1999).

In a study conducted by Eskilsson (2003) on the performance of SFE and PFE on extraction of oils and phenolic acids from various biological matrices, SFE has been found to be the technique of choice by many authors. This is mainly due to its high selective extraction, simplified by the fractionated extraction of different groups of polyaromatic hydrocarbons (Eskilsson, 2003; Ill'es *et al*., 1997). The method is very efficient for extraction of fatty acids from animal origin, however, problems associated with the use of this method is presented by hexane elimination after extraction and possible thermal degradation of the oil (Dauksas *et al.,* 2002; Somchit *et al.,* 1997). For extraction of essential oils, vegetable and animal oils, this approach has shown to be scientifically challenging. It is therefore critical to find applications that are industrially competitive with the traditional processes. However, in some cases its extraction efficiency of this technique has proved to be as good as the traditional organic solvent approach. The extraction and separation of phospholipids from tuna fish have been described using various concentrations of methanol in supercritical CO_2 and thus Good recovery of DHA-rich phospholipids were claimed in a series of industrial processes (Bhupesh, 1996).

2.5.2. Sample Storage, Autoxidation and Preservation of Lipids

Some of the major changes that occur during processing, distribution and final preparation of food are due to oxidation (Sant' Ana and Mancini-Filho, 2000). Oxidation of lipids initiates other changes which might therefore lead to alteration on fatty acids composition and quantity on fish.

Once fish samples have been collected, they should be individually stored or kept in a commercial polyethylene bags with a sealing clamp and maintained under dry ice condition (Sant' Ana and Mancini-Filho, 2000). Tissue samples removed from living organisms (e.g. Fish samples) should be extracted immediately after removal so that there is little opportunity for changes to occur to the lipid components. For tissues with high enzymatic activity such as plant and heart or brain tissues, rapid extraction is essential (Christie, 1993). When immediate extraction is not feasible, as a general rule tissue should be frozen as rapidly as possible either with dry ice or liquid nitrogen and stored in a sealed glass container at -20°C. In some cases storage temperature of as low as -60°C has been recommended (Christie, 1993; Brondz, 2002).

Similar precautions also need to be taken for storage of lipids after extraction from tissues. The principal danger is that, if not protected, polyunsaturated fatty acids will autoxidize very rapidly in air and sunlight and as a result this will lead to difficulties in obtaining an accurate analysis by chromatographic means. Autoxidation process involves attack by free radicals and is exacerbated by strong light and by metal ions which cause the reaction to proceeds autocatalytically (Christie, 1993). To prevent or eliminate autoxidation process, antioxidant such as tocopherol is added to afford some protection to the lipids extracts. Buckley and Morrissey (1992) indicate that because tocopherol is an integral part of the membrane, it consequently stabilises membrane lipids. Further antioxidants such as butylated hydroxytoluene (BHT) can be added to afford more protection. The main advantage of using this compound is that it does not interfere with chromatography, since it is very volatile and can be removed together with solvents when they are evaporated in the stream of nitrogen during pre-concentration; if it is not fully

taken out during evaporation, and based on the fact that it is non-polar, this antioxidant tends to elute at the solvent front, ahead of most lipids (Christie, 1993; Sant' Ana and Mancini-Filho, 2000).

To prevent fatty acids against various enzymatic activities, additional precautions which involve protection against oxygen by evacuation of gases from the storage bottle or the exchange of air for N_2, He or Ar can be applied (Brondz, 2002). During application of this, special precautions need to be taken to avoid contamination and the production of artefacts in handling of the samples.

2.5.3. Sample Derivatization

Direct analysis of fatty acids on the GC is problematic; this is because they have low volatility owing to polar groups which form strong intermolecular hydrogen bonding interaction (Braun, 1987). Unlike other compounds which are highly volatile, fatty acids are chemically modified by reacting with suitable reagents before any GC analysis. In this way, hydroxyl groups that form intermolecular interaction are blocked. This can be done in the off-line mode or simultaneously with liquid-liquid extraction (LLE) or solid phase extraction (SPE) (Tesarova and Pacakova, 1983). By modifying the functional groups, most acids can be well separated by GC. Several derivatization techniques have been developed for the analysis of medium and long chained fatty acids (Wang, 1997; Manning *et al*., 2006).

Usually, compounds of high polarity and low volatility tend to undergo adsorption during the chromatographic analysis. Extraction from aqueous phase into organic phase is also not easy because of their low partition coefficients into the latter (Faust, 1992).

Derivatization of fatty acids results in better separation on the GC columns as the ionisation of hydroxyl group is blocked, thus making derivatives to differ more in their physiochemical properties than the original fatty acids. A very important aspect of derivatization of fatty acids is the improvement of detection on chromatographic separation and analysis (Tesarova and Pacakova, 1983).

Despite the advantages of derivatization of fatty acids, this step can be an additional source of errors. Sterically hindered fatty acids may react incompletely and partial decomposition of derivatives may occur during their storage and GC analysis. Some reagents are also toxic, carcinogenic and explosive (Wang, 1997).

Different solvent or solvent mixtures have been tried by different researchers for Derivatization of fatty acid extracts from various origins (Faust, 1992).

2.5.3.1. Acid-catalysed esterification and transesterification

Through this derivatization method, free fatty acids are esterified and *O*-acyl lipids transesterified by heating with excess anhydrous methanol in the presence of an acidic catalyst. During this stage, the presence of water might disturb the reaction from going to completion (Christie, 1993). Because of this, it is recommended that before derivatization, the aqueous part of the fatty acids extracts must be evaporated and the remaining oil dissolved in a small volume of an organic solvent.

The most common, widely used and most frequently cited acid derivatization reagent for the preparation of methyl esters is 5% (w/v) anhydrous hydrogen chloride in methanol. This is most often prepared by bubbling hydrogen chloride gas slowly into dry methanol.

Many studies on the stability of this reagent have been cited. This has shown that half the titratable acid was lost at room temperature in six weeks, presumably by reaction between the acid and methanol to give methyl chloride and water. Similar findings were obtained with 1-butanol-hydrogen chloride (Christie, 1993).

In this esterification procedure, lipid sample is dissolved in a 100-fold excess of the reagent and the solution is refluxed for about few hours (2-4 hours) or overnight at about 50°C. All fatty acids are esterified at approximately the same rate by methanolic hydrogen chloride, so there are unlikely to be differential losses of specific fatty acids during the esterification step (Christie, 1993).

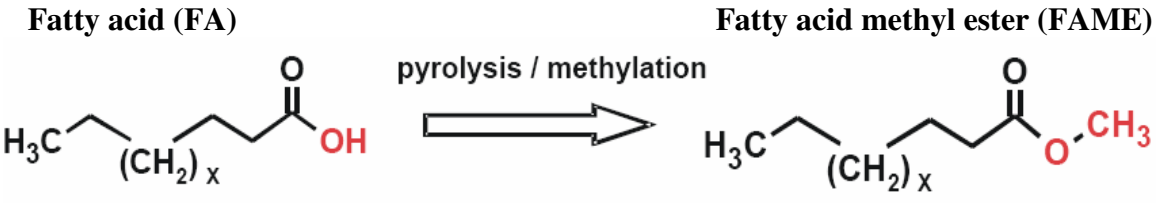


Figure 7. Schematic of general methylation reaction for free fatty acids (Gunstone, 1996).

Boron trifluoride in methanol 1: 2% (v/v) is another solvent mixture which has been highly researched and used on acidic esterification of fatty acids. It is regarded as a rapid means of esterifying free fatty acids (Christie, 1993). Good elution and detection levels were reported in the analysis of fish fatty acids esterified through the use of methanolic boron trifluoride (Zurain *et al.,* 2006).

2.5.3.2. Base-catalyzed transesterification

Fatty acid esters form an ionic intermediate which is transformed in the presence of a large excess of the alcohol into new ester derivatives. Free fatty acids are not subject to nucleophilic attack by alcohols or base and thus are not esterified in these conditions (Christie, 1993; Brondz, 2002). The main advantage of this derivatization technique is its derivatization speed and mild heating conditions. The use of a base-catalyzed derivatization technique is recommended for esterification of short-chain fatty acids or labile fatty acids. This is because since it uses mild heating conditions, incomplete esterification of some long chain fatty acids can result in major separation and identification problems in analysis of more complex lipids matrix.

The most useful transesterifying reagents are 1 to 2M Na or K methoxide in anhydrous methanol. The stability of this solution is high such that the solution can be stored for several months at 4℃ before the white precipitate of bicarbonate salt is formed (Brondz, 2002).

2.6. TECHNIQUES USED IN FATTY ACIDS ISOLATION AND IDENTIFICATION

Structures of known acids can be easily defined by comparison with a certified reference material (an authentic sample) or with compounds of related structure. This is generally made on the basis of its chromatographic behaviour (i.e. its retention time on one or more columns) (Gunstone, 1996).

Due to high quality and quantity of the outcomes as a result of high separation power of chromatographic techniques, these have made it to be an instrument of choice in lipids and fatty acid studies (Faust, 1992; Brondz, 2001). Based on the fact that the compounds are found in complex mixture, necessitating a separation step before detection in order to yield interferent free signals should be attained through chromatographic techniques (McCarthy, 2001).

2.6.1. Gas Chromatography (GC)

Gas chromatographic techniques use gas as the mobile phase (known as the carrier gas), with either solid (gas-solid chromatography) or a non-volatile liquid (gas-liquid chromatography) as the stationary phase (Faust, 1992). Although both techniques are used for lipids and fatty acids analysis, however, gas-liquid chromatography has been widely used (Braun, 1987). Braun (1987) further indicates that separation is primarily based upon the relative adsorption and solubility of the sample component on/ in the stationary phase.

For separation or identification, the sample must be either a gas or have an appreciable vapour pressure at the temperature of the column (Faust, 1992). The sample is injected into a small heating chamber where vaporisation takes place if necessary, since the sample should go into the column as a gas (AOAC International, 2000, McCarthy, 2001).

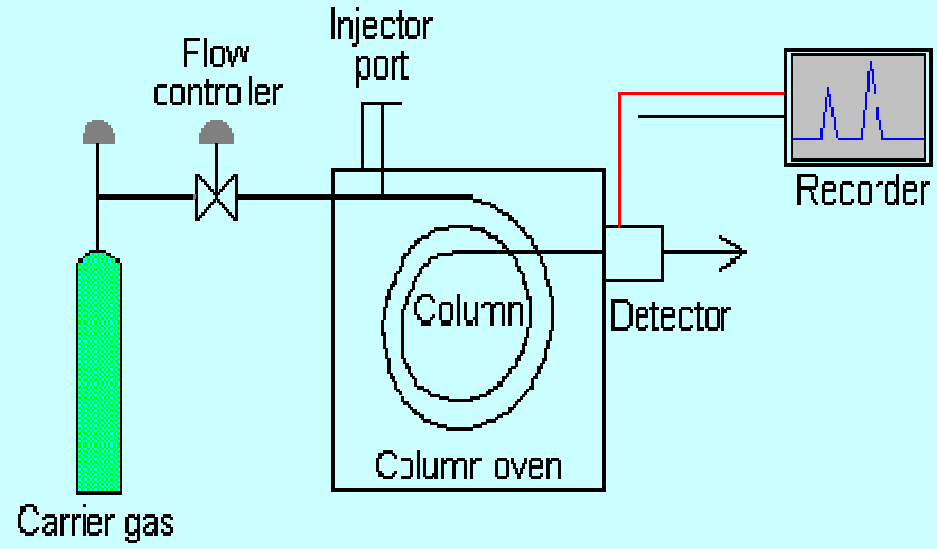


Figure 8. Schematic diagram of gas chromatography (McCarthy, 2001).

The main advantages that gas chromatography (GC) offers for separation of fatty acid compounds from a complex mixture are its separation efficiency, speed of analysis and many sensitive detectors to choose from (Braun, 1987; Erfanullah, 1998).

To improve separation and reduce tailing of the fatty acid, there is a need for derivatization. This is performed to increase the volatility of the lipid mixture and reduce its polarity (Brondz, 2002). There are many derivatization methods for fatty acids to enable analysis by GC (Brondz, 2002; McCarthy, 2001; Sant`Ana and Mancini-Filho, 1999). However, studies indicate that fatty acids are also suitable for analysis by GC without any derivatization as demonstrated by James and Martin, (1952).

Capillary columns with inner walls coated with thin film stationary phase (diethylene glycol, polysuccinate, OVI, polyglycol, etc) have been used for the analysis of the composition of mixtures of fatty acid methyl esters by GC (Plummer, 1987). Capillary

columns were found to be superior to the packed columns and offer high resolution and increased sensitivity of these compounds (Span and Wagner, 1996).

2.6.1.1. Detection

Sensitivity of GC detectors is quite high. The electron capture detector (ECD) and flame ionisation detector (FID) are commonly used in analysis of fatty acid composition of lipids (Brondz, 2002). FID is particularly useful for detecting organic compounds and is by far the most common GC detection system (Faust, 1992). Substances are pyrolised in a hydrogen-oxygen flame and produce ions in the process. Through FID, detection of fatty acids is quantifiable and only formic acid is difficult to analyse due to low sensitivity (Brondz, 2002).

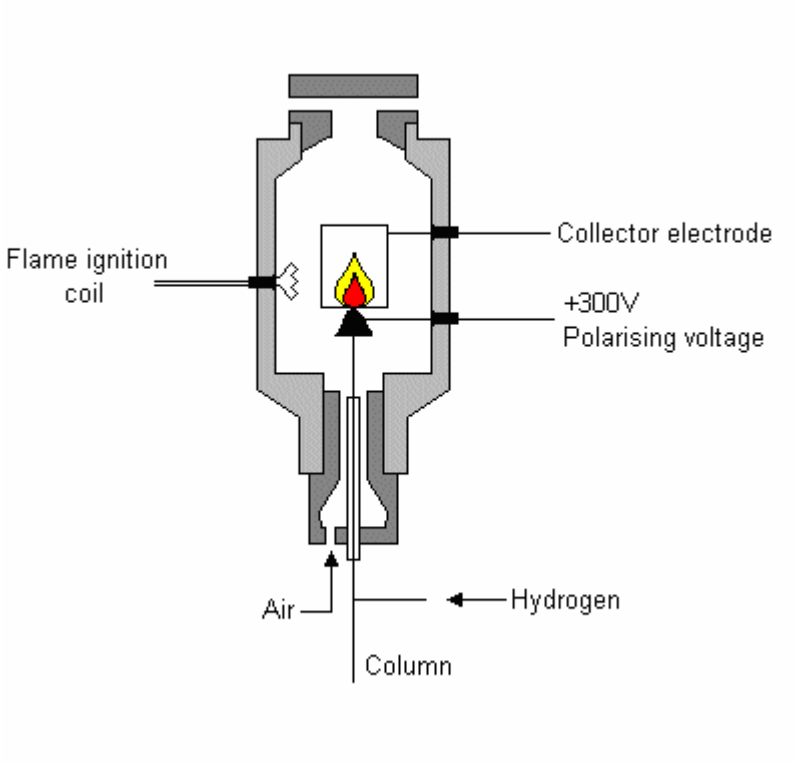


Figure 9. Schematic diagram of flame ionisation detector used on gas chromatography (McCarthy, 2001).

