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PROFILING AND MODELLING OF TRIGLYCERIDES AND VOLATILE COMPOUNDS IN SA CAPE HAKE (*MERLUCCIUS CAPENSIS* AND *MERLUCCIUS PARADOXUS)*

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BLOEMFONTEIN
JUNE 2011

DECLARATION OF INDEPENDENT WORK

DECLARATION WITH REGARD TO
INDEPENDENT WORK

I, HANITA SWANEPOEL, identity number [redacted] and student number 20227280, do hereby declare that this research project submitted to the Central University of Technology, Free State for the degree DOCTOR TECHNOLOGIAE: ENVIRONMENTAL HEALTH is my own independent work; and complies with the Code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the Central University of Technology, Free State; and has not been submitted before to any institution by myself or any other person in fulfilment (or partial fulfilment) of the requirements for the attainment of any qualification.

SIGNATURE OF STUDENT **DATE**

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SUMMARY

PROFILING AND MODELLING OF TRIGLYCERIDES AND VOLATILE COMPOUNDS IN SA CAPE HAKE (*MERLUCCIUS CAPENSIS* AND *MERLUCCIUS PARADOXUS)*

Apart from being the primary food source of many cultures around the world, fish contains notable amounts of essential fatty acids that are required by the human body, thus making fish a vital part of the human diet. In South Africa Cape hake is a well-known and highly consumed local fish species, which is transported from coastal areas countrywide where the fresh fish are displayed on ice in various retail stores. Fish is known to be highly susceptible to spoilage and, as a result, the maintenance of the cold-chain in related products is of particular importance. Additionally, recent trends showing a decline in natural fish resources have instigated growing concerns about the sustainability and optimal utilisation of fish as a food source. Against this backdrop, this study aimed at determining the influence of storage parameters on selected triglycerides and their possible metabolic pathways. Also applying prediction modelling of fatty acids and volatiles as instruments to assess exposure of Cape hake fillets to excessive microbial contamination and, in effect, be indicative of the environmental parameters (for example temperature) that may influence such contamination.

Randomly selected juvenile hakes were filleted and stored under various simulated retail storage conditions, under either controlled or uncontrolled environmental conditions. For each hake filleted, one fillet was inoculated with an increased load of autochthonous microbiota, and the corresponding fillet was kept at similar temperature conditions. All fillets were monitored over a ten day period, during which fatty acid and volatile samples were collected and analysed. From the resulting triglycerides a selection of fatty acids were profiled and their possible metabolic pathways investigated. Fish maturity, the distribution of the fatty acids and the implication thereof in the nutritional value were also assessed. Conventional chemometric

methods utilising mathematical expressions were subsequently utilised in order to predict contamination and whether the cold chain was sustained, while an artificial neural network (ANNs) were designed to predict excessive microbial contamination in the fillets.

The results showed that the nutritional value of fish differs notably with its maturity and size. Mathematical equations were furthermore found to be effective assessment instruments to indicate the percentage differences in storage temperature, as well as consequent microbial influences. Thus, this approach may introduce mathematical prediction modelling as a promising mechanism to assess Cape hake spoilage. An artificial neural network (ANN) was successfully designed, that succeeded in distinguishing between Cape hake fillets displayed and stored on ice that have been exposed to excessive contamination and those that have not been exposed. In the latter case, the selected variable was a fatty acid, hexadecanoic acid, used as biochemical indicator. This modulating approach may provide a platform for future shelf-life studies on related muscle tissue.

Ultimately, the study endeavoured to add to the body of knowledge regarding the biochemical and microbiological changes related to Cape hake storage, the prediction thereof via contemporary methods and contributing to the safety and effective utilization of this unique and declining South African nutritional resource.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL BACKGROUND

Quality of food products includes a series of factors related to safety, nutritional value, freshness, availability and edibility (Alimelli *et al.*, 2007). Aquatic tissue is highly perishable, even under refrigerated or frozen conditions (Frazier and Westhoff, 1988; Potter and Hotchkiss, 1997), resulting in a limited shelf life of products. According to Fennema (1985) processing, in addition, results in excessive chemical changes in food that could have a desirable, undesirable, or questionable consequence. Spoilage is usually recognised by changes in texture or off-flavours that develop during (bio)chemical and/or microbial actions (Huis in't Veld, 1996).

1.1.1 Seafood classification

Seafood is classified as vertebrate (fish) or invertebrate (shellfish), based on its anatomy. Fish (teleost fish) is the most abundant form of the five vertebrate classes, with at least 20 000 known species, of which more than 58% are found in the aquatic environment. However, no more than 200 of all known fish species are of commercial importance (Bennion, 1980). This study mainly focused on the Cape hake (*Merluccius capensis* and *Merluccius paradoxus*), which is of economical importance in South Africa (refer to section 1.2.2).

1.1.2 Fatty acids in fish

Fish is generally categorised according to fat composition (Bennion, 1980). The lipid fraction is the component in fish that shows the greatest variation, and also displays a characteristic seasonal curve within a certain species, with the minimum lipid content around the time of spawning (Huss, 1995). Lean fish contain less than five percent fat, while fat fish have up to 22 percent fat. Examples of lean fish include cod, haddock, halibut, sea trout, grouper and hake (Bennion, 1980). Fish is one of the components of the human diet that is rich in ω3 polyunsaturated fatty acids (PUFAs) (Hsieh and Kinsella, 1986). The ω3 fatty acids are considered ‗essential', since humans do not synthesize the ω3 structure and must ingest ω3 fatty acids with their diet. Marine lipids are characterised by their content of longer chain (up to C24) fatty acids (Marini, 1992), and the focus of lipid studies in fish is as a rule on the longer

chain unsaturated ω3 and ω6 essential fatty acids, because of its importance in the human diet. The nutritional importance of the longer chain unsaturated ω3 and ω6 essential fatty acids has been underscored by the fact that several countries, including those of Scandinavia and Canada, have established daily-recommended dietary intake values for ω3 fatty acids (Batista *et al.*, 2001). The basic organic acid classes of all fish muscle are phosphatidyl choline (lechitin), phosphatidyl ethanolamine (cephalin), triglycerides and free fatty acids (FFA). The fatty acid compositions that describe the two South African hake species (*Merluccius capensis* and *Merluccius paradoxus*), are listed in Table 1.1.

1.1.2.1 Nomenclature

The criterion for differentiation between fatty acids is according to the fatty acid distribution pattern in food (Belitz and Grosch, 1987). Fatty acids are divided into groups according to chain length, by number, position and configuration of their double bonds, and also by the occurrence of additional functional groups along the chain. The dominating lipids are the unsaturated lipids, which contain one, two or three allyl groups in their acyl residues. Fatty acids with isolated double bonds (a methyl group inserted between the two cis-double bonds) are usually denoted as isolenic or non-conjugated fatty acids. The structural relationship between the unsaturated and non-conjugated fatty acids that is derived from a common biosynthetic pathway, is distinctly revealed when the double bond position is determined by counting from the methyl end of the chain (Fig. 1.1) (Kinsella, 1986). Acids with similar methyl ends are then combined into groups. Three such family groups exist, namely ω3 (linolenic type), ω6 (linoleic type) and ω9 (oleic acid type).

Table 1.1 Comparison of the fatty acid composition of *Merluccius paradoxus* and *Merluccius capensis.*

Fatty Acids	Common name	*M. paradoxus*	*M. capensis*
C14: 0	Myristic acid	1.1	1.7
C16: 0	Palmitic acid	23.3	23.6
C16: 1	Palmitoleic acid	4.7	5.0
C18: 0	Stearic acid	3.8	4.4
C18: 1	Oleic acid	20.6	20.5
C18: 2 ω6	Linoleic acid	0.6	0.7
C20: 1	Eicosenoic acid	4.3	4.4
C20: 4 ω3	Arachidonic acid (AA)	2.0	Not detected
C20: 5 ω3	Eicosapentaenoic acid (EPA)	6.9	6.6
C22: 1	Erucic acid	Not detected	Not detected
C22: 6 ω3	Docosahexaenoic acid (DHA)	27.8	24.0

(Adapted from Méndez and González, 1997).

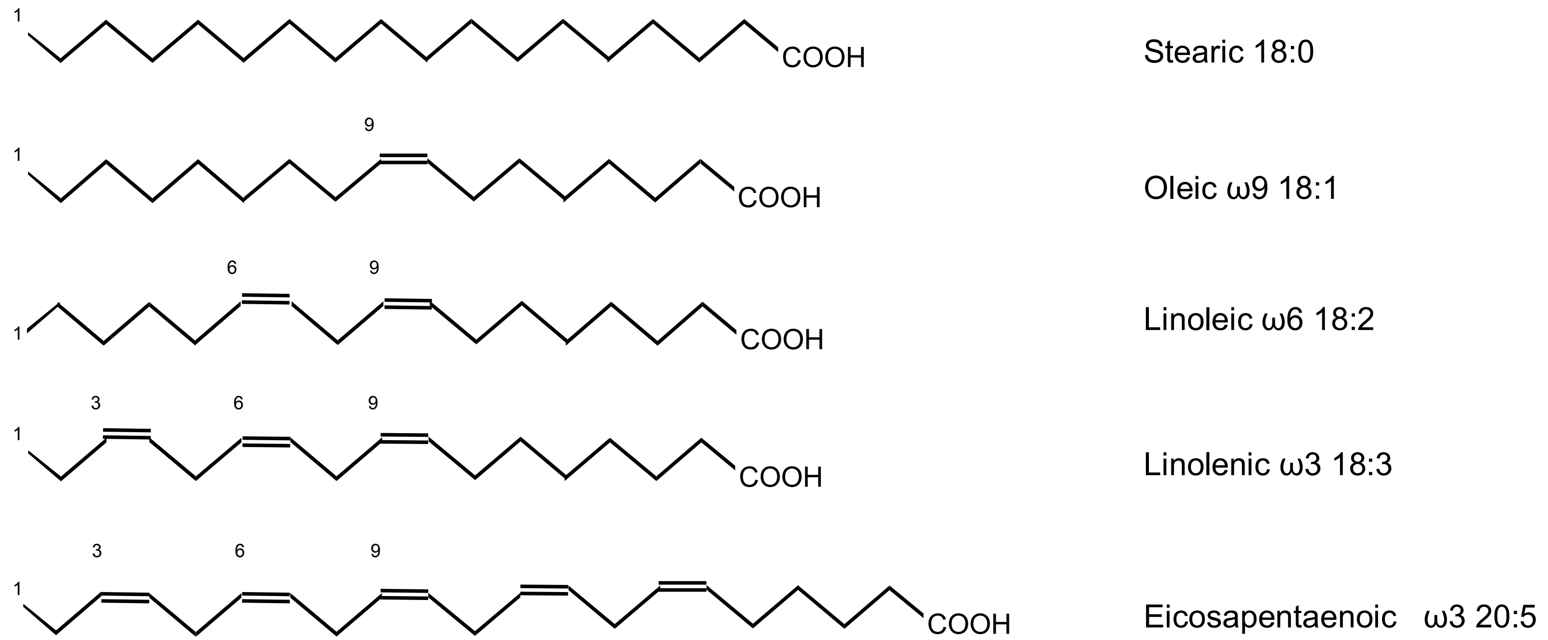


Figure 1.1 The principal families of fatty acids (Adapted from Kinsella, 1986).

1.2. SOUTH AFRICAN CAPE HAKE

1.2.1 *Merluccius capensis* and *Merluccius paradoxus*

Hakes or merlucciids belong to the genus *Merluccius* (family Merlucciidae) and are anacanthine fishes with one soft anal and two soft dorsal fins. Hakes are widely distributed in the world and are an important source in the world fisheries (Inada, 1981). Cape hake consists mainly of two morphologically similar species, the *Merluccius capensis* Castelnau and the *Merluccius paradoxus* Franca also known as the shallow and deep-water Cape hakes. It is difficult to visually distinguish between the two South African hake species whose distribution is depth and/or temperature dependent, since these are highly correlated (Fairweather *et al*., 2006). The two species overlap in intermediate waters between 150 and 400 m and are associated with the Benguela current system which is characterised by the upwelling of cold water along the west coast of southern Africa (Von der Heyden *et al*., 2007). The species maintain their integrity by spawning at different depths (Hutton, 2000). The shallow-water Cape hake (*Merluccius capensis)*, lives to approximately 400 m and can be found at latitudes between 0 and 35 ºS. The shallow-water Cape hake shows a type of selectivity in its food, and the size of the fish seem be to influenced the type of food eaten. The smaller (juvenile) fish of 25 cm up to 64 cm feeds on small crustaceans (mysidacea, Euphausiids), and small deep-sea fish (*Myctophum* sp. and *Maurolicus* sp.) (Inada, 1981).

The deep-water Cape hake (*Merluccius paradoxus)* lives close to the bottom of continental slopes to depths from 150 m to at least 850 m, occurring generally deeper in the north (450 m or more) than in the south (300 to 350 m), but more abundantly occur south of 22 ºS (Von der Heyden *et al.*, 2007). The deep-water Cape hake feeds on fish, mysid, euphausiid and squid. The young feed mainly on euphausiid, but the diet becomes polyphagous with growth (Inada, 1981). The compositional and nutritional properties of the edible muscle of fish are quite variable, depending on the season, degree of maturity (age), gender,

environment and in relation to feed intake, migratory swimming patterns and sexual changes due to spawning (Wessels and Spark, 1973; Huss, 1995; Méndez and González; 1997; Potter and Hotchkiss, 1997; Huynh and Kitts, 2009; Pacetti *et al*., 2010).

1.2.2 The impact of hake on the South African economy

South Africa has a 3 000 km coastline, extending from the mouth of the Orange River in the west, on the border with Namibia, to Ponta do Ouro in the east, adjacent to Moçambique. The majority of the total South African demersal catch is taken off the West Coast, and is dominated by the shallow-water hake *M. capensis Castelnau* and deep-water hake *M. paradoxus Franca* (Fairweather *et al.*, 2006). In Figure 1.2 the Cape hake samples used in this study.

Approximately one-third of the fresh and frozen catch is exported, and about two-thirds of the demersal catch is landed fresh and processed in extensive shore-based facilities (Hutton *et al.,* 1999). The Cape hake fishing sector is of considerable social and economic value for the South African demersal fisheries. The total allowable catch (TAC) was reduced from 166 000 ton in 2001 to only 130 532 ton in 2008 following declining stocks caused by extreme fishing pressure in the mid 1970s, when an estimated 1 115 000 million ton of hake were caught (Hutton and Sumaila, 2000; Butterworth and Rademeyer, 2005; Department: Environmental Affairs and Tourism, Republic of South Africa (DEAT RSA), 2007; Von der Heyden *et al.*, 2007).

Figure 1.2 Cape hake samples as received for this study.

The white fish industry has developed an extensive international market. With the development of high value products they are aiming at –better" utilisation of the catch, quality improvement, and processing more hake into value-added products, such as natural and processed hake, fillets, steak, loins, portions and sticks, breaded, battered, and sauced products (Kramer, 1997; Hutton *et al.*, 1999). Nationally, the major fishing companies, such as Irwin and Johnson Ltd. (I&J) play a dominant role as they have established a network of facilities for storage, processing, and marketing (Hutton *et al.*, 1999).

In South Africa, fish and other marine products are subjected to long distance transport from coastal areas to inland markets, since the fish are marketed countrywide (Hutton *et al.*, 1999). Being a highly perishable product, the maintenance of a continuous cold chain and the related expenditures in attempting to keep the product sensorially acceptable to the consumer, present many challenges to the industry.

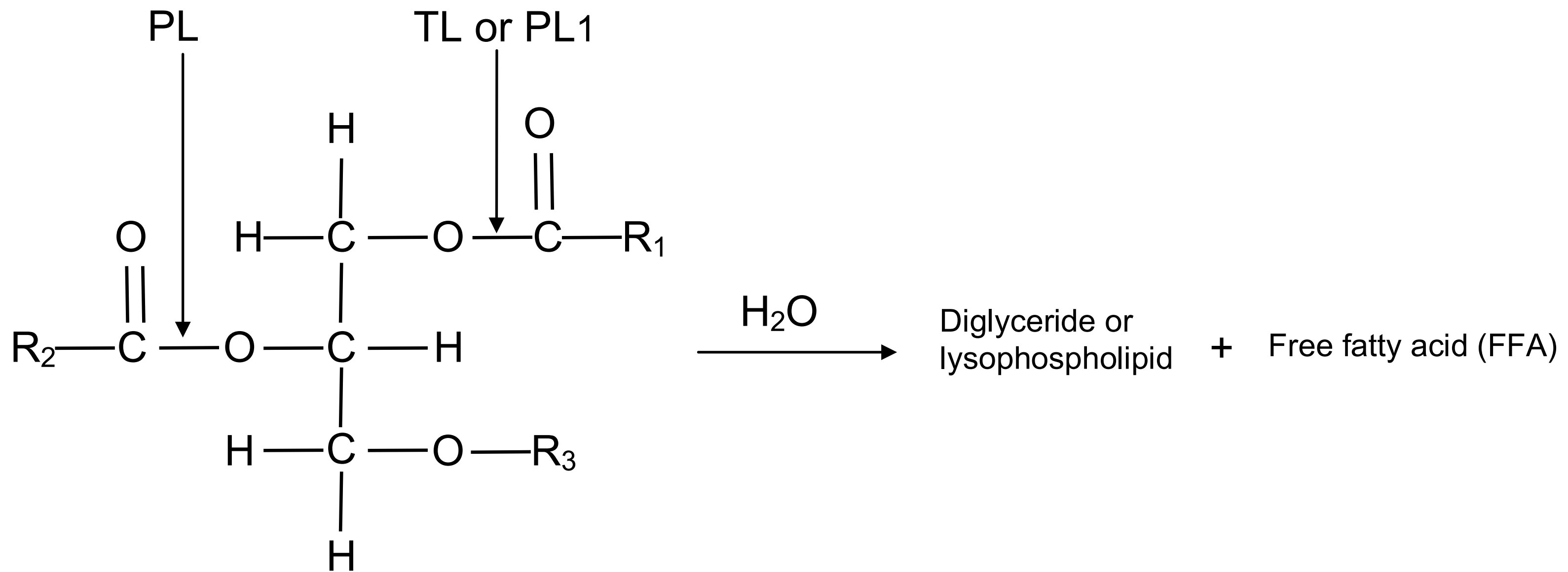
1.3 OXIDATION OF FISH LIPIDS

Lipids are, in general, produced through the major metabolic pathways: anabolism (the metabolic synthesis of proteins, fats, and other constituents of living organisms from molecules or simple precursors, using energy in the form of adenosine triphosphate otherwise known as ATP), and catabolism (the metabolic breakdown of large molecules in living organisms to smaller ones, with the release of energy). In fish, fatty acids arise from the *de novo* synthesis from non-lipid carbon sources from within, or directly from dietary lipid (Henderson, 1996). Lipid degradation is especially important in seafood, where highly-unsaturated lipids are present. During processing and storage, enzymatic and non-enzymatic lipid oxidation occurs that can directly influence the quality of the product (Aubourg, 1999a; Ross and Smith, 2006). The principle agents of decay are endogenous enzymes and bacteria, or more commonly, combinations of these to spoil fish by autolysis, oxidation or bacterial activity (Frazier and Westhoff, 1988).

1.3.1 Enzymatic and non-enzymatic lipid oxidation in fish tissue

Hydrolysis and oxidation are the two reactions of importance in fish lipids that result in the production of a range of substances among which some have unpleasant (rancid) taste and smell (Huss, 1995). Some of these substances also contribute to texture changes, by binding covalently to fish muscle proteins and therefore may have an influence on the quality deterioration of fish. These reactions are either non-enzymatic, or catalysed by microbial enzymes, or by autochthonous intracellular or digestive enzymes from the fish. The difference in the composition of fish tissue has a considerable effect on the pattern and the rate of these reactions. Besides that, the species, size, and condition during harvesting, processing, storage, distribution and preparation also have an influence on the quality deterioration (Jay, 1992; Ross and Smith, 2006).

Throughout storage, free fatty acid (FFA) concentrations increase (more intense in un-gutted than in gutted fish) as a result of digestive enzymes (Huss, 1995). During the process of FFA formation a triglyceride in the depot fat is cleaved by triglyceride lipase (TL in Figure 1.3) originating from the digestive tract or the excreta of certain micro-organisms. The production of FFAs occurs in lean fish even at low temperatures. Although cellular lipases play a minor role in hydrolysis, cellular phospholipases are believed to be the responsible enzymes (more specifically phospholipase A_2 [PL_2 in Figure 1.3]). However, a correlation between activity of these enzymes and the rate of appearance of FFA has not yet been confirmed. Hydrolysis often leads to increased oxidation since the fatty acids bound to phospholipids at glycerol-carbon atom 2 are most often of the polyunsaturated type. These fatty acids also cause a ―soapy” off-flavour (Huss, 1995).



Triglyceride or phospholipids depending on R_3

Figure 1.3 The primary hydrolytic reactions of triglycerides and phospholipids. Enzymes: PL_1 & PL_2 phospholipases; TL, triglyceride lipase (Adapted from Huss, 1995).

1.3.1.1 Enzymatic oxidation

Saltwater and freshwater fish have different odours arising from different enzyme activities (Aro *et al.,* 2003). As reported by Aubourg (1999b) and Melton (1983), one of the most important concerns in the oxidation of highly unsaturated lipids is the production of off-flavours and odours (also refer to volatiles - Section 1.4). Volatile compounds of fish and fish products have been studied intensively, although only a few have followed the changes in volatiles during storage (Aro *et al*., 2003).

Enzymes such as lipoxygenase, peroxidase and microsomal enzymes from animal tissue can potentially initiate lipid peroxidation (Hsieh and Kinsella, 1986). Lipoxegenase catalyses the addition of molecular oxygen to polyunsaturated fatty acids, in so doing generating a hydroperoxide (Winkler *et al*., 1991). A relatively high activity of lipoxygenase has been found in the gills and under the skin of many fish species (German and Kinsella, 1985; Huss, 1995). The enzyme is unstable and is probably important in fresh fish for lipid oxidation, given that cooking or freezing/thawing effectively destroys enzyme activity. Lipoxygenase is present in variable amounts in different fish tissues and acts as a catalyst to enzymatically form fatty acid hydroperoxides (Huss, 1995). These metabolites are analogous to the intermediates formed non-enzymatically in the free radical lipid autoxidation reactions that are likely to occur in all tissues containing polyunsaturated fatty acids. The hydroperoxides are reduced enzymatically to the corresponding hydroxyl analogs. Activity and specificity of lipoxygenase and its role in the deterioration and physiological metabolism of lipids in fish have been employed previously to investigate the role of lipid oxidation *in vivo* and in foods (Winkler *et al*., 1991). The bioactivities of the various lipoxygenase metabolites are widely distributed and include adhesion, the promotion of chemotaxis, possible action as second messengers, or at least, to modulate second messenger signalling by modifying membrane properties.

1.3.1.2 Autoxidation / non-enzymatic oxidation

Autoxidation is complex and involves a great number of interrelated reactions of intermediates. The rate of autoxidation is affected by fatty acid composition, the degree of unsaturation, the (partial) pressure of oxygen, and the nature of the surface being exposed to oxygen, as well as the storage conditions (temperature, light exposure, moisture content, etc.), and the presence and activity of pro- and antioxidants of fish (Belitz and Grosch, 1987). PUFA damages can lead to primary and secondary lipid oxidation products, which can react with amino constituents (proteins, peptides, free amino acids, and phospholipids) to produce interaction compounds. As a result, browning, flavour changes, and loss of essential nutrients may occur (Aubourg, 1999b). The PUFAs found in fish lipids are extremely susceptible to oxidation by an autocatalytic mechanism (Fig. 1.4). The oxidation process is initiated by abstraction of a hydrogen atom from the central carbon of the pentadiene structure found in most fatty acid acyl chains containing more than one double bond (Huss, 1995).

Metal ions are vital in the first step of lipid autoxidation - the initiation step - in catalysing the formation of reactive oxygen species, for example, the hydroxyl radical (OH•). This highly reactive radical immediately reacts with lipids or other molecules at the site where it is generated. FFAs have been found to be more susceptible to oxidation than the corresponding bound ones. The reason for this is that the amount of iron in the aqueous phase is presumably greater than the amount bound to the surface of cellular membranes and lipid droplets (Huss, 1995). As seen in Figure 1.4, the hydroperoxides, (also called peroxides or primary oxidation products) are readily broken down to secondary autoxidation products of shorter carbon chain-length. This reaction is also catalysed by heavy metal ions. These secondary products - mostly aldehydes, ketones, alcohols, small carboxylic acids and alkanes - give rise to a very broad odour spectrum and in some cases to a yellowish discolouration (Kinsella, 1986). Several of the aldehydes are referred to as "thiobarbituric acid-reactive substances".

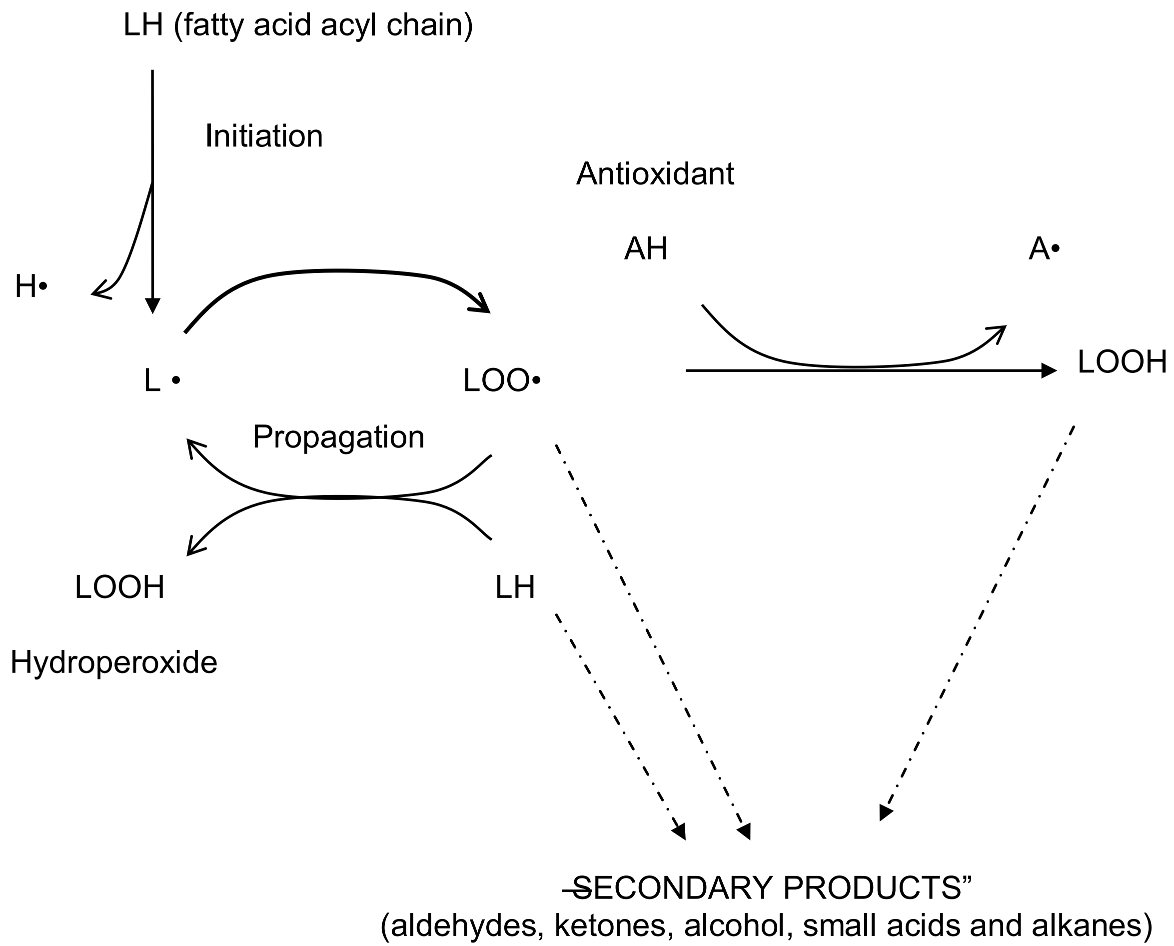


Figure 1.4 The autoxidation of polyunsaturated lipids (non-enzymatic) (Source: Huss, 1995).

In Figure 1.4 is illustrated how the lipid radical (L•) rapidly reacts with atmospheric oxygen, making a peroxy-radical (LOO•) that may remove hydrogen from another acyl chain resulting in a lipid hydroperoxide (LOOH) and a new radical L• (Huss, 1995). This reaction is called propagation (Melton, 1983). The hydroperoxides that are produced in relatively large amounts during propagation, are tasteless and therefore the "peroxide value" does not correlate well to sensorial properties (Huss, 1995). This process continues until the reaction reaches the termination step where one of the radicals is removed by reaction with another radical (Melton, 1983), or with an antioxidant (AH), resulting in a much less reactive radical A•. The phenolic compound, α-tocopherol (Vitamin E) is considered the most important natural antioxidant and carotenoids may also function as antioxidants (Huss, 1995).

The products formed during autoxidation of two of the PUFAs that occur in fish autoxidation pathways, oleic acid (C18: 1) and linoleic acid (C18: 2), are shown in Figure 1.5. Autoxidation of oleic acid yields four primary reaction products, namely (I) 11-Hydroperoxyoctadec-9-enoic acid, (II) 9-hydroperoxyoctadec-10-enoic acid, (III) 10-hydroperoxyoctadec-8-enoic acid, and (IV) 8-hydroperoxyoctadec-9-enoic acid. Thus, four products form because a hydrogen atom can be abstracted from either of the two allylic carbons, carbon-8 or carbon-11, resulting in two delocalised three-carbon allylic radicals. Since both ends of these intermediates have free-radical characteristics, oxygen has four potential sites at which to react, resulting in a mixture of four allylic hydroperoxides. It is further important to note that geometric isomers are formed since the radical can assume different stereochemical conformations under different reaction conditions.

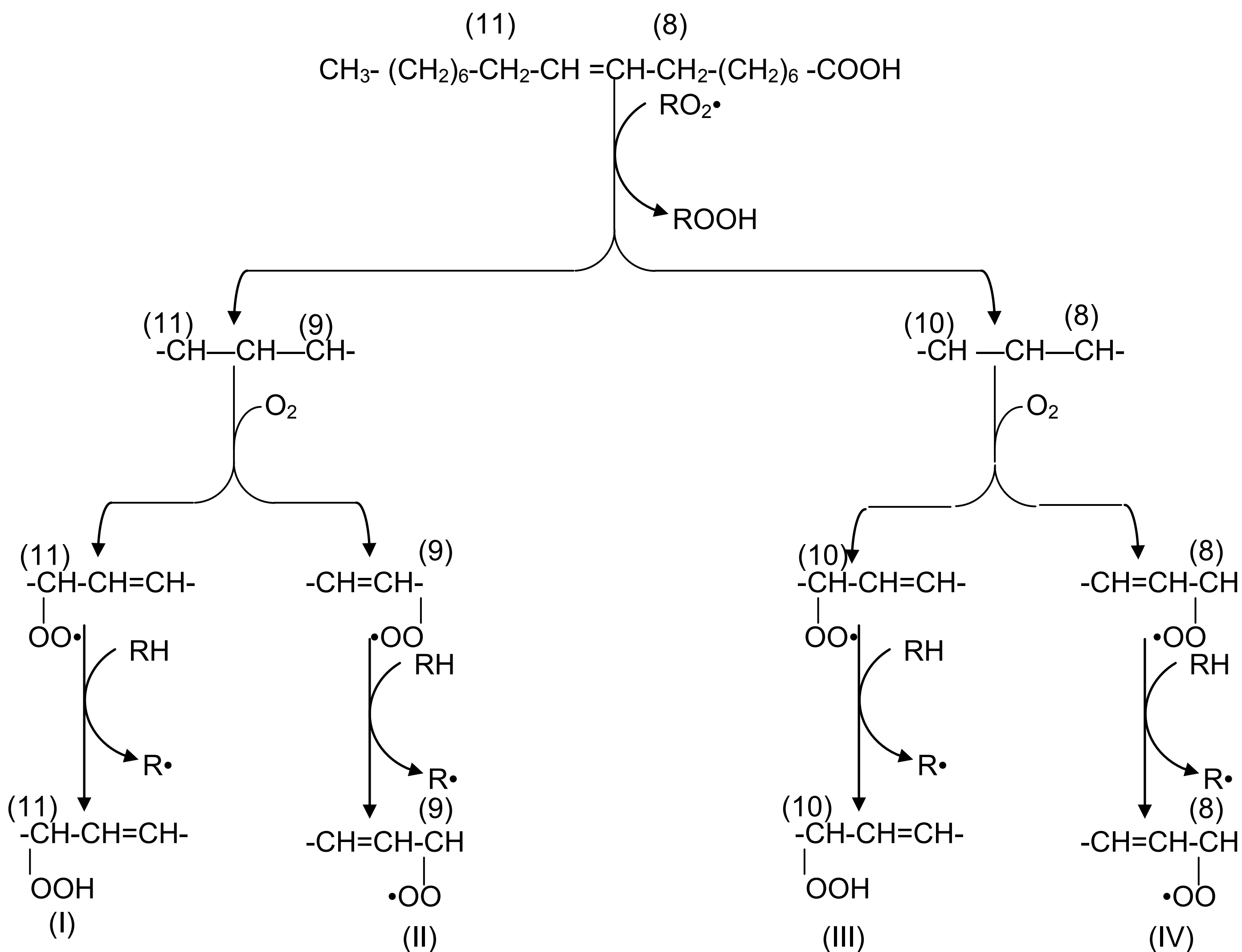


Figure 1.5 Primary reaction products formed during the autoxidation of oleic acid: (I) 11-Hydroperoxyoctadec-9-enoic acid, (II) 9-hydroperoxyoctadec-10-enoic acid, (III) 10-hydroperoxyoctadec-8-enoic acid and (IV) 8-hydroperoxyoctadec-9-enoic acid (Adapted from Belitz and Grosch, 1987).

