CHAPTER 1

FISH LIPIDS AND THE HUMAN DIET

1.1 General Introduction

During the twentieth century a shift has occurred from infectious to chronic diseases as the major cause of death in Western populations. The role that dietary intake has to play in the onset of chronic diseases has been investigated since the early 1970s. In 1977 a statement on the dietary goals for the United States (U.S. Senate Select Committee on Nutrition and Human Needs, 1977) claimed that consumption of diets high in fat (particularly saturated fat), cholesterol, simple sugars, salt and alcohol could be linked with the onset of six to ten of the leading chronic diseases contributing to mortality in the United States.

The consumption of diets rich in fish by various populations and groups participating in clinical trials has been associated with a wide range of positive health effects. Some of these are: low incidences of cardiovascular diseases and mortality from atherosclerosis and heart disease; reduced inflammation with rheumatoid arthritis and psoriasis; and low blood pressure and viscosity. These effects are duplicated when fish oils are used in place of flesh, and therefore are believed to be due to the lipid composition.

The distinctive feature of the lipids in fish and seafoods generally (as compared with other animal and plant food sources) is that they are rich in long-chain (twenty or more carbons) omega-3 (ω-3) polyunsaturated fatty acids. Fish
have long been major components in the diets of many peoples (including Greenland Eskimos and Japanese). In recent years the consumption of fish by Western populations has increased, largely in response to their reported therapeutic and preventative effects on many degenerative diseases.

Fish display a high level of variability, both in their lipid content and composition, between species and within the one species due to factors such as age, maturity, and sampling location and season. Most of the studies to date, however, have dealt with northern hemisphere species from temperate waters. Little is known about the nature and variability of fish from southern hemisphere waters, and most of the data that are available is from tropical samples. The production of the positive health effects attributed to the consumption of high fish diets may require stipulation of species and catch details. This would involve a more detailed knowledge of the lipid compositions of fish than currently exists.

The fatty acid composition and flesh lipid contents of a number of lean temperate Australian species of finfish have been investigated in this work, and their nutritional value assessed relative to the more comprehensively studied northern hemisphere and tropical Australian species. The extent of the effects of seasonal and locational variations on these factors have also been determined.

The trend towards greater fish consumption together with the rapid rate of increase in the world's population in this century has led to a dramatic depletion of the fish stock of oceans and inland waters. It has been suggested that the answer to this problem is to farm fish, as is common practice for other animals. Such a practice, however, with its intensive feeding and stock selection for rapid weight gain, may lead to a reduction in the nutritional value of the fish (by
decreasing their ratio of polyunsaturated to saturated fats) (Crawford et al., 1989). Until this problem is overcome more efficient use of the current catch is needed.

The ω-3 polyunsaturated fatty acids that make fish valuable from a nutritional perspective are also largely responsible for their high susceptibility to oxidation. This lowers their nutritional value (mainly by reducing the vitamin E content) and may render them organoleptically unacceptable, due to the accumulation of lipid oxidation products. By processing some of the catch to improve their keeping qualities waste can be reduced and a greater proportion of the catch made available for human consumption.

Fish are usually cooked or processed in some way before consumption. Such treatments initiate chemical reactions within foods, and thus alter their composition, and perhaps their nutritional value. The stability of the fatty acid compositions and vitamin E contents of fish during two preservation treatments has been investigated in this study, in order to enable an evaluation of the extent of retention of nutritional value. The methods chosen were the traditional one of smoking, and the newer and more controversial technique of gamma-irradiation.

Existing data indicate that lipid content and concentration, is to some extent characteristic of the species of fish. When considering fish for their nutritional value it is important that the appropriate species be selected for consumption. A reliable method of species identification is therefore required. Species identification is also desired for the setting of fair prices (as great variation in eating quality exists between species) and legal requirements (notable for labelling of a product for export). Fish are often purchased in some processed form, necessitating species identification of processed fish as well. Fish species
Identification, however, is complicated by the fact that an enormous number of edible species exist, many of which are morphologically very similar. This problem has been addressed by further development of a high performance liquid chromatographic method for species identification, based on the analysis of sarcoplasmic protein extracts. This enables the establishment of a database from which future identifications can be made. The applicability of the method to the identification of processed fish was also investigated.

1.2 Structures of Lipid Molecules

1.2.1 Lipid Classes

The term 'lipid' refers to the vast array of compounds in organisms that are soluble in organic solvents, and only sparingly soluble in water. Such a general definition ensures that many different structures fall within this category. They are commonly classified as follows:

1) The neutral (simple) lipids are dominated by the triglycerides, which are triesters of fatty acids with glycerol (Figure 1.1). Other members of this class include waxes, cholesterol esters, and vitamin A and D esters.

2) The polar (compound) lipids are esters of fatty acids and an alcohol with an attached polar group. The dominant forms are the phosphoacylglycerols. Other types include inositol phosphatides, cerebrosides and sphingomyelins (Figure 1.1).

3) The derived lipids are formed by hydrolysis of the more fundamental
types detailed above. They include free fatty acids, mono- and di-
glycerides, and numerous alcohols (including the sterols).

4) Other lipid-soluble components include the carotenoids, squalene
steroids, vitamins A, D, E and K and some aliphatic hydrocarbons.

1.2.2 Fatty Acids

The bound fatty acids are found within triglyceride and phospholipid
molecules. These lipid forms contain a maximum of three esterified fatty acids.
Fatty acid molecules are aliphatic monocarboxylic acids (RCOOH). Their
structures are diverse, differing in the nature of the alkyl chain (R), which varies in:-

1) chain length,
2) the number of double bonds (up to 6), and
3) the positions of the double bonds.

Fatty acids are classified according to their degree of unsaturation (or
number of double bonds) into the following groups:

1) saturated fatty acids (SFAs) - no double bonds,
2) monounsaturated fatty acids (MUFAs) - one double bond, and
3) polyunsaturated fatty acids (PUFAs) - more than one double
   bond.

Within the unsaturated groups (MUFAs and PUFAs) a further classification
is necessary to indicate the position of the double bond(s). This also serves to
group fatty acids with very similar physiological roles and biosynthetic pathways
together. Each group is given the prefix 'omega-' (ω-), or 'n-', followed by a
FIGURE 1.1
Structures of Some Lipid Molecules

Triglyceride

\[ \text{O} \]
\[ \text{H}_2\text{C-O-C-R}_1 \]
\[ \text{O} \]
\[ \text{HC-O-C-R}_2 \]
\[ \text{O} \]
\[ \text{H}_2\text{C-O-C-R}_3 \]

\( R_1, R_2, R_3 \) - alkyl chains

Phospholipids
Phosphoacylglycerols

\[ \text{O} \]
\[ \text{H}_2\text{C-O-C-R}_1 \]
\[ \text{O} \]
\[ \text{HC-O-C-R}_2 \]
\[ \text{O} \]
\[ \text{H}_2\text{C-O-P-O-PE/PC} \]

PE • Phosphatidyl ethanolamine (cephalin)

PC • Phosphatidyl choline (lecithin)

\[ \text{H}_2\text{C}_2\text{H}_4\text{N(CH}_3\text{)}_3 \]

Phosphatidyl inositol

\[ \text{O} \]
\[ \text{H}_2\text{C-O-C-R}_1 \]
\[ \text{O} \]
\[ \text{HC-O-C-R}_2 \]
\[ \text{O} \]
\[ \text{H}_2\text{C-O-P-O-} \]

\[ \text{H}_2\text{O} \]

\[ \text{H}_2\text{C}_2\text{H}_4\text{N(CH}_3\text{)}_3 \]

Sphingomyelin

\[ \text{H}_3\text{C-(CH}_2\text{)}_{12}-\text{CH}^+\text{OH-CH-CH-CH-CH}_2 \]

\[ \text{O} \]

\[ \text{O} \]

\[ \text{O} \]

\[ \text{O} \]

\[ \text{O} \]

\[ \text{O} \]

Cerebroside

\[ \text{H}_3\text{C-(CH}_2\text{)}_{12}-\text{CH}^+\text{OH-CH-CH-CH-CH}_2 \]

\[ \text{O} \]

\[ \text{O} \]

\[ \text{O} \]

\[ \text{O} \]

\[ \text{O} \]

\[ \text{O} \]

\[ \text{O} \]

\[ \text{O} \]

Adapted from Gruger (1967)
number indicating which carbon is involved in the double bond closest to the methyl end of the molecule (numbered from that end). For example, the molecule shown in Figure 1.2 has a double bond between the sixth and seventh carbon from the methyl group, and so is a member of the omega-6 class (also written as ω-6 or n-6).

Common naturally occurring classes include the n-7, n-9 and n-11 MUFAs, and the n-3 and n-6 PUFAs.

A number of naming systems are used to designate individual fatty acid molecules. The most concise and often used method is given by the general formula:-

\[ a:bwc \text{ (or } a:bnc), \]

where \( a = \) the number of carbon atoms,

\( b = \) the number of double bonds, and

\( c = \) the position of the double bond nearest the methyl end.

This system states only the position of the first double bond, and assumes that the others lie in a methylene (-CH\(_2\)-) interrupted configuration between it and the carboxyl group.

Systematic names are also used, but they are more cumbersome and can cause confusion, as the IUPAC (International Union of Pure and Applied Chemistry) convention requires the carbons to be numbered from the carboxyl end, contrary to the abbreviated notation.

Prefixes may be added to fatty acid names designated by either method to indicate the geometrical configuration of the double bonds (\textit{cis-}, or \textit{trans-}). This is not a common practice, however, as the naturally occurring form is the \textit{all cis},...
FIGURE 1.2
Structure and Naming of a Fatty Acid

\[
\begin{align*}
\text{H}_3\text{C} & \quad \text{CH}_2 \quad \text{CH}_2 \quad \text{CH} = \text{CH} \quad \text{CH} = \text{CH} \quad \text{CH}_2 \quad \text{CH}_2 \quad \text{CH}_2 \quad \text{CH}_2 \quad \text{CH}_2 \quad \text{CH}_2 \quad \text{O} \\
\text{OR} & \\
\text{CH}_3(\text{CH}_2)_4 \text{CH} = \text{CHCH}_2 \text{CH} = \text{CH(CH}_2)_7\text{COOH}
\end{align*}
\]

Systematic name: 9,12-octadecadienoic acid

Trivial name (abbreviation): Linoleic acid (L or LA)

Abbreviated notation: 18:2 ω6 (or 18:2n6)
and so this is usually assumed to be the case.

Trivial names and their abbreviations are also commonly employed, as most
are fairly well known and easily recognisable. Figure 1.2 shows a fatty acid named
by each method.

1.3 Importance of Lipids in the Human Diet

Reports that Greenland Eskimos experience a low incidence of
cardiovascular diseases (Ehrström, 1951; Rodahl, 1954) and low mortality rates
from atherosclerosis and coronary heart disease (CHD) (Høygaard, 1941; Brown,
1951) prompted the epidemiological studies on this community by Bang, Dyerberg
and colleagues in the 1970s (Bang and Dyerberg, 1972; Bang et al., 1980). The
incidence of ischemic heart disease amongst this population has been found to be
between 8% (Kromann and Green, 1980) and 30% (Dyerberg and Bjerregaard, 1987)
that of Eskimos who had migrated to Denmark. These findings were correlated
with a higher intake of seafood in the Greenland Eskimos (Dyerberg, 1981). Similar
studies on populations in Japan (Yamori et al., 1985) and Holland (Kromhout et al.,
1985) found inverse relationships between the incidence of CHD mortality and fish
consumption. Interest was thus generated in the possible hypolipidemic and anti-
atherogenic effects of n-3 PUFAs.

Such studies have always involved many variables, however, and so only
indicated a correlation without being able to pinpoint a cause. A large number of
controlled clinical trials feeding n-3 PUFAs to humans have since been carried
out. Variations in the findings were explained by Harris (1989) to be most likely
due to differences in the mode of dietary modification (by fish oil or flesh consumption), the dose involved, and the lipid characteristic of the subjects (normal or hyperlipidemic), as well as their age and sex. He summarised the various findings by stating that n-3 PUFAs act to lower plasma triglyceride levels, but the effects on low- and high-density lipoprotein (LDL and HDL, respectively) levels have not been unequivocally demonstrated.

Some physiological effects reported from increased n-3 consumption include reduced blood pressure and viscosity, increased bleeding times and greater membrane fluidity (Wolfram, 1990; Lampila, 1986). They have also been found to reduce inflammation associated with rheumatoid arthritis and psoriasis, as well as the symptoms of multiple sclerosis (Goodnight et al., 1982; Kinsella, 1988). Conversely, an increased incidence in the onset of mammary cancer as well as enhanced growth in existing tumours has been reported in rats fed diets rich in n-6 PUFAs (Kinsella, 1986). This prompted Kearney (1989) to recommend a high ratio of LN to LA to prevent these effects, and Kinsella (1986) to suggest that dietary n-3 PUFAs may reduce the risk of cancer.

The various findings resulting from diets rich in n-3 PUFAs led Granström (1990) to predict that many degenerative diseases will be able to be treated by dietary modification, without the serious side effects associated with many commonly used drugs.

The mode of action of polyunsaturated fatty acids on various physiological functions is via the production of a family of compounds called eicosanoids, which are among the most physiologically potent naturally occurring components known to man (Granström, 1990). They regulate secretory, reproductive and circulatory
functions, as well as inflammatory and immune responses, to name but a few effects (Kinsella, 1986). Many eicosanoids act as antagonists so as to maintain homeostasis. For example, arachidonic acid (20:4n6, AA) produces both the platelet aggregatory thromboxane TXA$_2$ and the anti-aggregatory prostacyclin PGI$_2$, which work together to regulate blood clotting (thrombotic activity).

The inclusion of n-3 PUFAs into the human diet has many marked effects on eicosanoid-regulated functions. Eicosapentaenoic acid (20:5n3, EPA) and docosahexaenoic acid (22:6n3, DHA) are incorporated into organ and tissue lipids in preference to AA (Knapp et al., 1986; Swanson and Kinsella, 1986). They thus inhibit the production of 2-series eicosanoids by decreasing the availability of their precursor.

For many years it was believed that the sole eicosanoid precursor was AA. The feeding of fish oils to humans (Fischer and Weber, 1983) not only revealed that TXA$_2$ production was selectively inhibited over that of PGI$_2$, but that a much weaker platelet aggregator (the 3-series thromboxane analogue TXA$_3$) was produced in its place. The 3-series anti-aggregatory prostacyclin PGI$_3$ was also isolated. Dyerberg et al. (1978) had previously hypothesised the existence of a 3-series of eicosanoids. The mode of action of EPA, therefore, is not only to limit the production of 2-series eicosanoids (in some cases selectively), but also to produce the bioactive 3-series.

As well as their importance in altering eicosanoid production, n-3 class PUFAs are essential structural components of nerve tissue (especially cerebral grey matter), sperm cells, seminal vesicles, retinal photoreceptors and the membranes of the endoplasmic reticulum and mitochondria (Kinsella, 1986; Salem
et al., 1986). They are particularly important in the foetus and neonate for the
development of visual acuity, learning ability and membrane structures.

Recently, however, the n-3 PUFAs have been considered to be essential
fatty acids, as they are not able to alleviate all of the symptoms of linoleic acid
(18:2n6, LA) deficiency. They are now believed to have their own physiological
roles, and as they cannot be synthesised by humans in vivo, dietary inclusion of
linolenic acid (18:3n3, LN), the major plant n-3, has been recommended. Many
authors believe, however, that this is insufficient, as the in vivo synthesis of EPA
and DHA from LN is not only slow (Leaf and Weber, 1988; Deslypere, 1990), but
the enzymes involved are competed for by LA, with AA formation favoured over
EPA (Dyerberg, 1986). For these reasons direct ingestion of the long-chain n-3
PUFAs is often recommended.

Some vegetable and seed oils (such as soybean, walnut, canola and linseed)
are high in n-3 PUFAs, but they are of the shorter-chain LN (18:3n3) form. The
health effects discussed above have been those associated with direct long-chain
n-3 PUFA or fish flesh consumption. These compounds appear to be more
physiologically active due to the role of EPA as an eicosanoid precursor, but some
mention should be given to the demonstrated ability of LA-rich margarine to
produce anti-aggregatory effects and depress blood pressure (Johnston, 1987).
1.4 Dietary Recommendations for Lipid Consumption

The inclusion of fats in the diet is necessary, as they have many important biological functions: they are major structural components of membranes; serve as energy stores; provide a solvent for vitamins A, D, E and K; control blood lipids; and regulate eicosanoid production. Most of these functions require hydrolysis reactions to release the bound fatty acid molecules prior to incorporation into human tissue lipids. The fatty acid composition of the diet plays a major role, therefore, in determining that of the tissue and serum (Bragdon and Karmen, 1961; Seyberth et al., 1975; Fischer and Weber, 1984), and ultimately the physiological effects produced. It is thus important to be aware of the composition of the dietary lipids, so that the required beneficial or therapeutic effects can be obtained.

The increase in the incidence of numerous degenerative diseases in Western populations during the early twentieth century prompted recommendations that individuals should reduce the amount of total lipid in the diet. Following findings that saturated fats were associated with the elevated cholesterol levels accompanying coronary arterial diseases (Ahrens et al., 1957; Keys et al., 1957) it was recommended that the ratio of polyunsaturated to saturated fats (P/S) be increased (Dyerberg, 1986). The term 'polyunsaturated fat' was at this stage considered to be synonymous with n-6 PUFAs, and so it was widely recommended to replace animal fats in the diet with vegetable oils. With the gradual realisation by nutritionists that the two major classes of PUFAs produce different physiological responses a further factor was included in dietary guidelines, that of
the omega-3/omega-6 (n3/n6) ratio (sometimes expressed as the reciprocal). Some authors now even recommend that within the n-3 PUFAs consumed there be adequate EPA and DHA (Galli and Simopoulos, 1989).

While numerous factors are perceived to be relevant to the physiological effects of lipid consumption few of them have actually been precisely quantified in terms of tissue values or dietary compositions. There is general agreement, however, that a P/S ratio of approximately 1.0 is desired for the diet. A ratio of n-3 to n-6 PUFAs for the overall diet of 0.1 to 0.25 has been recommended by Neuringer and Connor (1986). Galli and Simopoulos (1989) agreed with the higher value, while Kinsella (1988) recommended a value of 0.33. It appears that an optimum range may exist for this ratio in human lipids, as excessively high values are associated with prolonged bleeding times (Dyerberg and Bang, 1979), thrombocytopenia and vitamin E deficiency (Goodnight et al., 1982), as well as an increased propensity to bruise, low platelet counts and suppressed aggregation (Simopoulos, 1986). An appropriate range has yet to be determined.

Recommendations for daily n-3 consumption vary greatly. Galli and Simopoulos (1989) suggested a daily intake of at least 0.8g of combined EPA and DHA, while other sources suggest that higher doses may be beneficial, such as can be achieved by supplementing the diet with fish oil. Many clinical trials, undertaken to examine the physiological effects of increased n-3 consumption, have involved the feeding to human subjects of 0.5 to 30g of n-3 PUFAs daily (Harris, 1989). These large doses were based on the daily intake by Greenland Eskimos and Japanese populations, which were of the order of 7g (Dyerberg, 1986). Kromhout et al. (1985), however, concluded from their epidemiological survey of
Dutch males over a 20 year period that consumption of even very small amounts (less than 14g) of lean fish daily exerted a measurable preventative effect on CHD mortality. They concluded that the long term consumption of even one or two fish meals weekly may be of preventative value in CHD. Low levels of fish intake were also found to be significant by Nelson (1972), and were noted as possibly effective by Deslypere (1990). Ackman (1990) suggested that ingestion of only 300mg of long-chain PUFAs daily may produce significant effects. This level is known to result in significant changes in the serum fatty acid compositions of humans (Sinclair et al., 1987).

Fish are the most widely available and affordable seafood to Western populations, and as such represent the major n-3 PUFA dietary source. The consumption of fish flesh, rather than the extracted oils, is generally recommended, as the extraction process retains much of the vitamin D and A, but little vitamin E, which may lead to toxicity by the former two and deficiency of the latter (Dyerberg, 1986; Deslypere, 1990).
CHAPTER 2

LIPID CHARACTERISTICS OF SOME TEMPERATE AUSTRALIAN
MARINE FISH SPECIES

2.1 Lipids in Fish

The edible portion of fish is comprised of 0.5 to 25% lipid (Ackman, 1990), but rarely exceeds 10%. Most of the existing data on fish consumption, and clinical studies, have involved very few lean (below 2% lipid) species.

2.1.1 The Distinctive Nature of Fish Lipids

Positive health effects noted in epidemiological studies performed in the 1970s on populations consuming large amounts of fish generated the initial interest in fish lipids. Since then much work has been carried out to investigate the unusual lipid characteristics of fish compared with other human dietary components.

The lipids of fish and many other seafoods differ from those of terrestrial animals and plants in their fatty acid compositions. They are more polyunsaturated than other animals, and contain much greater amounts of n-3 PUFAs, which are dominated by the long-chain (20- and 22-carbon) forms. Numerous other aquatic organisms also contain high levels of long-chain n-3 PUFAs, but they do not significantly contribute lipid to the human diet (Table 2.1).

Some fatty acids commonly found in fish are listed in Table 2.2. It can be
TABLE 2.1
Polyunsaturated Fatty Acids in Some Natural Foods (%mass)

<table>
<thead>
<tr>
<th>Food source</th>
<th>LA</th>
<th>LN</th>
<th>EPA+DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terrestrial</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lamb</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Chicken</td>
<td>13</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Safflower oil</td>
<td>75</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>52</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Linseed oil</td>
<td>24</td>
<td>47</td>
<td>0</td>
</tr>
<tr>
<td>Beans</td>
<td>28</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>Peas</td>
<td>10</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Spinach</td>
<td>12</td>
<td>62</td>
<td>0</td>
</tr>
<tr>
<td>Aquatic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White fish</td>
<td>1.5</td>
<td>6</td>
<td>43</td>
</tr>
<tr>
<td>Fatty fish</td>
<td>2</td>
<td>1</td>
<td>17</td>
</tr>
<tr>
<td>Crustacea</td>
<td>2.5</td>
<td>3</td>
<td>34</td>
</tr>
<tr>
<td>Molluscs</td>
<td>1.3</td>
<td>2</td>
<td>21</td>
</tr>
<tr>
<td>Phytoplankton*</td>
<td>0.8</td>
<td>0.3</td>
<td>32</td>
</tr>
<tr>
<td>Algae*</td>
<td>1.2</td>
<td>2.6</td>
<td>17</td>
</tr>
</tbody>
</table>

*From Crawford et al. (1989)
(all other data taken from Regtop (1988))

seen that the vast majority have even numbers of carbon atoms, as they are synthesised by continual addition of 2-carbon acetate units, usually to shorter chain fatty acids with even numbers of carbons.

2.1.2 Lipid Location and Purpose in Fish

Phospholipids are located in cellular and subcellular membranes, where they are important structural components, and control the transport of small molecules across cell and organelle membranes. The fatty acids present in these molecules play a major role in both of these functions. Polyunsaturates produce more
<table>
<thead>
<tr>
<th>Abbreviated form</th>
<th>Systematic name</th>
<th>Common name</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>Tetradecanoic</td>
<td>Myristic (M)</td>
</tr>
<tr>
<td>15:0</td>
<td>Pentadecanoic</td>
<td>Pentadecylic</td>
</tr>
<tr>
<td>16:0</td>
<td>Hexadecanoic</td>
<td>Palmitic (P)</td>
</tr>
<tr>
<td>17:0</td>
<td>Heptadecanoic</td>
<td>Margaric</td>
</tr>
<tr>
<td>18:0</td>
<td>Octadecanoic</td>
<td>Stearic (St)</td>
</tr>
<tr>
<td>20:0</td>
<td>Eicosanoic</td>
<td>Arachidic (Ad)</td>
</tr>
<tr>
<td>22:0</td>
<td>Docosanoic</td>
<td>Behenic</td>
</tr>
<tr>
<td>24:0</td>
<td>Tetracosanoic</td>
<td>Lignoceric</td>
</tr>
<tr>
<td>14:1n9</td>
<td>5-Tetradecenoic</td>
<td></td>
</tr>
<tr>
<td>16:1n7</td>
<td>9-Hexadecenoic</td>
<td>Palmitoleic (Po)</td>
</tr>
<tr>
<td>18:1n9</td>
<td>9-Octadecanoic</td>
<td>Oleic (O)</td>
</tr>
<tr>
<td>18:1n7</td>
<td>11-Octadecanoic</td>
<td>Oleic (O)</td>
</tr>
<tr>
<td>18:1n5</td>
<td>13-Octadecanoic</td>
<td>Oleic (O)</td>
</tr>
<tr>
<td>20:1n11</td>
<td>9-Eicosanoic</td>
<td>Gadoleic</td>
</tr>
<tr>
<td>20:1n9</td>
<td>11-Eicosanoic</td>
<td>Gondoic</td>
</tr>
<tr>
<td>20:1n7</td>
<td>13-Eicosanoic</td>
<td>Gondoic</td>
</tr>
<tr>
<td>20:1n5</td>
<td>15-Eicosanoic</td>
<td>Gondoic</td>
</tr>
<tr>
<td>22:1n11</td>
<td>11-Docosenoic</td>
<td>Erucic (E)</td>
</tr>
<tr>
<td>22:1n9</td>
<td>13-Docosenoic</td>
<td>Erucic (E)</td>
</tr>
<tr>
<td>22:1n7</td>
<td>15-Docosenoic</td>
<td>Erucic (E)</td>
</tr>
<tr>
<td>22:1n5</td>
<td>17-Docosenoic</td>
<td>Erucic (E)</td>
</tr>
<tr>
<td>24:1n9</td>
<td>15-Tetracosanoic</td>
<td>Nervonic</td>
</tr>
<tr>
<td>16:2n6</td>
<td>7,10-Hexadecadienoic</td>
<td></td>
</tr>
<tr>
<td>16:2n4</td>
<td>9,12-Hexadecadienoic</td>
<td>Linoleic (L/LA)</td>
</tr>
<tr>
<td>18:2n9</td>
<td>6,9-Octadecadienoic</td>
<td>Linoleic (L/LA)</td>
</tr>
<tr>
<td>18:2n6</td>
<td>9,12-Octadecadienoic</td>
<td>Linoleic (L/LA)</td>
</tr>
<tr>
<td>20:2n9</td>
<td>8,11-Eicosadienoic</td>
<td>Linoleic (L/LA)</td>
</tr>
<tr>
<td>20:2n6</td>
<td>11,14-Eicosadienoic</td>
<td>Linoleic (L/LA)</td>
</tr>
<tr>
<td>18:3n3</td>
<td>7,10,13-Hexadecatrienoic</td>
<td>Gammar-Linolenic</td>
</tr>
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<td>6,9,12-Octadecatrienoic</td>
<td>Linolenic (Ln/LN)</td>
</tr>
<tr>
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<td>Linolenic (Ln/LN)</td>
</tr>
<tr>
<td>20:3n6</td>
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<td>Homo-gamma-Linolenic</td>
</tr>
<tr>
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<td>11,14,17-Eicosatrienoic</td>
<td>Homo-gamma-Linolenic</td>
</tr>
<tr>
<td>16:4n3</td>
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<td>Stearidonic</td>
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<td>Stearidonic</td>
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<td>Stearidonic</td>
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<td>Arachidonic (An/AA)</td>
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<tr>
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<td>7,10,13,16-Decatetraenoic</td>
<td>Arachidonic</td>
</tr>
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<td>Timnodonic (EPA)</td>
</tr>
<tr>
<td>22:5n6</td>
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<td>(DPA)</td>
</tr>
<tr>
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<td>7,10,13,16,19-Docosapentaaenoic</td>
<td>(DPA)</td>
</tr>
<tr>
<td>22:6n3</td>
<td>4,7,10,13,16,19-Docosahexaenoic</td>
<td>Cervonic (DHA)</td>
</tr>
</tbody>
</table>

18
permeable and fluid membranes, with the double bond position as well as degree of unsaturation governing these properties (Salem et al., 1986). Membrane fluidity (which is enhanced by long-chain n-3 PUFAs) is important in marine inhabitants as it allows them to tolerate low water temperatures.

Triglycerides make up the intercellular fat and most of the adipose tissue, or storage fat. They largely serve as an energy reserve for times of food shortage and as a pool of fatty acids for phospholipid incorporation.

The phospholipids of living organisms usually differ from their triglycerides in that they have higher levels of polyunsaturates (Brockerhoff et al., 1963), and lower monounsaturate contents (Ackman, 1980). The preferential incorporation and maintenance of PUFAs in phospholipids ensures a degree of stability in the desirable membrane characteristics produced.

The relative amounts of triglyceride and phospholipid in a fish depends mainly on the total amount of lipid in a given mass of tissue. The lipids located in the muscle of very lean fish are almost entirely composed of phospholipid, with any triglyceride present stored in the liver. Conversely, fatty fish store much of their triglyceride in the muscle. Lipids extracted from the muscle of fatty fish, therefore, would have significant contributions made to their fatty acid contents by the triglyceride – which, owing to the different compositions of the two lipid types, would be reflected in the total fatty acid profiles.

2.1.3 Sources of Fatty Acids to Fish

Unlike plants, animals (including fish) lack the enzymes necessary to bring about interconversion between the various classes of unsaturated fatty acids
(Lands, 1986). The long chain n-3 polyunsaturates found in fish, therefore, must be of dietary origin, either directly, or through the consumption of shorter-chain precursors. The major biosynthetic pathways to the various long-chain fatty acids within the fish are given in Figure 2.1.