Although sensitivity of the electron capture detector is very high for FAs analysis, this requires sufficient clean up since it is not a very selective detector. The ECD is based on measuring the decrease in the background current when electroactive molecules pass through it (Gutnikov and Streng, 1991).

2.6.2. Liquid Chromatography

Liquid chromatography uses liquids as a mobile phase, the stationary phases used are almost exclusively of the octadecylsilyl ("ODS") type, with an octyl phase being recommended occasionally as an alternative. The mobile phase is either acetonitrile (mainly) or methanol containing some water (Faust, 1992).

The efficiency of a separation increases if the particles in the stationary phase are made smaller. This is because the solute can equilibrate more rapidly between the two phases (Faust, 1992). Separation is based upon the relative abilities of the stationary phase to trap analytes and allow them to elute over time. As molecules of the sample components enter the column, they can either be adsorbed on the stationary phase or remain in the mobile phase. A strongly adsorbed sample component spends a greater proportion of its time within the column on the stationary phase than does a weakly adsorbed component. Consequently, the retention time or volume increases as the amount of adsorption on the stationary phase increases (Braun, 1987).

High performance liquid chromatography (HPLC) on columns of silica gel can be used for the analysis or isolation of fatty acids with polar functional groups, especially oxygenated moieties such as hydroperoxides. With care, isomers differing in the position of hydroperoxy or hydroxyl groups on an aliphatic chain can be separated (Christie,

1997). In reversed-phase HPLC, fatty acids are separated by both chain length and degree of unsaturation. The first double bond reduces the effective chain length by a little less than 2 carbon units. Christie (1997) indicates that an 18:1 fatty acid elutes just after C16:0. The second and further double bonds have smaller effects on retention so an C18:3 fatty acid elutes just before C14:0.

However, few studies on lipid chemistry have shown an interest in the use of HPLC on the analysis of fatty acid composition. Thus, many studies have used gas chromatography as an instrument of choice on lipid analysis. However Gunstone (1996) indicates that, good results are usually obtained with HPLC using a reverse-phase system on the analysis of fatty acids composition of fish oils. Hirsch and Ellingboe as cited by Brondz (2002) described the use of reverse -phase chromatography on the analysis of fatty acids. Acetic acid (ethanoic acid) CH_3COOH, known from ancient times can now be analysed successfully by HPLC (Bevilacqua and Califano; Chen *et al.,* as cited by Brondz, 2002).

If free fatty acids are analysed, a little acetic acid can be added to ensure sharp peaks. These solvents are transported to UV light at 205 to 210 nm, so that UV detection at such wavelengths can be used. However, derivatives of fatty acids such as phenacyl or related derivatives tend to increase sensitivity of the detector at high wave lengths (Christie, 1997).

Detection of underivatized fatty acids using traditional HPLC (UV) methodology is neither sensitive nor selective because these compounds generally do not contain suitable chromatophores. A number of derivatization methods have been developed, but this often increases analysis time and complicates the method (Alltech Associates, 1999).

Evaporative light-scattering detection (ELSD) can be used in fatty acid analysis. This is because it offers several advantages over traditional techniques for analysis of fatty acids. Through this technique, there is no need for post column derivatization and this greatly minimises the sample preparation time and removes complications associated with detection in the UV or lower range.

2.6.2.1. Detection

2.6.2.1.1. UV-Visible detector in liquid chromatography

The UV-Visible detector offers greater advantages on detection of complex fatty acids separated through liquid chromatographic techniques as these types of compounds can be absorbed in the UV region (Bronz, 2002). The use of photo diode array detectors is even more advantageous as it allows detection of each compound at the optimum wavelength (Böhm *et al.,* 1989). This makes it easier to detect and identify fatty acids through the use of high performance liquid chromatography.

Because UV detection may not be sufficiently sensitive on direct analysis of unconditioned (underivatized) fatty acid compounds, derivatization of fatty acids into other easily detected fat derivatives need to be done (Bronz, 2002). Determination of FAs such as phenacyl esters is a well established procedure. Phenacylbromide (2-bromoacetophenone) (PB) and other PBs are among other reagents used for FFAs. The derivatization of FAs to hydroxamic acids for HPLC analysis is an example of the use of non-phenacyl derivatives (Bougnoux, 1999).

2.6.3. Thin Layer Chromatographic Technique

Separation in ``good old´´ thin layer chromatography (TLC) is based on the interaction of sample molecules between the adsorbent covered plate (stationary phase) and the solvent flowing (mobile phase) up the plate by capillary action. Detection and quantification is achieved by spraying the dried plate with a chromatographic reagent to provide a visible spot for each separated component. Fatty acids absorbed in the UV region are capable of forming many coloured derivatives making them suitable by TLC separation and analysis (Gunstone, 1996).

The main advantages of TLC are that it is simple and offers a means for avoiding extensive sample clean up with selective post chromatographic derivatization of these compounds. TLC however, has a number of disadvantages that have resulted in it being surpassed by GC and LC. Its disadvantages are that the method can not easily be automated, it is not easy to quantify the analytes and reproducibility is marginal, the separation times are often longer and poorer than GC and LC (Gunstone, 1996). Many researchers have reported the use of TLC on fatty acids analysis as a means of fatty acids identification.

2.6.4. Gas Chromatography-Mass Spectrometry

Gas chromatography and mass spectrometry (GC-MS) make a good combination in chemical analysis for separation of compounds into their various components. This has opened a wide field of application in analysis of lipids. Since this invasion, a great many reports have been presented about analysis of fatty acids from lipids matrix. For determination of double bonds on fatty acids, this combination is highly used.

Dyer *et al*., (2002) and Phleger *et al.,* (1997), reported the use of GC-MS on separation of fatty acids from various plants and animal origin. Results obtained have shown some major improvements on detection and identification of fatty acids.

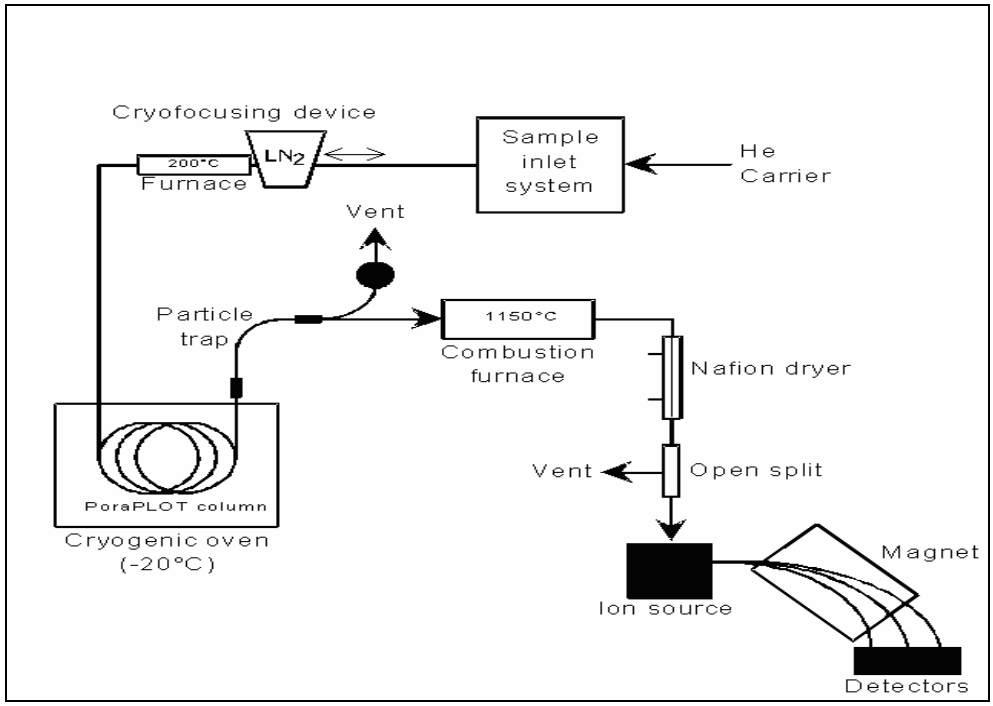


Figure 10. Gas chromatography-Mass spectrometry (Bell *et al.,* 2003)

2.7. SOURCES OF ERRORS IN FATTY ACIDS ANALYSIS

Sample preparation is probably the most important determinant of the quality of data obtained on chemical analysis (Connolly, 2005). Extraction of fatty acids from their natural complex matrix is problematic and very laborious. This requires more time and precautional measures to be undertaken. During this sample preparation stage, errors can be introduced thereby leading to poor and incorrect results.

When chloroform-methanol or any alcoholic extracts that contain lipids are heated or stored for long periods in the presence of small amount of sodium carbonate or bicarbonate of tissue origin, base-catalysed esterification can occur. Poor selection of extraction solvent(s) for use on fatty acids can lead to generation of artefacts which can therefore result in poor separation and identification of fatty acids (Levine, 1997). Very short chain fatty acids (length < C12) require special analytical methods. Their quantities can vary greatly due to random losses during the extraction procedure (Gopakumar, 1972; Grunger, 1964).

Frequently, fatty acids and leftover junk from a previous column injection might elute with another sample, creating spurious (false) peaks. This happens usually when elution from the previous runs were not complete. In sample analysis, particular attention to minimize these errors needs to be taken. Careful evaluation of the chromatogram and test results helps to identify these errors. One approach to prevent these spurious peaks is to clean the column after every sample analysis. The use of high amount or concentration of internal standard can cause this to remain or to be adsorbed in the column for a while before complete removal, and thus finally appear as small peaks specifically in subsequent sample injections that did not use such an internal standard (Gopakumar, 1972; Grunger, 1964).

Besides fatty acids, there might be some other substances that are measured during separation of fatty acids. Many of these substances are unknown. Some are by-products of oxidation of cholesterol and fatty acids, in part caused by the body and in part caused by the process of measuring or separation of fatty acids (Gopakumar, 1972; Grunger,

1964; Tesarova and Pacakova, 1983). A huge number of oxidation products of cholesterol and fatty acids appear on the chromatogram, usually close to the location of very long chain fatty acids such as 24:0, 24:1 and others. These fatty acids are in very small amounts and any junk left in the column or oxidation by-products appear as a larger peak. Poor storage and exposure of fatty acids to sunlight also have an important influence on decomposition and loss of some of the volatile fatty acids compounds (Levine, 1997).

In fatty acids analysis, proper purification and cleaning of fatty acids during the preparation stage is used as one major strategy used and highly effective on lipid chemistry for elimination of other non-essential substances which are of no value on lipid analysis or non fatty acids resulted from different stages of sample preparation.

CHAPTER 3: RESEARCH METHODOLOGY

3.1. REAGENTS/ CHEMICALS

All reagents used were of analytical grade or above. Fatty acid standards which include: myristic, palmitic, palmitoleic, stearic, oleic, linoleic, linolenic, arachidonic, eicosapentaenoic and docosahexaenoic acid used were purchased from Sigma-Aldrich (Steinheim, Germany). Chemically pure reagents such as petroleum ether, chloroform, methanol, butylated hydroxyl toluene and 36% hydrochloric acid were purchased from Merck (Saarchem, Wadeville, South Africa). All solvents were used without further purification. All water used was of ultra pure quality and obtained from a Milli-Q gradient (Millipore) water purification system.

3.2. PREPARATION OF SOLUTIONS AND REFERENCE STANDARDS

1000 mg/l stock solution was prepared through the use of known fatty acids standards. A mixture of 11 fatty acids including an internal standard were weighed and placed in a 100 ml test tube and dissolved in chloroform, which is the reagent that was also used for fatty acids extraction from the real samples. Reference standards prepared were very similar to those of the fish fatty acids to be analyzed. Working solutions of 10, 20, 50, 150 and 300 ppm containing eleven analytes including an internal standard were prepared by appropriate dilution of stock solutions with chloroform. A derivatizing solution of methanolic-HCl was prepared by addition of 5 ml of HCl in 100 ml of methanol.

Solutions were kept refrigerated at 4°C to minimise any chemical reaction resulting from room temperature storage.

3.3. EQUIPMENT/ INSTRUMENTATION

Analysis of fatty acids methyl esters was performed with the use of Hewlett-Packard 5890A gas chromatograph equipped with a flame ionization detector which is very popular in detection of organic compounds. A non-polar fused silica capillary column Omegawax-250^{TM} (30m x 0.25mm x 0.25µm film thickness) was used for analysis. A personal computer equipped with a Peak Simple version 3, 21 Chromatography Manager was used to process chromatographic data.

An Oxylaser solding burner (Oxyturbo, Desenzano del Garda, Brescia) was used for heating and closing of the test tube ampules after filling with sample to be derivatized. A vortex mixer (Bibby sterilin, United Kingdom) was used for mixing of derivatized sample with petroleum ether (extracting solvent) before any GC injection.

3.4. SAMPLE COLLECTION AND COLLECTION METHODS

Three (3) of the highly consumed fish species in South Africa such as *Oreochromis Mossambicus* (Mozambique Tilapia), *Clarias Gariepinus* (Sharptooth Catfish) and *Cyprinus Carpio* (Carp) were collected through the use of various methods. In the first phase fish samples were randomly collected (purchased) from the local suppliers at Thohoyandou (Limpopo Province) and around Johannesburg while in the second phase samples were collected from various fresh water systems such as Makuleke Dam found in Limpopo, Loskop in Mpumalanga and Vaal Dam in Gauteng Province. These

provinces were selected due to their differences in climatic conditions for which literature shows that they have an influence on fatty acid composition of lipids of the fish. Other fish samples were collected from the aquaculture (fish farm) system at the Agricultural Research Council (ARC) in Irene, South Africa. Fish samples were kept in an ice bath until refrigerated at about -20ºC.

For fish samples collected directly from the fresh water systems, experts on fishing were used in collecting fish samples. Multi-meshgillnets and various traditional methods (use of fish line) were used in collecting samples. Only target fish species were collected out of the water for analysis. Fish were collected in November and December 2006 and in January 2007. The reason for collecting samples during this season is that this is the period of the year in which fish are highly active and easier to catch from the water.