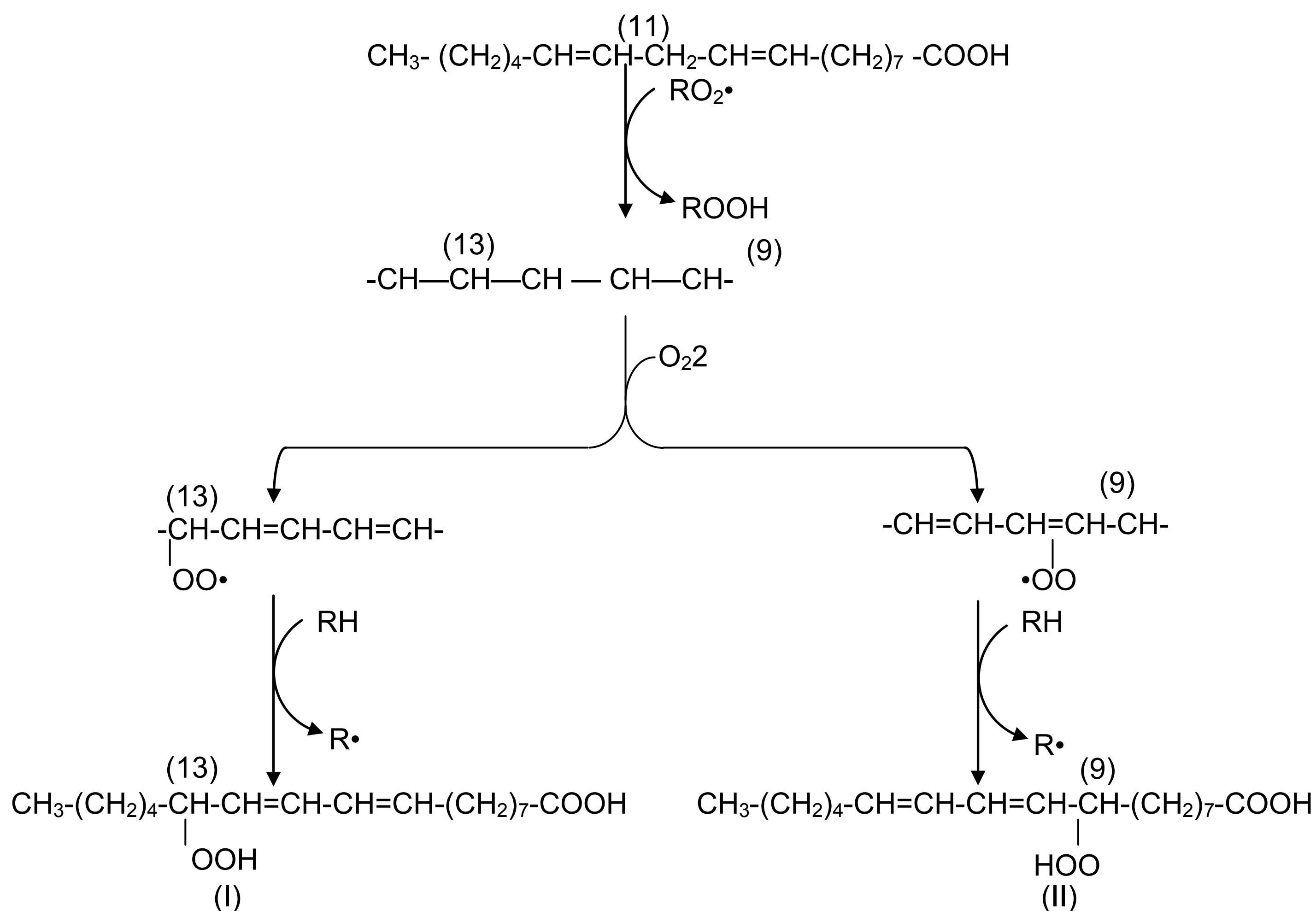


Figure 1.6 Primary reaction products formed during the autoxidation of linoleic acid: (I) 13-Hydroperoxyoctadeca-9,11-dienoic acid and (II) 9-hydroperoxyoctadeca-10,12-dienoic acid (Adapted from Belitz and Grosch, 1987).

When linoleic acid is oxidised (Fig. 1.6), the reactive hydrogen is on the bis-allylic methylene group at carbon-11. Because oxygen can react with either carbon-9 or carbon-13 on this formed pentadienyl radical, a mixture of two lipid hydroperoxides are formed, namely (I) 13-Hydroperoxyoctadeca-9,11-dienoic acid and (II) 9-hydroperoxyoctadeca-10,12-dienoic acid.

1.3.2 The influence of microbiota on lipid oxidation and spoilage of fish tissue

Bacterial pathogens that can grow at near freezing temperatures (between 4 ºC and 10 ºC), include enteropathogenic *Escherichia coli*, *Staphylococcus aureus*, Salmonellae, and *Vibrio parahaemolyticus* (Ahmed, 1992). These micro-organisms are a problem during transportation of seafood, if not cooled effectively at any time after harvesting. Thus, proper cooling of seafood during transportation becomes an important safety consideration.

Bacteria most often involved in the spoilage of fish are part of the natural microbiota of the external slime of fish and their intestinal contents (Frazier and Westhoff, 1988). The chemical compounds that develop in naturally spoiling fish, compared to sterile fish, are mostly the volatile compounds formed by bacteria (Huss, 1995). Many short chain alcohols, carbonyls, acids and aromatic compounds in fish are the products of spoilage bacteria (volatiles are elucidated under section 1.4).

During storage of lightly preserved fish, lipid hydrolysis caused by bacteria may be part of the spoilage profile (Huss, 1995). Bacterial attack on the lipid fraction of fresh fish contributes little to the spoilage profile and changes detectable in the lipid fraction are caused almost exclusively by chemical action, for example oxidation.

1.3.3 Bioactive lipids formed in fish tissue

Human population that maintains a high intake of especially fish, has a marked reduced incidence of, among others coronary artery disease (Das, 2000). These observations have led to intensive research indicating that ω3 fatty acids affect various and unique metabolic and functional biological functions (Galli and Marangoni, 1997; Das, 2000). The biological effects of fish oil are ascribed to their content of eicosapentaenoic acid 20:5ω3 (EPA), and docosahexaenoic acid 22:6ω3 (DHA) that reduce the content of arachidonic acid in phospholipids, both by direct incorporation and by inhibition of its synthesis.

The important metabolic functions of the essential fatty acids are mediated by their conversion to eicosanoids that include prostaglandins, thromboxanes, leukotrienes and lipoxins. Eicosanoids are local hormones, synthesised in response to cell specific proteolytic or hormonal stimuli, and not stored by cells (Smith, 1989). An increase in the concentration of free arachidonate is a direct effect of this stimulus.

Arachidonic acid can be converted to hydroxyl fatty acids via lipoxygenase, and these hydroxyl fatty acids, such as hydroxyl eicosatetranoic acid (HETE), may be further derivatized by dipeptides, such as glutathione to form leukotrine A4, which is then modified to form other leukotrines. The leukotrines are also bioactive modulators involved in pulmonary function (like asthma), allergies, macrophage functions, chemotaxis, chemokinesis, inflammatory responses, and immune functions (Smith, 1989). The different families of unsaturated fatty acids (ω3 and ω6) further affect the prostaglandin and leukotriene pathways as seen in Figure 1.7 (Kinsella, 1986; Brenner 1989).

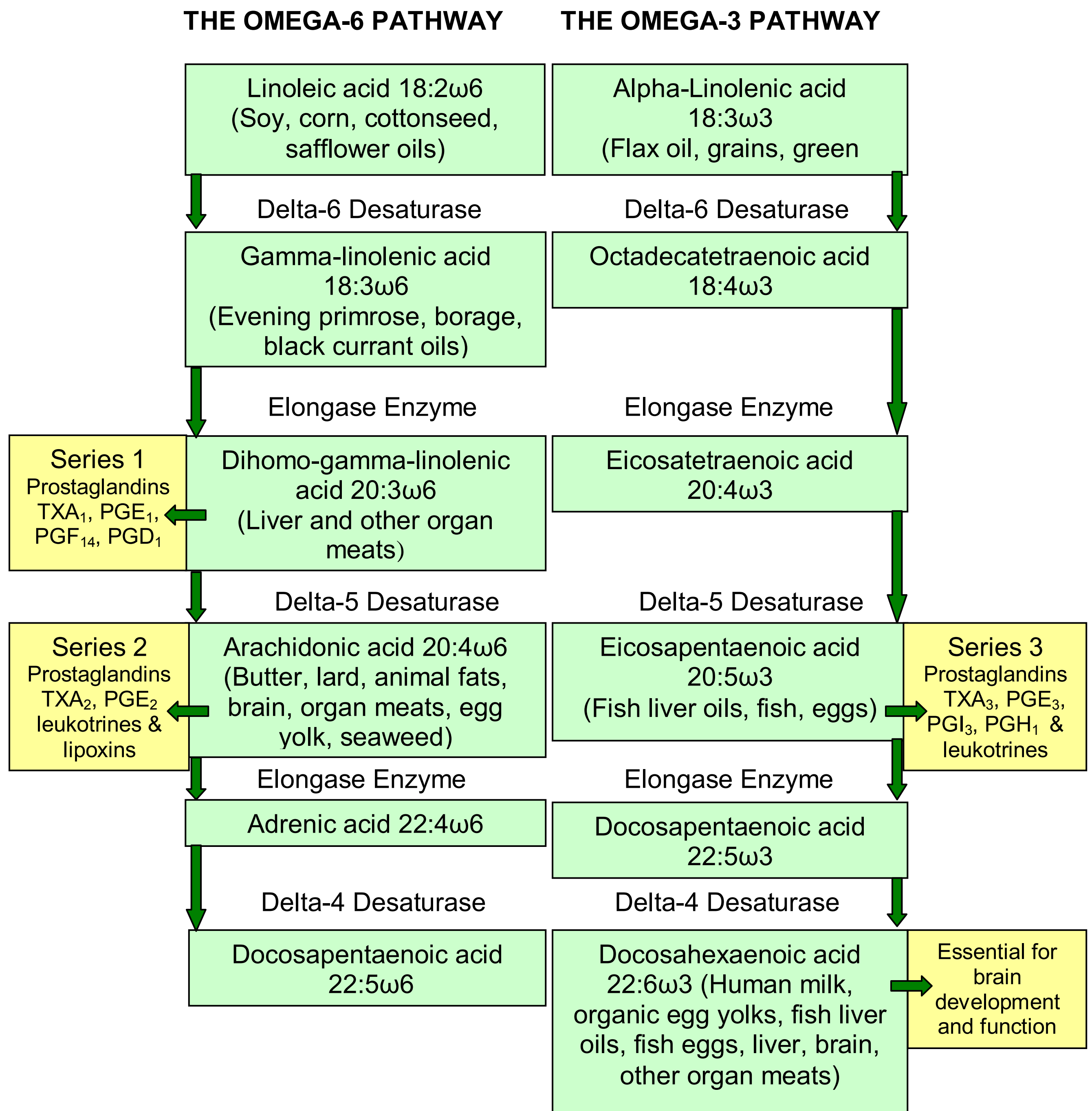


Figure 1.7 Conversion of essential fatty acids to biological active eicosanoids including various prostaglandins (PG), leukotrienes and thromboxanes (TX) via the omega-3 and the omega-6 pathways. (Adapted from Brenner, 1989).

Since prostaglandins and leukotrines are formed mainly from arachidonic acid free from phospholipids, ω3 fatty acids influence this formation by inhibiting arachidonic acid synthesis, by substituting arachidonic acid in the phospholipids, and by competing with arachidonic acid as substrates or inactivators in prostanoid formation (Larsen *et al*., 1997). The ω6 family - particularly arachidonic acid (20:4ω6) derived from dietary linoleic acid (18:2ω6) - are physiologically the most important precursors of eicosanoids.

1.3.4 Lipid oxidation during hake processing

In Figure 1.8 a typical hake processing schematic flow diagram is illustrated. During each processing step, chemical changes can occur, as discussed under section 1.3.1 and cross-contamination may occur if good manufacturing practises are not in place. This can result in a decrease in the quality of the raw fish. Cape hake is extremely perishable and tissue deterioration commences the instant that the fish dies and its original characteristics start to change, due to specific conditions of processing (Engelbrecht *et al*., 1996). Conditions such as storage on ice to slow down microbial activity are, therefore, applied to delay, but not inhibiting the spoilage of fish (Aubourg, 1999a). The hygienic handling of fish is of utmost importance from the moment fish are caught, in order to ensure good quality and long storage life (Huss, 1995). Fish are usually harvested from the ocean at great distances from processing facilities, and therefore preservation on the vessel should start immediately (Penfield and Campbell, 1990; Potter and Hotchkiss, 1997).

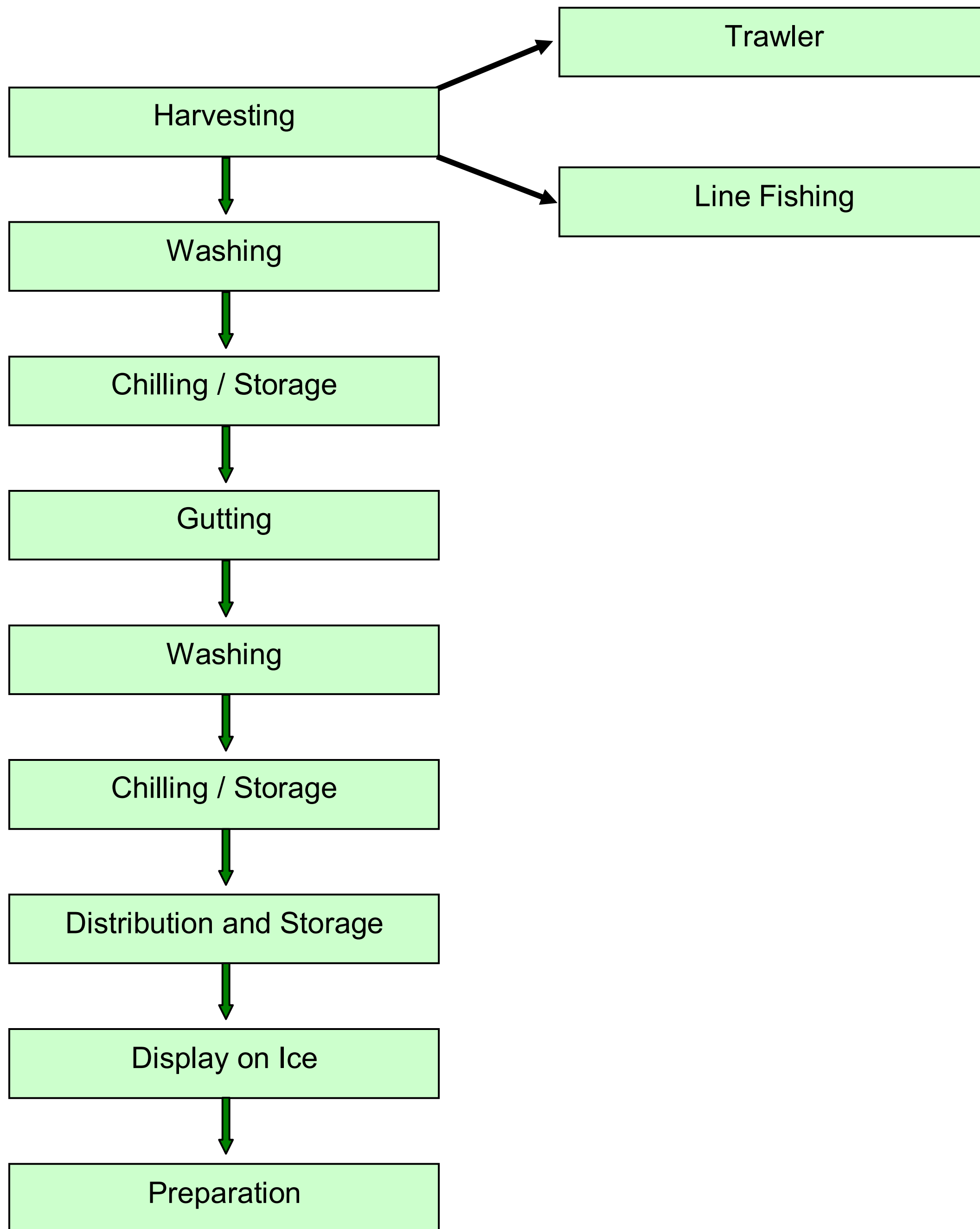


Figure 1.8 A schematic flow diagram illustrating the typical procedures of harvesting, processing, storage, distribution and preparation of fish.

Live fish muscle is generally recognised as sterile and the gills are known to be the most susceptible part of the fish to decay or to be infected (Jay, 1992). In addition, low rigor mortis enhances the enzymatic and microbiological changes, due to a lesser acid reaction in fish tissue which favours microbial growth (Frazier and Westhoff, 1988).

Cape hake, like most seafood, is an ideal substrate for "exogenous" bacterial contamination resulting from sewage polluted rivers, estuaries, bays and inshore waters, as well as poor sanitary conditions during processing, storage and handling (Venneman, 1996). Only a few rivers from the dry Southwest African subcontinent drain into the Atlantic Ocean, resulting in a low incidence of human bacterial pathogens on freshly caught Cape hake. The predominant microbial populations associated with cold and temperate marine waters (2 to 12 ºC) are mainly of the genera *Moraxella* and *Pseudomonas* (60 to 80 %), and *Corynebacterium* and *Micrococcus* (20 to 40 %) (Venneman *et al*., 1994).

Cape hake and most other deep-sea whitefish from cold water contains primarily *Moraxella*, *Pseudomonas, Alteromonas* (*Shewanella*), and some less frequently encountered (<20 %) Gram-negative genera such as *Flavobacterium* and *Vibrionaceae* (Venneman, 1996). The prominent Gram-positive bacteria groups include *Micrococcus* and Coryneforms and other less frequent encountered (<10 %) groups include *Bacillus* and *Clostridium*. Only limited *Vibrio* spp. have been isolated when removed from the influence of human inhabitation at the cold marine water temperatures of the Atlantic Ocean. *Vibrio* and *Clostridium* have rarely or never been isolated from South African hake (Venneman, 1996).

1.4 VOLATILES

Food is often seen as more than just a biological necessity for survival. Food can provide for a variety of psychological functions, for example, an aesthetic stimulus, an anesthetic or sedative or even a comfort. To provide these subtle human needs, food cannot be separated from its organoleptic values such as taste, flavour and texture. Several factors may contribute to specific volatiles or flavour of food. These include species-specific volatiles, chemicals and or conditions during processing, the gender of the animal, environmental pollutants (for example ―kerosene" in marine fish from oil spills), the growth of microorganisms, lipid oxidation or endogenous enzymatic decomposition (Wilkes *et al.*, 2000).

1.4.1 Volatiles in fish

The flavour characteristics of seafood are derived from both its volatile components and its non-volatile taste constituents. Not all volatile components are equally important and its contribution is dependent on their recognition threshold and concentration (Josephson, 1991). Various volatile carbonyls and alcohols (refer to Table 1.2) are derived from polyunsaturated fatty acids of fish lipids via specific lipoxygenase activity (Josephson, 1991; Durnford and Shahidi, 1998). Volatile aromatic compounds are derived primarily from the long chain fatty acids and the most prominent of these in seafood belong to the omega-3 fatty acids. These play a predominant role in the flavour of fish. Although most seafood have similar aroma compounds (Table 1.2) derived from common biochemical/chemical flavour generating mechanisms and precursors, additional flavour generating mechanisms characteristic of individual species are responsible for species-specific volatile aromatic compounds (Josephson, 1991). Natural odorous compounds that originate during metabolic pathways of amino acids and fats include carboxylic acids, alcohols, carbonyl compounds and lactones (Plutowska and Wardencki, 2007).

Table 1.2 Volatile aroma compounds enzymatically derived from fatty acids in freshly harvested seafood (Adapted from Josephson, 1991)

Compound	Aroma Description
2-Pentenal	Green, apple-like
1-Penten-3-one	Solvent-like
1-Pentanol	Wine-like, ethereal
1-Penten-3-ol	Grassy, green
Hexanal	Green, aldehyde
(E)-2-Hexenal	Green, stinkbug
(Z)-3-Hexenal	Green, apple-like
Hexanol	Green, fatty
(Z)-3-Hexen-1-ol	Green, leaf-like
3-Octanone	Resinous, ethereal
1-Octen-3-one	Mushroom
(Z)-1,5-Octadien-3-one	Geranium leaves
3-Octanol	Mushroom
1-Octen-3-ol	Mushroom
(Z)-1,5-Octadien-3-ol	Earthy, mushroom
(Z)-2-Octen-1-ol	Fatty, rancid
(E, Z)-2,5-Octadien-1-ol	Earthy, Mushroom
(E)-2-Nonenal	Cucumber, cardboard
(E)-3-Nonenal	Watermelon
(E)-6-Nonenal	Green-melon rind
(E,Z) – 2,6-Nonadienal	Cucumber peel
(E,Z) – 3,6-Nonadienal	Watermelon
(Z)-2-Nonen-1-ol	Green, waxy melon
(Z)-6-Nonen-1-ol	Green, waxy melon
(E,Z)-2,6-Nonadien-1-ol	Dry green, cucumber
(Z,Z)-3,6-Nonadien-1-ol	Cucumber, melon rind

Fresh fish are associated with mild, delicate and pleasant aromas. They are generally described as green, plant-like or melon-like and are provided by various volatile carbonyls and alcohols (Table 1.2), derived from polyunsaturated fatty acids of fish lipids via specific lipoxygenase activity (Josephson, 1991; Durnford and Shahidi, 1998). Volatile alcohols contribute smoother qualities, whereas volatile carbonyls contribute coarse, heavy aromas and contribute more to the overall fresh fish-like odours, because of their lower threshold values. Compounds known to occur in freshwater, as well as marine fish, are hexanal, 1-octen-3-ol, 1,5-octadien-3-ol, and 2,5-octadien-3-one. Unlike the eight-carbon compounds, the six-carbon and nine-carbon compounds have been identified in freshly harvested fish, but not in all seafood. During the deterioration process, the fresh odours may be masked or destroyed by microbial and autolytic activity, or newly produced compounds (Josephson, 1991; Durnford and Shahidi, 1998).

The most prominent volatiles produced in muscle foods during lipid oxidation are aldehydes, and include octanal, nonanal, pentanal and the most dominant hexanal (Ross and Smith, 2006). During lipid autoxidation of seafood, the volatiles generated are hexanal; the 2,4-heptadienals, the 3,5-octadien-2-ones, and the 2,4-decadienals. Some volatiles derived through enzymatic mechanisms, can be formed via autoxidation, for example, 2-nonenal, 2,6-nonadien, 1-octen-3-one, and 1,5-octadien-3-one. The particular volatile compounds produced by the lipoxygenase, are dependent on the substrate. If docosahexanoic acid or eicosapentanoic acid is the substrate, the flavour volatiles produced are 1,5-octadien-3-ol, (E,Z)-2,6-nonadienal, 2,5-octadien-1-ol and 3,6-nonadien-1-ol. Where arachidonic acid is the substrate, (E)-2-octenal, 1,-octen-3-ol, (E)-2-nonenal, (E)-2-octenol and (Z)-3-nonenol, flavour volatiles are produced (Josephson, 1991; Durnford and Shahidi, 1998).

Volatile sulphur compounds traditionally associated with seafood spoilage, can be produced by live seafood and include methyl mercaptan, dimethyl disulfide and dimethyl sulphide (Josephson, 1991; Durnford and Shahidi, 1998). Trimethylamine (TMA), volatile sulphur compounds, aldehydes, ketones, esters,

hypoxanthine, as well as other low molecular weight compounds, are categorised as spoilage odours. These are microbiologically produced from various substrates, including the carbohydrates (for example, lactate and ribose), nucleotides (for example, inosine mono-phosphate and inosine) and other molecules. The amino acids are particularly important substrates for the formation of sulphides and ammonia and, in addition, organic acids have been shown to play an inherent and very acute role in the establishment of off-flavours during the spoilage of fish. Deteriorated fish odours are associated with TMA and dimethylamine (DMA). During microbial spoilage, TMA is produced by the reduction of trimethylamine oxide (TMAO). DMA and formaldehyde are produced from the enzymatic breakdown of TMAO in the muscles of various fish species. TMAO has no odour, but TMA reacts with fat in the fish tissue to produce a potent fishy odour. In gadoid fish, such as hake, the formation of DMA and formaldehyde is more than that of TMA, and can be prevented, if microbial growth is repressed (Josephson, 1991; Durnford and Shahidi, 1998).

Fish, such as hake, develop characteristic off-flavours during prolonged or poor storage conditions. Hake has been shown to form formaldehyde during cold storage, which has a toughening effect to be associated with an “irreversible loosening of bound water”. This may be enhanced by variations in storage temperature, although it has been suggested that the toughening effect is present even in fish held at very low temperatures. Indications are that the aldehyde and similar compounds with less marked effects, originate from lipid oxidation. The application of chemometrics when using chemical markers such as volatiles to determine fish freshness, its storage life, quality and even safety, has been established in numerous studies (Ólafsdóttir *et al.,* 1997; Triqui and Bouchriti, 2003; Ross and Smith, 2006; Alimelli, *et al.*, 2007; Plutowska and Wardencki, 2007).

1.5 CHEMOMETRICS

Currently, modern analytical instruments produce great amounts of variables or features (information) for a large number of samples, that can be analysed in a relatively short time. This leads to multivariate data matrices that require mathematical and statistical procedures to efficiently extract the maximum useful information from the data. This science of extracting information from chemical systems by data-driven means, is known as chemometrics. The raw data used for chemometric treatments in food chemistry are usually chemical or physical data, consisting of total acidity, pH, conductivity, moisture, concentrations of specific chemical substances as measured in samples by analytical techniques such as high-performance liquid chromatography (HPLC) or gas chromatography (GC), fingerprinting data, such as chromatograms or spectroscopic measurements (specific signals or the complete spectra) as obtained by infrared (IR), nuclear magnetic resonance (NMR), mass spectrometry (MS), ultraviolet – visible or fluorescence spectrophotometry and also signals from sensor ranges, such as electronic tongues or noses and/or data from sensorial analysis of samples. It may be possible to select a specific signal or spectral regions based on knowledge as to which signals indicate the presence of specific chemical groups, or based on chemometric treatments (Berrueta, Alonso-Salces and Héberger, 2007).

The specific application of chemometrics, known as supervised pattern recognition, has been established in food quality assessment, including the determination of shelf life (quality monitoring during storage) (O'Farrell *et al.*, 2005; Siripatrawan and Jantawat, 2008). Several supervised pattern recognition techniques can be applied for product quality evaluation, such as linear discriminant analysis (LDA), partial least squares discriminant analysis (PLS), the *k*-nearest neighbours (*k*NN), soft independent modelling of class analogy (SIMCA), and artificial neural network (ANN) (Berrueta *et al.*, 2007; Siripatrawan *et al.*, 2009). Supervised pattern recognition procedures use a common strategy consisting of the following steps (Berrueta *et al.*, 2007):

(1) The selection of a training, a test and a calibration set, which consist of objects of known class membership for which variables are qualified. The calibration set is used for the optimization of parameters characteristic of each multivariate technique.

(2) The variable selection. Specific variables containing information for the aimed classification are kept, whereas variables encoding the noise and/or with no discriminating power, are eliminated.

(3) The model building using the training set. A mathematical model is derived between a certain number of variables qualified on the samples that constitute the training set and their known categories.

(4) Validation of the model using an independent test set of samples, in order to evaluate the reliability of the classification achieved.

Essentially the various kinds of pattern recognition procedures differ in the way they achieve classification. There are mainly two types of methods distinguished: those directed on discriminating among classes (including LDA, *k*NN and ANN), and those leaning towards modelling classes (such as SIMCA) (Berrueta *et al*., 2007).

1.5.1 Mathematical modelling

Mathematical modelling can be used in various applications in food science and has successfully been used for shelf life prediction of packed fish (Dalgaard, Mejlholm and Huss, 1997). Polynomial mathematics is used for prediction modelling and is also known as polynomial regression. In statistics it is a form of multilinear regression. In these equations the relationship between the independent variable x and the dependent variable y is modelled as an nth order polynomial. This is an example of curvilinear regression.

For example a polynomial equation has x raised to numeral powers such as x^2, x^3 and x^4. A parabola is produced by a quadratic equation (Eq. 1):

$$y = a + b_1 x + b_2 x^2 \quad (1)$$

where a is the Y-intercept and b_1 and b_2 constants. An S-shaped curve is produced by a cubic equation (Eq. 2):

$$y = a + b_1 x + b_2 x^2 + b_3 x^3 \quad (2)$$

and M (double positive) or W (double negative) shaped curves are produced by a quartic equation (Eq. 3):

$$y = a + b_1 x + b_2 x^2 + b_3 x^3 + b_4 x^4 \quad (3)$$

Different powers or orders of the variable x (x^2, x^3 and x^4) are used in polynomial regression to determine whether they increase the R^2 (correlation coefficient) significantly. The usual procedure is to first test the linear regression ($y = a + bx$), then the quadratic, then the cubic, until the best-fitting equation is chosen. The R^2 will always increase when you add a higher-order term that is possible to find the most suitable equation for the curved line, that best fits the data points for whichever equation. This allows for comparison of the fit of the more complicated equation to that of a simpler equation (for example the equation for a straight line) (McDonald, 2009).

1.5.2 Artificial neural networks (ANNs)

ANNs are accepted in many disciplines (including food analysis) for modelling real-world problems, because of its remarkable information processing characteristics (Basheer and Hajmeer, 2000). These characteristics include (i) highly refined non-linearity, capable of modelling exceptionally complex functions;

(ii) noise intensity, providing accurate prediction in the presence of fuzzy data and measurement errors; (iii) high parallelism, implying fast processing and hardware failure tolerance; (iv) learning and adaptability, allowing the system to modify its internal structure in response to environment changes; and (v) generalisation, allowing application of the model to unlearned data (Berrueta *et al*., 2007; Marini *et al*., 2009). Any given functional relation between a set of inputs and a corresponding set of outputs can be represented by choosing the right ANN architecture (Marini *et al*., 2009). Artificial neural networks (ANN) can be defined as structures comprised of densely interconnected adaptive simple processing elements, called artificial neurons (or nodes) that are capable of performing massively parallel computations for data processing and knowledge representation (Basheer and Hajmeer, 2000).

1.5.2.1 Artificial neurons

Findings surrounding the operation of the biological neuron has enabled early researchers to model the operation of simple artificial neurons (Siripatrawan *et al.*, 2009). Rosenblatt introduced the ‗perceptron' to solve problems in character recognition. As seen in Figure 1.9 an artificial processing neuron receives inputs as stimuli from the environment, combines them in a particular way to form a ‗net' input (ξ), passes that over through a linear threshold gate, and transmits the (output, y) signal forward to another neuron or the environment. Only when ξ is stronger than the neuron's threshold limit (also called bias, b), the neuron becomes activated (fired). Generally linear neuron dynamics are assumed for calculating ξ (Basheer and Hajmeer, 2000).

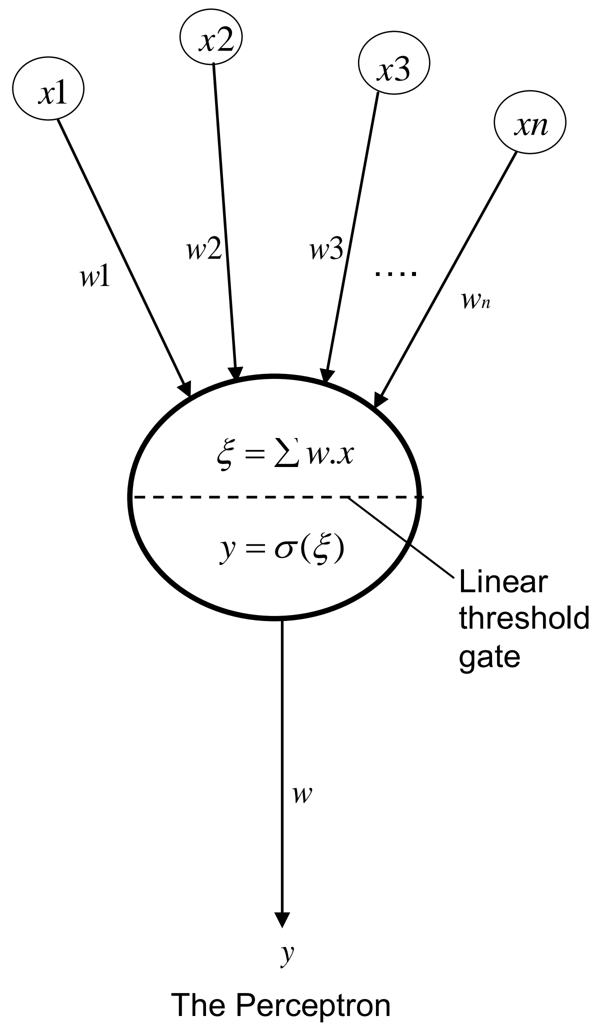


Figure 1.9 Summarising an artificial neuron comprising the single layer perceptron (adapted from Basheer and Hajmeer, 2000)

The net input is computed as the inner (dot) product of the input signals (x) interrupting on the neuron and their strengths (y). For n signals, the Perceptron neuron can be expressed as

$$y = \begin{Bmatrix} 1, \text{if} & \sum_{i=1}^{n} w_i x_i \geq b \\ 0, \text{if} & \sum_{i=1}^{n} w_i x_i < b \end{Bmatrix} \tag{4}$$

with 1 indicating ‗on' and 0 indicating ‗off', or class A and B, correspondingly, in solving classification problems. Positive connection weights ($w_i > 0$) enhance the net signal (ξ) and excite the neuron (this link is called excitory). Opposed to that, the negative weights reduce ξ and inhibit the neuron activity (this link is called inhibitory) (Basheer and Hajmeer, 2000). In Figure 1.9 the system called the Perceptron is indicated to consist of an artificial neuron and inputs. The Perceptron establishes a mapping between the inputs activity (stimuli) and the output signal. The neuron threshold in Equation (Eq.) (1) can be considered as an additional input node whose value is always unity (i.e. $x = 1$) and its connection weight is equal to b. In such case, the summation in Eq. (1) is run from 0 to n and the net signal ξ is compared to 0.

In order to cope with non-linearity separable problems, additional layer(s) of neurons placed between the input layer and the output neuron are needed, leading to multilayer perceptron (MP) architecture (Fig. 1.10).

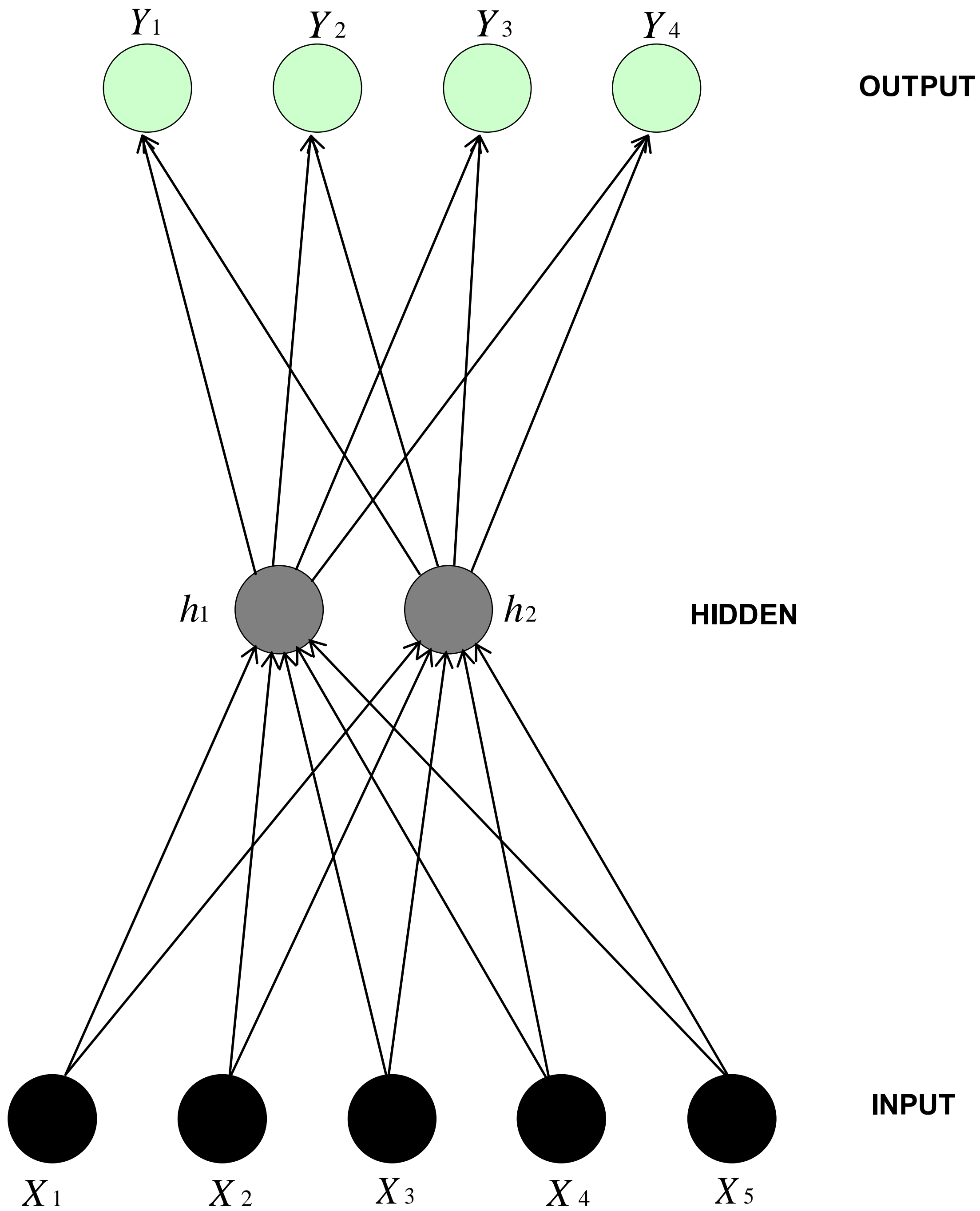


Figure 1.10 A schematic representation of a multilayered perceptron showing input, hidden and output layers and nodes with feedforward links (adapted from Marini *et al.,* 2009)

1.5.2.2 Multilayered perceptron networks

A feed-forward network ANN is composed of a large number of artificial neurons or perceptrons in a parallel structure (Fig. 1.10), consisting of (i) an input layer containing one neuron for each independent variable (X); (ii) one or more hidden layers (where the data are processed) (h); and (iii) an output layer, with one neuron for each dependent variable (Y). The data flows only forward into the network from input to output via synapses, which are associated with coefficients of connectivity namely weights (w as discussed above). The net input is computed as the inner product of the input variables, interrupting on the neuron and its weights. Once the node calculates this product, the result is passed to a differentiable non-linear transfer (activation) function, which transforms the weighted sum of all variables interrupting onto the neuron in order to get the output value. Several transfer functions can be used, for example, sigmoid function, variant logistic functions or hyperbolic tangent.

