PROTECTION OF THE LONG CHAIN N-3 POLYUNSATURATED FATTY ACIDS IN HAKE HEAD FLOUR AGAINST OXIDATION

by

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously, in its entirety or in part, submitted it at any university for a degree.

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ABSTRACT

Research on the oxidation level of hake head flour was preceded with refinement and standardisation of a production process developed by Melnyczuk Research and Development (CK 96/58741/23, Stellenbosch, South Africa). Hake head flour production entails the mincing of hake heads, with the neck flesh and teeth removed and the brain and eyes intact, followed by the drying and powdering thereof.

Fish are rich in the nutritionally important n-3 polyunsaturated fatty acids (PUFA’s), which are susceptible to oxidation. Oxidation reduces PUFA-levels and causes unpalatable rancidity. The oxidation level of the hake head flour as a function of the experimental storage age (days) of the heads (= x), stored on ice at ± 4°C, used to prepare the flour, as well as of the flour (= y), stored at ambient conditions, was therefore quantified. Peroxide values (PV’s) and absorption values at 532 nm/A_{532nm} (2-thiobarbituric acid test) were determined over 9 days of storage for three flours respectively produced from heads stored for 1, 5 and 9 days. An interaction (p ≤ 0.01) existed between x and y for both peroxide and A_{532nm} values. Two quadratic bivariate functions, which respectively predict PV’s (r^2 = 0.8976) and A_{532nm} values (r^2 = 0.9679) as functions of x and y, were therefore fitted.

Flour freshly prepared (D1 flour) from heads stored for 1 day had the highest peroxide and lowest A_{532nm} values (results at p ≤ 0.05). D1 flour prepared from heads stored for 9 days had the lowest PV. The A_{532nm} of D1 flour prepared from heads stored for 5 or 9 days did not differ significantly. The peroxide and A_{532nm} values decreased in flour stored for 9 days. The results indicated that an increase in the storage age of the heads or the flour increased the flour oxidation level, with an interaction between these two factors.

The efficiency of antioxidants in protecting the flour was then investigated. Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butyl hydroquinone
(TBHQ), Biored Pure, Ronoxan A, and a partially water-soluble treatment/WST (consisting of sodium ascorbate, dl-α-tocopherol and Emulfluide E/EE), with some of these combined with synergists, was initially compared by their protection factors (PF), calculated from their Rancimat induction times in cod liver oil. A TBHQ-citric acid (CA) combination gave the highest PF (5.23). The WST, preferred due to solubility characteristics and a more natural composition, gave a PF of 1.48. Greatly increased (x 722.16) concentrations of the WST constituents (WSTinc), except for EE, which was only increased 17.14 times in the Rancimat trials, gave a PF of 9.32. The WSTinc, with dl-α-tocopherol replaced by dl-α-tocopherol-acetate (WSTacetate), also applied with the reduced level of EE, gave the maximum measurable induction time (>48 hours).

Hake head mince was then, prior to drying, treated with CA (0.2 g/kg of flour lipid), combined with TBHQ (0.12 g/kg of flour lipid) and EE (at 0.14% add-in on mince weight), or with WSTacetate and water (respectively at 0.2 and 0.5% add-in on mince weight). Both the TBHQ-CA treated and a control flour (i.e. no antioxidants added) were oxidised after drying (PV > 40 meq/kg). WSTacetate-CA treated flour (PV of 0.79 meq/kg after drying) only reached a PV ≥ 10 meq/kg after ±140 h of storage (45°C), while a control was already oxidised after drying (PV of 33.88 meq/kg). Hake liver enriched mince treated with WSTinc or WSTacetate (0.2% add-in) resulted in flours with low PV’s after drying (3.03 and 1.73 meq/kg respectively), which were respectively stored at 60°C for ±10 and 32 h before reaching PV’s ≥ 10 meq/kg. This indicated antioxidant action relative to a control, which was oxidised after drying (PV of 18.03 meq/kg). Determinations of conjugated dienes and trienes, and the relative decrease in PUFA/C16:0 ratios after 149 h of storage (60°C), which was greatest for the control and least for the WSTacetate treated flour, supported this conclusion. WSTinc and WSTacetate therefore slowed down oxidation in hake head flour, with the latter being the most effective treatment.
OPSOMMING

Navorsing met betrekking tot die vlak van oksidasie in stokviskopmeel is vooraf gegaan deur die verfyning en standardisering van 'n produksie-proses, ontwikkel deur Melnyczuk Navorsing en Ontwikkeling (CK 96/58741/23, Stellenbosch, Suid-Afrika). Stokviskopmeel produksie behels die fynmaal van stokviskoppe, met die nekvleis en tande verwys, maar met die brein en oë intakt, gevolg deur die droging en verpoeiering daarvan.

Vis is ryk in die voedsame n-3 poli-onversadigde vetsure (POVS’e), wat geneig is tot oksidasie. Oksidasie verminder die POVS-vlakke en veroorsaak onaanvaarbare galsterigheid. Die oksidasie vlak van die stokviskopmeel as ‘n funksie van die eksperimentele bergingsouderdom van die koppe (= x), geberg op ys by ± 4°C, gebruik om die meel te vervaardig, asook van die meel (= y), geberg by kamertemperatuur, is dus gekwantificeer. Peroksied waardes (PW’s) en absorpsie waardes by 532 nm/A₅₃₂nm (TBA-toets) is oor 9 dae van berging vir drie meIe, onderskeidelik voorberei van stokviskoppe geberg vir 1, 5 en 9 dae, bepaal. ‘n Interaksie (p ≤ 0.01) tussen x en y is vir beide peroksied- en A₅₃₂nm-waardes waargeneem. Twee kwadratiese, twee-veranderlike funksies, wat onderskeidelik PW’s (r² = 0.8976) en A₅₃₂nm-waardes (r² = 0.9679) as funksies van x en y voorspel, is dus gepas.

Meel vars voorberei (D1 meel) van koppe geberg vir 1 dag het die hoogste peroksied- en laagste A₅₃₂nm-waarde getoon (resultate by p ≤ 0.05). D1 meel berei van koppe geberg vir 9 dae het die laagste PW gehad. Die A₅₃₂nm-waardes van D1 meel berei van koppe geberg vir 5 of 9 dae het nie verskil nie. Peroksied- en A₅₃₂nm-waardes het afgeneem in meel wat vir 9 dae geberg is. Hierdie resultate het aangedui dat ‘n toename in die bergingsouderdom van die koppe of die meel die meel oksidasie-vlak verhoog, en dat hierdie twee faktore ‘n interaktiewe invloed het.

Die effektiviteit van antioksidante in die meel is vervolgens vergelyk. Butiel hidroksianisool (BHA), butiel hidroksietolueen (BHT), tersière butielhidrokinon
(TBHQ), “Biored Pure”, Ronoxan A, en ‘n gedeeltelike water-oplosbare behandeling/WOB (bestaande uit natrium askorbaat, dl-α-tokoferol en Emulfluide E/EE), met sommige van die gekombineer met sinergiste, is eers vergelyk t.o.v. hul beskermingfaktore (BF), bereken vanaf hul Rancimat induksie-tye in kabeljou levertraanolie. ‘n TBHQ-sitroensuur (SS) kombinasie het die hoogste BF (5.23) gelewer. Die WOB, geselekteer weens oplosbaarheids-eienskappe en ‘n meer natuurlike samestelling, het ‘n BF van 1.48 gelewer. In ‘n tweede proef het baie verhoogde (x 722.16) konsentrasies van die WOB bestanddele (WOBverhoog), behalwe vir EE, wat slegs 17.14 keer verhoog is vir die Rancimat bepalings, ‘n BF van 9.32 gelewer. Die WOBverhoog, met dl-α-tokoferol vervang deur dl-α-tokoferol-asetaat (WOBasetaat), ook ingesluit met die verlaagde EE vlak, het die maksimum meetbare induksie-tyd (> 48 h) gelewer.

Gemaalde stokviskoppe is vervolgens voor droging behandel met SS (0.2 g/kg meelvet), gekombineer met TBHQ (0.12 g/kg meelvet) en EE (teen 0.14% van die vis se gewig ingesluit), of met WOBasetaat en water (teen onderskeidelik 0.2 en 0.5% van die vis se gewig ingesluit). Beide die TBHQ-SS behandelde en ‘n kontrole meel (d.w.s. geen antioksidante bygevoeg nie) was geoksideer na droging (PW > 40 meq/kg). WOBasetaat-SS behandelde meel (PW van 0.79 meq/kg na droging) het eers na ± 140 h van berging (45°C) ‘n PW ≥ 10 meq/kg bereik, terwyl ‘n kontrole reeds geoxideerde was na droging (PW van 33.88 meq/kg). Gemaalde viskoppe verryk met stokvislewer en behandel met WOBverhoog of WOBasetaat (teen 0.2% ingevoeg) het mele gelewer met lae PW’s na droging (3.03 en 1.73 meq/kg onderskeidelik) wat onderskeidelik vir ± 10 en 32 h by 60°C geberg was voordat PW’s ≥ 10 meq/kg bereik is. Dit dui antioksidant-werking relatief tot die kontrole aan, wat geoxideerde was na droging (PW van 18.03 meq/kg).

Resultate van gekonjugeerde dieen- en trieen-absorpsie bepalings, en die relatiewe afname in die POVS/C16:0 verhoudings na 149 h van berging (60°C), wat die grootste vir die kontrole en die kleinste vir die WOBasetaat behandelde meel was, ondersteun die gevolgtrekking. Alhoewel beide WOBverhoog en WOBasetaat dus oksidasie in stokviskopmeel vertraag, is WOBasetaat die effektiefste behandeling.
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LIST OF FREQUENTLY USED ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>A&lt;sub&gt;232nm&lt;/sub&gt;</td>
<td>absorbance values read at 232 nm to determine conjugated dienes</td>
</tr>
<tr>
<td>A&lt;sub&gt;268nm&lt;/sub&gt;</td>
<td>absorbance values read at 268 nm to determine conjugated trienes</td>
</tr>
<tr>
<td>A&lt;sub&gt;332nm&lt;/sub&gt;</td>
<td>absorbance values at 532 nm, determined with the 2-thiobarbituric acid test</td>
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<tr>
<td>AA</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>ALA</td>
<td>α-linolenic acid</td>
</tr>
<tr>
<td>AOCS</td>
<td>American Oil Chemists’ Society</td>
</tr>
<tr>
<td>AOM</td>
<td>accelerated oxygen method</td>
</tr>
<tr>
<td>ASLT</td>
<td>accelerated shelf-life test</td>
</tr>
<tr>
<td>BHA</td>
<td>butylated hydroxyanisole</td>
</tr>
<tr>
<td>BHT</td>
<td>butylated hydroxytoluene</td>
</tr>
<tr>
<td>BNF</td>
<td>The British Nutrition Foundation</td>
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<td>C/C’s</td>
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<td>CA</td>
<td>citric acid</td>
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<td>omega-3</td>
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<td>Description</td>
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<td>omega-6</td>
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<td>peroxide value/s</td>
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<td>RA</td>
<td>Ronoxan A</td>
</tr>
<tr>
<td>SMBS</td>
<td>sodium metabisulphite</td>
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<tr>
<td>TAG/TAG’s</td>
<td>triacylglycerol/s</td>
</tr>
<tr>
<td>TBA</td>
<td>thiobarbituric acid</td>
</tr>
<tr>
<td>TBARS</td>
<td>thiobarbituric acid reactive substances</td>
</tr>
<tr>
<td>TBHQ</td>
<td>tertiary butyl hydroquinone</td>
</tr>
<tr>
<td>UFA/UFA’s</td>
<td>unsaturated fatty acid/s</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>WST</td>
<td>(partially) water-soluble treatment</td>
</tr>
<tr>
<td>WST\text{inc}</td>
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<tr>
<td>WST\text{acetate}</td>
<td>WST\text{inc} with dl-\alpha-tocopherol</td>
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<tr>
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<tr>
<td></td>
<td>replaced with dl-\alpha-tocopherol-acetate</td>
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The guidelines for authors as prescribed by the International Journal of Food Science and
Technology (http://www.ifst.org, 2002) were used.
CHAPTER 1
Motivation for study

1.1 MOTIVATION

A fish waste utilisation project (FWUP), under which this study resorts, was initiated with the objective to utilise hake heads as a long chain n-3 polyunsaturated fatty acid (PUFA) rich food source in such a manner that the docking of the heads would become economically viable. Minnaar (2001) motivated that utilising the heads of the hake caught by the South African fishing industry (mainly the species Merluccius capensis and Merluccius paradoxus) in producing a food product, which could serve as a nutritional intervention tool, is quantitatively viable. Such utilisation would also provide a solution to the illegal practice of discarding the heads at sea in order to increase the cold storage space for higher value cuts, such as the fillets, a practise which has decreased in the last few years, but is still present (Minnaar, 2001).

Envisioned products from the heads include value-added products produced from the neck-flesh (the fleshy meat portion attached to the head after filleting), a fish flour produced from the head section, as well as a pet food, utilising off-cuts. The value-added commercial products should contribute to the financial viability of the project. The fish flour might be incorporated into a food carrier such as bread, and as such used as a nutritional intervention tool. Minnaar (2001) extensively discussed the literature surrounding malnutrition in South Africa, and concluded that, despite the lack of satisfactory national data on the prevalence of malnutrition amongst South African children, there is a need for nutritional intervention in South Africa, especially in the under-five age group and in black and rural primary school children. For example, a study on the fatty acid (FA) status of undernourished children in rural areas of Lebowa, South Africa, found a high prevalence of under-nutrition, associated particularly with a low energy intake and an imbalance of dietary n-6 to n-3 FA intake (Tichelaar et al., 1994).
The intake of n-3 FA's should also receive special attention as the Western diet has, since industrialisation, drastically increased in total fat intake, with a concurrent increase in n-6 and a decrease in n-3 FA intake (Leaf & Weber, 1987). Simopoulos *et al.* (2000) therefore recommend that dietary n-6 FA intake should be reduced with a simultaneous increase in n-3 FA intake. This is required to ensure optimal brain and cardiovascular health and function, and to reduce the possible adverse effects of a diet with a too high n-6:n-3 FA ratio (Simopoulos *et al.*, 2000). The n-3 FA's play an essential role in normal growth and development, and a potentially critical role in reducing the risk for or in treating various diseases, such as coronary artery disease, hypertension, arthritis, other inflammatory and auto-immune disorders, and cancer (Simopoulos, 1991; Bjerve *et al.*, 1988).

One way to augment n-3 FA intake is through increased fish consumption. Fish is a rich source of the n-3 FA's, specifically the long chain n-3 FA's, eicosapentaenoic acid (C20:5n-3/EPA) and docosahexaenoic acid (C22:6n-3/DHA), of which fish oil may contain up to 30% (Gunstone, 1996; Kaitaranta, 1992). Although hake is a lean fish species (Huss, 1988; Dassow & Beardsley, 1974), fish heads generally serve as fat depots (Huss, 1988). The lipids in yellowfin tuna heads and eye sockets have, for example, been shown to contain large quantities of n-3 FA's, respectively containing 22.0 and 25.3% DHA and 4.9 and 5.0% EPA (Panggat & Rivas, 1997). Hake heads could therefore conceivably be an unexploited source of n-3 FA's.

Therefore the main objective of the FWUP was to produce a hake head flour that can be used to enrich a staple food, such as bread, with n-3 polyunsaturated fatty acids (PUFA's). This enriched food product can then be used to address n-3 FA deficiencies and imbalances in the South African diet. Consequently a production process to produce flour from hake heads was developed by Melnychuk Research and Development (CK 96/58741/23, Stellenbosch, South Africa), and a basic processing line for hake head flour production already existed at the start of this study. This production process was however refined and standardised during the course of this study. The main purpose of this study was to protect the lipid fraction in the hake head flour against oxidation, since
oxidation leads to the undesirable loss of FA’s and fat-soluble vitamins, such as vitamins A, D and E, as well as to the development of unpleasant rancid odours and flavours (Dziezak, 1986; Buck & Edwards, 1997). Protection of the PUFA’s against oxidation is therefore vital in order to create an effective dietary intervention tool by ensuring PUFA bio-availability and by preventing or limiting the occurrence of unpalatable rancidity.

In order to develop effective protective measures against oxidation in the fish flour, objective indicators of the extent of oxidation had to be identified, and adapted where necessary, in order to follow the extent of lipid oxidation in the flour. The extent of oxidation present in the hake head flour was then first quantified as a function of both the storage age of the hake heads used to prepare the flour and of the flour. Suitable antioxidants with which to protect the hake head flour against oxidation during production and storage were then identified and evaluated for their efficiency.

1.2 REFERENCES


Huss, H.H. (1988). Fresh fish – quality and quality changes: A training manual prepared for the FAO/DANIDA training program on fish technology and quality control,


CHAPTER 2
Literature review

2.1 AN OVERVIEW OF LIPID NATURE, NOMECLATURE AND SYNTHESIS

2.1.1 Lipid nature and nomenclature

Lipids are water-insoluble, hydrophobic substances of animal or plant origin (Love, 1992). Lipids are a more heterogeneous group than proteins or carbohydrates, and are divided into two main types: lipoids (substances with the same solubility characteristics as lipids) and lipid propers (Alais & Linden, 1991). In foods, a sub-class of lipid propers, called triacylglycerols (TAG’s), is quantitatively predominant and normally represents more than 95% of the lipid weight in food fats and oils (Alais & Linden, 1991; Institute of Shortening and Edible Oils, 1988).

Acylglycerols are esters containing acid and alcohol components, with the most common alcohol being propane-1,2,3-triol (glycerol). Mono- and diacylglycerols are fatty acid (FA) mono- or di-esters of glycerol, and usually form less than 2% of the total lipid, while TAG’s are fully acylated derivatives of glycerol (Gunstone, 1996; Alais & Linden, 1991). The TAG’s are also known as “neutral lipids”, and are soluble in non-polar solvents (Love, 1992). Triacylglycerols are formed through the condensation of one molecule of glycerol with three molecules of FA’s to yield one molecule each of TAG and water (Stuckey, 1972). The TAG’s are classified as simple if the same FA is esterfied at all three positions on glycerol, and as mixed (the predominant form in nature) if two or more types of FA’s are esterfied with the glycerol molecule (Love, 1992; Stuckey, 1972).

The FA’s in a glyceride molecule contribute 94 to 96% of the molecule’s weight and are considered the reactive portion. The types and proportions of the FA’s present largely determine the chemistry of a fat (Love, 1992; Stuckey, 1972). A FA consists of a hydrocarbon chain of varying lengths (the melting point increases with an increase in
chain length) with a terminal carboxylate group. Fatty acids are saturated if they do not contain double bond carbon linkages, and unsaturated if they contain one or more of these linkages (Love, 1992). Four general statements can be made concerning the FA’s found in nature (Gunstone, 1996):

- they are straight chain compounds with an even number of carbon (C) atoms, of which the number is most commonly between 12 and 22
- monounsaturated (monoenoic) FA’s (MUFA’s) have one double bond, usually in a cis configuration, in one of a limited number of preferred positions, most commonly 9 (9C from the carboxyl group) or n-9 (9C from the methyl group)
- polyunsaturated (polyenoic) FA’s (PUFA’s) contain more than one double bond, with the bonds in cis configurations and mainly arranged in a methylene (CH₂) interrupted arrangement. This is the most common natural arrangement and is called a non-conjugated system. In a conjugated acid, the single and double bonds alternate between carbon atoms (Love, 1992)
- they seldom have other functional groups than the carboxyl group and the various types of unsaturation possible.

The cis configuration specified above refers to geometrical or spatial arrangement isomers. In the cis configuration, the hydrogen atoms on the C atoms joined by double bonds are on the same side of the C chain, and in the trans form (a form rarely found in nature), they are on opposite sides (Love, 1992).

The FA’s are named according to the hydrocarbon chain from which they originate, with the “e” in the hydrocarbon chain name replaced with “oic” in the FA name. If more than one double bond is present, this descriptor is further qualified by the number present, i.e. dienoic (two double bonds), trienoic (three), tetraenoic (four), etc. (Love, 1992). In the systematic name, the position of the double bonds are given before the name itself, with the position determined by counting from the carboxyl group C and identified by the lower number of the carbons it joins. Two shorthand notation forms, that both indicate the number of carbons and the presence of double bonds, exist (Gunstone, 1996). As an example, the systematic name of linoleic acid is 9,12-octadecadienoic, which indicates
that linoleic acid is an 18C carboxylic acid (oic) with two double bonds (dien), situated, counting from the carboxyl group C and identified by the lower number of the carbons it joins, between C9 and C10 and C12 and C13. In shorthand notation, it is written as C18:2Δ9,12. Alternatively, linoleic acid can be identified as C18:2n-6 or C18:2ω-6, which indicates a structure of 18 carbons, with two double bonds, with the first double bond at the sixth C counting from the methyl or omega (ω) end.

The FA’s of main interest in this study are the PUFA’s found in marine oils. Marine oils contain a mixture of TAG’s with various long chain FA’s, as well as small amounts of mono- and diglycerides. Marine oils differ from the various edible vegetable oils and animal fats in having a higher proportion of long chain PUFA’s with five or six double bonds, and in the position of the first double bond from the methyl group, which mostly occurs at the n-3 position as contrasted to the more usual n-6 position found in most other oils (Bimbo, 1987; Stansby, 1982). The long chain PUFA’s, such as eicosapentaenoic acid (EPA: C20:5n-3), docosapentaenoic acid (DPA: C22:5n-6) and docosahexaenoic acid (DHA: C22:6n-3) are primarily located at the second C in the TAG molecule (Shahidi & Wanasundara, 1995).

2.1.2 Lipid synthesis

Gunstone (1996) comprehensively describes FA biosynthesis as one of four processes: de novo synthesis of saturated FA’s, chain elongation, desaturation to MUFA’s, and desaturation to PUFA’s. De novo synthesis of FA’s mainly occurs via the reductive acetate-malonate pathway. Acetate (a 2C unit) is the building block of the reaction, explaining the predominance of FA’s with an even number of carbons. Acetate and carbon dioxide (CO₂) react in an enzymatically catalysed (acetyl-CoA carboxylase) reaction to form a reactive unit called malonate (3C). Malonate is the main extending unit during synthesis, but, on condensation with acetate or a FA, malonate loses the C originating from CO₂ so that effectively only acetate-originating C’s are incorporated. Stepwise, de novo synthesis involves the transfer of the acyl-CoA esters of acetate and malonate to acyl carrier proteins, after which condensation of these molecules occurs.
with the concurrent loss of CO$_2$. Further steps entail reduction and dehydration, with the enzymes responsible for this sequence called "FA synthetase". The netto effect of each such cycle is the elongation of the starting compound with two C's, and this continues until the FA is detached from the enzyme by a hydrolase.

Chain elongation occurs when a FA (saturated or unsaturated) reacts with acetyl- or malonyl-CoA through steps including condensation, reductions, and dehydration. The resulting FA has two additional C atoms at the carboxyl end of the molecule. This implies that, if the starting FA was unsaturated, the double bond does not change position with respect to the methyl group during elongation.

Desaturation to MUFA's in animals and plants most commonly occur via an aerobic pathway that requires oxygen and reduced nicotinamide adenine dinucleotide (NADH), or the phosphate form thereof (NADPH). The production of a monounsaturated fatty acid (MUFA) usually occurs by stereo- and regio-specific removal of hydrogen atoms from C9 and C10 in the saturated FA with the corresponding number of carbons to produce a cis-alkene with the double bond in the n-9 position. Further desaturation to PUFA's differs in plants and animals. In plants, additional cis-double bonds are mostly introduced in a methylene-interrupted pattern between the existing double bond and the methyl group, which allows the formation of α-linolenic acid (ALA: C18:3n-3) and linoleic acid (LA: C18:2n-6). Less commonly, double bonds can also be introduced on the carboxyl group side. The animal biosynthesis pathway cannot introduce double bonds on the methyl side of the n-9 double bond to form n-3 or n-6 PUFA's (Gunstone, 1996).

The FA's ALA and LA are therefore essential in the human diet (Gunstone, 1996; Krummel, 1996; Neuringer & Connor, 1986). Essential FA's (EFA's) are defined as those required for normal growth and physiological integrity that cannot be synthesised in adequate amounts by the human body (The British Nutrition Foundation/BNF, 1992). Once ingested, the human body can however convert ALA and LA to the long chain PUFA derivatives thereof by elongation and desaturation (Gunstone, 1996; Michelsen et
The limited position double bond introduction possible in human metabolism, i.e. only from the n-9 position towards the carboxyl terminus, explains why LA and ALA are not interconvertible and why they can only be converted to PUFA’s with a double bond in one of two positions relative to the methyl group, namely in the n-3 and n-6 position (Neuringer & Connor, 1986). Two biosynthetically related families of PUFA’s, called the n-3 and n-6 families, result, with ALA and LA, the FA’s with the shortest C chain, as the parent compounds (Figure 2.1).

**Figure 2.1. The conversion between polyunsaturated fatty acids with various desaturases and elongases**

### 2.2 NUTRITIONAL IMPORTANCE OF THE POLYUNSATURATED FATTY ACIDS

The EFA’s, and the biologically active FA’s synthesised from them, perform multiple functions in the body. These families of FA’s are the precursors of the eicosanoids, hormone-like substances that help to regulate blood pressure, heart rate, vascular dilation,
blood clotting, lipolysis and immune response (Krummel, 1996). The long chain PUFA’s, particularly arachidonic acid (AA) and DHA, are also important structural components of biological membranes (Neuringer & Connor, 1986).

In the absence of sufficient dietary intake of the EFA’s, deficiency symptoms develop. Linoleic acid (C18:2n-6) deficiency, for example, leads to dermatitis and poor growth in infants, while animals show reproductive failure and fatty livers (Krummel, 1996). Neuringer and Connor (1986) summarised the effects of n-3 FA deficiency recorded in various animal studies, and concluded that, in spite of the lack of human trials, there is growing evidence of the essentiality of the n-3 FA’s for normal development and functioning of the retina, and possibly the brain.

The EFA status is measured biochemically and expressed as the ratio of Mead’s acid (C20:3n-9) to AA (C20:4n-6), also known as the triene-tetraene ratio (Gunstone, 1996). The origin of this ratio can be explained as follows (BNF, 1992): the parent compounds of the n-3, n-6 and n-9 FA families all compete for the same 6-desaturase enzyme for the biosynthesis of other members of the family. Alpha linolenic acid (n-3) has the highest, LA (n-6) the intermediate and oleic acid (n-9) the lowest reaction rate with this enzyme. With sufficient LA intake, the relatively high affinity for the enzyme results in sufficient formation of the AA for body requirements. However, when the dietary LA intake is low, the n-9 FA family competes more successfully for the 6-desaturase enzyme. Consequently the elongation and desaturation of the commonly occurring n-9 FA, oleic acid (C18:1n-9), is enhanced, which results in an abnormal accumulation of its’ end product, Mead’s acid, usually present in trace amounts. Thus a disturbed ratio of Mead’s acid to AA indicates an EFA deficiency. An EFA deficiency is biochemically characterised by changes in the FA compositions of many cell membranes, whose functions are thus impaired, including metabolic energy production by mitochondrial beta-oxidation of FA’s (BNF, 1992).