**FIGURE 2.1**

Fatty Acid Biosynthesis in Animals

n-6 series 18:2 → 20:2 → 22:2

18:3 → 20:3 → 22:3

20:4 → 22:4

22:5

n-3 series 18:3 → 20:3 → 22:3

18:4 → 20:4 → 22:4

20:5 → 22:5

elongase

desaturase

22:6
The production of fatty acids via this scheme, however, is not as important to the overall lipid composition as the components directly ingested (Stansby, 1981).

Various external factors have been found to play a role in determining the fatty acid composition of fish. Some of these include the age, stage of the spawning cycle and sex of the fish, as well as the season, location and year of catch. It can be seen that many of these factors are environmental, acting via the alteration of the fatty acid composition of the diet and general availability of food (Ackman, 1967).

Diet, therefore, is the most important factor governing the fatty acid composition of individuals of a species of fish. It is also, in turn, the major determinant of their therapeutic effects in humans. Most of the fish consumed by the world’s human population, however, is from catches of wild fish from the oceans (87.7%) (Hilcoat, 1990). No control can be exerted over their all-important dietary intake and resultant fatty acid compositions. With a knowledge of the factors that effect the diets of fish, however, species selection for dietary incorporation can be made on the basis of those conditions known to favourably affect the profile.

2.1.4 Variability in Composition

The season and location of catch have been claimed to affect the fatty acid composition of fish species (Gruger, 1967; Exler et al., 1975), but the extent of their effects have not been given much attention.

Despite a generally accepted view that seasonal variations in fish fatty acid
compositions occur (Gruger, 1967; Exler et al., 1975), very few authors have even stated the season of catch of their samples. Fewer still have undertaken studies to assess the extent of the variations (Deng et al., 1976; Krzynowek et al., 1990).

Similarly, few studies have concentrated on lipid comparisons between individual fish of the same species from different locations. Problems arise in attempting to compare existing data from various locations, due to differences in the species involved, and general lack of information about the season or water temperature at the time of catch.

There appears to be a correlation between many desirable lipid characteristics, such as high P/S and n3/n6 ratios and low water temperature. Ackman (1990) suggested that fish from non-tropical locations are better for consumption. Another factor, however, has commonly been claimed to be important in selecting fish on the basis of their locality - that of the hemisphere of catch. The validity of this claim is difficult to substantiate because of the lack of standardisation in sampling factors (such as season and water temperature) for the data on which it is based. Much data exist on the lipid characteristics of fish from temperate northern hemisphere waters, as this is where the majority of the world’s human populations, and therefore markets, are located. Little is known, however, about the lipids of southern hemisphere species. Most of the relevant studies carried out have involved fish caught from tropical waters (north of 23°30'S) (Brown et al., 1989), involving very small sample sizes (often only one or two individual fish). The sampling sites on either side of the equator, therefore, have differed greatly in water temperature. Claims have been made on the basis
of the available data that fish from southern hemisphere waters off the Australian coastline are less nutritionally desirable than those from the opposing side of the equator, due to the higher AA and lower EPA contents, and therefore smaller n3/n6 ratios of their lipids (Gibson, 1983; Ackman, 1990). Comparison of northern and southern hemisphere fish is complicated by the fact that few if any species are distributed over both hemispheres, and their identification is hampered by a lack of standardisation in both the common and scientific names.

The fatty acid compositions and lipid contents of some common commercial fish species inhabiting temperate Australian marine waters have been investigated in this study, together with their dependence on catch location and season.

The findings are discussed with reference to existing data on Australian fish, with consideration of the differences in sampling factors.

This enabled conclusions on the nutritive value of the lipids in fish from temperate Australian waters relative to that of northern hemisphere species to be made, based on comparisons with a comprehensive sample of commercial species from a similar distance from the equator caught during the same season.
2.2 Experimental

2.2.1 Samples

Normal adult sized samples of common edible marine finfish species from eastern Australian temperate (south of the Tropic of Capricorn, 23°30'S) coastal waters were obtained (Table 2.3) from commercially netted catches. They were supplied by the Sydney Fish Marketing Authority (FMA) and species identifications were performed by the FMA biologist.

Fillets were removed and stored at -22°C for up to six months prior to thawing for analysis.

Water temperatures for Sydney were supplied by the staff of the Naval Weather Centre, H.M.A.S. Albatross (East Nowra, NSW). Those for Victorian and Queensland waters were obtained from the National Meteorological Centre (Melbourne, Vic.)

2.2.2 Sample Preparation and Extraction

Thawed fillets were minced individually, after removing any visible skin and dark flesh. A sample (5g) of the white muscle thus obtained was then removed and extracted by the Bligh and Dyer (1959) method, as adapted by Kinsella et al. (1977), with internal standards added (Figure 2.2). Calcium chloride was added (approximately 2mg) to ensure salting out of the more polar phospholipids from the aqueous phase.

Mass spectral analyses of some large peaks occurring after DHA (the final fatty acid detected) revealed contamination of samples with plasticizers. Care was
taken to prevent contact of subsequent samples with plastics (such as the stoppers of volumetric flasks and vials).

<table>
<thead>
<tr>
<th>Species Scientific name (Common name)</th>
<th>Location of catch</th>
<th>Time of catch</th>
<th>Water temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acanthopagrus australis</em> (Black/Silver bream)</td>
<td>Sydney, NSW (33°53'S)</td>
<td>Spring, 1989</td>
<td>16–20°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Autumn, 1990</td>
<td>20–23°C</td>
</tr>
<tr>
<td></td>
<td>Hervey Bay, Qld (24°40'S)</td>
<td>Spring, 1990</td>
<td>24–25°C</td>
</tr>
<tr>
<td></td>
<td>Lakes Entrance, Vic. (37°50'S)</td>
<td>Spring, 1990</td>
<td>12–14°C</td>
</tr>
<tr>
<td><em>Centroberyx affinis</em> (Nannygai/Redfish)</td>
<td>Wollongong, NSW (34°25'S)</td>
<td>Spring 1989</td>
<td>16–18°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Autumn, 1990</td>
<td>21–23°C</td>
</tr>
<tr>
<td><em>Zeus faber</em> (John dory)</td>
<td>Sydney, NSW (33°53'S)</td>
<td>Spring, 1989</td>
<td>16–20°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Autumn, 1990</td>
<td>20–23°C</td>
</tr>
<tr>
<td><em>Zenopsis nebulosus</em> (Mirror dory)</td>
<td>Ulladulla, NSW (35°21'S)</td>
<td>Spring, 1989</td>
<td>16–17°C</td>
</tr>
<tr>
<td><em>Genypterus blacodes</em> (Ling)</td>
<td>Ulladulla, NSW (35°21'S)</td>
<td>Spring, 1989</td>
<td>16–17°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Autumn, 1990</td>
<td>20–23°C</td>
</tr>
</tbody>
</table>
FIGURE 2.2
Lipid Extraction Procedure

5.0g fish + 15mL A\textsuperscript{a} + internal standard(s)\textsuperscript{b} + CaCl\textsubscript{2} (anhydrous) (approx. 2mg)

\[ \downarrow \]

homogenise 2 minutes

\[ \downarrow \]

add 5mL CHCl\textsubscript{3}

\[ \downarrow \]

homogenise 30 seconds

\[ \downarrow \]

add 5mL distilled deionised water

\[ \downarrow \]

centrifuge

\[ \downarrow \]

collect CHCl\textsubscript{3} layer

\[ \downarrow \]

dry over Na\textsubscript{2}SO\textsubscript{4} (anhydrous)

\[ \downarrow \]

overnight in freezer, under N\textsubscript{2}

\[ \downarrow \]

decant CHCl\textsubscript{3} layer and

\[ \downarrow \]

make up to 10mL

lipid extract in 10mL of CHCl\textsubscript{3}

\textsuperscript{a} Solution A: CH\textsubscript{3}OH:CHCl\textsubscript{3} (2:1)
\textsuperscript{b} 0.50 to 1.00mL of Trinonadecanoin and/or Phosphatidylcholine diheptadecanoyl
2.2.3 Internal Standards

Trinonadecanoin (99% pure, Sigma Chemical Co.) was included in the extraction (0.50 to 1.00mL of a 4mg/mL chloroform solution) for total fatty acid quantification. This is a triglyceride molecule containing three esterified saturated nonadecanoic acid (19:0) groups (Figure 2.3). This fatty acid is not generally reported to occur naturally in fish.

The samples also undergoing analysis of their phospholipid fatty acids had phosphatidylcholine diheptadecanoyl (98% pure, Sigma Chemical Co.) added (in a similar manner to the triglyceride standard). This is a choline phospholipid (Figure 2.3) containing two bound heptadecanoic acid (17:0) groups - a fatty acid that has generally been reported to occur in fish in only trace amounts.

**FIGURE 2.3**
Internal Standards

\[
\begin{align*}
\text{Trinonadecanoin} & \quad \text{Phosphatidylcholine diheptadecanoyl} \\
(C_{60}H_{110}O_8) & \quad (C_{40}H_{64}O_6PN)
\end{align*}
\]
2.2.4 Extract Fractionation

Isolation of the phospholipids was performed using aminopropyl bonded phase cartridges (500mg sorbent, Waters, MA) by the method of Kaluzny et al. (1985). This is a rapid (approximately 10 min), high purity separation technique that produces high yields (in excess of 95%). The process is outlined in Figure 2.4.

**FIGURE 2.4**

Lipid Fractionation

Aminopropyl bonded phase cartridge → 4mL hexane (conditioner) → 2mL lipid extract in chloroform → 4mL A^a → 4mL methanol → 4mL 2% acetic acid in diethyl ether → discard first 3 drops → Phospholipids

A^a = 2:1 chloroform:2-propanol
2.2.5 Methyl Ester Formation

Methyl esters of the bound fatty acids were formed by adding methanolic sodium methoxide (approximately 150μL, 0.5M) to the extract (1mL) and allowing it to react at room temperature for at least 5 minutes (Glass and Christopherson, 1969).

The mixture was then evaporated to dryness at room temperature under a stream of nitrogen (N₂). The methyl esters formed were then redissolved in chloroform (100μL) before GC analysis.

Fatty acids methyl esters (FAMEs) of the phospholipid fraction were formed as described in Figure 2.5.

FIGURE 2.5
Preparation of FAMEs from the Phospholipid Fraction

approx. 4mL phospholipids
\[ \downarrow \]
in methanol
neutralise with saturated NaOH/CH₃OH
\[ \downarrow \]
add 100μL NaOCH₃ solution
\[ \downarrow \] allow to stand 5 minutes
\[ \downarrow \] extract into 3mL hexane
\[ \downarrow \] evaporate to dryness
add 100μL CHCl₃
\[ \downarrow \] inject onto GC

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2.2.6 Total Lipid Content Determination

Aliquots (2mL) of the total lipid extracts were evaporated in a stream of nitrogen at room temperature in tared vials. Determination of the mass of residue enabled calculation of the lipid content (%lipid) in the muscle sample. Duplicate determinations were performed on each extract.

2.2.7 Gas Chromatographic Analysis

FAME solutions (1μL) were injected into an Hewlett-Packard 5890A gas-liquid chromatograph (GC), equipped with a flame ionisation detector (FID) and a DB-23 fused silica capillary column (30m x 0.32mm i.d. x 0.25μm, J&W Scientific). The carrier gas was hydrogen, at a linear velocity of 21.5cm/s. Split injection was used, with a split ratio of 1:60. Operating conditions were: detector, 220°C; injection port, 240°C. The column oven was held at 175°C for 12 minutes, then heated at 5°C/min to 220°C, and held for 9 minutes. The output was recorded and integrated using a computing integrator package (DAPA Scientific Software, Western Australia).

2.2.8 Data Handling

Peak areas were corrected for relative theoretical response factors of each FAME to FID (Craske and Bannon, 1988). They were then recorded as percentages of total fatty acid content. The total amount of fatty acid extracted from each flesh sample (mg/g) was calculated, as were P/S and n3/n6 ratios (after totalling the relevant fatty acid classes).

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2.2.9 Combined Gas Chromatography-Mass Spectrometry (GC-MS)

An Hewlett Packard gas chromatograph (HP 5890 Series II) with split injector (ratio 1:30) coupled to an Hewlett Packard mass selective detector (HP 5971A) was used to determine the identities of the FAMEs. A DB-23 fused silica capillary column (30m x 0.22mm i.d. x 0.25μm, J&W Scientific) was used, and heated as detailed in the previous section. Detector and injector temperatures were 280°C and 250°C, respectively.

The mass selective detector had an electron energy of 70 eV. The ion source was run in EI mode, at 190°C. The scan rate was 0.9 scans/second.

Mustang software (Hewlett Packard) was used for collection and analysis of the data.

2.2.10 Statistical Analysis

The mean fatty acid percentage compositions were calculated for each sample (from the ten individuals analysed), along with the standard deviations and coefficients of variability of the major components.

General comparisons of various sets of fatty acid percentage area profiles required multivariate analysis. The method chosen was Principal Components Analysis (PCA), as it enables visual comparisons to be made based on a reduced number of variables (components) that represent most of the original total variance. The data matrix was constructed from all variables (fatty acids) present at above 0.5 to 1.0% (depending on the number of observations (individual fish) and memory constraints). All components present at significant levels were retained by this selection process. The raw matrix was standardised during the procedure.
Two-dimensional scatterplots were produced from the first two principal components in each case, and were used to determine the existence of clusters for samples. Further information was obtained by the use of spider-web plots (Aishima, 1979) to display the appropriate number of principal components, as were determined by inclusion of all components with eigenvalues exceeding 1.0 (as such components each represent more than the average amount of the variance). This criterion was chosen as it was found to consistently require a large number of components in comparison with the other tests attempted (which included the Scree test, Bartlett's Significance Test and the number of components required to explain over 90% of the total variance).

Spider-web plots use radial axes corresponding to the component numbers on which the mean values (± standard deviation) for each group (sample) are plotted. They thus enable visual discrimination between groups while maintaining virtually all of the variance of the data set.

Biplots were formed by overlaying scatterplots with data vectors to display the variables correlating with each sample point. Spider-web plots gave similar information for the principal components (which are comprised of weighted combinations of the variables).

Comparisons of nutritionally important ratios and individual fatty acids between samples was carried out by both parametric and non-parametric statistical methods. In each case the null hypothesis was that the means of all groups were the same \( (H_0: \mu_1=\mu_2=\mu_3) \) at the 95% confidence level \( (\alpha=0.05) \).

Analysis of Variance (ANOVA) by the least square differences method (LSD) was performed, which assumed that the underlying populations had normal
distributions and equal variances. This test enabled visual discrimination - from the disjointedness of the error bars in the least squared differences plots. This is valid for sample sizes of ten or more (Browne, 1979), as was the case here.

Two-sample non-parametric comparisons were made with the Mann-Whitney test (Mann and Whitney, 1947), while those between more than two samples were made by the Kruskal-Wallis single factor analysis of variance (Kruskal and Wallis, 1952). Both of these tests utilise the rank order of the data, rather than the actual values themselves. Null hypothesis rejection in the Kruskal-Wallis test required a Tukey-type test (Zar, 1984) to determine where the differences lay, and their nature.

Simple regression analysis was used to determine the nature of the relationship between various factors and the total tissue contents of either fatty acids or lipid. Linear, hyperbolic (reciprocal), exponential, or multiplicative curves were fitted, with coefficients of determination stated.

ANOVA, PCA and regression analysis calculations and plots were carried out using a statistical software package (STATGRAPHICS, Statistical Graphics Corporation, Maryland, U.S.A.). All other calculations and tests were performed manually.
DISCUSSION

2.3 Development of a Method for Fatty Acid Methyl Ester (FAME) Preparation

Fatty acids are esterified to their methyl esters to ensure adequate volatility for GC analysis. Methyl esters of bound fatty acids were formed by reaction with sodium methoxide. The reaction is complete in approximately five minutes (Glass and Christopherson, 1969), and requires no heating (Shehata et al., 1970), thus reducing the likelihood of oxidation of highly polyunsaturated fatty acids. It was used on fish oils by Lamothe et al. (1988), where it was found to completely methylate their fatty acids. The method is a direct trans-esterification reaction (see mechanism, Figure 2.6), and, therefore, does not allow for the partial isomerisation or oxidation known to occur in fish lipids under some saponification conditions (Böttcher et al., 1959; Jamieson and Reid, 1965; Ackman, 1969). No removal of free fatty acids was necessary (Bannon et al., 1982), as their levels are very low in fish.

The total lipid extract was analysed, as this represents the fatty acid mix ingested when fish are eaten.

Phospholipid fatty acids were analysed to indicate the nature and variability in the membrane-bound lipids. The phospholipids were separated from the rest of the lipid extract by sorbent fractionation prior to trans-esterification for the separate analysis of their fatty acids. It was found that neutralisation of the phospholipid eluate from the lipid fractionation cartridge (with methanolic
potassium hydroxide) was necessary in order to provide favourable conditions for the trans-esterification reaction.

FIGURE 2.6
Transmethylation of Fatty Acid Esters by Sodium Methoxide

\[
\begin{align*}
R'\text{-C-OR''} + \text{Na}^+\text{OCH}_3^- & \overset{\text{Methanol}}{\leftrightarrow} \text{R'}\text{-C-OR''} + \text{Na}^+ \\
\text{OCH}_3^- & \\
\hline
R' = & \text{fatty acid chain} \\
R'' = & \text{rest of lipid molecule} \\
\hline
\text{R'-COOCH}_3^- + \text{Na}^+\text{OR''}^- & \\
\text{Fatty acid methyl ester}
\end{align*}
\]

Adapted from Bannon et al. (1982)

Initially both methyl ester extracts were reduced in volume, prior to injection onto the GC. This often caused sedimentation problems, due to the formation of sodium salts, such as the glyceroxide (Christopherson and Glass, 1969). A cleaner extract was produced by evaporating extracts to dryness and redissolving non-polar materials (mainly fatty acid methyl esters) in a small volume of chloroform. Injection of this reaction mixture onto the column at least 200 times over a two year period had no visible effect on its separating ability, as was also noted by Bannon et al. (1982) when using light petroleum ether as the solvent.
2.4 FAME Chromatograms and Peak Identification

The total lipid fatty acid chromatograms from the five species exhibited the same major peaks. There were also some prominent peaks characteristic to each species (Figures 2.7A and 2.7B). The fatty acids of the phospholipid fraction were remarkably similar in nature for all three samples (Figure 2.8).

The fatty acids detected in the Australian fish studied are listed in Table 2.4, along with the method of peak identification. Initially most fatty acid peaks were tentatively identified by comparison of their GC retention times (as methyl esters, FAMEs) with authentic compounds. The identities of peaks for which no such standards were available were tentatively assigned from the nature of the surrounding peaks, and the order of appearance established by other authors.

Mass spectral analysis was carried out on Bream and Redfish extracts to verify these assignments. Spectra obtained were compared with the available library, as well as studied visually for their fragmentation patterns, as detailed below.

The identification of saturated FAMEs by mass spectral analysis is usually straightforward. A molecular ion (M$^+$) peak, acylium ion (RCO$^+$, m/z=M-31), as well as a series of peaks for fragments of general formula $\text{CH}_2\text{O}_2\text{C(CH}_2\text{)}_n^+$ (Christie, 1982) are commonly observed. The base peak (B$^+$), occurring at m/z=74, is the result of a McLafferty rearrangement (Figure 2.9).

Distinction between mono- and poly-unsaturated FAMEs was made from their base peaks (m/z=55 and 79, respectively). Molecular ions were usually visible,
FIGURE 2.7 A  Fish Fatty Acid GC Chromatograms

Total Lipid Extracts
Black bream

Redfish

Ling

Retention Time (minutes)
FIGURE 2.7B  Fish Fatty Acid GC Chromatograms

Total Lipid Extracts

John dory

Mirror dory

Retention Time (minutes)
FIGURE 2.8  Fish Fatty Acid GC Chromatograms

Phospholipid Extracts

Black bream
Queensland

Black bream
Victoria

Ling
Sydney

Retention Time (minutes)
### TABLE 2.4
Fatty Acid Identities

<table>
<thead>
<tr>
<th>Peak Order</th>
<th>Identity</th>
<th>Basis for identification</th>
<th>Mass spectrum</th>
<th>Retention time*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14:0</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>15:0i</td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>15:0</td>
<td></td>
<td>+</td>
<td>+</td>
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<td>4</td>
<td>16:0i</td>
<td></td>
<td>+</td>
<td>-</td>
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<td>16:0</td>
<td></td>
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<td></td>
<td>+</td>
<td>+</td>
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<td>7</td>
<td>17:0i</td>
<td></td>
<td>+</td>
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<td>17:1n?</td>
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<td>17:1n8</td>
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<td>11</td>
<td>U1 (18:0i?)</td>
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</tr>
<tr>
<td>15</td>
<td>U2 (18:1n5?)</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>18:2n6 (LA)</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>17</td>
<td>U3 (18:2n3?)</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>18</td>
<td>18:3n6</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>19</td>
<td>18:3n3 (LN)</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>20</td>
<td>U4</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>21</td>
<td>18:4n3</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>22</td>
<td>20:0</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>23</td>
<td>20:1n9</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>24</td>
<td>20:1n7</td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>25</td>
<td>20:1n5</td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>26</td>
<td>20:2n9</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>27</td>
<td>20:2n6</td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>28</td>
<td>20:3n6</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>29</td>
<td>20:4n6 (AA)</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>30</td>
<td>U5 (20:3n3?)</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>31</td>
<td>20:4n3</td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>32</td>
<td>20:5n3 (EPA)</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>33</td>
<td>22:1n11</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>34</td>
<td>22:1n9</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>35</td>
<td>22:2n6</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>36</td>
<td>22:2n3</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>37</td>
<td>22:3n3</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>38</td>
<td>22:4n6</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>39</td>
<td>21:5n3</td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>40</td>
<td>22:5n3 (DPA)</td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>41</td>
<td>22:6n3 (DHA)</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*compared with that of an authentic standard

40
which, when compared with the region of the chromatogram that the FAME appeared in, enabled molecular formula determination. It is more difficult, however, to determine the positions of the double bonds from these mass spectra, as only slight differences exist between the various classes of unsaturates (Horman et al., 1989).

Characterisation of some of the FAMEs towards the end of the chromatograms was made difficult by the fact that their spectra were all very similar (originating from long-chain polyunsaturates), and the molecular ion peaks were sometimes not detected. Peak number 39 (Table 2.4) was tentatively identified as 21:5n3. This was done on the basis that it was a polyunsaturate (B⁺=79) and lacked ions characteristic of the 22-carbon PUFA spectra. Previous authors have usually assigned the peak occurring in this relative position to be the FAME of 22:5n6 (Gibson, 1983; Sinclair et al., 1983; Fogerty et al., 1986; Brown et al., 1989), or even 22:4n3 (Evans et al., 1986). The assignment here agrees with that of Beltrán and Moral (1991) and Ackman (1980). A more definitive
identification would require the availability of appropriate authentic fatty acids.

Odd chain 15- and 17-carbon saturated fatty acids were detected (as their FAMEs), along with their iso- and ante-iso- forms. (The ante-iso 17:0 is not listed, as its concentration was not significant.) These fatty acids probably originate from the endogenous intestinal bacteria in the fish (Hamid et al., 1978). Some iso- 16:0 was also found. Branched chain fatty acids were also reported to be in the fish oils analysed by Ratanayake et al. (1989).

Five minor peaks are listed as unknowns, as they were not detected in the extracts when analysed by mass spectrometry, and did not correspond to any authentic samples available. They usually only occurred at very low levels, and so have not been included in the data sets.
2.5 Fatty Acid Compositions of Australian Fish Species

2.5.1 Individuals Within a Single Sample

2.5.1.1 Total Lipid Extracts

Fatty acid compositions, lipid contents, and other nutritionally significant values are presented in Table 2.5 and Tables 1A and B (Appendix I). Each sample mean was calculated from the fatty acid chromatographic profiles of the total lipid extracts obtained from ten individual fish.

The tissue lipid content values (g lipid/100g flesh) were extremely low for some samples (namely John dory and Ling caught during Spring). While these levels are lower than those commonly believed to be necessary for the maintenance of membrane integrity, they are similar to those reported by various authors using similar extraction methods (including Gibson et al., 1984; Evans et al., 1986; Fogerty et al., 1986; Hearn et al., 1987; Ackman and McLeod, 1988; and Dunstan et al., 1988). They are also above the theoretical minimum tissue lipid content of approximately 0.20%, calculated using a Bream sample (see section 2.5.1.3).

A number of measures were taken to minimise any lipid loss during the commonly used Bligh and Dyer extraction procedure and to evaluate the reproducibility of the method. Bligh and Dyer (1959) estimated that their lipid recoveries were greater than 94%. The modified procedures adopted in this work to ensure good recoveries were:

1) replacement of the filtration step with centrifugation to prevent lipid loss via absorption on the filter paper and/or flesh. The
TABLE 2.5
Important Fatty Acid and Lipid Composition Factors for Total Fatty Acid Fraction (n=10) (with % variabilities)

<table>
<thead>
<tr>
<th>Factor</th>
<th>Black bream</th>
<th>Victoria</th>
<th>Queensland</th>
<th>Redfish</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>New South Wales</td>
<td>Spring</td>
<td>Autumn</td>
<td>Spring</td>
</tr>
<tr>
<td>%S</td>
<td>29.04 (6.68)</td>
<td>47.63 (12.0)</td>
<td>34.31 (31.8)</td>
<td>29.05 (17.4)</td>
</tr>
<tr>
<td>%M</td>
<td>26.21 (21.8)</td>
<td>35.31 (6.68)</td>
<td>30.57 (18.6)</td>
<td>19.54 (23.1)</td>
</tr>
<tr>
<td>%P</td>
<td>44.38 (14.7)</td>
<td>15.51 (28.4)</td>
<td>33.97 (31.2)</td>
<td>48.95 (16.7)</td>
</tr>
<tr>
<td>%n3</td>
<td>35.33 (21.1)</td>
<td>12.51 (33.6)</td>
<td>27.00 (33.0)</td>
<td>32.03 (24.0)</td>
</tr>
<tr>
<td>%n6</td>
<td>9.04 (25.3)</td>
<td>2.99 (20.2)</td>
<td>6.98 (27.1)</td>
<td>16.91 (14.5)</td>
</tr>
<tr>
<td>P/S</td>
<td>1.54 (18.0)</td>
<td>0.34 (43.2)</td>
<td>1.14 (46.9)</td>
<td>1.77 (31.5)</td>
</tr>
<tr>
<td>n3/n6</td>
<td>4.29 (37.7)</td>
<td>4.30 (31.1)</td>
<td>3.84 (19.4)</td>
<td>1.93 (26.3)</td>
</tr>
<tr>
<td>FA (mg/g)</td>
<td>10.10 (65.7)</td>
<td>12.30 (45.1)</td>
<td>4.54 (47.0)</td>
<td>2.00 (41.6)</td>
</tr>
<tr>
<td>%L</td>
<td>1.36 (38.1)</td>
<td>1.20 (49.8)</td>
<td>0.78 (31.5)</td>
<td>0.48 (18.0)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Factor</th>
<th>John dory</th>
<th>Ling</th>
<th>Mirror dory</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>New South Wales</td>
<td>Spring</td>
<td>Autumn</td>
</tr>
<tr>
<td>%S</td>
<td>33.91 (8.08)</td>
<td>38.65 (23.3)</td>
<td>35.53 (25.3)</td>
</tr>
<tr>
<td>%M</td>
<td>17.00 (14.6)</td>
<td>22.11 (19.9)</td>
<td>24.34 (12.4)</td>
</tr>
<tr>
<td>%P</td>
<td>48.78 (4.92)</td>
<td>36.05 (23.0)</td>
<td>38.12 (26.1)</td>
</tr>
<tr>
<td>%n3</td>
<td>43.06 (11.6)</td>
<td>31.83 (23.0)</td>
<td>34.18 (32.4)</td>
</tr>
<tr>
<td>%n6</td>
<td>5.72 (12.1)</td>
<td>4.22 (26.8)</td>
<td>5.80 (25.0)</td>
</tr>
<tr>
<td>P/S</td>
<td>1.46 (16.7)</td>
<td>1.02 (42.2)</td>
<td>1.17 (41.7)</td>
</tr>
<tr>
<td>n3/n6</td>
<td>7.67 (19.5)</td>
<td>7.67 (13.4)</td>
<td>6.29 (47.7)</td>
</tr>
<tr>
<td>FA (mg/g)</td>
<td>1.28 (38.0)</td>
<td>2.26 (59.0)</td>
<td>3.12 (17.7)</td>
</tr>
<tr>
<td>%L</td>
<td>0.28 (17.0)</td>
<td>0.39 (22.9)</td>
<td>0.35 (16.2)</td>
</tr>
</tbody>
</table>

* n=2 only
** n=3 only

resulting 'pad' of fish flesh formed at the phase boundary was dense and dry and was pressed firmly to expel all solvent.

2) The addition of calcium chloride to the aqueous phase of the extraction medium to lower solubility. The effectiveness of this modification was checked by subsequent GC analysis of this phase.
3) Duplicates from the same fillet, together with other checks on the various steps of the gravimetric procedure, showed only small variability (less than 6%).

The possibility that the low lipid content resulted from lipid hydrolysis during storage was explored by determining the free fatty acid levels in fish frozen for up to six months. Commonly only trace amounts were detected, representing much less than 5% of the total bound lipids. These results differed from those of Olley et al. (1969) for fattier fish (haddock and lemon sole). They reported hydrolysis to free fatty acids of up to one third of the phospholipid content when stored under similar conditions of temperature and time.