The learning process identifies the weights that generate the best fit of the predicted outputs over the complete training data set. The hidden layers are the most important in solving the non-linear classification problems (Marini, 2008).

Numerous networks, new or modified, are constantly being developed and although an estimated 50 different ANN types already exist, the following are the most frequently used in order of their discovery: Hopfield networks, Adaptive resonance theory networks, Kohonen networks, Backpropagation (BP) networks, Recurrent networks, Counterpropagation networks and Radial basis function networks. The most widely used ANN is the BP network, also known as the workhorse of ANNs (Basheer and Hajmeer, 2000).

The BP network uses supervised learning where the error-correction learning rule is applied (a learning rule defines how the weights of the network should be adjusted between successive training cycles). This rule basically uses the answer of the difference (error) between the ANN solutions at any stage during training to modify the connection weights to gradually reduce the overall network

error. Back propagation refers to the way the error computed at the output side is propagated backward from the output layer to the input layer via the hidden layer through the network (Basheer and Hajmeer, 2000; Berrueta *et al*., 2007; Marini *et al*., 2009).

Assigning random initial values to the weights (and thresholds) of all connections between neurons of a network should occur first. Thereafter, during the construction of a neural network, the following are important: (i) selection of the architecture, (ii) selection of the learning parameters, and (iii) network validation. During the training, the values for all the parameters involved in the learning process must be optimised, and the error between the net-predicted output and the correct output must be calculated. The size and number of the hidden layer and the number of training cycles (epochs) must be evaluated, by varying their values (trial and error) and checking the accuracy of the resulting prediction. The optimal number of training cycles for an ANN is attained when the error on the test set reaches a minimum. The number of hidden nodes is critical to the design of the network, because if too many hidden nodes are used, the network will memorise or over fit the training set data. Conversely, if too few hidden nodes are used, the network will become unstable and will fail to generalise. The optimal number of hidden nodes should be determined for each dataset. An approach to determine the best number, is to start with the simplest architecture, such as adding nodes one by one, until the network has learned the training set. This can, however, become time-consuming and it may be worthwhile to make use of some of the several rules of thumb available in literature. These relate hidden layer size to the number of nodes in input and output layers. At the end of the training phase, the network should be tested by using samples that were not present in the training set. The quality of the ANN architecture can be evaluated, using the root mean square error between the expected and the actual value of the output (Basheer and Hajmeer, 2000; Berrueta *et al*., 2007; Marini *et al*., 2009).

1.5.2.3 Concerns in ANN development

There are a number of concerns highlighted by Basheer and Hajmeer (2000) that should be addressed before initiation of any network training. Here is a summary of a few concerns, some relevant to only BP ANNs as well as a few applicable to all other types of ANN.

1.5.2.3.1 Database size and its partitioning

The development of ANN models are dependent on the size of the database on which they are constructed. Although neural networks can be developed from data of any size, its generalisation capability will be of great concern. Because ANN is required to generalise for the prediction of formerly unseen cases, prediction should be more reliable, if the developed ANN is used as interpolation tools rather than for extrapolation. It is, therefore, preferred that the data used for training should be large enough to cover the possible known variation in the problem domain. However, this requirement cannot be justified when it is really expensive, or time consuming to obtain additional data (Najjar *et al.,* 1997; Basheer and Hajmeer, 2000).

The development of an ANN requires partitioning of the initial database into three subsets, namely: training, test and validation. The training subset is used in the training phase to update the weights of the network, and should include all the data belonging to the problem domain. To check the network response for untrained data during the learning process, the test subset is used. This subset should contain data that is distinct from those in the training data, but should be within the training data margins. The architecture of the ANN model may be changed, and/or more training cycles applied based on the results of the test subset. The validation subset, or the third portion of the data (containing data different from the other two sets) is used to further examine the network, or to confirm its accuracy before it is implemented or delivered to the end user (Basheer and Hajmeer, 2000).

Although there are no mathematical rules for the determination of required sizes for each of the data subsets, some rules of thumb do exist. These have been derived from experience and analogy between ANNs and statistical regression. Furthermore, in database partitioning a large test subset may highlight the generalisation capacity, while the remaining smaller training subset may not be adequate to effectively train the network (Basheer and Hajmeer, 2000).

1.5.2.3.2 Data pre-processing, balancing and enrichment

To accelerate convergence, data should undergo several pre-processing techniques, before it is used for training. These include, amongst others, reducing input dimensionality and data transformation, noise removal, treatment of non-normally distributed data, data inspection and deletion of outliers. In classification problems it is important to balance the data. Training data should be distributed as evenly as possible between the various classes, to prevent the network from being biased to the over-represented classes. Some of the over-represented classes may therefore, be removed or extra examples pertaining to the under-represented class added. This may entail duplicating the under-represented input/output examples and adding random noise to their input data (while keeping the output class unchanged), and qualifying the degree of balance of the training database by using the information theory (Basheer and Hajmeer, 2000).

Another concern in ANN development is small database size, because of the inability to partition the database into fairly sized subsets for training, test and validation. Expanding the size of the database, when it is difficult or expensive to get new data conventionally, may require interjecting random noise in the available examples to generate new ones (Swingler, 2001). This addition of noise enhances the ANN robustness against measurement error (for example, noise =± instrument sensitivity), and is called data enrichment. If this is impossible, the leave-one-out method (or leave $-k$- out method) may be used for developing a network. With M exemplars available, a network is trained on $M-1$ (or $M-k$) exemplars, and tested on the one (or k) unused exemplar(s). This is repeated

M times, each with a new set of randomly initialised weights. To obtain a representative solution to the problem, the solutions of the M networks are averaged. Other techniques are also available to train and validate networks with limited data, such as grouped cross-validation, grouped jack-knife and bootstrap (Basheer and Hajmeer, 2000).

1.5.2.3.3 Data normalisation

It is essential to normalise (scale) the data within a uniform range (e.g. 0-1) to prevent (i) larger numbers overriding smaller ones and (ii) to prevent premature saturation of hidden nodes, which may hinder the learning process. Although there are no one standard procedure for normalising inputs and outputs, one way is to scale input and output variables (z_1) in intervals (λ_1, λ_2) corresponding to the range of the transfer function:

$$x_1 = \lambda_1 + (\lambda_2 - \lambda_1)\left(\frac{z_i - z_i^{\min}}{z_i^{\max} - z_i^{\min}}\right) \tag{5}$$

Where x_1 is the normalized value of z_1, and $z_i^{\max}$ and $z_i^{\min}$ are the maximum and minimum values of z_1 in the database.

To avoid saturation of the sigmoid function (that leads to slow or no learning) it is recommended that the data be normalised between slightly offset values such as 0.1 and 0.9 rather than between 0 and 1. Other more computationally involved techniques are available in literature. However, these more complicated techniques may not produce any better solution than that obtained using linear normalisation (Eq. 5). It may be beneficial to take the logarithm of data prior to normalisation for parameters with an exceptional large range (Basheer and Hajmeer, 2000).

1.5.2.3.4 Representation of input/output

Another determining factor in designing a successful ANN is proper data representation. Data inputs and outputs can be continuous, discrete, or a mixture thereof. For example, in a classification problem, where each of the input variables belongs to one of several classes and the output is also a class, all the inputs and outputs may be represented by binary numbers such as 0 and 1 (or 0.1 and 0.9 to prevent saturation). If two inputs (A and B) are to be designed to four levels of activation (for example, low, medium, high and very high), then each input may be represented by two binary numbers such as 00, 01, 10 and 11 to indicate the four levels. Another representation may assign four binary numbers such as 0001, 0010, 0100 and 1000 where 1 determines the type of activation of the input variable. Similar treatment applies to output variables. This representation increases the dimensionality of the input vector. In the above mentioned examples the two-digit representation converts the input vector into four inputs, and the four-digit representation into eight inputs. The value of binary inputs and outputs are in extracting rules from a trained network. A continuous variable may be replaced by binary numbers by partitioning its range into a number of intervals, each assigned to a unique class. Specialised algorithms also exist for discretising variables based on their distribution (Basheer and Hajmeer, 2000)

1.5.2.3.5 Network weight initialization

Part of initialisation of a network involves assigning initial values for the weights (and thresholds) of all connections links. However, current literature contradicts each other on whether weights initialisation can have an effect on network convergence, or not (Basheer and Hajmeer, 2000). In general, weights and thresholds are initialised uniformly in a relatively small range with zero-mean random numbers. An extremely small range can lead to very small error gradients, which may slow down the initial learning process. The choice of small numbers (e.g. -0.3 and +0.3) is vital to reduce the likelihood of premature neurons saturation. In addition, weight initialisation can be performed on a neuron-by-neuron basis by assigning values uniformly sampled from the range

($-r/N_j, +r/N_j$), where r is a real number depending on the neuron activation function, and N_j is the number of connections feeding into neuron j. Weight initialisation can force a hidden unit to learn efficiently (Basheer and Hajmeer, 2000).

1.5.2.3.6 BP learning rate (η)

Training will accelerate at a high learning rate *(η)* by changing the weight vector, W significantly from one cycle to the other (because of the large step). This may cause the search to oscillate on the error surface and never converge, thus increasing the risk of overshooting a near-optimal W. In contrast, a small learning rate drives the search steadily but slowly towards the global minimum. Throughout the training process a constant learning rate may be used. The following learning rates are suggested: η= 0.1 -10 or η=0.0 – 1.0, or η=0.3 – 0.6. Although the adaptive learning rate $[\eta(\mathrm{t})]$ varies along the course of training, it could also be used and can be effective in achieving an optimal weight vector for some problems. When the search is far from a minimum, larger steps are needed and smaller steps as the search approaches the minimum. Various heuristics have been proposed since the distance from a minimum cannot be predicted (Basheer and Hajmeer, 2000).

1.5.2.3.7 BP momentum coefficient (μ)

Commonly a momentum term is used in weight updating to assist in the search to escape local minima and reduce the likelihood of search instability. Weight change is determined via the modified delta rule, that can be written as:

$$\Delta w_{j_i}^{l} = \eta \delta_j^l x_i^{l-1} + \mu \Delta \mathrm{w}_{\mathrm{ji}}^{1(\mathrm{previous})} \tag{6}$$

where η is the learning rate that controls the update step size, μ is the momentum coefficient, and x_i^{l-1} is the input from the $l-1$ th interlayer.

The first part on the right hand side of Eq. 6 is the original delta rule. The added momentum term helps in directing the search on the error hyperspace to the global minimum, by allowing a portion of the previous updating (magnitude and direction) to be added to the current updating step. The momentum coefficient (μ) accelerates the weight updates when there is a need to reduce η to avoid oscillation. If μ is high, it will reduce the risk of the network being stuck in local minima, but it will increase the risk of overshooting the solution as does a high learning rate. When μ>1.0 it yields excessive contributions of the weight increments of the previous step, and may cause instability. In contrast, if μ is extremely small, it leads to slow training. It is possible to use both a constant and an adaptable momentum. In literature the following momentum coefficients are suggested: μ= 0.4 - 0.9 or μ=0.0 – 1.0 or $\mu \approx$0.1 or even that $\eta + \mu \approx 1$. Swingler (2001) uses μ= 0.9 and η= 0.25 for the solving of all problems, unless the solution obtained is not effective. However, the adaptive momentum involves varying μ with the training cycle [i.e. $\mu(t)$] in which the changes in μ are made in relation to error gradient information. Other methods relate μ to the adaptive learning rate η in such a way that μ decreases when learning speeds up. Addition of momentum should be considered with caution, because of the need of doubling computer space for storing weights of current and previous iterations (Basheer and Hajmeer, 2000).

1.5.2.3.8 Activation function (σ)

To determine a neuron's firing intensity, the activation or transfer function, σ, is necessary to transform the weighted sum of all signals impinging onto a neuron. Some functions are designed to only indicate whether a neuron can fire (step functions), despite the magnitude of the net excitation or signal (ξ). This is done by comparing ξ to the neuron threshold (Eq. 4). Most BPANNs applications employ a sigmoid function that possesses the distinctive properties of continuity and differentiability on (-∞,∞), essential requirements in BP learning. In literature success rates were reported with different transfer functions in relation

to data non-linearity and noisiness. However, the advantage of choosing a particular transfer function over another, is still unclear (Basheer and Hajmeer, 2000).

1.5.2.3.9 Convergence criteria

Training may be stopped using one of three different criteria. These include (i) training error ($\rho \leq \varepsilon$), (ii) gradient of error ($\nabla\rho \leq \delta$), and (iii) cross-validation, where ρ is the arbitrary error function, and ε and δ are small real numbers. The third and last criterion is more reliable, although it is computationally more demanding and often requires abundant data. Convergence is usually based on the error function ρ, exhibiting deviation of the predictions from the corresponding target output values, such as the sum of squares of deviations. Training will continue until ρ reduces to a desired minimum. The function ρ may further be expressed as the relative error of the absolute values of the deviations averaged over the subset. Another criterion is the coefficient-of-determination, R^2, representing the agreement between the predicted and the target outputs. There is other more involved methods for monitoring network training, and generalisation are often based on information theory. The sum-of-squared-errors (SSE) (Eq. 7) are the most commonly used stopping criterion in neural network training. SSE is calculated for the training or test subsets as

$$SSE = \frac{1}{N}\sum_{p=1}^{N}\sum_{i=1}^{M}(t_{pi} - O_{pi})^2 \qquad (7)$$

where O_{pi} is the actual and t_{pi} is the target solution of the i th output node on the p th example. N is the number of training examples and M is the number of output nodes.

Several SSE criteria include a measure of complexity of the network architecture. In general, the error on training data decreases indefinitely with increasing number of hidden nodes, or training cycles. The error on test subsets is monitored during ANN training, which normally shows an initial reduction and a

subsequent increase, due to memorisation and overtraining of the trained ANN. The optimal neural network architecture is obtained at the beginning of an increase in test data error.

In optimising network structure, other error metrics may be used and may perform equally well. For example, in classification problems (discrete-value output), the convergence criterion should be based on the hit (or miss) rate, representing the percentage of examples classified correctly (or incorrectly), or on confusion matrices rather than the absolute deviation of the network classification from the target classification (Basheer and Hajmeer, 2000).

1.5.2.3.10 Number of training cycles

To properly generalise the data, the number of training cycles may be determined by trial and error. The error in both the training and test data is monitored for each training cycle for a specific ANN architecture. Overtraining or memorisation is a phenomenon that occurs when training is done for such as long time, that results from a network can only serve as a look-up table. In theory, excessive training can result in near-zero error on predicting the training data (called ―recall"), though generalisation on test data, may degrade significantly. The test subset error continues to decrease initially with the number of training cycles. The error starts to build up after each epoch as the network loses its ability to generalise on the test data. Even though the error on the test data may not follow a smooth path, the beginning of a major increase in the error is considered to represent the optimal number of cycles for that ANN architecture (Basheer and Hajmeer, 2000).

1.5.3.11 Training modes

The network is presented with training examples in either one or a combination of the following two modes: (i) example-by-example training (EET), and (ii) batch training (BT). The weights are updated immediately after the presentation of each training example in the EET mode. At this point, the first example is presented to the network, and the BP learning algorithm, consisting of feed

forward and backward sweeps, is applied for either a specified number of iterations, or until the error drops to the desired level. Once the first example is learnt, the second is presented and the procedure is repeated. The advantages of the EET mode include smaller storage requirements for the weights as opposed to BT, and better stochastic search, which prevents entrapment in local minima. The disadvantage of the EET mode is associated with the fact that learning may become stuck in an initially bad example, which may force the search in the wrong direction. On the contrary, only after all training examples have been presented to the network, the BT mode requires that the weight updating is performed. To be exact, according to the BP learning law, the first learning cycle will include the presentation of all the training examples, the error averaged over all the training examples (e.g Eq. (4)), and then back propagated. Once completed, the second cycle includes another presentation of all examples, and so forth. Advantages of the BT mode include a more representative measurement of the required weight change and a better estimate of the error gradient vector. However, this training mode requires a large storage of weights and is more likely to be trapped in a local minimum. To optimise the search, the presentation of the training examples may be randomised between successive training cycles. Each mode‘s effectiveness can be problem specific (Basheer and Hajmeer, 2000).

1.5.2.3.12 Hidden layer size

One of the most critical tasks in ANN design is the determination of the appropriate number of hidden layers and hidden nodes (NHN). Initial starting is without prior knowledge of the number and the size of hidden layers unlike the input and output layers. One hidden layer is sufficient in most function approximation to approximate continuous functions. For learning functions with discontinuities, two hidden layers may be necessary. A network with too many hidden nodes will follow the noise in the data due to over parameterisation, leading to poor generalisation for untrained data. On the contrary, a network with too few hidden nodes would be incapable of differentiating between complex patterns leading to only a linear estimate of the actual trend. Training becomes

excessively time-consuming with the increasing number of hidden nodes. The optimal amount of HN essential for network generalisation may be a function of input/output vector sizes, size of training test subsets, and more crucially, the problem of non-linearity. In the literature several ―rules of thumb" are available which relate hidden layer size to the number of nodes in input (N_{INP}) and output (N_{OUT}) layers.

The modeller is forced to try networks with hidden layers that may not conform to any of the available ―rules of thumb", when facing unusual problems, such as those with high non-linearity and hysteresis. There are many approaches in finding the optimal number of hidden nodes, from using some of the known ―rules of thumb" as starting point, with trial and error, as well as beginning with a small number of hidden nodes and building on it, as needed to meet the model accuracy demand. Once again the cross-validation technique for determining the proper size of the hidden layer, involves monitoring the error on both the training and test subsets similar to that used to stop training. Another popular technique to optimise network size is the growing and pruning methods (Basheer and Hajmeer, 2000).

1.5.2.3.13 Parameter optimisation

BP training requires a good selection of values of several parameters, commonly through trial and error. Six parameters should not be set too low (small) or too high (large), and thus should be optimised or carefully selected. These parameters and their effect on both learning convergence and overall network performance, are listed in Table 1.3.

Table 1.3 The effect of extreme values of design parameters on training convergence and network generalisation.

Design parameter	***Too high or too large***	***Too low or too small***
Number of hidden nodes (NHN)	Over fitting ANN (no generalisation)	Under fitting (ANN unable to obtain the underlying rules embedded in the data)
Learning rate (η)	Unstable ANN (weights) that oscillates about the optimal solution	Slow training
Momentum coefficient (μ)	Reduces risk of local minima. Speeds up training. Increased risk of overshooting the solution (instability)	Suppresses effect of momentum leading to increased risk of potential entrapment in local minima. Slows training.
Number of training cycles	Good recalling ANN (i.e., ANN memorisation of data) and bad generalisation to untrained data	Produces ANN that is incapable of representing the data
Size of training subset (N_{TRN})	ANN with good recalling and generalisation	ANN unable to fully explain the problem. ANN with limited or bad generalisation
Size of test subset (N_{TST})	Ability to confirm ANN generalisation capability	Inadequate confirmation of ANN generalisation capability

(Adapted from Basheer and Hajmeer, 2000).

1.6 RATIONALE

Fish is an important part of the human diet, as it consists of various essential fatty acids required by the human body. The decline of fish resources is of a concern and this can have implications for the human diet. The concern is no longer only the quality of the fish that is consumed, but also the maturity of the fish and the impact on the nutritional value. After the fish has been harvested, it gets stored, transported and displayed at near freezing temperatures, that may only slow down and not eliminate oxidation of autochthonous fatty acids, or breakdown of fatty acids, when fish fillets are excessively contaminated. The focus of this study is on the leading cause of quality deterioration, namely lipid oxidation in specifically the Cape hake (*Merluccius capensis* and *Merluccius paradoxus*). Lipid oxidation produces a range of substances that influence taste and smell. It is, therefore, possible to use fatty acids and volatile components to determine fish freshness, storage life, quality and even safety (Josephson, 1991; Jay, 1992; Ross and Smith, 2006).

Prediction modelling has in the past been used to determine shelf life using chemical and/or analytical data. In this study both mathematical estimations, as well as artificial neural networks, are applied for prediction modelling of microbial contaminants, as well as the break in the cold chain. When taking this into consideration, in this study the following aims were formulated and investigated:

- Establishing triglycerides profiles in juvenile SA Cape hake (*M. capensis* and *M. paradoxus*) muscle tissue (Chapter 2)

- Mathematical predictions of temperature difference and microbial influences on fatty acids isolated from *Merluccius capensis* and *M. paradoxus* (Chapter 3)

- Mathematical predictions of temperature and microbial influences on selected volatiles in South African Cape hake (Chapter 4)

- Contamination predictions of Cape hake fillets during display and storage by artificial neural network modelling of Hexadecanoic acid (Chapter 5)

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CHAPTER 2

TRIGLYCERIDE PROFILES IN JUVENILE SA CAPE HAKE (*MERLUCCIUS CAPENSIS* AND *MERLUCCIUS PARADOXUS*) MUSCLE TISSUE

2.1 ABSTRACT

With the important social and economical value that Cape hake fishery contributes to South Africa and a decline in fish stocks, there is growing concern that more juvenile fish are being caught. Although the compositional and nutritional properties of the edible muscle of fish vary for various reasons, fish size, and therefore, its maturity is expected to have an influence on the distribution of triglycerides in fish. In this study the triglycerides of ten filleted juvenile fish (166±25 g) were extracted and analysed. Most of the fatty acid compounds were detected only in the larger of the juvenile fish. The three fatty acids mainly contributing to the total fatty acid composition, were palmitic acid (C16:0) (49.51 %), DHA (C22:6) (27.33 %) and stearic acid (C18:0) (10.01 %). Saturated fatty acid contribution to the total lipid content was found to be higher, in comparison to that reported in literature in other hake species and no EPA was detected. Furthermore, one of the fundamental fatty acids, namely eicosanoic acid, was detected only in the larger sized hake fillets, indicating reduced nutritional value in juvenile hake fillets. Size and age usually influence the total nutritional value of hake, as is also the case in other fish species. This highlights the potential future challenges of the fishing industry to not only control the sustainability of fish resources at a positive level, but also the size of the total allowable catch at an expected satisfactory nutritional level.

2.2 INTRODUCTION

South African Cape hake, also known as “stockfish”, consists mainly of two morphologically similar species, with the distribution of each species being depth dependent. The majority of the South African demersal total catch is harvested off the West Coast and is dominated by the shallow-water hake *Merluccius capensis* Castelnau (A), and deep-water hake *M. Paradoxus* Franca (B) (Fig. 2.1.) (Fairweather *et al*., 2006). In South Africa approximately one-third of the fresh and frozen catch is exported, and the same amount of the demersal catch reaches extensive shore-based facilities in a fresh or processed state (Hutton *et al.,* 1999).

The Cape hake fishery is a considerably social and economic asset to South Africa (Hutton and Sumaila, 2000), and the fishing industry has voiced serious concerns about the size and management of the catch. The total allowable catch (TAC) was reduced from 166 000 ton in 2001 to 130 532 ton in 2008, after a decline in stocks caused by extreme fishing pressure in the mid 1970s, when an estimated 1 115 000 ton of hake were caught (Hutton and Sumaila, 2000; Butterworth and Rademeyer, 2005; Department: Environmental Affairs and Tourism, Republic of South Africa (DEAT RSA), 2007; Von der Heyden *et al.*, 2007).

The supply of high quality fish with an acceptable storage life depends, amongst others, on continuous hygienic practices from harvest to plate (Huss, 1995). Hake is generally harvested at vast distances from processing facilities, necessitating preservation efforts immediately after fish are caught (Penfield and Campbell, 1990; Potter and Hotchkiss, 1997). Although live fish muscle is generally regarded as sterile, tissue deterioration commences the moment the fish dies. The principle agents of decay are the endogenous enzymes and contaminating bacteria (Frazier and Westhoff, 1988; Jay, 1992).

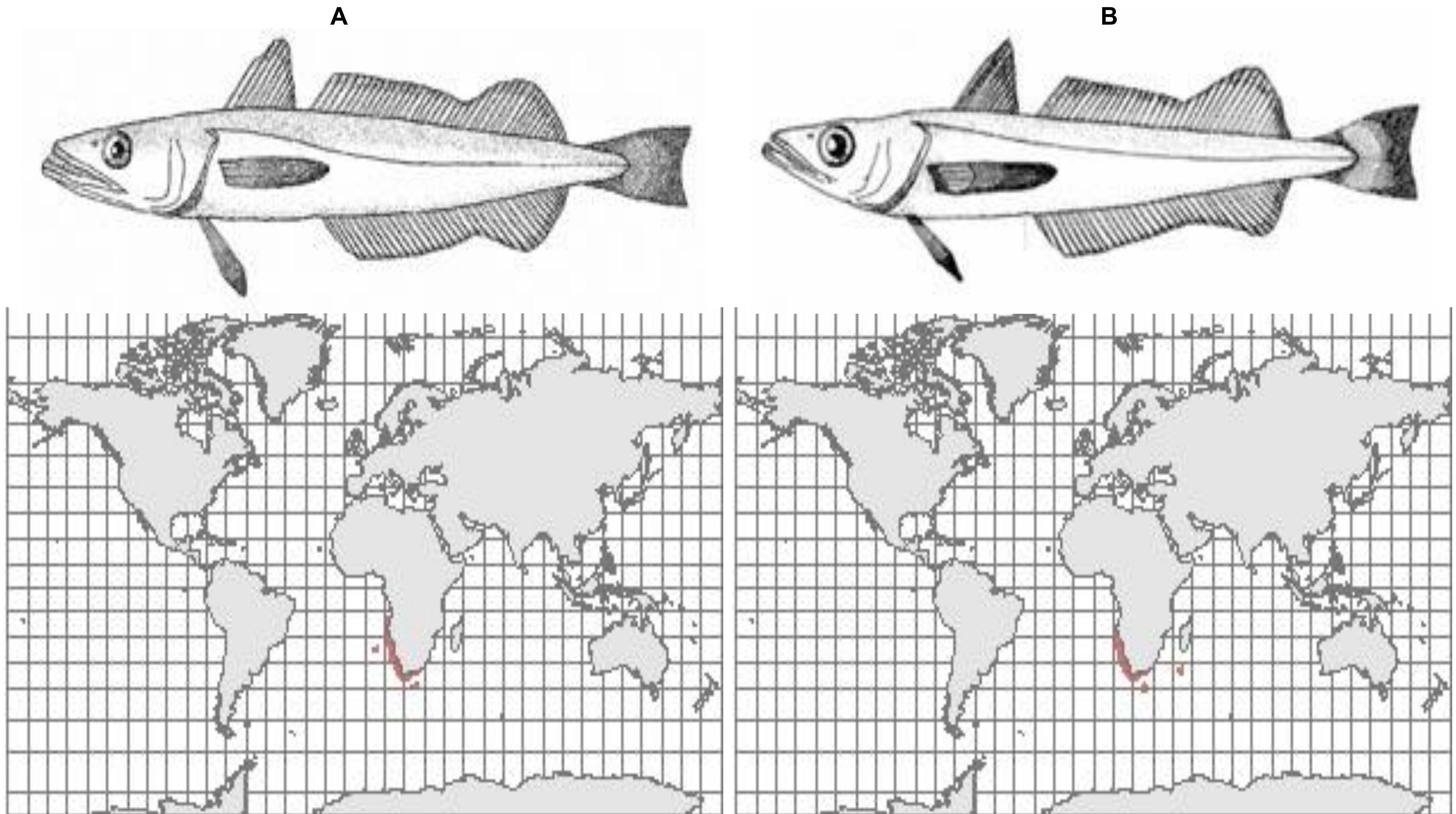


Figure 2.1 Geographical distribution maps of the South African shallow water hake (*Merluccius capensis*) (A) and the South African deep-water Cape hake (*Merluccius paradoxus*) (B) (Source: Inada, 1981).

During storage of fresh fish most of the macromolecules constituting the muscle tissue may suffer degradation and subsequently influence the organoleptic properties of the final product (Ackman, 1989; Aubourg, 1999; Baixas-Nogueras *et al*., 2002). Compared to most foods, fish boasts relative high levels of polyunsaturated fatty acids (PUFAs) in their muscle tissue, which form part of the macromolecules that degrade over time. In South Africa a large portion of marine products necessitates considerable transportation from coastal areas to inland markets, prolonging the time that the muscle associated lipids are exposed to intrinsic and extrinsic parameters, that could influence the tempo of decay (Hutton and Sumaila, 2000). Consequently, maintaining the cold chain and related expenditures in attempts to keep the product sensorally acceptable to the consumer, present a major challenge to the industry.

It is known that marine lipids are characterised by a content of longer chain (up to C24) fatty acids (Marini, 1992). One group of lipids present in fish muscle, namely the ω3 PUFAs is considered –essential” (required but not synthesised) and is acquired through the diet (Batista *et al*., 2001; Kiessling *et al.,* 2005; Huynh and Kitts, 2009; Rubio-Rodríguez *et al.,* 2010). Most culture groups in the world consume fish in some way or another and subsequently nourish their bodies with ω3 PUFAs (Hsieh and Kinsella, 1986). These are known precursors of metabolic products that play an important role in blood clotting, immune response and vascular tone, and include eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Das, 2000; Maki *et al*., 2003; Kiessling *et al.,* 2005).

In fish, fatty acids predominantly arise from synthesis *de novo* via non-lipid carbon sources within the animal, or directly from dietary lipid (Henderson, 1996). Lipid digestion, absorption and transport in fish are generally similar to that in mammals as described by Tocher (2003) in a comprehensive review of the metabolism of lipids. Numerous studies have also been published describing fish lipids and fatty acids, with special emphasis on the ω3 PUFAs of many fish species, including different species of hake (Wessels and Spark, 1973; Méndez

and González, 1997; Huynh and Kitts, 2009; Pacetti *et al*., 2010). Despite these studies there is limited information available on particularly the short and medium chain length fatty acids that form part of the total lipids of hake muscle tissue.

The aim of this study was to elucidate fatty acid metabolic pathways present in the muscle tissue of juvenile Cape hake, the influence of fish size on the distribution of selected fatty acids, and to compare these to relevant literature on similar species, in order to highlight possible changes in the nutritional value of Cape hake. The study should contribute to the knowledge base of triglycerides in juvenile fish and ultimately add to the sustainability of South African fish resources.

2.3 MATERIALS AND METHODS

2.3.1 Sample preparation

Both species *Merluccius capensis* and *Merluccius paradoxus* have similar morphological characteristics and are not distinguished commercially and as such, are sold under the generic name of ―hake". For purposes of this study samples from these species were, therefore, not separated (Herrero *et al.,* 2003). From a study conducted by Méndez and González (1997) it was evident that *M. paradoxus* muscle contained higher quantities of lipids during the month of February, when compared to other months. In the current study the hake was similarly harvested during the month of February from the South African shoreline close to the city of Cape Town. The fish were mechanically beheaded, gutted and kept on ice (average muscle temperature 7±0.5 ºC) for 24 hours prior to laboratory analysis. Ten fish samples, with an average weight of 166±25 g and length of 21±2 cm (beheaded) were randomly selected from the entire catch. Each fish sample was filleted and five 1 cm^3 sections of fish tissue were removed aseptically from different areas of the fillet.

2.3.2 Fatty acid extraction

Muscle cuts from 2.3.1 were homogenised, using mortar and pestle and 0.7 g of the homogenate was subjected to total lipid extraction, as described by Folsch (1957) using chloroform:methanol 2:1 (v/v) (Folsch *et al.,* 1957). All reagents, solvents and standards were of analytical grade (Merck, Midrand RSA and Separations, RSA), and stored in dark bottles.

2.3.3 Fatty acid analysis

Trans-esterification of the fish lipids was done by addition of trimethylsulphonium hydroxide (TMSOH, Merck, Midrand RSA) according to the method of Butte (1983). The fatty acid methyl esters (FAME) were analysed and separated on a Finnegan Focus GC equipped with a 30 m x 0.25 mm ZB-1 (Separations, RSA) glass capillary column. The column contained 100 % dimethyl polysiloxane (0.25 µm) with helium as carrier gas (constant flow – 3.0 ml. min^{-1}) and functioned in a splitless mode of injection. The temperature programme is summarised as follows: 40 ºC to 90 ºC at a rate of 8 ºC min^{-1}, followed by a ramp from 90 ºC to 280 ºC at 10 ºC min^{-1}. The column was attached to a Finnegan Focus DSQ mass spectrometer for mass detection of fragments with *m/z* smaller than 1000. Mass analysis was performed at eV with an ion source temperature of 200 ºC. Integration of the peaks was performed on the TIC (Total Ion Chromatogram), using Xcalibur software version 1.4 SR1 (Finnegan).