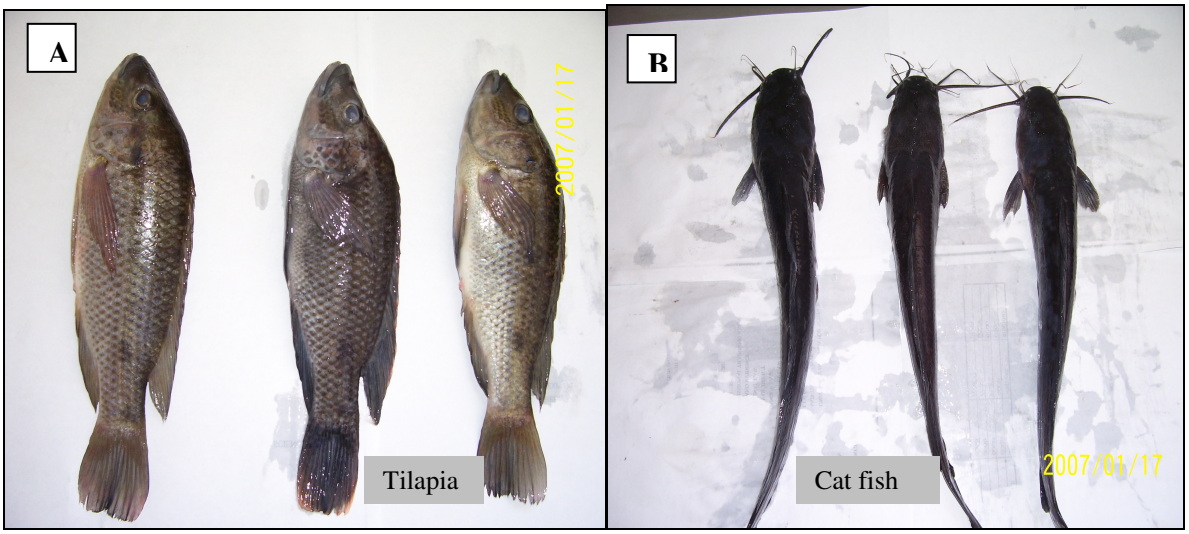




Figure 11. Three fish species of interest (A= Tilapia fish; B= Cat fish; C= Carp fish) collected from different sources.

About three fish samples of each species were collected from each fresh water system; this gave a total of nine fish samples collected from each water system and a combined total of twenty seven fish samples. Other two fish samples of each species were purchased from each of the two fish suppliers/ market selected around Thohoyandou and the other two selected around Johannesburg. Another three samples per each fish species were collected from the ARC aquaculture system.

3.5. SAMPLE PREPARATION AND ANALYSIS

3.5.1. Fish Filleting, Packaging and Storage of Samples

In fatty acids analysis, collected fish samples need to be properly filleted, packed and stored to prevent cross contamination and decomposition of samples. Once fish samples had reached the laboratory, they were filleted according to the method by Katikou *et al.,* (2001). The whole fillet was trimmed of the caudal and ventral fat depots, removing all the skin, bones and the fat attached to the skin, leaving the white muscles. Dark muscles

and any visible connective tissue were also removed as these have very different patterns of lipids deposition to the white muscle. The belly flap area was selected as the sampling area for analysis of fillets.

Packaging methodology of the fish fillets has a greater influence on the quality of fatty acids composition due to the influence of microbial contamination and oxidation of fatty acids overtime. In this study, after filleting the right fillets were individually packed in a vacuum bag and marked. These were refrigerated at about -10ºC and below as proposed by Christie (1993) and Lambertsen and Braekkan, (1965).

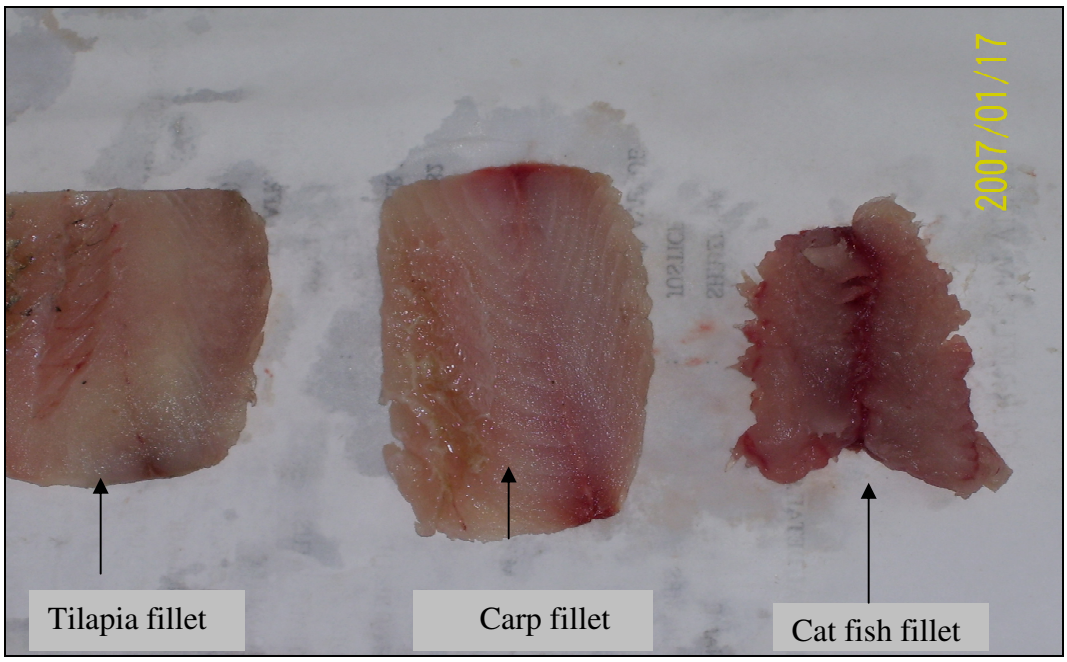


Figure 12. Different fish fillets extracted from different South African fresh water fish

3.5.2. Extraction Method Used

In nature, most fatty acids occur as part of more complex molecules. The broad spectrum of different substrates where fatty acids occur demands different but appropriate extraction method. Different extraction methods have been developed and used for extraction of fatty acids from the tissues of both animal and plant origin; this can be extracted either as bound or as free fatty acids. In lipids chemistry, the most popular extraction methods for fatty acids extraction are the Folch (1957) and Bligh and Dyer (1959) methods. These methods have a higher application in lipids extraction than in any other extraction methods.

In this research, the Folch (1957) extraction method was used. This was selected for use due to its high extraction efficiency, very little chance of loss of some groups of fatty acids, its ability to associate with water molecules and because it can be run at any scale that is technically feasible. The method involved mechanical homogenization of the fatty tissue with a 2:1 chloroform: methanol mixture to a final volume 20 times the volume of the tissue. From the whole fish fillet, 5 g of the fillet was weighed and extracted for the fatty acids. To minimise autoxidation, 10 ppm of butylated hydroxyl toluene was added. After extraction the solvent was washed with 4 ml of 20 ppm salt (sodium sulphate) solution (for complete recovery) for each 20 ml chloroform: methanol. After vortexing for some seconds, the mixture was allowed to separate into two phases. The lower chloroform phase containing lipids was collected and evaporated under a nitrogen stream to pre-concentrate the extracts before derivatization.

3.5.3. Derivatization Method Used

Because of high polarity, low volatility and availability of those that form hydrogen bonds, free fatty acids are difficult to be analyzed directly by gas chromatographic (GC) technique. Therefore, before any analysis by GC, it is necessary to convert them to low molecular weight non-polar derivatives, such as methyl esters. This therefore, improves peak shape and resolution.

Several derivatization techniques have been developed to increase volatility of fatty acids specifically long chain fatty acids. By modifying their functional groups, so as to make it easier for many fatty acids to be well separated by GC. In this research an acid-catalysed esterification method was used to increase volatility of the fatty acids extracts. This was done by hydrolysis of the lipid extract in 5 ml of the derivatization reagents (using 5 ml of 36% HCl added to 100 ml of methanol) and sealed in a test tube under nitrogen. The mixture was derivatized at 80°C for 5 to 6 hours, cooled and then extracted with 4 ml of petroleum ether before analysis by GC. The main advantage of this method is that all fatty acids are esterified at approximately the same rate, so there are unlikely to be differential losses of specific fatty acids during the esterification stage (Christie, 1993; McCullough and Lin, 2001).

3.6. GAS CHROMATOGRAPHIC ANALYSIS

For analysis, the operating conditions of the gas chromatographic method were as follows: the column temperature was initially set at 80°C for 5 minutes and then increased to 150°C at the rate of 10°Cmin^{-1} where it was held constant for 30 minutes; the temperature was further increased to 220°C at 5°Cmin^{-1} and finally held constant for

another 30 minutes. This is the optimum temperature programming for the best separation and run time. Helium was used as a carrier gas, at a constant flow rate of 1 mlmin^{-1}. The inlet (or injection port) and detector temperatures were kept at 250°C. For analysis, sample injection was at 1µl. Other than the carrier gas two supportive gases hydrogen (at flow rate of 250 kPa) and air (at flow rate of 320 kPa) were used. These gases are critical to control sensitivity of the detector.

3.7. QUALITY ASSURANCE

From the stock solution, different concentrations of the same fatty acids composition were prepared by appropriate dilution with chloroform, derivatized and analysed under similar chromatographic conditions employed during analysis of real samples. The precision or the accuracy of the instrument was determined by conducting three replicate injections of the same solution. To determine reproducibility of extraction and derivatization method, three samples extracted from the sample portion of the same fish were prepared and analysed under similar conditions. The relative standard deviations (%RSD) from both GC area counts and retention times were determined and compared for each analyte. Detection limits were calculated as that concentration corresponding to 3 times the noise level. Duplicate injections were made for each extract and the average value used.

3.8 FATTY ACIDS IDENTIFICATION AND QUANTIFICATION

Individual fatty acids were identified and quantified by comparison with retention times and peak areas of fatty acid methyl ester standards prepared and run under similar conditions as that of the real samples. The area percentage and weight (mg/g in oil) of each fatty acid methyl esters according to the AOAC official method 963.22 (AOAC, 1984), were calculated as follows: Area percent fatty $\text{acid}_x = [A_x/ (A_T\text{-}A_{IS})] \times 100$

Where A_x = area counts of methyl ester X; A_T = total area counts for chromatogram; and A_{IS} = area counts of internal standards.

While for weight in mg/g was as: weight in $\text{mg/g} = \frac{A_x \times W_{IS} \times CF_x}{A_{IS} \times W_S \times 1.04 \times 1000}$

Where A_x = area counts of methyl ester X; A_{IS} = area counts of internal standards; CF_X = theoretical detector correction factor for methyl ester X; WIS = weight of internal standard added to the sample, (mg); WS = sample weight, (mg); and 104 is a factor necessary to express results as mg fatty acid/g oil, rather than as methyl ester.

CHAPTER 4: RESULTS AND DISCUSSION

4.1. OPTIMIZATION OF THE SEPARATION METHOD

The initial step of the study was optimization of the analytical method to improve separation and detection of fatty acids analytes. The extraction and derivatization times were held constant, while temperature programme, run time and column choice which can provide best separation were investigated.

Different temperature ranges were applied, to obtain the best programme which can give best separation of fatty acids from the sample matrix. At low temperature, only short chained fatty acids were favoured. This caused all medium chained fatty acids to elute at a very long retention times with poor separation, while many of the long chained unsaturated fatty acids remained in the column without any elution.

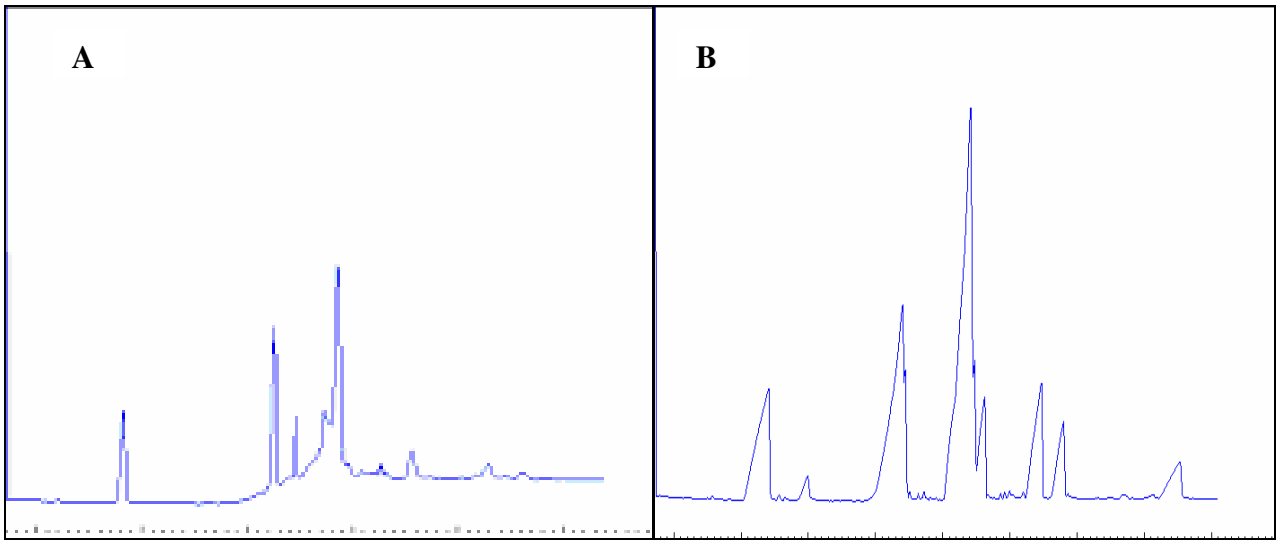


Figure 13. Chromatograms showing poor separation, A: due to poor temperature program specifically low temperature, B: shows poor separation due to column effects. (Nukol: 30m × 0.25 mm ×0.25 µm).

To obtain a better separation and elution of all fatty acids of interest, the separation method was optimized. To improve separation from figure 13 A, to figure 14, this was done by a gradient temperature programme with an increase on temperature from 80°C to 150°C at 10°Cmin^{-1} and from 150°C up to 220°C at 5°Cmin^{-1} to allow elution of fatty acids with different boiling points especially long chained fatty acids such as eicosapentaenoic and docosahexaenoic acids which requires high temperature and long retention times to elute. Other studies have shown good results under isothermal temperature condition, to obtain better results, Dayhuff and Wells (2005), used a gradient temperature programme with the oven temperature increased at 1°Cmin^{-1} from 170°C to 180°C and at 2°Cmin^{-1} from 180°C to 240°C. Gutierrez and da Silva (1993) separated fatty acids of seven Brazilian fresh water fish species with a constant temperature of 195°C. Different columns were also employed to find out the best column which can provide good separation of fatty acids. Fig 13 B shows poor chromatogram resulting from the use of capillary column (Nukol column 30m x 0.25mm x 0.25µm film thickness) which is not highly effective on separation of fatty acids. Best separations were obtained on a fused silica capillary column Omegawax-250^{TM} (Supelco, 30m x 0.25mm x 0.25µm film thickness). This is a special purpose column specifically designed for used on separation of fatty acid methyl esters. This column is coated with a less polar stationary phase (polyglycol based on carbowax) as compared to the other column used (Nukol), which is a general purpose column. These chromatographic traces lend support to Christie's view that Omegawax-250^{TM} is the best special purpose column, and it is recommened by this author to newcomers to the technique (Christie, 1989). Other than capillary column, Gutierrez and da Silva (1993) used a stainless steel column, 2m x 5mm,

packed with chromosorb W coated with 18% (by wt) of diethylene glycol succinate to obtain good separation of seven Brazilian fish species.