The long chain PUFA’s synthesised from LA and ALA could be classed as conditionally essential if circumstances prohibit the adequate synthesis thereof (BNF, 1992).
Michelsen et al. (2001) reports that animal and human studies have shown that the conversion of ALA to EPA and DHA is restricted. It has also been suggested that the activity of the enzyme responsible for the transformation of LA and ALA into the long chain PUFA’s may decrease with age (Alais & Linden, 1991), increasing the importance of consuming the long chain PUFA’s as such. Premature infants also cannot synthesise the long chain PUFA’s in sufficient amounts, rendering intake thereof essential (BNF, 1992). Consuming the longer chain n-3 and n-6 FA’s should also have a sparing effect on the EFA’s by reducing the need for ALA and LA conversion. Intake of n-3 and n-6 FA’s should therefore include consumption of the long chain PUFA’s synthesized from the parent n-3 and n-6 FA compounds.

In spite of the recognition of the essentiality of dietary intake of both the n-6 and n-3 FA’s, Neuringer and Connor (1986) identified the absence of dietary intake recommendations for the n-3 FA’s as an unresolved issue in human nutrition for several decades. These authors state the critical importance of n-3 PUFA intake during especially pregnancy, lactation, and infancy, and recommend a ratio of n-6:n-3 of 4-10:1 as a probable optimum in human nutrition.

A more recent international workshop on the essentiality of and recommended dietary intakes for n-6 and n-3 FA’s synthesised the extensive available data into dietary recommendations (Simopoulos et al., 2000). One of the main resulting recommendations was that the dietary intake of the n-6 PUFA’s should be reduced in favour of n-3 PUFA intake. This is necessary for optimal brain and cardiovascular health and function, and to reduce the probable adverse effects of a diet with a too high n-6:n-3 FA ratio. An n-6 rich diet negatively shifts the physiological status to a pro-thrombotic and pro-aggregatory state, which increases blood viscosity, vasospasm and vasoconstriction and decreases bleeding time (Simopoulos, 1991). Simopoulos et al. (2000) recommend that the intake of plant oils, the main source of the n-6 FA, LA, should be reduced to lessen the adverse effects of an excess of LA, which leads to an excess of AA and the eicosanoids produced thereof. The intake of the n-3 PUFA’s should simultaneously be
increased since the n-3 PUFA’s compete for the enzyme that converts LA, and thus inhibits the conversion of excess LA.

Increasing the n-6:n-3 ratio is challenging as the Western diet has, since industrialisation, drastically increased in total fat intake, with a concurrent increase in n-6 FA intake, and a decrease in n-3 FA intake (Leaf & Weber, 1987). This can in part be contributed to the increased consumption of dietary plant oils rich in the n-6 FA’s (Simopoulos et al., 2000). Compounding the problem is an insufficient dietary intake of n-3 FA’s, as is the case, for example, in the typical American diet (International Conference on the Health Effects of ω-3 Polyunsaturated Fatty Acids in Seafoods, 1990). Modern agricultural practices also contribute to the n-6:n-3 ratio imbalance, as is illustrated in the following two studies. A study comparing the n-3 FA level in fish showed that cultured fish were a significantly lower source of n-3 FA’s than wild fish (Van Vliet & Katan, 1990). The ratio of n-6:n-3 FA’s in the yolk of free-ranging chicken and standard U.S. supermarket eggs was 1.3 and 19.4 respectively (Simopoulos & Salem, 1989). The authors ascribed this to the modern emphasis on increased production. This has led to the development of chicken feeds rich in proteins and linoleic acid, which leads to increased egg production, but this diet is reflected in the egg composition and results in a high n-6:n-3 ratio.

The recommended intake levels for the n-6 and n-3 FA’s are at present receiving attention. Simopoulos et al. (2000) suggest adequate intake (AI) levels, defined as levels based on experimentally derived intake levels, or as approximations of observed mean nutrient intakes by a healthy group or groups of people. These levels should meet or exceed the amount needed to maintain a defined nutritional state or criterion of adequacy in all members of a specific healthy population. Adequate intake levels are stipulated in the absence of sufficient scientific evidence to calculate a recommended dietary allowance (Simopoulos et al., 2000). South African legislation (Regulations relating to the Labelling and Advertising of Foodstuffs, 2002) has recently been adapted and published and is currently open to comments. One of the adaptations already planned is that this legislation will contain dietary guidelines modeled on the most recent World Health Organisation guidelines. These guidelines are expected to include recommended
dietary intakes for n-6 and n-3 PUFA’s (personal communication, A. Booysen, 2002). A summary of the levels suggested by the cited sources is presented in Table 2.1.

The essential role of the n-3 FA’s in normal growth and development, and possible critical role in various diseases, such as coronary artery disease, hypertension, arthritis, other inflammatory and auto-immune disorders as well as cancer (Simopoulos, 1991) is further illustrated in a brief discussion of some of the health benefits of the n-3 FA’s.

### 2.2.1 Role in cardiovascular disease

Michelsen et al. (2001) report several cohort studies that have showed that marine food intake has a beneficial effect on coronary heart disease (CHD). As an example of such a study, Kromhout et al. (1985) found an inverse relation between fish consumption and CHD in a twenty year follow up longitudinal study on risk factors and chronic diseases. The mechanisms through which the n-3 FA’s may reduce the risk of CHD are numerous and include possible roles in atherosclerosis, thrombosis, blood pressure and rheology and cardiac function (Michelsen et al., 2001).

One example of how the n-3 FA’s may reduce the risk of CHD is that DHA and EPA have been shown to inhibit platelet aggregation and the accompanying release of atherogenic mitogens (Smith et al. 1989). The mechanism for this inhibitory action towards platelet aggregation possibly centers in the fact that the PUFA’s are precursors of the prostaglandins, prostacyclins and thromboxanes (Devadasan & Gopakumar, 1997; BNF, 1992; Kromhout et al., 1985). These are part of a range of highly biologically active substances, called “eicosanoids”, which are hydroxylated derivates of the C20 PUFA’s. Arachidonic acid (C20:4n-6) is a precursor of prostaglandin (PGE$_2$), prostacyclin (PGI$_2$) and thromboxane (TXA$_2$), also called the 2-series PG. The eicosanoid TXA$_2$ leads to platelet aggregation, which is balanced by PGI$_2$, which reduces it, and thus the likelihood of blood clotting and thrombosis. If disturbed, this balance cannot be rectified by alteration of the intake of AA, as that will simply maintain the disturbed equilibrium. Intake of EPA (C20:5n-3) leads to the synthesis of isomers of
Table 2.1. Suggested dietary intake levels of fat and selected fatty acids

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Adequate intake for adults&lt;sup&gt;1&lt;/sup&gt; (Simopoulos et al., 2000)</th>
<th>Adequate intake for infant formula or diet (Simopoulos et al., 2000)</th>
<th>Ranges of population nutrient intake goals&lt;sup&gt;6&lt;/sup&gt; (proposed South African legislation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Description</td>
<td>Adequate intake for adults&lt;sup&gt;1&lt;/sup&gt; (Simopoulos et al., 2000)</td>
<td>Adequate intake for infant formula or diet (Simopoulos et al., 2000)</td>
<td>Ranges of population nutrient intake goals&lt;sup&gt;6&lt;/sup&gt; (proposed South African legislation)</td>
</tr>
<tr>
<td>Linoleic Acid / LA (C18:2n-6)</td>
<td>4.44 Grams per day</td>
<td>2 Percentage of energy</td>
<td>10 Percentage of fatty acids</td>
</tr>
<tr>
<td>LA upper limit&lt;sup&gt;2&lt;/sup&gt;</td>
<td>6.67 Percentage of energy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arachidonic acid / AA (C20:4n-6)</td>
<td>-</td>
<td>0.5 Percentage of fatty acids</td>
<td></td>
</tr>
<tr>
<td>Total n-6 fatty acids (FA’s)</td>
<td>-</td>
<td></td>
<td>5-8</td>
</tr>
<tr>
<td>α-linolenic acid / ALA (C18:3n-3)</td>
<td>2.22 Grams per day</td>
<td>1 Percentage of energy</td>
<td></td>
</tr>
<tr>
<td>Docosahexaenoic acid / DHA (C22:6n-3)</td>
<td>0.22 minimum&lt;sup&gt;3&lt;/sup&gt; Grams per day</td>
<td>0.1 Percentage of fatty acids</td>
<td>0.35</td>
</tr>
<tr>
<td>Eicosapentaenoic acid / EPA (C20:5n-3)</td>
<td>0.22 minimum&lt;sup&gt;3&lt;/sup&gt; Grams per day</td>
<td>0.1 Percentage of fatty acids</td>
<td>upper limit &lt; 0.1</td>
</tr>
<tr>
<td>Total of DHA and EPA</td>
<td>0.65 Grams per day</td>
<td>0.3 Percentage of energy</td>
<td></td>
</tr>
<tr>
<td>Total n-3 FA’s</td>
<td>-</td>
<td></td>
<td>1-2</td>
</tr>
<tr>
<td>Total fat</td>
<td>-</td>
<td></td>
<td>15-30</td>
</tr>
<tr>
<td>Total polyunsaturated FA’s</td>
<td>-</td>
<td></td>
<td>6-10</td>
</tr>
<tr>
<td>Trans FA’s&lt;sup&gt;4&lt;/sup&gt;</td>
<td>2</td>
<td>1 Percentage of energy</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Saturated FA’s</td>
<td>-</td>
<td>&lt; 8 Percentage of energy</td>
<td>&lt; 7</td>
</tr>
<tr>
<td>Monounsaturated FA’s&lt;sup&gt;5&lt;/sup&gt;</td>
<td>-</td>
<td></td>
<td>by difference</td>
</tr>
</tbody>
</table>

1 based on a diet of 2000 kcal
2 enough scientific data exist to prescribe an upper intake limit
3 ensure 300 mg/day for pregnant and lactating woman
4 trans FA’s are not natural in foods other than dairy, and should preferably not be supplied by other sources, such as hydrogenated vegetable oils, in the diet
5 most dietary fat should come from monounsaturated FA’s, with total fat intake between 15-40%, depending on culture, and the need for weight and obesity control
6 personal communication, A. Booysen, 2002
PGI\(_2\) and TXA\(_2\), namely PGI\(_3\) and TXA\(_3\), also called the 3-series PG. The eicosanoid PGI\(_3\) also reduces platelet aggregation, but TXA\(_3\) differs from TXA\(_2\) in that it does not promote platelet aggregation. Thus the ingestion of the n-3 PUFA’s leads to suppression of the 2-series PG produced from AA, while the formation of the 3-series derivatives is elevated. This may help to maintain the platelet status in favour of preventing thrombosis. The FA’s EPA and DHA also decrease plasma concentrations of several atherogenic lipoproteins, and may thereby reduce the risk of coronary atherosclerosis.

The advantage of dietary marine oil intake is supported by results that the consumption of various marine oils led to changes in platelet membranes that are favourably anti-thrombotic (Vognild et al., 1998). Kromhout et al. (1985) however states that it is possible that other constituents than EPA in fish may also contribute to the anti-thrombotic effect of fish consumption.

Simopoulos (1991) summarised the role of the n-3 FA’s in atherosclerosis concisely: the n-3 FA’s alone will not eradicate atherosclerosis, but it is increasingly evident that dietary fish oil supplementation may help to prevent atherosclerosis or its’ thrombotic complications, and therefore most investigators advise dietary inclusion of fish several times a week.

2.2.2 Role in inflammatory and immune disorders and tumor growth

The dietary n-3 PUFA’s may reduce symptoms of disorders associated with suppression of the immune system such as rheumatoid arthritis, inflammatory bowel and skin diseases with an inflammatory component (Michelsen et al., 2001; Hughes, 1995). Hughes (1995) report that the mechanisms by which the n-3 PUFA’s suppress the immune system could possibly include their role in eicosanoid formation, in protein expression in cell membrane surfaces and in lymphocyte and monocyte function. For example, EPA and AA are, as reported, precursors of the eicosanoids. The eicosanoids modulate immune and inflammatory reactions. The body preferentially converts EPA to eicosanoids, which
are less potent than those produced from AA. This may be the reason why a diet rich in n-3 PUFA’s can lead to a depressed immune system (Hughes, 1995).

The n-3 PUFA’s may also play an important role in tumor management. Hughes (1995) reports that epidemiological studies prove an inverse relationship between n-3 FA intake and cancer prevalence. Animal tumor model studies also consistently prove that n-3 FA’s delay tumor appearance and decrease the growth rate, size, and number of tumors (Simopoulos, 1991). For example, the growth rate of a human mammary carcinoma was significantly depressed in mice fed fish oil relative to a corn oil-fed control group. This resulted in a mean tumor weight of less than half that found in the control group. The tumors of the fish oil-fed animals were also more sensitive to two anti-neoplastic agents than those of the control animals (Borgeson et al., 1989). Experimental studies therefore seem to indicate the definite benefit of consuming n-3 FA’s with regards to cancer management. In addition, PUFA’s of the n-6 series can promote cancer, and therefore reduction in total fat intake, as well as a modification to the intake of n-6 and n-3 PUFA’s in favour of n-3 PUFA’s is generally advised as prudent (BNF, 1992).

2.2.3 Role in growth

Bjerve et al. (1988) investigated growth and weight gain in a 7 year old girl with ALA deficiency who was fed by gastric tube alone and had showed almost no weight gain for 15 months. Her feeding formula was supplemented with a linseed and cod liver oil (CLO) mixture, increasing her ALA intake to 0.56 g and DHA plus EPA intake to 0.03 g per day. This change led to increased growth. A later change to only CLO decreased the ALA intake to 0.01 g and increased the DHA plus EPA intake to 1.04 g per day, and this led to an even more marked increase in weight gain and length. The authors concluded that n-3 FA’s are required for normal growth in man. An in-depth review by Simopoulos (1991) on the role of n-3 FA’s in health and disease and in growth and development supported this study in concluding that the n-3 FA’s are essential dietary inclusions for normal growth and development throughout the life cycle.
2.2.4 Neurological role

The most profuse FA's in the cellular membranes of the brain are AA, adrenic acid, and DHA (BNF, 1992). Particularly high concentrations occur in the membranes of neuronal synaptic terminals and in the retina photoreceptor cells (BNF, 1992; Bazan et al., 1993). This concentrated presence of the long chain PUFA's point to a definite neurological function.

Firstly, the availability of the long chain PUFA's are known to be critical during fetal growth to ensure brain development, as the human fetus obtains long chain PUFA's via the placenta, from where it is transferred to the fetal liver, and finally to the fetal brain (BNF, 1992). Intake of the long chain PUFA's remains critical during lactation, when rapid neural and vascular development takes place (BNF, 1992). A study conducted on baby mice (three days old) showed that intra-peritoneal injection of radio-labeled C18:3n-3 resulted in rapid accumulation of this FA in the liver, followed by a decline of labeled C18:3n-3 over time, with a concurrent increase in C22:6n-3 (DHA) synthesis and labeled C22:6n-3-levels. The brain showed a steady increase in labeled C22:6n-3 over time. These results indicate that the liver rapidly converts C18:3n-3 to C22:6n-3, and then supplies it via the blood stream to the brain and retina during postnatal development (Scott & Bazan, 1989). Human milk contains long chain PUFA's and is therefore a preformed source of long chain PUFA's such as DHA and AA (BNF, 1992).

A case study that illustrated the neurological role of the n-3 FA's in children is reported by Holman et al. (1982): a six year old girl fed on total parenteral nutrition was fed a diet that contained LA, but which was poor in ALA. The patient developed neurological abnormalities, such as numbness, pain in the legs, inability to walk, and blurring of vision. Increasing her ALA intake led to a concurrent disappearance of the neurological symptoms. This was attributed to the increased n-3 FA intake, since diagnostic analysis of the FA's in her serum lipids indicated that her LA deficiency had worsened during the change in formulation, indicating that the n-3 FA's rather than the n-6 FA's must have played the significant role in neurological symptom alleviation.
Secondly, the n-3 FA’s are required for retinal and brain function. Bazan et al. (1993) investigated the supply, uptake, and retention of DHA by the developing and mature retina and brain, and concluded that prolonged deprivation of DHA may lead to severe malfunctions. Neuringer and Connor (1986) report that DHA-rich phospholipids occur in the eye tightly bound to the photosensitive pigment rhodopsin, and also form a major component of the outer segment disk membrane in which rhodopsin rests. The authors conclude, from the results of the studies reviewed, that strong evidence exist that DHA is necessary for normal retinal function.

2.2.5 Summary

It is evident from the reported literature that the intake of n-3 FA’s is essential, and should receive special attention as the modern Western diet may lead to the intake of a disturbed, too high ratio of n-6:n-3 FA’s. One way to effectively bolster n-3 FA dietary intake is to increase fish consumption. Fish is considered the major source of the n-3 FA’s because fish oils are rich in the long chain n-3 PUFA’s, specifically EPA (C20:5n-3) and DHA (C22:6n-3). Up to 30% of the FA’s in fish oil may consist of a combination of EPA and DHA (Gunstone, 1996; Kaitaranta, 1992).

2.3 FISH AS A SOURCE OF POLYUNSATURATED FATTY ACIDS

2.3.1 Fish utilisation

As reported, the biological production of the long chain PUFA’s is restricted in humans (Michelsen et al., 2001; Shahidi & Wanasundara, 1995). Algae, which serve as a food source to marine animals, can readily synthesize n-3 long chain PUFA’s. These are efficiently transferred through the aquatic food chain, which is why marine fish and mammals, as well as shellfish, are rich sources of the n-3 FA’s, and a direct source of preformed DHA (Shahidi & Wanasundara, 1995; BNF, 1992; Neuringer & Connor, 1986).
Alternatives with which to introduce fish into the diet other than consuming fish as is exist. Fish oils are, for example, used in bulk during food preparation, such as for the manufacturing of salad oils, frying fats, margarines, shortenings, emulsifiers, etc. (Bimbo, 1987). Increasing attempts are also made to incorporate unhydrogenated fish oils with a low level of oxidation into popular food systems to serve as a source of EPA and DHA (Gunstone, 1996; Bimbo, 1987). Typically, fish used for reduction, i.e. to produce fish oil and meal, is classed in one of three categories (Bimbo, 1987):

- fish caught with the purpose of producing fish oil and meal thereof
- by-catch, i.e. fish caught incidentally
- fish offal or waste from the edible fish species

These sources are typically processed to produce fish oil and meal with the wet-reduction method, which includes cooking, pressing, separation of the oil and water emulsion to recover the oil, and drying of the residual protein material. The oil can then be treated, i.e. refined, winterised, degummed, neutralised, bleached, deodorised and hydrogenated (Bimbo, 1987).

Alternative fish utilisation methods to maximise the nutritional benefits to be gleaned from fish consumption are emerging. One method of growing interest to utilise the n-3 FA content of marine oil is to produce marine oil capsules that are used as a nutritional supplement. However, these capsules are expensive and not an affordable alternative to most people (Panggat & Rivas, 1997; Bimbo, 1987). There are also some reservations about the use of fish oils to bolster n-3 FA intake, summarised by Johnston (1987) as follows:

- high fish oil intakes may lead to possibly dangerous anti-clotting effects, e.g. during surgery
- some fish oils contain high levels of vitamins A and D that are toxic when consumed in excess
- fish oil concentrates could contain contaminants
- there may be a risk that the long-term consumption of highly unsaturated fish oils could possibly lead to increased peroxide formation in vivo.
It would therefore seem prudent to closely observe the recommended guidelines provided for the intake of the n-3 FA’s (summarised in Table 2.1), rather than consuming an excess of these n-3 FA’s. The use of a fish product to moderately enrich a staple food such as bread with n-3 FA’s may be more sensible than consuming fish oil concentrates as a source thereof as the latter may more likely be a possibly excessive source of the n-3 FA’s. Saldeen et al. (1998) showed that fish oil substituted for margarine in the preparation of bread (an equivalent of an intake of 1 g fish oil per day) effectively resulted, after only 2 weeks, in a significant increase in long chain n-3 FA’s in plasma phospholipids of an experimental group in comparison to a control group. After 4 weeks, EPA and DHA increased by 32 and 18% respectively, while serum TAG’s decreased with 17%.

2.3.2 The use of fish to address n-3 fatty acid intake deficiencies in South Africa

Minnaar (2001) extensively discussed the literature surrounding malnutrition in South Africa. As an example thereof, a study on the FA status of undernourished children in rural areas of Lebowa, South Africa, found a high prevalence of under-nutrition, associated particularly with low energy intake and an imbalance of dietary n-6:n-3 FA intake (Tichelaar et al., 1994). Minnaar (2001) concluded that, despite the lack of satisfactory national data on the prevalence of malnutrition amongst South African children, there is a need for nutritional intervention in especially the under-five age group and in black and rural primary school children. This age distinction is important as a child’s organs, tissues, blood, brain, bones, and physical potential are shaped from conception to the age of three (UNICEF, 1998).

Tichelaar et al. (1994) suggest the possibility of addressing the n-6:n-3 FA imbalance in pre-school children via existing primary health care systems through n-3 FA supplementation, i.e. with increased fish intake. Minnaar (2001) investigated the production of a fish flour from hake heads. This flour was used to fortify bread, a South African staple food. Newton and Snyder (1997) state that bread is an ideal medium for n-3 PUFA enrichment since the carbon dioxide produced during the proofing and baking of
bread helps to protect the oil from oxidation during the high temperature baking of the bread. The ultimate objective was to distribute the fortified bread with feeding schemes, attempting thus to address protein and n-3 PUFA needs (Minnaar, 2001).

Furthermore, fish flour enrichment of bread has been shown to increase the protein content of this mainly carbohydrate containing staple food (Irianto & Irianto, 1997). A similar product, “fish powder” (deboned and dried fish flesh, i.e. a product that contains all the nutrients of fish without the moisture) is in fact claimed to have the most nourishing protein combination naturally available to man (International Seafoods of Alaska, Inc., s.a.). Also, as discussed, the lipid fraction, if preserved, could make a very valuable nutritional contribution, which is substantiated by Minnaar (2001) in a review of the requirements, effects of deficiencies and advantages of consuming the long chain n-3 PUFA’s.

### 2.3.3 Hake head fish flour

The nutritional advantages of fish PUFA’s are increasingly a motivation to utilise low value fish (Reena et al., 1997). Minnaar (2001) motivated that utilising the heads of the hake caught by the South African fishing industry (mainly the species *Merluccius capensis* and *Merluccius paradoxus*) in producing a food product, which could serve as a nutritional intervention tool, is quantitatively viable.

The chemical composition of a given fish species varies, with the largest variation in the lipid component and its’ FA composition, and depends on the season, age, sex, as well as the maturity and feeding status of the fish. The yearly gonadal development, for example, is accompanied with low (or no) food intake, and therefore leads to a marked depletion of protein and lipid reserves in fish (Reena *et al.*, 1997; Huss, 1988). Hake is classified as a lean fish species (Huss, 1988; Dassow & Beardsley, 1974). Fish is classified as lean if reserve lipids are stored as TAG’s in the liver, and as fatty or oily if TAG’s are stored in the flesh (BNF, 1992). The head section is, however, one of the fat depots in fish (Huss, 1988). Panggat and Rivas (1997) report that the lipids in the heads
and eye sockets of Yellowfin tuna are noteworthy sources of the n-3 FA’s, respectively containing 22.0 and 25.3% DHA and 4.9 and 5.0% EPA. Hake heads may therefore plausibly be an unexploited source of n-3 FA’s. Fish trawlers are reported to illegally discard hake heads at sea to increase the cold storage space available for higher value portions, such as fillets (Minnaar, 2001). Utilising these heads in the production of flour, in such a manner that it increases the commercial value of the heads, would provide a satisfactory solution to this illegal discarding of heads at sea. The flour would find application in alleviating malnutrition and in correcting dietary imbalances of the n-6:n-3 intake ratio. The prototype of the flour studied by Minnaar (2001) was investigated further during this study.

Hake has been successfully used to produce a bland fish flour by means of the extraction method (Dreosti & Van der Merwe, 1961). The health benefits of fish oils, however, provide sound reasons against the extraction of the lipid component. Because the removal of the lipid component has previously resulted in a desirably bland product, it can be concluded that, if the lipid component is preserved because of potential nutritional benefits, it should be protected not only to maintain nutritional integrity but also to reduce a possible negative influence on the final product’s flavour. One of the disadvantage of unchecked lipid oxidation (see section 2.4.1.5.) is that it results in unpleasant rancid odours and flavours (Buck & Edwards, 1997; Dziezak, 1986).

The off-flavour of fish has been studied extensively, and has been ascribed to a variety of factors, including auto-oxidation, the formation of trimethylamine (TMA), and (Z)-4-heptenal and volatile acid production (Durnford & Shahidi, 1998). Wiechers and Rowan (1960) identified the lipid fraction as “the most important” contributor to the characteristic unpleasant smell of fish meal, although other factors, such as TMA and the products of scorching in direct hot air driers, were also acknowledged. Oxidation contributes to off-odours through the formation of foul smelling, volatile compounds, that include low molecular weight aldehydes and ketones, which, even at low concentrations, contribute to rancid odour (Alais & Linden, 1991). Not only does the unstable nature of fish oil lead to increased lipid oxidation, it also contributes to a fishy taste in the food in
which it is included (Saldeen et al., 1998). Oxidation studies have, however, proved the limited effect of preventing oxidation on fishy flavour elimination. Hamilton et al. (1998) state that, in spite of limiting oxidation to insignificant levels and maintaining long chain PUFA levels with a ternary blend of antioxidants, fishy flavours still developed rapidly.

The main purpose of this study was, however, to protect the long chain PUFA’s in hake head fish flour against oxidation in order to create an effective dietary intervention tool by ensuring FA bio-availability, rather than to improve the flavour profile of the product. A review of lipid deterioration via oxidation and the prevention thereof therefore follows.

2.4 LIPID DETERIORATION

Love (1992) ascribes lipid deterioration to one of three processes: thermal degradation, fat hydrolysis, or oxidation.

Thermal degradation in fats becomes pronounced at elevated temperatures, where complex changes take place and lipids can undergo thermal and oxidative reactions. The products include volatiles (the decomposition products of the unsaturated FA’s), but the greatest quantity of thermally altered material is dimers, polymers and cyclic compounds (Love, 1992).

Hydrolysis is the breaking of the ester bonds in fats in the presence of heat and moisture or by lipolytic enzymes to yield free FA’s (FFA’s) and glycerol or mono- and diacylglycerols (Love, 1992; Buck, 1991). Miller (1993) reports that acids and alkalis in oils can also catalyse hydrolysis. The FFA content is considered an indicator of the overall quality of the product (Love, 1992). The FFA’s can cause bitter or soapy off-flavours in some oils, often referred to as hydrolytic rancidity (Buck, 1991). Hydrolytic rancidity is mainly encountered in products based on lauric oils, such as palm kernel or coconut oils (Rossell, 1994). Fats with long chain PUFA’s (such as fish oils) seldom display off-flavours due to hydrolytic rancidity, even if high quantities of FFA’s are
present (Love, 1992). The use of food additives cannot prevent hydrolysis, which should be controlled through the use of high quality ingredients and good manufacturing practices (Buck, 1991).