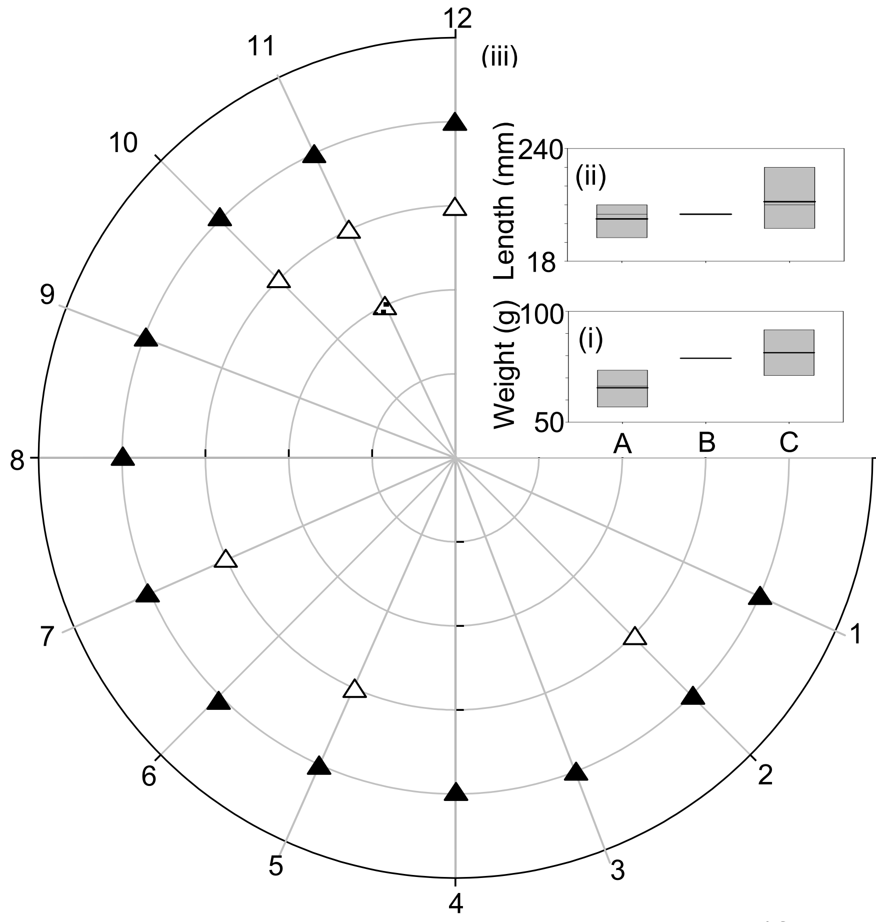
2.3.4 Data analysis

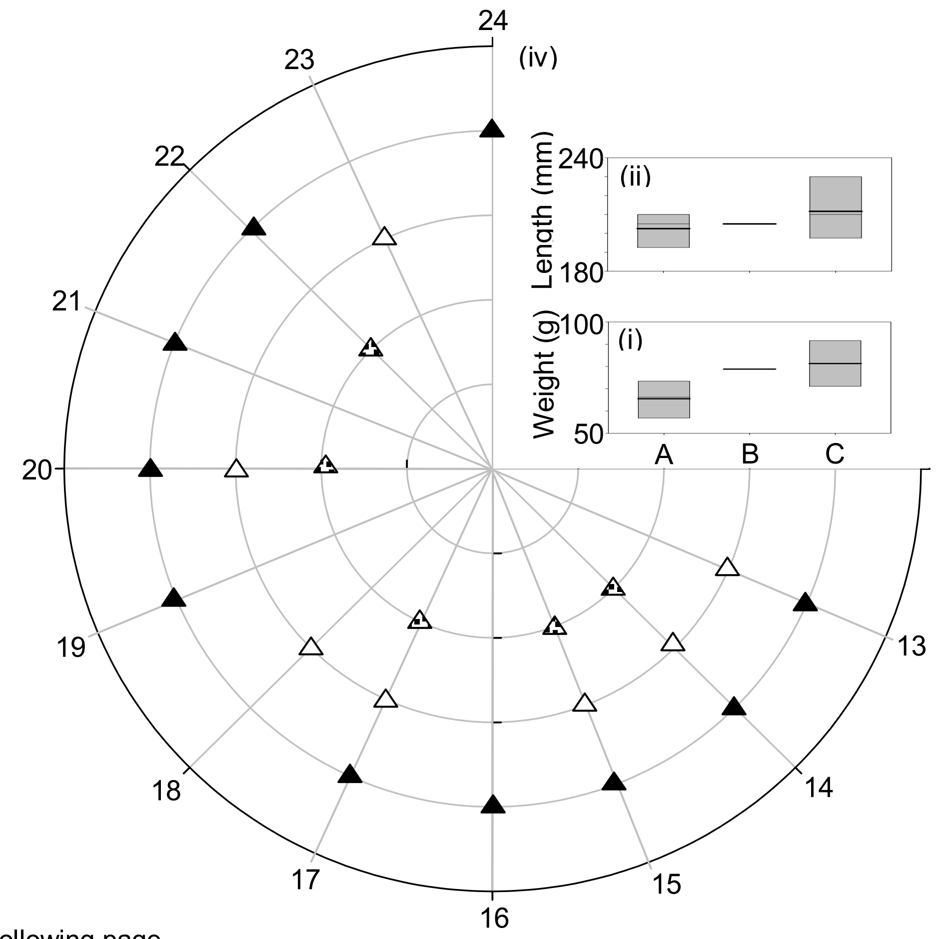
Every analysis was conducted at least in triplicate and the results are presented as the means.

2.4 RESULTS AND DISCUSSION

The hake fillets were divided into three categories A: small (60 – 70 g); B: medium (70 – 80 g) and C: large (80+ g), using the average weight of each fish's fillets. Sections i and ii (Fig. 2.2) indicate the average weight and length of the fish fillets. From these a strong positive correlation (0.77) between the average length, and the weight of the fish fillets emanated. The muscle tissue which consists primarily of moisture, proteins and fat, was the main contributor to the weight of the samples analysed.

Specific fatty acids were selected from the total extracted lipids based on a) their significance as reported in literature; b) their reported importance in biochemical and physiological processes; c) their possible relationships with quality, age, type and other variables in the fish tissue and; d) their links with environmental contaminants. The selected fatty acids are listed in Figure 2.2. Sections iii and iv (Fig. 2.2) indicate the absence/presence of the selected FAME found per category. The fatty acids are sequentially presented from low to higher carbon containing fatty acids, except for the glycine, cholesterol, 2-butanone, 3-hydroxy- and 2(3H)-furanone, dihydro-4-hydroxy. It is noteworthy that no compounds occurred only in the small-sized fish fillets (category A). When a component was present in category A, it also appeared in either the medium-sized (category B) or large-sized fillets (category C) or both B and C. A possible reason for this may be the fact that as the muscle weight increased, for example, as a result of a diet higher in lipids, the fat content (fatty acids) of the muscle also increased (Henderson, 1996; Kiessling *et al*., 2005).





*Caption on following page

Figure 2.2 Distribution of selected fatty acids in the different categories of fish. Section i represents the weight and ii indicates the Length of the fillets while iii and iv represents the absence/presence of the selected FAME (1- 24): A-Small, B-Medium and C-Large fish fillets. These compounds include 1.butanoic acid, 2-methyl-; 2. pentanoic acid, 3-methyl-, methyl ester; 3. hexanoic acid, methyl ester; 4. heptanoic acid, methyl ester; 5. 2-methylheptanoic acid; 6. octanoic acid, methyl ester; 7. nonanoic acid, methyl ester; 8. decanoic acid, methyl ester; 9. 9-dodecenoic acid, methyl ester, (E)-; 10. tridecanoic acid, 4,8,12-trimethyl-, methyl ester; 11. methyl tetradecanoate, 12. tetradecanoic acid, 10,13-dimethyl-, methyl ester; 13. pentadecanoic acid, methyl ester; 14. hexadecanoic acid, methyl ester; 15. 9-hexadecenoic acid, methyl ester, (Z)-; 16. heptadecanoic acid, methyl ester; 17. octadecanoic acid, methyl ester; 18. 9,15-octadecadienoic acid, methyl ester, (Z,Z)-; 19. eicosanoic acid, methyl ester; 20. 4,7,10,13,16,19-docosahexaenoic acid, methyl ester, (all-Z)-; 21. glycine; 22. cholesterol; 23. 2-butanone 3-hydroxy-, and 24. 2(3H)-furanone, dihydro-4-hydroxy.

2.4.1 Saturated Fatty Acids

Based on the structures of the short-chain fatty acids (component 1, butanoic acid, 2-methyl-, component 2, pentanoic acid, 3-methyl-, methyl ester, component 3, hexanoic acid, methyl ester, component 21 glycine and component 23 2-butanone 3-hydroxy in Fig. 2.2), as well as the medium-chain length fatty acids (component 4, heptanoic acid, methyl ester, component 5, 2-methylheptanoic acid, component 6, octanoic acid, methyl ester, component 7, nonanoic acid, methyl ester, component 8, decanoic acid, methyl ester in Fig. 2.2), each could be classified as saturated fatty acids. Although short-chain fatty acids are an important nutritive source to fish, limited information is available on the lipid transport mechanisms in fish intestines (Titus and Ahearn, 1988). Short-chain fatty acids are known products of anaerobic microbial fermentation in the hindgut of terrestrial vertebrates and are used as a blood fuel, either for energy purposes, or for lipid synthesis (Fig. 2.3) (Clements, Gleeson and Slaytor, 1994). In Figure 2.2 the short- and medium-chain compounds (C4-C10) were found to occur in all the large fillets and in none of the small fillets. The exception was components 2 (pentanoic acid, 3-methyl-, methyl ester), 5 (2-methylheptanoic acid) and 7 (nonanoic acid, methyl ester) that also occurred in the medium fillets, but not in the small fillets. The short-chain fatty acid that contributed most to the total selected fatty acid composition is component 3 (hexanoic acid), at 0.51 %. The medium chain fatty acid that contributed the most was found to be component 7, nonanoic acid at 0.66 %.

Component 21, glycine, also known as aminoacetic acid or G salt, occurred in category B (the medium sized fish fillets) as seen in Figure 2.2, and the total concentration of this component was 0.6 % of the total fatty acids. Glycine plays multiple biochemical roles, one being a precursor of acetyl and the other being bound to coenzyme A to form acetyl-CoA. Acetyl CoA is central to both lipid and carbohydrate metabolism (Fig. 2.3) (Mathews and Van Holde, 1990). Similar to glycine component 23 (2-butanone, 3-hydroxy) was only present in the medium sized fish fillets (category B of Fig. 2.2), with a total concentration of 0.21 %.

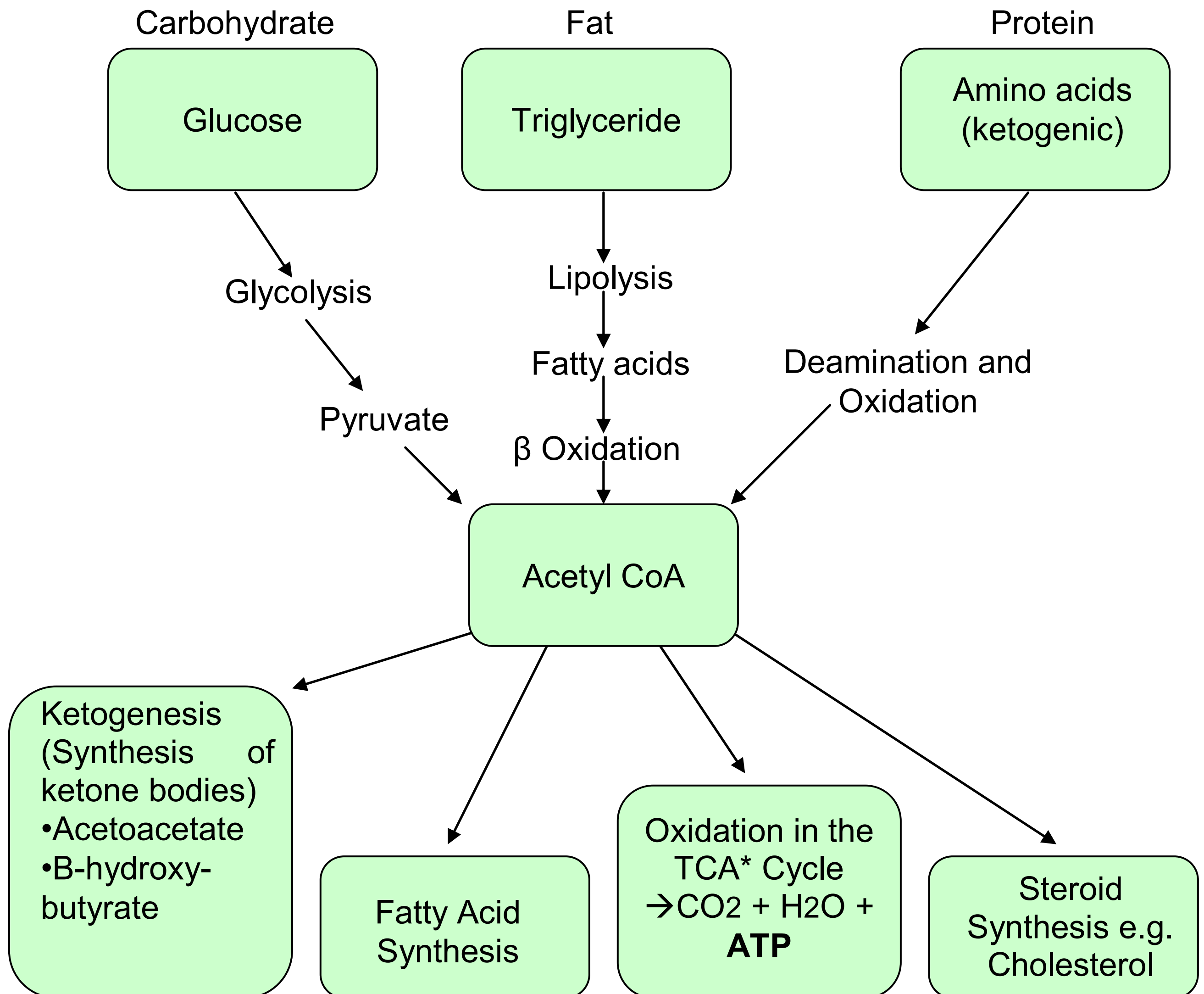


Figure 2.3 Acetyl CoA plays a central role in metabolism. An illustration of the main pathways that produce and utilise acetyl CoA (adapted from Roach and Benyon, 2003).* Tricarboxylic Acid Cycle.

2-Butanone, also known as acetoin, is an unusual, but important physiological metabolite and has been reported to be formed either through the catabolic 2,3-butanediol-formation route going through α-acetolactic acid from pyruvic acid in certain bacteria, or via the 2,3-butanediol cycle. Although not analysed, this may indicate that contamination by acetoin producing bacteria such as *Bacillus cereus*, *Bacillus subtilis* or *Micrococcus urea,* all which have been confirmed to utilise the 2,3-butanediol cycle (Ui *et al*., 2000). The saturated fatty acids identified during this study most probably resulted from either acetyl-CoA carboxylase (Fig. 2.3), or fatty acid synthetase (Henderson, 1996).

All even-numbered fatty acids (short-, medium- and long-chain) identified in this study, can be converted to acetyl-CoA (Fig. 2.3) through the β-oxidation pathway. The uneven carbon chains and branched chain molecules are unusual and require a different degradation pathway (α-oxidation). β-Oxidation of uneven fatty acids usually proceeds normally, until the last β-oxidation cycle, which yields one acetyl-CoA molecule and one propionyl-CoA. The latter is converted to succinyl-CoA and derives a substantial amount of energy, when fed into the citric acid cycle (Krebs cycle), to generate energy in the form of adenosine triphosphate (ATP) that can be used in energy requiring reactions (Mathews and Van Holde, 1990).

Component 10 (4,8,12-trimethyltridecanoic acid), also known as an isoprenoid fatty acid, only occurred in category C (the larger sized juvenile fillets). The total concentration of this component was 0.35 % of the total fatty acids. This component has chemotaxonomical significance for both marine and freshwater sponges and has shown to be derived from phytol, which in turn, is derived from chlorophyll (Dembitsky, Rezanka and Srebnik, 2003) and indicate the possibility of this component to be from a dietary source. Another fatty acid known to originate from the diet of fish, is myristic acid (component 11). Previous studies reporting on the fatty acids found in hake species, reported exclusively on fatty acids from medium-chain length of 14 carbons and higher. Referring to section iii and iv (Fig. 2.2), component 11 (myristic acid/methyl tetradecanoate C14:0) is

the first component reported to occur in all categories A, B and C. Occurrence of this component was slightly higher (2.48 %) than previously reported (Table 2.1). Myristic acid can originate, not only from the diet of fish, but can also be biosynthesised *de novo* by the fatty acid synthase pathway that produces predominantly palmitic acid (component 14) and minor amounts of myristic acid (Tocher, 2003).

Component 12, tetradecanoic acid (C14) occurred in both categories B (medium sized) and C (large sized fish fillets). The total concentration of this component was 0.23 % of the total fatty acids. Although limited information is available regarding this dimethyl branched fatty acid, it appears to have similar characteristics as component 10 (4,8,12-trimethyltridecanoic acid) and could possibly even be a precursor of component 10. Sponges contain large quantities of C14 up to C30 fatty acids, with branched, as well as odd chains. Tetradecanoic acid, although also a branched chain fatty acid, has not been reported in sponges, as has component 10 (4,8,12-trimethyltridecanoic acid). However, none of the previously mentioned studies on hake has referred to this component.

Branched chain fatty acids indicate the presence of the α-oxidation pathway that could result in the formation of tetradecanoic acid. Such branched fatty acid chains could also implicate bacterial activity (Carballeira *et al*., 1989; Dembitsky *et al*., 2003). It has been suggested that branched-chain fatty acids follow the α-oxidation pathway (Mathews and Van Holde, 1990). Another component indicating the α-oxidation pathway as the route of synthesis with its uneven carbon chain, is the pentadecanoic acid (component 13). This component occurred both in category B and C. Although pentadecanoic acid has previously been reported in hake, the total concentration of this component was found at a low total concentration of 1 % (Wessels and Spark, 1973; Pacetti *et al*., 2010).

Table 2.1 Comparison of the Fatty acid (wt %) of various hake species

Components as per Figure 2.2	Selected fatty acids	Cape hake (2010)[f]	Pacific hake (2009)[a]	European hake (2010)[b]	Cape hake (1973)[c]	Cape hake (1973)[d]	Atlantic hake (1979)[e]
11	C14:0	2.48	1.06	2	1.1	1.74	2.8
12	C14:0	0.23	-	-	-	-	-
13	C15:0	1.00	-	0.6	0.11	0.17	-
14	C16:0	49.51	21.7	18.3	23.28	23.57	18
15	C16:1 (9)	1.8	0.62	2.9	4.67	4.95	5.2
16	C17:0	0.92	1.08	0.8	0.43	0.68	-
17	C18:0	10.01	7.18	6.1	3.78	4.36	3.2
	C18:2 (9,12)	-	1.18	1.3	0.57	0.66	-
18	C18:2 (9,15)	0.7	-	-	-	-	-
19	C20:0	0.48	-	0.5	0.1	0.3	-
20	C22:6 (4,7,10,13, 16,19)	27.33	22.08	35	27.81	24.01	25.7

[a]Fillet of *Merluccius productus (Huynh and Kitts, 2009),* [b]Fillet of *Merluccius merluccius (Pacetti et al., 2010) ,*[c]*Merluccius paradoxus* (Wessels and Spark, 1973), [d]*Merluccius capensis* (Wessels and Spark, 1973), [e]*Merluccius hubbsi (Méndez and González, 1997),* [f]Fillet of Cape hake Cm:n (x); m=number of carbon atoms, n=number of double bonds, x=position of double bond(s).

The correct terminology for the biosynthetic reactions for the formation of new endogenous lipid, is lipogenesis (Tocher, 2003). Acetyl-CoA is the carbon source for the biosynthesis of new lipids (either from the decarboxylation of pyruvate or the oxidative degradation of various amino acids). The key pathway in lipogenesis has previously been characterised in fish and is catalysed by the cytosolic fatty acid synthetase multienzyme complex. The main products of fatty acid synthetase are palmitic acid (hexadecanoic acid and component 14; C16:0) and stearic acid (octadecanoic acid and component 17; C18:0). Palmitic acid is possibly the most common fatty acid found in nature. This acid contributes to almost half (49.5 %) of the total fatty acid concentration found in this study, and was found in categories A, B and C. In other studies done on hake, palmitic acid has also been found to be present at high concentrations (Wessels and Spark, 1973; Méndez and González, 1997; Huynh and Kitts, 2009; Pacetti *et al*., 2010).

The prevalence of octadecanoic acid (component 17), was higher (10.01 %) than previously reported (Table 2.1) and also occurred in all categories (small, medium and large sized fillets). Octadecanoic acid has been identified to occur at high concentrations in lean fish muscle, such as hake and is a known product of fatty acid synthetase (Tocher, 2003; Huynh and Kitts, 2009). Low amounts of arachidic acid have previously been detected, together with significant amounts of palmitic acid and stearic acid in phosphoglycerides, which constitute animal cell membranes (Tocher, 2003). Arachidic acid (C20:0), also known as eicosanoic acid (component 19), was also reportedly found in European hake and Pacetti *et al.* (2010) reported similar results of approximately 0.5 % of the total fatty acids.

2.4.2 Unsaturated Fatty Acids

It is important to note that, similar to humans, a marine fish‘s dietary intake determines the composition of unsaturated fatty acid in muscle tissue, since the essential polyunsaturated fatty acids are not synthesised *de novo* in teleost fish (Henderson, 1996; Tocher, 2003). Marine fish require the essential long-chain

unsaturated fatty acids EPA and DHA from its diet for optimal growth (Tocher and Ghioni, 1999).

9-Dodecenoic acid (C12:1) (component 9) is a mono-unsaturated fatty acid that only occurred in category C, which comprises the large sized fillets. The total concentration of this component constituted 0.27 % of the total fatty acids. Although previous investigations implicated cytochrome P450 mono-oxegenases pathway (predominant Phase I oxidation enzymes in vertebrates), relatively little is known regarding its function and regulation within aquatic organisms, such as fish. Cytochrome P450 mono-oxegenases enzymes are important in the bioactivation of certain toxins, as well as the oxidation of various fatty acids, such as arachidonate and lauric acid (Mosadeghi *et al.,* 2007). The occurrence of component 9 (9-dodecenoic acid) possibly indicates the exposure of hake to environmental chemical contaminants, for example, specific chlorinated herbicides, chlorinated solvents, constituents of bleached kraft mill effluent, perfluorinated acids and phtahalate esters (also collectively referred to as peroxisome proliferating agents). Environmental chemical contaminants are known to affect lipid metabolism in mammals by perturbation of fatty acid metabolism pathways, to adversely affect reproduction and development, and also to cause hepatocellular carcinogenesis after long-term exposures (Haasch, Henderson and Buhler*,* 1998).

Another mono-unsaturated fatty acid, 9-hexadecenoic acid (C16:1) (component 15) is known to occur in fish. Compared to other studies done on hake, less palmitoleic acid was detected than previously documented (1.8 % vs. 4.67 %) as seen in Table 2.1. Fish are capable, like most organisms, of desaturating certain saturated fatty acids, for example, C16:0 (palmitic acid) to C16:1 (palmitoleic acid) *via* an aerobic process, utilising CoA-linked substrates and requiring Nicotinamide-adenine dinucleotide phosphate (NADPH) and oxygen (Tocher, 2003).

In section iv (Fig. 2.2), component 18 (9,15-octadecadienoic acid a C18:2 polyunsaturated fatty acid), also known as mangiferic acid (first isolated from mango), was detected in category B (the medium sized fish fillets). In this study, aproximately 0.7% of the total fatty acids were from 9,15-octadecadienoic acid. This fatty acid was previously not recognised as a typical C18:2 fatty acid present in fish lipids, such as linoleic acid (9,12-octadecadienoic acid). However, 9,15-octadecadienoic acid has also been detected in a microalgae (*Scenedesmus obliquus*). Most of the PUFAs present in fish tissue are the result of the consumption of marine microalgae, which are considered to be the primary producers (Rasoul-Amini *et al*., 2009). 9,15-Octadecadienoic acid is a ω3 PUFA that may form part of the ω6 PUFAs pathways, which include linoleic and linolenic acid. 9,15-Octadecadienoic acid is also listed in this study to indicate that, although present in small quantities, interconversion and uncommon alteration of unsaturation of C18:2 ω6 do occur.

Component 20 (4,7,10,13,16,19-docosahexaenoic acid, (C22:6ω3)) is also known as DHA. On its own it contributes to almost one third of the total fatty acids (27.33 %) isolated from the fish tissue, and occurred in all categories. According to previous studies DHA in lean fish muscle, such as hake, are proportionally much higher than EPA (Huynh and Kitts, 2009). It is important to note that neither DHA nor EPA can be synthesised in fish. It is hypothesised that fish lipid depots mainly originate from the phytoplankton and zooplankton at the base of the marine food web (Tocher, 2003; Rasoul-Amini *et al*., 2009).

Cholesterol (component 22) is a well known 27 carbon simple lipid (not containing fatty acids), which originates from a two-carbon precursor, namely acetate, and is present in all animals and humans. Cholesterol was not detected in the medium sized fillets (category B), and the total concentration of this component constituted 0.75 % of the total fatty acids. Although cholesterol and other steroids cannot be degraded to smaller molecules, it is degraded primarily by conversion to bile salts, which facilitates the emulsification and absorption of dietary fat. Small amounts of cholesterol are used to synthesise powerful steroid

hormones, as indicated in Figure 2.3 (Mathews and Van Holde, 1990). Similarly, cholesterol in fish is obtained by dietary intake and is released from intracellular stores, or by *de novo* synthesis, and acts as a precursor to all steroid hormones (Sharpe *et al.,* 2006). However, it is believed that the triglyceride content is elevated in fish at the expense of cholesterol esters in teleost lipoproteins, and that fish has a higher concentration of circulating cholesterol. In contrast, the traditional detrimental effects associated with the high plasma cholesterol in humans, are absent in fish.

2(3H)-Furanone, dihydro-4-hydroxy (compound 24) was only present in category C and contributed to 0.36 % of the total fatty acids. 2-Furanone, also known as furan-2-one is a heterocyclic compound and a common component of natural products, synthesised through biological pathways in organisms (Alam *et al*., 2009). Furan fatty acids, previously detected in European hake, are 12,15-epoxy-13-methyleicosa-12,14-dienoic,12,15-epoxy-13,14-dimethyloctadeca-2,14 -dienoic and 12,15-epoxy-13,14-dimethyleicosa-12,14-dienoic. In this study only a single furanone component was detected. Studies have suggested that although no specific pathways connect a furan fatty acid and a furanone fatty acid, both are implicated in potential biological activities (Alam *et al*., 2009; Pacetti *et al.,* 2010). 2 (3H)-Furanone and its analogues have anti-inflammatory, as well as analgesic activity with lesser GI toxicity and reduced lipid peroxidation.

2.5 CONCLUSION

In marine teleost fish, including hake, the *de novo* synthesising and the catabolism pathways of dietary polyunsaturated fatty acids have previously been established. Most of the fatty acid compounds reported in this study were detected in the larger fish fillets. The three fatty acids that most contributed to the total fatty acid composition were palmitic acid (C16:0) (49.51 %), DHA (C22:6) (27.33 %) and stearic acid (C18:0) (10.01 %). The contribution of saturated fatty acids to the total lipid content was higher in comparison to studies

on other hake species, and no EPA was detected. Furthermore, one of the well-known fatty acids, namely eicosanoic acid, methyl ester (component nr 19), only occurred in the larger sized hake fillets. This indicates a reduced nutritional value of juvenile hake fillets, when compared to mature hake fillets (>500 g as per previous studies) harvested for retail stores. This study confirms that, concerning the fishing industry, the size of the catch matters in more than one way. The size and age can influence the total nutritional value of hake and most probably, in other fish species as well. Future challenges of the fishing industry, therefore, include effective management of sustainable fish resources, as well as maintaining the size of the total allowable catch at an expected satisfactory nutritional level, and preventing the fish resources from exposure to environmental contaminants.

2.6 ACKNOWLEGDEMENTS

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CHAPTER 3

MATHEMATICAL PREDICTIONS OF TEMPERATURE DIFFERENCE AND MICROBIAL INFLUENCES ON FATTY ACIDS FROM *MERLUCCIUS CAPENSIS* AND *MERLUCCIUS PARADOXUS*

3.1 ABSTRACT

This study was aimed at developing a mathematical model that would utilise selected fatty acid fluctuations in Cape hake fillets during various storage conditions, to determine the fillets' shelf life. The specific application of the mathematical equation is to determine whether the fillet has been exposed to excessive microbial contamination, and whether the degree of temperature fluctuated. The standard, as well as worse-case scenarios, regarding fish display in retail deli shops were simulated. Five randomly selected fishes were filleted, and one fillet of each fish was used for the shelf life study at a controlled 8 ºC on ice. The remaining set of five fillets was kept at the same temperature, but was inoculated with an increased load of autochthonous microbiota, found on hake (5.84×10^8 cfu.ml^{-1}). Similarly, the second randomly selected five Cape hakes were filleted, and used for the shelf life study at ambient temperature (25ºC) on ice. Fatty acid analysis was performed at 48 hour intervals, with a GC-MS for a total of 10 days. In conclusion, the combined equations might have potential in the retail industry to determine the hake (or similar lean fish) fillet's remaining shelf life, in terms of microbial spoilage and the integrity of the cold chain.

3.2 INTRODUCTION

In recent years the application of chemometric classification procedures, for example, pattern recognition techniques in food chemistry, has increased. Various literature studies are available in which several classes of chemical variables and sensory properties are considered. Different statistical tools are implemented in the classification of product brands and the quality of food origin, such as honey, olive oils, wines, milk, and also the differentiation of fish species (López *et al*., 1995). In addition to this, several mathematical models have been utilised, in concurrence with sensorial, microbial and chemical changes to determine the quality, or remaining shelf life (freshness) of food, which include fish (Barassi, *et al*., 1987; Baixas-Nogueras, *et al.,* 2003; Lougovois, Kyranas and Kyrana, 2003). In recent years, the retail industry is being audited to sell not just good, but consistent quality food products. This is to ensure food safety, to protect the product brand, and to comply with local and international food standards.

The Cape hake fishery, dealing mainly in the shallow-water hake *Merluccius capensis* Castelnau and deep-water hake *Merluccius paradoxus* Franca, is of considerable social and economic value to South Africa (Hutton and Sumaila, 2000; Fairweather *et al*., 2006). The local white fish industry (mostly fish with edible white muscle that includes hake) has developed an extensive international market with high value products, quality improvement, and value adding processed hake products (Hutton *et al*., 1999). In South Africa, as in many other countries, fish and marine products are caught at great distances from processing facilities, and are exposed to transportation across considerable distances from coastal areas to inland markets. Post-harvest biochemical and microbial changes in fish tissue depend on the concentration of substrates and metabolites in the live fish tissue, the activity of endogenous enzymes, microbial contamination, and also the post-catch storage and handling conditions (Frazier and Westhoff, 1988; Pacheco-Aguilar *et al*., 2000). A substantial increase in primary, secondary and tertiary lipid oxidation products have further been

reported in fish stored on ice (Undeland *et al*., 1999). Moreover, enzymatic and non-enzymatic lipid oxidation occurs during processing and storage and could directly influence the quality and freshness of fish (Aubourg, 1999).

Fish products are known to be highly perishable and freshness determines the shelf life of both raw and frozen fish, and is also essential for good quality fish and fish products (Ólafsdóttir *et al* 1997; Pacheco-Aguilar *et al.*, 2000). The Quality Index Method (QIM) has been developed for various species of raw fish (including Mediterranean hake, as well as for frozen Cape hake) and is a non-destructive tool for routine fish freshness evaluation. It is based on detailed descriptions grouped into distinct characteristics within general attributes, such as appearance, eyes, and gills. However, this type of analysis is inherently subjective, even when using a trained panel. Chemical methods have, therefore, been developed to determine fish freshness scientifically (Baixas-Nogueras *et al.,* 2003; Herrero *et al.*, 2003). Fatty acid metabolites have been regarded as good indicators of the freshness of fish and it is possible to use it as a parameter in chemometric classification, to determine fish quality (Baixas-Nogueras, *et al.,* 2003).

The aim of this study was to qualify the changes in selected fatty acid metabolites in Cape hake fillets over a period of ten days, under various storage conditions on ice, and to assess the potential influence on freshness and quality. These selected fatty acid metabolites were used as chemometric markers to predict the fillet freshness.

3.3 MATERIALS AND METHODS

Cape hake (*M. capensis* and *M. paradoxus* having similar morphological characteristics, and often caught together) were harvested during the month of February from the South African shoreline, close to the city of Cape Town. *M. paradoxus* muscle contains relatively higher quantities of lipids during the month of February, in comparison to other months (Méndez and González 1996).

3.3.1 Sample preparation

Mechanically beheaded and gutted fish were kept on ice (average muscle temperature 7±0.5 ºC) for 24 hours prior to laboratory analysis. The ten randomly selected hakes weighed 166±25 g, and had an average length of 21±2 cm (beheaded). The first group of five fishes were filleted and one fillet from each fish was used for the shelf life study at 8 ºC on ice – hereafter referred to as “C8” or “control”. The other set of five fillets were kept at the same temperature, but were inoculated with an increased load of autochthonous microbiota (5.84 x 10^8 cfu.ml^{-1}). This microbiota were found and enumerated from a purchased hake sample that was displayed on ice in a local store. These set of five fillets are hereafter referred to as “I8” or “inoculated”.

Both N8 and I8 simulate fillets which are displayed on ice, corresponding to retail stores that use display refrigerators with the outside temperature kept at 8 ºC. The second group of five Cape hake samples were filleted and used for the shelf life study, at ambient temperature conditions (25 ºC) on ice - hereafter referred to as “C25” and “I25”. This scenario simulated fillets displayed on ice in an unmonitored environment, corresponding with retail stores, where fish are displayed in the open. Fish fillets and whole fish are usually displayed on ice in shops during the day and refrigerated during night time. The standard duration of fish displayed on ice by major retail stores in South Africa, is seven days. According to the study done on Mediterranean hake the fish should be rejected after ten days of storage (Baixas-Nogueras *et al.*, 2003).

The second series of lipid extractions of the inoculated fillets were done 24 hours after the Cape hake samples were inoculated. The first samples were tested at the time when the fish arrived (T0), and both the –control" and –inoculated" samples shared the T0 results. The fish fillet samples were kept on ice throughout the duration of the study (ten days), and in the freezer between 8 pm and 8 am. These parameters were chosen to simulate the standard, as well as worse case scenarios on how many fish are displayed and sold in retail deli shops. Ice was replaced with fresh ice at 8 am and at 8 pm every day for all fillets, thus causing super chilling during night time when the fresh ice was under the fish, while the fish was refrigerated undisturbed through the night. Super chilling (or partial freezing) is known to extend the shelf life of fish products (Huss, 1995).

Every 48 hours, five 1 cm^3 sections of muscle tissue were removed aseptically from different areas of each fillet. Subsequent sample processing and lipid extraction were done, avoiding any physical, chemical or microbiological contamination.

3.3.2 Fatty acid extraction and analysis

For detailed methodology used for fatty acid extraction and analysis, refer to section 2.3.2 and 2.3.3.

3.3.3 Data analysis

Results are presented as the means of five repetitions, using the total signal (refer to Appendices I, II, III & IV). To determine the temperature percentage difference (TPD), each data point was calculated as follows for either the inoculated, or the control samples at each time interval:

$$TPDa = \left(\frac{\bar{x}c(t)8\,^{\circ}\mathrm{C}}{\sum d} \right) \times 100) - \left(\frac{\bar{x}c(t)25\,^{\circ}\mathrm{C}}{\sum d} \right) \times 100 \quad (1)$$

Where:

TPD	=	Temperature Percentage Difference
a	=	storage treatment (C or I)
$\bar{x}$	=	the mean of five samples
c	=	of each component
t	=	each time interval, either 0,2,4,6,8,10
d	=	the sum of all components

Similarly, to determine the Microbial Influence Percentage Difference (MIPD), each data point was calculated as follow for either the 8 ºC, or the 25 ºC samples for each time interval:

$$MIPDe = \left(\frac{\bar{x}c(t)C}{\sum d} \right) \times 100) - \left(\frac{\bar{x}c(t)I}{\sum d} \right) \times 100 \quad (2)$$

Where:

MIPD	=	Microbial Influence Percentage Difference
e	=	Temperature (8 ºC or the 25 ºC)
$\bar{x}$	=	the mean of five samples
c	=	of each component
t	=	for each time interval, b is either 0,2,4,6,8,10
C	=	Control
I	=	Inoculated
d	=	sum of all components

Polynomial curve fits were done for each individual component to the order of maximum 4 (also known as a quartic equation), whereas R^2 was calculated with Microsoft Office Excel 2003.

3.4 RESULTS AND DISCUSSION

Changes were observed in all the selected fatty acid metabolites over the ten days sampling period. There were notable differences amongst the samples with the selected fatty acid metabolite treatments, between the two environmental storage temperatures, and when fish fillets were exposed to increased microbial loads. As expected, both anabolic and catabolic reactions occurred during the storage period. Catabolic reactions could either contribute where a component decreased, because of the breakdown of the component, or because the component is used as a building block in a different pathway. In some components, more than one metabolic pathway played a role, indicating the array of metabolic reactions that may be encountered either, enzymatic, microbial or oxidative. Although the specific reactions, as well as the reasons for the changes in the fatty acid metabolites during the duration of sampling were not analysed, it is likely that such reactions are inherent metabolic reactions that could influence all raw filleted fish under similar conditions.

For the purpose of this study, the changes that occurred and are indicated by a percentage difference of either the temperature, or microbial influence over time, can be characterised under the following shapes, displayed by the polynomial graph: (1) a positive Bell curve (resembling the form of a bell), also known as a Gaussia-peak, (2) a negative Bell curve (resembling an upside down bell) or (3) a double curve – either positive or negative. With the polynomial equations to the 4th degree (a quartic equation), these double curves are expected from these graphs and are either in the shape of an M (double positive curve, when in fact it is a negative coefficient at the beginning of the polynomial equation that makes

both ends go to the bottom), or opposite to that of the W (Otto, 2007; McDonald, 2009).