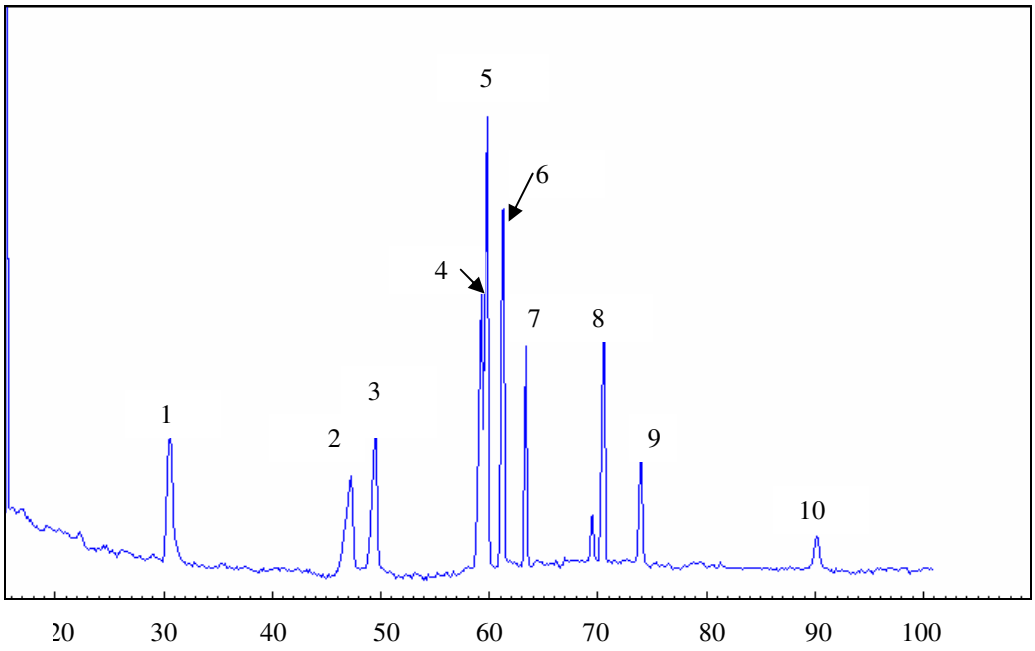


Figure 14. Chromatogram with 10 well separated fatty acid standards (Omegawax-250^{TM} column) (for names refer to figure 15).

Figure 14 shows a chromatogram with well separated fatty acids of interest. This was obtained through the use of an Omegawax capillary column which is a special purpose column designed for fatty acids analysis. This was also improved by the use of a well programmed column temperature. Under these conditions, higher detection limits were attained with good separation of fatty acid peaks. All peaks obtained were identified by analysis of individual fatty acid standards.

4.2. QUALITY ASSURANCE

4.2.1. Reproducibility of the Instrument (GC)

To determine reproducibility of the instrument, three replicate injections of the same 50 mgl^{-1} standard mixture of fatty acid methyl esters was conducted. This was done to find out whether there would be any serious deviation/ or variations on retention times and peak area of different compounds.

Table 4. Peak areas and relative standard deviations of three replicate injections of 50 mgl^{-1} fatty acids standards mixture.

	Fatty acid standards					
Fatty acid	**1st injection**	**2nd injection**	**3rd injection**	**Mean**	**SD**	**RSD %**
14:0	20.39	18.10	20.64	19.71	1.39	7
16:0	25.40	24.30	26.97	25.56	1.34	5
16:1 n-7	31.21	33.58	35.77	33.52	2.28	8
18:0	49.91	52.44	52.12	51.49	1.37	3
18:1 n-9	39.68	37.21	38.70	38.53	1.24	3
18:2 n-6	36.32	38.41	34.88	36.54	1.78	5
18:3 n-3	20.45	18.99	21.25	20.23	1.15	6
20:4 n-6	33.45	33.01	35.66	34.04	1.42	4
20:5 n-3	16.23	18.68	19.93	18.28	1.87	10
22:6 n-3	11.90	12.74	15.52	13.39	1.90	14

The results obtained show an agreement in repeated injection of the same standards mixture. There is a very low deviation of the fatty acids peak areas which is indicated by low percentage standard deviation (% RSD). The % RSD ranged between 3 to 14% proving good reproducibility of the gas chromatographic procedure.

4.2.2. Reproducibility of the Analytical Method (Extraction, Derivatization and Analysis)

The precision of the method was determined by conducting three replicate injections of three samples removed from the same portion of the same fish, extracted, derivatized and analysed under similar conditions. The mean peak area and percentage relative standard deviations (% RSDs) were determined for each analyte.

Table 5. Mean peak area and relative standard deviations of different fatty acids obtained from three replicate injections of each of the three fish specimens extracted from the same fish.

	Real fish sample					
Fatty acid	**Part 1**	**Part 2**	**Part 3**	**Mean**	**SD**	**RSD%**
14:0	20.32	18.71	16.60	18.54	1.861	10
16:0	120.63	125.09	131.08	125.61	5.24	4
16:1 n-7	15.11	11.37	13.31	13.26	1.88	14
18:0	26.13	22.08	20.11	22.77	3.07	14
18:1 n-9	61.30	58.08	45.98	55.12	8.076	15
18:2 n-6	118.20	112.48	101.96	110.55	8.24	7
18:3 n-3	31.02	29.82	26.84	25.98	7.73	29
20:4 n-6	19.92	23.48	17.08	20.16	3.21	15
20:5 n-3	15.58	17.02	17.81	16.80	1.13	7
22:6 n-3	46.01	43.90	37.53	42.48	4.42	10

From the results above, it is evident that the method had good reproducibility. There is a very low variation on the peak area with low relative standard deviations except for linolenic acid (18:3 n-3). The reproducibility of the retention times and area of the peaks made good identification of the fatty acids of interest. The results above clearly show that the extraction, derivatization and analysis methods used are precise and reproducible. Comparison of the % RSD in results on tables 4 and 5, in table 5 are slightly higher due to matrix interferences found in real samples as opposed to pure standards.

4.2.3. Identification of Fatty Acids

4.2.3.1. External calibration

Fatty acids methyl esters found in the fish were identified by comparing their retention times with those of commercially available individual purified standards. Both standards and the real fish lipids extracts were analysed under the same operating conditions.

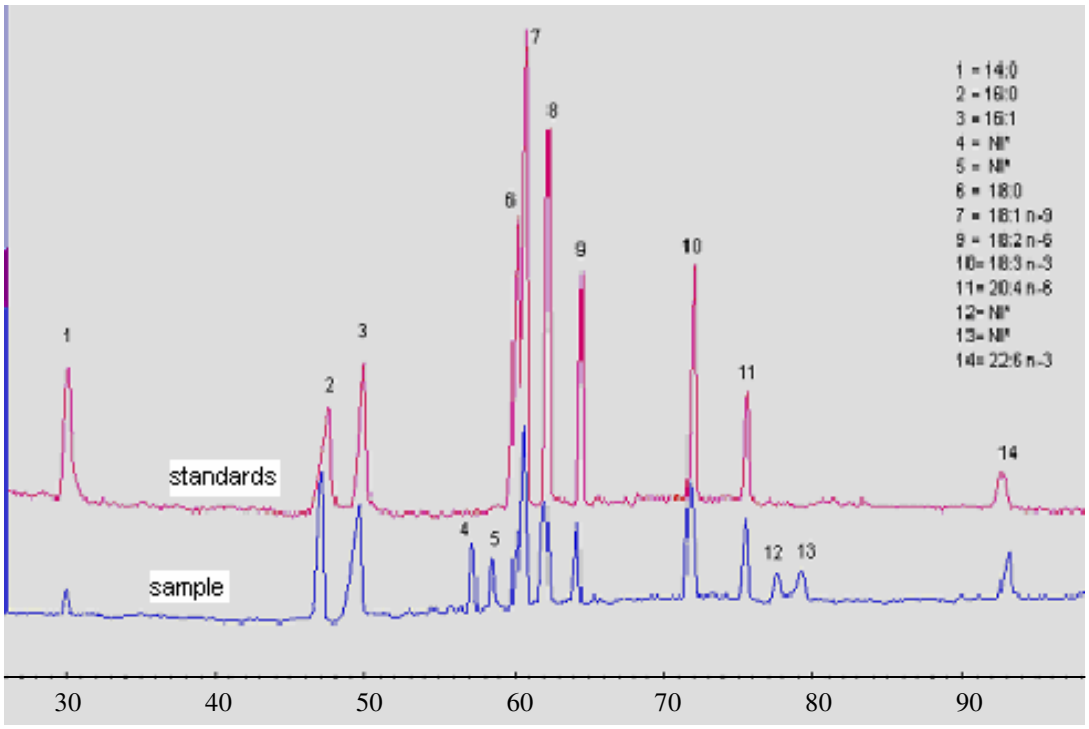


Figure 15. An overlay on the fatty acid standards and fish sample chromatographs indicating identified fatty acids from the lipids matrix.

Figure 15 shows different fatty acids separated and identified from the fish sample. All fatty acids of interest were found to be present in all three South African fish species under study. From the figure above, stearic acid (18:0) was found to co-elute with oleic acid (18:1 n-9) and this may have caused quantification of this two fatty acids to be

inaccurate. Pharmaceutically important fatty acids such as docosahexaenoic, ecosapentaenoic and arachidonic acid were also identified. From the sample matrix, other additional unknown peaks were obtained, which could be fatty acids too. This method was also used by Gutierrez and da Silva (1993); Erfanullah (1998) for identification of fatty acids from both fresh water and marine fish species.

4.2.3.2. Spiking

Although all fatty acids of interest were identified through external calibration, identification was further confirmed by spiking of the fatty acid methyl esters (FAMEs) prepared from the fish extracts with 25 mgl^{-1} of commercially available pure fatty acid standards.

Table 6. Fatty acids from different fish species before and after spiking

Fatty acid	**Carp [Area] before & after**		**Tilapia [Area] Before & after**	
14:0	3.61	8.21	7.33	11.94
16:0	26.69	32.55	38.49	46.03
16:1 n-7	14.74	18.93	8.68	13.56
18:0	5.19	11.71	20.29	26.72
18:1 n-9	17.96	24.63	34.06	39.49
18:2 n-6	18.84	26.15	20.37	28.61
18:3 n-3	5.12	9.83	12.37	15.81
20:4 n-6	6.91	10.10	9.05	15.03
20:5 n-3	4.03	8.71	4.51	7.83
22:6 n-3	6.17	9.44	11.58	15.95

Table 6 shows the area percentage of different fatty acids before and after 25 mgl^{-1} standard fatty acids mixture was spiked. The results have shown an increase in the area percentage of fatty acids of interest, while the area percentage of non-spiked compounds

remained constant (data not shown). The method was also used for identification of some compounds co-eluting with other fatty acids. This therefore confirms availability of fatty acids identified through external calibration and retention times. Spiking of fatty acids is critical in identification of fatty acids since some non-fatty acids contaminants can behave like FAMEs. For identification, this method has reportedly been used especially for low concentrated (minor fatty acids) and unknown peaks. For identification of conjugated linoleic and linolenic acids as component of fresh water fish lipids, Medez *et al.,* (1996); Manning *et al.,* (2006) used spiking of minor fatty acids for identification from other unknown sample peaks.

4.2.3.3. Detection limits

From the fatty acids methyl ester's chromatographs, detection limits of all fatty acids of interest were calculated as the concentration corresponding to three times the noise level. From duplicate injections made, the average value was used.

Table 7. Detection limits for different fatty acids methyl esters.

Fatty acid compounds	Detection limits [mg/l]
Myristic Acid	10
Palmitic Acid	10
Palmitoleic Acid	10
Stearic Acid	5
Oleic Acid	2.5
Linoleic Acid	4
Linolenic Acid	10
Arachidonic Acid	10
Eicosapentaenoic Acid	15
Docosahexaenoic Acid	20

From table 7, it is evident that the sensitivity of the procedure was good. This is shown by high detection limits obtained from analysis of fatty acid methyl esters. The lowest detection level ranged from 2.5 mgl^{-1} for oleic acid up to 20 mgl^{-1} for docosahexaenoic acid which is an essential fatty acid.

4.3. PROVINCIAL COMPARISON OF FATTY ACIDS COMPOSITIONS IN SOUTH AFRICAN FRESH WATER FISH

Table 8. Fatty acid composition of fresh water fish from Limpopo province.

	LIMPOPO PROVINCE		
Fatty acid	Cat Fish (wt %)	Carp Fish (wt %)	Tilapia Fish (wt %)
14:0	2.32	1.06	3.52
16:0	16.17	18.82	19.60
16:1 n-7	4.95	8.20	5.13
18:0	3.19	3.39	4.37
18:1 n-9	11.80	10.61	16.09
18:2 n-6	13.43	17.47	14.81
18:3 n-3	4.12	5.93	3.22
20:4 n-6	8.73	7.05	6.70
20:5 n-3	1.69	2.88	1.43
22:6 n-3	2.11	3.51	4.84

The table above indicate fatty acid composition of fish species collected from Limpopo province. The data show that the concentration of fatty acids ranged from 1.43 to 19.60 % for *Oreochromis Mossambicus* (Mozambique Tilapia), 1.06 to 18.82 % for *cyprinus carpio* (Carp) and 1.69 to 16.17 % for *Clarias Gariepinus* (Cat fish). Of all fatty acid groups, palmitic acid (16:0) was found to be the most dominant. Availability of high levels of palmitic acid supports the results obtained and published on many studies conducted on fresh water fish (Gutierrez and da Silva, 1993; Christie, 1993; Rasoarahona

et al., 2005). Higher levels of palmitic acid have been described as a characteristic of fresh water fish (Ackman, 1980). Highly important long chained fatty acids such as docosahexaenoic (22:6), eicosapentaenoic (22:5) and arachidonic acid (22:4) were found at significant levels. Linoleic acid (18:2) was found to be the most dominant polyunsaturated fatty acid, while linolenic acid (18:3) was found to be the most abundant Omega-3 fatty acid. From the medicinal point of view, tilapia fish species is of better quality as compared to others since it is characterised by the highest level of important Omega-3 fatty acids such as eicosapentaenoic (EPA) and docosahexaenoic acid (DHA).