Oxidation is considered the most common problem associated with the production, storage and usage of food oils and fats (Buck, 1991), and is therefore now discussed in greater detail.

2.4.1 Lipid oxidation

2.4.1.1 Definition

Lipid oxidation is a reaction between the unsaturated bonds of the FA’s in TAG’s and atmospheric oxygen (Gunstone, 1996; Dziezak, 1986).

2.4.1.2 Substrates

The main oxidation substrate is the lipid fraction that contains unsaturated bonds. Saturated fats also oxidize, which may result in the development of off-odours and flavours. Saturated fat oxidation is, however, a very slow process, and consequently it is not considered a noticeable contributor to food oxidation, where “rancidity” mostly refers to oxidation in the unsaturated fraction (Stuckey, 1972). Of the unsaturated fats, PUFA’s are more vulnerable to oxidation than MUFA’s because the methylene group (CH₂) between two C-C double bonds is extremely reactive. The rate of oxidation generally increases with the degree of unsaturation (Miller, 1993).

Fish is a rich source of n-3 PUFA’s, specifically the long chain n-3 FA’s, EPA and DHA. Up to 30% of the FA’s in fish oil may consist of a combination of EPA and DHA (Gunstone, 1996; Kaitaranta, 1992). This high PUFA content, together with the high amount of doubly allylic CH₂ groups present in the long chain PUFA, renders fish oils very susceptible to oxidation (Michelsen, et al., 2001; Gunstone, 1996; Kaitaranta,
The longer the CH₂-interrupted polyene pattern is extended, the more oxidation is accelerated. The rate of auto-oxidation relative to linoleate (= 1) is approximately equivalent to the number of doubly allylic CH₂ groups. Thus EPA (C20:5) and DHA (C22:6) are expected to oxidize four of five times faster than linoleate (Gunstone, 1996).

This is illustrated by the work of Cho et al. (1987), who compared the extent of oxidation of the methyl esters of EPA, DHA, ethyl linoleate (Lo) and ethyl linolenate (Ln), stored in the dark at 5°C, with various chemical parameters. The authors report that the esters of EPA and DHA oxidised rapidly, with an induction period of 3-4 days, while the Ln- and Lo-esters had induction periods of 20 and more than 60 days respectively.

Plant oil PUFA's typically have only half the amount of double bonds present than what is found in the long chain PUFA’s abounding in fish oil, i.e. two or three instead of up to six (Stansby, 1982), thus having fewer vulnerable sites for oxidation. Plant oils also contain much higher concentrations of natural antioxidants, such as tocopherols, which help protect against oxidative degeneration (Stuckey, 1972). In addition, Hamilton et al. (1997) report that fish oil have a much higher level of pro-oxidant metals than some plant oils (0.25 ppm of iron relative to the 0.06 ppm in maize oil), which also renders fish oil more susceptible to oxidation.

The problem of lipid oxidation can be of the same magnitude in foods containing small amounts of highly susceptible lipids than in fats and oils and high fat foods (Love, 1992). Fats become more susceptible to oxidation if their double bonds are in a cis- instead of a trans-configuration, if the double bonds are conjugated instead of non-conjugated, and if FFA’s are available instead of esterfied FA’s. The FA position on the glycerol molecule also influences the rate of oxidation (Michelsen et al., 2001; Love, 1992).

2.4.1.3 Mechanisms

Three mechanisms for oxidation exist. Oxidation can be of non-enzymic origin, which includes auto- and photo-oxidation, or it can occur due to enzyme action.
2.4.1.3.1 Auto-oxidation

Auto-oxidation is a radical chain process: "radical" because the intermediates are radicals, and a "chain process" since the reaction has an initiation step followed by a propagation sequence, which continues until termination occurs (Gunstone, 1996). During initiation, a hydrogen atom (H), i.e. a proton, is lost from the \( \alpha \)-methylenic C in an unsaturated FA (Sherwin, 1978). The hydrogen on a C atom taking part in a double bond is the most labile due to the nature of the electron distribution in the molecule (Hahm & Min, 1995). Hydrogen abstraction results in the formation of a free radical, which is very susceptible to attack by atmospheric oxygen to form an unstable peroxide free radical (Sherwin, 1978). A free radical is any chemical species having one or more unpaired electrons (Hamilton et al., 1997).

The nature of the initiation step that results in the formation of a free radical \((R')\) from the lipid is still not clearly elucidated. Although lipid oxidation is thermodynamically favourable, the reaction of PUFA's with molecular oxygen is kinetically unfavourable. An activating reaction must therefore occur to initiate oxidation (Saeed & Howell, 1999). Oxidation can, for example, be instigated by the presence of external energy sources (i.e. heat, light or high energy radiation), or by chemical initiation, with metal ions or metallo-proteins such as haem (Hamilton, 1994; Love, 1992). If the lipid contains peroxides or hydroperoxides, their decomposition may result in the formation of free radicals, which can also initiate oxidation (Gunstone, 1996; Love, 1992).

The steps of auto-oxidation (Figure 2.2) are widely described as:

- **step 1**: initiation, which is the production of a free radical \((R')\) through the abstraction of a H from a lipid molecule (RH)
- **step 2**: propagation, during which the reactive \(R'\) reacts with oxygen to form a lipid peroxy radical \((ROO')\). The \(ROO'\) catalyses further oxidation by extracting a H from a second lipid to form a hydroperoxide \((ROOH)\) and a \(R'\) from the other
Step 1: initiation (catalyst involved)

\[ RH \rightarrow R' + H^+ \]

\[ \downarrow \]

Step 2: propagation - a cyclical process (second lipid involved)

2.1. \( R' + O_2 \rightarrow ROO' \)

2.2. \( ROO' + RH \rightarrow ROOH + R' \)

\[ \downarrow \]

Step 3: termination, resulting in stable products

Reactions between radicals:

3.1. \( R' + R' \)

3.2. \( ROO' + ROO' \)

3.3. \( R' + ROO' \)

\[ \rightarrow \] stable, non-radical products

Figure 2.2. A simplified summary of the general steps of auto-oxidation

- lipid molecule. This newly formed \( R' \) can then repeat step 2, hence the cyclical nature of oxidation

- step 3: termination, which occurs when reactions that form only stable products take place.

Hamilton et al. (1997) describes an additional branching step, in which the breakdown of the hydroperoxides formed in step 2 is described as one of two reactions (ROOH = hydroperoxide; RO' = alkoxy radical, ROO' = peroxy radical, OH' = hydroxyl):

Reaction 1  \[ ROOH \rightarrow RO' + OH' \]

Reaction 2  \[ 2 ROOH \rightarrow ROO' + RO' + H_2O \]
Both breakdown reactions result in the formation of free radicals, which then aid the propagation step, further promoting the cyclical nature of oxidation (Hamilton et al., 1997; Stuckey, 1972). Thus the oxidation products catalyse the oxidation reaction, resulting in an increasing rate of oxidation over time (Dziezak, 1986).

Gunstone (1996) states that the rate of oxidation (as depicted by the chain sequence in Figure 2.2) is determined by the structure of the free radical formed, which is determined by the FA oxidised. In the propagation step, the reaction between the R' and oxygen (step 2.1) is rapid if oxygen is freely available, while the reaction of ROO' with another lipid molecule (step 2.2) is rate-determining.

In the presence of oxygen, the main termination reaction is that between two peroxy radicals (step 3.2), while the other termination reactions (steps 3.1 and 3.3) become important when the oxygen concentration is low (Love, 1992). Gordon (1990) states that, although the termination of auto-oxidation by the combination of two R' is favourable given the very low enthalpy of activation thereof, the occurrence of this termination reaction is limited by two factors. Firstly, steric factors necessitate the collision of two R' in the correct orientation for the termination reaction to occur, secondly, a relatively low concentration of R' exist, since the reaction of a R' with oxygen has an almost zero activation energy, resulting in much higher ROO' - than a R' -concentration.

2.4.1.3.2 Photo-oxidation

Photo-oxidation requires the presence of a sensitiser, such as chlorophyll, erythrosine, rose Bengal, or methylene blue, to, in the presence of light, produce highly reactive singlet oxygen from ordinary oxygen, which then reacts with a double bond in an unsaturated FA (Gunstone, 1996). Singlet oxygen can directly attack a double bond as double bonds have a high electron density and singlet oxygen is very electrophilic (Cuppett et al., 1997). No free radicals are involved in photo-oxidation, and therefore this form of oxidation is not self-perpetuating. The hydroperoxides formed can, however,
decompose to form free radicals, which can initiate auto-oxidation (Love, 1992). Although light also accelerates auto-oxidation, it is unlikely that this is due to photo-oxidation as described above, because unsaturated FA’s cannot absorb light energy of a wavelength smaller than 220 nm (Hamilton et al., 1997).

2.4.1.3.3 Enzymic oxidation

Enzymic oxidation is catalysed by a group of iron-containing enzymes, called lipoxygenases, which occur widely in plants, and have been found in animals (Love, 1992; Gunstone, 1996). Lipoxygenases catalyse oxidation in methylene interrupted PUFA’s with cis-double bonds (Forss, 1967). The resulting reaction is between oxygen and cis,cis-1,4-dienes, and leads to hydroperoxide formation (Gunstone, 1996). The principle oxidation products are the same as those obtained with auto-oxidation, except that the products from enzymic oxidation are optically active, which indicates an orientated rather than a random attack by oxygen (Forss, 1967). The hydroperoxides can be fragmented by hydroperoxide lyase into short-chain compounds, such as aldehydes, ketols, divinyl ether FA, etc. (Hamilton et al., 1997).

Lipids can be hydrolysed by endogenous or microbial lipases (Huss, 1988). The role of microbial lipases may be of importance if the heads used for flour preparation have pieces of intestine attached and are not stored at low temperatures, as it has been shown that a considerable amount of FFA’s can develop during the storage of un-eviscerated fish (Huss, 1988). Fatty acids are, as reported, more susceptible to oxidation when free than when esterified. This is also true for enzymic oxidation, since lipoxygenase reacts much more readily with free than bound FA’s (Hamilton et al., 1997). Microbes present in the intestines could therefore increase at favourable growth temperatures and accelerate lipid degradation by causing lipid hydrolysis. In a study on mechanically deboned fish, treatment with an antibiotic (chlorotetracycline HCl) did however not result in a significant reduction in the thiobarbituric acid (TBA) value, and the researchers postulated that microbial degradation did not contribute significantly to oxidative rancidity under the conditions employed (Lee & Toledo, 1977). These authors also refer
to previous research that indicated the insignificant role of extra-cellular bacterial enzymes in lipid hydrolysis and the incompatibility of bacterial growth with the production of rancid odours.

2.4.1.4 Oxidation products

The main oxidation products are double bond containing hydroperoxides, of which the position and/or configuration of the double bonds may have changed from those present in the PUFA's. Hydroperoxides may undergo further changes to form compounds of a higher (e.g. dimers or polymers) or lower molecular weight (e.g. volatiles, such as aldehydes), or compounds with the same chain length, i.e. rearrangement products (Gunstone, 1996).

Different fats result in the formation of structurally different hydroperoxides, each which results in specific breakdown products (Gunstone, 1996). The unstable lipid hydroperoxides break down to form an alkoxy radical, which can then decompose into various products (Hamilton, 1994; Love, 1992). Hydroperoxides do not possess any flavour (Shahidi, 1998), but their decomposition products include short-chain volatile compounds that contribute to odour and flavour, namely, ketones, alcohols, acids, esters, lactones, ethers, hydrocarbons, and aldehydes. Of these, the aldehydes are particularly significant in flavour studies (Gunstone, 1996). A complex mixture of aldehydes is released during fat oxidation because of the large number of hydroperoxides present for decomposition. Each hydroperoxide can decompose into two aldehydes, of which the short chain volatile member is considered the most significant in terms of odour. Aldehydes typically have very low threshold values and become noticeable at minute concentrations (Gunstone, 1996).

The hydroperoxides formed by the oxidation of the long chain PUFA's, which abound in fish, produce volatiles more readily than those formed from other oils because of a lower activation of decomposition (Michelsen et al., 2001). Controlled oxidation of steam-deodorised fish oil allowed a description of the compounds that develop during oxidation
and of their resulting aromas. Initially, "green flavours" develop, with mainly t,c-2,6-nonadienal, but also t-2-hexenal and l,c-5-octadien-3-one contributing. This is followed by a depletion of 2,6-nonadienal, an increase in c-4-heptenal, and concomitant formation of t,c,c- and t,t,c,-2,4,7-deactrienal. These compounds contribute to oxidised, fish or cod-liver oil like flavours. In addition, unidentified compounds that contribute to "trout- and minnow-bait-bucket-like odours" develop. Hexanal, 2,4-heptadienals and 2,4-decadienals all also contribute to general, oxidised, "painty" flavours (Karahadian & Lindsay, 1989).

2.4.1.5 Disadvantages of oxidation

Oxidation is known to have a significant effect on the nutritional quality of marine oils (Shahidi & Wanasundara, 1995). It destroys the nutritional value of food through the destruction of fat-soluble vitamins, such as A, D and E, through possible reduction of the caloric content of foods, and by decreasing the EFA (Buck & Edwards, 1997; Dziezak, 1986). Oxidation also leads to rancid odours and flavours, and reduces the distinctive flavour and odour of oils (Buck & Edwards, 1997; Dziezak, 1986). Oxidation also results in oil colour and viscosity changes. The colour can lighten or darken, depending on the substrate, while the viscosity tends to increase due to polymerisation (Buck, 1991).

A further disadvantage of oxidation is that the reactive products of lipid oxidation, such as free radicals and peroxides, are potentially damaging to cells. Free radicals may initiate tumorigenesis by lipid peroxidation or by direct reaction with DNA. Animal studies, however, indicate that very few of these reactive species are absorbed intact and are thus able to cause damage. The smaller molecular weight degradation products of lipid peroxides may, however, possibly be more readily absorbed and thus be potentially toxic (BNF, 1992).

Lipid oxidation products may also interfere with physiological functions. For example, Agerbo et al. (1992) report that secondary lipid auto-oxidation products found in oxidised fish oil inhibited the enzyme glucose 6-phophatase, although the concentration of
inhibitor necessary for a measurable effect was much higher than the level found in fish oil.

2.4.2 Prevention of lipid oxidation

Besides the degree and nature of unsaturation (position of the double bonds), oxidative stability also depends on storage conditions and on the antioxidant and pro-oxidant content of a food (Miller, 1993).

2.4.2.1 Methods to delay oxidation other than antioxidants

Firstly, low temperature storage is critical in limiting oxidation. High processing temperatures may also promote oxidation. In a study on mechanical de-boning of fish muscle, significantly higher TBA values were recorded after three days when de-boning at 25°C than when de-boning at a lowered drum temperature of 2-3°C (Lee & Toledo, 1977). Buck and Edwards (1997) report that a 10°C increase in temperature doubles the oxidation rate. Thus temperature control can limit oxidation.

The pro-oxidant content of oil refers to the presence of metal ions. Trace levels of metal ions significantly enhance lipid oxidation (Furia, 1972). Levels of only 0.05 ppm copper and 0.6 ppm iron, for example, halved the keeping time of lard at 98°C, which clearly illustrate the need to minimise metal ion concentrations (Gunstone, 1996).

Oxygen exposure should also be minimised during the processing of long chain PUFA-containing foods (Michelsen et al., 2001). Macerating of tissues incorporates oxygen and disrupts and intermixes tissue components, and can thus result in lipid oxidation even in non-fatty fish (Hultin et al., 1982). Macerating also releases lipolytic enzymes, which can release FFA’s (Hamilton, 1994), and unsaturated FA’s mostly oxidize faster when in a free state than when bound (Alais & Linden, 1991). Contact with air can be minimised by nitrogen blanketing and by avoiding splashing of the product (Gunstone, 1996). Light (wavelengths in the visible, ultraviolet and ionizing radiation region of the
electromagnetic spectrum) is also a potent initiator and catalyst of oxidation (Love, 1992; Buck & Edwards, 1997). Packaging that limits both oxygen and light exposure is therefore recommended to impede oxidation (Michelsen et al., 2001; Love, 1992).

An alternative method to inhibit oil oxidation is through the micro-encapsulation of oil. Micro-encapsulation entraps oil into a wall matrix that serves to protect the oil from oxygen, moisture, and light (Shahidi & Wanasundara, 1995). Yoshii et al. (1997) showed that encapsulating DHA oil into a powder form markedly protected the chemically reactive PUFA’s against oxidative degradation as measured with the peroxide value (PV). Thus the coating material protects the oil from oxidation during processing, masks odour and taste, and prolongs shelf-life in the final application (Michelsen et al., 2001).

2.4.2.2 The use of antioxidants

Besides the use of process control to protect against lipid oxidation, the use of antioxidants is often also required. Protection against oxidation is more readily assured if processing and packaging procedures aim to reduce all of the above factors, and use these measures in combination with antioxidants as well as other chemical preservatives (Dziezak, 1986). Antioxidant application is typically controlled through regulations, although not all countries stipulate similar guidelines – i.e. tertiary butyl hydroquinone (TBHQ) is allowed in the United States of America, but not in Europe (Gunstone, 1996). Antioxidants are utilised to slow oxidation as far as possible, but are unable to prevent or reverse oxidation. Antioxidants therefore simply serve to extend the inhibition period, during which oxidation is very slow (Gunstone, 1996; Dziezak, 1986).

2.4.2.2.1 Types of antioxidants

Antioxidants are classified as being either from a natural or synthetic origin. They are also classified according to their mode of action, as the broad term of antioxidant encompasses compounds with different functions in protecting against rancidity. The
following types of antioxidant compounds are identified (Kochhar & Rossell, 1990; Dziezak, 1986):

- free radical terminators, that interrupt the free-radical chain of oxidative reactions
- sequestering agents (chelators), that bind pro-oxidative compounds, such as metal ions
- oxygen scavengers, that react with oxygen
- secondary antioxidants, that break down hydroperoxides
- enzymic antioxidants, which remove dissolved or headspace oxygen, or remove highly oxidative species from food systems.

A broader antioxidant classification encountered is a division of antioxidants into primary and secondary antioxidants. In this classification, primary antioxidants are those that interrupt the auto-oxidation chain by converting free radicals to more stable species, while secondary antioxidants are any compounds that retard the rate of oxidation by any other means than that described for the primary antioxidants (Gordon, 1990). Secondary antioxidants would therefore include chelating agents, oxygen scavengers and the decomposers of hydroperoxides already mentioned, while Gordon (1990) also identify additional mechanisms of action for the secondary antioxidants, such as the absorption of ultraviolet (UV) radiation.

Often a synergistic effect is observed between various compounds. The term synergy describes the phenomenon that occurs when a mixture is more effective than what the sum of the activity of the individual components would suggest (Gunstone, 1996). The synergistic effects that can be obtained between antioxidants are critical in the stabilisation of the oxidation prone n-3 PUFA’s (Michelsen et al., 2001).

A brief discussion of antioxidant functions under the classification of antioxidants as being either primary or secondary follows.
2.4.2.1.1 Primary antioxidants: free radical terminators

Free radical terminators function through interference with the propagation cycle by promoting termination processes (Gunstone, 1996). The synthetic phenolic antioxidants butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tertiary butyl hydroquinone (TBHQ) and propyl gallate (PG), as well as the tocopherols and amines, and compounds with extensive unsaturation, such as carotenoids, all function as free radical terminators. Free radical terminators function by donating a hydrogen from a phenolic hydroxyl group to (Giese, 1996; Gunstone, 1996):

- a free radical, formed during step one of oxidation, to reform the fat molecule, i.e. the antioxidant functions in a sacrificial manner
- a peroxide free radical to form a hydroperoxide and an antioxidant free radical (i.e. the anti-oxidant without a hydrogen)

The antioxidant therefore functions in a sacrificial manner. Consequently oxidation is delayed until all or most of the antioxidant has reacted, at which point the induction period then ends. The antioxidant free radical breaks the oxidation chain reaction as the electron resonance structure of the aromatic ring of the antioxidant renders the antioxidant free radical very stable (Giese, 1996; Gunstone, 1996). These compounds therefore interrupt oxidation and propagation of the oxidation process without becoming highly reactive as a result.

2.4.2.1.2 Secondary antioxidants: sequestering agents, oxygen scavengers and hydroperoxide decomposers

Sequestering agents inhibit the chain-initiating process (Gunstone, 1996) by reacting with metal ions (i.e. copper, iron, zinc and calcium) that are considered pro-oxidant catalysts in almost all oxidation sensitive foods (Giese, 1996; Hughes, 1987; Furia, 1972). Sequestering agents contain an unshared electron pair in their structure that promotes metal ion complexion (Dziezak, 1986). By reducing the pro-oxidative effect of metal
ions through chelation, the activation energy of the oxidation initiation reactions is considerably raised (Gordon, 1990).

The selection of a sequestering agent is influenced by toxicological considerations, cost, and by colour-, flavour- and substrate-compatibility. The citrates, for example, have a distinct acidic flavour, of which the effect in the final food should be considered. The compatibility of the citrates with oils and fats can be enhanced if used in a solution with propylene glycol (Furia, 1972). The most commonly used sequestering agents are the citrates, phosphoric acid derivatives, ethylene diamine tetra-acetic acid (EDTA), and certain amino acids (Gunstone, 1996; Gordon, 1990; Dziezak, 1986). These compounds display a synergistic relationship with synthetic antioxidants such as BHT, BHA and PG, as well as with ascorbic and iso-ascorbic acid, tocopherols, phospholipids and thiopropionates (Furia, 1972).

Oxygen scavengers react with oxygen in a redox reaction and include ascorbyl palmitate, ascorbic acid, sulfites, glucose oxidase, and erythorbic acid and its’ salt, sodium erythorbate (Dziezak, 1986). Oxygen reacts with a scavenger such as ascorbic acid in preference to unsaturated lipids, oxidizing sodium ascorbate to dehydroascorbic acid (Love, 1992; Gordon, 1990; Dziezak, 1986). Thus the unsaturated lipids are protected from oxidation.

Hydroperoxides decomposers refer to compounds such as thiodipropionic acid and dilauryl thiodipropionate, which decompose hydrogen peroxides produced during lipid oxidation to form stable products (Dziezak, 1986).

### 2.4.2.2 Selection of antioxidants

The selection of the correct mix of antioxidants, synergists and sequestering agents to optimally protect a product against rancidity is a difficult task influenced by many factors. Firstly, the chosen mix should have regulatory approval in the country for which the product is intended (Michelsen et al., 2001; Stuckey, 1972). The antioxidant
compatibility and effectiveness within the particular product and its' potential for
dispersion in the product is also vital. The antioxidant should be soluble in the water/oil
phase of the product, and should disperse well through the food, especially if the product
has a low fat content (Michelsen et al., 2001; Dziezak, 1986).

Economical considerations also influence antioxidant selection (Michelsen et al., 2001;
Stuckey, 1972). The synthetic antioxidants, such as BHT, BHA and TBHQ, are typically
less expensive and more efficient at lower levels than the natural antioxidants (Gunstone,
1996; Jayasinghe et al., 1997). Buck (1991) proposes that the general performance trend
of antioxidants in animal fats and oils is TBHQ > propyl gallate > BHA > BHT >
tocopherols.

Although the synthetic antioxidants are approved for use in food products at specific
levels (Codex Alimentarius Commission, 1999), their use in foods for babies and young
children is not recommended (Hanssen, 1987). The preferential use of natural
antioxidants is in fact an increasing trend (Wanasundara & Shahidi, 1998; Jayasinghe et
al., 1997; Gunstone, 1996; Frankel, 1996). This trend can probably be ascribed to the
possible toxic effects attributed to the synthetic antioxidants (White & Xing, 1997). For
example, PG may cause gastric irritation, BHA can raise blood lipid and cholesterol
levels, and BHT can increase the rate of breakdown of vitamin D and possibly lead to
reproductive failures, behavioral effects and blood cell changes (Hanssen, 1987). Barlow
(1990) reports that recent studies showed that BHA and BHT could produce tumours in
animals, while TBHQ has shown mutagenicity in some studies. Besides health benefits,
replacement of the synthetics with natural antioxidants could have added functional
benefits, such as solubility in both oil and water (Moure et al., 2001).

Possible changes in the product flavour, colour and consistency because of antioxidant
addition should also be evaluated (Michelsen et al., 2001), as well as an antioxidant’s
carry through characteristics. “Carry through” is defined as the antioxidant’s stability
during processing steps, such as baking or frying, so that it can still function effectively in
the finished food (Giese, 1996).
Ideally, antioxidants should be added as early as possible during processing to protect the product against oxidation from as soon as possible (Dziezak, 1986; Sherwin, 1978). This is especially true if heat forms part of processing, in which case it is ideal to add the antioxidants prior to heat processing (Stuckey, 1972).

2.4.2.2.3 Specific antioxidants tested

2.4.2.2.3.1 Combinations of lecithin, sodium ascorbate/ascorbic acid and tocopherols

Burkow et al. (1995) illustrated the protective effect of lecithin against fish oil oxidation by determining induction times with the Rancimat for cod liver oil (CLO) treated with various substances. The induction time (IT) of a CLO control was 2.9 h. Treatment with 40% soybean and 60% egg lecithin increased the IT to 11.0 and 9.1 h respectively. Yi et al. (1991) also state that lecithin gave a lengthened IT relative to a control fish oil in a weak antioxidant effect. In a study on antioxidant properties in fish oil stored at 20°C, Hamilton et al. (1998) report that lecithin (0.5%) slightly improved oxidative stability, and acted in a synergistic fashion with ascorbyl palmitate, and in a pronounced synergistic fashion with a combination of ascorbyl palmitate and tocopherols.

Lecithin is also used in antioxidant blends because of its’ function as an emulsifying or surface active agent. Surface active agents, which consist of hydrophilic and hydrophobic parts, are substances that lower the interfacial tension and that form an energy barrier that prevent contact between emulsified drops (Setser, 1992). Surfactants can be used to enhance the uniform dispersion of water soluble antioxidants in oil. Han et al. (1990) report that the water soluble ascorbic acid can be used in oil systems by the addition of a surfactant to the oil, where after ascorbic acid, dissolved in water, is added to the oil mixture while stirring gently to form “reversed micelles”, obtaining uniform dispersion of the ascorbic acid-solution in the oil phase. Dispersing ascorbic acid thus was found to impart considerable oxidative stability to fish oil at only 0.02% concentrations, and the ascorbic acid also acted synergistically with δ-tocopherol (Han et al., 1990).
The tocopherols and ascorbic acid are the most important natural antioxidants used in commercial applications (Schuler, 1990; Stuckey, 1972).