The presence of very low levels of tissue lipid (which has commonly been reported for Australian fish in the past) along with the requirements for stable membranes requires further investigation.

It was expected that the intrasample variation in fatty acid compositions would be small, as the fish were of the same species, season and location of catch and at similar stages of maturity (as determined by size). The data, however, displays large variabilities for many components. This supports suggestions that individual fish rather than pooled samples should be analysed (Stansby, 1981; Brown et al., 1989) so that the data spread can be ascertained. Also, sample sizes as large as practicable should be used, rather than the common practice of using only a few individual fish (Pearson, 1977; Pearson, 1978; Gibson, 1983; Gibson et al., 1984; Fogerty et al., 1986; and Hearn et al., 1987).

Table 2.6 and Table 2 (Appendix I) present the mean phospholipid fatty acid
composition data of each sample for which this fraction was analysed.

TABLE 2.6
Important Fatty Acid and Lipid Composition Factors for Phospholipid Fractions (n=10) (with %variabilities)

<table>
<thead>
<tr>
<th>Factor</th>
<th>Black bream Victoria</th>
<th>Queensland Spring</th>
<th>New South Wales Spring</th>
<th>Autumn</th>
</tr>
</thead>
<tbody>
<tr>
<td>%S</td>
<td>25.76 (7.86)</td>
<td>22.50 (6.32)</td>
<td>31.09 (26.2)</td>
<td>24.67 (11.0)</td>
</tr>
<tr>
<td>%M</td>
<td>10.60 (18.5)</td>
<td>10.83 (12.3)</td>
<td>23.14 (13.7)</td>
<td>17.09 (10.3)</td>
</tr>
<tr>
<td>%P</td>
<td>63.17 (5.68)</td>
<td>66.49 (3.46)</td>
<td>45.65 (24.2)</td>
<td>57.73 (6.83)</td>
</tr>
<tr>
<td>%n3</td>
<td>50.19 (8.01)</td>
<td>46.62 (8.04)</td>
<td>39.41 (28.3)</td>
<td>50.61 (7.26)</td>
</tr>
<tr>
<td>%n6</td>
<td>12.98 (13.7)</td>
<td>19.86 (14.4)</td>
<td>6.23 (20.5)</td>
<td>7.12 (11.1)</td>
</tr>
<tr>
<td>P/S</td>
<td>2.48 (12.9)</td>
<td>2.97 (9.28)</td>
<td>1.64 (41.7)</td>
<td>2.38 (16.3)</td>
</tr>
<tr>
<td>n3/n6</td>
<td>3.95 (17.1)</td>
<td>2.41 (19.5)</td>
<td>6.66 (39.1)</td>
<td>7.17 (11.2)</td>
</tr>
</tbody>
</table>

2.5.1.2 Comparisons of Total Lipid and Phospholipid Composition

Comparisons of the phospholipid and total lipid fatty acid profiles of the spring Ling sample revealed only slight differences in total saturates, monounsaturates, PUFAs, n-3, n-6, AA, EPA, and DHA contents (Table 2.7). More numerous and significant differences were found between the total and phospholipid fatty acid profiles of the Victorian and Queensland Bream samples. This can be explained by the fact that these samples were higher in lipid content (%L) than the Ling (Table 2.5), and therefore would have contained a greater percentage of triglyceride, or storage fat (Ackman, 1990) contributing fatty acids to the total lipid extract profile. The Ling sampled in spring were so lean that they could probably be assumed to have all of their fatty acids bound in phospholipid molecules (Sinclair et al., 1983). This is supported by the fact that the mean phospholipid and total fatty acid profiles were nearly identical. The phospholipid and total lipid profiles of the autumn Ling sample differed to a far greater extent, reflecting the higher lipid (and therefore triglyceride) content.
### TABLE 2.7
Parametric and Non-parametric Statistical Comparisons Between Phospholipid (PL, n=10) and Total Fatty Acid (T, n=10) Profiles

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Black bream</th>
<th>Victoria</th>
<th>Ling</th>
<th>Autumn</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mann-Whitney</td>
<td>ANOVA</td>
<td>Mann-Whitney</td>
<td>ANOVA</td>
</tr>
<tr>
<td>%S</td>
<td>T &gt; PL</td>
<td>(p = 0.001)</td>
<td>T &gt; PL</td>
<td>(p = 0.001)</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.015</td>
<td></td>
<td>p &lt; 0.02</td>
<td></td>
</tr>
<tr>
<td>%M</td>
<td>T &gt; PL</td>
<td>(p &lt; 0.001)</td>
<td>T &gt; PL</td>
<td>(p &lt; 0.001)</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.01</td>
<td></td>
<td>p &lt; 0.01</td>
<td></td>
</tr>
<tr>
<td>%P</td>
<td>T &lt; PL</td>
<td>(p &lt; 0.001)</td>
<td>T &lt; PL</td>
<td>(p &lt; 0.001)</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.01</td>
<td></td>
<td>p &lt; 0.01</td>
<td></td>
</tr>
<tr>
<td>%n3</td>
<td>T &lt; PL</td>
<td>(p &lt; 0.001)</td>
<td>T &lt; PL</td>
<td>(p &lt; 0.001)</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.01</td>
<td></td>
<td>p &lt; 0.01</td>
<td></td>
</tr>
<tr>
<td>%n6</td>
<td>T &lt; PL</td>
<td>(p &lt; 0.001)</td>
<td>T &lt; PL</td>
<td>(p &lt; 0.001)</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.01</td>
<td></td>
<td>p &lt; 0.01</td>
<td></td>
</tr>
<tr>
<td>%AA</td>
<td>T &lt; PL</td>
<td>(p &lt; 0.02)</td>
<td>T &lt; PL</td>
<td>(p = 0.0286)</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.05</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>%DHA</td>
<td>T &lt; PL</td>
<td>(p = 0.0007)</td>
<td>T &lt; PL</td>
<td>(p &lt; 0.001)</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.002</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>%EPA</td>
<td>accept</td>
<td>(p &gt; 0.2)</td>
<td>T &lt; PL</td>
<td>(p &lt; 0.001)</td>
</tr>
<tr>
<td></td>
<td>p = 0.84</td>
<td></td>
<td>p &lt; 0.01</td>
<td></td>
</tr>
</tbody>
</table>

*accept = samples not rejected as being different at the 95% confidence level*
during this season, as compared with the same species sampled during spring.

Comparisons of the phospholipid and total fatty acid profiles for the two Bream samples show that the former fraction was richer in the major PUFAs, as was also reported for fish by Fogerty et al. (1986). An exception was EPA in Queensland bream, where the concentration in the phospholipid was not significantly different from that of the total lipid, which may indicate that the triglycerides of this sample contained similar relative amounts of EPA to the polar lipids. Some studies have actually found triglyceride EPA levels exceeding those of the phospholipid (Ackman, 1980). It may, however, have been a result of the smaller amounts of triglycerides present in these fish (as indicated by their lower tissue lipid contents than for the Victorian sample). The phospholipids were lower in saturated fatty acids, and much lower in MUFAs than the total lipid (as was also noted by Ackman (1980)), and thus also than the triglyceride.

2.5.1.3 Contribution of Triglyceride and Phospholipid to Total Bound Fatty Acids

The fatty acid data presented (Appendix I, Tables 1A, 1B and 2) are from phospholipid and total lipid extract fatty acids. The latter represent all of the bound fatty acids – including those of the phospholipids. The differences between the fatty acid compositions of the triglyceride and the phospholipid would, therefore, be much more pronounced than is evident here.

Variation in the recoveries of the fatty acid standards added (as the intact triglyceride and phospholipid) to the samples with low lipid levels precluded the possibility of absolutely quantifying the two fractions. This is most likely to have been a matrix effect, as repeated co-injection of standards gave a stable ratio.
Triglyceride and phospholipid fatty acid determination was consistent only in fatty fish. The lack of reproducibility in the results for lean fish may have been due to a matrix effect, the nature of which requires further investigation. As a consequence of this absolute results have not been reported for these species.

<table>
<thead>
<tr>
<th>Fish number</th>
<th>Total fatty acid (mg/g muscle)</th>
<th>%Fatty acids from phospholipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.15</td>
<td>45.8</td>
</tr>
<tr>
<td>2</td>
<td>5.50</td>
<td>32.8</td>
</tr>
<tr>
<td>3</td>
<td>2.45</td>
<td>77.4</td>
</tr>
<tr>
<td>4</td>
<td>2.38</td>
<td>62.9</td>
</tr>
<tr>
<td>5</td>
<td>4.91</td>
<td>93.0</td>
</tr>
<tr>
<td>6</td>
<td>7.51</td>
<td>28.4</td>
</tr>
<tr>
<td>7</td>
<td>8.81</td>
<td>20.8</td>
</tr>
<tr>
<td>8</td>
<td>5.18</td>
<td>66.4</td>
</tr>
<tr>
<td>9</td>
<td>4.44</td>
<td>60.2</td>
</tr>
<tr>
<td>10</td>
<td>2.44</td>
<td>100</td>
</tr>
</tbody>
</table>

By plotting the percentage of fatty acids from the phospholipids (PL) against the total amount of fatty acid extracted from a given mass (100g) of muscle (FA) (Table 2.8) a significant trend (p = 0.001) is observed (Figure 2.10). The resulting hyperbolic curve ($R^2 = 0.754$) indicates that, as noted earlier, fattier individuals contain relatively more neutral lipid (or less phospholipid) than their leaner counterparts. Further use can be made of this graph, by taking the mass of fatty acid corresponding to 100% phospholipid to estimate the minimum fatty acid content of the flesh, as shown below:

\[
\frac{1}{\%_{PL}} = 5.03 \times 10^{-3} \text{FA} - 2.32 \times 10^{-3}
\]

If $\%_{PL} = 100$,

FA = 2.45mg/g

49
FIGURE 2.10
Contribution of Phospholipid to Total Bound Fatty Acids
Victorian Black Bream

Phospholipid Fatty Acids (% of total)

Total Extracted Fatty Acids (mg/g)
As can be seen for this sample of Bream, a total fatty acid level of 2.45 mg/g would correspond to a fish containing no detectable triglyceride-bound fatty acids. This can be converted to a lipid content of 0.20 to 0.23% if it is assumed that 80 to 95% of the lipid mass is contributed by the fatty acids (Fogerty et al., 1986; Ackman, 1990). This value is far less than the mean lipid content for the sample, indicating that, in general, the Victorian bream had significant triglyceride contents.

2.5.1.4 Trends in Fatty Acid Composition with Increasing Lipid Content

The investigation of trends in fatty acid composition with increasing lipid content was enabled because a significant number of individual fish were analysed. The finding that fattier individuals had greater amounts of triglyceride (reported above), suggests that their total fatty acid profiles would tend to become more like those of the triglyceride and less like those of the phospholipid.

Significant correlations were found between the lipid level and nutritionally important factors in Redfish and Bream caught in spring off Sydney (Table 2.9).

Fewer correlations were determined between these values and the lipid contents for the same species in autumn. This may be a consequence of the fact that the autumn samples were taken at a time when a diet containing more saturated fat (lower P/S ratio) would probably have been consumed (following summer) (Kayama et al., 1963; Reiser et al., 1963; Ackman, 1967; Gruger, 1967; O'Dea and Sinclair, 1982), resulting in phospholipid compositions more closely resembling the triglyceride than in the previous sampling. The relative MUFA content of the Victorian Bream was the only one of these factors to correlate with
Table 2.9
Regression Analysis of Fatty Acid Composition Factors
with Lipid Content (n=10)

<table>
<thead>
<tr>
<th>Factor</th>
<th>Sydney</th>
<th>Victoria</th>
<th>Sydney</th>
<th>Sydney</th>
<th>Sydney</th>
</tr>
</thead>
<tbody>
<tr>
<td>%S</td>
<td>none</td>
<td>none</td>
<td>+, multiplicative 0.4442</td>
<td>- reciprocal 0.4366</td>
<td>none</td>
</tr>
<tr>
<td>%P</td>
<td>- reciprocal 0.7903*</td>
<td>none</td>
<td>- reciprocal 0.7316</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>%M</td>
<td>+, linear 0.7315</td>
<td>+, linear 0.4273</td>
<td>+, multiplicative 0.6678</td>
<td>none</td>
<td>+, multiplicative 0.5960</td>
</tr>
<tr>
<td>n3/n6</td>
<td>- reciprocal 0.6351</td>
<td>none</td>
<td>- reciprocal 0.4568</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>P/S</td>
<td>- reciprocal 0.6970</td>
<td>none</td>
<td>+, multiplicative 0.7373</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>AA</td>
<td>- exponential 0.4185</td>
<td>none</td>
<td>- exponential 0.7658</td>
<td>none</td>
<td>- exponential 0.4733</td>
</tr>
<tr>
<td>EPA</td>
<td>- reciprocal 0.7338</td>
<td>none</td>
<td>none</td>
<td>+, linear 0.4451</td>
<td>none</td>
</tr>
<tr>
<td>DHA</td>
<td>- reciprocal 0.0008</td>
<td>none</td>
<td>- multiplicative 0.7619</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>DHA+EPA</td>
<td>- exponential 0.0003</td>
<td>none</td>
<td>- multiplicative 0.7456</td>
<td>none</td>
<td>none</td>
</tr>
</tbody>
</table>

None: significance level of relationships below 95% (p>0.05)
+ : positive relationship
- : negative relationship
* : correlation coefficients
No correlations were evident for:
  John dory
  Mirror dory
  Black bream from Queensland during Spring
  Ling from Sydney during Autumn

(Insufficient lipid content data was available for Ling from Sydney during Spring)

The lipid level in that sample, while no relationships at all were found in the Queensland Bream. These findings may be explained by two facts. Firstly, that the ranges of muscle lipid contents for these samples were much smaller than for the Sydney Bream, thus providing inadequate spreads for accurate determination of trends. Secondly, the Queensland sample had a lipid content so low that very little triglyceride would have been present in even the fattiest individuals. Similarly, the extremely lean John dory and Ling samples yielded no significant correlations with
lipid level. Correlations between various fatty acid composition values and the amount of lipid within a sample of fish, therefore, may only be detected when significant amounts of triglyceride are present in most of the fish, producing a large range of lipid contents, and the sample size is adequate for regression calculations to be meaningful. This explanation assumes that the phospholipid fatty acid composition is fairly constant between individuals of the same sample, as was the case with the profiles from the fractions analysed (evidenced by the lower relative variabilities for the major fatty acids of this fraction, as shown in Table 2, Appendix I).

The trends in fatty acid compositions with lipid content for Bream and Redfish from the Sydney spring sample were consistent with those expected for keeping an approximately constant absolute amount of phospholipid, while increasing the triglyceride (with reference to the relative fatty acid compositions of these two fractions (see section 2.1.2)). Strong positive correlations between the lipid levels and percentage MUFA's (which is mainly present in the TG fraction) and negative correlations between lipid and percentage PUFAs (as found by Evans et al., 1986), particularly those of the n-3 class (which dominates the phospholipid) were found (as shown by the trends for Bream in Figures 2.11A, B and C). The nature of the relationships was determined by fitting the best linear, hyperbolic, exponential or logarithmic curve to those data sets that indicated a significant correlation (p<0.05). Brown et al. (1989) determined similar trends with lipid level for the saturated, monounsaturated and polyunsaturated fatty acid classes of seventy seven Australian species (fitting quadratic curves).

The nutritional significance of these trends is that fattier individuals of
FIGURE 2.11A
Trends with Increasing Tissue Lipid Content

I. Monounsaturated Fatty Acids (M)

\[ p = 0.0016 \]

Linear relationship

\[ r^2 = 0.7315 \]
FIGURE 2.11B
Trends with Increasing Tissue Lipid Content

II. Polyunsaturated Fatty Acids (P)

\[ p = 0.00078 \]
Reciprocal relationship
\[ r^2 = 0.7747 \]
FIGURE 2.11C
Trends with Increasing Tissue Lipid Content

III. DHA and EPA

\( p = 0.00027 \)

Exponential relationship

\( r^2 = 0.8256 \)
species containing significant triglyceride, when caught after winter, will have lower P/S and n3/n6 ratios, and therefore be less desirable for human consumption than their leaner counterparts from the same sample. Individuals from extremely lean species will exhibit much less variation in their fatty acid compositions.

2.5.2 Comparisons Between Samples from Different Seasons

The fatty acid profiles of each of the four species caught at similar locations during spring and autumn were subjected to principal components analysis. Distinct seasonal clusters were observed in the scatterplots of the first two principal components for Bream and Redfish (Figures 2.12A and 2.13A). When the scatterplots were overlayed with vectors representing the correlation of the variables with the components (to form the biplots shown in Figures 2.12B and 2.13B) both data sets showed correlation of the spring-caught fish with the major PUFAs, and the autumn samples with the monounsaturates and saturates.

Clusters were less distinct in the John dory and Ling samples (PC analyses not shown).

The effect of varying the season of catch on factors believed to be of nutritional importance was investigated by the non-parametric Mann–Whitney test, and by Analysis of Variance (ANOVA), both at the 95% confidence level (\( \alpha = 0.05 \)). The results are summarised in Table 2.10. As was found in the principal components analysis, the greatest number of significant inter-seasonal differences occurred in the Bream and Redfish samples. Some of these changes were also observed in the John dory, while only two factors had altered in the Ling (with one of them, the relative AA, showing a trend contrary to that in the other species).
FIGURE 2.12 A

Scatterplot for Principal Components Analysis of Black Bream from Autumn (A) and Spring (S)
Total Lipid Extract Fatty Acids

59.5% of total variance
FIGURE 2.13A
Scatterplot for Principal Components Analysis of Redfish from Autumn (A) and Spring (S) Total Lipid Extract Fatty Acids
64.6% of total variance
FIGURE 2.12B

Biplot for Black Bream from Two Seasons

(See Figure 2.12A)

Labels have been omitted from the scatterplot points for the sake of clarity.
FIGURE 2.13B
Biplot for Redfish from Two Seasons
(See Figure 2.13A)
<table>
<thead>
<tr>
<th>Table 2.10</th>
<th>Parametric and Non-parametric Statistical Comparisons Between Spring (S, n=10) and Autumn (A, n=10) Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total fatty acids</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bream</td>
</tr>
<tr>
<td>Quantity</td>
<td>Mann-Whitney</td>
</tr>
<tr>
<td>%S</td>
<td>S &lt; A (p &lt; 0.001)</td>
</tr>
<tr>
<td>%M</td>
<td>S &lt; A (0.002 &lt; p &lt; 0.005)</td>
</tr>
<tr>
<td>%P</td>
<td>S &gt; A (p &lt; 0.001)</td>
</tr>
<tr>
<td>PS</td>
<td>S &gt; A (p &lt; 0.001)</td>
</tr>
<tr>
<td>%n3</td>
<td>S &gt; A (p &lt; 0.001)</td>
</tr>
<tr>
<td>%n6</td>
<td>S &gt; A (p &lt; 0.001)</td>
</tr>
<tr>
<td>n3/n6</td>
<td>accept (p &gt; 0.2)</td>
</tr>
<tr>
<td>%AA</td>
<td>S &gt; A (p &lt; 0.001)</td>
</tr>
<tr>
<td>%DHA+EPA</td>
<td>S &gt; A (p &lt; 0.001)</td>
</tr>
<tr>
<td>%L</td>
<td>accept (p &gt; 0.2)</td>
</tr>
</tbody>
</table>

*Insufficient data available for Ling lipid comparisons
The smaller number of changes that occurred in the fatty acid compositions of the leaner species is probably a consequence of the greater contribution to the total fatty acids by the phospholipids than in the fattier fish. The phospholipids, as explained previously, tend to maintain their PUFAs during times of altered diet to a greater extent than the triglycerides.

PUFA maintenance in the phospholipid fraction was demonstrated by the Ling samples, where changes detected were contrary to those noted for the lipid extracts of the other species. This may be a precaution against PUFA depletion of the phospholipid (Ross and Love, 1979), which is known to occur in periods of food shortage (Ackman, 1967) in very lean fish, due to a lack of triglyceride stores (Reiser et al., 1963).

Univariate analyses of the total fatty acid extracts' profiles indicated that a shift to greater degrees of saturation during autumn had occurred in Black bream, Redfish and John dory (as evidenced by the lower levels of PUFAs and higher saturates and monounsaturates for the autumn samples, Table 2.10). This echoed the Principal Components Analysis correlations. For these species the relative amounts of n-3 and n-6 fatty acids was unchanged, except for Redfish.

The major factor affecting the fatty acids of fish is their relative proportions in the diet (Yu and Sinnhuber, 1972; Stansby, 1981), which overrides fatty acid anabolism occurring within the fish. Although the species studied here were all carnivorous, the original source of their tissue fatty acids is marine algae and plankton. Seasonal variations are known to occur in the fatty acid compositions of plankton (Lewis, 1962), which would cause a similar effect in the fish, which lie further along in the food chain. The original cause of these changes
is believed to be the variations in water temperature (as will be discussed later). The samples in this study were from spring (which followed a period of relatively cold water temperatures) and autumn (after exposure to warm summer waters).

In summary, the consumption of the moderately lean species from spring catches, as analysed here, would be more desirable than the same species from autumn, whereas the extremely lean species from both seasons would be equivalent.

2.5.3 Comparisons Between Samples from Different Locations

Since differences in the lipids of fish are believed to be caused by changes in those of their diet, as induced by water temperature variations, the largest sampling location effect would be expected to occur with latitude changes. In this study a species common to marine waters from three temperate locations were sampled during the same season. They came from a 13° latitude and 13°C water temperature range.

Principal components analysis performed on the total fatty acid profiles revealed some clustering between the Queensland and Victorian samples in the scatterplot of the first two principal components. Both of these, however, overlapped with the Sydney sample (Figure 2.14). This suggests that the Sydney sample was intermediate in the nature of its fatty acid profiles, as indeed was its location. A clustering is evident that excludes all fish having large negative correlations with the second principal component. This group excludes six of the Queensland, but only two of the Victorian Bream, suggesting that the Victorian fish were, in general, more similar to those from Sydney than those from

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FIGURE 2.14
Scatterplot for Principal Components Analysis of Black Bream from Queensland (Q), Victoria (V) and Sydney (S) Total Lipid Extract Fatty Acids
44.3% of total variance
Queensland.

Only 44.3% of the total variance was accounted for by this plot, necessitating the visual display of more principal components. This was achieved using spider-web plots (Aishima, 1979) (Figure 2.15). These plots also indicate (by the shapes and their orientation for each sample) that the Victorian sample was quite similar to that from Sydney. This is consistent with the fact that the Sydney sample was much closer in latitude to that from Victoria than the Queensland sample. Migratory habits of the species must also be taken into account. Black bream are not usually found in the colder Victorian waters during winter, but swim south in early spring. The fish caught in these waters, therefore, had not dwelt there long, and thus may not have had time to incorporate large amounts of fatty acids from the local food chain into their lipids. Profiles would, therefore, be expected to fairly closely resemble those of the Sydney sample. The results of the comparison of nutritional factors for the total lipid extracts are summarised in Table 2.11.

Again it is seen that the Victorian and Sydney samples' values were very similar, with greater differences occurring in most factors between these and the Queensland bream. The differences found are due to the decrease in n-6 PUFAs (mainly AA, as found by Sinclair et al., 1984; and Naughton et al., 1983) and increase in MUFA contents with increased latitude. The former effect is responsible for the lower PUFA content and P/S ratio at the greater latitudes. Strong linear relationships were determined for each of these factors (Table 2.12).

Similar multivariate statistical treatment of the phospholipid fatty acid profiles for the two extreme latitude samples (Queensland and Victoria) did not
FIGURE 2.15
Spider-web Plots of Black Bream
Principal Components (PC)

Queensland

Victoria

Sydney

Mean

Standard deviation

67
### TABLE 2.11
Parameteric and Non-parametric Statistical Comparisons Between Black Bream Samples from Three Locations (n=10 for each)

<table>
<thead>
<tr>
<th>Total fatty acid profiles</th>
<th>Phospholipid fatty acid profiles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Samples from Queensland (Q)</td>
</tr>
<tr>
<td></td>
<td>Victoria (V) and Sydney (S)</td>
</tr>
<tr>
<td>Quantity</td>
<td>Kruskal-Wallis</td>
</tr>
<tr>
<td></td>
<td>ANOVA</td>
</tr>
<tr>
<td>%S</td>
<td>accept (p = 0.6132)</td>
</tr>
<tr>
<td>%M</td>
<td>Q &lt; V (p = 0.0019)</td>
</tr>
<tr>
<td>%P</td>
<td>Q &gt; V (p = 0.0093)</td>
</tr>
<tr>
<td>P/S</td>
<td>accept (p = 0.0613)</td>
</tr>
<tr>
<td>%n3</td>
<td>accept (p = 0.0920)</td>
</tr>
<tr>
<td>%n6</td>
<td>Q &gt; S,V (p = 0.0000)</td>
</tr>
<tr>
<td>n3/n6</td>
<td>Q &lt; S,V (p = 0.0006)</td>
</tr>
<tr>
<td>%AA</td>
<td>Q &gt; S,V (p = 0.0001)</td>
</tr>
<tr>
<td>% (DHA + EPA)</td>
<td>accept (p = 0.1875)</td>
</tr>
</tbody>
</table>

### TABLE 2.12
Correlation of Fatty Acid Composition Factors with Latitude for Black Bream from Sydney (Spring, 1989)

<table>
<thead>
<tr>
<th>Factor</th>
<th>Probability level of correlation</th>
<th>Nature of relationship</th>
<th>Coefficient of Determination</th>
</tr>
</thead>
<tbody>
<tr>
<td>P/S</td>
<td>0.243</td>
<td>linear, negative slope</td>
<td>0.862</td>
</tr>
<tr>
<td>%MUFA</td>
<td>0.068</td>
<td>linear, positive slope</td>
<td>0.989</td>
</tr>
<tr>
<td>%PUFA</td>
<td>0.222</td>
<td>linear, negative slope</td>
<td>0.884</td>
</tr>
<tr>
<td>%n6</td>
<td>0.057</td>
<td>linear, negative slope</td>
<td>0.992</td>
</tr>
</tbody>
</table>
produce distinct clusters for the locations (Figure 2.16), even though a greater percentage of the variance was accounted for by the 2-dimensional scatterplot (64.6%). This indicated a very constant phospholipid fatty acid composition with changes in latitude. Further statistical analysis, however, revealed some differences (Table 2.11), which were largely consistent with those of the total lipid profiles.

The decrease in P/S ratio and total PUFAs with water temperature appears to be contrary to the expected trends, when considering the membrane fluidity requirements of fish in colder waters (Ackman, 1967). The expected increase in the polyunsaturated class, however, would be due to higher n-3 levels (as found by Sinclair et al., 1983), which were not present in the southernmost fish of this study, as the water temperature was still mild (12-14°C) and the fish were recent immigrants. Reiser et al. (1963) subjected fish to temperatures within a similar range to those encountered here (13-23°C), and concluded that there was no significant effect on the deposition or interconversion of PUFAs within the fish, unless food deprivation was enforced. The differences detected in n-6 PUFAs, therefore, would most likely be due to altered dietary fatty acid intake. The decrease in n-6 levels with increased latitude is consistent with previous findings (Sinclair et al., 1984; Naughton et al., 1983).

Current nutritional knowledge applied to these results leads to the conclusion that samples of a particular species from locations further from the equator would be more desirable for human consumption. This choice is based on the requirement for a high n3/n6 ratio. P/S ratios, although lower than for their
FIGURE 2.16
Scatterplot for Principal Components Analysis of Black Bream from Queensland (Q) and Victoria (V) Phospholipid Fatty Acids
64.6% of total variance
counterparts from warmer waters, were still acceptable.

2.5.4 Interspecies Comparisons

Having established the variations in fatty acid composition occurring within a species to be quite large, it remains to be determined whether or not the actual species chosen is an important dietary consideration.

In the discussion thus far it has been evident that the lipid content is an important factor governing the extent of variability in fatty acid composition within a species. Because the percentage lipid content of the flesh is an indicator of the amount of triglyceride present, and this portion of the lipid is mainly a storage of ingested lipid, it appears to be reasonable to expect that species with significant triglyceride contents sampled at the same time and place, and eating similar diets, would have very similar fatty acid profiles.

The fatty acid profiles of all fish analysed from the spring catch in Sydney were subjected to principal components analysis. The scatterplot produced from the first two principal components shows fairly distinct clusters for most of the species, even though only 43.9% of the variance is explained (Figure 2.17). Distinction between species was clearer using the spider-web plots of the first eleven components (Figures 2.18A and 2.18B), indicating that the variance was accounted for by species-characteristic combinations of the calculated principal components. Some similarity was observed between the Ling and John dory plots, as was evident from their overlap in the scatterplot. It is of interest to note that these were actually the two species lowest in fat among the five analysed, suggesting that the phospholipid profiles of different species may be similar, and
FIGURE 2.17
Scatterplot for Principal Components Analysis of Species Samples from Sydney (Spring, 1989)
Total Lipid Extract Fatty Acids
43.9% of total variance

Component 2

Component 1

BR = Black bream
JD = John dory
RF = Redfish
MD = Mirror dory
LI = Ling
FIGURE 2.18 A
Spider-web Plots of Spring Sample
Principal Components (PC)

Black bream

Redfish

Mean
Standard deviation
FIGURE 2.18b
Spider-web Plots of Spring Sample
Principal Components

John dory

Mirror dory

Ling

Mean

Standard deviation
the major differences occur in the triglyceride. This indicates that the enzyme systems responsible for fatty acid incorporation in phospholipids are similar in the different species, but fatty acid deposition in the muscle triglyceride varies.