Table 9. Fatty acid composition of fish from Gauteng province.

	GAUTENG PROVINCE		
Fatty acid	Cat Fish (wt %) cultured	Carp Fish (wt %)	Tilapia Fish (wt %) cultured
14:0	3.57	2.92	4.13
16:0	20.30	21.61	24.07
16:1 n-7	5.88	11.93	5.60
18:0	4.11	3.89	5.09
18:1 n-9	18.43	14.54	21.98
18:2 n-6	10.04	15.25	13.14
18:3 n-3	6.90	4.14	7.88
20:4 n-6	8.26	6.60	7.94
20:5 n-3	2.13	3.26	2.91
22:6 n-3	5.72	4.99	7.47

The saturated fatty acid percentages of the lipid extracted from the three fish species obtained from Gauteng (table 9) ranged from 2.92 to 24.07 %. Polyunsaturated fatty acid ranged from 2.13 to 15.25%. Of the saturated fatty acids, palmitic acid (16:0) has the greatest proportion as in other fish analysed from other provinces. These results are in

agreement with the fatty acid composition of fillet reported by Csengeri and Farkas, (1993). Oleic acid (18:1) is the main monounsaturated fatty acid in all the species analyzed. Carp fish showed the lowest proportion of oleic acid (14.45 %) and tilapia the greatest one (21.98 %). This fatty acid has exogenous origin and usually reflects the type of diet of the fish (Ackman *et al*., 1980; Ackman, 1989).

In comparing *Oreochromis Mossambicus* (tilapia) and *Clarias Gariepinus* (cat fish) which were both collected from an aquaculture system, tilapia fish species was found to consist of a high proportion of fatty acids when compared to the cat fish. Tilapia was found to consist of the highest level of docosahexaenoic acid which is the main essential fatty acid in lipid nutrition.

Table 10 shows the fatty acid composition of a single fish species obtained from Mpumalanga province.

	MPUMALANGA PROVINCE		
Fatty acid	Cat Fish (wt %)	Carp Fish (wt %)	Tilapia Fish (wt %)
14:0	N.A	1.84	N.A
16:0	N.A	20.33	N.A
16:1 n-7	N.A	6.50	N.A
18:0	N.A	4.72	N.A
18:1 n-9	N.A	12.16	N.A
18:2 n-6	N.A	15.29	N.A
18:3 n-3	N.A	6.43	N.A
20:4 n-6	N.A	7.91	N.A
20:5 n-3	N.A	2.62	N.A
22:6 n-3	N.A	4.03	N.A

N.A = Not analysed

The findings indicate that palmitic acid (16:0) is the most abundant fatty acid of all fatty acids obtained from the analysis. This is the case for the results obtained from other fish

species obtained from other provinces. Docosahexaenoic acid (22:6) was found in great quantity as compared to the eicosapentaenoic acid (20:5). Due to high levels of long chain polyunsaturated fatty acid such as DHA and EPA this type of fish can be used as nutritional supplement especially as a source of Omega-3 fatty acids.

Comparing fish from all provinces, the results shows that fish species from Gauteng Province are characterised by high concentrations of fatty acids. The reason for this high concentration might be attributed mainly to the diet since these were fed under special diet (cultured fish). Compared to the ones from Limpopo, Gauteng's *Oreochromis Mossambicus* species was found to be characterised by high amount of long chain polyunsaturated fatty acids docosahexaenoic (2.63%), eicosapentaenoic (1.48%) higher than that from Limpopo Province.

From all fish species analysed, cultured fish have proved to be of the best quality in terms of their fatty acid composition as compared to other fish species obtained from the natural river systems. Because of this, aquaculture systems can act as a useful system for supply of very nutritious fish species with good levels of essential fatty acid compositions. Although they have low levels of fatty acid composition as compared to the ones from Gauteng, the results show that fish species from Mpumalanga and Limpopo still possess significant amounts of essential fatty acids which can be used as diet supplement.

Table 11 provides data on fatty acids weight percentage composition of lipids of *Clarias Gariepinus* (Sharptooth Catfish), *Cyprinus Carpio* (Carp) and *Oreochromis Mossambicus* (Mazambique Tilapia).

	Fatty acids composition in South African fresh water fish species (mean ± RSD)		
Fatty acids	**Cat Fish (wt %)**	**Carp Fish (wt %)**	**Tilapia Fish (wt %)**
14:0	2.95 ± 0.3	1.94 ± 0.48	3.83 ± 0.11
16:0	18.24 ± 0.16	20.25 ± 0.06	21.84 ± 0.15
16:1 n-7	5.42 ± 0.12	8.88 ± 0.31	5.37 ± 0.06
18:0	3.65 ± 0.18	4.0 ± 0.17	4.73 ± 0.11
18:1 n-9	15.12 ± 0.31	12.44 ± 0.16	19.04 ± 0.22
18:2 n-6	11.74 ± 0.20	16.0 ± 0.08	13.98 ± 0.09
18:3 n-3	5.51 ± 0.36	5.50 ± 0.22	5.55 ± 0.59
20:4 n-6	8.50 ± 0.04	7.19 ± 0.09	7.32 ± 0.12
20:5 n-3	1.91 ± 0.16	2.92 ± 0.11	2.17 ± 0.48
22:6 n-3	3.92 ± 0.65	4.18 ± 0.18	6.16 ± 0.30

The fat content is influenced by species, geographical regions, age and diet (Piggott and Tucker, 1990). Of all fatty acids, palmitic (18.24 to 21.84 %) was found to be the most dominant of all fatty acid groups from all fish species under study, as it has been pointed out by Gopakumar and Nair (1972) for Indian fresh water fish. All three fish species contained arachidonic acid (20:4), which is a precursor for prostaglandin and thromboxane biosynthesis (Pompeia *et al.,* 2002). The level of arachidonic acid was high in all species with catfish consisting of the highest level (8.50 %). DHA and EPA had been shown to have preventive effects on human coronary artery disease (Leaf and Webber, 1988). Therefore, fish have been suggested as a key component for a healthy diet in humans (Leaf and Webber, 1988). Therefore, significant levels of EPA and DHA on fish species analysed clearly indicate that this can be used to supplement essential fatty acids in human diet. Comparing the three fish, it can be seen that tilapia fish was

found to be richest of all fish species analysed, also with the highest composition of docosahexaenoic acid (22:6). From all fish analysed, this fatty acid was found in higher levels than eicosapentaenoic acid. These data confirmed earlier observations of Katikou *et al.,* (2001); Gunstone *et al.*, (1978).

4.4. FATTY ACIDS COMPOSITION (SATURATION LEVELS) ON SOUTH AFRICAN FRESH WATER FISH

Further analysis of the fatty acids was conducted. From this the quality of fatty acids in terms of their saturation was determined and computed. Figure 16 indicates the total fatty acid compositions of South African fish interns of their level of saturation.

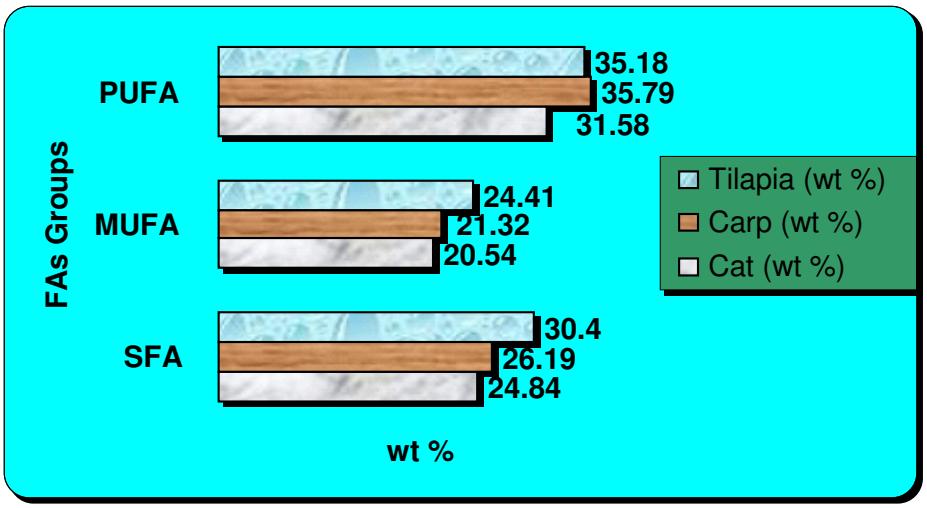


Figure 16. Fatty acid compositions on South African fish in terms of their level of saturation.

Among unsaturated fatty acids, polyunsaturated fatty acids were found to be the most abundant. These data confirmed earlier observations of Ackman (1967); Dayhuff and Wells, (2005) and Aras *et al*., (2003). In terms of the PUFA, the study revealed that *Cyprinius*

Carpio was found to be the richest fish with about 35.79 %. The second best was found to be *Oreochromis Mossambicus* with about 35.18 % and 31.58 % for *Clarias Gariepinus.* High levels of PUFA compositions in South African fish is a clear indication that they are of good quality and can be used as good nutritional supplement for human consumption. When compared to monounsaturated fatty acids, saturated fatty acids were found to be higher with Tilapia characterised by higher concentrations than in other fish species.

4.5. OMEGA-3 AND OMEGA-6 FATTY ACIDS COMPOSITION

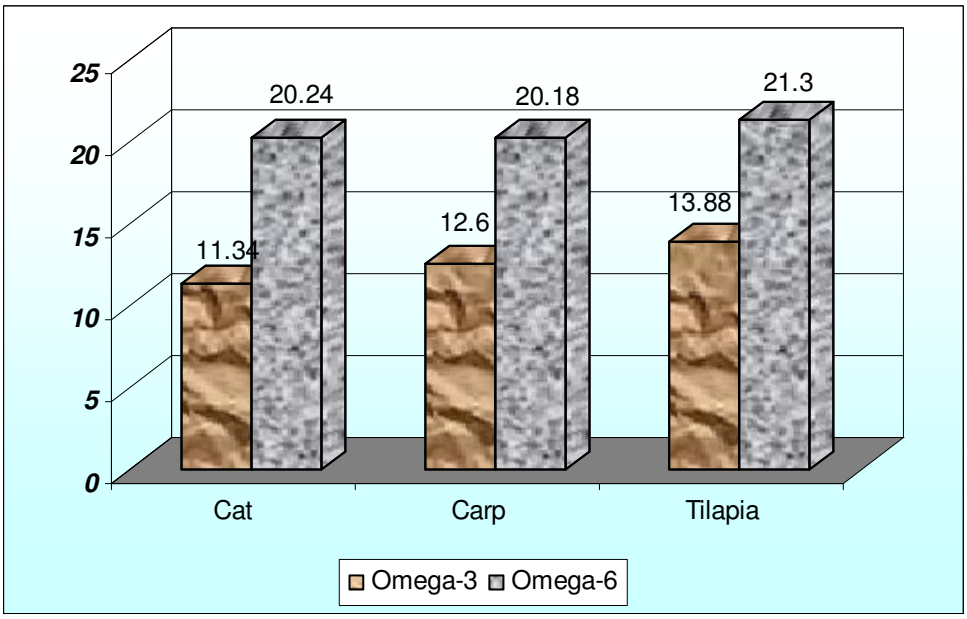


Figure 17. Representation of Omega-3 and Omega-6 fatty acids composition on fish.

The results above clearly indicate that all fish species analysed are characterised by high levels of Omega-6 fatty acids. The results are in accordance with the results obtained in other studies conducted on fresh water fish species. Other studies show that, fresh water fish are mainly characterised by high levels of omega-6 polyunsaturated fatty acids especially linoleic (18:2) and arachidonic (22:4) acid (Ackman, 1967; Cowey and

Sargent, 1972; Aras, 2003), but also substantial concentrations of eicosapentaenoic and docosahexaenoic acid.

From the fish studied, Omega-6 (n-6) fatty acid ranged from 20.18 % for carp, 20.24 % for catfish and 21.3 % for tilapia fish species. These results show no greater difference on the level of Omega-6 polyunsaturated fatty acids characterising such South African fresh water fish species.

The Omega-3/Omega-6 ratio has been suggested to be a useful indicator for comparing relative nutritional value of fish oils. It was suggested that the ratio of 1:1-1:5 would constitute a healthy human diet (Osman *et al*., 2001). When compared to Omega-6 fatty acids, all fish species analysed were found to be characterised by low levels of Omega-3 fatty acids. Because of the low content of Omega-3 fatty acids in fresh water fish, the ratio of total Omega-3 (n-3) to Omega-6 (n-6) fatty acids (essential fatty acid ratio) is much lower. All three fish had the Omega-3/Omega-6 ratio within the recommended ratio as suggested by Osman *et al*., (2001). Based on this, previous studies indicate that this is an opposite case for marine fish species which are mainly composed of Omega-3 and low levels of Omega-6 fatty acids (Steffens, 1997). Cat fish was found to be composed of the lowest amount of n-3 fatty acid as compared to carp and tilapia fish species. With respect to n-6 fatty acid, tilapia fish was found to be the richest on n-3 fatty acid. Although they are highly characterised by n-6 fatty acids, this fish species can also serve as a valuable source of essential n-3 fatty acids especially EPA and DHA.

4.6. COMPARISON OF FATTY ACIDS COMPOSITION IN SOUTH AFRICAN FISH WITH THOSE FROM OTHER COUNTRIES

South African fresh water fish has shown higher concentration of saturated, monounsaturated and polyunsaturated fatty acids. This therefore clearly indicates that they are of good quality. To support this, they are characterised by high levels of polyunsaturated fatty acids as compared to other fatty acids groups. Their high proportions of essential n-3 fatty acids such as EPA and DHA acid makes them a better source of essential fatty acids which are of greater medicinal importance since they are linked to reduced risk of cancer (Brondz, 2002; Steffens, 1997).

Table 10. Comparison of fatty acids compositions between South African fish and fish species from other countries

Types of fatty acid	**Wt % of fatty acid composition**				
	Mozambique tilapia (SA)	Carp fish (SA)	Cat fish (SA)	**Carp fish (Hungary)**	**Tilapia rendalli (Madagascar)**
14:0	3.83	1.94	2.95	**1.7**	**3.31**
16:0	21.84	20.25	18.24	**12.9**	**19.9**
16:1 n-7	5.37	8.88	5.42	**7.3**	**9.14**
18:0	4.73	4.0	3.65	**5.1**	**6.91**
18:1 n-9	19.04	12.44	15.12	**14.8**	**13.2**
18:2 n-6	13.98	16.0	11.74	**7.9**	**5.05**
18:3 n-3	5.55	5.50	5.51	**2.9**	**0.46**
20:4 n-6	7.32	7.19	8.50	**8.6**	**5.19**
20:5 n-3	2.17	2.92	1.91	**8.8**	**2.52**
22:6 n-3	6.16	4.18	3.92	**6.5**	**7.64**

Other studies on similar fish species were conducted in other countries. Observations have shown that all of similar species analysed are of higher quality (see on table 12). These fish species were found to be characterised by high levels of essential fatty acids such as DHA and EPA. High levels of palmitic and oleic acid in two South African fresh water fish have already been related to Hungarian carp and Madagascar's tilapia (fresh water fish), by Csengeri and Farkas (1993). The higher proportion of palmitic fatty acids in both South African and other fishes from other countries (Madagascar and Hungary) is an indication of their source of origin which is a characteristic for fresh water fish (Gutierrez and da Silva, 1993; Rasoarahona *et al*., 2005).