Free ascorbic acid, as well as bound forms thereof, such as sodium ascorbate, calcium ascorbate, ascorbyl palmitate and ascorbyl stearate, are used as food antioxidants (Schuler, 1990). Ascorbic acid (a white crystalline powder) and sodium ascorbate (a white to slightly yellowish crystalline powder) are water soluble at respectively 33 and 89 g/100 ml of water. Therefore ascorbyl palmitate, which is more fat soluble, is often used as an antioxidant (Gunstone, 1996; Schuler, 1990). The improved fat solubility of ascorbyl palmitate is ascribed to a less polar nature because of the FA esterification present (Dziezak, 1986). Ascorbyl palmitate is a white or yellowish white crystalline powder produced from ascorbic acid and palmitic acid (Giese, 1996). Although it is not a naturally occurring substance, hydrolysis of ascorbyl palmitate forms ascorbic and palmitic acid, which are both natural (Dziezak, 1986). Levels of 0.003–5% give antioxidant activity, although these levels exceed solubility (Dziezak, 1986).

Ascorbic acid and ascorbyl palmitate function through oxygen scavenging, regeneration of phenolic antioxidant radicals, metal inactivation, and by the reduction of hydroperoxides to produce stable alcohols (Frankel, 1996). Sodium ascorbate is a moderately strong reducing agent (Borenstein, 1972). As stated previously, oxygen reacts with sodium ascorbate, when its’ 2- and 3-positions are unbound, in preference to unsaturated lipids. Sodium ascorbate is thus oxidised to dehydroascorbic acid (Love, 1992; Gordon, 1990; Dziezak, 1986).

Regeneration of phenolic antioxidants occurs when ascorbic acid donates hydrogen atoms to spent antioxidants in what can be described as an antioxidant sparing activity (Gunstone, 1996; Love 1992). A study by Niki et al. (1984), in which the inhibition of methyl linoleate oxidation by vitamin E and vitamin C was investigated, illustrates this action. During the initial stages of oxidation, vitamin E was found to remain almost unchanged while vitamin C was consumed. Only once the vitamin C was exhausted did vitamin E start to decrease. It was concluded that vitamin E scavenges the peroxy radical.
more effectively than vitamin C, and that the resulting vitamin E radical then reacted with vitamin C to regenerate vitamin E.

Dziezak (1986) reports that ascorbic acid by itself has little antioxidant activity, but that it acts synergistically with other antioxidants in protecting against oxidation. The combination of tocopherols and ascorbic acid is in fact more effective in retarding oxidation than a combination of tocopherols with synthetics such as BHT or BHA (Dziezak, 1986). A combination of the phenolic tocopherols and ascorbic acid were indeed found to be synergistic in protecting ground beef against oxidation (Mitsumoto et al., 1991). Synergism between sodium ascorbate and tocoperol was also reported in a fish oil storage trial at 30°C. Ascorbic acid alone (dispersed with a surfactant) yielded a peroxide value (PV) of 10 meq/kg oil after approximately 18 days of storage, while a combination of ascorbic acid with δ-tocopherol gave a PV of only 3 meq/kg of oil after forty days of storage (Han et al., 1990). Yi et al. (1991) report that the synergistic effect between a fixed level of ascorbic acid and varying levels of δ-tocopherol was generally 100% or more. Ascorbyl palmitate, similarly to sodium ascorbate, also acts synergistically with tocopherol (Hamilton et al., 1998; Giese, 1996; Love, 1992).

If the use of synthetic antioxidants is not allowed by regulations regarding the use of additives in food, or is not favoured by consumers, tocopherols are valuable natural alternatives (Giese, 1996). The tocol group consist of the tocopherols and the tocotrienols, which have similar structures except that the tocopherols have a saturated trimethyltridecyl side chain while the tocotrienols have three double bonds in the side chain (Gunstone, 1996; Schuler, 1990). Each of these groups occurs as four naturally occurring homologues, namely α-, β-, γ- and δ-tocopherol, i.e. a total of 8 tocopherols exist, of which only the D-isomers occur in nature (Michelsen et al., 2001; Cuppett et al., 1997; Borenstein, 1972). The tocopherols are also synthetically produced, i.e. α-tocopherol is produced by the condensation of isophytol with 2,3,5-trimethylhydroquinone, forming a racemic mixture (designated dl-α-tocopherol or all-rac-α-tocopherol) of all eight possible stereo-isomers (Schuler, 1990). The tocopherols are quite fat-soluble due to their long side chains (Stuckey, 1972). They are clear,
viscous, pale yellow, oily substances that oxidize and darken on air and light exposure (Schuler, 1990). The tocopherols are more effective in animal fats than in some plant oils, and effective concentrations fall in the range of 0.01 – 0.02% (Dziezak, 1986).

The tocopherols have both vitamin and antioxidant activity. The order for vitamin E activity, usually expressed in α-tocopherol equivalents, is reported as α (1.0) > β (0.5) > γ (0.1) > δ (0.03), and this order is reversed for antioxidant efficiency (Moure et al., 2001; Hamilton et al., 1998; Gunstone, 1996). Giese (1996) and Buck (1991) also state that, although α-tocopherol exhibits some antioxidant potency, the γ- and δ-tocopherol homologues are more effective antioxidants.

Frankel (1996), however, reports that there is much discrepancy in the literature on the relative antioxidant efficiencies of the tocopherol homologues, and that this can be attributed to the wide differences in unsaturated substrates tested, the levels of oxidation used in the tests, the method used to follow oxidation and the test system. This is confirmed by Michelsen et al. (2001), who state that the relative antioxidant activity of the tocopherol homologues in the n-3 long chain PUFA's depends on the conditions. For example, α-tocopherol is very active, and is consumed quickly, but can be regenerated in the presence of ascorbic acid.

Burkow et al. (1995), for example, used the Rancimat to compare antioxidant efficiencies (1.5 mg of antioxidants/g of oil) in CLO. Induction times of 2.9 h for a CLO control oil, 4.7 h for an α-tocopherol treated oil, and 11.1 h in a δ-tocopherol treated oil were recorded, which illustrate the less significant protective effect yielded by the α- relative to the δ-tocopherol homologue. Ke et al. (1977) however, in a study on oxidation in mackerel skin lipids, report that d-α-tocopherol provided good protection against oxidation, better than BHA and BHT (order of efficiency of compounds tested: TBHQ > α-tocopherol > tempeh oil > BHA > BHT). The properties of the various forms of α-tocopherol also differ. Borenstein (1972) report that l-α-tocopherol has a lower vitamin
E activity (international units/mg) than d-\(\alpha\)-tocopherol, while the activity of dl-\(\alpha\)-tocopherol is intermediate (values of 0.51, 1.49 and 1.1 respectively).

The tocopherols derive their antioxidant function by acting either as free radical terminators or as singlet oxygen scavengers (Cuppert et al., 1997; Giese, 1996; Love, 1992). The free radical termination function of tocopherols is illustrated by the following general reactions (Schuler, 1990):

\[
\text{Reaction 1} \quad R^\cdot + AH_2 \rightarrow RH + AH^\cdot \\
\text{Reaction 2} \quad 2 AH^\cdot \rightarrow A + AH_2
\]

Reaction 1 forms a regenerated lipid molecule (RH). Reaction 2 consists of a reaction between two tocopheryl semiquinone radical molecules (AH\(^\cdot\)) to form one molecule each of regenerated tocopherol and tocopherylquinone (A). The singlet oxygen scavenging ability of the tocopherols occurs via quenching thereof, or through an irreversible reaction therewith to form a variety of products (Cuppert et al., 1997).

The acetate form of tocopherol does not normally function as an antioxidant since the active hydroxyl group is protected, resulting in excellent stability (Schuler, 1990; Borenstein, 1972). A slow hydrolysis, for example in acidic aqueous systems, can however release the bound tocopherol to act as an antioxidant (Schuler, 1990). Sahoo and Anjaneyulu (1997) report that 500 ppm sodium ascorbate, 10 ppm dl-\(\alpha\)-tocopherol-acetate and 0.5% sodium tripolyphosphate in buffalo meat nuggets significantly \((p < 0.05)\) lowered the FFA content in treated samples relative to a control with no antioxidant treatment. Kaitaranta (1992) investigated the effectiveness of \(\alpha\)-tocopherol-acetate \((0.03\%\) in fish oil and found that, although much less effective than the synthetic antioxidants tested, it did slightly retard oxidation relative to a control oil.

The tocopherols are marketed for antioxidant applications in oily product forms, i.e. as pure \textit{all-rac-}\(\alpha\)-tocopherol, or as mixed tocopherols, usually diluted in a vegetable oil, or as part of synergistic mixtures. The synergistic mixtures, which are usually pastes,
combine tocopherols with antioxidants, such as ascorbyl palmitate, synergists, such as lecithin and citric acid, and carriers (Schuler, 1990).

Newton and Snyder (1997) state that the production of n-3 rich oils for food applications typically includes stabilisation of the oil with a ternary antioxidant mix of tocopherols, ascorbyl palmitate and lecithin. Hamilton et al. (1998), who investigated the results of various accelerated oxidative stability studies in fish oil, concluded that the most notable effect in specifically fish oil stabilisation is indeed achieved with a ternary blend consisting of α- or γ-tocopherol, ascorbic acid or ascorbyl palmitate, and lecithin. They proceeded to illustrate the protective effect of the tocopherols and ascorbic acid and the synergism between them in a study on antioxidant properties in fish oil stored at 20°C.

The results can be summarised as follows (the concentrations reported refer to the amounts of concentrates added to the oil): ascorbyl palmitate (0.1%) had a slight initial pro-oxidant effect relative to a control, while lecithin (0.5%) slightly improved oxidative stability. Together these two components were strongly synergistic, greatly extending the induction period relative to a control. Alpha-tocopherol (0.2 and 2%) showed a pro-oxidant effect relative to a control, while γ/δ and δ-tocopherol additions improved oxidative stability increasingly with increasing concentrations (up to 2%). The pronounced synergistic nature of a ternary blend of lecithin, ascorbyl palmitate and tocopherols were confirmed. This ternary mixture gave a greater protection against auto-oxidation in fish oil than when the individual components were used alone or in binary mixtures. The ternary blend with δ-tocopherol (2%) gave the best protection, while the ternary blend with α-tocopherol was most effective with α-tocopherol at a 0.2% level (Hamilton et al., 1998). In the evaluation of antioxidant efficiencies in CLO (using the Rancimat) previously referred to (Burkow et al., 1995), where the ineffectiveness of the α- relative to the δ-tocopherol homologue was illustrated, Ronoxan A (RA), also a ternary blend of ascorbyl palmitate, lecithin and dl-α-tocopherol, gave an IT of 8.1 h. This is close to the IT yielded by the more effective antioxidant tocopherol homologues. The relative effectiveness of RA, in spite of utilising α-tocopherol, could therefore probably also be attributed to the high degree of synergy between its' components.
2.4.2.3.2 *Citric acid and sodium metabisulphite*

Citric acid (CA) is a chelating agent, and thus helps to eliminate or minimise the metal pro-oxidative effect, and allows higher antioxidant efficiency, typically giving a synergistic effect (Furia, 1972). Citric acid is one of the commonly used sequestering agents (Dziezak, 1986). As discussed, combinations of the synthetic antioxidants and CA function synergistically. For example, 200 mg TBHQ offered less protection than a 100 mg combination of TBHQ and CA (Du Plessis and Sudworth, 2001). Sodium metabisulphite releases sulphur dioxide in solution and acts as both an antioxidant and a preservative (Hughes, 1987).

2.4.2.3.3 *Synthetic antioxidants*

The synthetic antioxidants most frequently used to preserve food are BHA, BHT, TBHQ and PG (Moure *et al.*, 2001). These antioxidants are all mono- or polyhydric phenols with various ring substitutions (Love, 1992). They function as chain-breaking antioxidants (free radical terminators) by reacting with free radicals generated during oxidation to form antioxidant free radicals. Antioxidant free radicals are stabilised by extensive de-localisation of the resulting odd electron and therefore will not initiate or further propagate oxidation (Michelsen *et al.*, 2001; Giese, 1996; Gunstone, 1996; Sherwin, 1978).

Michelsen *et al.* (2001) states that BHA, BHT, TBHQ, PG and ascorbyl palmitate are all similarly effective in protecting the n-3 long chain-PUFA's against oxidation. The use of PG however has several disadvantages: PG is less soluble than BHA and BHT, it is heat labile, and therefore loses its' effectiveness under heat conditions, and it forms undesirable coloured complexes with metal ions (Gunstone, 1996; Giese, 1996; Love, 1992; Dziezak, 1986). Therefore PG was not considered for use in this study.

Tertiarty butyl hydroquinone (TBHQ) is a moderately fat and oil soluble beige powder that is also slightly water soluble. It does not form coloured complexes with iron or
copper, and displays good carry through in frying, but not in baking applications (Buck & Edwards, 1997; Giese, 1996; Love, 1992; Dziezak, 1986). It is considered the most effective antioxidant for most fats and oils, and especially so for oils with a high degree of unsaturation, such as fish oils (Giese, 1996; Love, 1992). This is illustrated by the work of Kaitaranta (1992). He reported that TBHQ (0.01%) offered the best protection against oxidation in a fish oil when compared to α-tocopherol acetate (0.03%), PG (0.01%), ascorbyl palmitate, ethoxyquin, BHT, BHA (all at 0.02%) and Anoxomer, a synthetic polymer with a phenolic nature (levels from 0.02-0.2%). Wanasundara and Shahidi (1998) also report that TBHQ was found to be the most effective of the antioxidants tested in restricting an increase in PV during an oven storage test of menhaden oil.

Butylated hydroxyanisole (BHA) is a mixture of two isomers, with the 3-isomer functioning as the more effective antioxidant, and therefore comprising at least 90% of commercially sold BHA (Buck & Edwards, 1997). Butylated hydroxyanisole is a white, waxy flake that is fat-soluble, volatile and steam-distillable, which has good carry-through characteristics, and which more successfully suppresses animal fat than vegetable oil oxidation (Dziezak, 1986). Butylated hydroxytoluene (BHT) is a white, crystalline solid, and is less fat-soluble than BHA, and not soluble in propylene glycol. It has a good, but slightly less effective carry through effect than BHA (Gunstone, 1996; Dziezak, 1986). A synergistic action exists between BHA and BHT, while BHA also functions synergistically with PG (Buck & Edwards, 1997; Gunstone, 1996; Dziezak, 1986).

The synthetic antioxidants TBHQ, BHT and BHA are commonly formulated with propylene glycol, mono- and diglycerides or vegetable oils as carriers to increase solubility and dispersion (Michelsen et al., 2001).
2.4.2.3.4 Biored Pure

Biored is a water and fat-soluble, free-flowing light brown powder. It is a bio-flavonoid rich plant extract, and the Biored Pure form contains a minimum of 80% flavonoids (Biorem BK, CK 86/22269/23, P.O. Box 565, Jan Kempdorp 8550, South Africa). Flavonoids have been used successfully in the protection of fish (sardine) oil against oxidation (Nieto et al., 1993). Biored is claimed to act as an antioxidant through free radical scavenging and by heavy metal chelation (Biorem BK, South Africa).

2.5 METHODS TO DETERMINE THE LEVEL OF OXIDATION

Comparing the effectiveness of antioxidants in protecting oils against oxidation necessitates the determination of oxidation indexes with which to objectively evaluate their efficiency. The measurement of an oil’s stability (i.e. its’ resistance to oxidation) is most accurate when monitoring the oil’s shelf-life under realistic storage conditions, but since this is time-consuming, many accelerated shelf-life tests (ASLT) have been developed. These include the frequently used active oxygen method (AOM), the Rancimat test and the Oxidograph (Michelsen et al., 2001).

If oil oxidation is followed experimentally by determining indexes of oxidation over time, a distinct two phase pattern of oxidation can be observed (Hamilton, 1994; American Oil Chemists’ Society/AOCS Cd 12b-92, 1997): during the first phase, oxidation is slow and occurs at a uniform rate. This phase is called the induction period or time. During the second phase, the oxidation rate accelerates rapidly until it is much greater than that observed during the induction period. Antioxidants can be used to extend the induction period.

Most methods quantifying the quality and stability of oils (i.e. indexes of oxidation) are based on measuring either a primary or secondary oxidation product, or a chemical or physical property that ensues from the change in the chemical balance brought on by oxidation (White, 1995). The following main methodologies are used to follow lipid
oxidation: gas chromatography of headspace samples, diene conjugation assays (UV absorption of conjugated diene hydroperoxides), peroxide assays (titration methods, UV-visible spectrophotometric methods, chemiluminescence methods and fluorescence methods), assays for carbonyl compounds and malondialdehyde and 4-hydroxynon-2-enal assays (Wheatley, 2000). Figure 2.3 summarises the changes during oxidation and the correlating test methods.

<table>
<thead>
<tr>
<th>Chemical reaction</th>
<th>Suitable test method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oils and fats</td>
<td>Accelerated tests: measured at elevated temperatures</td>
</tr>
<tr>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Primary oxidation products:</td>
<td>Peroxide value</td>
</tr>
<tr>
<td>hydroperoxides</td>
<td>Conjugated dienes</td>
</tr>
<tr>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Secondary oxidation products:</td>
<td>TBA test, Anisidine value,</td>
</tr>
<tr>
<td>mostly aldehydes</td>
<td>Conjugated trienes</td>
</tr>
<tr>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Tertiary oxidation products:</td>
<td>Rancimat</td>
</tr>
<tr>
<td>short-chain acids</td>
<td></td>
</tr>
</tbody>
</table>

*adapted from Gunstone (1996)

Figure 2.3. The relationship between the steps of oxidation and suitable test methods*

A discussion of the ASLT methods applied, and of the chemical indexes of oxidation determined during these storage tests to establish the induction periods of antioxidant treated oils, follows.
2.5.1 Accelerated shelf-life tests

Accelerated shelf-life tests (ASLT) provide a quick way to evaluate oil stability. It entails the determination of oxidation indexes in a product exposed to conditions that accelerate oxidation. Conditions commonly employed to accelerate oxidation include the elevation of the storage temperature, oxygen and/or light exposure, as well as the addition of oxidation initiators, such as metal ions, hydroperoxides or enzymes. The resulting oxidation is then measured by determining oxygen consumption, changes in free radical concentration, or in the concentration of different by-products generated during oxidation (Michelsen et al., 2001).

Temperature is typically used in ASLT to speed up oxidation since the rate of oxidation increases exponentially with an increase in temperature (Ragnarsson & Labuza, 1977). The induction period measured during the ASLT is then translated to a shelf-life for the product at ambient temperatures. However, Ragnarsson and Labuza (1977) state that the activation energy of oxidation is changed by factors such as temperature increases and antioxidant addition. These factors may lead to incorrect estimates of the shelf-life extension by antioxidants relative to a control at ambient temperatures on the basis of data collected at higher temperatures. For example, data gathered in ASLT tests conducted at 60-65°C leads to a predictable underestimation of shelf-life extension given by antioxidants at room temperature (Ragnarsson & Labuza, 1977). Whether a conservative estimate is a drawback in the prediction of a characteristic such as shelf-life is arguable. Also, the translation of ASLT results into ambient shelf-life is not necessarily required, as is the case when using an ASLT in the preliminary screening of antioxidants. Tests simulating actual shelf-life conditions, combined with for example sensory analysis, can then be performed on the product treated with the most effective antioxidants (as identified with ASLT) to determine actual shelf-life.

Two types of ASLT, the oil stability index/OSI (AOCS Cd 12b-92, 1997) and an oven storage test (AOCS Cg 5-97, 1997) will now be discussed in greater detail.
2.5.1.1 Oil stability index test

The OSI test (AOCS Cd 12b-92, 1997) measures the induction period of oils. Oxidation proceeds slowly until the oil’s natural resistance to oxidation has been overcome, at which point oxidation accelerates and becomes rapid. The length of time that elapses before the acceleration phase of oxidation occurs is an indication of the oil’s resistance to oxidation, and is referred to as the induction period. The OSI method accelerates the process of oxidation by exposing the oil samples to higher than ambient temperatures and to a constant flow of air through the samples. The effluent air from the oxidising oil contains volatile oxidation breakdown products that are accumulated in de-ionised water. This causes a change in the conductivity of the water, which is measured over time as an indication of the extent of oxidation. The OSI is defined as the point of maximum change of the rate of oxidation. Mathematically it is the maximum of the second derivative of the conductivity with respect to time (i.e. the end of the induction period). The OSI should be reported with the temperature used to accelerate oxidation, which may be 100, 110, 120, 130 or 140°C (AOCS Cd 12b-92, 1997). Results can also be expressed as a calculated value, namely the protection factor (PF), which allows comparison of the relative effectiveness of antioxidants. The PF is calculated through division of the stability of the antioxidant-containing sample with the stability of the control sample (Sonntag, 1979).

The Rancimat is a commercial instrument recommended for use in this test. This instrument has the advantage of being fully automated, and allows continuous measurement of oxidation without periodic analytical determinations requiring organic solvents (Hasenhuetti & Wan, 1992). In addition, although 5 g samples are normally used, samples of as little as 2.5 g can be used, and 6 samples can be tested simultaneously (Wan, 1995).

Although the use of the Rancimat has some disadvantages, e.g. the possibility of the escape of volatile components as well as antioxidants due to the air stream at the elevated temperature of the test, this method is used extensively (Burkow et al., 1995). The more
recent Rancimat models can also run at lower temperatures, e.g. at 50°C, which makes extrapolation of data to ambient shelf-life more convincing (Hasenhuetti & Wan, 1992). Use of the Rancimat to evaluate comparative antioxidant efficiencies in fish oils is frequently reported (Yi et al., 1991; Ganga et al., 1998; Burkow et al., 1995), even at such low temperatures as 60°C (Ganga et al., 1998).

### 2.5.1.2 Oven storage test

The oven storage test (AOCS Cg 5-97, 1997) measures the stability of oils during accelerated aging in an oven. Storage at temperatures higher than ambient (i.e. 25°C) are recommended as giving better reproducibility, since the increased storage time at ambient temperatures allows a greater probability that other environmental factors may influence the samples. However, a temperature lower than 80°C is suggested, since storage temperatures higher than 80°C cause a change in the oxidation mechanism, which makes the test non-representative of normal shelf-life conditions. A temperature of 60°C is recommended as ideal to limit the storage time while still allowing the accurate measuring of the level of oxidation. Oxidation indexes, such as PV’s, are then determined over a period of storage time to measure the oxidation level and to produce an induction curve. More than one index of oxidation should preferably be used, ideally one each for primary and secondary oxidation products (AOCS Cg 5-97, 1997).

Samples of the same surface to volume ratio should be stored in identical containers with a wide mouth since oxidation is oxygen dependent, and the surface area exposed to air influences the reaction rate. The samples can be covered or uncovered, and should preferably be in separate storage containers for each sampling time rather than removing aliquots. Oven storage should be conducted in the dark to eliminate the influence of light in a forced draft oven capable of maintaining a uniform sample temperature. The indicator tests should be performed immediately on sample removal, otherwise the samples should be flushed with nitrogen gas and stored below 0°C until analysis to prevent any further oxidation (AOCS Cg 5-97, 1997).
Since fish PUFA's are very susceptible to oxidation, lowering the storage temperature to closer to ambient temperatures during oxidation studies may be more suitable. However, ASLT in fish oils treated with various antioxidative compounds has been conducted at 60°C with clearly interpretable results (Kaitaranta, 1992). A temperature of 65°C has also been used with success during ASLT evaluation of antioxidant efficiency in marine oils (Wanasundara & Shahidi, 1998).

2.5.2 Chemical indexes of oxidation

2.5.2.1 Peroxide value

The determination of PV's is the most commonly used method to assess oxidative status (Michelsen et al., 2001; Gunstone, 1996). Peroxide values are also used to monitor the effect of processing and antioxidant addition on lipids, as a longer time span to reach a particular PV indicates a better preservative action by a particular treatment in comparison to other treatments that had a shorter time span (Shahidi, 1998).

Peroxide values are frequently used in studies to determine the level of oxidation and prevention thereof in fish oils (Yunizal, Basmal & Noor, 1997; Edirisinghe et al., 1997a, 1997b; Jayasinghe et al., 1997), and also to follow oxidation in fish flour (Petrescu, 1983, 1984). Shahidi (1998) reports that there is generally a significant relationship between PV's and sensory flavour scores.

Peroxide values reflect all substances, in terms of milliequivalents of peroxide per kilogram of sample, which oxidize potassium iodide under the test conditions (AOCS Cd 8-53, 1997). During the peroxide test, fat is dissolved in an acetic-acid-chloroform mixture, to which potassium iodide is then added. The iodide is liberated by any hydroperoxides present, and complexes with a starch indicator to yield a blue colour, which is titrated with a standard sodium thiosulfate solution to a colourless end point (Stuckey, 1972).
Hydroperoxides are intermediate oxidation products, and consequently hydroperoxide concentration increases initially as the lipid is oxidised, and then decreases as the hydroperoxides decompose into secondary oxidation products, typically volatiles (Hamilton et al., 1997; Coppen, 1994). The rapid development and decomposition of the peroxides in fish are ascribed to their highly polyunsaturated composition causing highly unstable structures (Rossell, 1994; Harris & Tall, 1994). This change in peroxide concentration as oxidation proceeds could lead to incorrect interpretations of the oxidation level (Hamilton et al., 1997). For example, if only the decomposition part of the peroxide curve is recorded, the resulting low peroxide values may be incorrectly interpreted as indicative of a low level of oxidation. This possible risk of misinterpreting peroxide values clarifies why the use of a combination of methods to assess both primary and secondary oxidative changes in oils is advised (Shahidi & Wanasundara, 1997).

Petrescu (1983, 1984) reports contradictory results on the age of fish flour at which peroxides start to decompose: in one study, the PV of untreated (no antioxidant added) fish meal rapidly increased to a maximum level within two days of storage, after which it gradually decreased to zero after 40 days (1983). A second study showed an increase in the PV of untreated fish meal during the first week (up to day 7) after production, followed by a decrease to zero after about one month of storage (1984).

A PV of 5 meq/kg is reported as the point where obnoxious oxidative rancidity flavours start to develop (Stansby, 1982). A fat is considered rancid at a PV of approximately 10 meq/kg (Gunstone, 1996). Miller (1993) ascribes flavours to specific PV’s, stating that “fishy” or “paint” flavours are slight at PV’s below 5 meq/kg, but becomes pronounced at values of 5-20 meq/kg. “Rancid” notes are perceived at PV’s of higher than 20 meq/kg. Michelsen et al. (2001) recommend that fresh fish oil should preferably have a PV of 2-3 meq/kg, with a maximum PV of 5 meq/kg. While a guideline of PV < 10 meq/kg may be sufficient for fish oils used as nutritional supplements, this PV is too high for fish oils used to enrich foods.
2.5.2.2 Thiobarbituric acid test

The thiobarbituric acid (TBA) test is one of the two most commonly used methods to monitor rancidity in fish (Harris & Tall, 1994). Irianto et al. (1997), for example, used TBA values as a chemical index of oxidation in products enriched with fish oil. Fernández et al. (1997) also state that several authors have reported good correlations between TBA values and sensory analysis results.