Species clusters produced on analysis of the autumn samples (Figure 2.19) indicated that the total lipid profiles exhibit some species-specificity that is maintained throughout the year.

The multivariate analyses referred to above are actually comparisons of the magnitudes of interspecies and intrasample differences. To determine the relative extents of the interspecies and interseasonal differences the data were combined and re-analysed. The resulting scatterplot (Figure 2.20) shows pronounced differentiation between the samples from the two seasons. This indicates that, for these moderately lean species (by Australian standards), the season of catch may be more nutritionally important than the actual species in influencing the fatty acid composition (as evidenced by the similar angular fatty acid correlations for samples from the same season). More overlap was observed in the Ling and John dory samples than for the Black bream or Redfish, suggesting that these species are not only similar to each other, but are also more stable over time in their fatty acid compositions (as was explained by their low triglyceride contents, mentioned previously).

Of the species analysed, consumption of Redfish flesh would be the most highly recommended for the human diet. This is because it contained greater levels of lipid of the highest quality found (where n3/n6 and P/S ratios are used as quality indices). Consumption of extracted oil (thus removing the factor of lipid content) would favour the selection of that taken from John dory. Extraction of
FIGURE 2.19
Scatterplot for Principal Components Analysis
of Species Samples from Sydney (Autumn, 1990)
Total Lipid Extract Fatty Acids
53.8% of total variance

BR = Black bream  JD = John dory
RF = Redfish      LI = Ling
FIGURE 2.20
Scatterplot for Principal Components Analysis of Species Sampled During Two Seasons (Sydney)
Total Lipid Extract Fatty Acids

61.8% of total variance

BR = Black bream
RF = Redfish
LI = Ling
JD = John dory
MD = Mirror dory
A = Autumn
S = Spring

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fish oil from such lean species is unlikely to be carried out, however, due to the obviously low yields possible from a given mass of flesh.

It should be noted that these conclusions are based solely on the limited data presented here, and only serve as an indication of the sampling factors to consider in dietary species selection. Absolute comparisons of the species-characteristic lipid profiles would require much larger sample sizes and more comprehensive sampling from different locations, seasons and years. Consideration should also be given to other factors, such as the sex, maturity, and spawning time of the fish, as well as their specific dietary intake and water depth inhabited (Deng et al., 1976; Brown et al., 1989).

The relative effects of geographical and species differences could not be explored using the present data. This would require the determination of many species common to waters over a wide range of latitudes.

2.5.5 Discussion

The lipid extracts of the species studied contained similar fatty acids. For extremely lean species (lipid content less than approximately 0.70%) the relative compositions were also found to be similar, even with differences in sampling season. Discrimination between the extremely lean species on the basis of their nutritional value would, therefore, not be necessary. Greater differences existed between the less lean species' profiles (Bream and Redfish), which although more significant than those between individuals of the same sample, were less so than the changes induced by different seasons. Species identification of fish for human consumption is generally recommended (Naughton et al., 1983; Brown et al., 1989),
however, as only a very small percentage of the world's fish species are so extremely lean.

The species sampled at different latitudes (Black bream) showed more pronounced changes in fatty acid compositions. This is consistent with the greater difference in water temperature between the extreme locations compared to that observed between the two seasons (13°C and 7°C, respectively). Major differences were found between the arachidonic acid contents of the Queensland and the two more southern samples. Arachidonic acid contents do not appear to be directly temperature-dependent, as the autumn water temperatures for Sydney were similar to those of Queensland waters when the Bream were caught (spring). All species sampled in Sydney during spring (including Black bream) were much lower in AA content than the equivalent Black bream from Queensland (Figure 2.21). They were also lower (with the exception of the very lean species, Ling) in their AA contents as compared to the same location in the colder season (spring) (as was seen in Table 2.10). This suggests that the dietary source of AA is native to more northerly (warmer) locations.

Variations in fatty acid compositions of fish are mainly due to changes in the dietary fatty acid profiles. The underlying cause of this is the differing temperatures of the surrounding water (Kayama et al., 1963; Reiser et al., 1963; Ackman, 1967; Gruger, 1967; O'Dea and Sinclair, 1982). Apart from its effect on the fatty acids of the food chain, water temperature changes are also known to alter the uptake of fatty acids into fish tissue. Reiser et al. (1963) showed that at low temperatures (13°C) no new saturated fatty acids were incorporated into fish tissue at all.

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FIGURE 2.21
Least Squared Difference ANOVA Mean Intervals of Queensland (Q) and Temperate (T) Samples' Arachidonic Acid (AA) Contents
Another factor which may be related to water temperature is the level to which the n-6 fatty acids permeate the food chain - an effect shown here to rely more on location than season.

In general, the results support the conclusion that fish for human consumption should be chosen from colder waters. This would favour the consumption of fish caught in more southern latitudes during late winter to early spring. Such specificity would usually require frozen storage of the fillets, which would not be expected to significantly affect their composition under normal freezing conditions and periods (Exler et al., 1975).

The results support the suggestions made by various authors (Stansby, 1981; Naughton et al., 1983; and Brown et al., 1989) that the season, location and species of catch should be clearly stated when presenting lipid composition data for fish samples. Also, a significant number of individual fish should be analysed in each sample, because of the large variations encountered. If an overall evaluation of the lipids of a given species is to be attempted, pooled samples from various locations, seasons, and years should also be analysed (Stansby, 1981).
2.6 Comparisons of the Temperate Australian Species with Existing Data

2.6.1 Tropical Australian Fish

Mean fatty acid compositions and nutritional factors for the fatty acids of each of the five species analysed from temperate Sydney waters were calculated, including data from both seasons, where available (Table 2.13, and Table 1, Appendix II). Parametric and non-parametric statistical comparisons with similar factors for samples from tropical waters (see Appendix II, Table 2) (analysed by Sinclair et al. (1983), Evans et al. (1986), and Fogerty et al. (1986)) were made. The results indicate that the temperate species were higher in MUFAs, while the tropical species were higher in AA (Table 2.14) (as also noted by Sinclair et al. (1984), and Evans et al. (1986)), causing their total n-6 contents and P/S ratios to also be larger and n3/n6 ratios smaller.

These findings indicate that the claim that Australian fish are higher in n-6 fatty acids than northern hemisphere species (Gibson, 1983), especially in AA (Ackman, 1990) may have been biased by the inclusion of many species from tropical waters. The review by Brown et al. (1989), for example, which presented data on seventy seven species of Australian marine fish included the fifty five species from latitudes north of 20°S used in the comparisons above. Fish from tropical waters other than Australian have also been found to contain unusually high AA levels, such as those from Malaysia, reported by Gibson et al., 1984.

A few species sampled from temperate Australian waters have displayed very high AA contents, but most of these species are herbivorous, and so feed
TABLE 2.13
Fatty Acid and Lipid Composition Factors for Temperate (New South Wales) Fish Samples

<table>
<thead>
<tr>
<th>Factor</th>
<th>Black bream (n=20)</th>
<th>Redfish (n=20)</th>
<th>John dory (n=20)</th>
<th>Ling (n=20)</th>
<th>Mirror dory (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>%S</td>
<td>36.34</td>
<td>32.78</td>
<td>36.28</td>
<td>34.65</td>
<td>37.38</td>
</tr>
<tr>
<td>%M</td>
<td>30.76</td>
<td>37.1</td>
<td>19.56</td>
<td>27.15</td>
<td>24.79</td>
</tr>
<tr>
<td>%P</td>
<td>29.94</td>
<td>27.16</td>
<td>42.41</td>
<td>24.01</td>
<td>36.84</td>
</tr>
<tr>
<td>%n3</td>
<td>23.92</td>
<td>23.85</td>
<td>37.45</td>
<td>20.28</td>
<td>32.53</td>
</tr>
<tr>
<td>%n6</td>
<td>6.02</td>
<td>3.31</td>
<td>4.97</td>
<td>3.73</td>
<td>4.31</td>
</tr>
<tr>
<td>P/S</td>
<td>0.94</td>
<td>0.92</td>
<td>1.24</td>
<td>0.81</td>
<td>1.06</td>
</tr>
<tr>
<td>n3/n6</td>
<td>4.30</td>
<td>6.72</td>
<td>7.67</td>
<td>5.63</td>
<td>8.04</td>
</tr>
<tr>
<td>FA (mg/g)</td>
<td>11.17</td>
<td>5.71</td>
<td>1.77</td>
<td>2.23</td>
<td>5.44</td>
</tr>
<tr>
<td>%L</td>
<td>1.28</td>
<td>0.78</td>
<td>0.34</td>
<td>0.48</td>
<td>0.53</td>
</tr>
</tbody>
</table>

TABLE 2.14
Parametric and Non-parametric Statistical Comparisons with Five Temperate Australian Fish Species

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Mann-Whitney</th>
<th>ANOVA</th>
<th>Southern (SH) and Northern (NH) Hemispheres**</th>
</tr>
</thead>
<tbody>
<tr>
<td>%S</td>
<td>accept</td>
<td>accept (0.10&lt;p&lt;0.20)</td>
<td>SH &gt; NH (p &lt; 0.001)</td>
</tr>
<tr>
<td></td>
<td>(p = 0.4033)</td>
<td></td>
<td>SH &gt; NH (p = 0.0230)</td>
</tr>
<tr>
<td>%M</td>
<td>Te &gt; Tr</td>
<td>Te &gt; Tr (p = 0.0001)</td>
<td>accept (p = 0.20)</td>
</tr>
<tr>
<td></td>
<td>(p &lt; 0.001)</td>
<td></td>
<td>accept (p = 0.4451)</td>
</tr>
<tr>
<td>%P</td>
<td>accept #</td>
<td>accept (p = 0.0798)</td>
<td>accept (p = 0.20)</td>
</tr>
<tr>
<td></td>
<td>(p = 0.05)</td>
<td></td>
<td>accept (p = 0.9768)</td>
</tr>
<tr>
<td>P/S</td>
<td>Te &lt; Tr</td>
<td>Te &lt; Tr (p = 0.0353)</td>
<td>accept (0.10&lt;p&lt;0.20)</td>
</tr>
<tr>
<td></td>
<td>(p = 0.02)</td>
<td></td>
<td>accept (p = 0.2369)</td>
</tr>
<tr>
<td>%n3</td>
<td>accept #</td>
<td>accept (p = 0.4209)</td>
<td>accept (p = 0.20)</td>
</tr>
<tr>
<td></td>
<td>(p = 0.20)</td>
<td></td>
<td>accept (p = 0.4889)</td>
</tr>
<tr>
<td>%n6</td>
<td>Te &gt; Tr #</td>
<td>Te &lt; Tr (p = 0.0248)</td>
<td>accept (0.10&lt;p&lt;0.20)</td>
</tr>
<tr>
<td></td>
<td>(p = 0.005)</td>
<td></td>
<td>accept (p = 0.1800)</td>
</tr>
<tr>
<td>n3/n6</td>
<td>Te &gt; Tr #</td>
<td>Te &gt; Tr (p = 0.0043)</td>
<td>accept (0.05&lt;p&lt;0.10)</td>
</tr>
<tr>
<td></td>
<td>(0.005&lt;p&lt;0.01)</td>
<td></td>
<td>accept (p = 0.3001)</td>
</tr>
<tr>
<td>%AA</td>
<td>Te &lt; Tr</td>
<td>Te &lt; Tr (p = 0.0246)</td>
<td>accept (p &gt; 0.10)</td>
</tr>
<tr>
<td></td>
<td>(p = 0.01)</td>
<td></td>
<td>accept (p = 0.4125)</td>
</tr>
<tr>
<td>%EPA</td>
<td>accept</td>
<td>accept (p = 0.9687)</td>
<td>accept (0.05&lt;p&lt;0.10)</td>
</tr>
<tr>
<td></td>
<td>(p = 0.20)</td>
<td></td>
<td>accept (p = 0.0822)</td>
</tr>
<tr>
<td>%DHA</td>
<td>accept</td>
<td>accept (p = 0.2862)</td>
<td>accept (0.05&lt;p&lt;0.10)</td>
</tr>
<tr>
<td></td>
<td>(p = 0.20)</td>
<td></td>
<td>accept (p = 0.1098)</td>
</tr>
<tr>
<td>%DHA+EPA</td>
<td>accept</td>
<td>accept (p = 0.0895)</td>
<td>accept (p &gt; 0.20)</td>
</tr>
<tr>
<td></td>
<td>(0.05&lt;p&lt;0.10)</td>
<td></td>
<td>accept (p = 0.6335)</td>
</tr>
<tr>
<td>%L</td>
<td>Te &gt; Tr</td>
<td>accept (p = 0.2215)</td>
<td>SH &lt; NH (p &lt; 0.001)</td>
</tr>
<tr>
<td></td>
<td>(0.005&lt;p&lt;0.01)</td>
<td></td>
<td>accept (p = 0.0816)</td>
</tr>
</tbody>
</table>

Data used for comparisons:
* Sinclair et al. (1983) (10 species),
  and Evans et al. (1986) (27 species)
# Also includes Fogerty et al. (1986)
  (18 species)
** NH: Hearn et al. (1987)
from the beginning of the food chain. An example is the Leatherjacket from Adelaide waters (35°S) that was found to contain 15.8% of its fatty acids as AA (Gibson, 1983).

This study has shown with the comparison of Bream from cool temperate and warm temperate (sub-tropical) waters, that the dietary AA sources appear to be confined to the warmer latitudes, and therefore do not form part of the diet of species from cool temperate waters, even during summer.

Very little data is available on the fatty acid compositions of organisms towards the beginning of Australian marine food chains. It has been found, however, that many species of red and brown macro-algae from both temperate and tropical Australian marine environments are extremely high in AA (Johns et al., 1979). This feature is characteristic of shellfish caught in tropical waters (O’Dea and Sinclair, 1982) as well as the local fish (including herbivores, omnivores and carnivores). In the waters of Bass Strait (38°S) cephalopods and the phytoplankton they mainly feed on are both low in AA (Dunstan et al., 1988), suggesting that this food chain is AA-poor. These workers determined that the fish with the highest level of AA for this region were the macro-algae consumers (the omnivorous bony fish), while the lowest levels were in those feeding mainly on phytoplankton (pelagic and benthopelagic carnivores). Demersal carnivores, which feed from both webs, were found to have intermediate AA levels. These findings suggest that AA more generally pervades the food chains found in tropical waters than temperate. It appears, therefore, that the generalisations made about the high AA contents of Australian fish should be modified to specify that this may
be a characteristic of species from tropical waters, as well as those feeding on the macro-algae food chain in temperate waters.

The species analysed in this work were all demersal carnivores, feeding mainly on small fish, molluscs and crustaceans, whereas the tropical species used for comparison also included herbivores and omnivores.

The nutritional status of the majority of temperate Australian fish species appears to be higher than previous authors have claimed. The evaluation of individual species for human consumption, however, needs much more data to be collected (consisting of very large sample sizes from various locations, times and species). Only an indication of their properties can be gleaned from the data of this study. It was seen (Table 2.5) that the factor believed to be most nutritionally important (the n3/n6 ratio) ranged from 3.88 to 9.58 (mean of 6.21) for the samples used in these comparisons. Dietary recommendations suggest a value for this ratio in the overall diet (0.1 to 0.33). The values in Table 2.5 were considerably higher, and so would favourably affect the ratio in the overall diet. They were also much higher than the n3/n6 ratio for the tropical species. A more general comparison (that with northern hemisphere species) will be made in the following section.

The P/S ratios were greater than the generally recommended value of 1.0, except for the two fattier species (Bream and Redfish) in the warmer season (autumn).

To ingest the 300mg/day of combined EPA and DHA recommended by Ackman (1990) from the temperate samples analysed, about 120g to 1.0kg of flesh would need to be consumed daily. One average sized fish meal (containing about 85
200g of flesh) would provide adequate n-3 from three of the samples measured (Table 2.15). Diets based on the other samples would need a greater level of consumption, or supplementary n-3 sources to be consumed. According to the findings of Kromhout et al. (1985) moderate consumption of these species would produce some benefit to the diet if eaten over an extended period. The inclusion of the extracted oils in the diet would eliminate the concern over low flesh lipid contents.

<table>
<thead>
<tr>
<th>Sample (n=10)</th>
<th>% (DHA+EPA)</th>
<th>Total Fatty Acids (mg/g)</th>
<th>Consumption for 300mg PUFA (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bream Spring</td>
<td>25.0</td>
<td>10.1</td>
<td>119</td>
</tr>
<tr>
<td>Autumn</td>
<td>7.78</td>
<td>12.3</td>
<td>313</td>
</tr>
<tr>
<td>Victoria*</td>
<td>19.1</td>
<td>4.54</td>
<td>346</td>
</tr>
<tr>
<td>Redfish Spring</td>
<td>31.0</td>
<td>6.44</td>
<td>150</td>
</tr>
<tr>
<td>Autumn</td>
<td>8.16</td>
<td>4.99</td>
<td>737</td>
</tr>
<tr>
<td>John dory Spring</td>
<td>36.5</td>
<td>1.28</td>
<td>642</td>
</tr>
<tr>
<td>Autumn</td>
<td>28.2</td>
<td>2.26</td>
<td>471</td>
</tr>
<tr>
<td>Ling Spring</td>
<td>28.2</td>
<td>3.12</td>
<td>341</td>
</tr>
<tr>
<td>Autumn</td>
<td>22.3</td>
<td>1.34</td>
<td>1004</td>
</tr>
<tr>
<td>Mirror dory Spring</td>
<td>27.7</td>
<td>5.44</td>
<td>199</td>
</tr>
</tbody>
</table>

* All other samples were from Sydney
2.6.2 Temperate Northern Hemisphere Fish

As previously mentioned, comparisons of fish from different areas is complicated by the lack of common species, as well as inconsistent naming. The species selected for comparison between the two hemispheres have been chosen because they represent commonly available commercial species from their respective locations. This provides a basis for comparison of the nutritional value of the fish in the diets of the local populations.

The lipid contents and compositions of the five species sampled from temperate Australian waters (33°53'S to 35°21'S) during spring (Table 2.5 and Appendix I, Tables 1A and 1B) were compared with those for the forty-one species analysed by Hearn et al. (1987) (see Appendix II, Table 3) from a similar absolute latitude north during the northern spring.

The fatty acids present in both data sets were generally similar. Minor components were excluded from both.

Various 20:1 and 22:1 isomers are reported for the Australian species, whereas most studies have not differentiated between them. The total levels of long-chain monounsaturates ranged from 0.50 to 6.96%, with a mean of 3.26%. Both Mann-Whitney and ANOVA revealed no differences between these values for the southern and northern hemisphere species, whereas ANOVA of the values quoted by Brown et al. (1989) lead to rejection of the Australian species' levels as being lower (p = 0.0003). In particular, 22:1n11 cannot be biosynthesised by fish, but is obtained through the diet from fatty alcohols of the same chain length in the wax esters of marine invertebrates (Ackman, 1990). The finding that comparable amounts of these fatty acids exist in temperate fish from the two
hemispheres contradicts previous suggestions that Australian fish are poor in the long-chain monoenoics, due to their lack of availability in the local food chain (Brown et al., 1989). Again the inclusion of a large number of tropical species may have biased their results.

The results of the various parametric and non-parametric analyses are summarised in Table 2.14. The Australian samples were found to have higher levels of saturated fatty acids than the northern hemisphere species. This, however, did not significantly lower the P/S ratio, and so is not considered to be nutritionally important. The n3/n6 ratios of both sampling locations were also comparable, with no significant differences found in either the n-3 or n-6 PUFAs that comprise this ratio.

Contrary to the general belief that Australian fish species are higher in AA than their northern hemisphere counterparts, the levels of this fatty acid were not significantly different in the data compared here.

It was reported previously (Armstrong et al., 1991) from the results of this study that the Australian samples had slightly lower (0.02<p<0.05) EPA levels in their lipids than the northern hemisphere species of Hearn et al. (1987), as determined by the non-parametric Mann-Whitney test. The same analysis re-applied to the data with corrected fatty acid identities, following mass spectral analysis, however, failed to reject the Australian samples' EPA levels. The result, however, was just within the 95% confidence limit, and is, therefore, not considered to be conclusive.

Australian species have been noted because their DHA levels generally
exceed their EPA (Pearson, 1978; Gibson, 1983; Sinclair et al., 1983; Evans et al., 1986; Fogerty et al., 1986; Dunstan et al., 1988), as indeed was the case for the five species analysed here. The data of Hearn et al. (1987), however, included only five species in which EPA exceeded DHA contents. Ackman and McLeod (1988) also found this to be true for the minority of their fish (six of the twenty four species from Nova Scotia). The widely accepted belief that EPA is the major fatty acid of northern hemisphere species (Evans et al., 1986; Ackman, 1990), therefore, may not be generally correct.

Previous workers have equated high EPA content with desirable lipid quality, as it is the direct precursor of the 3-series eicosanoids. Such a narrow criterion for nutritional assessment of lipids, however, may not be adequate. Ackman and Burgher (1964) regarded DHA and EPA to be interchangeable in the phospholipids of fish flesh. This was supported by von Schacky and Weber (1985), who found that DHA could be retroconverted in vivo to EPA, suggesting that DHA may be a storage form. Very little is known about the relative effects of DHA and EPA, as most feeding trials have involved mixtures of the two (Poisson, 1990). It has been noted, however, that DHA was readily incorporated into human lipids, since supplementing the diet with DHA caused a significant increase in its concentration in platelets, plasma, serum and red blood cells (Salem et al., 1986).

It has been found to inhibit both platelet and prostaglandin biosynthesis (Morisaki et al., 1982), and causes a decrease in plasma low-density lipoprotein cholesterol (LDL-C) that is not duplicated by EPA (Childs et al., 1988). The physiological importance of DHA, thus demonstrated, has prompted some authors to suggest
that the level of this fatty acid should be considered along with EPA (Gibson et al., 1984; Ackman and McLeod, 1988). It appears, therefore, more appropriate to consider the combined EPA and DHA level rather than EPA alone when assessing the nutritional quality of lipids. Distinction between DHA and EPA may be necessary if more carefully controlled studies reveal differences of their in vivo effects. Comparison of the combined EPA and DHA levels of the northern and southern hemisphere studies made above indicates very similar levels.

An inverse relationship between AA and DHA levels in cardiac membranes has been found, and linked with an increased incidence of myocardial necrosis (Gudbjarnason and Hallgrimson, 1975; Gudbjarnason and Oskarsdottir, 1977). Recent work by Weber (1989) has indicated that the ratio of AA to EPA in human tissue may be related to the incidence of coronary heart disease mortality. By combining these two findings it appears that the ratio of AA to the sum of DHA and EPA may be important in the diet, for its effects on the tissue composition. On comparing this ratio calculated for the southern and northern hemisphere samples it was found that they were very similar (Table 2.14).

Mann-Whitney comparison revealed that the five Australian species were significantly lower in lipid content than the northern hemisphere species (p<0.001). This is consistent with the findings of Brown et al. (1989). Specifically, the mean lipid content was found to be about one-sixth that of the fish analysed by Hearn et al. (1987). ANOVA, however, lead to the conclusion that the two lipid ranges were not significantly different (as displayed by the slight overlap in their least squared difference plot (Figure 2.22). This is due to the large spread of lipid
FIGURE 2.22
Least Squared Difference ANOVA Mean Intervals of Northern Hemisphere (NH) and Southern Hemisphere (SH) Samples’ Tissue Lipid Contents (%L)
contents in the Australian species, and the fact that even though the mean value for the northern samples was much higher, seventeen of the forty-one species would be classified as being lean (Exler et al., 1975; Ackman, 1990), as less than 2% of their wet mass was comprised of lipid. The five Australian species analysed here were all lean. Many Australian species, however, have lipid contents greatly exceeding these levels - ten low fat (2-4%L), seven medium (4-8%L), and two high fat (>8%L) species were included in the review by Brown et al. (1989). More data needs to be collected before the overall lipid levels typical of Australian species can be assessed. If it is true, however, that they are lower in fat than those from the northern hemisphere, it would mean that although greater quantities would have to be consumed to mirror the magnitude of the positive health effects of fattier species, some benefits would be expected with long-term consumption of moderate amounts. Leaner fish may even be desirable for populations consuming large amounts of fish flesh, to avoid the production of excessive bleeding times (Dyerberg and Bang, 1979). Species selection between lean fish for such populations could then safely be done on the basis of maximum n3/n6 ratio. Consumption of the oils extracted from temperate Australian species would obviate the concern with tissue lipid content.

2.6.3 Conclusion and Dietary Relevance

The oils from the Australian and Northern Hemisphere species were found to be very similar in fatty acid composition, as would be expected for samples taken from waters of similar temperature. No inherent hemisphere-specific characteristics, therefore, distinguished the species. Inclusion of the oils from
temperate Australian fish species in the diet, on the basis of the findings here, would be equally beneficial as that of species from similar temperate northern hemisphere waters.

Major differences, however, were found between fish from tropical and temperate waters of the same hemisphere. High AA levels, in particular, may be a characteristic of fish from tropical waters in general, rather than the Australian tropics in particular.

In summary, it appears to be more important to know the approximate absolute latitude (distance from the equator) of fish for dietary inclusion than the hemisphere of catch.
CHAPTER 3

THE EFFECTS OF PROCESSING ON THE LIPIDS AND VITAMIN E IN

AUSTRALIAN FISH

3.1 Processing of Fish

All processing methods (including cooking) initiate chemical reactions within foods. The extent of these may be significant in either the reduction of nutrients, or the generation of toxic products (Armstrong and Bergan, 1992).

Fresh, raw fish muscle contains highly polyunsaturated fat (chapter 2). This does not necessarily mean that the lipids oxidise readily, as high antioxidant levels may also be present (Enser, 1987). Fish, however, is rarely eaten raw by consumers of a normal Western diet, but is cooked, and may also be processed to enhance its keeping ability. Such treatments of food can reduce the vitamin E content by up to 50% (Parker, 1989).

Fish tissue that has undergone significant lipid oxidation is usually rejected for consumption on the grounds of noticeable 'off' odours and flavours, characteristic of rancid lipid. Although rancidity may not be noticeable, major vitamin E depletion may have occurred. Consumption of fish in this condition may provide inadequate vitamin E to protect against oxidative damage to the polyunsaturated fatty acids ingested, which would result in the production of lipid free radicals and peroxides. Accumulation of these chemical species can lead to cellular aging, cancer and alteration of the genetic material (Walton and Packer, 1980).
3.2 Lipid Oxidation

The major type of damage to lipids during food processing is oxidative. Lipid oxidation is initiated by free radicals, whose production is enhanced by exposure to heat, light and ionising radiations (gamma-rays and x-rays) (Schaich, 1980).

Lipid oxidation by free radicals (autoxidation) is generally classified into three stages: initiation, propagation and termination, as reviewed by Labuza, 1971.

Substrates for lipid oxidation contain one or more of the methylene interrupted diene systems (1,4-cis,cis-pentadiene) commonly found in polyunsaturated fatty acids. Initiation of oxidation occurs with the abstraction of an hydrogen radical from this configuration (Figure 3.1). Once lipid free radicals have been produced they react with molecular oxygen to propagate throughout the lipid phase (Figure 3.2). Quenching reactions compete with the propagation, and eventually terminate the chain-reaction, as they give rise to non-radical products (Figure 3.3).

Many primary and secondary oxidation products are formed which alter the flavour of the food.

3.3 Protection Against Lipid Oxidation

Polyunsaturated fatty acids are protected against oxidation by lipid-soluble antioxidants, which may be primary or secondary in their mode of action.

Primary antioxidants quench non-lipid free radicals before initiation of lipid
FIGURE 3.1
Initiation

\[
\begin{align*}
\text{LH} & \xrightarrow{\text{Initiator}} \cdot^{	ext{H}} \\
& \quad \text{(Lipid molecule with the 1,4-cis,cis-pentadiene system)} \\
& \quad \text{(Lipid free radical)}
\end{align*}
\]

FIGURE 3.2
Propagation

\[
\begin{align*}
\text{L}^\cdot + \text{O}_2 & \rightarrow \text{LOO}^\cdot \\
\text{LOO}^\cdot + \text{L}^1\text{H} & \rightarrow \text{LOOH} + \text{L}^1\cdot
\end{align*}
\]

FIGURE 3.3
Termination

\[
\begin{align*}
2 \text{L}^\cdot & \rightarrow \text{LL} \\
2 \text{LOO}^\cdot & \rightarrow \text{O}_2 + \text{LOOL} \\
\text{LOO}^\cdot + \text{L}^\cdot & \rightarrow \text{LOOL}
\end{align*}
\]
oxidation can occur. They include many enzymes, such as superoxide dismutase and glutathione peroxidase, (Witting, 1980).

Secondary antioxidants (AH below) reduce the incidence of free radical chain propagation reactions, by competing with them for unpaired electrons:

\[ \text{LOO}^\cdot + \text{AH} \rightarrow \text{LOOH} + \text{A}^\cdot \]

When all of the available antioxidants have been consumed free radical propagation takes over.

Secondary antioxidants include vitamin E and β-carotene (Burton and Ingold, 1981).

3.4 Vitamin E

3.4.1 Structure and Biological Role

Vitamin E is the major lipid antioxidant in living organisms (Tappel, 1980). It consists of a group of molecules that are polyisoprenoid chromanol ring derivatives. There are eight naturally occurring forms (Pennock et al., 1964) (Figure 3.4), of which α-tocopherol is the most abundant and bioactive (Burton and Ingold, 1981). It mainly exists \textit{in vivo} as the acetate (Figure 3.5).

Tocopherols darken on oxidation, which is accelerated by exposure to light, heat, oxygen, alkaline pH, and Fe^{2+} and Cu^{2+} ions (Diplock, 1985).