3.4.1 Temperature percentage difference (TPD)

Changes in the selected short-chain (Fig. 3.1), medium- and long-chain (Fig. 3.2) fatty acid metabolites were evident for both the “control” samples (C8 fatty acid values in percentage subtracted from the C25 fatty acid values in percentage as per Eq. 1, also refer to Appendix I), and “inoculated” samples (I8 fatty acid values in percentage subtracted from the I25 fatty acid values in percentage as per Eq. 1, also refer to Appendix II). No linear temperature percentage difference (TPD) was observed in Figure 3.1 or Figure 3.2.

3.4.1.1 *Changes in selected short chain fatty acid metabolites over time*

The graphs of the short-chain fatty acid metabolites (Fig. 3.1) for the fillet samples at different temperatures that were not exposed to excessive micro-organisms (“control”) generally displayed the most prominent percentage difference at the onset stages of the experiment (Time 0). In Figure 3.1 the most short-chain fatty acid metabolite graphs initially start (at Time 0) with a negative Bell curve, (either with one clearly visible curve, or a double curve – not always as clear). The “control” graphs that start with a negative Bell curve, include the following short-chain fatty acid metabolites: glycine (a), hexanoic acid (c), octanoic acid (f), nonanoic acid (g), and decanoic acid (h). The exception short-chain fatty acid metabolites that started with a positive Bell curve, were pentanoic acid (b), heptanoic acid (d) and 2-methylheptanoic acid (e). Although these were exceptions, the latter graphs (b, d and e) correspondingly displayed the most prominent percentage difference at Time 0, at the commencing step of the experiment. The only short short-chain fatty acid metabolite that displayed a double positive Bell curve (M shape), was Decanoic acid (graph i), with a similar percentage difference at the beginning and end of the sampling period (Fig. 3.1).

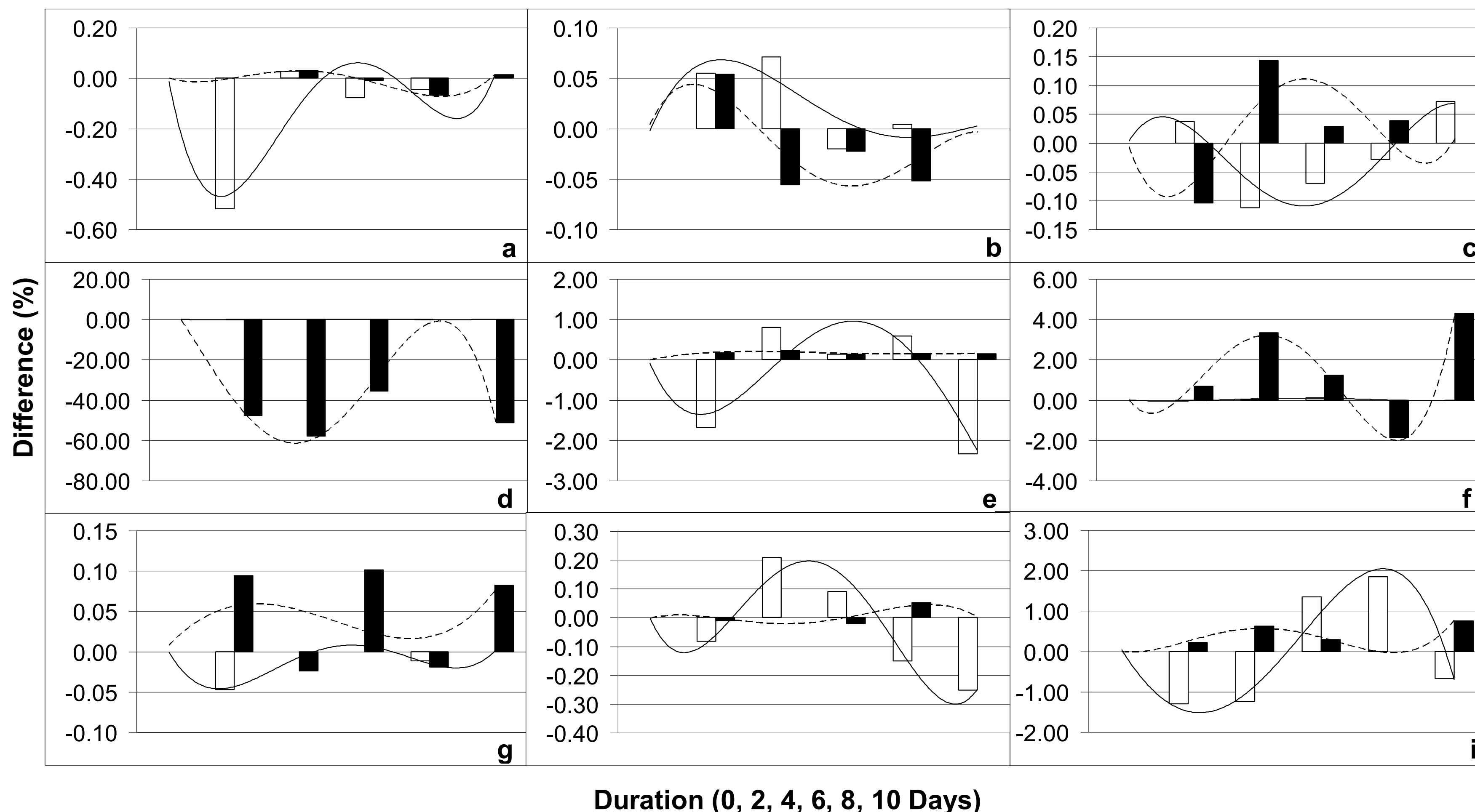
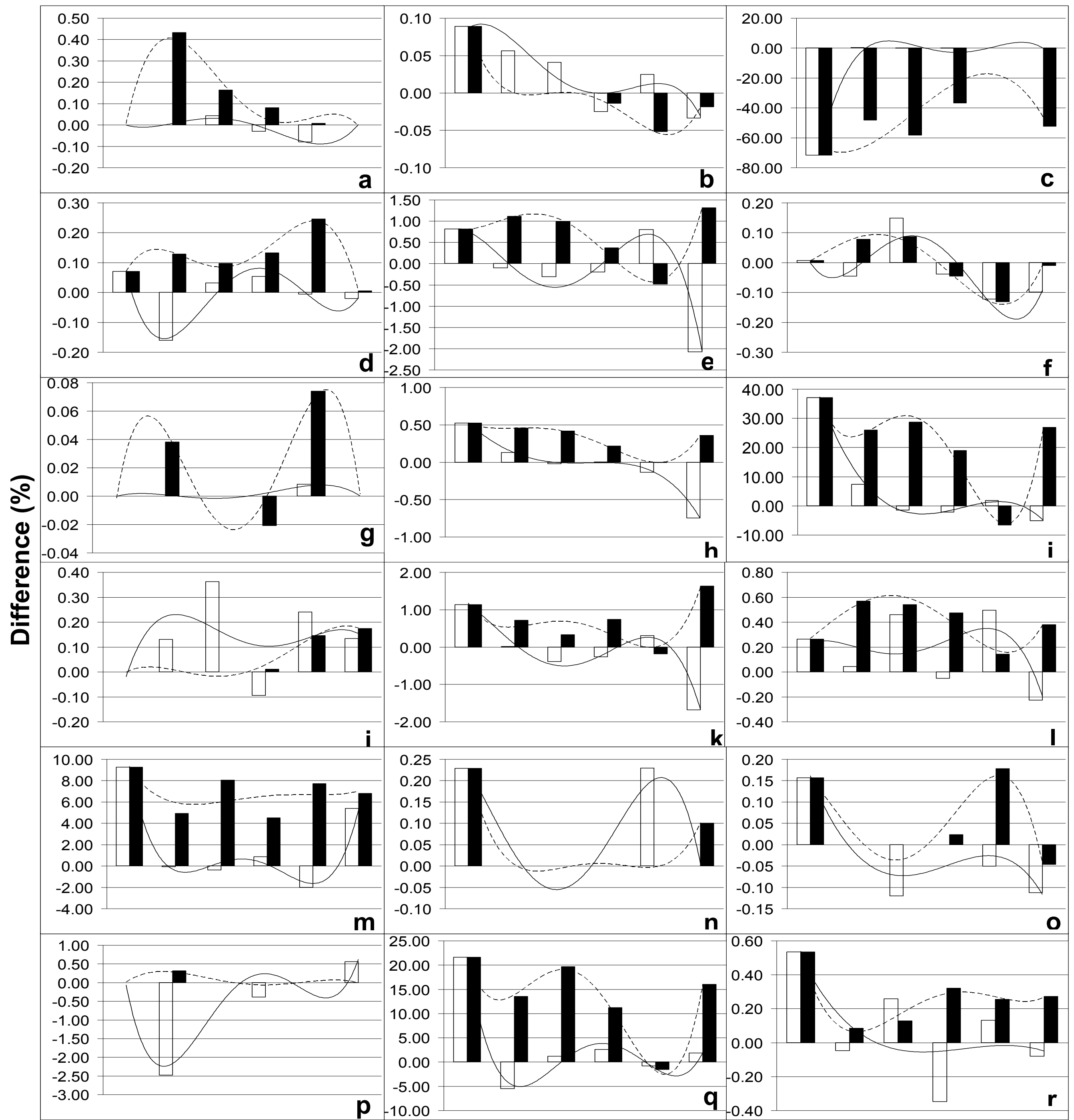


Figure 3.1 Temperature percentage difference of the changes in saturated short-chain methylated fatty acids for control (□) and inoculated (■) hake fillets over a period of ten days. The curve fits are indicated for control (—) and inoculated (---), where a = glycine, b = pentanoic acid, 3-methyl-, c = hexanoic acid, d = heptanoic acid, e = 2-methylheptanoic acid, f = octanoic acid, g = nonanoic acid, h = decanoic acid and I = decanoic acid, 2-methyl.



Duration (0, 2, 4, 6, 8, 10 Days)

*Caption on following page

Figure 3.2 Temperature difference (%) of the changes in saturated and unsaturated medium- to long-chain methylated fatty acids for control (☐) and inoculated (■) hake fillets over a period of ten days. The curve fits are indicated for control (—) and inoculated (---), where a = 9-dodecenoic acid, b = dodecanoic acid, c = tridecanoic acid, d = tridecanoic acid, 4,8,12-trimethyl-, e = methyl tetradecanoate, f = tetradecanoic acid, 10,13-dimethyl-, g = tetradecanoic acid, 12-methyl-, h = pentadecanoic acid, I = hexadecanoic acid, j = 6-hexadecenoic acid, 7-methyl, k = 9-hexadecenoic acid, l = heptadecanoic acid, m = octadecanoic acid, n = 9,15-octadecadienoic acid, o = 11-eicosenoic acid, p = eicosanoic acid, q = 4,7,10,13,16,19-docosahexaenoic acid, r = cholesterol.

Temperature percentage difference (TPD) can be observed from the “inoculated” polynomial graphs (the short-chain fatty acid metabolites for the fillet samples that were exposed to excessive micro-organisms at the different temperatures). The excess micro-organisms influenced the changes of the fatty acid metabolites during the sampling period, when observing the graphs in Figure. 3.1. Overall the metabolites for the “inoculated” short-chain fatty acid metabolites displayed more than one curve (to be expected from quartic polynomial equation), with the most prominent difference similarly at the beginning stages of the experiment. In Figure 3.1 a (glycine), f (octanoic acid) and g (nonanoic acid) are the only fatty acid metabolite graphs with one curve, either positive (f) or negative (a and g). At the end of the sampling period the only fatty acid metabolite that had opposite percentage differences at the end, was Decanoic acid (graph i).

3.4.1.2 *Changes in selected medium and long chain fatty acid metabolites*

In Figure 3.2 most of the “control” fatty acid metabolite graphs displayed a double Bell curve, as the norm would be for polynomial equations to the 4th order. These double curves formed by the percentage changes in the fatty acid metabolites in the various graphs in Figure 3.2, can be separated into those resembling an M, or double positive Bell curve (b, c, e, j, k, l and n), and those resembling a W, or double negative Bell curve (a, d, f, m, p and q). No single Bell curve is displayed in Figure 3.2, but there are a number of graphs representing the “control” samples, that are not clearly discernable (g, h, I, o and r).

The “inoculated” fillets‘ fatty acid metabolites did not signify an inclination to a positive or negative trend at the onset of the experiment. Nine fatty acid metabolites displayed a double Bell curve, with an initial negative trend at the beginning of the experiment (similar to the majority of the “control” fillets). These included b (dodecanoic acid), c (tridecanoic acid), i (hexadecanoic acid), k (9-hexadecenoic acid), m (octadecanoic acid), n (9,15-octadecadienoic acid), o (11-eicosenoic acid), q (4,7,10,13,16,19-docosahexaenoic acid) and r (cholesterol) in Figure 3.2. The other nine that displayed a portion of, or a positive Bell curve at

the beginning of the experiment in Figure 3.2 included: a (9-dodecenoic acid), d (tridecanoic acid, 4,8,12-trimethyl), e (methyl tetradecanoate), f (tetradecanoic acid, 10,13-dimethyl), g (tetradecanoic acid, 12-methyl), h (pentadecanoic acid), j (6-hexadecenoic acid, 7-methyl), I (heptadecanoic acid) and p (eicosanoic acid). From the latter graphs the following had prominent TPD changes at the end of the experiment: c, d, e, g, h, I, j, k, l, m, q and r. At the end of the sampling period the following fatty acid metabolites had opposite percentage differences: e (methyl tetradecanoate), h (pentadecanoic acid), k (9-hexadecenoic acid) and I (heptadecanoic acid).

3.4.1.3 Prediction of remaining hake fillet shelf life by mathematical estimations, using temperature percentage differences

Mathematical equations describing the temperature percentage differences (TPD) of the fatty acid metabolites over a ten day period, were defined by polynomial equations as shown in Table 3.1. All the fatty acid metabolites for both the ―control", as well as the ―inoculated" fillets were defined by 4-grade polynomial equations, except for one (tridecanoic acid). These equations may contribute towards a model to calculate the remaining fillet shelf life, by using the fatty acid data as variable. However, when considering the correlation coefficient (R^2) in Table 3.1, as well as the TPD in Figures 3.1 and 3.2, it is clear that certain fatty acids could be better indicators of the remaining shelf life than others and therefore, a combined formula of more than one fatty acid that relate the information of the selected fatty acids, should give a more accurate estimate of the remaining shelf life. The following fatty acid equations where selected for the ―control" and the ―inoculated" fatty acid metabolites, based on the R^2 (≥0.90) for both C and I: glycine, pentanoic acid, 3-methyl, hexanoic acid, heptanoic acid, 2-methylheptanoic acid, dodecanoic acid, tridecanoic acid, 4,8,12-trimethyl, methyl tetradecanoate, pentadecanoic acid, hexadecanoic acid and 4,7,10,13,16,19-docosahexaenoic acid.

Relevant to hake investigated in this study, the following two formulae are proposed for inoculated and non-inoculated (control), as a collective representation of the changes in fatty acid metabolites occurring during the storage of hake fillets on ice:

Control:

$$11y = 0.08x^4 - 15.90x^3 + 98.35x^2 - 254.87x + 231.83 \tag{3}$$

Inoculated:

$$11y = 3.48x^4 - 45.42x^3 + 200.88x^2 - 356.62x + 258.42 \tag{4}$$

Where x is the predicted remaining shelf life and y is the TPD for the selected component. Conversely, it should be possible to select a certain fatty acid from Table 3.1, and to do a prediction on the remaining shelf life by solving an individual equation.

Table 3.1. Mathematical equations representing the patterns of selected fatty acid metabolite TPD during the storage of control (C) and inoculated (I) hake fillets on ice

Figure	Fatty Acid Metabolites	Storage condition	Equation: $y = Ax^n + bx^n - c$	R^2
Fig. 3.1 a	Glycine	C	$y = 0.0128\,x^4 - 0.1915\,x^3 + 1.0008\,x^2 - 2.1258\,x + 1.5281$	$R^2 = 0.9743$
		I	$y = 0.0019\,x^4 - 0.0305\,x^3 + 0.2011\,x^2 - 0.6336\,x + 0.6821$	$R^2 = 0.9951$
Fig. 3.1 b	Pentanoic acid, 3-methyl	C	$y = -0.0038\,x^4 + 0.0582\,x^3 - 0.3181\,x^2 + 0.6871\,x - 0.3861$	$R^2 = 0.9278$
		I	$y = 0.0162\,x^4 - 0.2217\,x^3 + 1.0277\,x^2 - 1.8563\,x + 1.0754$	$R^2 = 0.9422$
Fig. 3.1 c	Hexanoic acid	C	$y = 0.0035\,x^4 - 0.0561\,x^3 + 0.3263\,x^2 - 0.8x + 0.6918$	$R^2 = 0.9952$
		I	$y = -0.0133\,x^4 + 0.1953\,x^3 - 0.9758\,x^2 + 1.833\,x - 0.8686$	$R^2 = 0.9197$
Fig. 3.1 d	Heptanoic acid	C	$y = -0.0006\,x^4 + 0.0096\,x^3 - 0.056\,x^2 + 0.1373\,x - 0.1187$	$R^2 = 0.9952$
		I	$y = -0.0025\,x^4 + 0.0377\,x^3 - 0.2011\,x^2 + 0.4308\,x - 0.2927$	$R^2 = 0.9264$
Fig. 3.1 e	2-Methyl heptanoic acid	C	$y = -0.0024\,x^4 + 0.0338\,x^3 - 0.1598\,x^2 + 0.2634\,x - 0.0632$	$R^2 = 0.9623$
		I	$y = 0.0022\,x^4 - 0.035\,x^3 + 0.1944\,x^2 - 0.4449\,x + 0.3545$	$R^2 = 0.9886$
Fig. 3.1 f	Octanoic acid	C	$y = 0.0006\,x^4 - 0.0138\,x^3 + 0.1104\,x^2 - 0.3462\,x + 0.3475$	$R^2 = 0.975$
		I	$y = -0.0034\,x^4 + 0.0548\,x^3 - 0.3093\,x^2 + 0.6679\,x - 0.3078$	$R^2 = 0.7898$

Fig. 3.1 g	Nonanoic acid	C	$y = -0.003\,x^4 + 0.0509\,x^3 - 0.2472\,x^2 + 0.2828\,x + 0.2622$	$R^2 = 0.8685$
		I	$y = 0.0083\,x^4 - 0.1176\,x^3 + 0.5808\,x^2 - 1.228\,x + 1.901$	$R^2 = 0.9697$
Fig. 3.1 h	Decanoic acid	C	$y = 0.0019\,x^4 - 0.0306\,x^3 + 0.1781\,x^2 - 0.4366\,x + 0.3775$	$R^2 = 0.9952$
		I	$y = -0.0221\,x^4 + 0.3301\,x^3 - 1.6932\,x^2 + 3.3593\,x - 1.8748$	$R^2 = 0.8495$
Fig. 3.1 i	Decanoic acid, 2-methyl	C	$y = -0.004\,x^4 + 0.056\,x^3 - 0.2706\,x^2 + 0.514\,x - 0.2952$	$R^2 = 0.9961$
		I	$y = 0.0056\,x^4 - 0.0755\,x^3 + 0.342\,x^2 - 0.5942\,x + 0.3242$	$R^2 = 0.7577$
Fig. 3.2 a	Dodecanoic acid	C	$y = 0.0055\,x^4 - 0.0696\,x^3 + 0.287\,x^2 - 0.4486\,x + 0.2244$	$R^2 = 0.9465$
		I	$y = -0.0173\,x^4 + 0.2729\,x^3 - 1.501\,x^2 + 3.2536\,x - 2.003$	$R^2 = 0.9535$
Fig. 3.2 b	9-Dodecenoic acid	C	$y = 0.0032\,x^4 + 0.044\,x^3 - 0.2006\,x^2 + 0.32422\,x - 0.078$	$R^2 = 0.8616$
		I	$y = 0.0041\,x^4 - 0.0589\,x^3 + 0.2953\,x^2 - 0.6246\,x + 0.473$	$R^2 = 0.9996$
Fig. 3.2 c	Tridecanoic acid	C	$y = -1.5089\,x^4 + 24.46\,x^3 - 142.08\,x^2 + 347.82\,x - 300.12$	$R^2 = 0.9951$
		I	$y = -3.0363\,x^4 + 28.452\,x^2 - 64.74 - 26.348$	$R^2 = 0.6622$
Fig. 3.2 d	Tridecanoic acid, 4,8,12-trimethyl	C	$y = 0.015\,x^4 - 0.2248\,x^3 + 1.159\,x^2 - 2.3438\,x + 1.4626$	$R^2 = 0.9517$
		I	$y = -0.0122\,x^4 + 0.159\,x^3 - 0.7056\,x^2 + 1.2511\,x - 0.623$	$R^2 = 0.9895$
Fig. 3.2 e	Methyl tetradecanoate	C	$y = -0.0911\,x^4 + 1.0791\,x^3 - 4.106\,x^2 + 5.3671\,x - 1.4622$	$R^2 = 0.9724$
		I	$y = 0.0616\,x^4 - 0.7129\,x^3 + 2.625\,x^2 - 3.5667\,x + 2.4142$	$R^2 = 0.9899$

Fig. 3.2 f	Tetradecanoic acid, 10,13-dimethyl	C	$y = 0.0132\,x^4 - 0.1775\,x^3 + 0.7922\,x^2 - 1.3461\,x + 0.7181$	$R^2 = 0.7846$
		I	$y = 0.005\,x^4 - 0.0524\,x^3 + 0.1461\,x^2 - 0.0687\,x - 0.025$	$R^2 = 0.9902$
Fig. 3.2 g	Tetradecanoic acid, 12-methyl	C	$y = -0.0005\,x^4 + 0.0067\,x^3 - 0.029\,x^2 + 0.0489\,x - 0.0262$	$R^2 = 0.881$
		I	$y = -0.0079\,x^4 + 0.1085\,x^3 - 0.5088\,x^2 + 0.9318\,x - 0.5253$	$R^2 = 0.9001$
Fig. 3.2 h	Pentadecanoic acid	C	$y = -0.0049\,x^4 + 0.0266\,x^3 + 0.0985\,x^2 - 0.8088\,x + 1.214$	$R^2 = 0.9666$
		I	$y = 0.0163\,x^4 - 0.1989\,x^3 + 0.8042\,x^2 - 1.3359\,x + 1.239$	$R^2 = 0.9996$
Fig. 3.2 i	Hexadecanoic acid	C	$y = -0.0577\,x^4 - 0.7661\,x^3 + 15.637\,x^2 - 69.974\,x + 92.171$	$R^2 = 0.9986$
		I	$y = 2.1023\,x^4 - 27.436\,x^3 + 121.26\,x^2 - 215.71\,x + 157.1$	$R^2 = 0.9895$
Fig. 3.2 j	6-Hexadecenoic acid, 7-methyl	C	$y = -0.0092\,x^4 + 0.1454\,x^3 - 0.7984\,x^2 + 1.7652\,x - 1.1227$	$R^2 = 0.2614$
		I	$y = -0.005\,x^4 + 0.0685\,x^3 - 0.3043\,x^2 + 0.5192\,x - 0.2802$	$R^2 = 0.9763$
Fig. 3.2 k	9-Hexadecenoic acid	C	$y = -0.0581\,x^4 + 0.66\,x^3 - 2.2724\,x^2 + 2.0198\,x + 0.7743$	$R^2 = 0.9917$
		I	$y = 0.0688\,x^4 - 0.8975\,x^3 + 4.058\,x^2 - 7.560\,x + 5.50521$	$R^2 = 0.8353$
Fig. 3.2 l	Heptadecanoic acid	C	$y = 0.0159\,x^4 - 0.1901\,x^3 - 0.7512\,x^2 + 1.1295\,x - 0.3218$	$R^2 = 0.265$
		I	$y = 0.0113\,x^4 - 0.1221\,x^3 + 0.3502\,x^2 - 0.0969\,x + 0.1258$	$R^2 = 0.9288$

Fig. 3.2 m	Octadecanoic acid	C	$y = 0.4546x^4 - 6.4637x^3 + 32.687x^2 - 69.401x + 52.054$	$R^2 = 0.9857$
		I	$y = 0.0692x^4 - 1.1322x^3 + 6.6444x^2 - 16.162x + 19.635$	$R^2 = 0.3842$
Fig. 3.2 n	9,15-Octadeca-dienoic acid	C	$y = -0.0096x^4 + 0.1086x^3 - 0.355x^2 + 0.2526x + 0.2266$	$R^2 = 0.8927$
		I	$y = 0.0069x^4 - 0.1019x^3 + 0.5489x^2 - 1.2622x + 1.0365$	$R^2 = 0.9985$
Fig. 3.2 o	Eicosanoic acid	C	$y = -0.0009x^4 - 0.001x^3 + 0.0964x^2 - 0.4536x + 0.5206$	$R^2 = 0.8918$
		I	$y = -0.0079x^4 + 0.0881x^3 - 0.2898x^2 + 0.234x + 0.1289$	$R^2 = 0.9316$
Fig. 3.2 p	11-Eicosenoic acid	C	$y = 0.1506x^4 - 2.2292x^3 + 11.437x^2 - 23.073x + 13.652$	$R^2 = 0.8285$
		I	$y = -0.0196x^4 + 0.2952x^3 - 1.5292x^2 + 3.0922x - 1.8324$	$R^2 = 0.881$
Fig. 3.2 q	4,7,10,13,16,19-Docosa hexaenoic acid	C	$y = 1.0378x^4 - 15.797x^3 + 84.484x^2 - 184.82x + 136.57$	$R^2 = 0.9922$
		I	$y = 1.32x^4 - 17.446x^3 + 78.151x^2 - 139.84x + 99.339$	$R^2 = 0.9974$
Fig. 3.2 r	Cholesterol	C	$y = 0.0004x^4 - 0.0234x^3 + 0.2571x^2 - 1.0138x + 1.2832$	$R^2 = 0.5111$
		I	$y = 0.0143x^4 - 0.2304x^3 + 1.3049x^2 - 2.9842x + 2.4322$	$R^2 = 0.9793$

$R^2 =$ correlation coefficient

3.4.2 Microbial influence percentage difference (MIPD)

In Figures 3.3 and 3.4 the microbial influence percentage differences (MIPD) are indicated by graphs, representing the difference of the fatty acid metabolite between the control and inoculated fillets of both temperatures (8 ºC and 25 ºC), as previously shown in Eq 2 (for more detail also refer to Appendices III and IV). MIPD are observed in the changes of the short-chain (Fig. 3.3), medium- and long-chain (Fig. 3.4) fatty acid metabolites. The high average death rate of $\Delta\bar{x}$ =- 5.6×10^{3} cfu.day^{-1} $\pm1.428x10^{3}$ for both the inoculated samples (I8 and I25), indicates that these percentage differences originate from non-culturable micro-organisms from the fatty acid metabolites of hake triglycerides, or are associated with fatty acid metabolites from the micro-organisms themselves.

The high death rate may be explained by super chilling the fish fillets during the night, because, as discussed earlier, a temperature shock radically influences microbial growth (Theron *et al.*, 2003). The majority of the graphs for the MIPD showed double Bell curves. Three equations associated with the fillets at 25 ºC MIPD resulted in y=0 (linear on zero), as observed in Figures 3.3 and 3.4.

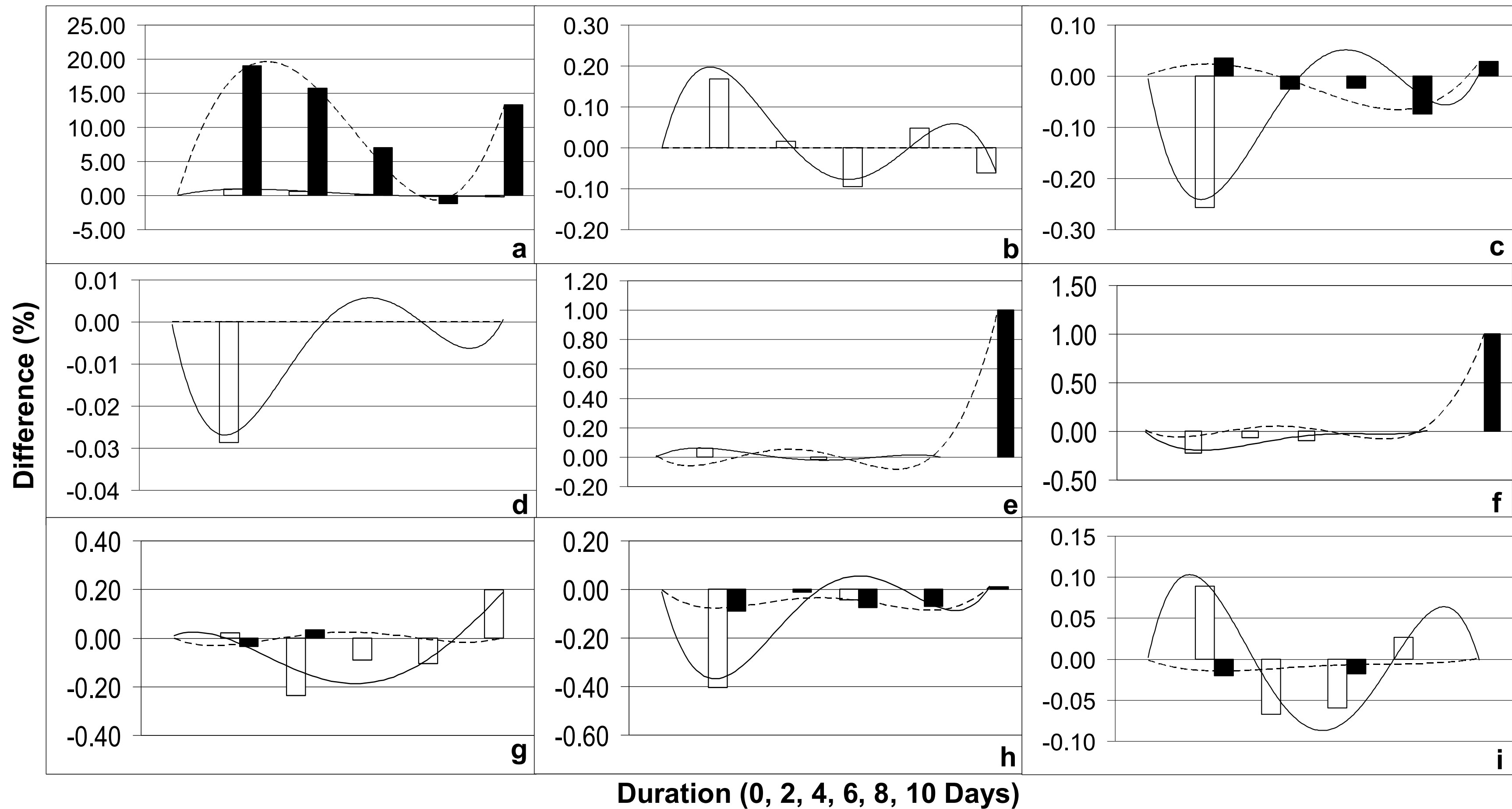
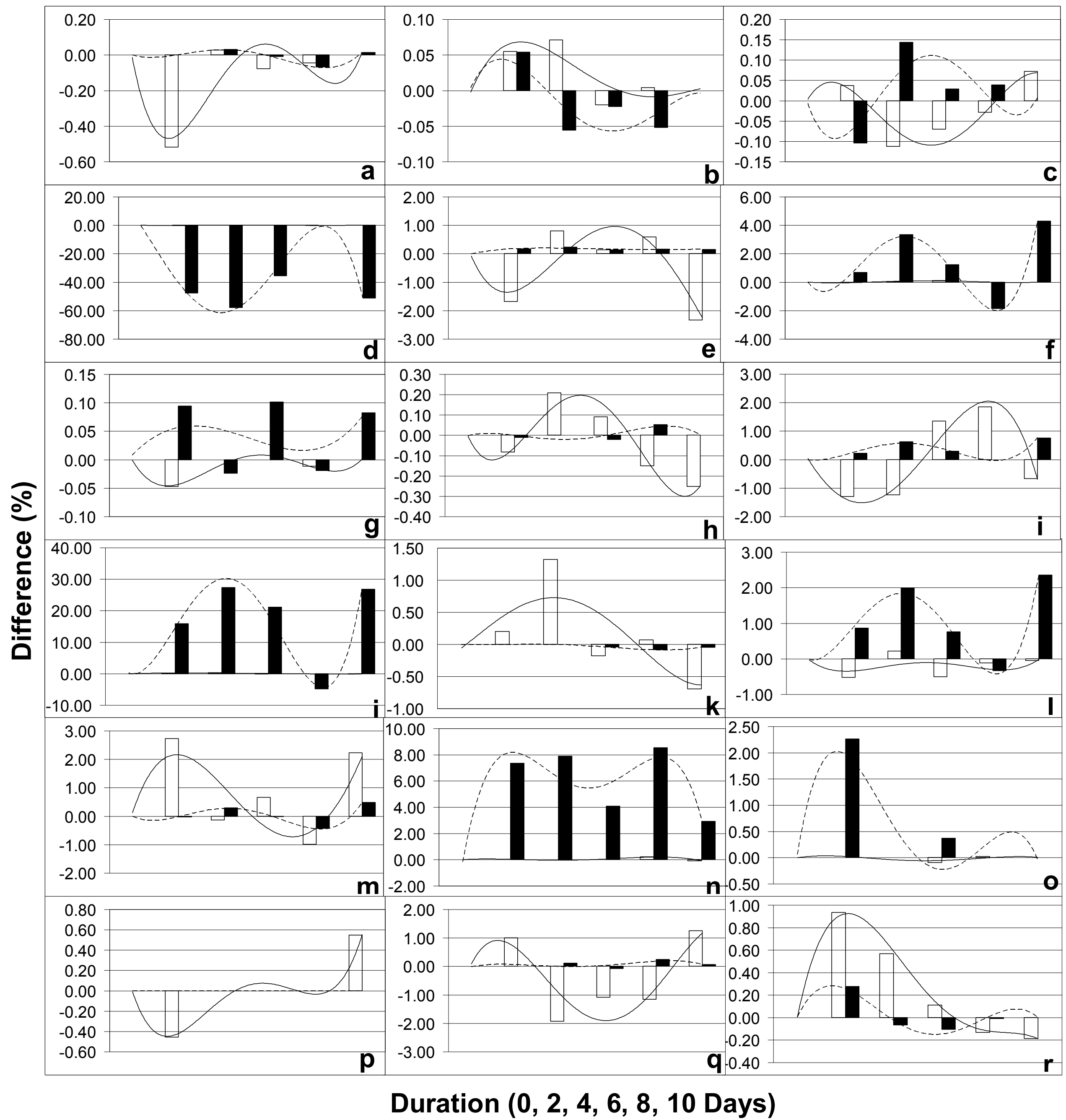


Figure 3.3 Microbial influence percentage difference (MIPD) of the changes in saturated short-chain methylated fatty acids in hake fillets stored at 8 ºC (☐) control minus inoculated and stored at 25 ºC (■) control minus inoculated fillets over a period of ten days. The curve fits are indicated for 8ºC (—) and 25ºC (---), where a = glycine, b = pentanoic acid, 3-methyl-, c = hexanoic acid, d = heptanoic acid, e = 2-methylheptanoic acid, f = octanoic acid, g = nonanoic acid, h = decanoic acid and i = decanoic acid, 2-methyl



*Caption on following page

Figure 3.4 Microbial influence percentage difference (MIPD) of the changes in saturated and unsaturated medium- to long-chain methylated fatty acids in hake fillets stored at 8 ºC (☐) control minus inoculated and stored at 25 ºC (■) control minus inoculated fillets over a period of ten days. The curve fits are indicated for 8 ºC (—) and 25 ºC (---), where a = 9-dodecenoic acid, b = dodecanoic acid, c = tridecanoic acid, d = tridecanoic acid, 4,8,12-trimethyl-, e = methyl tetradecanoate, f = tetradecanoic acid, 10,13-dimethyl-, g = tetradecanoic acid, 12-methyl-, h = pentadecanoic acid, i = hexadecanoic acid, j = 6-hexadecenoic acid, 7-methyl, k = 9-hexadecenoic acid, l = heptadecanoic acid, m = octadecanoic acid, n = 9,15-octadecadienoic acid, o = 11-eicosenoic acid, p = eicosanoic acid, q = 4,7,10,13,16,19-docosahexaenoic acid, r = cholesterol.