Of all saturated fatty acids, palmitic fatty acid was found to be the highest. There is a minimal difference between Mozambique tilapia and Tilapia rendalli (Madagascar) in terms of their palmitic fatty acid composition, with Mozambique tilapia consisting of 1.94 wt % higher than its Madagascan counterpart. Hungarian carp was found to consist of 7.35 wt % higher than the South African carp. From all fish species, Oleic acid was found to be the most abundant monounsaturated fatty acid with South African fish consisting of levels higher than their counterparts. The fatty acid profile of the analysed fish species generally fits into the typical pattern for fresh water fish, where palmitic and oleic are usually the major constituents.

Arachidonic acid (20:6 n-6) was detected in significant amount in all species analysed. This is also the case for findings obtained by other researchers for similar fish species from other geographical location. Hungarian carp was found to be the richest fish in terms of the level of arachidonic concentration (8.6 %), while tilapia rendalli consisted of

the lowest level (5.19). Long chained Omega-6 polyunsaturated fatty acids such as EPA and DHA were discovered in all fish. These types of fatty acids are used as indicators of fish quality (Osman *et al*., 2001). When compared to fish species from other countries, South African fish were found to be consisting of low levels of essential fatty acids especially DHA (22:5 n-3) and EPA (20:5 n-3). Hungarian carp have shown to be the richest fish in terms of the level of eicosapentaenoic fatty acid composition; other findings show that it consist of 8.8 wt % with 5.88 wt % higher than that of South African Carp (2.92 %). The level of EPA obtained from the lipids of Mozambique tilapia are in line with the findings obtained from Madagascar's tilapia rendalli.

The results obtained showed a considerable amount of polyunsaturated fatty acids in fresh water lipid fractions. Although not typical, the results obtained in the study are in agreement with many other published results on fatty acid content in different fish species originating from different locations in the world (Csengeri and Farkas 1993; Varljen, *et al*., 2003). Generally, the fatty acid composition of Mozambique tilapia and tilapia rendalli, and South African carp and Hungarian carp show similar patterns, although Hungarian carp showed more favourable fatty acid composition, containing more polyunsaturated fatty acids.

4.7. CONCLUSION AND FUTURE PROSPECTS

Gas chromatography was employed for analysis of the lipids of fish fillet oil. Detailed qualitative and quantitative analyses were performed on three South African fresh water fish species. The fish oil isolated from three different fish provided interesting data regarding the fatty acid composition of the total lipids classes. From the data obtained, it is evident that fatty acid mixtures were of a complex nature for all fish analysed.

From all fish species analysed, different fatty acid groups (saturated, monounsaturated and polyunsaturated) were identified. Palmitic fatty acid was found to be the most abundant fatty acid with the highest concentration levels from all fish species. This is a common characteristic for fresh water fish. Essential fatty acids (medicinally important) such as eicosapentaenoic (EPA) and docosahexaenoic (DHA) were identified, these were found at significant concentrations. In all fish analyzed, DHA was found to be greater than EPA. Although there is a small variation, when compared to other species tilapia was found to be the richest fish with higher concentrations of many essential fatty acids especially which are essential for human nutrition.

From the data obtained, higher proportions of Omega-6 PUFA were observed. The ratio of Omega-3 to Omega-6 fatty acids is much lower in all species. Although they are highly characterised by Omega-6 fatty acids, these fish species can also serve as a valuable source of essential Omega-3 fatty acids especially docosahexaenoic and eicosapentaenoic acids which were found to be available in significant quantities.

The distribution of fatty acids composition was found to be in accordance with observations on similar fish species from Hungary and Madagascar. South African fresh water fish was found to be the richest as compared to other similar fresh water fish. This might be attributed to their diet source more than to other factors such as temperature, age, environmental conditions, etc. Species analysed were found to be of high nutritional value. Because of this, they can be used as an alternative source of essential polyunsaturated fatty acid (Omega-3) especially in poor rural communities where the majority of people can not afford to buy encapsulated pharmaceutical fish oil (Omega-3 rich). In summary, all fresh water fish analysed are good source of essential polyunsaturated fatty acids especially Omega-3 PUFA and can be used to provide essential fatty acids which can not be synthesised in the human body, by inclusion in the formulation of highly unsaturated low-fat diets.

FUTURE WORK

Fatty acids exist in nature as complex matrices consisting of a large number of short, medium and long chain fatty acids. It is well known that a fish lipid consists of large number of short and long chain fatty acids. This study identified a few fatty acids. A broader study can be undertaken to identify all or as many fatty acids in the fish lipids especially short and very long chain fatty acids which are overlooked in many research studies.

The present study evaluated local fresh water fish resources for total fatty acids and fatty acid types, especially PUFA and Omega-3 fatty acids. Fish species under study were found to be a good source of essential fatty acids. There is therefore a need to investigate

the main factors influencing fatty acids composition in South African fish as this will be useful for fish farming in growing them under such conditions.

5. REFERENCES

Ackman, R. G. (1989). Fatty Acids. In: *Marine Biogenic Lipids, Fats and Oils.* R.G Ackman (Ed.), CRC Press, Boca Raton, 145–178.

Ackman, R.G (1980). Fish lipids, part 1. *In advances in fish sciences and technology*. (J.J. Connell, Ed.), Fishing news books Ltd. Farnham, Surrey, 86 -103.

Ackman, R.G. (1967). *Characteristics of the fatty acid composition and biochemistry of some fresh-water fish oils and lipids in comparison with the marine oils and lipids.* Comparative biochemistry and physiology, 22, 907-922.

Ackman, R.G., Sebedio, J.L., and Kovacs, M.I.P (1980). *Role of eicosenoic and docosenoic fatty acid in fresh water and marine lipids*. Marine chemistry, 9, 157-164.

Alltech associates. (1999). *Evaporative light scattering detection simplifies fatty acid analysis*. www.altechweb.com/productioninfo/technical/app/0045SE.pdf (Accessed: April, 2006).

American Heart Association. (2006). *Trans fatty acids. AHA*. http://www.americanheart.org/presenter.jhtml?identifier=3030450 (Accessed: September, 2005).

Anton para, (2006). *Solvent extraction using the multiwave solvent and rotar solvent.* www.anton-paar.com. (Accessed: July 2006).

AOAC. (1984). *Methyl esters of fatty acids in oils and fats: gas chromatographic method*. AOAC, 963, 22.

Aras, N.M., Haliloğlu, H.I., Yetim, H., and Ayik, Ö. (2003). *Comparison of Fatty Acid Profiles of Different Tissues of Mature Trout (Salmo trutta labrax, Pallas, 1811) Caught from Kazandere Creek in the .oruh Region, Erzurum, Turkey*. Turk J. Veterinary Animal Science, 27, 311-316.

Bang, H.O and Dyerberg, J. (1972). *Plasms lipids and lipoproteins in greenkandic west coast Eskimos*. Acta medical scand, 192, 85-94.

Bell, M.V., James R. Dick, J.R., Alexander E.A., and Porter, A.E.A. (2003). *In vivo assays of docosahexaenoic acid biosynthesis in fish.* Marine research, Norway.

Beyer, P.L. (2001). Nutrient consideration in inflammatory bowels disease and short bowels syndrome. In: A.M. Coulston., C.L. Rock., and E.R. Monsen (editor). *Nutrition in the prevention and treatment of disease.* Academic press, London, U.K. 577-599.

Bhupesh, C.R. (1996). *Extraction and separation of phospholipids from Tuna fish*. Food science technology, 31, 137.

Bligh, E. G., and Dyer, W. J. (1959). *A rapid method of total lipid extraction and purification*. Canadian J. Biochemistry, 37, 911-17.

Boggio, S.M., Hardy, R.W., Babbitt, J.K., and Brannon, E.L. (1985). *The influence of dietary lipid source and alpha-tocopheryl acetate level on product quality of rainbow trout (salmo gairdneri).* Aquaculture, 51, 13-24.

Böhm, H.B., Feltas, J., Volner, D., and Levsen, K. (1989). *Food and health influences*. Chromatography A, 478, 399.

Bougnoux, P. (1999). *N-3 polyunsaturated fatty acids and cancer*. Curr opion clinical nutrition metabolic care, 2, 121-126.

Braun, R.D. (1987). *Introduction to instrumental analysis*: Chemistry series. McGraw-Hill international, New York, U.S.A.

British nutrition foundation. (1992). *Unsaturated fatty acids: nutritional and physiological significance*. Champman and Hall, London, U.K.

Brondz, I., Olsen, M., and Greibrok, T. (1983). *Lipids separation and analysis*. Chromatography A, 274, 299.

Brondz, I. (2002). *Development of fatty acid analysis by high-performance liquid chromatography, gas chromatography and related techniques*. Analytica Chimica Acta, 465, 1-37.

Buckley, D.J., and Morrissey, P.A. (1992). *Vitamin E and meat quality.* Roche and Hoffmann, London, UK.

Burr, G.O., and Burr. M.M. (1930). *On the nature and role of the fatty acids essential in nutrition*. University of Minnesota, Minnespolis.

Carlson, L.A. (1985). *Advances in applied lipid research*. Clinical chimica Acta, 149, 89-93.

Carter, S.J. (2000). *Lipids: fats, oils, waxes, etc*. University of Cincinnati, Ohio, U.S.A http://biology.clc.uc.edu/courses/bio104/lipids.htm (accessed: May, 2006).

Christie, W.W. (1989). *Gas chromatography and lipids.* Oily press, Bridgwater. U.K.

Christie, W.W. (1993). Preparation of ester derivative of fatty acids for chromatographic analysis, in: W.W. Christie (Ed), *Advances in lipid methodology*. Oily Press, Dundee, 2, 69-111.

Christie, W.W. (1997). *Analysis of fatty acids by high-performance liquid chromatography*. Lipid Technology, 9, 124-126.

Collins, A. (2005). *Saturated fat in food*. http://www.annecollins.com/saturated-fat.htm (Accessed: February, 2006).

Connolly, J.R. (2005). *Sample preparation and systematic diffractometer errors: introduction to X-Ray powder diffraction*, http://www.macaulay.ac.uk/spraydrykit/index.html (Accessed: October, 2006).

Connor, W.E. (2000). *Importance of n-3 fatty acids in health and disease*. American Clinical Nutrition, 71, 1715-1755.

Cowey, C.B. (1993). Some effects of nutrition on flesh quality of cultured fish. In : S.J. Kaushik and P. Luquet (editors). *Fish nutrition in practice*. INRA, Paris, France. 227-236.

Cowey, C.B. and Sargent, J.R. (1972). *Lipid nutrition in fish*. Comp.Biochemistry Physiology, 57, 269-73.

Csengeri, I., and Farkas, T. (1993). *Effects of essential fatty acid deficient diets on the carcass acids and membrane viscosity in the common carp*. Proceedings of EIFAC workshop on methodology for determination of nutrient requirements in fish, July 1993, Eichenau, Hungary.

Csengeri, I., Müller, F., Olâh, J., Majoros, F., Farkas, T., Joó, I., Tóth, S., Garai, I., and Huszka, T. (1988). *Fish consumption and cardiovascular diseases*. Presented at, 12th Hungarian conference on fisheries sciences, 9-10 July 1988, Szarvas, Hungary.

Dauksas, E., Venskutonis, P.R., Sivik, B., and Nillson, T. *(2002). Effect of fast CO2 pressure changes on the yield of lovage (Levisticum officinale Koch.) and celery (Apium raveolens L.) extracts*. Supercritical Fluids, 22, 20–2101.

Day, D.T. (1897). In: Proceedings of the first international petroleum congress, Paris, as cited by Brondz, I. 2002. *Development of fatty acids analysis by high-performance liquid chromatography, gas chromatography, and related techniques*. Analytica Chimica Acta, 465, 1-37.

Dayhuff, L and Wells, M.J.M. (2005). *Identification of fatty acids from fishes collected from Ohio river using gas chromatography-mass spectrometry in chemical ionization and electron impact modes*. Chromatography A, 1098, 144-149.

de Decker, E.A. (1999). *Possible beneficial effects of fish and fish n-3 polyunsaturated fatty acids in breast and colorectal cancer.* European. j. Cancer Preview, 8, 213-221.

Dyer, J.M., Chapital, D.C., Kuan, W.J., Mullen, R.T., Turner, C., McKeon, T.A and Pepperman, A.B. (2002). *Molecular Analysis of a Bifunctional Fatty Acid Conjugase/Desaturase from Tung. Implications for the Evolution of Plant Fatty Acid Diversity.* Plant Physiology, 130, 2027–2038.

Erfanullah, A.K.J. (1998). *Effect of dietary carbohydrate-to-lipid ratio on growth and body composition of walking catfish* (*Clarias batrachus*). Aquaculture, 161, 159-168.

Eskilsson, S.C. (2003). *Recent extraction techniques with emphasis on supercritical fluid extraction and microwave-assisted extraction,* Ph.D. Thesis, Lund University, Luna, Sweden.

Faust, C.B. (1992). *Modern chemical techniques*. The royal society of chemistry. London, U.K.

Fawkes, J., Albro, W.P., Walters, B.D., and McKinney, J.D. (1982). *Comparison of extraction methods for determination of polybrominated biphenyl residues in animal tissue.* Analytical Chemistry, 54, 1866-1871.

Fifield, F.W. (1993). Separation techniques. In: F.W. Fifield., and P.J. Haines (editors) *Environmental analytical chemistry*. Blackie academic and professional, London, U.K. 81-104.

Folch, J., Lee, M., and Stanley, G.H.S. (1957). *A simple method for isolation and purification of total lipids from animal tissue*. Biochemistry, 226, 497-509. www.cyberlipid.org/fattyt/fttooo1.htm (Accessed: April, 2005).

Francisco J. (2002). *On the extraction of essential oils by supercritical carbon dioxide and its influence on the mesomorphic phase structures.* Ph.D. Dissertation, Lund University, Luna, Sweden.

Gopakumar, K., and Nair, M.R. (1972). *Fatty acid composition of eight species of Indian marine fish*. Science of Food and Agriculture, Essex, 23, 493-496.

Grunger, E.H., Nelson, R.W., and Stansby, M.E. (1964). *Fatty acid composition of oils from 21 species of marine fish, freshwater fish and shellfish*. Americal Oil Chemist's Society. Champaign, 41 (10), 662-667.