The TBA test entails a reaction on heating of an oxidation product, malonaldehyde (MDA), with thiobarbituric acid, in a 1:2 molar ratio, to form a red coloured complex with absorption at a wavelength band of 532-535 and 245-305 nm (Wheatley, 2000). Absorbance is usually measured at 532 nm because this is the wavelength where the chromogen has a peak absorbance (Love, 1992). Malondialdehyde is primarily formed through hydroperoxides from PUFA’s with three or more double bonds and is a three C dialdehyde with carbonyl groups on C1 and C3 (Fernández et al., 1997). This methylene interrupted dialdehyde form easily undergoes enolisation to form an enolic tautomer, which undergoes molecular rearrangement to form open s-cis-, s-trans-, and chelated forms. In aqueous solutions, an anion form is also encountered (Kwon & Watts, 1963).

Malondialdehyde is generally bound to biological materials, and therefore the TBA test typically contains an acid treatment step to release MDA before quantification (Shahidi, 1998). Other substances than MDA can also react with TBA, yielding an adduct with an identical spectrum to that of MDA. Consequently results are quantified as the content of TBA reactive substances (TBARS) rather than just MDA (Shahidi, 1998; Fernández et al., 1997).

There are various TBA test procedures with different advantages and disadvantages. The TBA can react directly with a whole food sample, with a portion of distillate, with a previously extracted lipid or with an aqueous acid extraction of the sample. A spectrofluorometric TBA test procedure also exists which requires lipid extraction (Fernández et al., 1997). The TBA test has many shortcomings, some specific to the
particular method followed. With the whole sample method, for example, interference by other food components is problematic, although adaptations to reduce interference have been developed. The distillation method reduces interference, and is most frequently used (Fernández et al., 1997). However, Fernández et al. (1997) states that several authors have reported a high correlation between results obtained with the distillation and extraction method, although the latter typically yields lower TBA values. The variations in results with the different TBA test methods necessitate the use of relative rather than absolute values in comparative work (Shahidi, 1998). A study comparing three TBA methods recommend the use of a faster, easier modified aqueous extraction method rather than the distillation method when quick analysis of many samples is needed (Pikul et al., 1989).

General disadvantages of the TBA test, as summarised by Wheatley (2000) and Shahidi (1998), include the following:

- the test is not specific, as TBA reacts with several TBARS (such as hydroperoxides and conjugated aldehydes) to form chromogens
- the main reaction product, MDA, is labile
- MDA concentration may more accurately reflect the presence of PUFA’s in a fat than the fat’s oxidation level
- the TBA values of cooked muscle foods typically increase with storage to a maximum, and then decline, with the disadvantage that a given TBA value may correspond with two points during storage time.

The TBA test is theoretically well suited for fish lipids since fish lipids are rich in PUFA’s with three double bonds, of which the oxidation products, as reported, include MDA. Whether the TBA test is suitable for monitoring lipid oxidation in a dehydrated fish flour is yet uncertain. Kwon and Watts (1963) state that lipid oxidation may already be far advanced in dehydrated foods with little or no accumulation of MDA. This can possibly be ascribed to the presence of MDA in a volatile, chelated form (which allows escape thereof) in dehydrated foods. Very little MDA was for example found in highly rancid flour (Kwon & Watts, 1963).
2.5.2.3 Conjugated diene and triene values

The conjugated diene value (percentage of conjugated dienoic acid) expresses the level of oxidation as indicated by the presence of primary oxidation products, namely conjugated dienes (CD). During one of the first steps in the oxidation of PUFA’s with 18 or more carbons, a hydrogen atom is lost from the methylene group positioned between two double bonds in the original 1,4-pentadiene-configuration. This loss produces a pentadienyl radical intermediate, which can form one of two possible conjugated diene structures (White, 1995). The extent of this double bond displacement is correlated with the degree of peroxidation occurring (Farmer et al., 1943). The resulting conjugated diene systems strongly absorb UV light at 232-234 nm (White, 1995; Love, 1992), which provides a way to quantify the formation thereof. The CD value initially increases rapidly as conjugated dienes are formed from the PUFA’s, and then levels off as the oil deteriorates and less PUFA’s are available for conversion to CD (Duve & White, 1991).

Double bonds normally occur in a non-conjugated position, and when occurring in the conjugated position, certain types of chemical reactivity are increased, yielding the fat more liable to oxidation and polymerisation (Alais & Linden, 1991; Institute of Shortening and Edible Oils, Inc., 1988). The resulting conjugated diene structures therefore react with molecular oxygen to form conjugated hydroperoxides (White, 1995). The correlation coefficient between PV (which measures the level of hydroperoxides) and UV light absorbance at 232 nm (which measures the conjugated dienes before they react with oxygen to form hydroperoxides) has been reported as 0.993 (Hahm & Min, 1995).

Conjugated triene systems also form as the result of PUFA oxidation, and these triene systems absorb UV light at 268 nm (Love, 1992). Ultraviolet absorbance values at 232 and 268 nm therefore respectively indicate the conjugated diene and triene contents (Yoon et al., 1985), which are respectively primary and secondary oxidation products (Noor & Augustin, 1984). Oxidation studies that have used UV absorbance at 232 and 268 nm to indicate the conjugated diene and triene-contents include work by Yoon et al., (1985) and Noor & Augustin (1984).
While conjugated diene and triene measurements have the advantage of being rapid and simple (Love, 1992), these measurements have to be interpreted with caution. The potential rise in CD is greater the more PUFA’s oil contains, and differs with various types of unsaturated FA’s. The magnitude of change can therefore not be related to the degree of oxidation, although it can be used as a relative measurement of oxidation in a particular oil (White, 1995; Love, 1992).

White (1995) summarised the advantages and disadvantages of the CD method as follows: CD correlates reasonably well with PV, but is an easier and faster method that requires no chemical reaction, and uses very small (100 mg) samples. Conversely, CD values of different oils (with varying PUFA’s) cannot be compared and CD is more suitable to the highly unsaturated oils. Samples should also be free from contaminating material to prevent absorption disturbances. The CD value, which measures a primary oxidation product, also becomes ineffective as an oxidation level predictor once reaching the plateau where decomposition is proceeding rapidly.

2.5.2.4 Gas chromatographic determination of changes in fatty acid methyl esters

Measurement of the change in the concentration of the substrate for oxidation, i.e. the FA’s, can be used to quantify the extent of oxidation (Shahidi, 1998). In other words, the rate of disappearance of the FA’s susceptible to oxidation is an indication of the degree of oxidation (Noor & Augustin, 1984). Changes in the relative area percentage of FA ester peaks can be used in antioxidant efficiency evaluation studies, in that the higher the relative percentage of unsaturated FA’s (UFA’s) that remain after a certain time, the better the antioxidant protected the UFA’s from thermal and oxidative breakdown (Duve & White, 1991).

The rate of UFA disappearance is, however, only an indicator of oxidative damage in the later stages of storage when oxidation has proceeded significantly (Noor & Augustin, 1984). The relative percentages of the EFA’s can be expressed in a format similar to that previously used in oil deterioration studies (Duve & White, 1991; Augustin et al., 1987;
Noor & Augustin, 1984), i.e. as a ratio to a saturated FA (EFA/C16:0). Since the saturated FA palmitic acid (C16:0) occurs in considerable quantities in nearly all fish oils, typically forming 30% or more of the total FA’s (Stansby, 1982), it is a suitable saturated FA to use in this ratio. Alternatively, the polyene index, which is the relative ratio of the n-3 PUFA’s to the total of saturated FA’s can be reported to reflect the effect of antioxidant treatments on FA composition (Boyd et al., 1993).

2.6 CONCLUSION

The importance of an increased consumption of the n-3 FA’s to health has been discussed (section 2.2). The Fish Waste Utilisation Project (FWUP), under which this study resorted, investigated the use of hake heads, a possible unexploited source of n-3 FA’s, to produce an n-3 rich hake head flour. Effective utilisation of the n-3 FA content of the flour as a dietary intervention tool, however, requires the protection of these PUFA’s against oxidation, which is the focus of this study. The extent of oxidation present in the hake head flour should first be quantified using the indexes of oxidation discussed. The efficiency of the selected antioxidants reviewed (section 2.4.2.2.3) must then be investigated to compare their ability to slow down oxidation in the flour. The Rancimat can be used in a preliminary screening of the efficiency of the antioxidants in slowing oxidation in fish oil. Oven storage of hake head flours, treated with the most suitable antioxidants (as identified in the Rancimat), with periodic determinations of some of the indexes of oxidation discussed, eg. PV’s, UV absorbance at 232 and 268 nm, and relative percentage changes in the PUFA’s/C16:0 ratio, can then used to compare the efficiency of the antioxidants in protecting the flour against oxidation (# 5). A detailed description of the methods used and the antioxidants evaluated in this study are reported in # 3.

2.7 REFERENCES


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CHAPTER 3
Product development, materials and methods

3.1 INTRODUCTION

The main objective of this study was the protection of the n-3 fatty acid (FA) content of hake head flour against oxidation. Before work on this aspect could commence, the hake head flour production process first had to be standardised. Melnychuk Research and Development (CK 96/58741/23, Box 862, Stellenbosch, 7599, South Africa) had already developed a product concept at the start of this study, and an unrefined processing line for flour production from hake heads existed. This production process and the processing equipment used were however substantially refined during the course of this study in order to limit oxidation, increase the production efficiency and standardise the procedure. This fish flour production process may be patented in the future, and details thereof remain undisclosed. Only a brief description of the flour production process as used in this study is therefore given in this chapter.

The objective of this study was to protect the hake head flour lipid fraction against oxidation. The protection of the n-3 FA’s against oxidation is critical to ensure FA bioavailability and to limit unpalatable rancidity (see # 2, section 2.4.1.5). This study investigated the use of antioxidants to protect the lipid fraction. In order to quantify the level of oxidation in the hake head flour, and to compare the efficiency of antioxidants in slowing down oxidation in the flour, objective indexes of the extent of oxidation were identified. The methods used to determine these indexes were adapted where necessary for use in determining the level of oxidation in the hake head flour. These methods are described in section 3.3.1. The extent of oxidation present in the hake head flour was then first quantified as a function of both the storage age of the hake heads used to prepare the flour and of the flour.

Secondly, antioxidants suitable for protecting a fish flour against oxidation during production and storage were selected after a literature review of the subject (# 2, section
2.4.2.2. The efficiency of these antioxidants was compared in a screening procedure (section 3.3.2.1). The most effective and suitable combinations were then applied to hake head mince prior to drying, and their antioxidant efficiency in the flour produced thereof was compared during accelerated shelf-life tests (ASLT), described in section 3.3.2.2. Periodic determinations of the selected objective indexes of oxidation were used during the flour ASLT to determine the induction times (# 2, section 2.5) yielded by each antioxidant combination. The levels and methods of application used for the antioxidants investigated are described in sections 3.2.2 and 3.2.3.

3.2 MATERIALS

3.2.1 Hake head flour

3.2.1.1 Raw material

Hake heads, reserved from the last day’s catch, were obtained, according to availability, from either Irvin and Johnson (Cape Town, South Africa) or Sea Harvest (Saldanha, South Africa). These suppliers stored the heads on ice in large cooler boxes from harvesting until collection by the researcher. Upon receival, the heads were either used as soon as possible, or packaged and frozen at approximately -18°C until used.

3.2.1.2 Production process

The mince used for flour production was prepared according to standard techniques (FWUP, patent pending). A brief discussion thereof follows.

The main challenge of utilising hake heads to produce a flour was to cut the heads in such a manner that all teeth, including the ridges of teeth present deep in the mouth cavity, were removed. The neck flesh also had to be removed, while the brain and the eyes had to remain intact, since these are possible rich sources of n-3 FA’s (# 2, section 2.3.3). The resulting head sections were minced finely with various industrial cutting machines.
The order of use and type of cutting machines were refined over time to increase the capacity and effectiveness of the production line, as well as to obtain a small particle size that would allow quick drying. This particle reduction had to be achieved with the minimum labour input and processing steps, while also minimising the temperature increase of the product in a precautionary step to slow down oxidation.

A brief description of the hake head flour production process follows: firstly, as used for the experiment used to determine the effect of the storage age of both the heads and the flour on the flour oxidation level (see # 4), and secondly, as used for the experiment that investigated the protection of the flour lipid fraction against oxidation (see # 5).

3.2.1.2.1 Initial hake head flour production process

Eighty kilograms of hake heads, reserved from the last day’s catch of a commercial trawler from Irvin and Johnson, was used for the experiment conducted to determine the effect of the storage age of both the heads and the flour produced thereof on the flour oxidation level (# 4). Due to production limitations, the heads were packaged in closed plastic bags on receipt and frozen at approximately -18°C until the start of the experiment. The heads were then defrosted overnight at ± 8°C, and then stored on ice at ± 4°C. Storage containers that allowed drainage were used and the ice was replenished as necessary. Heads of three storage ages (1, 5 and 9 days of storage) were used to prepare flour.

Heads stored for longer than 9 days were not considered suitable for use in a product intended for human consumption since the heads were deteriorating rapidly by then, displaying a disagreeable mottled surface and emitting a strong, fishy odour. Since the heads were reportedly stored on ice from the catch harvested during the last day at sea, and were frozen the next day, this experimental storage age of 9 days translated to an actual storage age of approximately 10 days since harvesting. This storage age was shorter than expected. Oehlenschläger (1992) reports that fish storage on ice maintains the fish temperature at 0°C, while the melting ice washes out low molecular weight
compounds that could contribute to off-flavours and -odours, and that a shelf-life of 15 to 20 days can thus be obtained. The South African Code of Practice for the Handling of Chilled and Frozen Foods prescribes fish storage at 0°C-2°C, and reports that these conditions allow for a storage life (from time of catching) of 8-12 days (SABS 0156, 1979). Dassow and Beardsley (1974) also reported that fresh (less than 24 h old) hake fillets, stored at a temperature of 1°C, remained in an acceptable condition for up to two weeks. The faster rate of deterioration observed in the present study in spite of storage on ice could possibly be attributed to the absence of cleaning of the heads at sea, which caused visible gut-contamination of the heads. Huss (1988) reports that the quality and storage life of many fish species decrease if gutting does not occur. Although Oehlerenschläger (1992) reports that storing fish on ice maintains the fish temperature at 0°C, a lower environmental storage temperature than 4°C may possibly have extended the shelf-life. The freezing of the heads prior to the start of the experiment may also have influenced the shelf-life of the heads once defrosted.

Prior to flour preparation, the heads were washed with cold tap water and cut to yield a triangular top head section, neck flesh, and jaw-piece. Only the head triangles were used for flour preparation. The head triangles were finely minced, and the mince mixed with 6% sodium alginate (Tranarc, 18 Arbroath Avenue, Hurling Ham, Sandton, South Africa), included into the mince at a 10% level. This mixture was then extruded through a thin die (1.2 mm) into a brine solution, which consisted of 94.05% water, 4.2% calcium lactate (Chempure, Meiring Naudé Road, CSIR Building 41, Lynwood, Pretoria, South Africa) and 1.75% lactic acid at a concentration of 80% (Petrow Chemicals, PO Box 37183, Chempet, 7442, South Africa). This procedure set the mince into strips on contact with the brine. This strip production step improved further handling of the mince since the well-set outer skins of the strips retained both the liquid and solid phase of the mince, and consequently produced a manageable product. The strips were then washed with cold tap water and transferred onto dry, perforated trays for drying.

Drying took place in a closed stainless steel cabinet connected to a Munters dessicant unit (Tobo, Switzerland). This unit dries ambient air drawn from the environment over a
rotating silica wheel. Although the drier had a fan to distribute the desiccant air, the fan’s position on the back wall of the drier was not ideal as the desiccant air entered the drier from the bottom. The fan should ideally be in the pipe introducing the desiccant air. The possible positional effect during drying due to the uneven distribution of the desiccant air was addressed by rotating and turning the drying trays (each containing the same weight of strips, with a total of 3.87 kg strips dried at a time) at approximately 30 min intervals.

The two batches of flour prepared from the heads stored for 1 or 5 days respectively were dried for 4 h 43 min each. At the end of this drying time, the air in the oven cavity was recorded as having slightly different relative humidities for the two batches of flour (5.9 and 8.8%). This could possibly be ascribed to the fact that the drier used ambient air as a starting point, and the temperature and humidity of this air would vary along with factory conditions. The third batch was therefore dried until the air coming of the product also reached a relative humidity of ± 8.8% (4h 25 min). The temperature in the drier at the end of the drying period for the three batches of flour varied between 35.6 and 38.7°C. A further problem experienced during drying was that the air stream dislodged the strips, especially during the rotation of the trays. Resting metal grids on top of the trays solved this problem as the grids kept the strips in place while still allowing air circulation. After drying, the strips were powdered to produce a flour-like product.

### 3.2.1.2.2 Refined hake head flour production process

Refinement of the hake head flour production method took place between the first experiment (# 4) and the experiment investigating the protection of the lipid fraction in the flour against oxidation (# 5).

Firstly, although the heads were still cut into three sections, the cutting line was changed so that the top vertebra were retained as part of the head section, thus ensuring that no brain material was lost during cutting. The resulting head triangles were minced only once (13 mm mincing plate) and the same weight of mince was packed in plastic bags and heat-sealed. The bags were filled with only a thin layer of mince to ensure quick and
even freezing (-18°C). The mince was kept in frozen storage (-18°C) until the start of the experiment. For the first trials, in which the mince was treated with either tertiary butyl hydroquinone (TBHQ) combined with citric acid/CA (trial 1: T1) or with a partially water soluble treatment (WST\text{inc}, see section 3.2.2) combined with CA (trial 2: T2), the mince was frozen for approximately four and two weeks respectively before production could take place. In the third trial (T3), in which mince enriched with hake liver was treated with WST\text{inc} or WST\text{acetate} (WST\text{inc} with the dl-\(\alpha\)-tocopherol therein replaced with the acetate form thereof), the freezing time of the mince was decreased to one day to limit the possibility of oxidation of the finely cut mince during a long freezing period. In an attempt to minimise the effect of heat build-up during mincing, which would accelerate oxidation, the heads were also submerged in ice water before mincing to lower the raw material starting temperature.

Mince prepared on the same day from the same batch of heads (i.e. originating from one catch, from one supplier) or from well-mixed batches of heads (i.e. originating from more than one supplier) was used for all the batches of flour produced in a trial. In each trial, the weight of mince required per flour to be prepared (kept constant within a trial) was randomly selected and defrosted at ± 2-4°C until it could just be broken into chunks by hand. The defrosting time before using the mince for flour preparation was kept constant as far as possible within a trial.

A preliminary trial to investigate the effect of the point of antioxidant addition on the level of flour oxidation showed that the addition of dl-\(\alpha\)-tocopherol after drying resulted in a higher starting peroxide value (PV) in the flour than when adding the antioxidant prior to drying. Therefore all antioxidants were mixed into the mince before drying. Mixing times were kept constant within a trial, and equipment were washed and dried between treatments.

In T1 and T2, the antioxidants were mixed into the defrosted mince by hand, followed by another brief mix in a bowl cutter. The antioxidant treated mince was then passed through a micro-cutter to produce small particles (≤ than 2 mm) suitable for drying. This
process was adapted slightly for T3, both to improve the antioxidant distribution and because 4% hake liver (add-in on the weight of mince) was included into the mince used to prepare the flour. The liver was added in an attempt to increase the FA content of the flour since the livers of lean fish serve as lipid stores (The British Nutrition Foundation/BNF, 1992; Stansby, 1982). Since the hake heads frequently had liver attached, the addition of hake liver to the mince to increase the lipid content of the flour was a viable alternative. The nutritional content of the hake head flour with and without 4% hake liver addition (reported in Tables 3.1 and 3.2) was determined by the CSIR (Lower Hope Road 15, Rosebank, Cape Town, South Africa). As can be seen from the results, hake liver inclusion did increase the lipid content of the flour, with a concurrent increase in the levels of the nutritionally beneficial (see # 2, section 2.2) polyunsaturated FA’s (PUFA’s), docosahexanoic acid (DHA) and eicosapentanoic acid (EPA). The liver used in T3 had been reserved, washed and frozen during the preparation of the mince for this trial, and was defrosted along with the mince prior to flour preparation. The preparation procedure followed for T3 was as follows: the hake head mince was first cut in a micro-cutter (yielding particles ≤ than 2 mm) to produce a fine mince. The antioxidants were blended into the pre-weighed liver (Magimix Compact 3), and this antioxidant-liver mixture was then hand-mixed into the mince. The resulting mixture was again passed through the micro-cutter.

In each trial, a control flour (no antioxidants added) was prepared on the same day from the same batch of mince as the antioxidant treated flours, using the identical processing steps and mixing times as used when adding antioxidants.

The drying procedure initially used (section 3.2.1.2.1) could compromise experimental control since the procedure utilised ambient air, which introduced a variable into the drying procedure since the factory environment (relative humidity and temperature) varied with the weather conditions. In order to achieve better experimental control the drier was changed into a closed system in which the air present in the drier chamber when the product was placed into the drier was continuously circulated: after blowing over the product, this air passed the silica wheel to dry before re-entering the drier. The silica
Table 93.1. Nutritional content of flour prepared from hake head mince treated with 0.2% WST

<table>
<thead>
<tr>
<th>Sample*</th>
<th>Proximate analysis</th>
<th>Minerals</th>
<th>Cholesterol</th>
<th>Fatty acids (FA’s)</th>
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<td>Fat (%)</td>
<td>Ash (%)</td>
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<tr>
<td>Std deviation</td>
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<td>0.6</td>
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<td>0.4</td>
</tr>
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</table>

*fish used for preparation of flour samples were sterilised with 4% hydrogen peroxide; **not calculated since some totals, due to small experimental errors, exceeded 100

Table 3.2. Nutritional content of flour prepared from hake head mince enriched with 4% hake liver and treated with 0.2% WST

<table>
<thead>
<tr>
<th>Sample*</th>
<th>Proximate analysis</th>
<th>Minerals</th>
<th>Cholesterol</th>
<th>Fatty acids (FA’s)</th>
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<td>Fat (%)</td>
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<tr>
<td>Std deviation</td>
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<td>0.6</td>
<td>0.2</td>
<td>0.7</td>
</tr>
</tbody>
</table>

*fish used for preparation of flour samples were sterilised with 4% hydrogen peroxide
wheel was in turn dried by air from the environment, which was heated, run over the wheel, and then removed with an extraction fan that is located outside the factory.

Thermostatically controlled heaters were also installed in the drier. These maintained a pre-set temperature with a ±2°C variation. The flours were dried at 38.5°C (±2°C). A cooling device, which consisted of a system of watertight pipes, running through a large water-filled container, was also incorporated. This was used to cool the hot air coming from the drying cabinet before it entered the desiccant unit, as warm air could have reduced the effectiveness of the silica in removing the moisture from the air (personal communication, M. Melnyczuk, Melnyczuk Research and Development, CK 96/58741/23, Box 862, Stellenbosch, 7599, South Africa).

The preparation of the mince for drying was also changed from the strip-production previously used. A flexible template with small, evenly sized and spaced holes was positioned on a solid drying tray and a thin layer of mince was smoothed over the template. The template was then lifted, the drying tray slid out, and the mince remaining on the tray dried as such into small pellets. Mince not immediately used for flour production was refrigerated until used. This pellet production procedure was both easier and quicker than the strip production procedure previously used. The very thin pellets also dried faster and more evenly than the strips.

The drying procedure took place as follows: during drying, one tray was added in the bottom position of the pre-heated oven every 8 min, displacing the other trays one position upwards. Each tray was removed and the pellets scraped off after having dried for 8 min in the top (ninth) rung of the drier. As far as possible, only pellets dried in an oven filled to capacity were retained to ensure that the flour batches used for the ASLT were dried under the same conditions. The dried pellets for each batch produced were powdered and the resulting flour stored overnight in a sealed plastic bag to allow the moisture in the flour to equalise. The flour was then divided into samples for the start of the ASLT (see section 3.3.2.2.) the day after production.
3.2.2 **Antioxidants screened in the Rancimat**

Due to the time-consuming nature of the hake head flour production process, as well as to the large quantity of possible antioxidants available with which to protect the lipid fraction in the flour, several commonly applied antioxidants were first screened through evaluation of their effectiveness in protecting fish oil against oxidation in the Rancimat (discussed in section 3.3.2.1).

Commercially available Alpha Cod Liver Oil B.P., packaged in dark–brown plastic bottles (Allied Drug Company (Pty) Ltd., 27 Hannar Road, Congella, Durban, South Africa) was used for all Rancimat trials. Synthetic antioxidants screened included butylated hydroxytolune (BHT), butylated hydroxyanisole (BHA), and tertiary butyl hydroquinone (TBHQ), all supplied by Fluka, Sigma-Aldrich, South Africa. Combinations of the synthetic antioxidants are synergistic, as are combinations with citric acid/CA, while sodium metabisulphite/SMBS also acts as an antioxidant (see # 2, section 2.4.2.2.3.2). Therefore combinations of the synthetics, as well as combinations of the antioxidants investigated with CA or SMBS (CA and SMBS both from Protea Chemicals, Berrange Road, Wadeville, Germiston, South Africa) were investigated.

A natural, flavonoid rich product, Biored Pure (Biorem BK, P.O. Box 565, Jan Kempdorp 8550, South Africa), declared to act as an antioxidant, was also evaluated, as well as a commercial antioxidant formulation, Ronoxan A/RA (Chempure, Meiring-Naudé Road, Ringwood, Pretoria, South Africa). Ronoxan A was selected for screening by the product developer, M. Melnyczuck, and is a commercial ternary formulation that consists of 25% ascorbyl palmitate, 5% dl-α-tocopherol and 70% lecithin. As discussed in the literature review (# 2, section 2.4.2.2.3.1), a ternary combination of tocopherols, ascorbic acid or ascorbyl palmitate and lecithin has the most notable effect in the protection of fish oil against oxidation.

A formulation modeled on RA was also developed by substituting the ascorbyl palmitate used in RA with the water soluble sodium ascorbate (Protea Chemicals, Berrange Road,
Wadeville, Germiston, South Africa), and by using Emulfluide E [Chemimpo South Africa (Pty.) Ltd, Roggebaai, P.O. Box 7057, 8012] as a lecithin. Emulfluide E (EE) is an enzymatically hydrolysed, liquid soybean lecithin, claimed to have high emulsifying and dispersing properties in aqueous systems (Chemimpo, South Africa). The resulting partially water soluble formulation (WST) therefore consisted of 25% sodium ascorbate, 5% dl-α-tocopherol [Roche Products (Pty) Ltd, South Africa] and 70% EE, and was formulated since water-solubility was expected to facilitate dispersion in the watery fish mince. The principle of ascorbic acid dispersion reported by Han et al. (1990) was applied to distribute the sodium ascorbate in both the fish oil used during screening and later in the fish mince: a lecithin, i.e. the EE, was used to incorporate a solution of ascorbic acid in water into the medium used through the formation of a water and oil micro-emulsion.

The antioxidants screened were applied at levels prescribed by the World Health Organisation (WHO) for edible oils and fats not covered by individual standards (Codex Alimentarius Commission, 1999). The prescribed levels of the antioxidants relevant to this study are summarised in Table 3.3.