3.4.2.1 *MIPD* **changes in selected short chain fatty acid metabolites over time**

As expected from polynomial equation to the 4th order, there were largely more than one curve exhibited for the 8 ºC short-chain fatty acid metabolite graphs, as viewed in Figure 3.3. Most of the short-chain fatty acid metabolites started with a positive Bell curve. The potential important role played by microbes in the changes of short chain fatty acid metabolites, depicted as the most prominent microbial influence percentage difference (MIPD), was clearly visible on the second day (T1) of sampling. Furthermore, because of the high death rate of the microbes, the percentage difference is expected at the second day, when the first sampling of the inoculated samples took place. These graphs include the following fatty acid metabolites: glycine (a), pentanoic acid, 3-methyl (b), 2-methylheptanoic acid (e) and decanoic acid, 2-methyl (i). The only exception in the short chain fatty acid metabolite graphs that started with a positive Bell curve, was nonanoic acid (g) that had the most prominent percentage difference on day six (T2). The rest of the 8 ºC short-chain fatty acid metabolite graphs in Figure 3.3 all started with a negative Bell curve, again with the most prominent MIPD at day two, these being hexanoic acid (c), heptanoic acid (d), octanoic acid (f), and decanoic acid (h).

It is possible that the higher uncontrolled environmental temperature gave the micro-organisms more time to influence the fatty acid metabolites on the inoculated samples and the super chilling effect is, therefore, less visible in the 25 ºC graphs, while the percentage difference that occurred for the 25 ºC short-chain fatty acid metabolites differ from the 8 ºC. Generally, the metabolites for the 25 ºC short-chain fatty acid metabolites displayed mainly small Bell curves, with the exception of glycine (graph a) in Figure 3.3. Differences are noticed at the end of the experiments for graphs a, e and f. However, in the following cases it was observed that the short-chain fatty acid metabolites end together (b, c, d, h and i). Therefore, with a zero ―0" percentage difference, indicating that, although

differences were observed for the duration of the sampling period, at the end no significant difference is displayed by the short-chain fatty acid metabolite graphs.

3.4.2.2 *MIPD changes in selected medium and long chain fatty acid metabolites*

Fatty acid metabolites for the 8 ºC fillets displayed more than one curve, although no visible trend could be distinguished. Of the 18 medium and long-chain fatty acid metabolite changes, depicted on graphs, 50 % of them had an initial negative trend at the beginning, and 50 % had an initial positive trend (Fig. 3.4). Most of the positive Bell curve is displayed on the longer chain fatty acid metabolites: b (dodecanoic acid), c (tridecanoic acid), j (6-hexadecenoic acid, 7-methyl), k (9-hexadecenoic acid), m (octadecanoic acid), n (9,15-octadecadienoic acid), o (11-eicosenoic acid), q (4,7,10,13,16,19-docosahexaenoic acid) and r (cholesterol). The most prominent differences were evident on day two and four.

Fatty acid metabolites for the 25 ºC fillets generally displayed a higher percentage difference than the 8 ºC fillets, indicating that the higher temperature caused the fatty acid metabolites to have more changes. The majority had an initial positive Bell curve (Fig. 3.4), with the exception of: a (9-dodecenoic acid), c (tridecanoic acid), d (tridecanoic acid, 4,8,12-trimethyl), f (tetradecanoic acid, 10,13-dimethyl), i (heptadecanoic acid), k (9-hexadecenoic acid), l (heptadecanoic acid) and m (octadecanoic acid). Heptadecanoic acid is also the only fatty acid that displayed opposite results at the end of the experiment for the two different storage conditions.

3.4.2.3 Prediction of the remaining hake fillet shelf life by means of mathematical estimations using microbial influenced percentage differences

Mathematical equations describing the patterns of the microbial influenced percentage differences (MIPD) of the fatty acid metabolites over a ten day period, were defined by polynomial equations (Table 3.2). Most of the fatty acid metabolites for both the environmental storage temperatures were defined by 4-grade polynomial equations. These equations may contribute towards a model to calculate the remaining fillet shelf life, by using the fatty acid data as variable. However, if considering the correlation coefficient (R^2) in Table 3.2, as well as the MIPDs in Figures 3.3 and 3.4, it is clear that certain fatty acids may be more accurate indicators of the remaining shelf life than others.

A combined formula of more than one fatty acid that relates the information of the selected fatty acids should, therefore, give a more accurate estimate of the remaining shelf life. The following fatty acid equations for both the temperature formulae where selected, based on the R^2 (≥0.90): glycine, 2-methylheptanoic acid, tridecanoic acid, 4,8,12-trimethyl-, methyl ester, hexadecanoic acid, methyl ester and cholesterol. Relative to the hake investigated in this study, the following two formulae (8 ºC and 25 ºC) are proposed as a collective representation of the percentage difference in fatty acid metabolites occurring during the storage of hake fillets on ice, that were exposed to excessive micro-organisms:

8 ºC:

$$5y = -0.08x^4 + 0.89x^3 - 3.56x^2 + 5.85x - 3.07 \tag{5}$$

25 ºC:

$$5y = -1.85x^4 + 21.96x^3 - 79.74x^2 + 61.68x - 26.51 \tag{6}$$

Table 3.2. Mathematical equations representing the patterns of selected fatty acid metabolites. Microbial influenced percentage difference changes during the storage of hake fillets on ice at 8 ºC and 25 ºC.

Figure	**Fatty Acid Metabolites**	**Temperature**	**Equation:** $y = Ax^n + bx^n - c$	R^2
Fig. 3.3 a	Glycine	8	$y = -0.0257\,x^4 + 0.4378\,x^3 - 2.6092\,x^2 + 6.0692\,x - 3.8696$	$R^2 = 0.9986$
		25	$y = 0.1127\,x^4 + 0.6652\,x^3 - 16.785\,x^2 + 62.316\,x - 46.2$	$R^2 = 0.9914$
Fig. 3.3 b	Pentanoic acid, 3-methyl	8	$y = -0.018\,x^4 + 0.2617\,x^3 - 1.3027\,x^2 + 2.5282\,x - 1.4714$	$R^2 = 0.9704$
		25	$y = 0$	$R^2 = \#NA$
Fig. 3.3 c	Hexanoic acid	8	$y = 0.0161\,x^4 - 0.2419\,x^3 + 1.2531\,x^2 - 2.5339\,x + 1.5015$	$R^2 = 0.881$
		25	$y = 0.001\,x^4 - 0.0059\,x^3 - 0.0132\,x^2 - +0.0863\,x - 0.0659$	$R^2 = 0.8329$
Fig. 3.3 d	Heptanoic acid	8	$y = 0.0018\,x^4 - 0.027\,x^3 + 0.1397\,x^2 - 0.2824\,x + 0.1674$	$R^2 = 0.881$
		25	$y = 0$	$R^2 = \#NA$
Fig. 3.3 e	2-Methylheptanoic acid	8	$y = -0.0045\,x^4 + 0.067\,x^3 - 0.3451\,x^2 + 0.6901\,x - 0.407$	$R^2 = 0.9816$
		25	$y = 0.0114\,x^4 - 0.154\,x^3 + 0.7083\,x^2 - 1.2691\,x + 0.7143$	$R^2 = 0.9848$
Fig. 3.3 f	Octanoic acid	8	$y = 0.0074\,x^4 - 0.1167\,x^3 + 0.6493\,x^2 - 1.4336\,x + 0.888$	$R^2 = 0.9848$
		25	$y = 0.0114\,x^4 - 0.154\,x^3 + 0.7083\,x^2 - 1.2691\,x + 0.7143$	$R^2 = 0.7966$

Fig. 3.3 g	Nonanoic acid	8	$y = 0.0114\,x^4 - 0.154\,x^3 + 0.7083\,x^2 - 1.269\,x + 0.7143$	$R^2 = 0.8059$
		25	$y = 0.0036\,x^4 - 0.0516\,x^3 + 0.25\,x^2 - 0.4664\,x + 0.2623$	$R^2 = 0.5622$
Fig. 3.3 h	Decanoic acid	8	$y = 0.0236\,x^4 - 0.3544\,x^3 + 1.8409\,x^2 - 3.7422\,x + 2.2224$	$R^2 = 0.8188$
		25	$y = 0.0066\,x^4 - 0.0911\,x^3 + 0.4354\,x^2 - 0.8406\,x + 0.4869$	$R^2 = 0.7894$
Fig. 3.3 i	Decanoic acid, 2-methyl	8	$y = -0.0125\,x^4 + 0.1786\,x^3 - 0.8652\,x^2 + 1.6124\,x + 0.9117$	$R^2 = 0.9638$
		25	$y = 0.0005\,x^4 - 0.0076\,x^3 + 0.0417\,x^2 - 0.0926\,x + 0.0569$	$R^2 = 0.3545$
Fig. 3.4 a	Dodecanoic acid	8	$y = 0.0332\,x^4 - 0.4911\,x^3 + 2.5073\,x^2 - 5.0182\,x + 2.9553$	$R^2 = 0.7853$
		25	$y = 0.0054\,x^4 - 0.0694\,x^3 + 0.294\,x^2 - 0.4763\,x + 0.246$	$R^2 = 0.9977$
Fig. 3.4 b	9-Dodecenoic acid	8	$y = -0.0016\,x^4 + 0.0286\,x^3 + -0.1792\,x^2 + 0.4312\,x - 0.2817$	$R^2 = 0.7329$
		25	$y = 0.0034\,x^4 + 0.0532\,x^3 - 0.28\,x^2 + 0.5521\,x - 0.3184$	$R^2 = 0.6405$
Fig. 3.4 c	Tridecanoic acid	8	$y = 0.0067\,x^4 + 0.0995\,x^3 - 0.4889\,x^2 - +0.8878\,x - 0.4885$	$R^2 = 0.8839$
		25	$y = 0.0113\,x^4 - 0.1635\,x^3 + 0.7969\,x^2 - 1.476\,x + 0.8239$	$R^2 = 0.569$
Fig. 3.4 d	Tridecanoic acid, 4,8,12-trimethyl	8	$y = 0.0242\,x^4 - 0.3295\,x^3 + 1.5207\,x^2 - 2.7023\,x + 1.4826$	$R^2 = 0.9258$
		25	$y = -1.9916\,x^4 + 21.591\,x^3 - 63.871\,x^2 + 24.316\,x + 19.697$	$R^2 = 0.995$

Fig. 3.4 e	Methyl tetradecanoate	8	$y = 0.0597\,x^4 - 1.0662\,x^3 + 6.1456\,x^2 - 13.071\,x + 7.8509$	$R^2 = 0.8005$
		25	$y = -0.0026\,x^4 + 0.0467\,x^3 - 0.3036\,x^2 + 0.8087\,x - 0.5523$	$R^2 = 0.917$
Fig. 3.4 f	Tetradecanoic acid, 10,13-dimethyl	8	$y = 0.0084\,x^4 - 0.1211\,x^3 + 0.587\,x^2 - 1.0796\,x + 0.608$	$R^2 = 0.8523$
		25	$y = 0.3543\,x^4 - 4.5195\,x^3 + 19.088\,x^2 - 30.172\,x + 15.233$	$R^2 = 0.9972$
Fig. 3.4 g	Tetradecanoic acid, 12-methyl	8	$y = 0.0037\,x^4 - 0.0535\,x^3 + 0.2692\,x^2 - 0.5307\,x + 0.3106$	$R^2 = 0.9292$
		25	$y = 0.0003\,x^4 + 0.0028\,x^3 - 0.0505\,x^2 + 0.1764\,x - 0.1215$	$R^2 = 0.1888$
Fig. 3.4 h	Pentadecanoic acid	8	$y = 0.0217\,x^4 - 0.307\,x^3 + 1.4401\,x^2 - 2.5532\,x + 1.3942$	$R^2 = 0.9666$
		25	$y = -0.0034\,x^4 + 0.0446\,x^3 - 0.1928\,x^2 + 0.3192\,x - 0.1696$	$R^2 = 0.6664$
Fig. 3.4 i	Hexadecanoic acid	8	$y = -0.0437\,x^4 + 0.2807\,x^3 + 0.482\,x^2 - 4.2786\,x + 3.5972$	$R^2 = 0.96$
		25	$y = 0.0398\,x^4 - 0.4948\,x^3 + 1.9998\,x^2 - 2.871\,x + 1.3211$	$R^2 = 0.9829$
Fig. 3.4 j	6-Hexadecenoic acid, 7-methyl	8	$y = -0.0012\,x^4 + 0.0401\,x^3 - 0.3445\,x^2 + 1.0037\,x - 0.7154$	$R^2 = 0.5044$
		25	$y = 1.8898\,x^4 - 23.645\,x^3 + 95.289\,x^2 - 134.47\,x + 61.207$	$R^2 = 0.9802$
Fig. 3.4 k	9-Hexadecenoic acid	8	$y = 0.0162\,x^4 - 0.1953\,x^3 + 0.5991\,x^2 - 0.1144\,x + 0.3654$	$R^2 = 0.5946$
		25	$y = 0.0025\,x^4 - 0.0307\,x^3 + 0.1182\,x^2 - 0.1767\,x + 0.0865$	$R^2 = 0.9981$

Fig. 3.4 l	Heptadecanoic acid	8	$y = 0.0269x^4 - 0.3781x^3 + 1.8415x^2 - 3.5838x + 2.0563$	$R^2 = 0.2024$
		25	$y = 0.1311x^4 - 1.6033x^3 + 6.3129x^2 - 8.7264x + 3.8699$	$R^2 = 0.9888$
Fig 3.4 m	Octadecanoic acid	8	$y = -0.0406x^4 + 0.8827x^3 - 5.8806x^2 + 14.115x - 8.9624$	$R^2 = 0.6874$
		25	$y = 0.0507x^4 - 0.6506x^3 + 2.789x^2 - 4.5897x + 2.3983$	$R^2 = 0.9973$
Fig. 3.4 n	9,15-Octadecadienoic acid	8	$y = -0.0156x^4 + 0.1997x^3 - 0.8578x^2 + 1.4376x - 0.7686$	$R^2 = 0.897$
		25	$y = -0.433x^4 + 6.2614x^3 - 31.856x^2 + 66.56x - 40.659$	$R^2 = 0.881$
Fig. 3.4 o	Eicosanoic acid	8	$y = -0.0052x^4 + 0.0756x^3 + -0.3674x^2 + 0.6767x - 0.384$	$R^2 = 0.4862$
		25	$y = -0.126x^4 + 1.8963x^3 - 9.8654x^2 + 20.117x - 11.962$	$R^2 = 0.7815$
Fig. 3.4 p	11-Eicosenoic acid	8	$y = 0.0398x^4 - 0.5619x^3 + 2.7588x^2 - 5.3548x + 3.1112$	$R^2 = 0.9764$
		25	$y = 0$	$R^2 = \#NA$
Fig. 3.4 q	4,7,10,13,16,19-Docosahexaenoic acid	8	$y = -0.0897x^4 + 1.4226x^3 - 7.4079x^2 + 14.127x - 7.9708$	$R^2 = 0.7957$
		25	$y = -0.0121x^4 + 0.1642x^3 - 0.7512x^2 + 1.3587x - 0.7717$	$R^2 = 0.397$
Fig. 3.4 r	Cholesterol	8	$y = -0.0257x^4 + 0.4378x^3 - 2.6092x^2 + 6.0692x - 3.8696$	$R^2 = 0.9986$
		25	$y = -0.0238x^4 + 0.3532x^3 - 1.7902x^2 + 3.5065x - 2.0417$	$R^2 = 0.9551$

$R^2 =$ correlation coefficient

Where x is the predicted remaining shelf life and y is the MIPD in the selected fatty acid. It is also possible to select a certain fatty acid from Table 3.2 and to do a prediction on the remaining shelf life, by solving the individual equation.

3.5 CONCLUSION

Equations represented in this study are suitable only to hake, stored under conditions as stipulated in the materials and methods. However, the possibility to use a single, or the combined formula, may have potential in the retail industry to determine whether the hake has been subjected to excessive microbial contamination, or if the cold chain integrity has been kept. Using prediction mathematical estimations (equations 3-6), could also establish if the hake has been subjected to excessive microbial spoilage, or whether the environmental temperature was properly controlled, or not. Furthermore, it may be useful to determine whether it is possible to use the same approach on other hake species or even on other lean fish species. During this study the high death rate was unexpected for the microbial populations on the fillets, exposed to excessive microbial contamination. Noteworthy, and possibly valuable to pursue further, is the effect of super chilling, in combination with the storage of fish fillets on ice, on autochthonous microbiota contamination. It may be concluded that temperature shocks, including super chilling, reduced the microbial contamination for the duration of this study.

3.6 ACKNOWLEDGEMENTS

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CHAPTER 4

MATHEMATICAL PREDICTIONS OF TEMPERATURE AND MICROBIAL INFLUENCES ON SELECTED VOLATILES IN SOUTH AFRICAN CAPE HAKE

4.1 ABSTRACT

This study was aimed at developing a mathematical equation, that would use selected changes in volatile formation in Cape hake fillets during various storage conditions, in order to determine the fillets' shelf life. The specific application of the mathematical prediction was to determine whether the fillet was exposed to excessive microbial contamination, and whether the environmental temperature was controlled. Retail fish fillet display and storage conditions were simulated in terms of on-ice, using display refrigerators and kept at 8 ºC, with the outside temperature monitored and fillets displayed on ice in an unmonitored environment. These conditions corresponded with retail stores, where fish are displayed in the open. Analysis of volatiles was performed using a GC-MS with 48 hour intervals over a ten day period. Many components were identified, but for this study the focus was on the volatiles, which formed specific peaks in all the chromatograms (including nitrogen, ethyl acetate, ethanol, trichloromethane and water). The formulae determined in this study may have potential in the retail industry to determine fish fillet remaining shelf life, in terms of excessive microbial spoilage and environmental temperature control.

4.2 INTRODUCTION

It is known that protein degradation and lipid oxidation give rise to a great number of volatile components; consequently volatiles can be used to determine fish freshness, storage life, quality and even safety. Volatiles are present on freshly harvested fish, but additional volatiles may originate at each production step, including storage. The aromatic compounds found in food are the result of a number of reactions occurring between specific components. Fish aroma can, therefore, also vary considerably among species (Josephson, 1991). Volatile compounds in fish can be divided into three groups based on their origin (Fig. 4.1).

According to Figure 4.1 fresh fish are associated with mild, delicate and pleasant aromas, generally described as green, plant-like or melon-like, and are provided by various volatile carbonyls and alcohols, derived from polyunsaturated fatty acids of fish lipids via specific lipoxygenase activity (Josephson, 1991; Durnford and Shahidi, 1998). Volatile alcohols contribute to smoother qualities, whereas volatile carbonyls contribute to coarse, heavy aromas and also contribute more to the overall fresh fish-like odours, because of their lower threshold values. Compounds known to occur in freshwater as well as marine fish are hexanal, 1-octen-3-ol, 1,5-octadien-3-ol, and 2,5-octadien-3-one. Unlike the eight-carbon compounds, the six-carbon and nine-carbon compounds have been identified in freshly harvested fish, but are not found in all seafood. During the deterioration process fresh odours may be destroyed by microbial and autolytic activity, or the new compounds that are produced will possibly mask the fresh aromas (Josephson, 1991; Durnford and Shahidi, 1998).

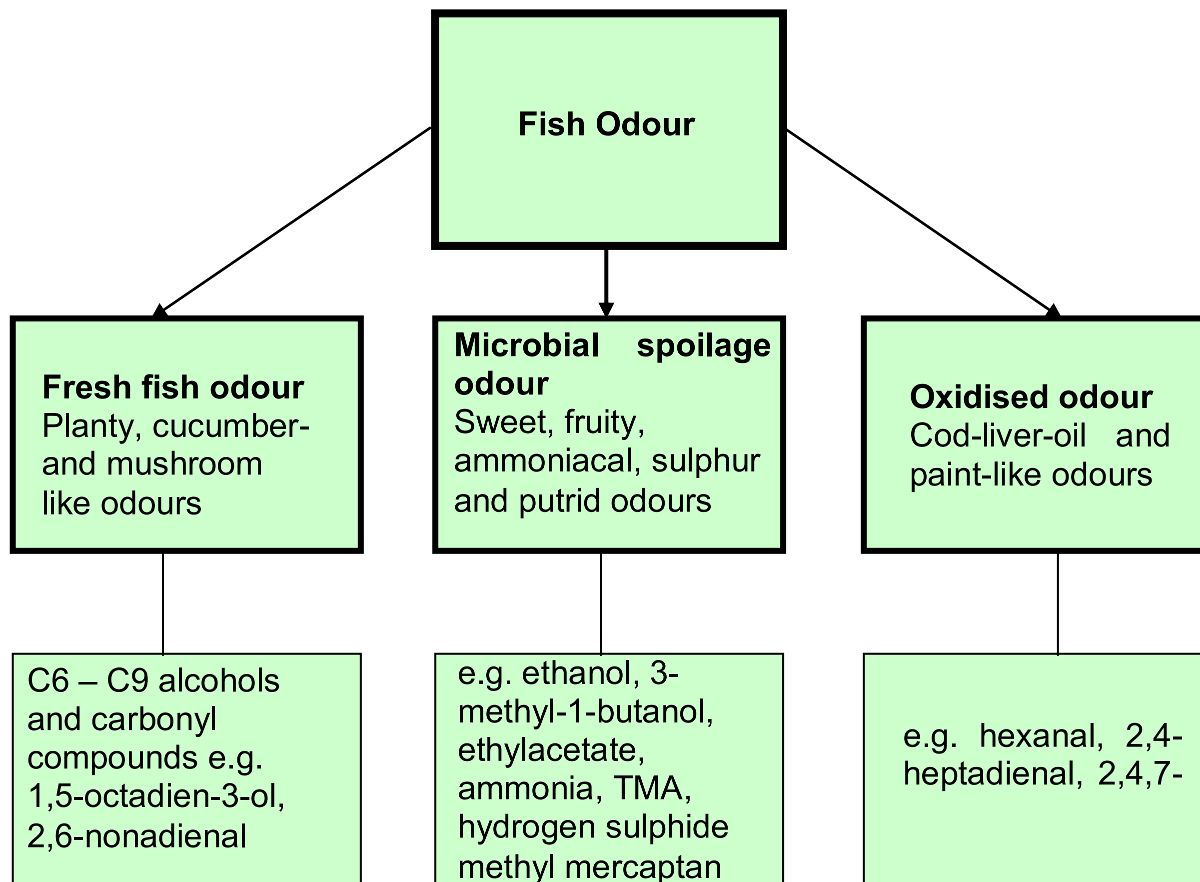

Figure 4.1 Categorisation of fish aromas and the volatile compounds contributing to the characteristic aroma of fresh, spoiled and oxidised fish (Ólafsdóttir *et al.,* 1997).

The most prominent volatiles produced in muscle foods during lipid oxidation are aldehydes, including octanal, nonanal, pentanal and hexanal, being the most dominant (Ross and Smith, 2006). During the oxidation of seafood the volatiles generated are hexanal, the 2, 4-heptadienals, the 3,5-octadien-2-ones, and the 2,4-decadienals (Fig. 4.1). Some volatiles derived through enzymatic mechanisms can be formed via autoxidation, for example, 2-nonenal, 2,6-nonadienal, 1-octen-3-one, and 1,5-octadien-3-one. The particular volatile compounds produced by the lipoxygenase are dependent on the substrate. If docosahexanoic acid or eicosapentanoic acid is the substrate, their flavour volatiles produced are 1,5-octadien-3-ol, (E,Z)-2,6-nonadienal, 2,5-octadien-1-ol and 3,6-nonadien-1-ol. In cases where arachidonic acid is the substrate, (E)-2-octenal, 1,-octen-3-ol, (E)-2-nonenal, (E)-2-octenol and (Z)-3-nonenol flavour volatiles are produced (Josephson, 1991; Durnford and Shahidi, 1998).

Volatile sulphur compounds traditionally associated with seafood spoilage, can be produced by living aquatic flora and fauna and include methyl mercaptan, dimethyl disulfide and dimethyl sulphide (Josephson, 1991; Durnford and Shahidi, 1998). Deteriorated fish odours are associated with trimethylamine (TMA) and dimethylamine (DMA). During microbial spoilage TMA is produced by the reduction of trimethylamine oxide (TMAO), while DMA and formaldehyde are produced from the enzymatic breakdown of TMAO in the muscles of various fish species. TMAO has no odour, but TMA reacts with fat in the fish tissue to produce a potent fishy odour. In gadoid fish, such as hake, the formation of DMA and formaldehyde lead that of TMA, which can be prevented if microbial growth is repressed (Josephson, 1991; Durnford and Shahidi, 1998).

When microbial growth is not suppressed efficiently, the fish muscle will start to decay from the moment the fish is killed (Jay, 1992). On any decaying carcass, including fish, consisting of the main substrates carbohydrates, amino acids, glycerol and fatty acids, the microbial metabolic pathways may lead to the formation of the following volatile components: ethanol, acetaldehyde, acetone,

2-propanol, 1-propanol, 1-butanol, isobutanol, isoamyl alcohol, *d*-amyl alcohol, acetate, propionate, butyrate, isobutarate and ethyl esters, such as ethyl acetate (Boumba, Ziavrou and Vougiouklakis, 2008).

To evaluate the quality of food products the analysis of volatile organic molecules via headspace analysis has been chemometrically applied in a number of studies (Ólafsdóttir *et al.,* 1997; Triqui and Bouchriti, 2003; Ross and Smith, 2006; Alimelli, *et al.*, 2007; Plutowska and Wardencki, 2007). Over the past years the use of various supervised pattern recognition tools in food analysis (a specific application of chemometrics) has been exponentially increasing (Berrueta, Alonso-Salces and Héberger, 2007). The aim of this study was to construct an initial volatile profile, with specific emphasis on the selected peaks, to qualify the changes in these selected volatiles over a period of ten days in juvenile Cape hake fillets, under various storage conditions on ice, and to assess the influence of these changes and the possibility of using these changes for prediction modelling to determine the fish fillets' shelf life.

4.3 MATERIALS AND METHODS

In this Chapter the same Cape hake (*M. capensis* and *M. paradoxus*) sampling protocol was used, as described in Chapters 2 and 3 and sample preparation was performed as described in Chapter 3. Every 48 hours five 1 cm^3 sections of each fillet's muscle tissue were aseptically removed from different areas of each fillet. Subsequently, the samples were processed and handled aseptically to avoid any physical, chemical or microbiological contamination.

4.3.1 Volatile sampling and analysis

Muscle tissue cuts of 1 cm^3 were homogenised with a mortar and pestle and 1 g of the homogenate placed in a glass vial and sealed with petrifilm (3M, SA). These samples were refrigerated until analysis. Sealed samples were placed in an oven at 50 ºC for 1 hour, prior to analysis. Volatile analysis was done by the injection of a compressed 0.2 ml headspace sample (0.8 ml compressed to 0.2 ml) into a Finnegan Focus DSQ mass spectrometer, for mass detection of fragments with a *m/z* smaller than 1000. Mass analysis was performed at 70 eV with an ion source temperature of 200 ºC. Integration of the peaks was performed on a TIC (Total Ion Chromatogram), using Xcalibur software (Finnegan). All the results used in the analysis were the means of five repetitions.

4.3.2 Mathematical estimations

The results presented were the means of at least five repetitions. To determine the Temperature Percentage Difference (TPD), each data point was calculated as follows, for either the inoculated, or the control samples for each time interval:

$$TPDa = \left(\frac{\bar{x}c(t)8\,^{\circ}\mathrm{C}}{\sum d} \right) \times 100) - \left(\frac{\bar{x}c(t)25\,^{\circ}\mathrm{C}}{\sum d} \right) \times 100 \qquad (1)$$

Where:

TPD	=	temperature percentage difference
a	=	storage treatment (C or I)
$\bar{x}$	=	the mean of 5 samples
c	=	each selected volatile
t	=	each time interval, either 0,2,4,6,8,10
d	=	the sum of all the selected volatiles

Similarly, to determine the microbial influence percentage difference (MIPD), each data point was calculated as follows for either the 8 ºC or 25 ºC samples at each time interval:

$$MIPDe = \left(\frac{\bar{x}c(t)C}{\sum d} \right) \times 100) - \left(\frac{\bar{x}c(t)I}{\sum d} \right) \times 100 \qquad (2)$$

Where:

MIPD = microbial influence percentage difference

e = Temperature (8 ºC or the 25 ºC)

$\bar{x}$ = mean of five samples

c = each selected volatile

t = each time interval, b is either 0,2,4,6,8,10

C = Control

I = Inoculated

d = sum of all the selected volatiles

Polynomial curve fits were performed on each individual component, to the order of maximum 4 and R^2 with Microsoft Office Excel 2003.

4.4 RESULTS AND DISCUSSION

Figure 4.2 shows the chromatogram of one of the initial samples (Time 0), all of which had a similar “footprint” of five peaks. This footprint has been visible in all the chromatograms from the onset throughout the sampling, until the end of the experiment. This typical structure (footprint) has specific peaks, identified as 1: nitrogen (N_2) with Chemical Abstract Service (CAS) registry number 7727379, 2: ethyl acetate with CAS registry number 141786, 3: ethanol with CAS registry number 64175, 4: trichloromethane with CAS registry number 67663 and 5: water with CAS registry number 7732185. These were slightly changed in the relative

abundance of each component per peak over the time, and with slight differences for the different temperatures, and if it were excessively contaminated (I) or not (C). A composite graph of the TIC that indicates the main peaks at the final sampling of the experiment from all the variables, namely two chromatograms for T5 (day 10) at 8 ºC for both "control" and "inoculated", as well as two chromatograms for T5 (Day 10) at 25 ºC for both "control" and "inoculated", is displayed in Figure 4.3.

As expected, changes occurred in all the volatile components over the sampling period of ten days, and there were also visible differences between the two environmental storage temperatures, as well as when the fish fillets were exposed to excessive micro-organisms. As indicated in Figure 4.1, changes in volatiles do occur and can be the result of, or influenced by numerous reactions. Although the specific reactions that took place and the reasons for the changes in the volatiles during the duration of sampling were not analysed, it may be that such reactions are inherent metabolic reactions, that may influence all raw filleted fish under similar conditions. Josephson (1991) as well as Durnford and Shahidi (1998) have explained the reactions that influence and change volatile components of fish.

The percentage difference of the temperature and microbial changes that occurred during the study, are for the purpose of this study and are characterised under the following shapes, as displayed by the polynomial graph: (1) a negative Bell curve (resembling an upside down bell), (2) a positive Bell curve (resembling the form of a bell), also known as a Gaussia-peak, or (3) a double Bell curve – either positive and or negative. With the polynomial equations to the 4th degree (also known as a quartic equation), these double Bell curves are expected from these graphs and either in the shape of an M (double positive) or W (double negative), depending on whether the constant is positive or negative (Otto, 2007; McDonald, 2009).

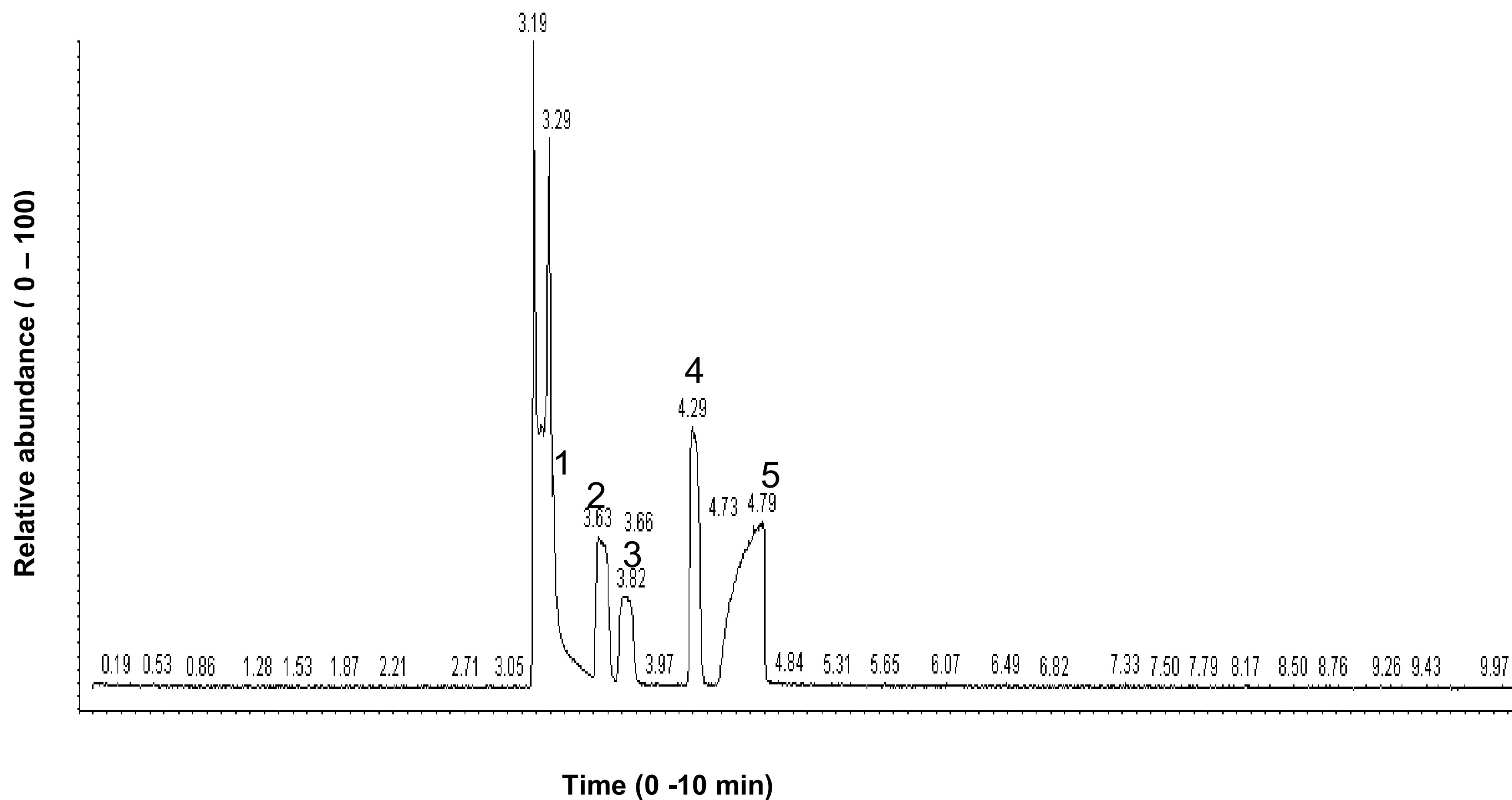

Figure 4.2 A TIC chromatogram indicating the main peaks of one of the samples at Time 0, (the start of the experiment). The Peaks are identified as: 1 = nitrogen, 2 = ethyl acetate, 3 = ethanol, 4 = trichloromethane and 5 = water

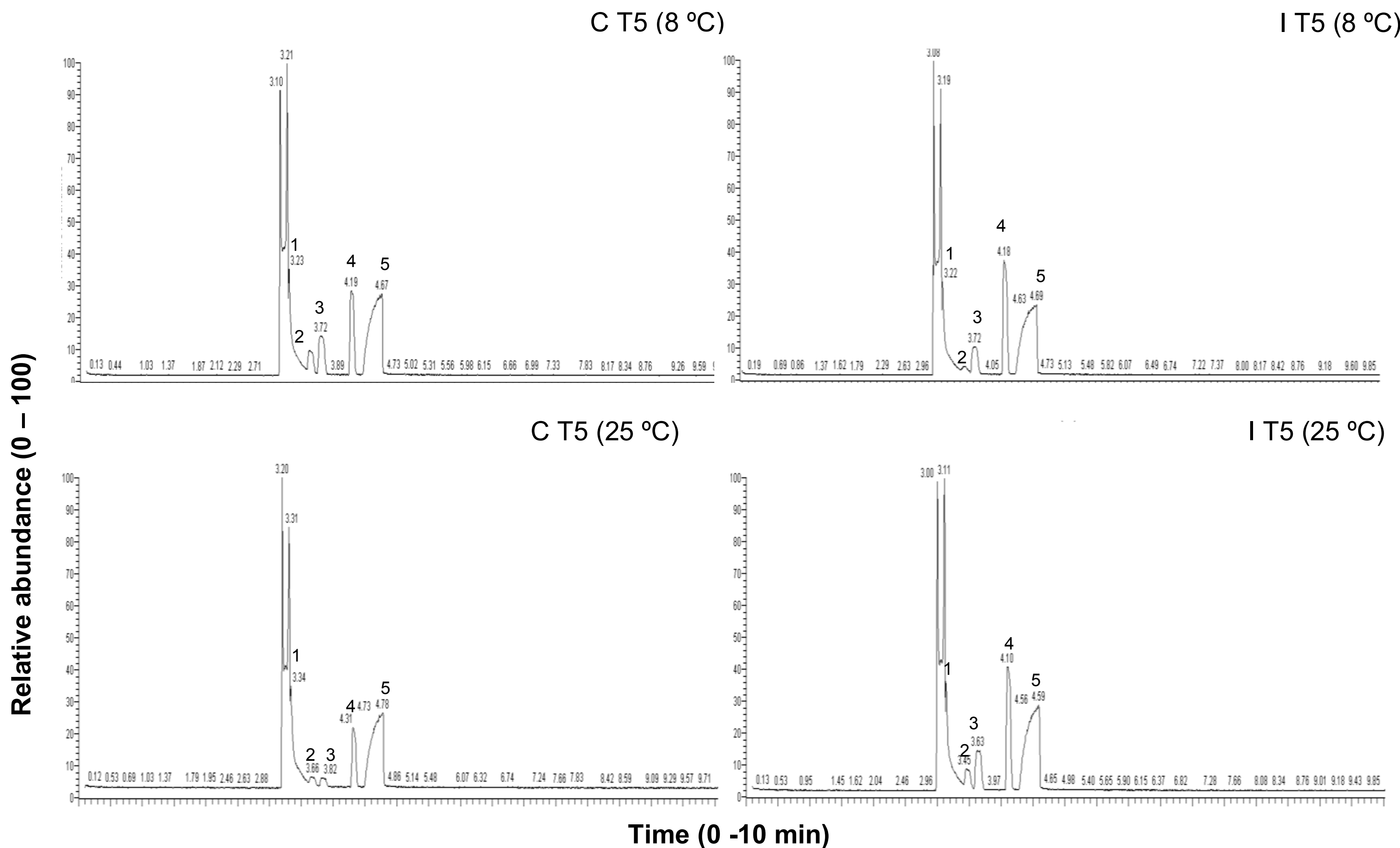

Figure 4.3 A composite graph of reconstructed TIC chromatograms that indicate the main peaks at the final sampling point (T5) for each control (C) and inoculated (I) at 8 ºC and 25 ºC. Peaks are identified as: 1 = nitrogen, 2 = ethyl acetate, 3 = ethanol, 4 = trichloromethane and 5 = water

4.4.1 Temperature percentage difference (TPD)

Changes in the selected “footprint” volatiles (Fig. 4.4) were evident for both the “control” (C8 volatile component values in percentage subtracted from the C25 volatile component values in percentage) and “inoculated” (I8-I25) samples. No linear temperature percentage difference (TPD) was observed (Fig. 4.4).