Vitamin E also acts \textit{in vivo} to suppress thromboxane synthesis, while
FIGURE 3.4
Vitamin E Structures

\[
\begin{align*}
&\text{OH} & \text{CH}_3 \\
&\text{R}^1 = -(\text{CH}_2\text{CH}_2\text{CH}-\text{CH}_2)_g\text{H} \\
&\text{R}^2 = -(\text{CH}_2\text{CH}=\text{C}-\text{CH}_2)_g\text{H}
\end{align*}
\]

<table>
<thead>
<tr>
<th>Tocopherol (R(^1))</th>
<th>Tocotrienol (R(^2))</th>
<th>Methyl Group Positions</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\alpha)-T</td>
<td>(\alpha)-T3</td>
<td>5,7,8</td>
</tr>
<tr>
<td>(\beta)-T</td>
<td>(\beta)-T3</td>
<td>5,8</td>
</tr>
<tr>
<td>(\gamma)-T</td>
<td>(\gamma)-T3</td>
<td>7,8</td>
</tr>
<tr>
<td>(\delta)-T</td>
<td>(\delta)-T3</td>
<td>8</td>
</tr>
</tbody>
</table>

FIGURE 3.5
Alpha-tocopherol and Alpha-tocopherol acetate

\[
\begin{align*}
&\text{OH} \\
&\text{Alpha-tocopherol} \\
&[2,5,7,8\text{-tetramethyl}-2\text{-}(4,8,12\text{-trimethyltridecyl}-6\text{-chroman})]
\end{align*}
\]

\[
\begin{align*}
&\text{O} \\
&\text{H}_3\text{CO} \\
&\text{Alpha-tocopherol acetate}
\end{align*}
\]
boosting that of prostacyclins. It is suspected of lowering lipoxygenase and
cyclooxygenase activities, and is essential in preventing tissue damage by
intermediates of eicosanoid synthesis.

3.4.2 Dietary Requirements

Because vitamin E protects against lipid oxidation its requirement in the
diet is given in relation to the PUFA intake. Horwitt et al. (1961) suggested a
level of 0.6mg α-T/g PUFA, as well as a daily total for adults of 5.0mg. Draper
(1980) stated that only 0.18 to 0.52mg α-T/g PUFA is required.

Vitamin E levels in fish vary with sexual maturity, location and season of
catch and water temperature (Kinsella, 1982), but are usually within the range of
0.16 to 1.1mg α-T/g PUFA (Syväoja et al., 1985). Even populations with highly
polyunsaturated diets (such as the Greenland Eskimos) very rarely exhibit
deficiency symptoms, due to the accompanying high vitamin E levels in the
seafoods consumed. Vitamin E protection, however, may be inadequate in fish that
are stale or have been processed.

Deficiency of vitamin E is rare (usually only occurring in infants). It is
characterised by cellular aging, the accumulation of age pigments (lipofuscin and
ceroids), tissue lesions, and disorders of the reproductive, nervous and
cardiovascular systems.

3.4.3 Antioxidant Action

As mentioned in the previous section vitamin E terminates free radical
propagation reactions in the lipid phase. It does this by donating a phenolic

99
hydrogen radical (H'). The remaining unpaired electron is delocalised over the chroman ring. Many oxidation products have been described for α-T (including dimers, trimers, and tocopheroxides). The dominant route is given in Figure 3.6.

The levels of Vitamin E may not undergo significant reduction until the supply of vitamin C is depleted. Vitamin C has the capacity to regenerate vitamin E from the tocopheroxy radical, as has been demonstrated for a model solution by Packer et al. (1979).

3.4.4 Site of Lipid Oxidation and Vitamin E Protection

Vitamin E is a lipid antioxidant that is located within membranes. It acts there as an antioxidant for membrane-bound phospholipids.

The exact distribution of vitamin E within membranes, and its mode of action are not known. Diplock and Lucy (1973) proposed that the phytlyl side-chain may interact with phospholipid fatty acids within the bilayer, by fitting its branches into the spaces offered by two methylene interrupted cis- double bonds, stabilising the system (Figure 3.7).

Ingold (1983) stated that this model is insufficiently dynamic to account for vitamin E oxidation. It is generally agreed, however, that the bioactive chromanol rings are located at the membrane surface, and an interaction between phytlyl side-chains and phospholipid fatty acids occurs in the non-polar membrane interior.

Little work has been carried out to determine the influence of processing on the vitamin E levels of fish since that of Bunnell (1965).
FIGURE 3.6

Free Radical Scavenging by Alpha-tocopherol

\[ \text{Alpha-tocopherol} \xleftrightarrow{\text{LOO}^* \text{ LOOH}} \text{Alpha-tocopheroxy radical} \]

\[ \text{Alpha-tocopheryl quinone} \]
FIGURE 3.7
Possible Nature of Polyunsaturated Fatty Acid Protection by Vitamin E

Adapted from Diplock (1985)
3.5 Preservation of Fish by Gamma Irradiation

3.5.1 The Nature of Gamma Radiation

Gamma-(γ) radiation is very short wavelength electromagnetic radiation (3x10^{-11} to 3x10^{-9} cm, representing 0.04 to 4MeV of energy), produced by the spontaneous decay of certain nuclides. The source most commonly used for food irradiation is cobalt-60 (^{60}Co). It has a steady energy output (1.247MeV), and a relatively long half-life (5.27 years) (Spinks and Woods, 1976).

Gamma radiation is a form of ionising radiation, as the energies involved are so low that only the valence electrons of substrate atoms undergo interaction (Simic, 1983), thus forming ions.

The amount of radiation that a sample is exposed to depends on the dose-rate of the source as well as the time of exposure. It is measured in grays (Gy) - 1Gy involves the deposition of 1J of energy in each kilogram of sample. This unit has replaced the rad (1Gy = 100rad, or 1kGy = 0.1Mrad).

The actual amount of energy absorbed depends also on the nature of the sample. Variations in tissue composition and the presence of small bones is not considered important for normal doses applied to foods (Lovell, 1979).

3.5.2 World Use of Food Irradiation

The adoption of food irradiation by various countries has generally been the result of safety statements by international committees. Approximately nine countries were irradiating a limited range of foods in 1969. Following the Joint FAO/IAEA/WHO Expert Committee on Food Irradiation (JECFI) of 1976 an extra
seven countries were added to this list (van Kooij, 1982).

Currently thirty six countries perform some food irradiation, of which twenty do so on a commercial scale. More than forty nine foods are now treated (Pszczola, 1990).

Approximately six thousand tonnes of seafood per year is irradiated worldwide, which represents only about 6% of the total amount for human consumption (Australian Consumers' Association, 1987).

3.5.3 Application to Seafoods

Fish for human consumption are exposed to radurizing doses of gamma radiation. This involves doses defined to be sufficient to delay microbial spoilage (by increasing the lag-time) such that refrigerated storage is significantly extended. It has been estimated (Nickerson et al., 1983) that a dose of only 1kGy extends the shelf-life of unfrozen fish by approximately 1 week.

The mode of preservation is by non-selective reduction of the total microbial count, via damage to their chromosomes. It is, therefore, a form of cold sterilisation analogous to pasteurisation in that all micro-organisms are not killed (Nickerson et al., 1983).

For organoleptic as well as safety reasons doses are kept to a minimum. An optimum dose represents a balance between adequate shelf-life extension and maintenance of flavour and odour. Such a value needs to be determined for each fish species to be treated, as wide variations in tissue composition are known to exist. Nickerson et al. (1983) reviewed the results of the irradiation of several
species of fish and concluded that optimum doses lie between 1.0 and 2.5kGy.

The control of various physical factors can significantly reduce unwanted organoleptic changes, while maintaining the same degree of bacterial reduction. These include the use of low temperatures (slightly above 0°C), short high dose-rate exposures, and vacuum packing. Subsequent effects are minimised by refrigerated storage and maintenance of the vacuum. Storage time and temperature after radiation exposure also alter the sensitivity of lipids to damage (Coleby, 1959).

Benefits of Irradiating Seafoods

By increasing the interval of time during which the product is acceptable for consumption the radius of distribution is increased (Nickerson et al., 1983). This would enable communities isolated from fish sources to consume them in significant quantities. Thus not only would the domestic market be expanded, but the health benefits of high seafood consumption (as discussed in chapter 1) would be more widely enjoyed. The product could also be exported in fresh form. Many countries presently irradiate imported fresh fish. A higher quality product could be obtained if this were performed immediately after catch.

More efficient use of the world’s catch would be possible if radiation preservation were undertaken on a wider scale. Currently, large quantities of fish are dumped or relegated to fish meal due to the early onset of spoilage.

Many retailers understock with fish to avoid spoilage losses. A more stable product would also lead to greater availability to consumers in regions where fresh fish are already consumed.
Gamma-irradiation of foods can often be utilised to replace chemical preservatives. The cost of irradiation is greater than that of common additives, but use may be made of the technique to replace chemicals associated with health risks – such as sodium nitrite in fish preservation (Loaharanu and Urbain, 1983).

Problems with Food Irradiation

The main problem that has delayed the wider use of gamma radiation to preserve foods (including seafoods) is the poor opinion held by the general public of the technique. These attitudes were summarised by Nickerson et al. (1983) to involve a widespread fear of radiation and toxin production, as well as of the possibility of abuse.

It is quite commonly believed that irradiated food is actually radioactive itself. The energies involved (less than 2MeV), however, are well below those required to trigger secondary radioactivity (in excess of 15MeV) (Nickerson et al., 1983). The safety of foods in terms of induced radioactivity is, therefore, assured.

The production of toxins is a far more controversial issue. The very complexity and variability of the food matrix, even within fish, is so great that it is highly unlikely that all products of irradiation will ever be determined. Indeed, this has never been done for any more conventional preservation or cooking method. Many volatile compounds have been found to arise as a result of exposure to gamma radiation, and destruction of a proportion of some nutrients is known to occur. Nickerson et al. (1983) compared these effects to those produced by heat

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sterilisation, and concluded that although the mechanisms may differ, their nature and magnitude are very similar. Schubert (1978) estimated the consumption of heat-processed foods to contribute 50 to 500 times the decomposition products to the diet that irradiated foods would. The actual amount of energy absorbed by food during γ-irradiation is much less than during cooking. In fact a dose of 10kGy exposes a food with the heat capacity of water (4.184J/°C) to as much energy as a 2.4°C rise in temperature (Joint FAO/IAEA/WHO Expert Committee on the Wholesomeness of Irradiated Food (JECIF), 1980). It was concluded by the 1980 JECIF that irradiation of any food at doses up to 10kGy presents no toxicological hazard, and that toxicological testing is no longer required (FAO/IAEA/WHO, 1981).

The most convincing evidence available to demonstrate the safety of the consumption of γ-irradiation preserved fish comes from animal feeding trials. Neither carcinogenicity nor toxicity have been reported for various animals fed irradiated cod, ocean perch, or tuna (Nickerson et al., 1983). Irradiated foods, in general, are routinely fed to humans with suppressed immune systems, astronauts and clinical study volunteers (Pszczola, 1990). The 1987 European Scientific Committee for Food report stated that no further animal feeding studies are needed to establish the safety of consumption of foods irradiated at up to 10kGy (Scientific Committee for Food, 1987).

The consumer concern over abusive handling of irradiated foods is unfounded. Gamma radiation cannot reverse spoilage that has already occurred, nor can it mask its effects (Pszczola, 1990). The claims that irradiation kills spoilage organisms while allowing pathogens to proliferate have been largely
dismissed by experimental findings. Irradiated fish have been found to spoil, and therefore become organoleptically unacceptable, before becoming toxic (Learson et al., 1970).

In summary, irradiation is probably the most comprehensively studied method of food preservation known (Ghosh, 1989; Pszczola, 1990). Many economical, nutritional and ecological advantages could be enjoyed if its use were more widespread. Foods thus treated have been tested on both animals and humans without adverse effects, and the process has been endorsed by the major international health organisations (Pszczola, 1990).

3.5.4 Chemical Effects

The energy possessed by incoming γ-rays is sufficient to disrupt any chemical bonds (Rosen, 1972) and cause lesions in macromolecules when applied directly. The in vivo effects, however, are much less severe - with not even the inactivation of autolytic enzymes achieved. This is due to shielding and dilution effects of the surrounding tissue, as well as the presence of natural antioxidants. The nature of the matrix, therefore, is critical in determining the extent of radiation damage (Nawar, 1983).

Effects on Lipids

Of major concern to producers of radiation-preserved fish is the generation of undesirable ("burnt" or "irradiated") flavours and odours, although radurization doses are generally believed to be too low to produce them, except in very fatty fish (Delincée, 1983a). It has been found that the volatiles responsible for these
effects are lipid breakdown products similar to those from more widely practiced treatments.

The major reactions induced by $\gamma$-irradiation of lipid molecules, as listed by Thomas (1988), include:

* H abstraction
* $\text{H}^+$ and $\text{OH}^-$ addition
* isomerisation (both structural and stereochemical)
* hydrolysis
* polymerisation
* dehydration
* decarboxylation
* cross-linking.

These are the same as those identified in heated foods (Nawar, 1983; Thomas, 1988). Such a diverse range of reaction types leads to a similarly wide range of products (including hydrocarbons, aldehydes, peroxides, ketones and lactones) (Urbain, 1986). It should be noted that these observations have been made on model systems (which do not provide any matrix shielding) when exposed to extremely high doses - for example, 60kGy (Le Tellier and Nawar, 1972), and 250kGy (Merritt and Vajdi, 1982; Vajdi et al., 1983). At the low doses of irradiation the accumulation of these products is not significant, as assessed organoleptically.

Of major concern in the irradiation of fish lipids is the loss of fatty acids, which are known to be very oxidatively labile, by nature of their high degree of unsaturation.
Radiation-induced lipid oxidation is the result of increased free radical production. Water comprises approximately 80% of the mass of fish muscle, and is believed to be the main free radical source in irradiated tissue. Free radicals are generated in reactions such as those shown in Figure 3.8.

![Figure 3.8: Radiolysis of Water](image)

The hydroxyl radical (·OH) is believed to be the major initiator of lipid (LH) oxidation, as follows:

\[
\text{LH} + \cdot\text{OH} \rightarrow \text{L}^- + \text{H}_2\text{O}
\]

Oxidation is substantially increased when molecular oxygen (O$_2$) is present, via the formation of lipid peroxy free radicals, as was shown in Figure 3.2. Some O$_2$ is present in cells, but its concentration is substantially increased when irradiation is performed in air. This is a consequence of the low dose rates used for gamma-irradiation of food allowing enough time for oxygen to diffuse into the
sample (Diehl, 1990).

It is generally recommended that fish be vacuum-packed prior to exposure to gamma radiation, to prevent such oxidation. Adam et al. (1982) detected no polyunsaturated fatty acid destruction in herring fillets, even when irradiated at 50kGy under vacuum and stored for twenty one days. Although this practice has been found to prevent lipid oxidation, it may not be necessary in lean fish species, and actually may be undesirable, as stated by Delincée (1983a), in that it causes deterioration in appearance. As oxidation is probably only of concern in the irradiation of very fatty fish it was noted that air packaging may be preferred for radurization to maintain physical characteristics.

Irrespective of the presence of oxygen, γ-irradiation is known to cause significant radiolysis, releasing a wide range of products (such as hydrocarbons, aldehydes, and methyl and ethyl esters). Triglyceride cleavage occurs preferentially at electron-deficient sites (Urbain, 1986), such as the ester linkage, as has been determined by irradiation of model systems (Williams, 1962). Little is known about the radiolytic reactions of phospholipids. Nawar and Handel (1978), however, irradiated phospholipids and triglycerides and found that the products were similar but produced in far smaller quantities from the former.

Lipid cleavage is the result of direct radiolytic attack on intramolecular bonds. This effect is highly dependent on the amount of matrix shielding afforded by the tissue.

**Effects on Other Components**

Losses of various vitamins (including vitamins A, B group (B₁, B₂, B₆, and
B₁₂), C, D, and K) have been reported as a result of radurization performed in air. The 1977 JECIF report stated that the reduction in nutritional value should be compared with other processes (FAO/IAEA/WHO, 1977). This was taken a step further by the 1980 JECIF, which concluded that there is no cause for concern over nutritional losses caused by food irradiation (FAO/IAEA/WHO, 1981). Nickerson et al. (1983) asserted that vitamin destruction in irradiated foods occurs to an extent no greater than in heat treated foods.

In order to address these issues the lipids and vitamin E of lean fish were analysed before and after exposure to radurizing doses of gamma radiation. The extent of any changes, and their nutritional significance will be assessed by determining the extent of fatty acid and vitamin E retention.

3.5.5 Experimental

3.5.5.1 Samples

Nine normal adult sized Black bream (Acanthopagrus australis), and nine Redfish (Centroberyx affinis) were obtained from the catches in New South Wales waters during Autumn, 1990 (section 2.2.1).

The two fillets (skin intact) from each fish were individually sealed in plastic bags and stored at -22°C. All fillets were placed on ice 24 hours prior to irradiation, which was performed on one fillet from each fish. All samples were returned to frozen storage, as before, within 2 hours of completion of irradiation.
Triplicate samples of both species were irradiated on ice at each of three doses (1, 2 and 6kGy). Gamma-irradiation, at a dose rate of $3.596 \times 10^3$Gy/s was performed in the Underwater Calibration Facility (UCF) at the Australian Nuclear Science and Technology Organisation (ANSTO) facility at Lucas Heights (Sydney).

### 3.5.5.2 Vitamin E Analysis

#### Sample Preparation

A portion (10g) of minced fillet also used for lipid extraction (section 3.5.5.3) was subjected to saponification and extraction into non-polar solvent (hexane) by the method of Syvänöja et al. (1985) (Figure 3.9). Care was taken throughout the procedure to exclude oxygen (by blanketing in N$_2$) and light (by wrapping flasks in aluminium foil, and by working in a darkened room). Solvents were of analytical (ethanol, and hexane for extraction) or HPLC grade (water, and the final 1mL of hexane). The temperature was kept at below 40°C even during solvent evaporation (Söderhjelm and Andersson, 1978).

#### Internal Standard

The internal standard, γ-tocopherol (95% pure, Eastman-Kodak), was added as a solution in hexane (0.23mg/mL, 0.40mL) at the beginning of the saponification. This solution was stored at low temperature (-17°C) under N$_2$ in a darkened container, and replaced every two weeks (Indyk, 1988).
FIGURE 3.9
Vitamin E Saponification and Extraction

10g fish + 0.6g ascorbic acid + 20mL water + 50mL ethanol + internal standard + 10 mg BHT

stand 30min under N₂

add 10mL KOH (50%)

stir magnetically overnight at room temperature

extract 3 times into hexane (50mL)

wash 3 times with water (50mL)

dry over Na₂SO₄ (anhydrous) for 2 hours in freezer

evaporate solvent under N₂ at room temperature

add 1mL hexane

BHT = Butylated hydroxytoluene

Internal standard = 0.40mL of 0.23mg/mL γ-tocopherol
HPLC Analysis

Analyses were performed on an Altex isocratic HPLC system equipped with a fixed loop injector (20μL) and a tunable UV detector (model 484, Waters Associates, Milford, MA). A silica DYN Microsorb normal-phase column (5μm particles, 100Å pore size, 4.6x250mm, Rainin Instruments Inc., MA) was used, with a silica guard column (which was replaced every 10-12 injections).

The mobile phase was isopropanol (2-propanol) (0.40%) in hexane, and was degassed with helium prior to use. The column was conditioned (to remove polar compounds) and stored in methanol after each day of use (Carpenter, 1979).

UV detection was at 295nm, 0.10AUFS. The analysis time was approximately 30 minutes.

Data were recorded and integrated using the DAPA computing integrator package (DAPA Scientific Software, Perth, WA).

3.5.5.3 Lipid Analysis

Lipid Extraction and Derivatisation

Lipid extraction, separation and methyl ester formation of the total lipid and phospholipid fraction was performed on all of the thirty-six fillets (9 controls and 9 irradiated of both species) by the methods outlined in sections 2.2.2 and 2.2.4.

Free fatty acid (FFA) methyl esters of lipids from the Black bream fillets treated at 6kGy and their controls were also prepared. Their selective formation from the total lipid extract was performed by a method adapted from that of Tserng et al. (1981) (Figure 3.10).
FIGURE 3.10
Free Fatty Acid Methyl Ester Formation

200\(\mu\)L Lipid extract + 1mL DMP + 200\(\mu\)L conc. HCl

\[\text{Stand 15 minutes at room temperature}\]

add 100\(\mu\)L pyridine

\[\text{Centrifuge briefly}\]

add 0.50mL \(\text{H}_2\text{O}\) + 0.50mL iso-octane

\[\text{Remove iso-octane layer}\]

\[\text{Evaporate to dryness}\]

add 50\(\mu\)L iso-octane

DMP = dimethoxypropane

Adapted from Tserng \textit{et al.} (1981)
Internal Standards

Triglyceride and phospholipid internal standards were as described in section 2.2.3. Tricosanoic acid (23:0) was the internal standard for the free fatty acid analyses (99% pure, Sigma Chemical Co.).

Determination of Lipid and Water Contents

The lipid content of all fillets (%L) was determined by the gravimetric method outlined in section 2.2.6.

The water content (%H₂O), by mass, in each of the Redfish fillets was determined by drying in a Mettler LP12 Infrared Moisture Balance (setting 6).

Gas Chromatographic Analysis and Data Handling

GC analyses and data handling were carried out for all three fractions as detailed previously (sections 2.2.7 and 2.2.8).

Statistical Analysis

Statistics were performed using STATGRAPHICS (see section 2.2.10). Fatty acid profiles were used directly in all statistical procedures (without mean profile calculation). Multivariate and univariate statistics as those described previously (section 2.2.10) were used. Where sample sizes were small only ANOVA was performed. (This was for the comparisons made between fatty acid ratios within each dose level.)

Species data matrices for PCA were constructed from the major fatty acids of all eighteen profiles, and performed as in section 2.2.10.
3.5.6 Development of Preparation and Analysis Procedures for Vitamin E

Reversed-phase high performance liquid chromatography (RP-HPLC) analysis of vitamin E requires a fluorescence detector (Ball 1988a), unless further extract purification is carried out (Cohen and Lapointe, 1980). As this was not available, normal-phase (NP-) HPLC was employed. The poorer selectivity of UV detection dictated that a more specific sample preparation (than the usual extraction followed by saponification that is used for fluorescence detection) was needed. A saponification step was necessary to release the free forms of vitamin E from their esters (Figure 3.11). By performing this step prior to the extraction a cleaner extract was obtained, as any polar lipid residues were discarded with the aqueous phase. Less trouble with emulsion formation, and more complete release of tocopherols from their esters are other advantages of this method - which gave 92-95% \( \alpha \)-tocopherol recovery as compared with 85-89% when extraction precedes saponification (McMurray et al., 1980).

The saponification-extraction method of Syväöja et al. (1985) was employed, which the authors claimed provides 85-101% recovery of the various vitamin E forms. Unnecessary oxidation was avoided during the procedure by using very clean glassware, keeping the reaction mixture under nitrogen, and protecting it from as much light as possible, as well as the addition of vitamin C (ascorbic acid) (Niki, 1988) and butylated hydroxytoluene (BHT) (Speek et al., 1985) as antioxidants.
Internal standard selection was limited by the fact that the commonly used compound, tocol (2-methyl-2-(4',8',12'-trimethyltridecyl)chroman-6-ol) and the tocotrienols (which only occur naturally in very small quantities) are unstable in alkaline solutions (Chow et al., 1969), and so could not have been added until after the extraction step. The use of a natural tocopherol was decided upon. The α- and β-forms have been reported to commonly occur in fish (Syvāoja et al., 1985) and so were rejected. δ-Tocopherol was suspected of also being unstable to alkaline pH (Syvāoja et al., 1985). This left γ-tocopherol as the only possible choice from these compounds. This form is known to be more stable under alkaline conditions than the δ-form, and so was added prior to commencement of the saponification. No γ-tocopherol was detected in Bream or Redfish extracts performed without its addition.

Hexane mobile phases with 1% (Indyk, 1988), and 1.5% isopropanol (Carpenter, 1979; Pozo et al., 1990) have been specified for use in normal phase
HPLC separation of vitamin E forms. Both of these were tested, and although a number of peaks were resolved, it was found that on lowering the alcohol concentration (reducing the solvent polarity) a larger number of peaks appeared, indicating that the initial conditions gave inadequate resolution. Resolution was acceptable when 0.4% isopropanol in hexane (at a flow rate of 1.1mL/min) was used. This extended the analysis time from approximately 12 to 30 minutes (for all small peaks to elute). (Absolute retention times varied greatly from day to day and even between runs, which is a disadvantage of using NP-HPLC (Ball, 1988b).)

The concentrated nature of the extracts obtained from this method obviated concern over the poor sensitivity of UV detection – stated by Thompson and Hatina (1979) to be ten times lower than for fluorescence detection of the same compounds. Nelis et al. (1985) claimed that 60ng of α-tocopherol can be observed by using UV detection. The extract with the smallest α-tocopherol content resulted in an injection containing at least 400ng and provided a substantial peak.

NP-HPLC offers the advantage over reversed phase (RP-) HPLC of being able to resolve all eight forms of vitamin E by isocratic elution. The order of elution, paralleling that of increasing polarity, is as follows:-

\[
\alpha-T \rightarrow \alpha-T3 \rightarrow \beta-T \rightarrow \gamma-T \rightarrow \beta-T3 \rightarrow \gamma-T3 \rightarrow \delta-T \rightarrow \delta-T3
\]

least polar

most polar

Although a number of peaks were observed (Figure 3.12) only α-tocopherol was quantified. This was because authentic standards of the other forms were not
available and the traces were dominated by the α-form, which is also by far the most bioactive.

3.5.7 Development of a Method for Free Fatty Acid Methyl Ester Preparation

The free fatty acid (FFA) methylation method of Tserng et al. (1981) was chosen for use, as it is selective for these molecules (thus removing the need for prior fractionation) and overcame many of the problems of other methods (such as the formation of pyrolazines from unsaturated fatty acids, which occurs with diazomethane). It is rapid (15 minutes) at room temperature reduced the risk of polyunsaturated oxidation, and efficiently esterified even very long chain acids. The final extraction into iso-octane ensured a clean sample for GC injection.

The esterification process involves the scavenging of water by dimethoxypropane (DMP) to form methanol, which reacts with the FFAs forming their methyl esters. The methylation step is an equilibrium, which is pushed towards completion by scavenging of the water produced by DMP:

\[ \text{RCOOH} + \text{CH}_3\text{OH} + \overset{H}{\overset{+}{\text{H}}^+} \rightarrow \text{RCOOCCH}_3 + \text{H}_2\text{O} \]

where RCOOH is the free fatty acid and RCOOCH\(_3\) is the fatty acid methyl ester. The reaction is terminated by the inactivation of remaining DMP by pyridine.
FIGURE 3.12
HPLC Chromatogram of Fish Vitamin E Extract

Internal standard
\( \gamma \)-Tocopherol

\( \alpha \)-Tocopherol

Detector Response

Retention Time (minutes)
Tserng et al. (1981) developed this method for use with blood plasma, which is comprised of approximately 200μg FFA/mL. The 50μL plasma samples used by these authors, therefore, contained approximately 10μg of FFAs. The amount of fish lipid extract used for this reaction was initially determined from the fact that about 1% of fatty acids in the extracted lipid from fresh fish are present in the free form. The use of a 200μL aliquot was estimated to provide 10μg of free fatty acids.

The extent of trans-esterification of bound fatty acids occurring during this reaction was stated by Tserng et al. (1981) to depend upon the acid concentration and reaction time (with extended times increasing the efficiency of the reaction). Trials were conducted with various amounts of concentrated hydrochloric acid (40-250μL) and pyridine (20-150μL), and varying reaction times (5-20minutes). Determination of the extent of transmethylation was facilitated by the addition of trinonadecanoin (the triglyceride standard). It was found that the greatest ratio of methylated free to triglyceride-bound fatty acids (as assessed by the 23:0/19:0 ratio) resulted when 200μL of concentrated HCl, 100μL of pyridine and a 15 minute reaction time were used.
3.5.8 Irradiated Fish

3.5.8.1 Physical Observations

An initial γ-irradiation trial was carried out at 1, 2 and 6kGy on Black bream and Redfish fillets that had been vacuum-packed. This was found to produce a pink discolouration at all doses. The red pigment produced was probably the result of interactions between lipid hydroperoxides (or their products) and existing pigments (such as the carotenoids) (Hsieh and Kinsella, 1989). Lukton and Mackinney (1956) found that losses of carotenoids on irradiation of various foods were often lower when performed in air rather than nitrogen. They suggested that this may be due to a competition between oxygen and the carotenoids for lipid free radicals. The subsequent irradiation was performed on fillets packaged in air (at the same doses), with alleviation of this problem.

Lipid and vitamin E extraction and analyses were performed on the fillets packaged in air.

Some of the irradiated fillets (irrespective of packaging environment) exhibited a smell judged to be like that of hexenal.

3.5.8.2 Bound Fatty Acids

No qualitative differences were observed between any of the control and irradiated fillets' total lipid fatty acid chromatograms.