<table>
<thead>
<tr>
<th>Food additive</th>
<th>Stipulated level/kg of fat/oil (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbyl palmitate</td>
<td>500</td>
</tr>
<tr>
<td>Alpha-tocopherol</td>
<td>GMP**</td>
</tr>
<tr>
<td>Synthetic delta-tocopherol</td>
<td>GMP</td>
</tr>
<tr>
<td>Tertiary butyl hydroquinone (TBHQ)</td>
<td>120</td>
</tr>
<tr>
<td>Butylated hydroxyanisole (BHA)</td>
<td>175</td>
</tr>
<tr>
<td>Butylated hydroxytoluene (BHT)</td>
<td>75</td>
</tr>
<tr>
<td>Any combination of gallates, BHA and BHT and/or TBHQ</td>
<td>200, but limits above not to be exceeded</td>
</tr>
<tr>
<td>Citric acid</td>
<td>GMP</td>
</tr>
</tbody>
</table>

*Codex Alimentarius Commission (1999)
**Good Manufacturing Practise

All the antioxidants used during this study were applied within the levels prescribed by the Codex Alimentarius Commission (1999). Antioxidants for which only good
manufacturing practices (GMP) levels are recommended were used at levels commonly suggested by the suppliers thereof. The antioxidant combinations investigated in this study during the screening in the Rancimat, as well as their application levels, are summarised in Table 3.4.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ingredients</th>
<th>Dosage level (g/kg of oil)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ronoxan A (RA)</td>
<td>RA</td>
<td>0.500</td>
</tr>
<tr>
<td>RA &amp; citric acid (CA)</td>
<td>RA</td>
<td>0.500</td>
</tr>
<tr>
<td></td>
<td>CA</td>
<td>0.200</td>
</tr>
<tr>
<td>Partially water soluble treatment (WST)</td>
<td>WST</td>
<td>0.500</td>
</tr>
<tr>
<td>WST &amp; CA</td>
<td>WST</td>
<td>0.500</td>
</tr>
<tr>
<td></td>
<td>CA</td>
<td>0.200</td>
</tr>
<tr>
<td>RA &amp; sodium metabisulphite (SMBS)</td>
<td>RA</td>
<td>0.500</td>
</tr>
<tr>
<td></td>
<td>SMBS</td>
<td>0.010</td>
</tr>
<tr>
<td>WST &amp; SMBS</td>
<td>WST</td>
<td>0.500</td>
</tr>
<tr>
<td></td>
<td>SMBS</td>
<td>0.010</td>
</tr>
<tr>
<td>Tertiary butyl hydroquinone (TBHQ) &amp; CA</td>
<td>TBHQ</td>
<td>0.120</td>
</tr>
<tr>
<td></td>
<td>CA</td>
<td>0.200</td>
</tr>
<tr>
<td>Butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), TBHQ &amp; CA</td>
<td>BHT</td>
<td>0.0375</td>
</tr>
<tr>
<td></td>
<td>BHA</td>
<td>0.0875</td>
</tr>
<tr>
<td></td>
<td>TBHQ</td>
<td>0.060</td>
</tr>
<tr>
<td></td>
<td>CA</td>
<td>0.200</td>
</tr>
<tr>
<td>Biored Pure</td>
<td>Biored Pure</td>
<td>0.900</td>
</tr>
</tbody>
</table>

Many of the antioxidants investigated required mixing with other ingredients prior to addition to the oil in order to facilitate a homogeneous dispersion in the oil. These antioxidants were either first dissolved to form stock solutions, of which the correct volume to yield the required weight of antioxidant was then incorporated into the oil, or mixed with EE, which facilitated incorporation into the oil.

The methods of incorporating the antioxidants used into the oil were determined during preliminary trials as follows:

- the fat-soluble tocopherols were mixed with EE, followed by the gradual addition of oil
• the active component of Biored Pure was extracted by mixing first with ethanol (Biored Pure:ethanol weight ratio 1:22.22), then with distilled water (Biored Pure:water weight ratio at 1:22.22), and, after an hour, with EE (Biored Pure:EE weight ratio at 1:10.11). Oil was then gradually introduced into the correct weight of stock.

• sodium ascorbate and SMBS were dissolved in distilled water in a water to antioxidant ratio of 3:2. Citric acid, BHA and TBHQ were dissolved in propylene glycol (Protea Chemicals) in ratios of 1:5.7, 1:11.3 and 1:2 respectively, which allowed all particles to dissolve with continuous stirring while heating over a water bath (temperature at 40-50°C). The weight of each solution required to yield the level of that antioxidant needed in the oil volume prepared was then incorporated into the oil while continuously stirring over a water bath (40-50°C). In EE-containing formulations the emulsifying characteristics of the EE were used to incorporate the solutions.

• BHT, which is not soluble in propylene glycol (Gunstone, 1996) and RA, according to manufacturer’s directions, were not dissolved prior to addition to the oil. These antioxidants were added directly to approximately 5 ml of oil, and heated over a water bath (40-50°C) until dissolved. This antioxidant-oil combination was then mixed into the remainder of the oil.

The WST was also later evaluated in CLO in the Rancimat at application levels greatly increased from the levels tested during the initial screening. The product developer, M. Melnyczuck, reported the successful reduction of flour rancidity during preliminary trials by using an approximate 0.2% add-in level of WST into the weight of fish mince. Assuming that the antioxidants completely reached the lipid fraction, the levels of antioxidants/kg of lipid were calculated (using a recorded 18.5% flour yield and an average minimum expected flour fat content of 3%) as 90.27 g sodium ascorbate, 18.05 g dl-α-tocopherol, and 252.76 g of EE/kg of oil. This calculated application level of WST was greatly increased (722.16 times) from what was initially screened in the Rancimat, and is henceforth referred to as WST_{inc}. However, the EE quantity of 252.76 g/kg of oil required for the WST_{inc} would not stay in suspension in the oil, and was therefore
reduced to 6 g/kg of oil for this trial (i.e. only 17.14 times the level of EE tested in the first Rancimat trial). This allowed the effective formation of a temporary emulsion in the oil. The sodium ascorbate was dissolved in distilled water in the same ratio as used before (2:3). The WST\textsubscript{inc} was also investigated with one ingredient thereof, dl-\(\alpha\)-tocopherol, replaced with dl-\(\alpha\)-tocopherol acetate [Roche Products (Pty.) Ltd, South Africa], henceforth referred to as WST\textsubscript{acetate} (also applied with the reduced level of EE in the Rancimat trial). Therefore, besides determining the effect of the increased levels of WST on the degree of protection provided against oxidation, this trial also compared the influence of the two tocopherol forms (dl-\(\alpha\)-tocopherol or -acetate) on the effectiveness of the WST\textsubscript{inc}. Since the synergistic effect in the preliminary trial was found to be minimal, no CA was added to either treatment.

### 3.2.3 Antioxidants evaluated in hake head flour

Due to cost as well as time and practical constraints, only a limited number of the most effective antioxidants screened could be selected for evaluation in the hake head flour. Besides effectiveness, other factors, namely the origin and solubility of the antioxidants, were also considered in the selection of the antioxidants to be evaluated in the hake head flour.

Since the hake head flour is to serve as a nutritional intervention tool, the use of natural antioxidants was preferred because of the possible negative health effects increasingly associated with the synthetic antioxidants (see # 2, section 2.4.2.2.2). The successful combination of TBHQ and CA, which yielded the highest protection factor (PF) during the initial screening, was however also selected for flour application as a benchmark against which to compare the effectiveness of the natural antioxidants. Fresh TBHQ (Sigma-Aldrich, South Africa) was obtained for the flour trial (trial l/T1). The TBHQ and CA were respectively applied at 0.12 and 0.2 g/kg of lipid in the flour, i.e. the same levels of antioxidants per kg of flour lipids as was screened per kg of oil in the Rancimat, using a 18% flour yield and a minimum expected average flour lipid content of 3% for calculations. The TBHQ and the CA were dissolved in propylene glycol prior to addition.
to the hake head mince in the same ratios as used in the Rancimat screenings. The
emulsifier EE was also included at an add-in level of 0.14% into the fish mince in order
to improve the dispersion of the mostly fat-soluble TBHQ in the watery fish mince.

It was postulated that, of the natural antioxidants, the WST should distribute better than
the fat-soluble RA in the water-laden fish mince, and consequently protect the fat
missiles more effectively. WST\textsubscript{inc} and WST\textsubscript{acetate}, which both gave a very good protection
against oxidation in the second Rancimat trail, were therefore selected for application in
the hake head flour. WST\textsubscript{acetate}, combined with CA, was first applied to the hake head
flour to confirm the effective antioxidant behaviour observed by WST\textsubscript{acetate} in the
Rancimat (trial 2/T2). The WST\textsubscript{acetate} was applied at the WST levels reported successful
by the product developer, i.e. at a 0.2% add-in level into the hake head mince. The
sodium ascorbate was, on recommendation of the product developer, dissolved in water
used at a 0.5% add-in into the fish mince. Citric acid, dissolved in propylene glycol in a
1:5.7 ratio, was added at 0.2 g/kg of flour lipid (i.e. at the same concentration as was
screened in the Rancimat), using a 18.5% flour yield and an average minimum expected
flour lipid content of 3% for calculations.

A third trial (T3) was completed to compare the efficiency of the WST\textsubscript{inc} and WST\textsubscript{acetate}
in the hake head flour. For this trial, the hake head mince was enriched with a 4% hake
liver inclusion. Although the liver inclusion increased the flour lipid content (see Tables
3.1 and 3.2), the treatments were still added at a 0.2% add-in level (calculated on the total
weight of the mince and liver) since the purpose of this trial was to compare the relative
efficiency of the two treatments. The sodium ascorbate in each treatment was dissolved
in water in a 2:3 ratio, as was used in the Rancimat trials, as the 0.5% add-in level of
water used in T2 resulted in an unnecessarily large volume of water. CA was not added
in this trial due to the minimal synergistic effect observed by the addition thereof in the
Rancimat.
3.3 METHODS

3.3.1 Chemical methods

The majority of the chemical analyses were conducted at the Department of Animal Sciences (University of Stellenbosch, Private Bag X1, Matieland, 7602, South Africa). Gas chromatographic work was conducted at the CSIR (Rosebank, Cape Town, South Africa). Rancimat analyses were performed at the CSIR (Meiring-Naudé Road, Lynwood, Pretoria, South Africa).

3.3.1.1 Fat extraction

The method reported by Lee et al. (1996) was adapted as follows to obtain approximately 0.5 g of lipid/sample for use in the peroxide and conjugated diene test: flour samples (20 g) were extracted with 200 ml chloroform:methanol (1:2, as indicated for lean fish). Sodium chloride (80 ml of a 0.5% solution) was used for phase separation. Instead of using a Corning hot plate for solvent evaporation, ± 40 ml of the solvent was thoroughly flushed with nitrogen gas before suspending it in a water bath placed within a fume cupboard, with nitrogen gas constantly blowing onto the solvent, as recommended by Ulberth (1998). Nitrogen flushing of a sample before incubation protects against heat-induced auto-oxidation through oxygen depletion (Ulberth, 1998), and this principle was applied before and during solvent evaporation to prevent artificial peroxidation due to the elevated temperature. The elevated water bath temperature was used to accelerate solvent evaporation. However, peroxides are heat labile compounds and break down at elevated temperatures (Ulberth, 1998). The degree of breakdown in the samples should, however, have been relatively constant as the solvent volumes and water bath temperature were kept the same within each experiment. Initially, a water bath temperature of ± 60°C was used, but this was reduced to ± 40°C when the drying process became fixed at 38.5°C. When the fish flour was enriched with hake liver (4% level), the fat content increased sufficiently to allow the use of smaller flour samples (± 10 – 16 g) to extract the required
amount of lipids for the chemical analyses. Solvent ratios and procedures were, however, kept the same.

A similarly adopted extraction method (Lee et al., 1996) was used to obtain fat containing solvent for fatty acid methyl ester (FAME) determinations, with the following adaptations: a smaller quantity of flour (± 6-10 g) was extracted using 100 chloroform-methanol and 40 ml 0.5% sodium chloride solution. The solvent was flushed with nitrogen gas and frozen and stored at -18°C in dark bottles until tested.

3.3.1.2 Peroxide value

Peroxide values were determined with the acetic acid-chloroform method of the American Oil Chemist’s Society/AOCS (AOCS Cd 8-53, 1997), using 0.01 N sodium thiosulphate for titration. Solvent volumes were adapted for the 0.5 g lipid sample to 10 ml acetic acid-chloroform and distilled water each. Results reflect all substances, in terms of millequivalents of peroxide present per kilogram of sample, which oxidize potassium iodide under the test conditions (AOCS Cd 8-53, 1997).

3.3.1.3 Thiobarbituric acid test

For this test, a fast, modified aqueous extraction method, recommended when quick analysis of many samples is needed (Pikul et al., 1989), was used with the following adaptations: 49 ml of 4% perchloric acid and 0.75 ml 0.1% BHT in ethanol were added to 4 g flour, blended with a Bamix M22 hand blender (high speed, 1 min) and filtered into a 100 ml Erlenmeyer flask; the rest of the method was used as reported. Samples and a reagent blank were heated in a boiling water bath for 1 h and cooled under running tap water for ± 4 min. Since comparison between treatments for samples of the same weight was the objective, the A_{532} values were not multiplied with the constant K, but reported as is, an alternative way previously used to report TBA test results (Roozen, 1987).
3.3.1.4 Ultraviolet absorbance by conjugated dienes and trienes

The lipid quantity required (approximately 0.01 g per test) for the determination of the ultraviolet (UV) absorbance by the conjugated diene and triene contents of the lipid was removed from the fat extracted for the peroxide test. The absorbance of the samples was determined with the ISO/DIS 3656 method (The Draft International Standard, 1989), using trimethylpentane (iso-octane) as a solvent. Absorbance was, however, only read at 232 and 268 nm. Absorbance at both wavelengths were determined on at least duplicate samples for each variation and averaged.

3.3.1.5 Gas chromatographic determination of changes in fatty acid methyl esters

3.3.1.5.1 Sample preparation

The extracted lipid containing solvent (section 3.3.1.1) was rotor-evaporated (50°C) and used to prepare methyl esters according to the method reported by De Koning et al. (1985). Reflux times after each reagent addition were increased to 5-10 min, and anhydrous sodium sulfite drying was not used.

3.3.1.5.2 Gas chromatography

A Hewlitt Packard HP 6890 Series gas chromatograph, with a capillary column HP5 (cross-linked 5% PH Me Siloxane, 30 m x 0.32 mm x 0.25 μm), was used. Inlet and detector temperatures of respectively 250 and 300°C were used, and the starting column temperature was 150°C. This was increased by 4°C /min to 280°C and then maintained for 8 min to ensure that all volatiles pass through the column length. Nitrogen gas (flow rate: 40 ml/min) was used as a carrier for the volatilised molecules. Detection was done by flame ionisation and the relative percentages of each FA ester peak were quantified using a Spectra Physics recorder. Peaks were identified through comparison with the retention times of a fish oil control (CSIR, Rosebank, Cape Town, South Africa).
3.3.2 **Accelerated shelf-life tests**

3.3.2.1 **Oil stability index test**

The Rancimat is a commercial instrument recommended for use in the oil stability index (OSI) test (AOCS Cd 12b-92, 1997). It allows determination of the induction period of oils. Oxidation proceeds slowly until the oil’s natural resistance to oxidation has been overcome, at which point oxidation accelerates and becomes rapid. The length of time that elapses before the acceleration phase of oxidation occurs is an indication of the oil’s resistance to oxidation, and is referred to as the induction period. The Metrohm Rancimat (Model 679) was used to perform the oil stability index test (AOCS Cg 5-97, 1997) and to determine the induction periods of oils spiked with the antioxidants to be evaluated for use in the hake head flour. The induction times were used to calculate each combination’s protection factor (PF), which allows comparison of the relative effectiveness of antioxidants (# 2, section 2.5.1.1).

Hake oil extracted directly from the target section would have been the ideal oil to use for this antioxidant screening, as it would have had the exact FA profile that must be protected in the final product. However, because of hake’s low fat content and the large quantities of oil needed to prepare the antioxidant treated oils for use in the Rancimat, extraction of sufficient quantities of oil from hake heads was impractical.

Alternatively, oil with a FA profile similar to that of the lipid fraction in hake head flour could be used for the screening of antioxidants. The use of hake liver oil (HLO) was next considered, as HLO is present in sufficient quantities to make extraction for use in the antioxidant screening viable. Hake livers were collected on three occasions and frozen until used. The oil was cold hexane extracted by drying first with anhydrous sodium sulphate, and the solvent evaporated under vacuum in a rotor evaporator. The three batches of oil were mixed, flushed with nitrogen gas, and frozen at -20°C. The oil was defrosted under running tap water just before use in the Rancimat.
Several problems were encountered with the Rancimat screenings performed on the HLO. The oil bubbled on heating, with bubbles rising right to the top of the oil-containing vessels. This could lead to contamination of the de-ionised water. Adjusting the rate of airflow from 20 to 15 l/h did not reduce the problem, and consequently the airflow was changed back to 20 l/h. The bubbling was effectively reduced by insertion of a glass rod into each vessel to disrupt the surface tension, and by reducing the sample size to 2.5 g (instead of the recommended 5 g). The induction time could, however, not be determined because of a very slow, gradual gradient in all the treatments, with even the untreated control not displaying a sharp inflection point. Various temperatures and adjustments to the conductivity range were investigated in order to obtain a sharper gradient, but with no success. This was not expected since Méndez et al. (1997) reported that partially refined HLO was used successfully in determining induction periods in Rancimat tests at a conductivity range of 250 $\mu$S.cm$^{-1}$ and a range of temperatures (55, 60, 70, 80 and 90°C). When the use of various Rancimat temperature and conductivity range settings did not improve the interpretability of this study’s results, it was postulated that the HLO extraction method could have contributed volatiles that, due to a gradual escape, caused the slow gradient reported. Although not stated, the HLO successfully used in the Rancimat by Mendez et al. (1997) may have been cold-pressed and not extracted. Therefore the HLO was kept under vacuum in a desiccator in an attempt to reduce possible traces of extraction chemicals. Although this resulted in foaming and weight loss, the resulting HLO still did not yield a clear induction period in the Rancimat.

The next oil investigated for possible use in the Rancimat was oil extracted from frozen whole commercial hake fillets with a chloroform-methanol extraction adapted from an altered Bligh and Dyer method (De Koning et al., 1985). A sample of 100 g defrosted fish was flaked, and since the fish contained approximately 80% water, the method was adapted as follows: 70 ml distilled water was blended (using an Ultra-Turrax T25, N.T. Laboratory supplies (PTY) Ltd.) with the flaked fish, followed by 150 ml methanol. Then 75 ml of both chloroform and distilled water were blended in. This mixture was centrifuged (Sorvall RC 5Bplus centrifuge) for 15 min at 4000 RPM, and then filtered through 1 PS (phase separator) silicone treated filter paper using sodium sulphate.
crystals. Evaporation of the solvent took place in a rotary evaporator under vacuum. The induction curve of the extracted hake flesh oil gave a slightly sharper turn over time than the HLO, but the turn was still not sharp enough to identify the end of the induction period (i.e. the induction time). This result confirmed the theory that chemically extracted oils may possibly not be suitable for use in the Rancimat. Extracting sufficient oil from the hake fillets for the preparation of the Rancimat samples would also have been impractical since the above extraction yielded less than 1 g of fat/100 g of fillet.

Other fish oils were therefore investigated as carrier oils for the antioxidants to be screened. Firstly, anchovy oil (designated as “crude oil”) was sourced by the CSIR (Rosebank, Cape Town, South Africa) from South African Sea Products. This anchovy oil was produced during fishmeal production by pressing the fish after cooking, and then separating the oil by centrifuging. This anchovy oil also did not yield interpretable induction times on testing in the Rancimat. Commercially available cod liver oil (Alpha BP) was also tested (Allied Drug Company (Pty) Ltd., South Africa). The cod liver oil (CLO) gave clear induction periods at a conductivity range of 100 μS/cm, with other settings as used by Burkow et al. (1995), i.e. a temperature setting of 80°C and an airflow speed of 20 l/h. These results indicated that not only may the oil extraction procedure influence the success of Rancimat trials, but also that every type of oil seems to require the determination of a temperature and conductivity range combination at which the rate of volatiles released results in a clearly interpretable induction period.

Due to the interpretable Rancimat results and the ease and low cost of procuring CLO, this oil was used as a medium for all further antioxidant trials at the above stated settings. The same production lot, or thoroughly mixed production lots of oil, was always used within a trial. Samples of 2.5 g were weighed, and always tested with a control sample (i.e. CLO with no antioxidants added). A glass rod was inserted in each sample vial to reduce bubbling. Samples were made up and tested immediately or frozen in the dark and analysed as soon as possible (within 2 days). The frozen samples were defrosted and mixed thoroughly prior to testing. Results (calculated induction time) are given on a print out by the built-in printer and were used to calculate the PF (# 2, section 2.5.1.1) of each
treatment. Each treatment investigated was prepared and tested at least twice. The PF’s reported in # 5 are therefore the average for each treatment.

Du Plessis and Sudworth (2001), however, reported that monounsaturated and polyunsaturated oils displayed different PF’s when treated with the same antioxidants. Therefore GC analysis of the relative FA composition of CLO and HLO was conducted at the CSIR (Rosebank, Cape Town, South Africa) to determine if the CLO was an acceptable alternative for use in the antioxidant screening procedure (results reported in Table 3.5).

Table 3.5. Relative area percentages of some fatty acid ester peaks in cod and hake liver oil

<table>
<thead>
<tr>
<th>Fatty acid percentage</th>
<th>Cod liver oil</th>
<th>Hake liver oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPA (C20:5n-3)</td>
<td>14.35</td>
<td>5.92</td>
</tr>
<tr>
<td>DHA (C22:6n-3)</td>
<td>9.04</td>
<td>13.60</td>
</tr>
<tr>
<td>Total polyunsaturated fatty acids</td>
<td>33.39</td>
<td>24.63</td>
</tr>
</tbody>
</table>

Since the principal reason for antioxidant use in this study was to preserve the n-3 PUFA’s, DHA and EPA should be present in comparable quantities in the oil used for the antioxidant screening than in the hake head flour to which the antioxidants will be applied. A GC analysis was used to compare the FA content of CLO and HLO, with the latter used instead of hake head oil because of the ease of extraction of sufficient quantities thereof for the Rancimat screenings. As reported in Table 3.5, the CLO had a higher content of EPA than HLO (14.35 vs. 5.92%), and a lower DHA content (9.04 vs. 13.60). However, since CLO had the highest total amount of the n-3 FA’s, and of the oxidation susceptible PUFA’s (33.39% vs 24.63%), it was postulated that CLO was particularly well suited to evaluate antioxidant efficiency.

3.3.2.2 Oven storage test

The oven storage test for accelerated aging of oils (AOCS Cg 5-97, 1997) was adapted for a storage test for the hake head flour to measure the stability of the lipid fraction. The
oven storage test recommends a storage temperature higher than ambient temperatures (±25°C), but lower than 80°C. A temperature of 60°C is the recommended storage temperature for oils (AOCS Cg 5-97, 1997). A lower storage temperature of 45°C (±2°C) was used for T1 and T2 because the high PUFA content of the fish flour’s lipid component was expected to oxidise rapidly. The oven temperature was increased to 60°C (±2°C) for T3, thus accelerating the reactions.

The practices recommended for this test were closely adhered to (AOCS Cg 5-97, 1997): flour samples of the required size per test (kept constant within a trial) were stored in identical, wide mouthed, uncovered glass containers in random order in a dark, fan-fitted oven. Sample containers were removed at regular intervals and tested for chemical indexes of oxidation. As recommended, samples were tested immediately or vacuum-sealed (an alternative to displace oxygen instead of the nitrogen flushing recommended for oils), frozen (-18°C) and tested as soon as possible. Similar sized samples of each variation were tested as far as possible on each day to keep the lipid quantity extracted comparable and to eliminate any role the test procedures may have on the oxidation status of a varying quantity of lipid. Three indexes of oxidation were determined on lipid extracted from the flours: peroxide and $A_{232\text{nm}}$ values, which measure primary oxidation products (#2, sections 2.5.2.1 and 2.5.2.3), $A_{268\text{nm}}$ values, which measures secondary oxidation products (#2, section 2.5.2.3), and changes in the relative percentages of DHA and EPA to C16:0 over time, which indicate the extent of oxidative damage in the later stages of storage when oxidation has proceeded significantly (#2, section 2.5.2.4). The first point of measurement (0 h) was before the start of oven storage (i.e. the day after flour production). The other measurements were made at regular intervals thereafter as appeared necessary in order to record the induction curves of the flours.

3.4 REFERENCES


CHAPTER 4

Hake head flour oxidation as a function of the storage age of both the heads and the flour


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Hake head flour is a possible source of the nutritionally important n-3 polyunsaturated fatty acids (PUFA’s). These fatty acids (FA’s) are susceptible to oxidation, which reduces their levels and causes rancidity. The level of oxidation in hake head flour as a function of both the experimental storage age (days) of the heads (= x), stored on ice at ± 4°C, used to prepare the flour, as well as of the flour (= y), stored at ambient conditions, was quantified. Peroxide values (PV’s) and absorption values/A₅₃₂nm (2-thiobarbituric acid test) were determined over 9 days of storage for 3 flours respectively produced from heads stored for 1, 5 and 9 days. Interactions (p ≤ 0.01) existed between x and y for both peroxide and A₅₃₂nm values. Quadratic bivariate functions, which respectively predict peroxide and A₅₃₂nm values as functions of x and y, were fitted. Flour freshly prepared (D1 flour) from heads stored for 1 day had the highest peroxide and lowest A₅₃₂nm value (all results at p ≤ 0.05). D1 flour prepared from heads stored for 9 days had the lowest PV. The A₅₃₂nm of D1 flour prepared from heads stored for 5 or 9 days did not differ significantly. The peroxide and A₅₃₂nm values for all 3 flours decreased during 9 days of storage. The results indicated that an increase in the storage age of the heads or the flour increased the flour oxidation level, with a significant, interactive influence between these two factors.

KEY WORDS: oxidation, fish flour, hake, n-3 polyunsaturated fatty acids, peroxide value, 2-thiobarbituric acid test

4.1 INTRODUCTION

Fish is a rich source of the polyunsaturated fatty acids (PUFA’s), particularly of the long chain n-3 fatty acids (FA’s), eicosapentaenoic acid (C20:5n-3/EPA) and docosahexaenoic acid (C22:6n-3/DHA). Up to 30% of the FA’s in fish oil may consist of a combination of

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EPA and DHA (Gunstone, 1996; Kaitaranta, 1992). Although hake is classified as a lean fish species (Huss, 1988; Dassow & Beardsley, 1974), fish heads generally serve as fat depots (Huss, 1988). Panggat and Rivas (1997) report that the lipids in the heads and eye sockets of Yellowfin tuna are noteworthy sources of the n-3 FA’s, respectively containing 22.0 and 25.3% DHA and 4.9 and 5.0% EPA. Hake heads, or the products produced thereof, could therefore plausibly be an unexploited source of the n-3 FA’s.