4.4.1.1 Temperature percentage difference (TPD) changes in volatile component values over time

Among the samples taken from fillets exposed to excessive microbiota (“inoculated”), the most prominent temperature percentage difference (TPD) changes of the volatile components nitrogen (a) and trichloromethane (d) are displayed during the 3rd (day six) and 4th (day eight) sampling period. However, these changes are opposite since “a” is displayed as a positive Bell curve and “d” as a negative Bell curve. The volatile components “b” (ethyl acetate) and “e” (water) both have an initial negative percentage difference for the inoculated volatiles. As expected from quartic polynomial equations, most of the volatile graphs displayed more than one curve. For the inoculated samples, the only clear double positive double curve (in the shape of an M) was found with ethyl acetate (b). The most prominent TPD for the “control” volatiles (fillets that were not exposed to excessive microbial contamination) occurred at day four, as seen in graphs “a” (nitrogen), “c” (ethanol), “d” (trichloromethane) and “e” (water), with “b” (ethyl acetate) as the exception at the beginning of the experiment. More double curves were distinguished for the control graphs. These are observed in graphs “c” (ethanol), ”d” (trichloromethane) and “e” (water), which had an M shape (double positive curve), while additionally graph “a” (nitrogen), had a W shape (double negative curve). At the end of the sampling period ethyl acetate “b” had opposite TPD, while nitrogen “a” was similar.

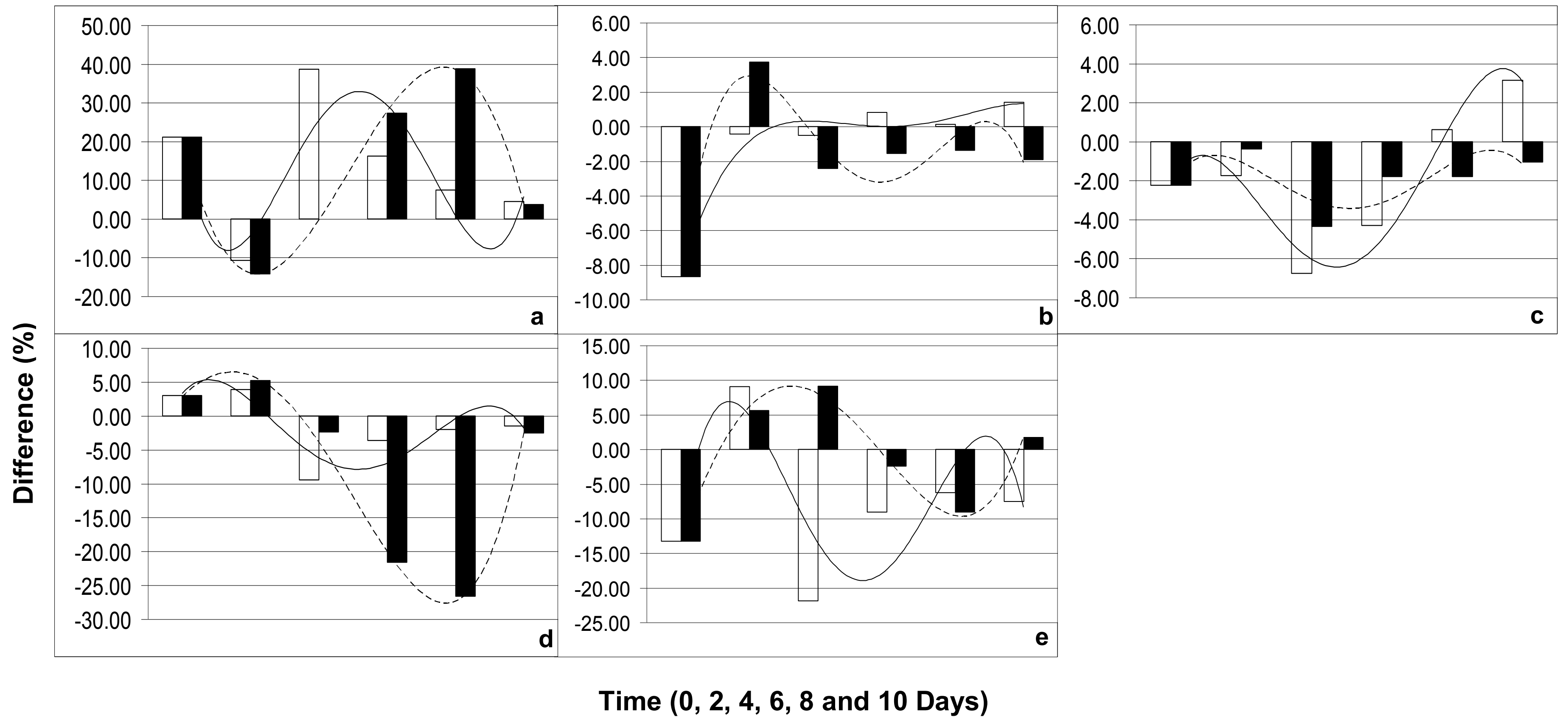

Figure 4.4 Percentage difference of the changes in volatiles in hake fillets stored at 8 ºC minus 25 ºC for control (☐) and similarly for inoculated (■) fillets over a period of ten days. The curve fits are indicated for control (—) and inoculated (---), where a = nitrogen, b = ethyl acetate, c = ethanol, d = trichloromethane, e = water.

4.4.1.2 Remaining hake fillet shelf life prediction by mathematical estimations

The mathematical equations describing the temperature percentage differences of the volatiles over a ten day period, were defined by polynomial equations, as shown in Table 4.1. All the volatiles for both the ―control", as well as the ―inoculated" fillets were defined by 4-grade polynomial equations. However, when considering the correlation coefficient (R^2), certain volatiles may be better indicators of the remaining shelf life than others, and a combined formula of more than one volatile, which relates the information of the selected volatiles, should give a more accurate estimate of the remaining shelf life. For the ―control" and the ―inoculated" formulae the only volatile equation, where both C and I have a $R^2 \geq 0.90$, is ethyl acetate (peak 2). Relevant to the hake investigated in this study, the following two formulae (control and inoculated) are proposed as collective representation of the changes in fatty acid metabolites, occurring during the storage of hake fillets on ice:

Control:

$$y = -0.1197x^4 + 2.056x^3 - 12.647x^2 + 33.1x - 30.989$$

Inoculated:

$$y = -0.5344x^4 + 8.0944x^3 - 42.787x^2 + 91.159x - 64.449$$

Where *x* is the predicted remaining shelf life and *y* is the TPD for the selected volatile.

It is also possible to select a certain volatile from Table 4.1 and to perform a prediction on the remaining shelf life, by solving the associated equation. These equations can contribute towards a model to calculate the remaining fillet shelf life, by using the volatile data as variable.

Table 4.1 Mathematical equations representing the patterns of selected volatiles TPD during the storage of control and inoculated hake fillets on ice

Figure	Difference Table	Contaminated	Equation: $y = Ax^n + bx^n - c$	R^2
Fig. 4.2 a	Nitrogen	C	$y = 3.0209x^4 - 43.42x^3 + 211.82x^2 - 400.86x + 249.31$	$R^2 = 0.6871$
		I	$y = 0.1188x^4 - 6.9141x^3 + 62.889x^2 - 177.18x + 142.23$	$R^2 = 0.9998$
Fig. 4.2 b	Ethyl acetate	C	$y = -0.1197x^4 + 2.056x^3 - 12.647x^2 + 33.1x - 30.989$	$R^2 = 0.9766$
		I	$y = -0.5344x^4 + 8.0944x^3 - 42.787x^2 + 91.159x - 64.449$	$R^2 = 0.9147$
Fig. 4.2 c	Ethanol	C	$y = -0.372x^4 + 5.2134x^3 - 23.984x^2 + 41.106x - 24.13$	$R^2 = 0.9783$
		I	$y = -0.188x^4 + 2.6841x^3 - 12.907x^2 + 23.805x - 15.5$	$R^2 = 0.5046$
Fig. 4.2 d	Trichloromethane	C	$y = -0.6316x^4 + 8.8047x^3 - 40.704x^2 + 68.892x - 33.064$	$R^2 = 0.7662$
		I	$y = 0.348x^4 - 2.3548x^3 - 1.0765x^2 + 17.639x - 11.742$	$R^2 = 0.9933$
Fig. 4.2 e	Water	C	$y = -1.8976x^4 + 27.346x^3 - 134.48x^2 + 257.76x - 161.13$	$R^2 = 0.6585$
		I	$y = 0.2556x^4 - 1.5095x^3 - 6.1188x^2 + 44.573x - 50.539$	$R^2 = 0.9919$

R^2 = correlation coefficient

4.4.2 Microbial influence percentage difference (MIPD)

Figure 4.5 indicates the microbial influence percentage difference (MIPD) for both the 8 ºC and the 25 ºC samples, as explained be equation 2. Although MIPD in the selected volatiles are observed, the high average death rate of $\Delta\bar{x}$ =- 5.6×10^{3} cfu.day^{-1} $\pm1.428x10^{3}$ for both the inoculated samples (I8 and I25), indicate that these percentage differences are from the volatile components, originating from non-culturable micro-organisms, rather than from the volatile components of the hake, or may be volatile components from the micro-organisms themselves. The high death rate could be explained by the super chilling of the fish fillets during the night time, as has been previously observed (Theron *et al.*, 2003).

4.4.2.1 Microbial influence percentage difference (MIPD) changes in volatile component values over time

In general, the volatiles displayed more than one curve, as is the norm for quartic polynomial equations in Figure 4.5. Prominent microbial influence percentage difference (MIPD) are visible for the 8 ºC volatiles (those fillets that were not exposed to excessive microbial contamination at 8 ºC subtracted, from those that were exposed at 8ºC) at day two for ethyl acetate (graph “b”), day four for both nitrogen and water (graphs “a” and “e”). All the 8 ºC volatiles graphs display evident double curves. Similar to the control, graphs “c” (ethanol), “d” (trichloromethane) and “e” (water) had an M shape (double positive curve). Graph “a” (nitrogen) as well as “b” (ethyl acetate) had a W shape (double negative curve). The most prominent microbial influence percentage difference (MIPD) changes of the 25 ºC volatile graphs, volatile components are displayed on the 8th day for graphs “a” (nitrogen), “c” (ethanol), “d” (trichloromethane) and “e” (water), with ethyl acetate being the only exception that had the most prominent MIPD on day six.

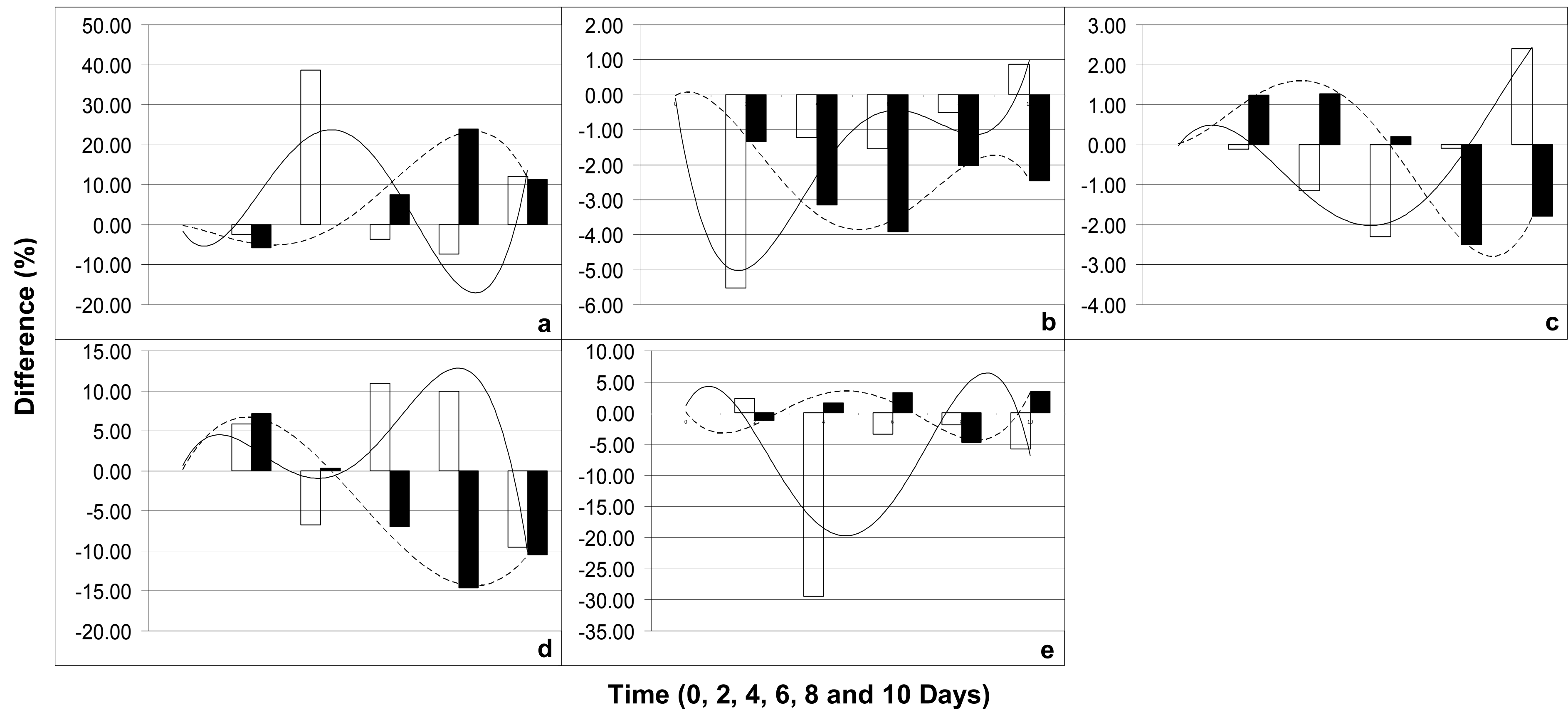

Figure 4.5 Microbial influence percentage difference (MIPD) in volatiles in hake fillets stored at 8 ºC (☐) control minus inoculated and stored at 25 ºC (■) control minus inoculated fillets over a period of ten days. Curve fits are indicated for 8ºC (—) and 25ºC (---), a = nitrogen, b = ethyl acetate, c = ethanol, d =trichloromethane, e = water.

The only apparent double positive double curve (in the shape of an M) was found with ethyl acetate (b), and the only negative double curve (in the shape of a W) was found with water (e). At the end of the sampling period ethyl acetate (b), ethanol (c) and water (e) had opposite MIPD and nitrogen (a), while trichloromethane was similar. However, none was found at equal percentage differences.

4.4.2.2 Remaining hake fillet shelf life prediction by mathematical estimations

Mathematical equations describing the microbial influence percentage differences of the volatile components over the ten day sampling period, were defined by polynomial equations (Table 4.2). All the volatiles for both storage methods were defined by 4-grade polynomial equations. As reported in 4.4.1.2 these equations may contribute towards a model to calculate the remaining fillet shelf life, by using the volatile data as variable. However, when considering the correlation coefficient (R^2) as well as the MIPD (Fig. 4.5), it is clear that certain volatiles could be better indicators of the remaining shelf life. A combined formula of more than one volatile, that relate the information of the selected volatiles should, therefore, give a more accurate estimate of the remaining shelf life. For the "control" and the "inoculated" formulae, the only volatile equation where both C and I have a R^2 ≥0.90, is ethanol.

Table 4.2 Mathematical equations representing the patterns of selected volatiles MIPD changes during the storage of control and inoculated hake fillets on ice at 8 ºC and 25 ºC

Figure	**Difference Table**	**Temperature (ºC)**	**Equation:**	R^2
Fig. 4.3 a	Nitrogen	8	$y = 2.3257x^4 - 30.119x^3 + 128.28x^2 - 202.21x + 100.19$	$R^2 = 0.6006$
		25	$y = -0.5764x^4 + 6.3876x^3 - 20.643x^2 + 21.475x - 6.8905$	$R^2 = 0.9724$
Fig. 4.3 b	Ethyl acetate	8	$y = 0.2798x^4 - 4.1902x^3 + 21.917x^2 - 45.494x + 27.379$	$R^2 = 0.8807$
		25	$y = -0.135x^4 + 1.8481x^3 - 8.2227x^2 + 12.565x - 6.0822$	$R^2 = 0.9818$
Fig. 4.3 c	Ethanol	8	$y = -0.081x^4 + 1.2872x^3 - 6.5447x^2 + 11.948x - 6.6457$	$R^2 = 0.9719$
		25	$y = 0.103x^4 - 1.2422x^3 + 4.5328x^2 - 5.354x + 1.985$	$R^2 = 0.9875$
Fig. 4.3 d	Trichloromethane	8	$y = -1.0107x^4 + 12.789x^3 - 54.574x^2 + 91.703x - 48.324$	$R^2 = 0.7686$
		25	$y = -0.0311x^4 + 1.6298x^3 - 14.946x^2 + 40.45x - 27.002$	$R^2 = 0.9922$
Fig. 4.3 d	Water	8	$y = -1.5138x^4 + 20.233x^3 - 89.082x^2 + 144.06x - 72.598$	$R^2 = 0.5548$
		25	$y = 0.6395x^4 - 8.6233x^3 + 39.28x^2 - 69.136x + 37.99$	$R^2 = 0.8816$

$R^2 =$ correlation coefficient

Relative to the hake investigated in this study, the following two formulae (8 ºC and 25 ºC) are proposed as a collective representation of the microbial influence percentage difference in fatty acid metabolites, occurring during the storage of hake fillets on ice, that were exposed to excessive micro-organisms:

8 ºC:

$$y = -0.081x^4 + 1.2872x^3 - 6.5447x^2 + 11.948x - 6.6457$$

25 ºC:

$$y = 0.103x^4 - 1.2422x^3 + 4.5328x^2 - 5.354x + 1.985$$

Where *x* is the predicted remaining shelf life and *y* is the MIPD in the selected component. It should also be possible to select a certain volatile from Table 4.2, and to perform a prediction on the remaining shelf life, by solving the individual equation.

4.5. CONCLUSION

Although the equations in this Chapter may be suitable only to volatiles of hake stored under conditions as stipulated, the applicability in the retail industry to use a single or a combined model, may have potential in determining whether the hake was subjected to excessive microbial spoilage, or whether the environmental temperature was controlled (was the cold chain integrity kept or not?). It would be helpful to determine the possibility of utilising the same volatile formula on other hake species, or even on other lean fish species. As indicated in Chapter 3 it may be valuable to further pursue the effect of super chilling. The combination of normal storage of fillets on ice, with super chilling significantly lowered the autochthonous microbial colonies on the hake.

Another application has been identified for some of the components highlighted in this study. These components correspond to those in a decaying corpse, as reported by Boumba, Ziavrou and Vougiouklakis (2008). This is the first time that ethyl acetate has been reported in decaying fish, and also the first time reported where the parallel is being drawn between human decay (a corpse), and muscle tissue decay. This possibility may influence the way that decay, quality and shelf life of muscle foods are determined in the future.

4.6 ACKNOWLEGDEMENTS

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CHAPTER 5

CONTAMINATION PREDICTIONS OF CAPE HAKE FILLETS DURING DISPLAY AND STORAGE BY ARTIFICIAL NEURAL NETWORK MODELLING OF HEXADECANOIC ACID

5.1 ABSTRACT

The aim of this study was to design an artificial neural network (ANN) that could distinguish between Cape hake fillets, displayed and stored on ice, that have been exposed to excessive contamination, and those that were not. The selected variable was a biochemical indicator, and fatty acid hexadecanoic acid. Cape hake fillets with and without excessive contamination were kept on ice, and analysed every second day, over a period of ten days. A novel ANN was designed and applied, which provided an acceptable prediction on the contaminated fillets, based only on the hexadecanoic acid changes during day 8 (T4) and day 10 (T5). The ANN consisted of a multilayered network, with supervised training arranged into an ordered hierarchy of layers, in which connections were allowed only between nodes in immediately adjacent layers. The network consists of two inputs T4 and T5, connected to two neurons. These two neurons are connected to one output neuron that indicates a prediction on contamination of the fillets. The model sets the stage for the development of alternative quality control measures for retailers and buyers of fish and other food, that contain fatty acids like hexadecanoic acid, to provide consumers with safer food.

5.2 INTRODUCTION

Artificial neural networks (ANNs) have recently seen an explosion of interest and application in numerous fields, including Piscimetrics and food analysis, to model complex real-world problems. Piscimetrics were initiated when neural networks and other chemometrics were applied in fisheries research. This entails the life cycle of fish, fish identification, fish stock, and factors affecting it. However, no ANN has as yet been applied to the final product made available to retail shops, and specifically in predicting the remaining shelf life (Suryanarayana *et al.*, 2008). ANNs' attractiveness to food science is its ability to model the kinds of data encountered in food science. A limited number of "clean" variables are qualified on a suitable number of samples with a basic linear, or at least mildly non-linear model to those, where many variables (possibly noisy or highly correlated) are qualified on a small number of samples and the functional relation is heavily non-linear (Basheer and Hajmeer, 2000; Marini, 2009). In ANN the development of a small database size is a concern, because of the inability to partition the database into manageable sized subsets for training, testing and validation. To expand the size of the database, due to difficulty or expensiveness to obtain new data in a conventional manner, is to interject random noise in the available examples, to generate new ones (Swingler, 2001). This addition of noise enhances the ANN's robustness against measurement error (for example, noise = ± instrument sensitivity), and is called "data enrichment" (Basheer and Hajmeer, 2000).

ANNs, classified as artificial intelligence (AI), is a family of mathematical models, where the main algorithmic features are inspired by the functioning of the human brain, simulating human intelligence. However, currently a neural network is predominantly a mathematical, rather than a biological model (Callan, 2003; Marini, 2009). ANN is based on collections of neurons or nodes that are connected in a tree model to permit communication (Callan, 2003). A single node computes by combining the input signals with an activation function to produce an output signal (Fig. 5.1) (Callan, 2003).

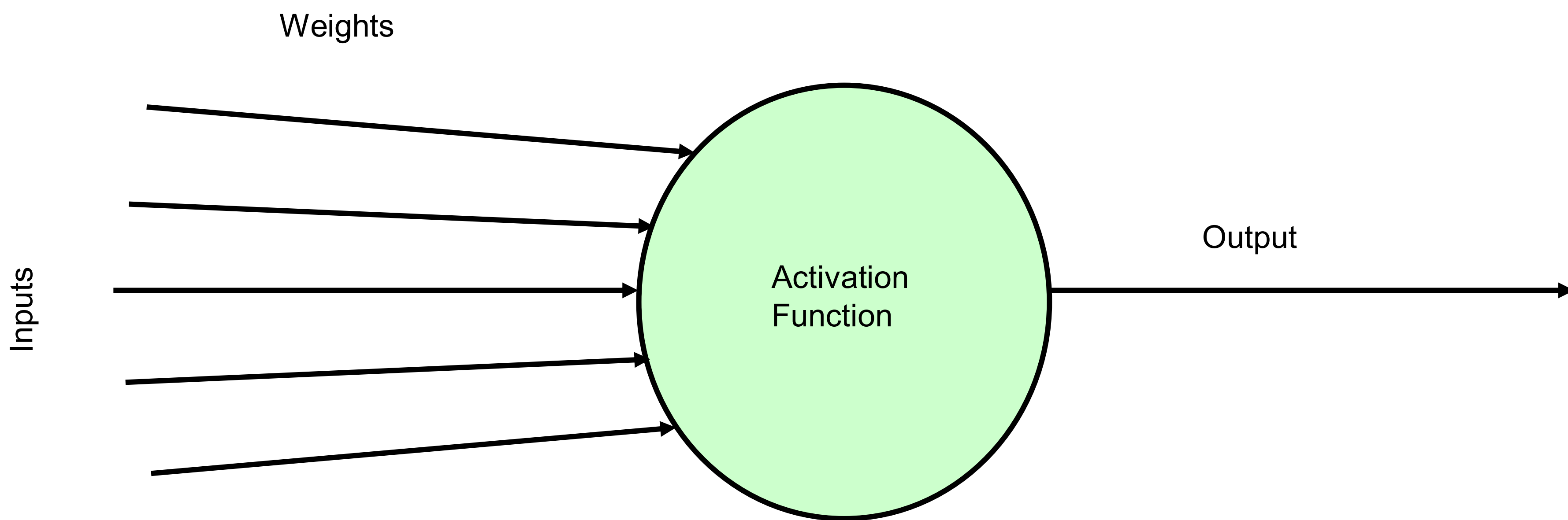

Figure 5.1 Layout of a single network node (adapted from Callan, 2003).

These nodes are interconnected with weighted connections – weight being a multiplying constant for the connection's input. In isolation, these nodes are limited in operation, but interconnection in a multilayered network gives them the ability to perform complex tasks in food analysis and food safety. These include food authentication, prediction whether a foodstuff is contaminated or not, as well as the identification of the kind of microbial contamination and ultimately, to determine the freshness or shelf life of food products (Bertone *et al.*, 1996; Siripatrawan and Jantawat, 2008; Limbo *et al.*, 2009; Marini, 2009; Siripatrawan *et al.*, 2009).

Freshness of fish during storage depends on various factors, including storage temperature, the fish species and its physiological condition, initial microbial load, contamination, and physical handling (Huss, 1995; Raatikainen *et al.*, 2005). Deterioration of fish lipids are primarily caused by two distinct reactions, namely hydrolysis and oxidation - generally from endogenous enzymes and contaminant bacteria (Refer to Chapter 1) (Frazier and Westhoff, 1988; Jay, 1992; Huss 1995). These will influence amongst others, the organoleptic properties and therefore, the shelf life of the final product (Ackman, 1989; Huss, 1995; Aubourg, 1999; Baixas-Nogueras *et al.*, 2002). Sensory scoring methods, such as the Quality Index Method (QIM), have been developed for the evaluation of fish freshness and chemical and biochemical parameters have been used in numerous studies. These parameters include pH, trimethylamine, K value, peroxide index and free fatty acids (Pacheco–Aguilar *et al.*, 2000; Baixas-Nogueras *et al.*, 2003; Herrero *et al.*, 2003). Free fatty acid levels in hake muscle correlated well with sensory scoring methods from previous studies, and a conclusion was formed that the free fatty acid level could be used, instead of sensory scoring methods to determine hake freshness (Barassi *et al.*, 1987).

Prediction of remaining shelf life of whole and filleted fish has been investigated, using numerous chemometric applications (Barassi *et al.*, 1987; Limbo *et al.*, 2009). However, free fatty acids have not been used in combination with ANN to

predict if Cape hake fillets have been exposed to excessive contamination. The aim of this study was to apply a custom-designed ANN to a basic variable, namely hexadecanoic acid to be applied in producing and validating a recognition pattern, that may be used to predict whether fish fillets were exposed to excessive microbial contamination originating from, amongst others, improper handling or storage.

5.3 MATERIALS AND METHODS

Freshly caught Cape hake (*M. capensis* and *M. paradoxus)* were obtained from the South African shoreline close to Cape Town. The sampling of the fish was done similar to described in Chapter 3 (Herrero *et al.* 2003). Please refer to section 3.3.1.

5.3.1 Fatty acid extraction

Fatty acid extraction was done as described in Chapters 2 and 3 by means of the Folsch methodology (Folsch *et al.,* 1957).

5.3.2 Fatty acid analysis

The fatty acid analysis method used was similar to Chapter 2 section 2.3.3.

5.3.3 Data analysis

Total signal results of all the fatty acids were used to determine the final data used in the ANN. The raw data underwent several pre-processing techniques, for example, reducing input dimensionality and data transformation, treatment of non-normally distributed data, data inspection and deletion of outliers to accelerate convergence, before it could be used for training in an ANN. The starting point was the same raw data for hexadecanoic acid used in Chapter 3. Every individual fatty acid's total signal was divided by the total of all the fatty acids to render the data between 0 and 1. All calculations were done using Microsoft Office Excel 2003.

It was complex to create an ANN, with the available hexadecanoic acid results to determine both the difference between the temperature, as well as excessively contaminated samples. In order to increase the number of hexadecanoic acid results, an ANN was created to differentiate only between excessively contaminated samples, or non-contaminated samples. With two examples of contaminated and two of non-contaminated in a training set, more data were required to effectively teach the neural network. Random noise of up to +- 20 % were, therefore, added to the data set at each learning cycle, artificially extending the data set. This provided a larger training set ,as well as a model with more robust generalisation properties (Swingler, 2001).

It is important to balance the data in classification problems. Training data were distributed as evenly as possible between the various classes, to prevent the network from being biased to the over-represented classes. Some of the over-represented classes may, therefore, be removed or extra examples added, pertaining to the under-represented class. Alternatively, under-represented input/output examples may be duplicated and random noise added to their input data, while keeping the output class unchanged (Basheer and Hajmeer, 2000).

5.3.4 Artificial neural network modelling

By calculating the error at each net or node, followed by the adjustment of weights accordingly to produce all the required outputs, a multilayered network with supervised training was designed, to be able to learn a required function. This process can be mathematically simulated with the formula of the neuron, as follows (Callan, 2003):

$$net_j := \sum_{i=1}^{N} x_{i,j} w_{i,j} \qquad (1)$$

Where:

N	=	number of inputs
i	=	node number for a specific input
j	=	number of the net
x	=	input value
w	=	weights or constants

This is commonly put through a sigmoid function, as follows (Callan, 2003):

$$f_j := \frac{1}{1 + \left[e^{\left(-net_j\right)}\right]} \qquad (2)$$

Where:

net	=	output of the net
j	=	number of the net

To calculate the error, the network applies a generalisation of the delta rule by starting at the last layer with (Chauvin and Rumelhart, 1995; Callan, 2003):

$$\delta_j := (t_j - o_j) \cdot o_j (1 - o_j) \quad (3)$$

Where:

t	=	required output
o	=	net output
j	=	number of the net

Subsequently, the error at the hidden layers is calculated as follows (Callan, 2003):

$$\delta_j := o_j (1 - o_j) \cdot \sum_k \delta_k w_{j,k} \quad (4)$$

Where:

o	=	net output
j	=	number of the net
k	=	number of the net from where the error originates
δ_k	=	error from the previous layer
l	=	number of that specific path

The weight change for each node is then calculated with (Callan, 2003):

$$\Delta w_{i,j} := \eta \cdot \left(x_{i,j} \cdot \delta_j \right) \quad (5)$$

Where:

η	=	learning rate
I	=	node number for a specific input
j	=	number of the net
x	=	input value
δ	=	error from each layer

Thereafter the weights are adjusted as follows (Callan, 2003):

$$W_{i,j} := w_{i,j} + \Delta w_{i,j} \quad (6)$$

Where:

Δw	=	weight change
w	=	old weight

A training data set was mapped that simulates a real-world problem. This training data set consisted of inputs with the corresponding outputs, that were fed to the neural network for weight adaptation. It is advantageous to randomise the order of the presentation for each training sample (Callan, 2003; Gurney, 2003; Marini, 2009).

5.4 RESULTS AND DISCUSSION

A multilayered network with supervised training was arranged into an ordered hierarchy of layers, in which connections are allowed only between nodes in immediately adjacent layers, which coded for evaluations. The network consists of two inputs T4 (data from day 8) and T5 (data from day 10), connected to two neurons. These two neurons are connected to one output neuron. The output neuron produces one output, that indicates a prediction on contamination. This is a network with two layers of weights, capable of approximating any continuous functional mapping. The inputs of T4 and T5 of hexadecanoic acid, methyl ester were used to train the network. The output would be a percentage of probability of contamination at 8 °C.

The sigmoid‘ s best resolution for an output is between 0.9 and 0.1. The input values are between these parameters and need no further processing. The output is converted from “yes” and “no” to a probability of “yes” (contaminated). The output range would be from 0.1 of 0 % probability and 0.9 for a 100 % probability. To ease interpretations, the output is stepped through a function that would give the probability as a percentage. This function is as follows:

$$y = \frac{(x - 0.1)}{0.8} \times 100 \qquad (7)$$

Where:

y = output probability in percentage

x = output of the network in the range of 0.1 for 0 % to 0.9 for a 100 %

The “yes” prediction is converted to 0.9 and a no prediction to 0.1, and the data divided into training and evaluation sets as per Table 5.1.