Gunstone, F. (1996). *Fatty acid and lipid chemistry*. Blackie Academics and Professional. London, U.K.

Gunstone, F.D., Wijesundera, R.C., and Scrimgeour, C.M. (1978). *The component acids of lipids from marine and fresh water species with special reference to furan-containing acids.* Science of Food and Agriculture, Essex, 29, 539-550.

Gutierrez, L.E., and da Silva, R.C.M. (1993). *Fatty acid composition of commercially important fish from Brazil*. Science, Agricultura, Piracicaba, 50, 478-483.

Tapiero, H., Nguyen Ba, G., Couvreur, P., and Tew, K.D. (2002). *Polyunsaturated fatty acids (PUFA) and eicosanoids in human health*. Biomedical Pharmacother, 56, 215–222.

Halver, J.E. (2000). *Lipid and fatty acids*. University of Washington, Seattle, Washington DC, U.S.A.

Harris, W.S. (1997). *n-3 fatty acids and serum lipoproteins*: *human studies*. Clinical Nutrition, 65, 1645-1654.

Higgs, G.A. (1986). The role of eicosanoids in inflammation. Prog. Lipid res, 25, 555-561.

Hoffman, D. R., Birch, E. E., and Birch, D. G. (1993). *Lipids and fatty acids.* American. J. Clinical Nutrition*, 57 (5),* 807–812.

Hyötyläinen, T. (2006). Solutions for online coupling of extraction and chromatography in the analysis of food and agricultural samples. In: C. Turner, (eds), 2006. *Modern Extraction techniques:Food and agricultural samples*. Library of congress cataloguing. Washington DC, U.S.A.

Ibańez, E., Herrero, M., Martín-Álvarez., Señoráns, J.F., Reglero, G and Cifuentes, A. (2006). Accelerated solvent extraction: a new procedure to obtain ingredients from natural sources. In: C. Turner, (eds), 2006. *Modern Extraction techniques: Food and agricultural samples*. Library of congress cataloguing. Washington DC, U.S.A.

Jensen, C.L., Liorente, A.M., Voigt, R.G., Prager, T.C., and Heird, W.C. (1999). *Presented on the workshop on the essentiality of and dietary reference intakes (DRI) for Omega-3 and Omega-6 fatty acids.* The cloisters, National institure of health, USA.

Kang, J.X., and Wang, J. (2005). *A simplified method for analysis of polyunsaturated fatty acids.* BCM Biochemistry, 6, 5.

Karmeli, R.A. (1987). *Omega-3 fatty acids and cancer:* A review. In: W.E.M. Lands (editor), proceedings of AOAC short course on polyunsaturated fatty acids and Eicosanoids, American oil chemical society, Champaign, Illinois. 222-231.

Katikou, P., Hughes, S.I., and Robb, D.H.F. (2001). *Lipid distribution within atlantic salmon (salmo salar) fillets,* Aquaculture, 202, 89-99.

Kingston, H.M., and Jassie, L.B. (1988). *Introduction to microwave sample preparation,* American Chemical Society, Washington DC, U.S.A.

Kontogiannea, M., Gupta, A., and Ntanios, F. (2000). *Omega-3 fatty acids decrease endothelial adhesion of human colorectal cercinome cells.* Surgical Resources, 92, 201-205.

Kris-Etherton, P.M., Taylor, D.S., Yu-poth, S., Huth, P., Moriarty, K., Kishell, V., Harbrone, R.L., Zhao, G., and Etherton, T.D. (2000). *Polyunsaturated fatty acids in the food chain in the united states.* American. J. Clinical Nutrition, 71, 179-188.

Lambertsen, G., and Brækkan, O. (1965). *The fatty acid composition of cod liver oil*, series teknologiske undersøkelser. 11, 3-14.

Leaf, A., and Webber, P.C. (1988). *Cardiovascular effects of n-3 fatty acids*. New England J. of Medicine, 318, 549-555.

Levine, B.S. (1997). *Most frequently asked questions about DHA*. Nutrition Today, 132, 248-249.

Lloyd, A.H and., Young, K.Y. (1999). *Health benefits of docosahexaenoic acid (DHA).* Pharmacological Research, 40(3), 211-225.

Manning, B.B., Menghe, H.L., Robinson, E.H., and Peterson, B.C. (2006). *Enrichment of channel catfish (Ictalurus punctatus) fillets with conjugated linoleic acid and Omega-3 fatty acids by dietary manipulation.* Aquaculture, 261, 337-342.

McCarthy, A. (2001). *Methods for analysis and detection*. Cambridge university press, United Kingdom.

McCullough, M., and Lin, P. (2001). Nutrition, diet and hypertension. In A.M. Coulston., C.L. Rock., and E.R. Monsen (editor). *Nutrition in the prevention and treatment of disease.* Academic press, London. U.K. 78- 93.

Mendez, E., Ruth, M.G., Gustavo, I., Horacio, G., and Grompone, M.A. (1996). *Lipid content and fatty acid composition of fillet of six fishes from the Rio de la Plata.* Food Composition and Analysis, 9, 163-170.

Mendez, E., Gonzalez, R.M., Gustavo, I., Gludice, H., and Grompone, M.A. (1996). *Lipid content and fatty acid composition of fillets of six fishes from the Rio de la Plata*. Food Composition and Analysis, 9, 163-170.

Meed, J.F., and Kayama, M. (1967). Lipid metabolism in fish. *In: Fish oils*, edited by M.E. Stansby. Westport, Conn., Avi Publ. Co, 289-99.

Menong, T. (2005). *Dread disease clain in South Africa*: article. City press, July 2005.

Mensink, R., Janssen, M., and Katan, M. (1988). *Effect on blood pressure of two diets differing in total fat but not in saturated and polyunsaturated fatty acids in healthy volunteers.* American. J. Clinical Nutrition, 47, 976-980.

Microbial ID. (2000). *Fatty acid profiling by gas chromatography*.

Morris, M., Sacks, F., and Rosner, B. (1993). *Does fish oil lower blood pressure? A meta-analysis of controlled trials.* Circulation, 88, 523-533.

Osman, H., Zurría, A.R., and Law, E.C. (2001). *Fatty acid composition and colesterol contento f selected marine fish in malaysian waters.* Food Chemistry, 73, 55-60.

Pepping, J. (1999). *Omega-3 essential fatty acids*. American. J. Health-System Pharmacy, 56, 719-724.

Phleger, C.F., Nichols, P.D., and Virtue, P. (1997). *The lipid, fatty acid and fatty alcohol composition of the myctophid fish Electrona antarctica: high level of wax esters and food-chain implications.* Antarctic Science 9 (3), 258-265.

Piggot, G.M., and Tucker, B.W. (1990). *Effects of technology on nutrition*. Newyork, USA, Marcel Dakker.

Plummer, D.T. (1987). *A practical guide to biochemistry,* 3rd ed. Mc Graw-Hill, London, U.K.

Pompeia, C., Freitas, J.S., Kim, J.S., Zyngier, S.B., and Curi, R. (2002). *Arachidonic acid cytotoxicity in leukocytes: Implications of oxidative stress and eicosanoid síntesis*. Biology of the Cell, 94 (4), 251-265.

Pratt, S.G., and Matthews, K. (2004). *Super foods RX: fourteen foods that will change your life.* William Morrow, New York, U.S.A.

Quillet, E., Guillou, S.L., Aubin, J., and Fauconneau, B. (2005). *Two- way selection for muscle lipid content in pan-size rainbow trout (oncorhynchus mykiss).* Aquaculture, 45, 49-61.

Span, R., and Wagner, W. (1996). *A new equation of state for carbon dioxide covering the fluid region from the triple-point temperature to 1100K at pressures up to 800MPa*, Physical Chemistry Data, 25, 1509–1596.

Rasoarahona, R.E., Bernathan, G., Bianchini, J.P., and Gaydou, M.E. (2005). *Influence of seasons on the lipid content and fatty acids profiles of three tilapia species, (Oreochromis Niloticus, O. Macrochir and Tilapia Rendalli) from Madagascar*. Food Chemistry, 91(4), 683-694.

Reverchon, E., and De Marco, I. (2006). *Supercritical fluid extraction and fractionation of natural matter*. Supercritical Fluids, 38, 146-166.

Richter, B.E., Ezzell, J.L., Felix, D., Roberts, K.A., and Later, D.W. (1995). *Pressurized fluid extraction*. American Lab, 27, 24-28.

Robert, G.J. (1996). *The lipids in human milk*. Prog. Lipid Research, 35, 1, 53-92.

Rovessac, F., and Rovessac, A. (1992). *Chemical analysis: modern instrumentation methods and techniques.* John Willey and sons, Newport.

Saint' Ana, L.S., and Mancini-Filho, J. (2000). *Influence of the addition of antioxidants in vivo on the fatty acid composition of fish fillets*. Food Chemistry, 68, 175-178.

Salem, N., Greiner, R., Moriguchi, T., Woods, J., and Uauy, R. (1999). *Evidence for the essential nature of DHA in the human and rat nervous system:* A paper presented on the workshop on the essentiality of the dietary reference intakes (DRIs) for Omega-6 and Omega-3 fatty acids. National institute of health, Bethesda, U.S.A.

Schwalme, K., Mackay, W.C., and Clandinin, M.T. (1993). *Seasonal dynamics of fatty acids composition in female northern pike (Esox Lucius)*. Comparative Physiology, 144, 77-93.

Serhan, N.C., Gotlinger, K., Hong, S., and Arita, M. (2004). *Review Resolvins, docosatrienes and neuroprotectins, novel Omega-3-derived mediators, and their asprin-triggered endogenous epimers: an overview of their protective roles in catabasis.* Prostaglandins and Other Lipid Mediators, 73, 155-172.

Smith, R.M. (1999). *Supercritical fluids in separation science—the dreams, the reality and the future,* Chromatography. A, 856, 83–115.

Smith, R.M. (2003). *Before the injection-modern methods of sample preparation for separation techniques,* Chromatography. A, 1000, 3-27.

Somchit, N., Rajion, M.A., Zakaria, Z.A., and Mat Jais, A.M. (2005). *Fatty acid and amino acid composition of three local Malaysian Channa fish species.* Food Chemistry, 97, 674-678.

Stahl, E., Quirin, K.W., and Gerard, E. (1988). *Dense gases for extraction and refining*. Springer, Berlin, Heidlberg.

Stansby, M.E. (1990). Nutritional properties of fish oil for human consumption-early developments. In: M.E. stansby (editor). *Fish oils in nutrition*. Van nostrand reinhold, Newyork, U.S.A. 268-288.

Stansby, M.E. (1967). *Fatty acid pattern in marine, fresh water and anadromous fish*. American Oil Chemistry Society, 44, 64.

Stansby, M.E., Schlenk, H., and Gruger, E.H. (1990). *Fatty acid composition of fish*. In: M.E. Stansby (editor), *Fish oils in nutrition*. Van nostrand Rein hold, New York, U.S.A. 39-63.

Steffens, W. (1997). *Effects of variation in essential fatty acids in fish feeds on nutritive value of fresh water fish for humans*. Aquaculture, 151, 97- 119.

Steffens, W., Rennert, B., Wirth, M., and Kruger, R. (1999). *Effects of two lipid levels on growth, feed utilization, body composition and some biotic chemical parameters of rainbow trout,* Applied Chemistry, 15, 159-164.

Steffens, W., Wirth, M., Mieth, G., and Lieder, U. (1993). *Fresh water fish as a source of n-3 poly unsaturated fatty acids and their application to human nutrition*. In: S.J Kaushik and P. Luquet (editors), *fish nutrition in practice*. INRA, Paris, France. 469-474.

Tesarova, E., and Pacakova, V. (1983). *Solid phase extraction*. Chromatographia, 17, 269.

Thais, F., and Stahl, R.A.K. (1987). Effects of dietary fish oil on renal function in immune mediated glomerular injury. In: W.E.M. Lands (editor), *proceedings of AOAC short course on polyunsaturated fatty acids and Eicosanoids, American oil chemical society*, Champaign, Illinois, U.S.A. 123- 126.

Theodoridis, G.A., Zacharis, C.K., and Voulgaropoulos, A.N. (2006). *Automated sample treatment by flow techniques prior to liquidphase separations*: A review, Biochemical and Biophysical Methods, 453, 345-353.

Thies, F., Kokkema, M.R., and Eritsland, J. (2001). *Dietary supplement with eicosapentaenoic acid, but not with other long chain-3 or n-6 polyunsaturated fatty acids, decrease natural killer cell activity in healthy subjects aged > 55 years*. Clinical Nutrition, 73, 539-548.

Tissue, B.M. (1996). *Gas chromatography*. Science hypermedia, www.scimedia.com (Accessed: February, 2006).

Tocher, D.R., and Sargent, J.R. (1990). *Effects of temperature on the encorporation into phospholipids classes and metabolism via desaturation and elongation of n-3 and n-6 polyunsaturated fatty acids in fish cells in culture.* Lipids, 25, 435-442.

Tswett, M.S. (1903). Varshavskogo obschestva estestvoispsytatelei, Otd. Bio. 14. as cited by Brondz, I. 2002. *Development of fatty acids analysis by high-performance liquid chromatography, gas chromatography, and related techniques.* Analytica Chimica Acta, 465, 1-37.

Turner, C. (2006). *Agricultural simples: in Overview of modern extraction techniques for food and agricultural simples.* C. Turner, (editor.). Library of Congress Cataloging, 3-19.

Turner, C., Eskilsson, C.P., and Björklund, E. (2001). *Collection in analytical-scale supercritical fluid extraction*. Chromatography A, 947, 1-22.

Uauy, R., and Valenzuela, A. (1992). *Marine oils as a source of Omega-3 fatty acid in the diet: How to optimise the health benefits.* Food Nutrition Science, 16, 199-243.

Ill´es, V., Szalai, O., Then, M., Daood, H.G., and Perneczki, S. (1997). *Extraction of hiprose fruit by supercritical CO2 and propane*, Supercritical Fluids, 10, 209–218.

Varljen, J., Šulic, S., Brmalj, J., Batičić, L., Obersnel, V., and Kapović, M. (2003). L*ipid classes and fatty acid composition of Diplodus vulgaris and Conger conger originating from Adriatic Sea.* Food Technol. Biotechnology, 42 (2), 149-156.

Walker, A. (2003). *Getting the right balance of Omega-3 and 6. Health span. Advanced nutrition*. www.healthspan.co.uk/articles/article.aspx?/d=167 (Accessed: October, 2006).

Wang, Y. (1997). *Sample preparation/ concentration for trace análisis in GC-MS: a study of solid phase microextraction and headspace sampling*. Ph.D. Dissertation, Virginia Polytechnic Institute and State University, U.S.A.