The n-3 FA’s have many physiological functions, i.e. in normal growth and development (Bjerve et al., 1988) and in preventing neurological abnormalities (Holman et al., 1982). Docosahexaenoic acid is also required for normal retinal function (Neuringer & Connor, 1986). A study on the FA status of undernourished children in rural areas of Lebowa, South Africa, found a high prevalence of under-nutrition, associated particularly with a low energy intake and an imbalance of dietary n-6:n-3 FA intake (Tichelaar et al., 1994). An n-3 PUFA rich fish food product could be an important nutritional intervention tool in such instances. Minnaar (2001) motivated that utilising the heads of hake caught by the South African fishing industry (mainly of the species Merluccius capensis and Merluccius paradoxus) to produce a food product is quantitatively viable, while such utilisation would also provide a solution to the reported illegal practice of fishing vessels discarding the heads at sea in order to increase the cold storage space for higher value cuts, such as the fillets.

However, the high PUFA content, together with the large amount of doubly allylic methylene groups present in the long chain PUFA’s, render fish oils very susceptible to oxidation (Michelsen et al., 2001; Gunstone, 1996; Kaitaranta, 1992). Oxidation is a reaction that occurs between the unsaturated bonds of triacylglycerides and atmospheric oxygen (Gunstone, 1996; Dziezak, 1986). It results in off-flavours and odours, often described as rancidity, as well as in the loss of essential FA’s and vitamins A, D and E (Fernández et al. 1997; Buck & Edwards, 1997; Hamilton, 1994; Alais & Linden, 1991; Dziezak, 1986). Oxidation should therefore be limited as far as possible in order to create an effective dietary intervention tool.
The influence of the storage age of the hake heads used to prepare a flour on the flour's oxidation level was uncertain. Although freshness and spoilage indices of white fish species stored on ice have previously been correlated with the number of storage days, oxidation levels were not determined (Oehlenschläger, 1992), possibly because oxidation in iced fish is considered limited, and therefore not a good freshness indicator. The limited oxidation in iced fish has been ascribed to a relationship between increasing bacteria counts and a reduction in the oxidation rate (Stansby, 1982). Hamilton (1994), however, states that a low storage temperature does not prevent oxidation. Increasing 2-thiobarbituric acid (TBA) values with an increase in storage time at a temperature of 1°C for some fish species support this view (Mendenhall, 1972). Therefore, the effect of the storage age of hake heads on the level of oxidation in flour produced thereof was investigated. The level of oxidation in the flour was expected to increase during flour storage since studies on the level of oxidation in stored fish flour previously showed an increase in oxidation (indicated by an increase in PV's) over time (Petrescu, 1983, 1984).

This study determined the effect of two factors, the experimental storage age of hake heads on ice used to prepare hake head flour and the storage age of the flour produced thereof, on the oxidation level of the flour. The two most frequently used methods to measure rancidity in fish, i.e. peroxide values (PV's) and absorbance values (determined with the TBA test) were selected as oxidation indicators (Harris & Tall, 1994).

4.2 MATERIALS AND METHODS

4.2.1 Materials

Eighty kilogram of hake heads, reserved from the last day’s catch of a commercial trawler (Irvin and Johnson, Cape Town, South Africa), was used. Due to production limitations, the heads were packaged, and then frozen and stored at approximately -18°C until the experiment commenced. The heads were defrosted overnight at ± 8°C, and then stored on ice at ± 4°C for the duration of the experiment. Storage containers that allowed drainage were used and the ice were replenished as necessary. Heads stored for 1, 5, and
9 days respectively were used to prepare flours. At each of these times of storage, 20 kg of heads were randomly selected and each lot was used to prepare a batch of flour, i.e. three batches of flour were prepared in total. Flour production took place according to a standardised procedure on the premises of Melnyczuk Research and Development (CK 96/58741/23, Box 862, Stellenbosch, 7599, South Africa). This fish flour production process may be patented in the future, and details thereof remain undisclosed. Only a brief description follows: the top section of hake heads, with brain and eyes intact, were cut from washed heads and minced finely. This mince was mixed with 6% sodium alginate (Tranarc, 18 Arbroath Avenue, Hurling Ham, Sandton, South Africa), included into the mince at a 10% level. This mixture was extruded through a 1.2 mm die into a brine solution, which consisted of 94.05% water, 4.2% calcium lactate (Chempure, Meiring Naudé Road, CSIR Building 41, Lynwood, Pretoria, South Africa) and 1.75% lactic acid at a concentration of 80% (Petrow Chemicals, PO Box 37183, Chempet, 7442, South Africa). The mince was set into strips on contact with the brine, and these were then dried in a system that passed ambient air through a desiccant unit. A total of ± 3.87 kg of strips were evenly spaced over drying trays and rotated and turned at approximately 30 min intervals. The two batches of flour prepared from heads stored for 1 and 5 days respectively were dried for 4 h 43 min, which, at the end of this time, resulted in slightly different humidities in the air coming off the dry product (5.9 and 8.8%). This was probably because the drier used ambient air, which would vary along with the daily factory conditions. The third batch was therefore dried until the air coming of the product also reached a relative humidity of ± 8% (4h 25 min). The temperature in the drier at the end of the drying period for the three batches of flour varied between 35.6 and 38.7°C. The dried strips were cut (13 mm mincing plate), then powdered in a hammer-mill and stored in sealed plastic bags at ambient conditions. Peroxide and A_{532nm} values were determined in triplicate for each flour batch on a randomly selected, individually packaged sample on days 1, 3, 5, 7 and 9 of flour storage (day 1 being the day after preparation).
4.2.2 Chemical indexes of oxidation

The method of Lee et al. (1996) was adapted as follows to obtain a 0.5 g lipid sample: approximately 20 g of flour was extracted with 200 ml chloroform:methanol (1:2, as indicated for lean fish). Sodium chloride (80 ml of a 0.5% solution) was used for phase separation. A solvent volume of ± 40 ml was collected and thoroughly flushed with nitrogen gas before suspending it in a water bath (temperature ± 60°C) placed within a fume cupboard. Nitrogen gas was continuously blown onto the solvent during heating since nitrogen flushing protects against heat-induced auto-oxidation through oxygen depletion (Ulberth, 1998). The water bath temperature of 60°C accelerated solvent evaporation, but it is known that peroxides are heat labile compounds that break down at elevated temperatures (Ulberth, 1998). The degree of breakdown should, however, have been relatively constant for all the samples as the solvent volume and water bath temperature remained constant. The PV’s were determined with the acetic acid-chloroform method of the American Oil Chemists’ Society/AOCS (AOCS Cd 8-53, 1997), using 0.01 N sodium thiosulphate for titration. Solvent volumes were adapted for the 0.5 g lipid sample used to 10 ml for both acetic acid-chloroform and distilled water. Results reflect all substances, in terms of millequivalents of peroxide present per kilogram of sample, which oxidize potassium iodide under the test conditions (AOCS Cd 8-53, 1997). Absorption values at 532 nm (A\textsubscript{532nm}) were determined with a fast, modified aqueous extraction TBA method (Pikul et al., 1989), recommended when quick analysis of many samples is needed, with the following adaptations: 49 ml of 4% perchloric acid and 0.75 ml 0.1% BHT in ethanol were added to 4 g flour, blended with a Bamix M22 hand blender (high speed, 1 min) and filtered into a 100 ml Erlenmeyer flask. Samples and a reagent blank were heated in a boiling water bath for 1 h and cooled under running tap water for ± 4 min. Since comparison between treatments for samples of the same weight was the objective, the A\textsubscript{532nm} values were not multiplied with the constant K, but reported as is, an alternative way previously used to report TBA test results (Roozen, 1987).
4.2.3 Data analysis

A 3 x 5 factorial analysis of variance (ANOVA) was conducted (SAS, 1999). The three factors refer to the three storage ages of the hake heads and the five factors to the five storage ages of the flour investigated. Each main effect was broken down into single degree polynomial functions to determine the order of the polynomial function that best fitted the data. The Shapiro-Wilk test was performed to test for non-normality (Shapiro & Wilk, 1965). The Student’s t-least significant difference (LSD) was calculated to compare treatment means at a 5% significance level. Bivariate functions were fitted with Tablecurve® 3D software (version 3.12, AISN Software).

4.3 RESULTS AND DISCUSSION

Hake heads of three storage ages (1, 5 and 9 days of storage) were used for flour preparation. By 9 days of storage the heads were deteriorating rapidly, displaying a disagreeable mottled surface and a strong, fishy odour, and therefore heads stored for longer than 9 days were not considered suitable for use in a food product. Since the fish were reportedly reserved on ice from the last day’s catch and frozen the next day, this experimental storage age of 9 days translated to an actual age of approximately 10 days, which was shorter than expected. Oehlenschläger (1992) reports that fish storage on ice maintains the fish temperature at 0°C, while the melting ice washes out low molecular weight compounds that could contribute to off-flavours and -odours, and that a shelf-life of 15-20 days can thus be obtained. The South African Code of Practice for the Handling of Chilled and Frozen Foods prescribes fish storage at 0°C-2°C, and reports that these conditions allow for a storage life (from time of catching) of 8-12 days (SABS 0156, 1979). Dassow and Beardsley (1974) also reported that fresh (less than 24 h old) hake fillets, stored at a temperature of 1°C, remained in an acceptable condition for up to two weeks. The faster rate of deterioration in the present study could possibly be ascribed to the absence of cleaning of the heads at sea, which caused visible gut-contamination of the heads. Huss (1988) reports that the quality and storage life of many fish species decrease if gutting does not occur. Although Oehlenschläger (1992) reports that storing fish on ice
maintains the fish temperature at 0°C, a lower environmental storage temperature than 4°C may possibly have extended the shelf-life. The freezing of the heads prior to flour production may also have decreased the shelf-life of the heads once defrosted.

Data were found to be in a normal distribution. The results of the ANOVA analysis are reported in Table 4.1.

Table 4.1. Results of 3x5 factorial analysis of variance

<table>
<thead>
<tr>
<th>Factor</th>
<th>Df</th>
<th>Percentage variation explained</th>
<th>Variable: Absorbance (532 nm)</th>
<th>Percentage variation explained</th>
<th>Variable: Peroxide value</th>
<th>MS²</th>
<th>p</th>
<th>Percentage variation explained</th>
<th>Variable: Peroxide value</th>
<th>MS²</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of heads (x)</td>
<td>2</td>
<td>980.368</td>
<td>490.183785</td>
<td>0.611</td>
<td>0.3053</td>
<td>&lt;0.01</td>
<td>0.3053</td>
<td>&lt;0.01</td>
<td>490.183785</td>
<td>0.611</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Linear</td>
<td>1</td>
<td>90.766</td>
<td>889.83719</td>
<td>&lt;0.01</td>
<td>71.463</td>
<td>0.4363308</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quadratic</td>
<td>1</td>
<td>9.234</td>
<td>90.530379</td>
<td>&lt;0.01</td>
<td>28.537</td>
<td>0.17424</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age of flour (y)</td>
<td>4</td>
<td>4228.517</td>
<td>1057.129263</td>
<td>&lt;0.01</td>
<td>0.1505</td>
<td>0.03762928</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linear</td>
<td>1</td>
<td>91.850</td>
<td>3883.877898</td>
<td>&lt;0.01</td>
<td>96.736</td>
<td>0.14560444</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quadratic</td>
<td>1</td>
<td>7.420</td>
<td>313.754056</td>
<td>&lt;0.01</td>
<td>1.459</td>
<td>0.00219584</td>
<td>0.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cubic</td>
<td>1</td>
<td>0.720</td>
<td>30.449785</td>
<td>0.07</td>
<td>0.374</td>
<td>0.00056</td>
<td>0.32</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quatric</td>
<td>1</td>
<td>0.010</td>
<td>0.435</td>
<td>0.82</td>
<td>1.431</td>
<td>0.00215433</td>
<td>0.06</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(x) x (y)</td>
<td>8</td>
<td>269.643067</td>
<td>&lt;0.01</td>
<td>0.00281493</td>
<td>&lt;0.01</td>
<td>0.00054442</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>30</td>
<td>8.588139</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Corrected total</td>
<td>44</td>
<td>8.588139</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Shapiro Wilk</td>
<td>-</td>
<td>-</td>
<td>0.3605</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.1193</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

1 degrees of freedom; ² mean square

For both factors (storage of heads on ice = x, age of flour = y) and both variables (PV’s and A₅₃₂nm), the linear and quadratic polynomial functions contributed significantly (p ≤ 0.05) to explain 100% of the variation in x for both variables and 99.3 and 98.2% of the variation in y for peroxide and A₅₃₂nm values respectively. For both variables, a highly significant interaction (p ≤ 0.01) existed between the factors. Bivariate functions were therefore fitted to yield equations 1 and 2, which respectively predict two indicators of the flour oxidation level, PV’s (r² = 0.8976) and A₅₃₂nm values (r² = 0.9679), as functions
of the experimental storage age (days) of the heads used to prepare the flour (= x) and of the storage age (days) of the flour (= y):

1. \[ PV = 86.6496 - 2.5363x - 10.2849y - 0.1881x^2 + 0.3945y^2 + 0.6111xy \]
   \[
   (\pm 6.665) \quad (\pm 1.998) \quad (\pm 2.222) \quad (\pm 0.181) \quad (\pm 0.204) \quad (\pm 0.148)
   \]

2. \[ A_{532nm} = 0.5463914 + 0.1120979x - 0.0310997y - 0.0082500x^2 + 0.0010437y^2 + 0.0001104xy \]
   \[
   (\pm 0.0385188) \quad (\pm 0.0115490) \quad (\pm 0.0128385) \quad (\pm 0.0010456) \quad (\pm 0.0011783) \quad (\pm 0.0008537)
   \]

Equations 1 and 2 are illustrated in Figure 4.1 and Figure 4.2.

![Figure 4.1. Function predicting the peroxide values (PV's) in hake head flour as a function of both the storage time of the heads used to prepare the flour and of the flour](image)
Figure 4.2. Function predicting the absorbency values at 532 nm ($A_{532\text{nm}}$) in hake head flour as a function of both the storage time of the heads used to prepare the flour and of the flour

The interaction means for the peroxide and $A_{532\text{nm}}$ values within the factor storage age of the flour (day 1 old/freshly prepared flour) are reported in Table 4.2.

Table 4.2. Interaction means for peroxide values (PV's) and absorbance values at 532 nm ($A_{532\text{nm}}$) within storage age of the flour (freshly prepared/one day old flour)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Storage of heads on ice (days)</th>
<th>LSD** (p ≤ 0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>PV* in day 1 flour</td>
<td>80.161&lt;sup&gt;a&lt;/sup&gt;</td>
<td>62.252&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>$A_{532\text{nm}}$* in day 1 flour</td>
<td>0.633&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.853&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Means within a row followed by different superscripts differ significantly
**Least Significant Difference

Peroxide values reflect the level of hydroperoxides, which are intermediate oxidation products. The peroxide concentration therefore increases to a maximum, and then
decreases as the hydroperoxides decompose into secondary oxidation products (Hamilton et al., 1997; Coppen, 1994). This change in peroxide concentration as oxidation proceeds could lead to incorrect interpretations of the oxidation level (Hamilton et al., 1997). For example, if only the decomposition part of the peroxide curve is recorded, the low PV’s recorded may be incorrectly interpreted as indicative of a low level of oxidation. This possible risk of misinterpretation clarifies why the use of a combination of methods to assess both primary and secondary oxidative changes in oils is advised (Shahidi & Wanasundara, 1997). The PV’s recorded should therefore be interpreted in conjunction with the A_{532nm} values, as the latter indicates the levels of a secondary oxidation product, malonaldehyde/MDA (Shahidi, 1998).

Although the oxidation rate in iced fish is slow (Stansby, 1982), the oxidation level was expected to increase in heads stored for an increasing period of time. This increasing level of oxidation should then have been detectable in the flour freshly prepared from heads of increasing storage ages. The PV’s of the freshly prepared flour (one day/D1 old flour) however decreased significantly (p ≤ 0.05) with an increase in the storage age of the heads used to prepare it (Figure 4.1 and Table 4.2). This decrease should however be considered in the light of the intermediate nature of the hydroperoxides measured in the PV test, which decompose after formation (Hamilton et al., 1997; Coppen, 1994). Hydroperoxides formed from fish have a particularly rapid development and decomposition curve, ascribed to their highly polyunsaturated nature. This leads to highly unstable structures with low activations of decomposition (Mitchelsen et al., 2001; Rossell, 1994; Harris & Tall, 1994). Considering this known fast decomposition rate, it appears as if the increase in PV’s associated with the start of oxidation was never recorded in this study, and that even the fresh (D1) flour prepared from heads stored for only 1 day was already oxidised to such an extent that peroxide decomposition exceeded formation. The increasingly lower PV’s in the D1 flours prepared from heads of increasing storage ages therefore probably indicate a more advanced stage of peroxide formation and decomposition, and thus oxidation, in the flours, and therefore in the heads from which it was prepared.
This interpretation of the oxidation status of the D1 flours is supported by the increase in the $A_{532\text{nm}}$ values recorded for the D1 flours prepared from heads of an increasing storage age (Figure 4.2 and Table 4.2). The $A_{532\text{nm}}$ values in the D1 flour were significantly higher ($p \leq 0.05$) when prepared from heads stored for 5 days than when prepared from heads stored for 1 day. The $A_{532\text{nm}}$ values reflect the levels of MDA, a 3-carbon dialdehyde with carbonyl groups on carbons 1 and 3 (Fernández et al., 1997), which is a final oxidation product (Shahidi, 1998). Therefore, it appears as if the heads became more oxidised during storage up to 5 days, and that the increasing levels of MDA in the heads as oxidation progressed were transferred to the flour prepared thereof, resulting in the increase in $A_{532\text{nm}}$ values recorded in the D1 flour. There was no significant difference in the $A_{532\text{nm}}$ values of D1 flour prepared from heads stored for 5 or 9 days, which suggests that the oxidation level in the heads reached a plateau by 5 days of storage.

The opposite trends in the peroxide and $A_{532\text{nm}}$ values of the D1 flours prepared from heads of an increasing storage age further verify the above conclusion, since MDA primarily originates on the decomposition of hydroperoxides formed from PUFA’s with three or more double bonds (Fernández et al., 1997). The recorded decrease in hydroperoxides in the D1 flours should therefore have led to a concurrent increase in $A_{532\text{nm}}$ values, which was indeed the case.

The interaction means for the peroxide and $A_{532\text{nm}}$ values within the factor head storage on ice (days) are reported Table 4.3.

During the storage of the flour (1 to 9 days) the oxidative deterioration showed the following trends: the PV’s of all three flour batches decreased as the storage age of the flour increased, with the PV of the 9-day old (D9) flour of a specific batch always significantly lower ($p \leq 0.05$) than the PV of D1 flour (Figure 4.1 and Table 4.3). These decreasing PV’s again indicate that oxidation has reached the point where peroxide decomposition exceeded formation, i.e. the decreasing PV’s indicate increased oxidation, as more oxidised flours have had more extensive decomposition of peroxides. In spite of
Table 4.3. Interaction means for peroxide value (PV) and absorbance values at 532 nm (A$_{532\text{nm}}$) within head storage age on ice (days)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Head storage on ice (days)</th>
<th>Age of flour (days)</th>
<th>LSD** (p ≤ 0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>PV*</td>
<td>1</td>
<td>80.161$^a$</td>
<td>57.964$^b$</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>62.252$^a$</td>
<td>47.299$^b$</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>40.530$^a$</td>
<td>38.930$^{ab}$</td>
</tr>
<tr>
<td>A$_{532\text{nm}}$*</td>
<td>1</td>
<td>0.633$^a$</td>
<td>0.564$^b$</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.853$^a$</td>
<td>0.842$^a$</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>0.850$^a$</td>
<td>0.823$^a$</td>
</tr>
</tbody>
</table>

*Means within a row followed by different superscripts differ significantly
**Least significant difference

the disadvantage of peroxide decomposition, the peroxide test could still be used to compare the level of oxidation of different treatments if raw material with a constant oxidation level are used and tests are repeated over sufficient days to establish the curve of peroxide formation.

Petrescu (1983), however, reports that the PV’s of untreated (no antioxidant added) fish meal rapidly increased to a maximum level within two days of storage, after which it gradually decreased to zero after 40 days. In another study, the PV’s of untreated fish meal increased during the first week (up to day 7) after production, followed by a decrease to zero after about 1 month of storage (Petrescu, 1984). The faster fish flour oxidation rate recorded in the present study (as indicated by the already decreasing PV’s during the first days of flour storage) could possibly be attributed to a higher flour PUFA lipid content since there was no lipid extraction during flour preparation. Both the high PUFA content and the structure of the PUFA’s present in fish oil render it (and therefore the hake head flour) very susceptible to oxidation (Michelsen et al., 2001; Gunstone, 1996; Kaitaranta, 1992). The possible influence of the frozen storage of the heads prior to flour preparation on the oxidation status of the flour should also be considered since oxidation does proceed, albeit slowly, in frozen fish exposed to air (Stansby, 1982). The extent of oxidation in the heads (and therefore in the flour) may have been less had fresh fish been used for flour preparation. The high level of oxidation in even the D1 flour
prepared from heads stored for 1 day can possibly also be attributed to the processing conditions. The mincing of the heads during flour production both enlarges the exposed surface areas and incorporates oxygen. Maceration of flesh also releases lipolytic enzymes, which liberate free FA’s (Hamilton, 1994), and unsaturated FA’s mostly oxidize faster when in a free than when in a bound state (Alais & Linden, 1991). An increase in temperature during processing may also promote oxidation. Buck and Edwards (1997) report that a 10°C increase in temperature doubles the oxidation rate. Significantly higher TBA values were, for example, recorded after 3 days when de-boning at 25°C in comparison to de-boning at a lowered drum temperature of 2-3°C (Lee & Toledo, 1977). In the current production process, the mincing of the heads generates heat, while greatly increasing the surface areas exposed to incorporated oxygen. The mince is also dried with air at room temperature. These factors could all have contributed to the high level of oxidation in the freshly prepared flour.

The A532nm values declined for all three flours during storage, with the A532nm value of the D9 flour always significantly lower (p ≤ 0.05) than that of the D1 flour (Figure 4.2 and Table 4.3). As discussed, MDA primarily originates on the decomposition of hydroperoxides formed from PUFA’s with three or more double bonds (Fernández et al., 1997). Since fish is a rich source of the n-3 PUFA’s (Gunstone, 1996; Kaitaranta, 1992), the TBA test was expected to be particularly well-suited to determine the extent of oxidation in the fish flour. The decrease of a final oxidation product, MDA, with an expected increase in oxidation was therefore unexpected, more so since the hydroperoxides, from which MDA is hypothetically formed, was shown to decrease with an increase in the flour storage age (see Figure 4.1), and a concurrent increase in MDA levels was therefore expected.

The recorded MDA decrease during flour storage could possibly be ascribed to the dehydrated nature of the flour. The methylene interrupted dialdehyde form of MDA is known to undergo enolisation to form an enolic tautomer, which undergoes molecular rearrangement to form open s-cis-, s-trans-, and chelated forms (Kwon & Watts, 1963). In dehydrated foods, lipid oxidation may be far advanced with little or no MDA
accumulation due to MDA being present in its' volatile, chelated form. Very little MDA was for example found in highly rancid flour since the MDA could not be held in the food by metal chelation (Kwon & Watts, 1963). If the rate of MDA escape exceeded the rate of MDA formation, the $A_{532}$ would decrease in spite of an increased formation of MDA from decomposing hydroperoxides. The decrease in MDA concentration in the aging flour is therefore not, as with peroxides, simply an indicator of the transitory nature of the product measured, but also of the influence of the treatment (dehydration) of the material tested, and may result in an incorrect impression of oxidation level of the flour.

The decrease in $A_{532nm}$ values in the flours during storage was in contrast with the reported increase in the $A_{532nm}$ values in D1 flours prepared from heads of an increased storage age. Possibly the increasing MDA levels recorded in the D1 flours prepared from heads of an increasing storage age were transferred to the flours from the heads, which became more oxidised and thus retained higher MDA levels as their storage time increased. These levels of MDA were then recorded in the D1 flours before it could escape significantly due to the flour’s dehydrated state. If the age, and therefore supposedly the oxidation level, of the hake heads is constant, the $A_{532nm}$ values in the freshly prepared flour could therefore still be used as an indicator of the level of flour oxidation as a function of the raw material’s treatment. The $A_{532nm}$ values in the flour, however, decreased during storage because the MDA appeared to escape faster from the dehydrated flour than its’ rate of formation from hydroperoxides.

### 4.4 CONCLUSION

The experimental storage age of hake heads used to prepare hake head flour as well as the storage age of this flour had a significant, interactive influence on the extent of oxidation in the flour. An increase in either factor caused an increased level of flour oxidation. Consequently, in fish flour oxidation studies, the storage age of the fish used to prepare the flour as well as the storage age of the flour should be taken into consideration to allow an assessment of the flour’s oxidation status. The TBA test proved largely ineffective in measuring oxidation in the fish flour since the main oxidation product measured, MDA,
appeared to escape, possibly due to being present in a volatile, chelated form in the dehydrated flour. Further research on the rate of MDA escape, and of its' relationship with peroxide formation and decomposition and with the level of oxidation in the product (as pertaining to a dehydrated product) is required to determine whether the TBA test can be used successfully to assess the extent of lipid oxidation in a dehydrated fish flour.

4.5 ACKNOWLEDGEMENTS

Appreciation to Mr. F. Calitz (ARC-Biometry Unit, Stellenbosch, South Africa) for statistical analysis of data, Melnyczuk Research and Development for assistance with flour production, and to the DACTS Innovation Fund (project number 32348) for funding of this project.

4.6 REFERENCES


CHAPTER 5
Protection of hake head flour against lipid oxidation

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The antioxidant efficiency of butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butyl hydroquinone (TBHQ), Biored Pure, Ronoxan A, and a partially water soluble treatment/WST (containing sodium ascorbate, dl-α-tocopherol and Emulfluide E/EE) in cod liver oil was compared by their protection factors (PF’s), calculated from their Rancimat induction times. A TBHQ-citric acid (CA) combination gave the highest PF (5.23). The WST, preferred due to solubility characteristics and a more natural composition, gave a PF of 1.48. Greatly increased (x 722.16) concentrations of the WST constituents (WSTinc), except for EE, which was only increased 17.14 times in the Rancimat trials, gave a PF of 9.32. WSTinc with dl-α-tocopherol replaced by dl-α-tocopherol-acetate (WSTacetate) gave the maximum measurable induction time (> 48 h). Hake head mince, prior to drying to produce flour, was then treated with CA (0.2 g/kg of flour lipid), combined with TBHQ (0.12 g/kg of flour lipid) and EE (at 0.14% add-in on mince weight), or with WSTacetate and water (respectively at 0.2 and 0.5% add-in on mince weight). Both the TBHQ-CA treated and a control (no antioxidants added) flours were oxidised after drying (peroxide values/PV’s > 40 meq/kg). WSTacetate-CA treated flour (PV of 0.79 meq/kg after drying) only reached a PV ≥ 10 meq/kg after ± 140 h of storage (45°C), while a control was already oxidised after drying (PV of 33.88 meq/kg). Hake liver enriched mince treated with WSTinc or WSTacetate (0.2% add-in) resulted in flours with low PV’s after drying (respectively 3.03 and 1.73 meq/kg), which were respectively stored for ± 10 and 32 h at 60°C before reaching PV’s ≥ 10 meq/kg. This indicated antioxidant action relative to a control, which was oxidised after drying (PV of 18.03 meq/kg). This conclusion was supported by determinations of conjugated dienes and trienes, and by the relative decrease in polyunsaturated fatty acid/C16:0 ratios after 149 h of storage (60°C), which was greatest for the control and least for the WSTacetate treated flour. WSTacetate appeared the most effective formulation.