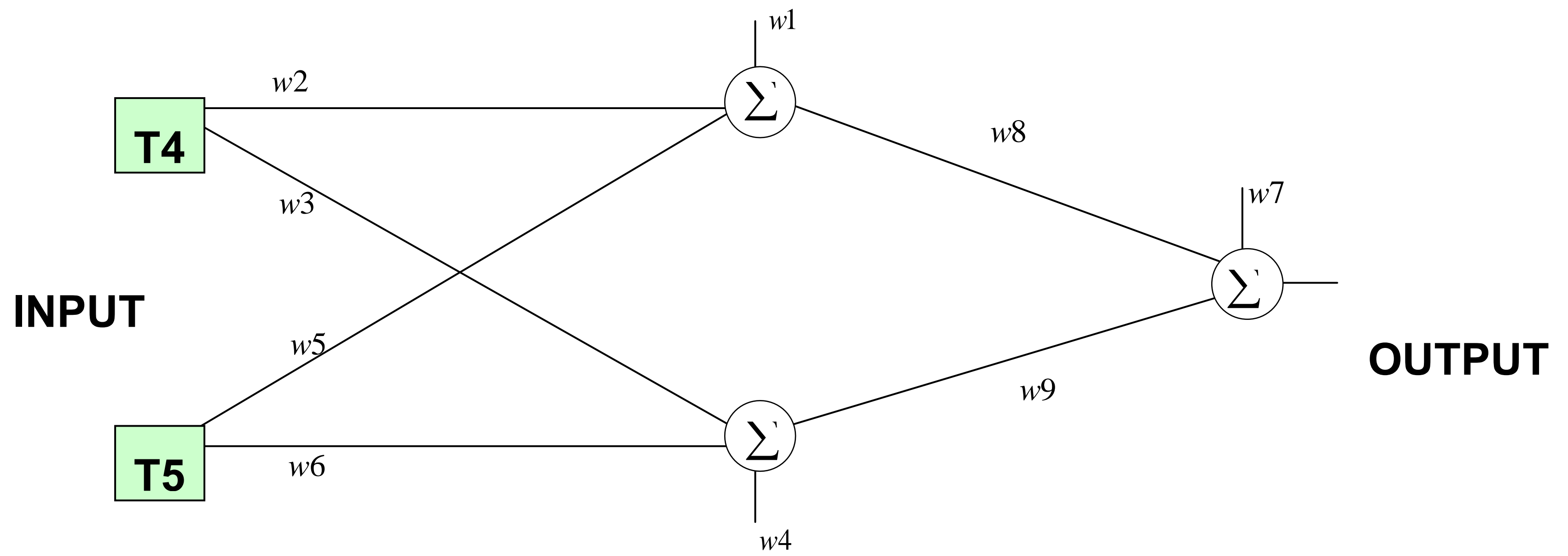

Figure 5.2 Multilayered network for the two analogue inputs to two nodes to one node to the one output.

Table 5.1 The data sets used in the neural network, divided into the necessary training and evaluation sets.

Training data set	**1**	**2**	**3**
T4	0.551429	0.541386	0.551429
T5	0.487019	0.518307	0.487019
Control	0.1	0.1	0.1
T4	0.541386	0.522321	0.522321
T5	0.518307	0.515228	0.515228
Control	0.1	0.1	0.1
T4	0.497288	0.506668	0.497288
T5	0.50756	0.516287	0.50756
Contaminated	0.9	0.9	0.9
T4	0.506668	0.498248	0.498248
T5	0.516287	0.502239	0.502239
Contaminated	0.9	0.9	0.9
Evaluation set	**1**	**2**	**3**
T4	0.522321	0.551429	0.541386
T5	0.515228	0.487019	0.518307
Control	0.1	0.1	0.1
T4	0.498248	0.497288	0.506668
T5	0.502239	0.50756	0.516287
Contaminated	0.9	0.9	0.9

There are only two examples of contaminated and two examples of non contaminated (control) in a training set. This is not enough to effectively teach the neural network. To expand this, random noise of up to approximately 20 % was added to the data set at each learning cycle. Artificially extending the data set would provide a larger training set, as well as a model with more robust generalisation properties (Swingler, 2001).

A training cycle would be as follows:

- Read randomly in a sample from the training set (T4, T5 and the corresponding output).
- Add random noise of up to +- 20 % to T4 and T5.
- Compare the network output with the required output, calculate the error and adjust the weights.

Start again.

This is done until acceptable results are produced. The evaluation is fed in to evaluate (it was never part of the training set), and the neural network is evaluated. The neural network is evaluated on the learning set, plus noise, including the evaluation set. The network is trained with a training set, that will consist of T4, T5 with their corresponding outputs. This is randomised and the noise is added (Table 5.2).

Since random noise is added, it is not possible to over-train the network, whereas 500 000 training cycles, at a learning rate of 0.5, would be sufficient training. Training took 232 seconds on an Intel Pentium 4 CPU 3.40 GHz, 2.87 GB Ram.

The ANN model was able to predict correctly, whether the fillets were contaminated or not (control), after all three training sets during the evaluation set. Even on the first set where the prediction was high (40 %), it was still under the 50 % split, suggesting the control fillet, which is correct.

Table 5.2 Six examples of each sample (T4 and T5) within each training data set (1, 2 and 3) plus 20 % noise after the neural network was taught for 500 000 cycles at a rate of 0.5, including the evaluation data set and the results.

Training data set 1	**1st sample**	**Control**	**2nd sample**	**Control**	**3rd sample**	**Contaminated**	**4th sample**	**Contaminated**
T4	0.5514	0.1	0.5414	0.1	0.4973	0.9	0.5067	0.9
T5	0.4870		0.5183		0.5076		0.5163	
Six examples of input values from each set plus random ±20 % noise								
Input / Output	**1st set**	**Output**	**2nd set**	**Output**	**3rd set**	**Output**	**4th set**	**Output**
T4	0.4741	-11.9581	0.4806	2.2766	0.4330	97.8478	0.4582	97.1088
T5	0.4187		0.4601		0.4419		0.4669	
T4	0.5758	-12.1758	0.4451	4.4470	0.4219	97.4932	0.5741	99.6816
T5	0.5085		0.4261		0.4306		0.5850	
T4	0.6222	-12.2235	0.4529	3.9397	0.4490	98.3333	0.4627	97.2353
T5	0.5495		0.4336		0.4583		0.4715	
T4	0.5132	-12.0681	0.6106	-3.1895	0.4152	97.2726	0.4086	95.5912
T5	0.4533		0.5846		0.4237		0.4163	
T4	0.5801	-12.1811	0.5288	-0.1423	0.4618	98.7015	0.4882	97.9036
T5	0.5123		0.5063		0.4713		0.4975	

T4 T5	0.4730 0.4178	-11.9545	0.4627 0.4429	3.3266	0.4470 0.4563	98.2759	0.5188 0.5286	98.6195
Evaluation data set 1			**Status**			**Trained net output**		**Prediction**
T4 T5	1st set	0.5223 0.5152	Control			40.3814		✓
T4 T5	2nd set	0.4982 0.5022	Contaminated			84.4114		✓
Training data set 2	**1st sample**	**Control**	**2nd sample**	**Control**	**3rd sample**	**Contaminated**	**4th sample**	**Contaminated**
T4 T5	0.5414 0.5183	0.1	0.5223 0.5152	0.1	0.5067 0.5163	0.9	0.49825 0.50224	0.9
Six examples of input values from each set plus random ± 20 % noise								
Input / Output	**1st set**	**Output**	**2nd set**	**Output**	**3rd set**	**Output**	**4th set**	**Output**
T4 T5	0.5880 0.5630	-12.4453	0.5880 0.5630	3.4874	0.4523 0.4609	109.2513	0.5127 0.5168	98.9813
T4 T5	0.4518 0.4325	-12.2487	0.4518 0.4325	6.3607	0.4286 0.4367	108.7255	0.5707 0.5753	101.2974

T4 T5	0.4601 0.4404	-12.2727	0.4601 0.4404	3.1107	0.5796 0.5906	110.9931	0.5700 0.5746	101.2706
T4 T5	0.6109 0.5848	-12.4566	0.6109 0.5848	5.8861	0.5839 0.5950	111.0299	0.4045 0.4078	93.7142
T4 T5	0.5103 0.4885	-12.3735	0.5103 0.4885	4.4205	0.4228 0.4309	108.5856	0.5978 0.6026	102.2628
T4 T5	0.5077 0.4860	-12.3697	0.5077 0.4860	8.1403	0.5251 0.5351	110.4236	0.4535 0.4571	96.2505
Evaluation data set 2			**Status**			**Trained net output**		**Prediction**
T4 T5	1st set	0.5514 0.4870	Control			-12.5000		✓
T4 T5	2nd set	0.4973 0.5076	Contaminated			110.6229		✓
Training data set 3	**1st sample**	**Control**	**2nd sample**	**Control**	**3rd sample**	**Contaminated**	**4th sample**	**Contaminated**
T4 T5	0.5514 0.4870	0.1	0.5223 0.5152	0.1	0.4973 0.5076	0.9	0.49825 0.50224	0.9
Six examples of input values from each set plus random ±20 % noise								
Input /	**1st set**	**Output**	**2nd set**	**Output**	**3rd set**	**Output**	**4th set**	**Output**

Output								
T4	0.5056	-12.5000	0.4212	2.9666	0.5103	109.9299	0.5842	97.2802
T5	0.4465		0.4154		0.5208		0.5889	
T4	0.4518	-12.5000	0.5223	-1.1512	0.4797	109.3321	0.4067	86.0592
T5	0.3990		0.5152		0.4896		0.4100	
T4	0.5899	-12.5000	0.5325	-1.4766	0.4601	108.8758	0.4556	89.5618
T5	0.5210		0.5253		0.4696		0.4592	
T4	0.6604	-12.5000	0.5657	-2.4413	0.3999	106.9862	0.4235	87.2979
T5	0.5833		0.5580		0.4082		0.4269	
T4	0.6418	-12.5000	0.5614	-2.3239	0.5718	110.7972	0.4980	92.3508
T5	0.5669		0.5538		0.5837		0.5020	
T4	0.6433	-12.5000	0.4262	2.7193	0.4483	108.5647	0.4052	85.9470
T5	0.5682		0.4204		0.4575		0.4084	
Evaluation data set 3			**Status**			**Trained net output**		**Prediction**
T4	1st set	0.5414	Control			-12.4513		✓
T5		0.5183						
T4	2nd set	0.5067	Contaminated			109.0538		✓
T5		0.5163						

5.5. CONCLUSION

This study confirms the possibility to use a selected fatty acid, for example, hexadecanoic acid, in an ANN model to effectively predict whether a fillet has been exposed to contamination. The neural network created, trained and tested during this study, ensured an objective and reliable prediction of the freshness of the Cape hake fillets under given conditions. The model supports the development of alternative quality control measures for retailers and buyers of fish and other food that contain fatty acids, such as hexadecanoic acid. This could contribute in providing safer food to consumers, and to improve the due diligence that a supplier has in providing safe foods. However, the ANN presented in this reseaarch is less suitable for endpoint sampling, as growth was followed over 10 days. A network designed to incorporate more than one biological indicator, in addition to chemical indicators should, therefore, be considered.

5.6 ACKNOWLEGDEMENTS

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CHAPTER 6

CONCLUSIONS

6.1 GENERAL CONCLUSIONS

This study focussed on Cape hake, since this fishing sector is of considerable social and economic value to the South African demersal fisheries industry, and the Cape hake plays an important role in the diet of many South Africans. Fish, including lean fish such as the Cape hake, contains various essential fatty acids required by the human body. In South Africa, Cape hake and other marine products are marketed countrywide, and subjected to long distance transport from coastal areas to inland markets (Hutton and Sumaila, 2000). Fish is known to be a highly perishable product, and the maintenance of a continuous cold chain and related expenditures in attempting to keep the product sensorially acceptable to the consumer, present many challenges to the industry.

Protein degradation and lipid oxidation give rise to a considerable number of volatile components, which can be used to determine fish freshness, shelf life, quality and even safety. Volatiles are present on freshly harvested fish, but additional volatiles originate at each production step including storage. During storage, transportation and displaying of fresh fish, the oxidation of autochthonous fatty acids and the break-down of fatty acids, when fish fillets are excessively contaminated, will only be delayed and not eliminated at near freezing storage temperatures.

Several chemometric applications have been reported in the prediction of remaining shelf life of whole and filleted fish (Barassi *et al.*, 1987; Limbo *et al.*, 2009), and various supervised pattern recognition tools (a specific application of chemometrics) are increasingly used in food analysis (Berrueta, Alonso-Salces and Héberger, 2007). Specific aims and objectives were set in this study as seen in Chapter 1. The outlay was, therefore, structured into chapters that correspond with the addressing of each aim. The chapter outlay from Chapter 2 with the aims and main conclusions of each chapter is as follows:

Chapter 2: Establishing profiles of triglycerides in juvenile SA Cape hake (*M. capensis* and *M. paradoxus*) muscle tissue.

- The aim of this Chapter was to elucidate possible fatty acid metabolic pathways, present in the muscle tissue of juvenile Cape hake; to determine the influence of fish size on the distribution of selected fatty acids, and to compare those to relevant literature (old and new) on similar species, in order to highlight possible changes in the nutritional value of Cape hake.

The contribution of saturated fatty acids to the total lipid content was found to be higher, in comparison to literature in other hake species and no EPA was detected. Furthermore, one of the fundamental fatty acids, eicosanoic acid, only occurred in the larger sized hake fillets, indicating a reduced nutritional value in juvenile hake fillets. Size and age influence the total nutritional value of hake, as may be the case in other fish species. This highlights the future challenges of the fishing industry to manage, not only the sustainability of fish resources at a positive level, but also the size of the total allowable catch at an expected satisfactory nutritional level.

Chapter 3: Mathematical predictions of temperature difference and microbial influences on fatty acids isolated from *M. capensis* and *M. paradoxus*.

- The aim of this Chapter was to qualify the changes in selected fatty acid metabolites in Cape hake fillets over a period of ten days under various storage conditions on ice, and to assess the influence on freshness and quality. In addition, these selected fatty acid metabolites were used as chemometric markers to predict the fillet freshness.

The combined equations may have a potential in the retail industry to determine the hake (or similar lean fish) fillet's remaining shelf life, in terms of microbial spoilage and the integrity of the cold chain.

Chapter 4: Mathematical predictions of temperature and microbial influences on selected volatiles in South African Cape hake.

- The aim of this Chapter was to construct the initial volatile profile with specific emphasis on the selected peaks, and to qualify and assess the influence of changes in these selected volatiles over a period of ten days in juvenile Cape hake fillets, under various storage conditions on ice, and to investigate the best prediction modelling.

The combined formulae may have potential in the retail industry, to determine the hake (or even other lean fish) fillet's remaining shelf life, since it could be used to establish if the hake was subjected to excessive microbial spoilage, or whether the environmental temperature was controlled. Additionally, ethyl acetate is indicated as one of the volatile components in fish.

Chapter 5*:* Contamination prediction of Cape hake fillets during display and storage by artificial neural network modelling of hexadecanoic acid.

- The aim of this Chapter was the application of a custom-designed ANN to a selected relevant basic variable Hexadecanoic acid (assessable through laboratory analysis), to be applied in producing and validating a recognition pattern (model), that could be used to recognise if a fish fillet was exposed to excessive microbial contamination.

This study confirms the possibility to use a selected fatty acid, such as hexadecanoic acid, in an ANN model to effectively predict whether a fillet was exposed to contamination. The neural network created, trained and tested during

this study ensured an objective and reliable freshness prediction of the Cape hake fillets under given conditions. The model supports the development of alternative quality control measures for retailers and buyers of fish and other food that contain fatty acids, such as hexadecanoic acid, to provide consumers with safer food and improve the due diligence that a supplier has in providing safe foods. However, the ANN presented in this research is less suitable for endpoint sampling as growth was followed over 10 days. A network was, therefore, designed to incorporate more than one biological indicator, in addition to chemical indicators.

6.2 CONCLUDING REMARKS

6.2.1 Mathematical models

Many mathematical models have been used on various fish species for determining either shelf life, or quality. Not many mathematical models have been designed for Cape hake and this study, therefore, determines percentage differences, using polynomial equations, which describe changes in selected fatty acid metabolites. This study peruses the possibility to use either single, or combined polynomial equations to construct mathematical models, with potential use in the retail industry and to determine whether excessive contamination, or temperature fluctuation (break in cold chain integrity) occurred during storage (as discussed in Chapters 3 and 4). If either occurred, it should be possible to prevent the sales of potentially unsafe fish to the consumer and to optimise resources. Combined equations presented a more precise indicator, whether excessive contamination or temperature fluctuation occurred, rather than single formulae for all the types of compounds (fatty acids or volatiles) (Table 6.1).

Table 6.1 Consolidated Equations representing each of the selected different components (e,g fatty acids and volatiles) in Microbial Influenced Percentage Difference, as well as Temperature Percentage Difference respectively.

Chapter	**Component**	**Percentage Difference**	**Equation**
3	Fatty Acid	Microbial Influence (8)	$5y = -0.08x^4 + 0.89x^3 - 3.56x^2 + 5.85x - 3.07$
3	Fatty Acid	Microbial Influence (25)	$5y = -1.85x^4 + 21.96x^3 - 79.74x^2 + 61.68x - 26.51$
3	Fatty Acid	Temperature (C)	$11y = 0.08x^4 - 15.90x^3 + 98.35x^2 - 254.87x + 231.83$
3	Fatty Acid	Temperature (I)	$11y = 3.48x^4 - 45.42x^3 + 200.88x^2 - 356.62x + 258.42$
4	Volatile	Microbial Influence (8)	$y = -0.081x^4 + 1.2872x^3 - 6.5447x^2 + 11.948x - 6.6457$
4	Volatile	Microbial Influence (25)	$y = 0.103x^4 - 1.2422x^3 + 4.5328x^2 - 5.354x + 1.985$
4	Volatile	Temperature (C)	$y = -0.1197x^4 + 2.056x^3 - 12.647x^2 + 33.1x - 30.989$
4	Volatile	Temperature (I)	$y = -0.5344x^4 + 8.0944x^3 - 42.787x^2 + 91.159x - 64.449$

6.2.2 Artificial neural networks (ANNs)

The concept of artificial neural networks (ANNs) has far reaching potential in the application of food science. In this study it became evident that there is much scope in doing further research on ANN, using biochemical, chemical and microbiological variables in predicting the quality of foodstuffs. Similar to mathematical models, this concept has potential in the retail industry, especially since suppliers should be able to demonstrate due diligence in supplying food products with the highest possible quality, as enforced by the recently promulgated Consumer Protection Act (South Africa. Consumer Protection Act 2008). Biochemical and chemical analysis are costly and therefore, the use of ANN, as well as mathematical models are indispensable in processing data into usable information, to make informed decisions and predictions, regarding the safety and or freshness of fish or any other foodstuffs.

6.2.3 Future Research

A number of research questions, which could be used for future research evolved as a result of this study. These include:

- Investigating the effect of super chilling, in combination with normal storage of fish fillets on ice, on autochthonous microbial colonies on any type of fish, since the excessive microbial load has significantly decreased during the study period.

- Investigating the possibility of farming with Cape hake, to make it a more sustainable protein and fish source for the future generations.

- With the global concern in sustainability and the research showing that mature Cape hake has higher nutritional value, it would be interesting to determine the nutritional difference of farmed Cape hake, versus wild Cape hake, and to establish at what age its nutritional value reaches an optimum.

- Looking into the possibility to use the mathematical models and ANN on other fish species, to correctly predict if fish have been exposed to excessive microbial contamination.

- In future it may be possible to combine one or more biochemical indicators (and or chemical indicators) into neural networks, to predict whether other foodstuffs has been contaminated.

- Similarly, in the future nanotechnology may be applied to determine if a product has been exposed to higher temperatures than it should be, again minimising manual chemical analysis and preventing loss of product, due to sampling.

- Determining changes in nutritional value of Cape hake, when cooked with various domestic methods and various domestically used oils.

- Further investigations into the similarities between human corpse decay, and muscle tissue decay of foodstuffs and to improve the determination of shelf life.

- Rigorous testing under controlled factory/shop floor conditions to determine if the mathematical models are practical workable.

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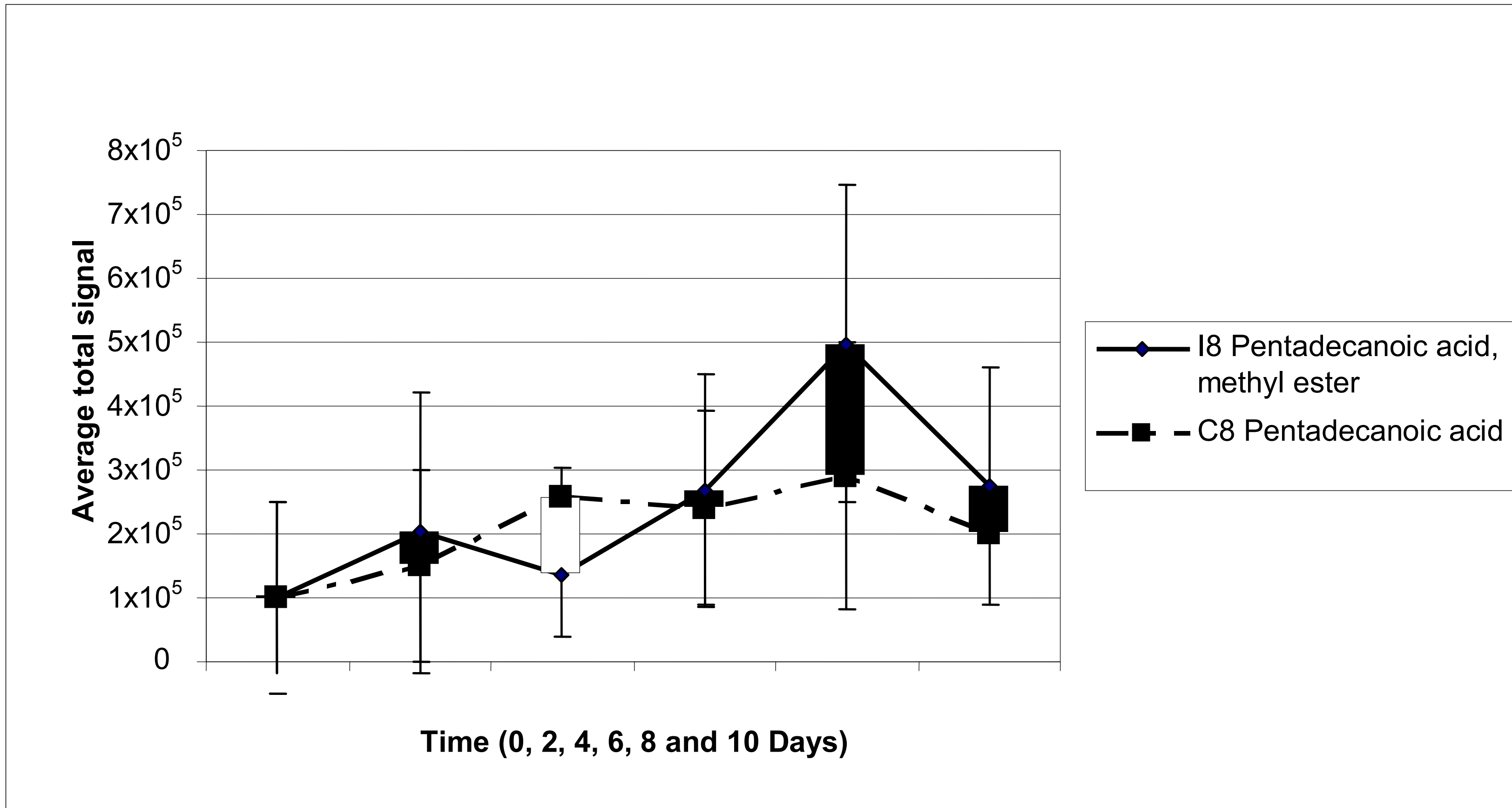
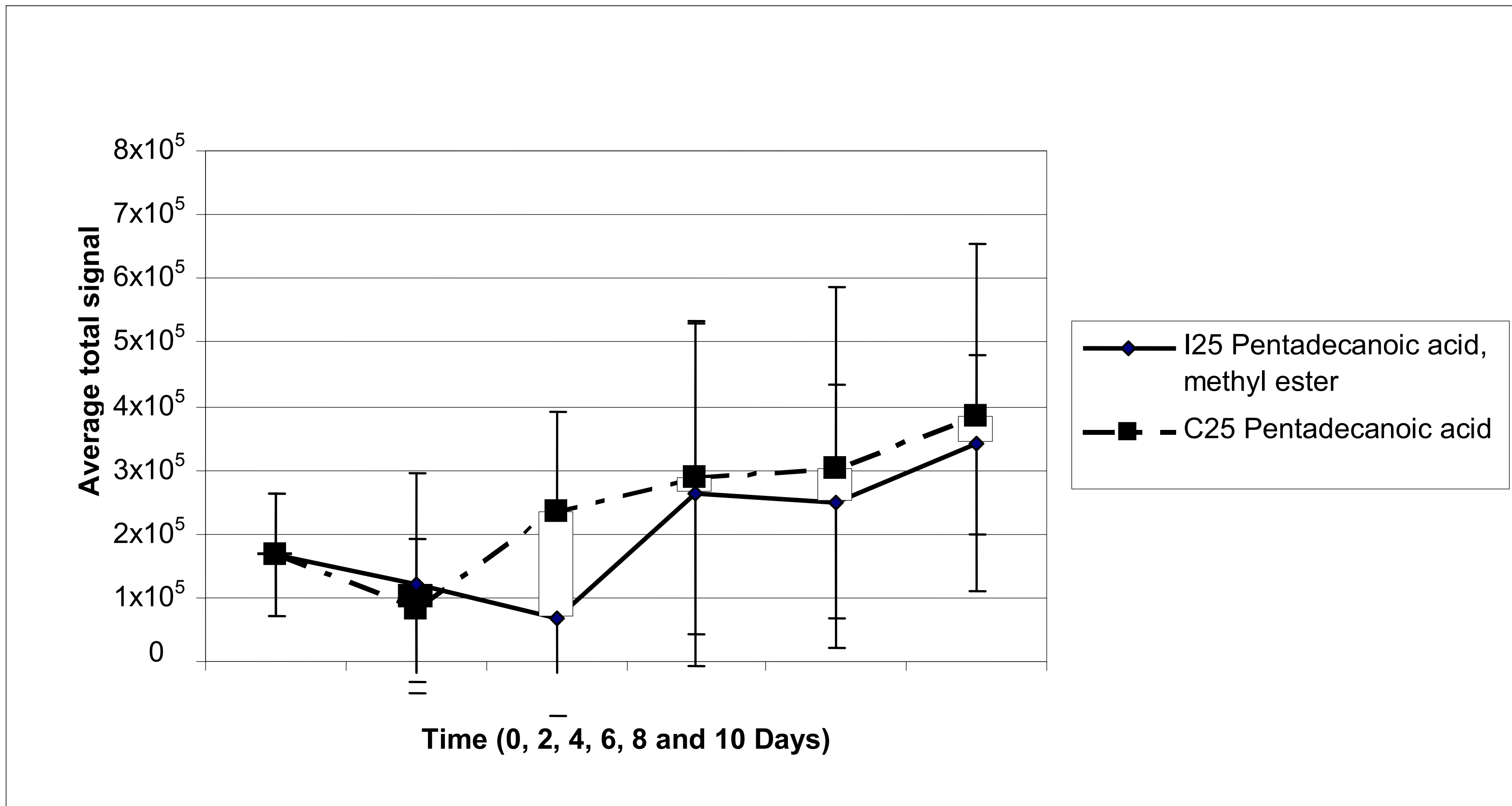
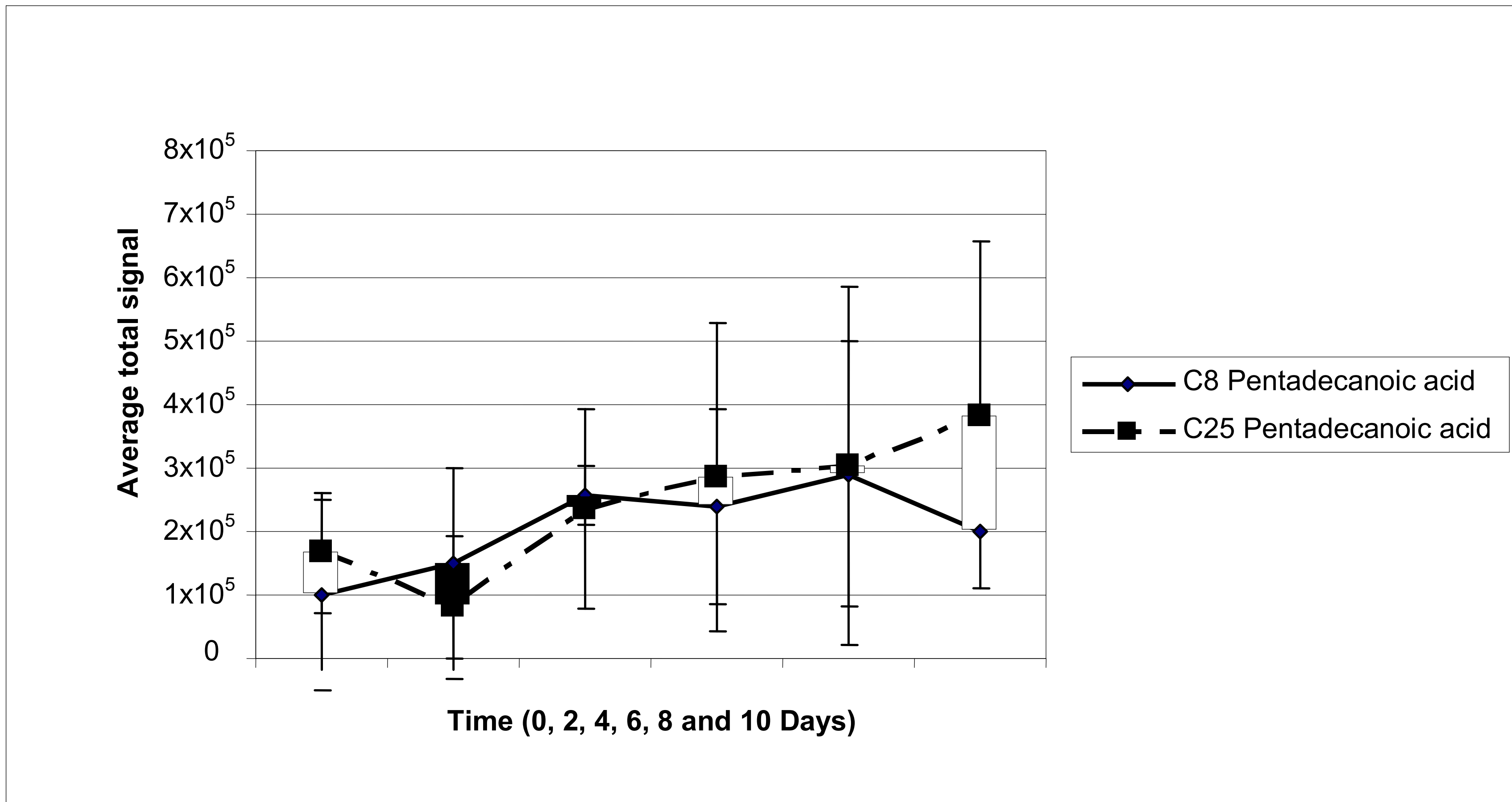
APPENDICES



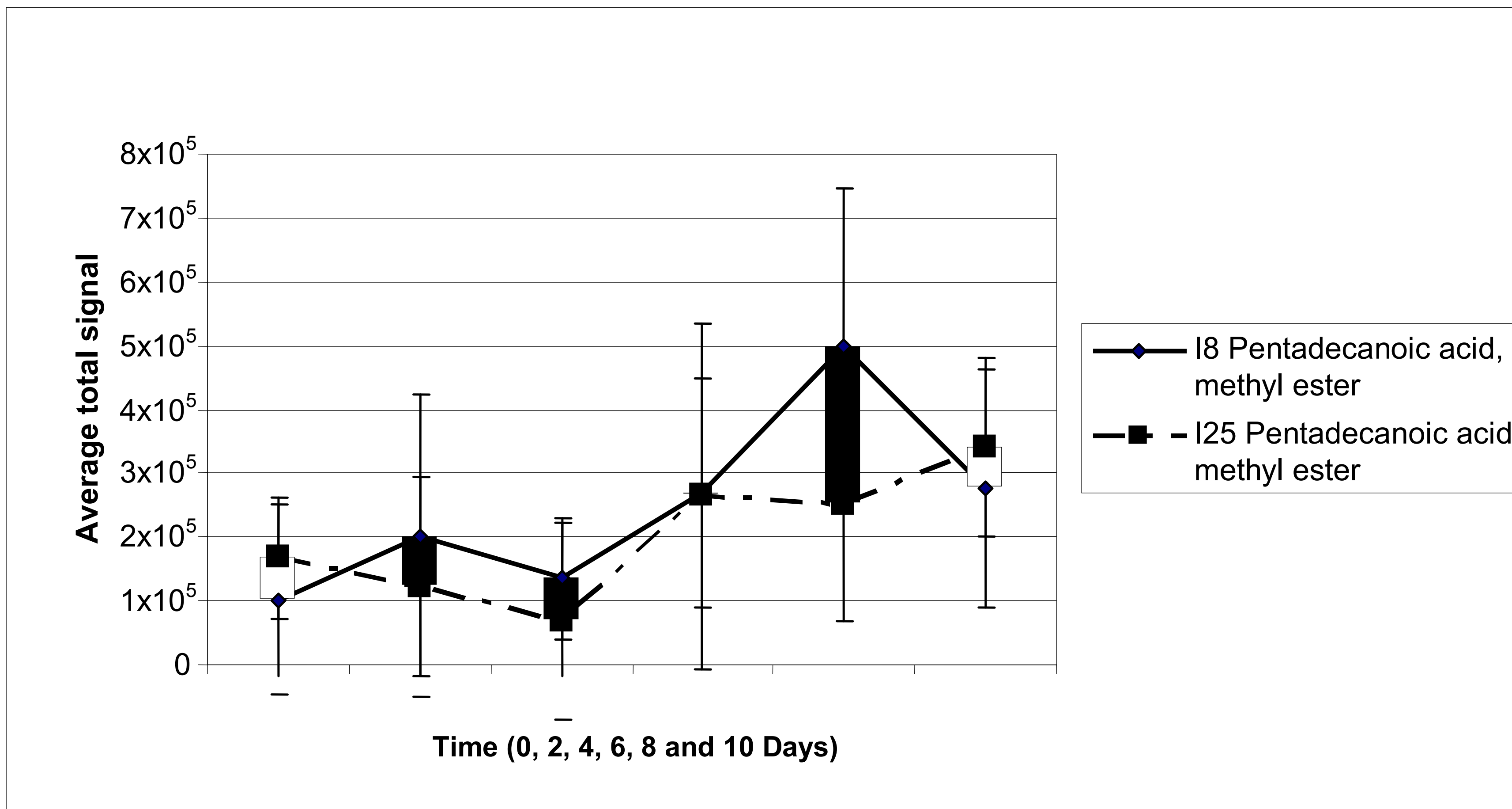
Appendix I The average of the total signal for one example of the selected fatty acids, e.g. Pentadecanoic acid for the control as well as the inoculated samples at 8 ºC. Equation 1 in Chapter 3 uses the percentage difference of these results to determine the temperature percentage difference (TPD).



Appendix II The average of the total signal for one example of the selected fatty acids, e.g. pentadecanoic acid for the control as well as the inoculated samples at 25 ºC. Equation 1 in Chapter 3 uses the percentage difference of these results to determine the temperature percentage difference (TPD).



Appendix III Average of the total signal for one example of the selected fatty acids, e.g. Pentadecanoic acid for the controlled samples at 8 ºC and 25 ºC. Equation 2 in Chapter 3 uses the percentage difference of these results to determine the microbial influence percentage difference.



Appendix IV The average of the total signal for one example of the selected fatty acids, e.g. pentadecanoic acid for the inoculated samples at 8 ºC and 25 ºC. Equation 2 in Chapter 3 uses the percentage difference of these results to determine the microbial influence percentage difference.