Watanabe, T. (1982). *Lipid nutrition in fish*. Biochemistry physiology, 73, 3-15.

Wilkipedia. (2005). *Fatty acids*. http://en.wikipedia.org/wiki/Fatty_acid (Accessed: October, 2006).

Williams, R.S. (1997). *Nutrition and diet theraphy*, 8th ed. Mosby, Newyork, U.S.A. www.microbialid.com (Accessed: July, 2006).

Zahler, P., and Niggli, V. 1977. *Lipids extraction and purification*. Methods in Membrane Biology, 8, 1-50.

Zuraini, A., Somchit, M.N., Solihah, M.H., Goh, Y.M., Arifah, A.K., and Zakaria M.S. (2005). µChemLab Technology Team, "*Autonomous Micro-Chemical Analysis Laboratory (µChemLab Technologies),"* Sandia Report, SAND2005-2001, Sandia National Laboratories, Albuquerque.

APPENDIX

Fatty acid standard mixture calculations and weighing for preparation of 1000ppm stock solution.

For all Solid standards: Myristic, Palmitic, Stearic, fatty acids

1000ppm = 1000mg/l
1000mg → 1 liter
1g = 1000ml
X = 50ml

$$X = \frac{1g \times 50ml}{1000ml}$$

X = 0.05g

0.05g to mg
0.05g × 1000
= 50 mg

For each solid fatty acid standard, 50 mg was weighed and dissolved in 50 ml of chloroform, which is the extracting solvent.

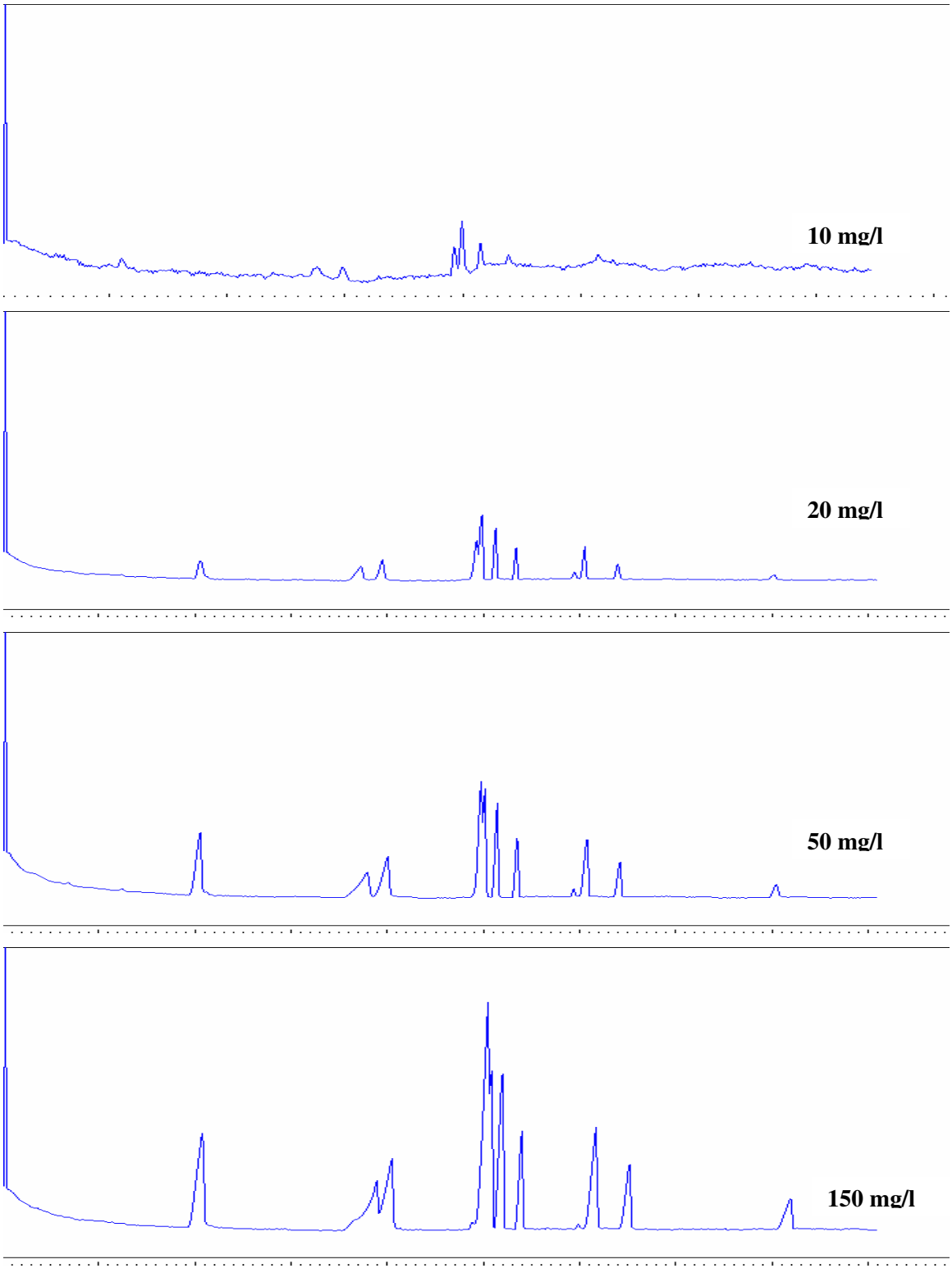
For liquid fatty acids standards: Palmitoleic, Oleic, Linoleic, Linolenic, arachidonic, Eicosapentaenoic and Docosahexeanoic acid.

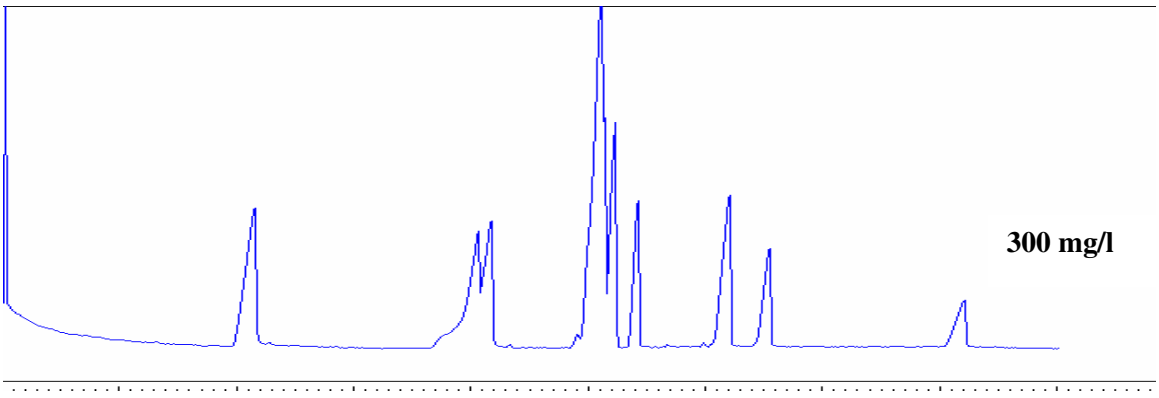
OLEIC: $V = \frac{m}{e}$

$= \frac{0.05\ g}{0.891\ g/cm^3}$

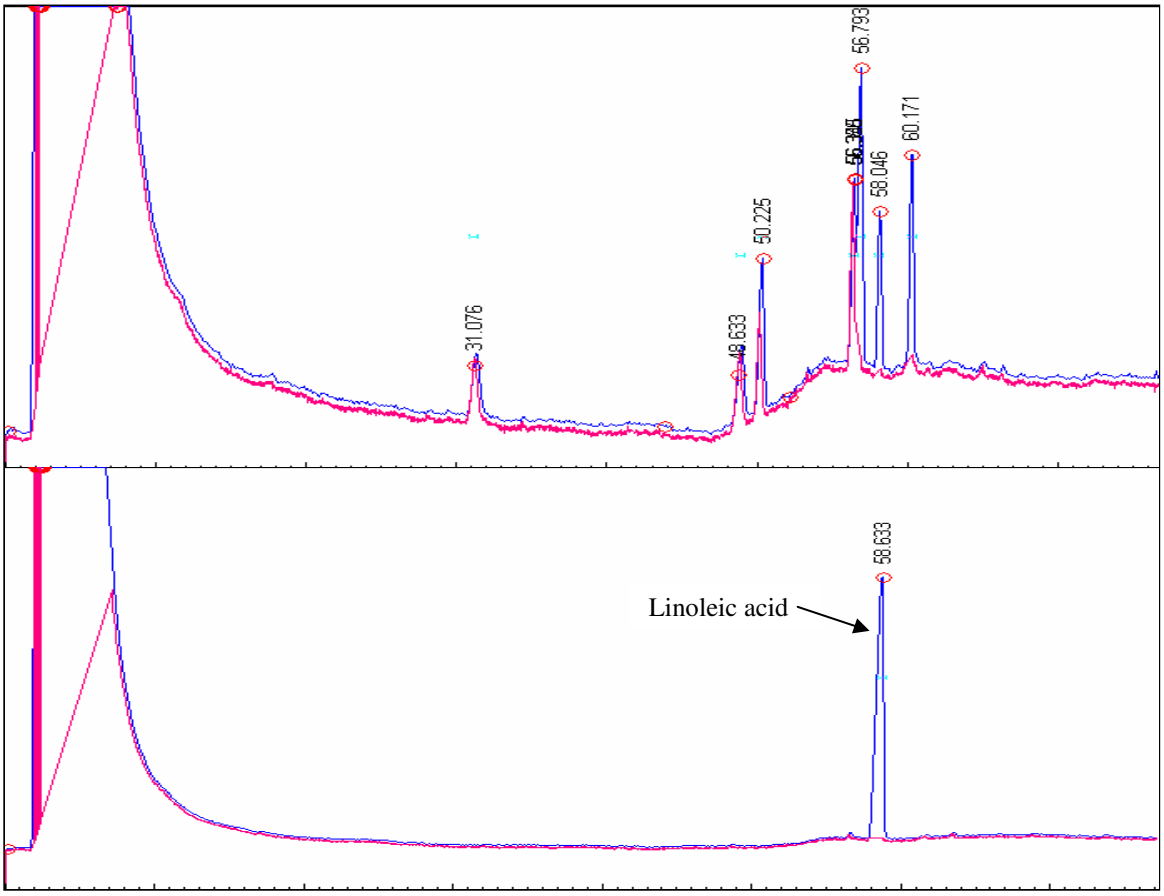
= 0.056 ml
= 56µl

Fatty acid Standards	Measured (µl)
PALMITOLEIC	55.93
OLEIC	56.0
LINOLEIC	55.4
LINOLENIC	54.6
ARACHIDONIC	54.2
EICOSAPENTAENOIC	53.0
DOCOSAHEXAENOIC	53.1

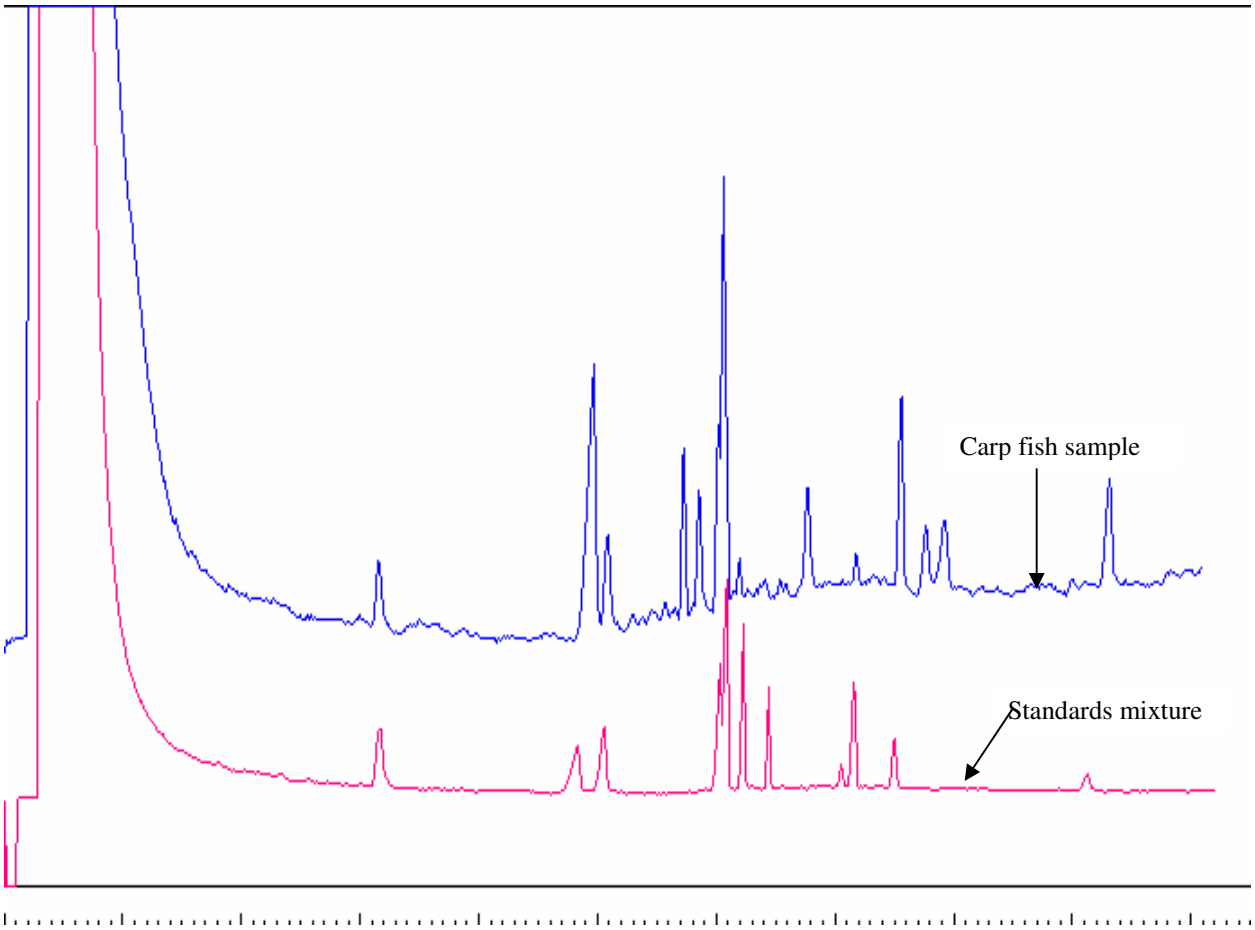

10 mg/l
20 mg/l
50 mg/l
150 mg/l



Chromatograms showing fatty acids standard mixtures at the concentrations of 10, 20, 50, 150, 300 mg/l.



Linoleic fatty acid identification through the use of an individual fatty acid analysis method.



Fatty acid identification from the carp fish through the use of fatty acids standard mixture.