Principal components analysis of the fatty acids from total lipid fractions of each species produced no clusters in the 2-dimensional scatterplots (Figures 3.13 and 3.14) (the presence of which would have indicated differences between control and irradiated fillets).
FIGURE 3.13
Scatterplot for Principal Components Analysis of Irradiated (I) and Control (C) Black Bream Total Lipid Extract Fatty Acids
49.0% of total variance

Gx = xkGy

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FIGURE 3.14
Scatterplot for Principal Components Analysis of Irradiated (I) and Control (C) Redfish Total Lipid Extract Fatty Acids
63.6% of total variance

$G_x = xkGy$
Univariate analyses of fatty acid ratios from the total lipids of the irradiated and control fillets of each species revealed no significant changes on irradiation (Table 3.1). Comparisons at each dose by ANOVA only (due to insufficient data for Mann-Whitney testing) revealed only one Redfish to have an altered n3/n6 ratio, which was probably an experimental error (Table 3.2). These results are consistent with those of Rosinvalli et al. (1971), where the irradiation of Cod and Haddock at 2kGy was reported to produce only small changes in the fatty acid profiles, which were probably within the error of the method.

TABLE 3.1
Parametric and Non-parametric Statistical Comparisons Between Gamma-Irradiated (n=9) and Control (n=9) Fish Fillets

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Mann-Whitney</th>
<th>ANOVA</th>
<th>Mann-Whitney</th>
<th>ANOVA</th>
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</thead>
<tbody>
<tr>
<td>Total lipid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P/S</td>
<td>accept</td>
<td>(p &gt; 0.20)</td>
<td>accept</td>
<td>(p = 0.963)</td>
</tr>
<tr>
<td>n3/n6</td>
<td>accept</td>
<td>(p &gt; 0.20)</td>
<td>accept</td>
<td>(p = 0.601)</td>
</tr>
<tr>
<td>%M</td>
<td>accept</td>
<td>(p &gt; 0.20)</td>
<td>accept</td>
<td>(p = 0.564)</td>
</tr>
<tr>
<td>Phospholipid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P/S</td>
<td>accept</td>
<td>(p &gt; 0.20)</td>
<td>accept</td>
<td>(p = 0.408)</td>
</tr>
<tr>
<td>n3/n6</td>
<td>accept</td>
<td>(p &gt; 0.20)</td>
<td>accept</td>
<td>(p = 0.688)</td>
</tr>
</tbody>
</table>

3.5.8.3 Phospholipid Fatty Acids

No significant differences were observed between the phospholipid fatty acid chromatograms of control and irradiated samples. Principal components analysis of the phospholipid extracts of the two species also revealed no evident clustering (Figures 3.15 and 3.16). Both species did, however, exhibit altered correlations with one of the displayed components between the fillets irradiated
FIGURE 3.15
Scatterplot for Principal Components Analysis of Irradiated (I) and Control (C) Black Bream Phospholipid Fatty Acids
38.7% of total variance

Gx = xkGy
FIGURE 3.16
Scatterplot for Principal Components Analysis of Irradiated (I) and Control (C) Redfish Phospholipid Fatty Acids
49.4% of total variance

\[ G_x = xkG_y \]
at 6kGy and their controls. In both cases this was found to signify a poorer correlation between irradiated samples and saturated and monounsaturated fatty acids than with the controls. (This was shown by the decreased correlation of Black bream with its first, and the Redfish with its second principal component.) This is an unusual result, as the major effect was expected to be oxidation of polyunsaturates. It was, however, consistent with the findings of Dubravcic and Nawar (1969) for volatile lipid oxidation products in Mackerel oil irradiated at up to 60kGy. They reported the major components produced to be those known to originate from saturated and monounsaturated fatty acids (14:0, 18:1, 20:1 and 22:1), while insignificant quantities of polyunsaturated fatty acid products (from 18:4, 20:5 and 22:6) were detected. This effect cannot be attributed to the membrane-bound nature of the phospholipid fatty acids, nor their higher proximity to vitamin E, as the extracted oil was irradiated. The authors explained the results by noting the compactness of the PUFA chains, which reduces hydrocarbon formation, as well as the fact that the single bonds are located alpha to double bonds, and are thus stabilised relative to those in more unsaturated fatty acids.

Univariate analyses of combined controls compared with irradiated fillets yielded no significant differences in phospholipid fatty acid properties on irradiation (Table 3.1). ANOVA analyses at each dose level also showed no differences (Table 3.2). The fact that the phospholipid fatty acids of fillets treated at 6kGy did not differ from their controls suggested that the trends mentioned above were not nutritionally significant. The trends may actually have been a result of experimental variations, rather than of the radiation treatment.
TABLE 3.2
ANOVA Comparisons Between Phospholipid Fractions of Gamma-irradiated (I, n=6) and Control (C, n=6) Fish Fillets

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Black bream (n=6)</th>
<th>Radfish (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1kGy</td>
<td>2kGy</td>
</tr>
<tr>
<td>Total lipid</td>
<td>accept</td>
<td>accept</td>
</tr>
<tr>
<td>P/S</td>
<td>(p = 0.889)</td>
<td>(p = 0.421)</td>
</tr>
<tr>
<td>n3/n6</td>
<td>accept</td>
<td>accept</td>
</tr>
<tr>
<td></td>
<td>(p = 0.800)</td>
<td>(p = 0.316)</td>
</tr>
<tr>
<td>%M</td>
<td>accept</td>
<td>accept</td>
</tr>
<tr>
<td></td>
<td>(p = 0.434)</td>
<td>(p = 0.965)</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>accept</td>
<td>accept</td>
</tr>
<tr>
<td>P/S</td>
<td>(p = 0.063)</td>
<td>(p = 0.963)</td>
</tr>
<tr>
<td>n3/n6</td>
<td>accept</td>
<td>accept</td>
</tr>
<tr>
<td></td>
<td>(p = 0.529)</td>
<td>(p = 0.906)</td>
</tr>
</tbody>
</table>

3.5.8.4 Free Fatty Acids

No consistent changes were determined in the free fatty acid profiles as a result of gamma-irradiation at 6kGy. Principal components analysis was not possible, due to the small number of samples analysed.

3.5.9 Absolute Quantification of Lipid, Fatty Acids and Vitamin E Before and After γ-Irradiation

This set of experiments was designed to evaluate the influence of γ-irradiation on the lipid, fatty acids and vitamin E in two species of fish.

3.5.9.1 Lipid and Fatty Acid Contents

Large differences were observed in the total fatty acid contents between control and irradiated fillets of both species. Smaller, but still significant, variations occurred in the lipid contents (%L). These findings prompted an investigation into the water contents of the fillets after freezing, to determine
if drip loss, occurring during thawing (which would contain mostly water, with negligible lipid) was the cause of the variations. As can be seen from Table 3.3 little variation in (fillet) water contents was evident for Redfish. (Water contents of Black bream could not be determined, as insufficient flesh remained after the other analyses.)

**TABLE 3.3**

Fillet Composition in Irradiated and Control Redfish

<table>
<thead>
<tr>
<th>Sample</th>
<th>%Water</th>
<th>%Lipid</th>
<th>Fatty acids (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1kGy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>78.4</td>
<td>1.06</td>
<td>8.70</td>
</tr>
<tr>
<td>Irradiated 1</td>
<td>77.7</td>
<td>1.07</td>
<td>8.40</td>
</tr>
<tr>
<td>Control 2</td>
<td>79.8</td>
<td>0.43</td>
<td>3.18</td>
</tr>
<tr>
<td>Irradiated 2</td>
<td>80.2</td>
<td>0.52</td>
<td>5.11</td>
</tr>
<tr>
<td>Control 3</td>
<td>78.9</td>
<td>0.56</td>
<td>6.70</td>
</tr>
<tr>
<td>Irradiated 3</td>
<td>79.3</td>
<td>0.77</td>
<td>7.25</td>
</tr>
<tr>
<td>2kGy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>78.1</td>
<td>1.08</td>
<td>7.77</td>
</tr>
<tr>
<td>Irradiated 1</td>
<td>80.2</td>
<td>1.26</td>
<td>12.0</td>
</tr>
<tr>
<td>Control 2</td>
<td>80.6</td>
<td>1.14</td>
<td>11.0</td>
</tr>
<tr>
<td>Irradiated 2</td>
<td>79.8</td>
<td>1.01</td>
<td>5.04</td>
</tr>
<tr>
<td>Control 3</td>
<td>79.5</td>
<td>0.90</td>
<td>5.44</td>
</tr>
<tr>
<td>Irradiated 3</td>
<td>80.5</td>
<td>0.84</td>
<td>5.19</td>
</tr>
<tr>
<td>6kGy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>78.1</td>
<td>1.17</td>
<td>5.12</td>
</tr>
<tr>
<td>Irradiated 1</td>
<td>79.2</td>
<td>1.04</td>
<td>3.72</td>
</tr>
<tr>
<td>Control 2</td>
<td>77.3</td>
<td>1.48</td>
<td>10.2</td>
</tr>
<tr>
<td>Irradiated 2</td>
<td>78.1</td>
<td>1.38</td>
<td>9.31</td>
</tr>
<tr>
<td>Control 3</td>
<td>78.9</td>
<td>1.37</td>
<td>10.2</td>
</tr>
<tr>
<td>Irradiated 3</td>
<td>79.0</td>
<td>1.58</td>
<td>8.47</td>
</tr>
</tbody>
</table>

The variation in the flesh lipid content were initially thought to be due to the error inherent in its gravimetric determination. The total fatty acid content values, however, were obtained by summing the FAME peaks in each GC chromatogram, and were expected to be more accurate. The source of error in this procedure was probably the same as was mentioned in section 2.5.1.3, namely
incomplete lipid extraction and inconsistent recovery of the triglyceride internal standard.

On the basis of these results no significant effects of $\gamma$-radiation on either the total lipid or fatty acid contents of the fish can be concluded.

3.5.9.2 Vitamin E

The vitamin E values and their retentions on irradiation are presented in Tables 3.4 and 3.5.

Some fillets actually gave increased vitamin E levels on $\gamma$-irradiation, which may have been due to a loss of other components to drip causing a concentration effect on the vitamin E.

More than half of the fillets irradiated exhibited lower vitamin E contents than their controls. This was probably due to radiation-induced tocopherol oxidation. The extent of the losses could not be correlated with dose. This result is probably due to variations in the levels of other components, such as the amounts of other antioxidants (such as vitamin C) and PUFAs.

Tissue vitamin E levels in nine Bream from the same catch were determined (Table 3.5A). They were found to be lower than those found in low-fat Finnish fish by Syvänäja et al. (1985) and to bound the range reported by Bieri (1984). The biological variability in vitamin E content between individuals was quite large (29% variability).

3.5.10 Discussion

Analyses of the fatty acid compositions revealed that differences between
individual control fillets were as significant as any irradiation-induced effects. No differences in nutritionally important fatty acid ratios (P/S and n3/n6) were displayed between the treated and untreated groups. (Lower values would be

<table>
<thead>
<tr>
<th>Sample</th>
<th>Vitamin E (mg/100g muscle)</th>
<th>% Vitamin E retained</th>
</tr>
</thead>
<tbody>
<tr>
<td>1kGy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>0.798</td>
<td></td>
</tr>
<tr>
<td>Irradiated 1</td>
<td>0.936</td>
<td>117%</td>
</tr>
<tr>
<td>Control 2</td>
<td>0.368</td>
<td></td>
</tr>
<tr>
<td>Irradiated 2</td>
<td>0.301</td>
<td>82%</td>
</tr>
<tr>
<td>Control 3</td>
<td>0.786</td>
<td></td>
</tr>
<tr>
<td>Irradiated 3</td>
<td>0.501</td>
<td>65%</td>
</tr>
<tr>
<td>2kGy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>0.470</td>
<td></td>
</tr>
<tr>
<td>Irradiated 1</td>
<td>0.512</td>
<td>109%</td>
</tr>
<tr>
<td>Control 2</td>
<td>0.882</td>
<td></td>
</tr>
<tr>
<td>Irradiated 2</td>
<td>1.143</td>
<td>130%</td>
</tr>
<tr>
<td>Control 3</td>
<td>0.765</td>
<td></td>
</tr>
<tr>
<td>Irradiated 3</td>
<td>0.512</td>
<td>67%</td>
</tr>
<tr>
<td>6kGy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>0.495</td>
<td></td>
</tr>
<tr>
<td>Irradiated 1</td>
<td>0.510</td>
<td>103%</td>
</tr>
<tr>
<td>Control 2</td>
<td>0.988</td>
<td></td>
</tr>
<tr>
<td>Irradiated 2</td>
<td>0.565</td>
<td>57%</td>
</tr>
<tr>
<td>Control 3</td>
<td>0.751</td>
<td></td>
</tr>
<tr>
<td>Irradiated 3</td>
<td>0.371</td>
<td>49%</td>
</tr>
</tbody>
</table>
TABLE 3.5
Vitamin E (alpha-tocopherol) Retention in Irradiated Bream

<table>
<thead>
<tr>
<th>Sample</th>
<th>Vitamin E (mg/100g muscle)</th>
<th>%Vitamin E retained</th>
</tr>
</thead>
<tbody>
<tr>
<td>1kGy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>0.332</td>
<td></td>
</tr>
<tr>
<td>Irradiated 1</td>
<td>0.246</td>
<td>74%</td>
</tr>
<tr>
<td>Control 2</td>
<td>0.284</td>
<td></td>
</tr>
<tr>
<td>Irradiated 2</td>
<td>?*</td>
<td>?*</td>
</tr>
<tr>
<td>Control 3</td>
<td>0.538</td>
<td>93%</td>
</tr>
<tr>
<td>Irradiated 3</td>
<td>0.502</td>
<td></td>
</tr>
<tr>
<td>2kGy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>0.329</td>
<td></td>
</tr>
<tr>
<td>Irradiated 1</td>
<td>0.274</td>
<td>83%</td>
</tr>
<tr>
<td>Control 2</td>
<td>0.334</td>
<td></td>
</tr>
<tr>
<td>Irradiated 2</td>
<td>0.381</td>
<td>114%</td>
</tr>
<tr>
<td>Control 3</td>
<td>0.326</td>
<td></td>
</tr>
<tr>
<td>Irradiated 3</td>
<td>0.203</td>
<td>62%</td>
</tr>
<tr>
<td>6kGy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>0.437</td>
<td></td>
</tr>
<tr>
<td>Irradiated 1</td>
<td>0.253</td>
<td>58%</td>
</tr>
<tr>
<td>Control 2</td>
<td>0.372</td>
<td></td>
</tr>
<tr>
<td>Irradiated 2</td>
<td>0.314</td>
<td>84%</td>
</tr>
<tr>
<td>Control 3</td>
<td>0.495</td>
<td></td>
</tr>
<tr>
<td>Irradiated 3</td>
<td>0.511</td>
<td>103%</td>
</tr>
</tbody>
</table>

*Data not available

---

TABLE 3.5A
Tissue Vitamin E Levels for a Single Sample of Black Bream (mg/100g flesh)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0.628</td>
<td>0.730</td>
</tr>
<tr>
<td>0.527</td>
<td>0.613</td>
</tr>
<tr>
<td>0.945</td>
<td>0.827</td>
</tr>
<tr>
<td>0.329</td>
<td>0.496</td>
</tr>
<tr>
<td>0.796</td>
<td></td>
</tr>
</tbody>
</table>

mean: 0.655mg/100g
variability: 29%
expected from selective oxidation of the more polyunsaturated fatty acids.) The therapeutic effects believed to stem from these ratios in dietary lipid would, therefore, be maintained when replacing fresh fish with irradiated fish.

The stability of the polyunsaturated fatty acids exhibited is consistent with the high level of protection afforded by vitamin E in the fresh fillets. This antioxidant is bound within membranes, and so protects the phospholipid-bound polyunsaturates. This suggests that any oxidation would occur in the triglyceride fatty acids (as they lack this protection), which also have high degrees of polyunsaturation. The fillets used in this study, however, were extremely lean, and as such would have contained only very small amounts of this type of lipid. Oxidation of neutral lipid-bound fatty acids, therefore, would not have contributed significantly to the overall PUFA loss.

The nutritional significance of the vitamin E destruction needs to be assessed in terms of the dietary requirement for this component relative to ingested polyunsaturated fatty acids, which reflects its role in the protection of polyunsaturates against free radical attack. This level has been quoted to be, at most, approximately 0.6mg α-T/g PUFA, as mentioned previously (section 3.4.2). The values for fillets before and after irradiation (Table 3.6) exceeded this requirement in all fillets (in most cases by very large factors). The differences in this ratio between control and irradiated fillets was determined not to be significant, by univariate analyses (Table 3.7).
TABLE 3.6
Vitamin E Relative to PUFA Content of Control and Irradiated Fish (mg alpha-tocopherol/g PUFA)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Bream</th>
<th>Redfish</th>
</tr>
</thead>
<tbody>
<tr>
<td>1kGy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>3.00</td>
<td>7.16</td>
</tr>
<tr>
<td>Irradiated 1</td>
<td>3.44</td>
<td>9.36</td>
</tr>
<tr>
<td>Control 2</td>
<td>0.89</td>
<td>14.3</td>
</tr>
<tr>
<td>Irradiated 2</td>
<td>?</td>
<td>5.36</td>
</tr>
<tr>
<td>Control 3</td>
<td>1.84</td>
<td>9.31</td>
</tr>
<tr>
<td>Irradiated 3</td>
<td>1.61</td>
<td>5.23</td>
</tr>
<tr>
<td>2kGy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>1.25</td>
<td>5.71</td>
</tr>
<tr>
<td>Irradiated 1</td>
<td>2.45</td>
<td>3.41</td>
</tr>
<tr>
<td>Control 2</td>
<td>15.5</td>
<td>6.49</td>
</tr>
<tr>
<td>Irradiated 2</td>
<td>8.50</td>
<td>16.1</td>
</tr>
<tr>
<td>Control 3</td>
<td>1.65</td>
<td>9.38</td>
</tr>
<tr>
<td>Irradiated 3</td>
<td>2.38</td>
<td>6.67</td>
</tr>
<tr>
<td>6kGy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>1.22</td>
<td>4.93</td>
</tr>
<tr>
<td>Irradiated 1</td>
<td>1.16</td>
<td>8.73</td>
</tr>
<tr>
<td>Control 2</td>
<td>1.76</td>
<td>6.99</td>
</tr>
<tr>
<td>Irradiated 2</td>
<td>0.62</td>
<td>7.18</td>
</tr>
<tr>
<td>Control 3</td>
<td>2.62</td>
<td>6.40</td>
</tr>
<tr>
<td>Irradiated 3</td>
<td>1.11</td>
<td>3.00</td>
</tr>
</tbody>
</table>

TABLE 3.7
Parametric and Non-parametric Comparisons of Vitamin E Protection of Polyunsaturated Fatty Acids Between Control (n=9) and Irradiated (n=9) Fish (mg alpha-tocopherol/g PUFA)

<table>
<thead>
<tr>
<th></th>
<th>Black bream</th>
<th>Redfish</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mann-Whitney</td>
<td>ANOVA</td>
</tr>
<tr>
<td>accept</td>
<td>accept</td>
<td>accept</td>
</tr>
<tr>
<td>(p &gt; 0.20)</td>
<td>(p=0.735)</td>
<td>(p &gt; 0.20)</td>
</tr>
</tbody>
</table>

137
3.5.11 Conclusion

The results obtained indicate that radurization of Black bream and Redfish resulted in no significant changes in fatty acid profiles of the bound lipid fractions. This was the case even when performed in air at three times the recommended maximum dose for fish. Vitamin E losses were evident in some fillets, but this nutrient was still present at levels above those required to act as an antioxidant for the PUFAs.

The resistance of these species to radiation-induced lipid oxidation is thought to be a consequence of their extremely low lipid contents (in particular triglyceride) combined with a high degree of vitamin E protection for the phospholipids.

The implications of these findings for Australian fish in general (which are commonly very lean) are that they can be irradiated at normal radurization doses without the need for vacuum-packing, while retaining the nutritionally beneficial characteristics of their lipids. Gamma-irradiation preservation, therefore, appears to be particularly suited to fish from temperate Australian waters.

Despite the lack of significant changes in the fatty acid compositions of the lipid fractions, changes in colour and aroma were detected (see section 3.5.8.1). Analysis of the volatile products of lipid gamma-irradiation may be a more sensitive indicator of radiation-induced changes. While the production of these compounds may not have direct nutritional significance in terms of fish composition, they may lead to rejection for consumption on the grounds of organoleptic changes.
3.6 Preservation of Fish by Smoking

3.6.1 Smoking of Fish

Smoked fish are consumed throughout the world for their enhanced and characteristic flavours. The main and original use of this processing method, however, is for the preservation of fish in tropical regions (such as South East Asia, India and West Africa), where the fresh product has an extremely short keeping time (Rawson, 1966).

The process generally involves a combination of brining, heating, drying and smoking, although much flexibility exists as to how these steps are carried out (Poulter, 1988). The precise conditions markedly affect the degree of preservation afforded.

Microbial spoilage is delayed by a reduction in the water content of the fish, as well as the action of antimicrobial agents from the smoke. Antioxidants in the smoke prevent rancidity of the lipid. The keeping ability of smoked fish is, therefore, improved by a combination of the prevention of microbial spoilage and rancidity.

3.6.2 Chemicals Deposited in Smoked Foods

Very little work has been carried out to establish the precise chemical effects on foods of smoking them. It is known, however, that deposition in the flesh of compounds from the smoke, although occurring to only a small extent (approximately 10mg/100g flesh (Burt, 1988)), have dramatic effects.

The most obvious effect of the smoking process to the consumer is the
strong smoky flavour of the food. This comes from complex mixtures of volatile flavour constituents (mainly phenolic compounds and aldehydes with low flavour thresholds) being present in the flesh (Gilbert and Knowles, 1975). The exact composition, and thus the flavour produced, varies with the type of wood used.

Smoked fish also exhibit a yellow to brown colour, which is believed to be due to carbonyl-amino browning reactions.

A factor not obvious to the consumer is the presence of carcinogenic polycyclic aromatic hydrocarbons (such as 3,4-benzopyrene) within the food. These compounds are mainly present in the extreme outer layers of smoked foods, as their large molecular structures ensure poor penetrability (Gilbert and Knowles, 1975).

The phenolic compounds responsible for much of the characteristic flavour of smoked foods also serve as lipid antioxidants. Their production is via pyrolysis of the major wood components (cellulose, hemicellulose, and lignin (Goos, 1952)). The structures of some of the more common phenolics are given in Figure 3.17.

The initial soaking of fish to be smoked in brine (sodium chloride solution) usually results in tissue salt contents of 2 to 5% (Burt, 1988). Salt is used to reduce the water content of fillets such that subsequent drying will only need mild heating to reduce the water activity enough to significantly delay spoilage. It also acts as a pro-oxidant.
FIGURE 3.17
Some Common Phenolics in Wood Smoke

- 2-Methoxyphenol (Guaiacol)
- 2,6-Dimethoxyphenol (Syringol)
- 2-Methoxy-4-methylphenol (4-Methylguaiacol)
3.6.3 Effects on Lipid and Vitamin E Content of Each Processing Step

3.6.3.1 Soaking in Brine

Exposing the surface of fish muscle to sodium chloride results in an overall uptake of salt, as well as water, due to the formation of salt-protein complexes (Hultin, 1985). At the final tissue levels involved in the brining of fish prior to smoke exposure, salt acts as a pro-oxidant (Castell et al., 1965; Koizumi et al., 1980; Nambudiry, 1980). The mechanism of their action is not known, but is suspected of either being through the catalytic effects of haem protein degradation products, or the direct attack on lipids by chloride ions (Love and Pearson, 1971).

Although this pro-oxidant effect of salt has been noted no reports have been found in the literature concerning losses of the labile endogenous antioxidants (such as vitamin E) as a result of brining.

3.6.3.2 Heating

Heating of fish is known to cause lipid oxidation. The temperatures commonly used, however, greatly exceed those involved in smoking (up to only 80°C) (Armstrong and Bergan, 1992). This factor alone, therefore, may not be sufficient to induce significant lipid oxidation.

The drying effect of hot-smoking of fish causes lipid oxidation - the extent of which depends on the final water content (Woolfe, 1975).

At low water contents oxidative degradation is enhanced by high temperatures (Bligh et al., 1988). The two factors, therefore, work together to alter the fatty acid compositions.
3.6.3.3 Smoke Exposure

The phenolics of wood smoke act as antioxidants in the same way as vitamin E (by donating a hydrogen radical and resonance stabilising the remaining phenoxy radical’s unpaired electron over the aromatic ring). Although this effect is widely acknowledged, little work has been carried out to determine the extent of the protection against lipid oxidation of these compounds. In one study of hot-smoked Atlantic mackerel (Scomber scombrus L.) Bhuiyan et al. (1986) reported that no changes were found in fatty acid profiles.

Hobbs (1982) stated that the antioxidative effect of smoke compounds on fish are more than compensated for by the pro-oxidative effects of brining.

3.6.3.4 Conclusion

The antioxidative action of smoke phenolics, together with the mild heat exposure, suggest that hot smoking is probably a very gentle preservation treatment. Thus, from a nutritional viewpoint, it appears to be a suitable process to subject fish to, as their high degree of lipid polyunsaturation may not be significantly reduced. Conclusions at this stage must be tentative, as there is a paucity of data available on the actual effects of the process on fish lipids.

Changes occurring in the lipids, as well as loss of vitamin E from lean fish fillets as a result of brining and hot smoking were determined.
3.6.4 Experimental

3.6.4.1 Samples

Four Black bream (*Acanthopagrus australis*) and four Redfish (*Centroberyx affinis*) were obtained from the same catch (autumn, NSW, 1990) as described in section 2.2.1.

Each fish (skin intact) was filleted and labelled, with one fillet used as a control and the other immersed in an 80 sal brine (20% NaCl w/v). Two of the salted fillets from each species were then further treated in a smoking cabinet and exposing them to wood smoke (15min, 80C), followed by extended mild cooking (35min, 80C).

3.6.4.2 Lipid Extraction and Analysis

Lipid extraction, internal standard addition, fractionation and methyl ester formation were all performed as described previously (section 2.2). The total lipid, phospholipid and free fatty acid methyl esters formed were then analysed by capillary gas-chromatography (see section 2.2.7).

3.6.4.3 Statistical Analysis

Matrices for principal components analyses were constructed from the eight fatty acids profiles of each fraction (4 controls, 2 salted, and 2 smoked extracts). The analyses were performed as in section 2.2.10.

ANOVA and Kruskal-Wallis univariate comparisons were carried out on the P/S and n3/n6 ratios, as well as monounsaturated fatty acid contents (%M) of the total 48 profiles.

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ANOVA of the vitamin E percentage retentions on treatment (both species combined) was performed to detect any differences between salting and smoking (preceded by salting) retentions.

3.6.5 Changes in Smoked Fish

3.6.5.1 Bound Fatty Acids

Principal components analyses of the eight fillets' (four controls, two salted and two smoked) total lipid extract fatty acid profiles did not reveal clusters that indicated effects from the treatments in either species (Figures 3.18 and 3.19). It was evident that the differences between the profiles of untreated and treated fillets were no greater than between individual untreated (control) fillets. In many cases the profile correlating most closely with that of a particular control was that from the salted or smoked fillet from the same fish. These findings indicate that individual variations were greater than those induced by salting or smoking.

Univariate analyses failed to detect any nutritionally important differences between the three groups (control, salted and smoked), as determined by comparisons of their P/S and n3/n6 ratios, as well as the monounsaturated fatty acid content (%M) (Table 3.8).

3.6.5.2 Phospholipid Fatty Acids

The Black bream PCA 2-dimensional scatterplot revealed that the control and treated fillets from each fish had almost identical phospholipid fatty acid profiles, as the four pairs of data points (representing the four fish) show (Figure 3.20). This indicated that the membrane-bound phospholipids were protected from
FIGURE 3.18
Scatterplot for Principal Components Analysis
of Smoked (SM), Salted (SA) and Control (C)
Black Bream
Total Lipid Extract Fatty Acids
82.5% of total variance
FIGURE 3.19
Scatterplot for Principal Components Analysis of Smoked (SM), Salted (SA) and Control (C) Redfish Total Lipid Extract Fatty Acids

74.3% of total variance
FIGURE 3.20
Scatterplot for Principal Components Analysis of Smoked (SM), Salted (SA) and Control (C) Black Bream Phospholipid Fatty Acids

71.6% of total variance
TABLE 3.8
Parametric and Non-parametric Statistical Comparisons Between Control (n=4), Salted (n=2) and Smoked (n=2) Fish

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Total fatty acids</th>
<th>Phospholipids</th>
<th>Free fatty acids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kruskal-Wallis</td>
<td>ANOVA</td>
<td>Kruskal-Wallis</td>
</tr>
<tr>
<td>Bream</td>
<td>accept</td>
<td>accept</td>
<td>accept</td>
</tr>
<tr>
<td>P/S</td>
<td>(p=0.5698)</td>
<td>(p=0.7041)</td>
<td>(p=0.7788)</td>
</tr>
<tr>
<td>n3/n6</td>
<td>accept</td>
<td>accept</td>
<td>accept</td>
</tr>
<tr>
<td>(p=0.3456)</td>
<td>(p=0.3836)</td>
<td>(p=0.9394)</td>
<td>(p=0.7391)</td>
</tr>
<tr>
<td>%M</td>
<td>accept</td>
<td>accept</td>
<td>accept</td>
</tr>
<tr>
<td>(p=0.1271)</td>
<td>(p=0.1131)</td>
<td>(p=0.5134)</td>
<td>(p=0.3782)</td>
</tr>
<tr>
<td>Redfish</td>
<td>accept</td>
<td>accept</td>
<td>accept</td>
</tr>
<tr>
<td>P/S</td>
<td>(p=0.2191)</td>
<td>(p=0.3200)</td>
<td>(p=0.1353)</td>
</tr>
<tr>
<td>n3/n6</td>
<td>accept</td>
<td>accept</td>
<td>accept</td>
</tr>
<tr>
<td>(p=0.1271)</td>
<td>(p=0.2798)</td>
<td>(p=0.2476)</td>
<td>(p=0.3504)</td>
</tr>
<tr>
<td>%M</td>
<td>accept</td>
<td>accept</td>
<td>accept</td>
</tr>
<tr>
<td>(p=0.2347)</td>
<td>(p=0.4329)</td>
<td>(p=0.1026)</td>
<td>(p=0.0672)</td>
</tr>
</tbody>
</table>

any processing effects.

The Redfish scatterplot (Figure 3.21) displayed no clusters, but it appeared to have consistent shifts in the two smoked fillets' profiles, causing decreased correlations with the first and increased correlations with the second principal components. When the weightings of these components were investigated it was found that this was indicative of poorer correlations with the major saturated and monounsaturated fatty acids. These results are unusual, but are consistent with those discussed in section 3.5.8.3.

Univariate analyses of this fraction also failed to reveal processing effects on nutritional factors (Table 3.8).
FIGURE 3.21
Scatterplot for Principal Components Analysis of Smoked (SM), Salted (SA) and Control (C) Redfish Phospholipid Fatty Acids

79.9% of total variance
3.6.5.3 Free Fatty Acids

The scatterplots for the free fatty acid traces of the two species also displayed no effects that could be attributed to processing (figures 3.22 and 3.23). Again, however, a consistent shift occurred with two of the smoked fillets (Black bream). This arose because of better correlation with the second principal component. This component was weighted heavily in 18:1n9 and 16:1n7, which are major triglyceride components. This result was probably caused by cleavage of fatty acids from triglyceride molecules.

Univariate analyses revealed no nutritionally important changes in this fraction (Table 3.8). (It must be noted that these changes would have been relevant from a compositional point of view only, as the contribution of this fraction to the total ingested fatty acids is negligible.)

3.6.5.4 Absolute Quantification

Correction Factors

Comparisons of the total amounts of fatty acids from each fraction, and vitamin E, were complicated by the mass fluctuations resulting from both treatment processes. For each fillet the multiplicative factor necessary to convert the initial mass to the final was calculated:

\[
\text{Mass factor} = \frac{\text{final fillet mass (g)}}{\text{initial fillet mass (g)}}
\]

The total amounts of fatty acids and vitamin E (each converted to a percentage of tissue mass, g/100g) determined initially were divided by this factor (as
FIGURE 3.22
Scatterplot for Principal Components Analysis of Smoked (SM), Salted (SA) and Control (C) Black Bream Free Fatty Acids
66.2% of total variance
FIGURE 3.23
Scatterplot for Principal Components Analysis of Smoked (SM), Salted (SA) and Control (C) Redfish Free Fatty Acids
65.5% of total variance
decreased fillet mass would increase their contributions, and vice versa). These values were compared with those determined from the processed fillets to investigate the nature of any changes.

Comparisons of absolute amounts of fatty acids extracted from each fraction could not be made, as relative errors between them occurred in quantification (as was discussed in section 2.5.1.3).

Fillets Soaked in Brine

All fillets soaked in brine gained mass (3 to 7%), indicating that sodium chloride had been absorbed, thus causing slight dilutions of tissue components.

No consistent changes were found in the total amounts of extracted fatty acids from any of the fractions.

All salted fillets contained significantly less vitamin E than their controls (with only 24-58% retained), indicating that oxidation had occurred (Table 3.9).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mass factor</th>
<th>Total bound</th>
<th>Phospholipid</th>
<th>Free fatty acids</th>
<th>Vitamin E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bream</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salted 1</td>
<td>1.07</td>
<td>204</td>
<td>173</td>
<td>167</td>
<td>24</td>
</tr>
<tr>
<td>Salted 2</td>
<td>1.03</td>
<td>96</td>
<td>142</td>
<td>93</td>
<td>58</td>
</tr>
<tr>
<td>Smoked 3</td>
<td>0.78</td>
<td>420</td>
<td>198</td>
<td>107</td>
<td>132</td>
</tr>
<tr>
<td>Smoked 4</td>
<td>0.80</td>
<td>-</td>
<td>148</td>
<td>118</td>
<td>77</td>
</tr>
<tr>
<td>Redfish</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salted 1</td>
<td>1.05</td>
<td>73</td>
<td>88</td>
<td>38</td>
<td>42</td>
</tr>
<tr>
<td>Salted 2</td>
<td>1.03</td>
<td>147</td>
<td>103</td>
<td>60</td>
<td>46</td>
</tr>
<tr>
<td>Smoked 3</td>
<td>0.71</td>
<td>186</td>
<td>216</td>
<td>71</td>
<td>88</td>
</tr>
<tr>
<td>Smoked 4</td>
<td>0.75</td>
<td>654</td>
<td>300</td>
<td>71</td>
<td>85</td>
</tr>
</tbody>
</table>
Smoked Fillets

Fillets subjected to smoking exhibited mass losses of 20 to 30%. This meant that the mass increases expected from the uptake of sodium chloride from the brine and compounds from the smoke were more than compensated for by the drying effect of the hot smoking. By assuming that all of the mass lost was from water, and that it initially comprised 80% of the total mass (as determined on fish from the same catch, shown in Table 3.3) it was calculated that the smoked fillets contained approximately 70% water (after correction for salt absorption). The smoking process, therefore, involved only a mild drying of the fillets.

Smoked fillets from both species yielded greater amounts of fatty acids from the total lipid and phospholipid fractions than expected from the concentration effect of water loss. This was probably caused by variations in the efficiency of the lipid extraction procedure, as discussed previously (section 2.5.1.3).

The free fatty acid contents remained constant for the smoked Bream, and were slightly lower for the Redfish. It was earlier determined, however, that triglyceride cleavage had probably occurred in the Redfish. Excess free fatty acids, therefore, were lost to the drip.

Only small changes in Vitamin E levels were determined in the smoked fillets (with a minimum of 77% retention), indicating that oxidation was not significant.

3.6.5.5 Discussion

When the amounts of vitamin E retained in the smoked and salted fillets
were compared, the latter were found to have been depleted to a greater extent
($p = 0.0087$) (Figure 3.24).

It should be realised that the smoked fillets had first been salted, and so
would have lost much of their original vitamin E (section 3.6.5.4.2). The high
retention in the smoked fillets compared to the untreated controls suggests that
any oxidation caused by the salt absorption was reversed by the smoking process.

The initial step in the oxidation of vitamin E involves the abstraction of an
hydrogen radical to form a tocopheroxy free radical. The antioxidants in smoke,
which are mainly phenolic compounds, undergo similar abstraction, and in doing
so reconvert tocopheroxy radicals to the parent tocopherol. This reaction is
outlined in Figure 3.25 for the major natural form, \( \alpha \)-tocopherol.

Adequate vitamin E was present, even in the salted fillets, to protect
against PUFA oxidation (>0.60mg \( \alpha \)-T/gPUFA) (Table 3.10). (This ratio was not
calculated for Bream, as although consistent quantities of base were added,
enabling \%\( \alpha \)-T retention calculations, the amount was not enough for complete
saponification.)

3.6.5.6 Conclusion

Smoking of Black bream and Redfish produced no detectable changes in the
fatty acid profiles of the total bound lipids, or phospholipids. Changes in vitamin
E levels were also not significant.

The smoked fish exhibited greatly increased flesh lipid contents, which
would be to the advantage of consumers seeking health benefits from fish fatty
acids through consumption of flesh. In terms of fatty acid compositions and
FIGURE 3.24
Least Squared Difference ANOVA Mean Intervals of Salted (SA) and Smoked (SM) Fish

% Vitamin E Retained

% Vitamin E

SA (n=4)  SM (n=4)
FIGURE 3.25
Alpha-tocopherol Regeneration by Wood Smoke Phenolics

Alpha-tocopherol
Phenoxy radical

LOO• ↔ LOOH

Alpha-tocopherol radical
contents, therefore, smoking of lean Australian fish would actually increase their nutritional value for the consumption of a given mass of fish.

### TABLE 3.10
Alpha-tocopherol protection of Polyunsaturated Fatty Acids (PUFA) in Redfish

<table>
<thead>
<tr>
<th>Sample</th>
<th>Alpha-tocopherol/PUFA (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9.87</td>
</tr>
<tr>
<td>Control</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>9.70</td>
</tr>
<tr>
<td>2</td>
<td>9.44</td>
</tr>
<tr>
<td>3</td>
<td>13.0</td>
</tr>
<tr>
<td>4</td>
<td>5.58</td>
</tr>
<tr>
<td>Salted</td>
<td>3.97</td>
</tr>
<tr>
<td>1</td>
<td>5.91</td>
</tr>
<tr>
<td>2</td>
<td>1.57</td>
</tr>
<tr>
<td>Smoked</td>
<td>4</td>
</tr>
</tbody>
</table>

#### 3.7 General Conclusion

No nutritionally significant changes were found in the fatty acid compositions of gamma-irradiated or smoked fish. It appears that Australian fish are very resistant to oxidative degradation of their lipids, even though they are very polyunsaturated. This is most likely a result of their low lipid contents and high degrees of vitamin E protection.

Vitamin E loss was detected for some irradiated fillets, although no correlation with dose was apparent. It was also depleted in fillets soaked in brine, but appeared to be restored during the subsequent smoking process. None of the
losses were sufficient to lower the protection against PUFA oxidation to unacceptable levels.

In the case of smoked fish the increased tissue lipid content (as a result of mild drying) is actually beneficial to the consumer, as smaller quantities of fish flesh would need to be consumed to obtain positive health effects from the fatty acids.
CHAPTER 4

THE USE OF HPLC PROTEIN PROFILES FOR

FISH SPECIES IDENTIFICATION

4.1 The Need for Fish Species Identification

Knowledge of the species of fish destined for human consumption is necessary for economic, legal and nutritional reasons. These include the adherence to fair pricing policies (Sumner and Mealy, 1983; Ashoor and Knox, 1985), the meeting of quality control requirements and export labelling regulations, as well as enabling species selection for dietary inclusion based on known lipid compositions (Naughton et al., 1983; Brown et al., 1989).

The very large number of different species of finfish make species identification based on physical characteristics more difficult than with other phyla consumed by humans. This has resulted in confusion and deliberate misrepresentation - such as was exposed in the fish species substitution scandal in Australia (Australian Consumers' Association, 1982; Sumner and Mealy, 1983), in which many inexpensive species were being commonly retailed as more expensive ones (such as Spotted ling, Orange roughy and other species for Barramundi, and Orange roughy and Oreo dory for John dory).

4.2 Current Methods of Species Identification

Fish are usually identified on the basis of their physical appearance. This
requires them to be whole, and so cannot be performed on processed, minced or filleted samples. For closely related species, however, morphological identification is often a difficult task requiring an experienced person.

Chemical means of fish species identification are based on the fact that the water-soluble proteins of the sarcoplasm have structures and relative compositions characteristic of each species. Within a species they are invariant under changes in such factors as age, sex and physiological condition (Mackie, 1980).

Electrophoresis was the first chemical technique applied to species identification (Connell, 1953). Much work has since been published on the further development and applications of various electrophoretic techniques. Methods have been established to identify the species of raw (both fresh and frozen), dried, salted, smoked, ripened, canned and cooked fish (Rehbein, 1990a). Electrophoresis is clearly a useful and widely applicable technique for species identification.

The need for another method arose from consideration of the disadvantages involved in the electrophoretic methods. These include: poor reproducibility (and therefore need for simultaneous analysis of the suspected authentic species); the large amount of time required for preparation and analysis (although many samples can be run at once); and the need for a densitometer, as well as the extra step of using it.

High performance liquid chromatography (HPLC) is a technique that is fast, produces stable profiles, and uses equipment commonly available in most analytical laboratories. It was first applied to the separation and quantification of the sarcoplasmic proteins of fish to produce species-characteristic profiles by Ashoor and Knox (1985). Only two subsequent studies have appeared in the
literature: those by Osman et al., 1987 and Rehbein, 1990b.

Many areas remain to be addressed in the use of this technique. One of these is the extent of intraspecies variation apparent in the profiles, both between samplings (from different locations and seasons), and within a sample of adequate size (greater than the maximum of three individuals that have been analysed to date). Another issue requiring attention is the development of a comprehensive identification procedure that takes into account minor variations in chromatography, and that can be generally applied. The ability to identify the species of processed fish, as has been extensively developed for electrophoretic methods, also remains to be investigated (as will be discussed in chapter 5).

Despite considerable variations in the chromatographic parameters between the three published works, the chromatograms have all contained fewer peaks than the numbers of bands obtained in electrophoretograms. Although this indicates that the resolution was poorer for HPLC, it may actually be an advantage in this instance, as species identification may be facilitated from a smaller number of variables. It may also avoid problems arising from polymorphism (Keenan and Shaklee, 1985).

The issue of intraspecies variability in sarcoplasmic protein HPLC profiles is addressed in this study, in order to determine the validity of the method for species characterisation. A refining of the procedures for extraction and analysis was also carried out, and a general procedure for species identification established that can be applied to the library of profiles generated, or any other appropriate data.
4.3 Experimental

4.3.1 Samples

Ten individuals from each of five species (*Acanthopagrus australis* (Black bream), *Zeus faber* (John dory), *Centroberyx affinis* (Redfish/Nannygai), *Genypterus blacodes* (Ling) and *Zenopsis nebulosus* (Mirror dory)) were analysed. They were caught in the spring of 1989 from the coastal waters of New South Wales, and the sampling repeated at the same locations in the following autumn (1990). A further ten Black bream were also taken from both Victorian and Queensland waters during spring 1990. For more information on catch details see section 2.2.1.

Other fish analysed included *Hoplostethus atlanticus* (Orange Roughy) (5), *Glaucosoma scapulare* (Pearl Perch), *Argyrosomus hololepidotus* (Jewfish), *Mugil cephalus* (Mullet), *Chrysophrys auratus* (Red Bream/Schnapper), *Hyporhampus australis* (Garfish), *Sillago ciliata* (Whiting), *Paracaeos pedleyi* (Banana fish), *Pseudocaranx dentex* (Trevally) and *Helicolenus percoides* (Ocean perch) (3 of each) (May & Maxwell, 1986). These were caught in eastern Australian coastal waters (NSW and Qld), and purchased in Sydney fish markets.

4.3.2 Sample Preparation

Fillets were stored at -22°C prior to thawing and mincing.

Minced white muscle (5g) was homogenised at high speed for 30 seconds in Milli Q water (20mL) using a Waring blender. The supernatant was filtered through
a 0.45μm membrane prior to refrigeration. Analysis was carried out within 4 hours of preparation.

4.3.3 Internal Standard

Various peptides (including trypsinogen, lysozyme, β-lactoglobulin, pepsin, bovine serum albumin (BSA) and ovalbumin) were tested for their suitability as internal standards. Quantitative trials were made with BSA (Sigma Chemical Co., MO) which was added for a final concentration of approximately 8mg/mL.

Fifteen aromatic organic compounds (analytical grade) were trialed. Of these, 2,6-dimethylphenol (DMPh) was selected for quantitative addition (0.10mg/mL).

4.3.4 HPLC Analysis

Analyses were performed on a Waters HPLC system equipped with two model 501 pumps, a model U6K injector, model 720 programmable system controller and a model 484 tunable UV detector (Waters Associates, Milford, MA), fitted with a 250mm x 4.4mm Hi-Pore RP-304 C-4 column (Bio-Rad Laboratories, Richmond, CA), and a C-18 guard column.

Data were recorded and integrated using the DAPA computing integrator package (DAPA Scientific Software, Perth, WA).

The HPLC mobile phase was obtained from 30% (solvent A) and 70% (solvent B) aqueous solutions of acetonitrile in 0.1% trifluoroacetic acid. The extract (20μL) was run using a gradient of 17 to 55% solvent B (36.8-52.0% acetonitrile) over 60min, during which time the absorbance was recorded at
280nm, 0.10 AUFS. A further 30 minutes was then required for washing the column with up to 100% solvent B, then return to initial conditions and equilibration.

When not in use the column was stored under 10% isopropyl alcohol (2-propanol).

4.3.5 Statistical Analyses

Mean peak percentage areas for each sample (10 profiles) are given, along with the corresponding percentage variabilities.

Star-symbol plots and chi square statistics were generated using the STATGRAPHICS package of statistical software (Statistical Graphics Corporation, Maryland, USA), using the HPLC peak percentage areas. Profiles were rejected as being significantly different if the probability level of their chi-square value was below 0.05.
DISCUSSION

4.4 Method Development

The general sample preparation and analysis procedures developed by Ashoor and Knox (1985) were initially adopted and refined, as detailed in the following sections.

4.4.1 Sample Preparation

Fish were filleted and any visible dark muscle removed prior to mincing. Portions were then removed for analysis. This was carried out to avoid any effects due to a lack of homogeneity in composition over the fillets, although they were not expected to be significant (Hultin, 1985; Kyriacou, 1988).

The sample preparation was scaled down for use with 5g of fish muscle. Extractions on many fish that had been stored for various times and temperatures lead to the observation that the addition of a large volume of water was necessary to avoid gel formation. When gel formation occurred it hindered filtration, in some cases entirely. The use of 20mL of water avoided this problem when homogenising 5g of fish.

The preservative (sodium azide) was omitted, as extracts were analysed within a few (maximum of 4) hours of preparation. Refrigerated storage for times exceeding this produced noticeable turbidity in the extracts (Kyriacou, 1988).
4.4.2 HPLC Analysis

A reversed phase C-4 column was used, as with previous work. Rehbein (1990b) trialed three columns (one ion exchange and two reversed-phase - including the one used here), and concluded that the best separation was achieved with the C-4 column.

The gradient used by Ashoor and Knox (1985) (38-66.5% acetonitrile over 90 minutes) gave broad peaks on the system used in this study, especially towards the end of the profiles, while the more polar components were inadequately retained on the column. Various modifications to this gradient were tested in an attempt to improve peak shape and resolution. A gradient of 36.8–52% acetonitrile over 60 minutes was eventually determined to give the best results. In comparison with gradients used by other authors this was very gradual, and involved only a small increase in acetonitrile composition in the mobile phase (Table 4.1).

<table>
<thead>
<tr>
<th>Acetonitrile gradient</th>
<th>Change in acetonitrile concentration</th>
<th>Time for gradient (min)</th>
<th>Analysis time (min)</th>
<th>Rate of change in gradient (%/min)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>38.0 - 66.5%</td>
<td>20.6%</td>
<td>90</td>
<td>65</td>
<td>0.32</td>
<td>Ashoor and Knox (1985)</td>
</tr>
<tr>
<td>36.0 - 66.5%</td>
<td>20.3%</td>
<td>90</td>
<td>60</td>
<td>0.34</td>
<td>Osman et al. (1987)</td>
</tr>
<tr>
<td>19.0 - 66.5%</td>
<td>47.5%</td>
<td>40</td>
<td>40</td>
<td>1.19</td>
<td>Rehbein (1990b)</td>
</tr>
<tr>
<td>36.8 - 52.0%</td>
<td>15.2%</td>
<td>60</td>
<td>60</td>
<td>0.25</td>
<td>This work</td>
</tr>
</tbody>
</table>

Peak shape towards the end of the chromatograms was improved by eluting proteins before the acetonitrile concentration significantly exceeded 50%, as excessive interaction with uncapped silanol groups was avoided (Engelhardt, 1986).

A gradual decrease in mobile phase polarity aided in the separation of
peaks, as adequate time was allowed for interactions with the C-4 stationary phase. It also reduced the likelihood of protein precipitation on the column.

The acetonitrile concentration was increased to 70% over the 30 minutes immediately following data collection, to remove any extremely non-polar material from the column.

4.4.3 Internal Standard Use

Most previous reports on the use of HPLC profiles of sarcoplasmic protein extracts for fish species identification (Ashoor and Knox, 1985; Osman et al., 1987; Kyriacou, 1988) have used bovine serum albumin (BSA) as an internal standard, even though in all cases overlap with the native peaks of at least one species was noted. BSA was trialed in this study and found to be unsatisfactory for use as it not only overlapped with the proteins of many species, but exhibited a small peak tailing the main (BSA) peak (Figure 4.1). The relative areas of these peaks was not constant. (The dual peaks were also evident when newly purchased BSA was injected.)

Various peptides were substituted for BSA in an attempt to find a suitable internal standard. These included trypsinogen, lysozyme, β-lactoglobulin, pepsin and ovalbumin. All of these were found to be unacceptable, as peaks (which were often multiplets) were produced at undesirable retention times.

Ribonuclease, which was used by Rehbein (1990b) was not tested, but its reported elution well before the fish protein peaks suggested that at the lower polarity of the initial mobile phase in this work it would not have been adequately retained on the column.
The problem in using a peptide internal standard with these conditions is that the broad peaks produced must overlap with those of many fish species, as the latter cover virtually all of the chromatogram. This was overcome by the selection of a small organic molecule as an internal standard. The much sharper peaks produced by these molecules were easily distinguished from protein peaks. They could thus be used for quantification even if some overlap occurred.

A total of fifteen organic compounds with a range of polarities were tested. They were required to be polar enough for adequate water solubility, but not so polar as to be unretained by the column. The most suitable compound tested was found to be 2,6-dimethylphenol (DMPh). This was added to fish extracts at 0.10mg/mL. It was tested with numerous fish species and found to be easily distinguished from the native peaks, and to usually elute without overlapping them (Figure 4.2).

DMPh was found to give reproducible retention times and detector responses (±0.70%) on storing for 10 days at room temperature. A linear response to UV detection was determined over the mass range of 0.43–4.3μg ($r^2=0.9996$). Each extract was injected with 2.00μg of DMPh, which was well within this range. Only 69% was recovered when addition was made prior to homogenisation. This was probably due to interaction with the matrix, as recovery was increased to 97.5% when the standard was added to an aliquot of homogenate.

Despite the apparent benefits of using DMPh as an internal standard, problems arose when attempting to compare peak areas relative to it between profiles of fish from the same species. Large differences in the total amounts of sarcoplasmic proteins extracted were evident. The viscosity of the filtrate during
extraction was also observed to vary. Four extracts of the same Redfish injected consecutively displayed a 9% variability in total sarcoplasmic contents (as determined from the total area of all peaks in each chromatogram), while ten Redfish from the same sampling gave a 13% variability. This suggests that variations in absolute quantification were probably due to random experimental errors for fish stored under the same conditions. Also, retention times of later peaks varied in ways that were not mirrored by the internal standard.

These problems lead to the decision to abandon the use of an internal standard altogether. While quantitative species identification was desired, it should be realised that HPLC facilitates species identification through pattern recognition, and as such needs only to compare peak positions and relative areas.

Direct visual comparisons of profiles was not of much diagnostic use, as absolute retention times, peak widths and resolution varied significantly even between consecutive injections of the same sample. These changes are believed to arise from slight variations in mobile phase composition, gradient generation and temperature, and prompted the original use of an internal standard by Ashoor and Knox (1985).

The problem was then to find a way to correct for the variations described such that profiles could be more easily compared.

4.4.4 Profile Comparison Without the Use of an Internal Standard

Comparison of two profiles with matching peaks can be made by simply comparing the calculated percentage areas of peaks from both. The difficulty in identifying fish species in this way lies in the initial step of determining whether
or not the peaks are the same in each profile (eluting at the same position) when retention times for common peaks are not reproducible.

It was conceived that this problem could be overcome by co-injecting a large number of pairs of species extracts to determine the relative peak positions for all species of concern. It was envisaged that by doing this a combined data base of species-characteristic profiles could be established.

4.5 Characteristic Species Profiles

The HPLC profiles of sarcoplasmic protein extracts from numerous individuals belonging to 15 species of Australian marine fish were recorded. One chromatogram from each species is presented in Figures 4.3A to E. Similar numbers of peaks and chromatographic resolution were obtained to the work of Ashoor and Knox (1985) and Osman et al. (1987). This was far greater than the resolution achieved by Rehbein (1990b), owing to his use of a much steeper gradient and shorter analysis time, as discussed previously (section 4.4.2).

4.6 Combined Species Data

A total of 130 peaks were used in the characterisation of the 15 species profiles. Co-injections of pairs of species extracts revealed that most of these peaks were common to a number of species. After 50 such co-injections it was determined that in fact only 28 peaks were resolved under these chromatographic conditions, with each species profile consisting of a unique combination of up to
FIGURE 4.3A
Fish Species HPLC Protein Profiles

Ling

Redfish

Black bream

Retention Time (minutes)
FIGURE 4.3B
Fish Species HPLC Protein Profiles

Detector Response

Retention Time (minutes)

Orange roughy

Mirror dory

John dory
FIGURE 4.3C
Fish Species HPLC Protein Profiles

Red bream

Trevally

Pearl perch

Retention Time (minutes)
FIGURE 4.3D
Fish Species HPLC Protein Profiles

Whiting
Banana fish
Garfish

Retention Time (minutes)
FIGURE 4.3E
Fish Species HPLC Protein Profiles

Jewfish

Mullet

Ocean perch

Detector Response

Retention Time (minutes)
10 of these peaks. Running of the other 55 possible co-injections (as 105 ways exist of selecting pairs from 15 species) was not necessary, as those already performed enabled the total run time to be characterised, and the relative peak positions of all species to be determined. The resulting combined species profile data are presented in Table 4.2.

Relative retention indices were calculated with reference to a peak (peak number 6) common to many (seven) of the species. It was found that the most reproducible indices were obtained by correcting all peaks for an appropriate time value such that this peak was shifted to a constant retention time (which was 19.0 minutes on the system used here) before calculation of the indices. Those species not containing peak 6 required co-injection of their extracts with ones that did prior to retention index calculations.

4.7 Profile Comparisons

No comprehensive procedure for the use of sarcoplasmic protein HPLC profiles in species identification has been devised to date.

Osman et al. (1987) presented a table of the percentage peak areas and relative positions for their thirty one species, but only used the percentage areas of the three largest peaks found in the unknown profiles for identification. All other authors have relied on visual comparisons only.

Visual species identification from the profiles presented here was usually possible. Chromatographic variations in some profiles, however, hindered this. It is envisaged that attempts to visually identify the species of a sample by
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<th>Orange Roughy (n=5)</th>
<th>Pearl Perch (n=3)</th>
<th>Trevally Bream (n=3)</th>
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Complex Peaks: Broad peak (B) Doublet (D) # Peak that may not be resolved from the previous one
comparison of its profile with those from a larger library would be more difficult.

The problem with quantitatively comparing the peak areas of two profiles is that not all peaks are present in both (unless they are of the same species), and selection of just a few peaks (as performed by Osman et al. (1987)) will probably be inadequate for comparisons with large libraries of profiles. Some criterion needed to be established as a basis for evaluating a possible match to tolerate variations both between fish, and in chromatographic conditions.

Chi-square ($x^2$) analysis was used to compare the corresponding peaks of profiles suspected or known to be of the same species. There are inherent problems with the use of the chi-square test in this situation, however, due to the relative rather than absolute nature of the data. Absolute peak area counts could not be used as they varied greatly with sample extractability (as a result of gel formation), thus scaling was imperative. The $x^2$ value, however, depends on the magnitude of the data, and so any scaling could effect the conclusions made. It was decided to use the peak percentage areas, as they are generally quoted and provide for easy interpretation. The strictures of Cochran (1954) that no expected frequency (%area) should be below 1.0 and no more than 20% should be less than 5.0 were adhered to by these values, indicating that their use for chi-square analysis was acceptable. The null hypothesis was that samples had equal frequencies in each class (meaning that corresponding peaks had the same percentage areas).

4.8 Graphical Species Identification

A method for visually comparing profiles that reduces the complexity of the
data but retains its relative nature was desired. Star-symbol plots were chosen to satisfy this need. This graphical technique has been previously used by Vasconcelos and Chaves das Neves (1990) to classify wines made from eight different varieties of grapes, based on their free amino acid compositions.

Stars consist of radial lines originating from a common centre, each representing one of the chromatographic peaks. The length of each line is in proportion to the corresponding peak's percentage area. A polygon surrounding each star is formed by joining the tips of adjacent peaks (spokes). These are only apparent, however, when connecting adjacent non-zero radii. A star-symbol plot representing the (hypothetical) case of a fish containing all 28 possible peaks with equal areas would thus approximate a circle, as shown in Figure 4.4 (see also Appendix III).

The beauty in using star-symbol plots to represent chromatograms lies in the fact that they eliminate the visual complications of peak broadening and resolution variations, while retaining the relative quantitative information. They thus provide compact, easily recognisable shapes based on complex multivariate data.

Unique stars were generated from the mean profiles of each species analysed (Figure 4.5). Distinction between even closely related species (or subspecies) is evident (as seen by the distinctive shapes of the Red and Black bream stars; the Mirror and John dory; and the pearl and Ocean perch). Orange roughey profiles produced stars easily distinguishable from those of John dory, a species for which it is commonly substituted.
FIGURE 4.4
General Star-symbol Plot
FIGURE 4.5
Stars for Australian Fish Species

JEWFISH  WHITING  GARFISH  BANANA FISH  OCEAN PERCH

ORANGE ROUGHY  PEARL PERCH  TREVALLY  RED BREAM  MULLET

BLACK BREAM  REDFISH  LING  JOHN DORY  MIRROR DORY
4.9 Profile Variability

Multiple extractions from the same fish gave only small percentage variabilities in peak areas (Table 4.3), and thus very similar stars (Figure 4.6). Differences between profiles, therefore, are most likely to be mainly due to variations in the extracts' compositions rather than in the chromatography or inhomogeneities in the fillet.

Intraspecies variation was investigated by the HPLC analysis of extracts from ten individual fish, and from the use of samples caught at different locations and seasons. The total intraspecies variations are expressed as percentage variabilities in the mean percentage areas (Table 4.4). Many of the values are very high and would be expected to detract significantly from the species-characteristic nature of the profiles. The star-symbol plots, however, were very similar within the one sample as well as between samples from different locations and seasons. Those for Black bream (which was sampled the most comprehensively) are presented in Figures 4.7A and B. It can be seen that the species-characteristic shapes of the stars were maintained over two seasons (spring and autumn) and three locations (that were 12°30', or approximately 1500km apart) of sampling. It is concluded that fish species identification by the use of star-symbol plots constructed from water-soluble protein profiles is enabled irrespective of the season or location of catch.

The relatively invariant nature of the star-symbol plots obtained within samples consisting of 10 individuals justified the reduction of sample sizes to three for all subsequent species analysed.
TABLE 4.3
Percentage Variability in Peak Areas for Four Extractions of the Same Redfish

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FIGURE 4.6
Stars for HPLC Profiles of One Redfish
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Percentage Variability in Protein Profile Peaks

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<td>41.1</td>
<td>4.7</td>
<td>12.4</td>
<td>52.8</td>
<td>9.0</td>
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<td>35.7</td>
<td>19.0</td>
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<td>23.1</td>
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<td>44.7</td>
<td>12.4</td>
<td>6.1</td>
<td>2.5</td>
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<td>20.0</td>
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<td>17.6</td>
<td>14.6</td>
<td>36.2</td>
<td>45.0</td>
<td>30.7</td>
<td>11.9</td>
<td>2.4</td>
<td>23.4</td>
<td>7.2</td>
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<td>25.4</td>
<td>11.9</td>
<td>28.5</td>
<td>15.1</td>
<td>6.3</td>
<td>28.4</td>
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<td>12.3</td>
<td>-</td>
<td>64.6</td>
<td>-</td>
<td>75.0</td>
<td>-</td>
<td>12.6</td>
<td>-</td>
<td>9.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
</tbody>
</table>
FIGURE 4.7A
Stars for Individual Black bream
Sampled Over Two Seasons
From Sydney


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FIGURE 4.7B
Stars for Individual Black bream Sampled From Two Locations

QUEENSLAND QUEENSLAND QUEENSLAND QUEENSLAND QUEENSLAND

QUEENSLAND QUEENSLAND QUEENSLAND QUEENSLAND QUEENSLAND

VICTORIA VICTORIA VICTORIA VICTORIA VICTORIA

VICTORIA VICTORIA VICTORIA VICTORIA VICTORIA
4.10 Species Identification Procedure

A procedure for the identification of unknown samples' species was developed and is presented in Figure 4.8. The use of such a scheme is necessary when authentic samples of the suspected matching species are not available, or the performing of an extra injection is not desirable. (Co-injection of the two extracts would obviously prove or disprove the match.)

The identification procedure was tested on twenty one unlabelled fillets, representing twelve of the library species. Only one injection of sarcoplasmic extracts was sufficient in each case to facilitate species identification in agreement with those made morphologically. The method is faster, makes more comprehensive use of the data, and is more sophisticated than that of Osman et al. (1987), which required two to three injections and only considered the three largest peaks, resulting in nineteen correct identifications out of twenty.

4.11 General Application of the Method

It is envisaged that any laboratory following the experimental procedure described here will be able to directly use the species data provided. Minor chromatographic variations are not relevant, as the data is presented with relative retention indices. Extension to include other species would be possible after only a few co-injections with defined library species, due to the complete coverage of the profile time range by the twenty eight defined peaks. Their addition would simply involve the determination of which of these possible peaks are present,
FIGURE 4.8
Species Identification Procedure

Obtain HPLC profile of sample
↓
Visually compare with library (Fig. 4.3)

Profile match?

YES
Construct a star-symbol plot of percentage areas
Visually compare stars (Fig. 4.5)

Star match?

YES
Species identified
NO
Co-inject with a library species containing peak 6
Calculate relative retention indices to determine which of the 28 peaks are present

NO
Non-library species (library extension needed)
along with calculation of the mean peak percentage areas.

Star-symbol plots can be used with any profile data, including that collected by an analyst, or existing published data. That of Osman et al. (1987) has been used to construct thirty one species stars that are easily distinguished from each other, even for very closely related species (as evidenced by, for example, the Largemouth, Striped and White bass stars) (Figures 4.9A and B).

4.12 Conclusion

A method for the rapid identification of fish species by HPLC analysis of water soluble sarcoplasmic protein extracts was devised that removed the need for the use of an internal standard. Star-symbol plots of unknown species' profiles were constructed and compared with the given library of the same for 15 common edible species of Australian marine fish. The method is applicable for use with any such profile data, or the library can be easily extended.

The analytical procedure usually requires only a single sixty minute HPLC run, following a ten minute extraction procedure.

The HPLC method is much faster than electrophoretic techniques, but does not provide the facility of running many samples at once. The two methods should be viewed as complementary, and the choice for their use made on the basis of the analyst's specific requirements.
FIGURE 4.9A
Stars Constructed from the Data of
Osman et al. (1987)

RED DRUM  SILVER HAKE  SMOOTHEAD SCORPIONFISH  SWORDFISH  WALLEYE

HADDOCK  LAKE WHITEFISH  OCEAN PERCH  POLLOCK  RAINBOW TROUT

BLUEFISH  BUTTERFISH  COMMON CARP  CUSK  DEEPSEA SOLE

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FIGURE 4.9B
Stars Constructed from the Data of Osman et al. (1987)

BLACK CRAPPIE

STEELHEAD SALMON AMERICAN PLAICE ATLANTIC BONITA ATLANTIC COD ATLANTIC Mackerel

WITCH FLOUNDER LARGEMOUTH BASS STRIPED BASS WHITE BASS PINK SALMON

CANYON ROCKFISH YELLOWEYE ROCKFISH CHANNEL CATFISH FLATHEAD CATFISH ARROWTOOTH FLOUNDER
CHAPTER 5

SPECIES IDENTIFICATION OF PROCESSED FISH

5.1 Introduction

5.1.1 Current Methods

Fish are often purchased in a processed form - for example, smoked or cooked. These treatments usually cause a toughening of the muscle, and loss of water, and so would be expected to affect our ability to identify the species using methods involving comparisons between water-soluble protein extracts.

Isoelectric focusing (IEF) has been successfully used for this purpose in cooked, smoked, dried, salted and irradiated fish (Rehbein, 1990a). Often, however, it is necessary to solubilise denatured components before analysis. This is achieved by the addition of urea, sodium dodecyl sulphate (SDS) or 2-mercaptoethanol. When this step is not performed bands are produced from the more heat-stable proteins only, though these are often adequate for species identification purposes.

The use of HPLC protein profiling for the identification of processed fish has to date been investigated for only a few heat treatments - including boiling in water or salt solution, canning (Osman et al., 1987) and microwave heating (Rehbein, 1990b). In each case only a few peaks remained after the treatment. These, unlike IEF bands, were insufficient for species identification. It was concluded by both studies that denatured proteins need to be solubilised, as with some IEF applications, to retain the facility of species identification by HPLC. The need for an attempt to identify processed samples from many different
species is suggested by the finding of Poulter et al. (1985) that the stability of sarcoplasmic proteins shows some species dependence. Differences, however, are not expected to be very great.

Investigations into the effects on HPLC species identification of treatments not involving heat have not been reported.

The use of HPLC for species identification does not require a knowledge of the chemical nature of the proteins. However, consideration of their chemistry will enable a better understanding of the range of processing methods to which the usual extraction and analysis procedure can be applied.

5.1.2 The Sarcoplasm

Fish have been found to be comprised of up to 25% protein (w/w), of which 16-22% is water-soluble (the sarcoplasm) (Love, 1970). Sarcoplasmic proteins of white fish muscle are mainly enzymes - including glycolytic ones (such as glyceraldehyde-3-phosphate dehydrogenase), those of the pentose phosphate shunt, AMP deaminase and creatine kinase.

The stability of this fraction to a number of processing practices will be considered below.

5.1.3 Processing Methods Involving Heat

5.1.3.1 Effect of heat on the Sarcoplasm

When fish is heated the flesh firms as a result of myofibrillar (structural) protein denaturation. Poulter et al. (1985) found that sarcoplasmic proteins were more stable to heat than myofibrillars. The onset of changes in the former was
determined to occur at 30° to 40°C, with a gradual decrease in total solubility reported as the temperature was increased. The authors acknowledged that this effect was not unexpected, as it indicated a wide range of heat stabilities for the enzymes. Hultin (1985) stated that enzymes are deactivated by temperatures below 60° to 70°C. Fish are generally considered to be cooked when their internal temperature has reached 70°C (Gall et al., 1983; Rehbein, 1990b). Normal cooking, therefore, would inactivate enzymes (denature sarcoplasmic proteins). This effect explains the loss of the ability to identify species in the profiles of heated fish reported by Osman et al. (1987) and Rehbein (1990b).

IEF has been used to identify the species of cooked fish with reference to only a few bands emanating from heat-stable proteins (such as parvalbumins). This technique, however, separates a very large number of proteins, such that the loss of the more heat-labile components does not greatly effect the species-characteristic nature of the electrophoretograms.

5.1.3.2 Microwave Cooking

Microwaves raise the temperature of a food by generating heat within it (by inducing oscillations of polar molecules about their axes). The high surface temperatures common to other cooking methods, therefore, are not produced. It is generally considered to be a mild cooking process, and so may cause less damage to the sarcoplasm than others. The maximum temperature of a food during cooking, however, is largely responsible for the nature and extent of the chemical changes produced (Armstrong and Bergan, 1992), a factor that is the same for microwaved fish as those cooked by other methods. This suggests that the changes
within microwaved fish may be comparable to those in conventionally cooked samples. The HPLC protein profiles of microwaved fish recorded by Rehbein (1990b) were not adequate for species identification. His profiles, even for raw samples, resolved only a small number of peaks. If better resolution had been achieved, however, the ability to identify the species of microwaved fish samples may have been retained.

5.1.3.3 Smoking

Fish preservation by smoking involves an initial brining step to remove water and inhibit micro-organism proliferation (section 3.6.3.3). This step reduces the fillets' sarcoplasmic protein content, by salting them out into the brine.

The subsequent smoke exposure is usually performed at elevated temperatures (70-80°C), and so would be expected to denature the remaining sarcoplasmics.

5.1.4 Processing Methods Not Involving Heating of Samples

5.1.4.1 Infrared Drying

Many different drying techniques are used for fish. They usually involve heat and, therefore, would cause denaturation, as previously mentioned (section 5.1.3.1). The effects of milder methods, such as long wavelength infrared radiation drying performed at ambient temperatures, have not yet been reported in the literature.
5.1.4.2 γ-Irradiation

Numerous effects of γ-irradiation on proteins have been reported - such as peptide bond scission, deamination, and aggregation (Delincée, 1983b). These changes, however, require higher doses than are involved in food radurization (which are less than 10kGy).

Some studies have been made as to the effects of radurization on the proteins of muscle foods. Uzunov et al. (1972) reported a 30% lowering in solubility of sarcoplasmic proteins in beef at 10kGy. High molecular weight (>465000amu) complexes were found in the sarcoplasmic extracts of beef and pork samples irradiated at 10kGy by Radola (1969). Delincée (1983b), on the other hand, stated that only minimal chemical changes are induced in proteins when foods containing them are radurized, and that autolytic enzymes are not inactivated.

The extent of damage to the sarcoplasmic components of fish when exposed to radurization doses of γ-radiation is not precisely known, and may in fact vary between species. Species identification by IEF has been shown to be possible for fish treated at up to 20kGy without the need for the addition of solubilising agents (Salmon et al., 1983).

5.1.5 Conclusion

HPLC analysis using the conditions of Osman et al. (1987) and Rehbein (1990b) did not enable species identification of cooked and canned fish.

The effects of processing methods not applying heat to or generating it within the fish have yet to be determined. Two such treatments were investigated
in this study: γ-irradiation preservation; and infrared drying. They were expected to cause less damage to the sarcoplasmic proteins than those involving heat.

Two mild heat treatments (microwave cooking and smoking) were investigated in this study to determine the extent of peak loss, as well as to assess the possibility of species identification under the conditions developed in chapter 4.

The aim of these trials was to broaden the range of applications of the HPLC method of fish species identification developed.
5.2 Experimental

5.2.1 Sample Processing, Extraction and Analysis

Fish were filleted, with one fillet from each fish used as a control, and the other processed by one of the methods outlined below.

Protein extracts were prepared and analysed as described in section 4.3.

5.2.2 Microwave Cooking

Duplicate Redfish (*Centroberyx affinis*) fillets were microwaved to an internal temperature of 65\(^\circ\)C (Sharp model R-9510E domestic microwave with turntable, Sharp Corporation of Australia Pty Ltd) on full power (650W). A final temperature of 70-71\(^\circ\)C was reached on standing (as determined using the Sharp temperature probe (thermistor) provided with the microwave).

5.2.3 Smoking

Four Redfish and Black bream (*Acanthopagrus australis*) fillets were soaked in brine, and two of these from each species were smoked (as in section 3.6.4.1).

5.2.4 Infrared Drying

Duplicate John dory (*Zeus faber*), Redfish, and Mirror dory (*Zenopsis nebulosus*) fillets were subjected to mild drying (15 hours at 25\(^\circ\)C, to final water contents of 42-56%) in a far infrared radiation vacuum dryer (prototype developed by Dr M. Kurumazuka and Showa Manufacturing, Tokyo).
5.2.5 γ-Irradiation

Triplicate vacuum-packed fillets of Black bream and duplicate of Redfish were irradiated at 2 and 6kGy. Samples of the same species, in triplicate, were packaged in air and irradiated at 1, 2, and 6kGy, as in section 3.5.5.1. Protein profiles were run on extracts from the irradiated samples only for the air-packaged trial.

Irradiations were performed by ANSTO (Lucas Heights, Sydney), as mentioned in section 3.5.5.1.

5.2.6 Statistical Analysis

Star-symbol plots were constructed from individual profiles or means using STATGRAPHICS (see section 4.3.5).

Chi-square analyses were also performed on STATGRAPHICS.
DISCUSSION

5.3 Analysis of Processed Fish

5.3.1 Heat Treatments

5.3.1.1 Microwave Cooking

Protein profiles from microwaved Redfish differed dramatically from their controls (Figure 5.1). Only four of the original peaks were apparent (Redfish peaks 1, 3, 4 and 8). These peaks were not the largest in the control fillets' profiles, indicating that they were probably from the more heat-stable proteins. They were spread well over the chromatograms, and so had very different polarities.

Rehbein (1990b) reported the appearance of new very polar peaks in the profiles of microwaved Saithe (Pollachius virens). The lower mobile phase polarity at the start of the runs performed in this study suggested that if these were present in the samples investigated they were obscured by the large initial peak containing unretained material.

The loss of all distinctive Redfish protein profile characteristics meant that the species identification of microwave cooked fish was not possible by this HPLC method, as was also concluded by Rehbein (1990b) for the conditions he employed.

5.3.1.2 Smoking

The initial brining step (which involved no heating) yielded Redfish profiles that had lost many peaks (Figure 5.2) but Bream profiles in which nearly all of the original peaks were present. The relative sizes of the peaks, however, varied

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FIGURE 5.2
Smoked, Salted and Control Redfish HPLC Profiles

Retention Time (minutes)

Detector Response
greatly. As with microwaved fillets, the peaks lost were not confined to any one section of the chromatogram, and therefore represented proteins of very different polarities. A large amount of material from the salted Bream extracts was not retained by the column, which suggests that the denatured proteins had become more polar by interacting with the absorbed salt.

Both species exhibited large peaks at the ends of their profiles on salting. These are from very non-polar proteins, which would be expected to be least effected by the uptake of sodium chloride.

Star-symbol plots of the salted Bream (as these were the only group of this processing method to retain most peaks) are presented (Figure 5.3). They reveal great changes in shape, and show that the ability to identify the species of origin was lost. Chi square analysis also lead to rejection of the treated fillets' profiles as belonging to the same species as their controls ($\chi^2 = 155.5, 101.5; p = 0.00, 0.00$).

Profiles from the smoked fish had fewer peaks than the brined, indicating that further destruction of the sarcoplasm had occurred (Figure 5.4), which was probably the result of the heat applied during smoking. Again large peaks were observed at ends of the profiles.

As the facility for species identification was lost in the initial brining step, and further changes occurred on smoking, it is concluded that this method of smoking fish destroys the species-characteristic nature of the sarcoplasmic proteins, and therefore precludes their use for identification by HPLC using the method employed in this study.

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FIGURE 5.3
Star-symbol Plots of Salted (S) and Control (C) Black Bream

c1  s1

c2  s2
FIGURE 5.4
Smoked and Control Black Bream HPLC Profiles

Retention Time (minutes)
5.3.1.3 Conclusion

Both of the processing treatments involving heat (hot smoking and microwave cooking), as well as the brine soaking caused a loss of sarcoplasmic proteins to an extent where species identification based on their HPLC profiles was no longer possible.

The appearance of a peak at the end of each smoked fish profile, representing very non-polar proteins may have been due to the fact that the salt had not noticeably effected these proteins, rather than any greater inherent heat-stability, as the final peak was not selectively retained in the microwaved Redfish. It could also represent stable aggregate formed from the original proteins.

5.3.2 Non-heat Treatments

5.3.2.1 Infrared Drying

Profiles (Figures 5.5 and 5.6) and star-symbol plots (Figure 5.7) of the two control fillets' profiles, and those from the same fillets after mild infrared drying show that the species-characteristic shapes were retained for both species (Redfish and John dory). Chi-square analysis lead to acceptance of the dried fillets as being of the same species as their controls (Table 5.1).

Species identification of fish subjected to mild infrared drying at ambient temperatures is, therefore, still facilitated by comparison of sarcoplasmic protein HPLC profiles with those of raw samples. The conditions employed were, however, were very mild and may not be adequate for most preservation requirements.
FIGURE 5.5
Infrared Dried and Control Redfish HPLC Profiles

Retention Time (minutes)
FIGURE 5.6
Infrared Dried and Control John Dory HPLC Profiles

Detector Response

Retention Time (minutes)

Infrared Dried

Control
FIGURE 5.7
Star-symbol Plots of Infrared Dried (D) and Control (C) Fish

RF = Redfish
JD = John dory

CJD1  DJD1  CJD2  DJD2

crf1  drcf1  crf2  drcf2
TABLE 5.1
Chi-Square Analysis of HPLC Profiles from Infrared Dried Fish

<table>
<thead>
<tr>
<th>Sample</th>
<th>Degrees of Freedom</th>
<th>Chi-square</th>
<th>Probability level</th>
</tr>
</thead>
<tbody>
<tr>
<td>John dory 1</td>
<td>7</td>
<td>0.75</td>
<td>0.998</td>
</tr>
<tr>
<td>John dory 2</td>
<td>8</td>
<td>1.34</td>
<td>0.995</td>
</tr>
<tr>
<td>Redfish 1</td>
<td>7</td>
<td>7.30</td>
<td>0.399</td>
</tr>
<tr>
<td>Redfish 2</td>
<td>7</td>
<td>1.09</td>
<td>0.993</td>
</tr>
</tbody>
</table>

5.3.2.2 γ-Irradiation

HPLC protein profiles of Black bream and Redfish fillets irradiated in the absence of air at 2 and 6kGy, as well as their corresponding controls, were recorded, and star-symbol plots constructed (Figures 5.8 and 5.9). Stars of fillets from the same fish were remarkably similar, regardless of the treatment or dose, enabling unhindered species identification. These results were mirrored by the chi-square conclusions (Table 5.2).

Fillets irradiated in air at 1, 2, and 6kGy had their profiles compared with the mean species profiles of ten fish from the same sample (Autumn, 1990) (Figure 5.10), as the corresponding control profiles were not available. Again, no changes in star-symbol plots were apparent, and acceptance as the same species by chi-square analysis was concluded in all cases (Table 5.3), as was expected.

5.4 General Conclusion

HPLC sarcoplasmic protein profiles were found to be appropriate for use
FIGURE 5.8
Star-symbol Plots of Black Bream
Gamma-Irradiated in a Vacuum

\( xIy \) = fillet \( y \) irradiated at \( x \)kGy

\( C = \) Control

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FIGURE 5.9
Star-symbol Plots of Redfish
Gamma-Irradiated in a Vacuum

$\Delta I_y = \text{fillet } y \text{ irradiated at } xk\text{Gy}$

$C = \text{Control}$
FIGURE 5.10
Star-symbol Plots of Black Bream and Redfish
Gamma-Irradiated in Air (with Controls)

Irradiated stars = means of 3 profiles
Control stars = means of 10 profiles

Redfish  1kGy Redfish  2kGy Redfish  6kGy Redfish

Bream  1kGy Bream  2kGy Bream  6kGy Bream
# Table 5.2
Chi-Square Analysis of HPLC Profiles from Fish Irradiated in a Vacuum

<table>
<thead>
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<th>Degrees of Freedom</th>
<th>Chi-square value</th>
<th>Probability level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bream 2kGy</td>
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<td></td>
<td></td>
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<tr>
<td>Bream 1</td>
<td>9</td>
<td>5.78</td>
<td>0.762</td>
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<tr>
<td>Bream 2</td>
<td>9</td>
<td>6.55</td>
<td>0.684</td>
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<tr>
<td>Bream 3</td>
<td>9</td>
<td>1.13</td>
<td>0.999</td>
</tr>
<tr>
<td>6kGy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bream 1</td>
<td>9</td>
<td>1.15</td>
<td>0.999</td>
</tr>
<tr>
<td>Bream 2</td>
<td>9</td>
<td>1.31</td>
<td>0.998</td>
</tr>
<tr>
<td>Bream 3</td>
<td>9</td>
<td>2.91</td>
<td>0.968</td>
</tr>
<tr>
<td>Redfish 2kGy</td>
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<td></td>
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</tr>
<tr>
<td>Redfish 1</td>
<td>7</td>
<td>2.98</td>
<td>0.887</td>
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<tr>
<td>Redfish 2</td>
<td>7</td>
<td>2.05</td>
<td>0.957</td>
</tr>
<tr>
<td>6kGy</td>
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<td></td>
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<tr>
<td>Redfish 1</td>
<td>7</td>
<td>1.11</td>
<td>0.993</td>
</tr>
<tr>
<td>Redfish 2</td>
<td>7</td>
<td>0.79</td>
<td>0.998</td>
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# Table 5.3
Chi-Square Analysis of mean HPLC Profiles from Fish Irradiated in Air

<table>
<thead>
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<th>Sample</th>
<th>Degrees of Freedom</th>
<th>Chi-square value</th>
<th>Probability level</th>
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<tbody>
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<td>Bream</td>
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<td></td>
<td></td>
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<tr>
<td>1kGy (n=2)</td>
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<td>8.97</td>
<td>0.441</td>
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<tr>
<td>2kGy (n=3)</td>
<td>9</td>
<td>8.01</td>
<td>0.533</td>
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<tr>
<td>6kGy (n=3)</td>
<td>9</td>
<td>7.76</td>
<td>0.559</td>
</tr>
<tr>
<td>Redfish</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1kGy (n=3)</td>
<td>7</td>
<td>0.42</td>
<td>0.9997</td>
</tr>
<tr>
<td>2kGy (n=3)</td>
<td>7</td>
<td>1.01</td>
<td>0.995</td>
</tr>
<tr>
<td>6kGy (n=3)</td>
<td>7</td>
<td>0.81</td>
<td>0.997</td>
</tr>
</tbody>
</table>

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in species identification of fillets that have been γ-irradiated at radurization doses up to 6kGy (in air or a vacuum), as well as for fillets dried with infrared radiation.

The method is not able to be directly applied to microwaved or hot-smoked fish, as insufficient characteristic peaks remain. Species identification of these fish by HPLC may be enabled after denatured proteins are made soluble (as is performed prior to IEF), but would require the development of new analysis conditions. Heat is most likely to be the agent causing the protein damage during these processes. It is predicted that any treatment involving a cooking temperatures will cause sarcoplasmic protein loss to a degree where species identification by the method described here will no longer be possible.


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TABLE 1
Fatty Acid Compositions of Temperate (New South Wales) Fish Samples
(\% of total fatty acid mass)

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TABLE 2
Patty Acid Compositions (and Variabilities) of Tropical Australian Fish (% mass)

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<th>Sinclair et al. (1986) 10 species, n=2 to 8</th>
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<td>0.0 (-)</td>
<td>0.0 (-)</td>
</tr>
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</tr>
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<td>11.0 (29.7)</td>
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<td>0.0 (-)</td>
</tr>
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### TABLE 3
Fatty Acid Compositions (and Variabilities)
of Northern Hemisphere Fish (% mass)

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APPENDIX III

Star-Symbol Plots

The use of STATGRAPHICS to construct star-symbol plots is complicated by the in-built scaling practice of the program. It was discovered that each radial line had its length scaled relative to the range of values entered for that spoke. This resulted in distortion of the stars in a way that was dependent on the nature of the other observations in the data set. One outcome of this was the loss of the smallest value for each peak over the data set, as it was scaled to be equal to zero - causing dramatic peak loss in cases where a particular peak is present at a high level in all stars of a data set, as the smallest, even though it may be a large peak compared with others, will be lost.

Correction for this scaling facility was made by entering two constant value 'dummy' stars in each data set with values bounding those of the protein peak values. An artificial range of 'spoke' lengths was thus imposed. Characteristic stars were then maintained over different data sets irrespective of the nature of the accompanying stars in each.
PUBLICATIONS FROM THE WORK PRESENTED IN THIS THESIS


CHEMICAL ANALYSIS OF NUTRITIONALLY IMPORTANT COMPONENTS IN TEMPERATE AUSTRALIAN FISH

by

Sharyn G. Armstrong

A Thesis Submitted to the Faculty of Science and Technology

in Partial Fulfilment of the Requirements for the Degree of

Doctor of Philosophy

THE UNIVERSITY OF WESTERN SYDNEY, HAWKESBURY

1992
PLEASE NOTE

The greatest amount of care has been taken while scanning this thesis,

and the best possible result has been obtained.
ABSTRACT

The lipid composition of five species of marine finfish from temperate Australian waters was determined. In particular, claims that the lipids of Australian fish contain high levels of omega-6 (ω-6) fatty acids, and arachidonic acid (AA) were investigated. Individual fish were analysed from samples collected at three locations and two seasons, to determine the extent of variability in composition within each species.

The Australian fish were found to have fatty acid compositions of similar nutritional value to those from northern hemisphere temperate waters, as assessed by their polyunsaturated to saturated fatty acid (P/S) and ω-3/ω-6 ratios. Levels of AA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were also found to be comparable.

The variability in fatty acid compositions was greater between samples taken from different locations than from different seasons. This may explain the previous claims that Australian fish are of less nutritional value to the human diet than northern hemisphere species, since formerly the data were collected primarily on fish from tropical waters.

Lipid contents and, to a lesser extent, compositions were found to exhibit some species-characteristic nature, indicating a need for accurate species identification to enable selection for dietary inclusion. To this end a high-performance liquid chromatographic (HPLC) method for fish species identification was developed. A general identification procedure was proposed that was successful for all twenty one samples tested. The procedure uses star-symbol plots
of chromatographic data to facilitate easy visual discrimination between species. Its application using new and published data is described.

Fish are usually consumed after being exposed to some form of cooking or preservation technique, which would be expected to alter their lipid compositions, and thus nutritional value. Fish muscle fatty acid compositions were found to be unaffected when fillets were preserved by gamma-irradiation or smoking. Significant vitamin E loss was recorded, although all fillets retained adequate amounts to protect against polyunsaturated fatty acid oxidation.

The application of the HPLC species identification method to processed fish was investigated. It was found to be suitable for gamma-irradiated and infrared dried fish, but not for those that had been smoked or microwave cooked.
DECLARATION

This is to certify that the work presented in this thesis has not been submitted previously to any other university or institution for a degree or award.

Sharyn Armstrong

Sharyn Armstrong
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understanding, love, support, encouragement and, most of all, tolerance during the
course of this study.
**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>α-T</td>
<td>Alpha-tocopherol</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary Heart Disease</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>DPA</td>
<td>Docosapentaenoic acid</td>
</tr>
<tr>
<td>DMP</td>
<td>Dimethoxypropane</td>
</tr>
<tr>
<td>DMPh</td>
<td>2,6-Dimethylphenol</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty acid</td>
</tr>
<tr>
<td>FAME</td>
<td>Fatty acid methyl ester</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>FID</td>
<td>Flame ionisation detector</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>IEF</td>
<td>Isoelectric focussing</td>
</tr>
<tr>
<td>%L</td>
<td>g Lipid/100g flesh</td>
</tr>
<tr>
<td>LA</td>
<td>Linoleic acid</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LDL-C</td>
<td>Low density lipoprotein cholesterol</td>
</tr>
<tr>
<td>LN</td>
<td>Linolenic acid</td>
</tr>
<tr>
<td>LSD</td>
<td>Least squared difference</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>M/MUFA</td>
<td>Monounsaturated fatty acid</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>n3/n6</td>
<td>Omega-3/omega-6 ratio</td>
</tr>
<tr>
<td>P/PUFA</td>
<td>Polyunsaturated fatty acid</td>
</tr>
<tr>
<td>P/S</td>
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</tr>
<tr>
<td>PC</td>
<td>Principal component</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal components analysis</td>
</tr>
<tr>
<td>PL</td>
<td>Phospholipid</td>
</tr>
<tr>
<td>S/SFA</td>
<td>Saturated fatty acid</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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