KEY WORDS: antioxidants, fish flour, hake, shelf-life, Rancimat, polyunsaturated fatty acids

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5.1 INTRODUCTION

Dietary intake of fatty acids (FA’s) with n-6 or n-3 double bonds is essential because the human body cannot synthesise these FA’s (Devadasan & Gopakumar, 1997). Dietary intake recommendations for these FA’s however advise that intake of the n-6 FA’s should be reduced in favour of n-3 FA intake to ensure optimal brain and cardiovascular health and function, and to reduce the possible adverse effects of a too high n-6:n-3 intake, a common occurrence in the diets of Western industrialised countries, which contain too much dietary plant oils rich in n-6 FA (Simopoulos et al., 2000). The n-3 FA’s have an essential role in normal growth and development, and a possible critical role in various diseases, such as coronary artery disease, hypertension, arthritis, other inflammatory and auto-immune disorders, and cancer (Bjerve et al., 1988; Simopoulos, 1991).

Fish is a rich source of the long chain n-3 polyunsaturated FA’s (PUFA’s) eicosapentaenoic acid (C20:5n-3/EPA) and docosahexaenoic acid (C22:6n-3/DHA). As much as 30% of the FA’s in fish oil may consist of a combination of EPA and DHA (Gunstone, 1996; Kaitaranta, 1992). Although hake is a lean fish species (Huss, 1988; Dassow & Beardsley, 1974), Panggat and Rivas (1997) report that the lipids in the heads and eye sockets of Yellowfin tuna are noteworthy sources of the n-3 FA’s, respectively containing 22.0 and 25.3% DHA and 4.9 and 5.0% EPA. Hake heads could therefore be an unexploited source of n-3 FA’s. South African fish trawlers are reported to illegally discard hake heads at sea to increase the cold storage space for higher value cuts, such as fillets (Minnaar, 2001). A process that utilises the heads in the production of flour in such a way that it increases the commercial value of the heads could limit this practise. This flour could then enrich a staple food with primarily n-3 PUFA’s (Minnaar, 2001), and the ensuing staple food could be used to address PUFA needs and imbalances in the human diet.

The high PUFA content of fish oil, however, results in an increased susceptibility to oxidation (Kaitaranta, 1992). Oxidation leads to unpleasant rancid odours and flavours, and to the undesirable loss of PUFA’s and fat-soluble vitamins, such as vitamins A, D
and E (Buck & Edwards, 1997; Dziezak, 1986). Protection against oxidation is therefore vital in order to create a PUFA-rich dietary intervention tool. Possible protective measures include process control (minimisation of pro-oxidising factors, e.g. heat, catalysts, and light), as well as the use of antioxidants (Alais & Linden, 1991). Antioxidants can slow down but not reverse oxidation, and can only protect against auto-oxidation (Dziezak, 1986). Synthetic antioxidants, such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tertiary butyl hydroquinone (TBHQ), are typically less expensive and more effective at lower inclusion levels than natural antioxidants (Gunstone, 1996). Although approved for use in food products at specific usage levels (Codex Alimentarius Commission, 1999), the use of synthetic antioxidants in foods for babies and young children is not recommended (Hanssen, 1987). Preferential use of the natural antioxidants is becoming an increasing trend (Gunstone, 1996; Frankel, 1996), which can probably be ascribed to the possible toxic effects attributed to the synthetic antioxidants (White & Xing, 1997). In selecting antioxidants, efficiency should be assessed along with dosage level, cost, and health implications.

The objective of this study was to determine which of a selection of antioxidants added to hake head mince best protected the flour produced thereof against oxidation during an accelerated shelf-life test/ASLT (oven storage). Due to practical constraints, the performance of only a limited number of antioxidants could be evaluated in the flour. Therefore, the efficiency of commonly used antioxidants was first screened in fish oil in the Rancimat, a commercial instrument used in the oil stability index (OSI) test. The Rancimat accelerates oxidation by exposing oils to higher than ambient temperatures and a constant air flow (American Oil Chemist’s Society/AOCS Cd 12b-92, 1997), and allows continuous measurement of oxidation without periodic analytical determinations requiring organic solvents (Hasenhuetti & Wan, 1992). It records induction times, which is the time before oxidation, which initially proceeds slowly until the oil’s natural resistance to oxidation is overcome, starts to accelerate (AOCS Cd 12b-92, 1997). The most effective and preferred antioxidant combinations were then applied to the mince, and the flours produced thereof were subjected to oven storage (AOCS Cg-97, 1997). Periodic determinations of peroxide values (PV’s) and of the ultraviolet (UV) absorbance
by the conjugated dienes and trienes were used during the flour storage to record the induction curve of each flour. Conjugated diene levels and PV's are commonly used to follow oxidation during antioxidant efficiency studies (Duve & White, 1991; Noor & Augustin, 1984), and the latter is one of the most frequently used methods to determine rancidity in fish (Harris & Tall, 1994), and has previously been used in fish flour (Petrescu, 1983, 1984). Since the preservation of the n-3 PUFA's was the main objective, the relative decreases in the ratios of the relative percentages of DHA and EPA to C16:0 were also determined.

5.2 EXPERIMENTAL PROCEDURES

5.2.1 Materials

5.2.1.1 Oil used for Rancimat screenings

Commercial cod liver oil [Alpha BP, Allied Drug Company (Pty) Ltd., 27 Hannar Road, Congella, Durban, South Africa] was used for the Rancimat tests as it was the only fish oil tested during preliminary trials that gave a clear induction period. One production lot or thoroughly mixed production lots of oil was always used within a trial.

5.2.1.2 Antioxidants screened in the Rancimat

Synthetic antioxidants screened included BHT, BHA and TBHQ (Fluka, Sigma-Aldrich, South Africa). Combinations of the synthetics, such as BHA combined with BHT, are synergistic (Buck & Edwards, 1997; Gunstone, 1996; Dziezak, 1986), as are combinations with citric acid (CA). For example, 200 mg TBHQ offered less protection than a 100 mg combination of TBHQ and CA (Du Plessis & Sudworth, 2001). Therefore combinations of the synthetics, as well as combinations of the antioxidants investigated with CA or sodium metabisulphite/SMBS (both Protea Chemicals, Berrange Road, Wadeville, Germiston, South Africa) were screened. Sodium metabisulphite acts as an antioxidant by the release of sulphur dioxide in solution (Hughes, 1987). A natural,
flavonoid rich product, Biored Pure, (Biorem BK, P.O. Box 565, Jan Kempdorp 8550, South Africa), was also screened, as well as a commercial formulation, Ronoxan A (RA), which consists of 25% ascorbyl palmitate, 5% dl-α-tocopherol and 70% lecithin (Chempure, Meiring-Naudé Road, Ringwood, Pretoria, South Africa), and a partially water soluble treatment (WST), formulated since water solubility was expected to facilitate dispersion in the water-rich fish mince. The WST was modeled on the RA, using 70% Emulfluide E (EE) as a lecithin and substituting the ascorbyl palmitate in RA with 25% of the water soluble sodium ascorbate [EE: Chemimpo South Africa (Pty) Ltd, Roggebaai, P.O. Box 7057, 8012; sodium ascorbate: Protea Chemicals; dl-α-tocopherol (5%): Roche Products (Pty) Ltd, South Africa]. Emulfluide E is an enzymatically hydrolysed, liquid soybean lecithin that has high emulsifying and dispersing properties in aqueous systems (Chemimpo). The EE functions as a surfactant, enhancing the uniform dispersion of water soluble antioxidants, such as ascorbic acid, in oil (Han et al., 1990). Lecithin has also been found to improve oxidative stability in fish oil (inclusion level of 0.5%), and acts synergistically with ascorbyl palmitate, and in a pronounced synergistic fashion with a combination of ascorbyl palmitate and tocopherols (Hamilton et al., 1998).

Antioxidants were used within the levels prescribed by the World Health Organisation (Codex Alimentarius Commission, 1999). Antioxidants for which only good manufacturing practice (GMP) is recommended were used at levels suggested by manufacturers. The application levels of the antioxidant combinations investigated are given in Table 5.1.

Some of the antioxidants were dissolved prior to addition to the oil in order to facilitate a homogeneous dispersion in the oil. Sodium ascorbate and SMBS were dissolved in distilled water in a 2:3 ratio. Propylene glycol (Protea Chemicals) was used as a solvent for CA, BHA and TBHQ in ratios of 1:5.7, 1:11.3 and 1:2 respectively, with these ratios set during preliminary trials to allow all particles to dissolve during brief heating over a water bath (temperature 40-50°C) while stirring.
Table 5.1. Dosage levels of antioxidant variations screened

<table>
<thead>
<tr>
<th>Variation</th>
<th>Ingredients</th>
<th>Dosage level (g/kg of oil)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ronoxan A (RA)</td>
<td>RA</td>
<td>0.500</td>
</tr>
<tr>
<td>RA &amp; citric acid (CA)</td>
<td>RA</td>
<td>0.500</td>
</tr>
<tr>
<td></td>
<td>CA</td>
<td>0.200</td>
</tr>
<tr>
<td>Partially water soluble treatment (WST)</td>
<td>WST</td>
<td>0.500</td>
</tr>
<tr>
<td>WST &amp; CA</td>
<td>WST</td>
<td>0.500</td>
</tr>
<tr>
<td></td>
<td>CA</td>
<td>0.200</td>
</tr>
<tr>
<td>RA &amp; sodium metabisulphite (SMBS)</td>
<td>RA</td>
<td>0.500</td>
</tr>
<tr>
<td></td>
<td>SMBS</td>
<td>0.010</td>
</tr>
<tr>
<td>WST &amp; SMBS</td>
<td>WST</td>
<td>0.500</td>
</tr>
<tr>
<td></td>
<td>SMBS</td>
<td>0.010</td>
</tr>
<tr>
<td>Tertiary butyl hydroquinone (TBHQ) &amp; CA</td>
<td>TBHQ</td>
<td>0.120</td>
</tr>
<tr>
<td></td>
<td>CA</td>
<td>0.200</td>
</tr>
<tr>
<td>Butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), TBHQ &amp; CA</td>
<td>BHT</td>
<td>0.0375</td>
</tr>
<tr>
<td>Biored Pure</td>
<td>Biored Pure</td>
<td>0.900</td>
</tr>
</tbody>
</table>

The weight of each solution required to yield the level of that antioxidant needed in the oil volume prepared was then incorporated into the oil while continuously stirring over a water bath (40-50°C). The emulsifying characteristics of EE were used to incorporate the solutions in EE-containing formulations.

Of the other antioxidants investigated, BHT, which is not soluble in propylene glycol (Gunstone, 1996), and RA, according to manufacturer’s directions, were added directly to ±5 ml of oil, heated over a water bath until dissolved (40-50°C), and then mixed with the remainder of the oil. The fat-soluble tocopherols were mixed with EE, followed by gradual addition of the oil. The active component of Biored Pure was extracted by mixing first with ethanol (Biored Pure:ethanol weight ratio 1:22.2), then with distilled water (Biored Pure:water weight ratio at 1:22.2), and, after an hour, with EE (Biored Pure:EE weight ratio at 1:10.1), followed by gradual addition of the oil.
A second Rancimat trial to evaluate the effectiveness of greatly increased levels of the WST was also completed. The developer of the hake head flour (Melnyczuck Research and Development, CK 96/58741/23, Stellenbosch, South Africa) reported the successful use during preliminary trials of an approximate 0.2% add-in level of WST into the fish mince to reduce flour rancidity. Assuming that this level of WST completely reached the flour lipid fraction, the levels of antioxidants per kilogram of lipid were calculated (using an average 18.5% flour yield and a minimum expected flour lipid content of 3%) as 90.27 g sodium ascorbate, 18.05 g dl-α-tocopherol, and 252.76 g of EE. This level of WST, which was greatly increased from what was initially screened (times 722.16), was tested in the Rancimat to determine how much this increased level (referred to as $\text{WST}_{\text{inc}}$) improved the PF. The increased level of EE would, however, not remain in suspension in the CLO and was reduced to 6 g/kg of oil for the Rancimat trials to allow a temporary emulsion in the oil (i.e. only 17.14 times the level screened in the first Rancimat trial). The sodium ascorbate was dissolved in water as before, i.e. in a 2:3 ratio. The $\text{WST}_{\text{inc}}$ was also investigated with the dl-α-tocopherol therein replaced with the same quantity of dl-α-tocopherol acetate (Roche, South Africa), referred to as $\text{WST}_{\text{acetate}}$ (also applied with the reduced level of EE in the Rancimat trial). Since the synergistic effect in the first trial appeared minimal, no CA or SMBS were added to either treatment.

5.2.1.3 Antioxidant tested in the hake head flour

Selected antioxidants were applied to hake head mince to compare the induction times obtained during ASLT of flours produced from antioxidant-treated mince with that prepared from untreated mince (i.e. a control flour). Since the flour is intended as a nutritional intervention tool, the more natural antioxidants were preferred to the synthetics in spite of their lesser efficiency, which was also confirmed during the Rancimat screenings, because of the possible negative health effects increasingly associated with the synthetics (White & Xing, 1997). It was postulated that, of the mainly natural antioxidant formulations investigated, WST, which contained both water- and fat-soluble components, should distribute better in the water-laden fish mince than RA, which is fat-soluble, and should therefore protect the fat missiles more effectively.
Therefore the WST\textsubscript{inc} (i.e. applied with the full amount of EE) was selected for evaluation in the flour. However, the TBHQ-CA combination gave the highest PF during the initial screening, and was therefore, although synthetic, evaluated in the flour during a preliminary trial (Trial 1 / T1) in order to provide a benchmark to compare the effectiveness of the other antioxidants. The TBHQ (freshly procured from Sigma-Aldrich, South Africa) and CA were applied to hake head mince at levels that yielded 0.12 and 0.2 g/kg lipid in the flour (i.e. levels/kg lipid as screened in the Rancimat), using a recorded 18% flour yield and a minimum expected flour lipid content of 3% for calculations. The TBHQ and CA were dissolved in propylene glycol in the same ratios as used in the Rancimat screenings, and EE was also included (add-in level of 0.14% into the fish mince) in order to facilitate the dispersion of the mostly fat-soluble TBHQ in the watery fish mince.

A second preliminary trial (T2) investigated the antioxidant action of the WST\textsubscript{acetate} in the flour. This trial would confirm if the effectiveness of the WST\textsubscript{acetate} (with the reduced quantity of EE) observed in the Rancimat would be present in flour produced from hake head mince treated with WST\textsubscript{acetate}. The WST\textsubscript{acetate} (i.e. with full levels of EE) was applied at the WST-levels reported successful by the product developer, i.e. at 0.2% add-in into the mince, dissolving the sodium ascorbate component in water, used at a 0.5% add-in level. Citric acid (dissolved in propylene glycol in a 1:5.7 ratio) was used at 0.2 g/kg of lipid in the flour (i.e. at the same level/kg lipid as was screened in the Rancimat), using a 18.5% flour yield and an minimum expected flour lipid content of 3% for calculations.

The efficiency of WST\textsubscript{inc} and WST\textsubscript{acetate} (both with full level of EE) in the flour was compared in a final trial (T3). The antioxidants were applied to hake head mince enriched with 4% hake liver (add-in) at a 0.2% add-in level calculated on the total weight of the fish and liver. Although the liver addition was expected to increase the flour lipid content, the objective was to compare the relative efficiency of the treatments, and therefore the antioxidant application level was kept at 0.2% add-in. The sodium ascorbate was dissolved in water in the 2:3 ratio used in Rancimat trials since the 0.5%
add-in water level used in T2 resulted in an unnecessarily large volume of water. Citric acid was omitted due to the minimal synergistic effect thereof with WST observed in the Rancimat.

5.2.1.4 Hake head flour preparation

Flour preparation took place with standardised techniques on the premises of Melnyczuk Research and Development. This production process may be patented in the future, and therefore details thereof remain undisclosed. Only a brief description of the flour production process follows.

The top sections of hake heads, with brains, eyes and top vertebra intact, were minced (13 mm mincing plate), and similar weights of mince were packaged in plastic bags, heat sealed, and frozen and stored at approximately -18°C until the experiment commenced. For T1 and T2, the mince was respectively frozen for ±4 and 2 weeks before production could take place. The freezing time was decreased to 1 day for T3, and the heads used were also briefly submerged in ice water prior to mincing, with both actions undertaken to reduce the starting oxidation level. Within a trial, the control (no antioxidant added) and variation flours were produced on the same day from mince prepared from the same or from thoroughly mixed batches of heads. The mince was randomly divided for the preparation of the control and variation flours, and the weight (kept consistent within a trial) needed for the production of each flour was defrosted at ±2–4°C for approximately the same time before using it for flour preparation.

A preliminary trial on the influence of the point of antioxidant addition on the flour oxidation level showed that dl-α-tocopherol addition after drying gave a higher starting PV than when adding the antioxidant prior to drying. Therefore antioxidants were added to the mince prior to drying. In T1 and T2, antioxidants were mixed into the mince, and this mixture was passed through a micro-cutter to produce a homogenous mince (particles ≤2 mm). The production process used in T1 and T2 was adapted for T3, both to improve the antioxidant distribution and since 4% hake liver was included into the mince. The
liver used in T3 had been reserved, washed and frozen during the preparation of the mince for T3, and was defrosted along with the mince prior to flour production. The mince was first passed through a micro-cutter (yielding particles ≤ than 2 mm). The antioxidants were blended with the liver (Magimix Compact 3), and this blend was then hand-mixed into the mince. This mixture was again passed through the micro-cutter.

Mixing times were kept constant within a trial, and all equipment was cleaned between treatments. In each trial, a control flour (no antioxidants added) was prepared on the same day as the antioxidant treated flour/s, using exactly the same processing steps and mixing times as used when adding antioxidants. Pellets of fish mince were dried with desiccant air according to a standardised procedure in a closed system at 38.5°C (± 2°C), powdered, and kept overnight in sealed plastic bags to allow moisture equalisation before the commencement of storage tests.

5.2.2 Methods

5.2.2.1 Rancimat evaluations of antioxidant efficiencies in cod liver oil

The Metrohm Rancimat instrument (Model 679) was used (temperature: 80°C, airflow: 20 l/h, conductivity range: 100 μS/cm) to determine the induction periods of antioxidant treated (spiked) oils relative to that of a control oil (i.e. CLO with no antioxidants added). Spiked oils were either tested directly after preparation or frozen until analysis as soon as possible after preparation (within 2 days), and the oils were always mixed thoroughly to ensure even distribution of the antioxidants before removing a 2.5 g sample for testing. A glass rod was inserted in each vial to reduce bubbling. Each antioxidant combination investigated was prepared and tested at least twice. The induction periods were used to calculate a protection factor (PF) for each antioxidant combination by dividing the stability of the antioxidant-containing sample with the stability of the control sample (Sonntag, 1979). Averages of the calculated PF’s per antioxidant combination are reported.
5.2.2.2 Accelerated shelf-life storage tests of antioxidant treated flours

Control and antioxidant treated hake head flours were subjected to oven storage (AOCS Cg 5-97, 1997), during which indexes of oxidation, namely peroxide, $A_{232nm}$ and $A_{268nm}$ values (section 5.2.2.3) were measured on lipid extracted from the flours in order to record the oxidation level and the induction curve of each flour. Indexes reported at time 0 were determined before the commencement of flour oven storage (equal to ± 16-24 h after flour production), after which the storage time reflects the hours of oven storage. Measurements during oven storage were made at regular intervals as appeared necessary to record the induction curve of each flour. Gas chromatographic determinations of the relative percentages of fatty acid methyl esters (FAME) were done on lipid extracted from the flour before the commencement of the ASLT and at regular intervals thereafter during the ASLT.

The oven storage test should be conducted at a storage temperature higher than ambient temperatures (± 25°C) in order to accelerate the oxidation reactions, but lower than 80°C in order to ensure that the oxidation mechanisms at ambient temperatures are represented. A temperature of 60°C is recommended for plant oils (AOCS Cg 5-97, 1997). Storage at 45°C was selected for T1 and T2 since the high PUFA content of the fish lipid was expected to oxidize rapidly. The storage temperature was increased to 60°C for T3, which accelerated the reactions. A temperature of 65°C for 144 h has previously been used successfully during accelerated shelf-life evaluation of antioxidant efficiency in marine oils (Wanasundara & Shahidi, 1998). Flour samples of the required size per test (kept constant within a trial) were stored in a random order in similar, wide mouthed, uncovered glass containers in a dark, fan-fitted oven. Samples were removed at regular intervals and tested immediately, or vacuum-sealed, frozen and stored at -18°C, and tested as soon as possible. Similar sized samples per variation were tested on a day to keep the lipid quantity extracted relatively constant and to reduce any influence the test procedures could have on the recorded oxidation status of varying quantities of lipids.
5.2.2.3 Chemical indexes of oxidation determined during flour storage tests

The method of Lee et al. (1996) was adapted as follows to obtain a 0.5 g lipid sample: ± 20 g of flour, 200 ml chloroform:methanol (1:2) and 80 ml sodium chloride (0.5%) were used. Solvent volumes of ± 42 ml were collected in similar containers, flushed with nitrogen gas, and suspended in a water bath (40°C), placed within a fume cupboard, to evaporate. Nitrogen gas was continuously blown onto the solvent during heating as nitrogen flushing protects against heat-induced auto-oxidation through oxygen depletion (Ulberth, 1998). The extracted fat was used for chemical determinations. A similarly adopted extraction method (Lee et al., 1996) was used to obtain lipid containing solvent for FAME determinations, with the following adaptations: a smaller quantity of flour (± 6-10 g) was extracted using 100 ml chloroform-methanol and 40 ml 0.5% sodium chloride. The solvent was decanted into a dark bottle, flushed with nitrogen gas and tested as soon as possible, or frozen and kept at -18°C until tested. Samples were rotor-evaporated (50°C) and used to prepare methyl esters according to the method reported by De Koning et al. (1985), with the following adoptions: reflux times were increased to 5-10 min after each reagent addition, and anhydrous sodium sulfite drying was not used. A Hewlitt Packard HP 6890 Series gas chromatograph, with a capillary column HP5 (cross-linked 5% PH Me Siloxane, 30 m x 0.32 mm x 0.25 μm), with inlet and detector temperatures of respectively 250 and 300°C, was used. The starting column temperature was 150°C, and was increased by 4°C /min to 280°C, and then maintained for 8 min. Nitrogen gas (flow rate: 40 ml/min) was used as carrier for the volatilised molecules. Detection was by flame ionisation and the relative percentage of each FA ester peak was quantified using a Spectra Physics recorder. Peaks were identified by comparison with the retention times of a fish oil control (CSIR, Lower Hope Road, Rosebank, South Africa). Peroxide values (PV’s) were determined on the lipid extracted from the flour with the acetic acid-chloroform AOCS method (AOCS Cd 8-53, 1997), using 0.01 N sodium thiosulphate. Solvent volumes were adapted for the 0.5 g lipid sample to 10 ml acetic acid-chloroform and distilled water each. Ultraviolet (UV) absorbance of the extracted lipid samples, using iso-octane as a solvent, was determined with the ISO/DIS 3656 method (The Draft International Standard, 1989). Absorbance was, however, only
read at 232 nm (A_{232nm}) and 268 nm (A_{268nm}). Absorbance and peroxide values were determined on at least duplicate samples for each variation and averaged.

5.2.2.4 Data analysis

Rancimat data were compared through the PF’s calculated for the treatments (Sonntag, 1979). The peroxide, A_{232nm} and A_{268nm} values were examined relative to each flour’s storage time to determine induction periods. The efficiency of the antioxidant treatments was then evaluated by comparing the induction periods of the antioxidant treated flours with that of the control flours. The relative percentages of DHA and EPA are reported as done in previous oxidation studies, i.e. as a ratio to C_{16:0} (Duve & White, 1991; Noor & Augustin, 1984; Augustin et al., 1987). The percentage decrease in this ratio relative to the initial value was compared for the different flours.

5.3 RESULTS AND DISCUSSION

5.3.1 Preliminary antioxidant efficiency evaluations in the Rancimat

Du Plessis and Sudworth (2001) report that mono- and polyunsaturated oils had different PF’s when treated with the same antioxidants. Hake head oil would have given the exact profile of FA’s present in the flour that required protection, but extracting sufficient quantities for the screening of the antioxidants was not viable. Hake flesh oil (chloroform-methanol extraction) was an alternative, but this oil did not yield clearly interpretable results on the Rancimat during preliminary trials. Hake liver oil (HLO), which was cold hexane extracted by first drying with anhydrous sodium sulphate, also did not yield interpretable results, which was unexpected since Méndez et al. (1997) report that partially refined HLO was used to determine Rancimat induction times at a conductivity of 250 μS.cm^{-1} and a range of temperatures (55-90°C). When varying the temperature and conductivity range for the HLO in the present study did not improve the Rancimat results, it was postulated that the HLO used successfully by Mendez et al. (1997) may have been cold-pressed, while the HLO extraction procedure used in this
study could have contributed volatiles, which escaped gradually, causing a slow gradient and no clear end to the induction period. The HLO was therefore kept under vacuum in a desiccator to reduce possible traces of extraction chemicals. This resulted in foaming and weight loss, but the HLO still did not yield a clear induction period. Since fish oils are generally highly unsaturated, alternative fish oils for use in the screening procedure were investigated. Cod liver oil (CLO) was found to yield interpretable results in the Rancimat. Since the principal reason for antioxidant use in this study was to preserve the n-3 PUFA’s, DHA and EPA should be present in comparable quantities in the oil used for the antioxidant screening than in the hake head flour to which the antioxidants will be applied. A GC analysis was used to compare the FA content of CLO and HLO, with the latter used instead of hake head oil because of the ease of extraction of sufficient quantities thereof for the Rancimat screenings. The CLO had a higher content of EPA than HLO (14.35 vs. 5.92%), and a lower DHA content (9.04 vs. 13.60). However, since CLO had the highest total amount of the n-3 FA’s, and of the oxidation susceptible PUFA’s (33.39% vs 24.63%), it was postulated that CLO was particularly well suited to evaluate antioxidant efficiency.

The average PF’s obtained for CLO spiked with the antioxidant treatments investigated are presented in Figure 5.1. The synthetic antioxidants were the most effective (i.e. had the highest PF’s). The TBHQ and CA combination gave the best PF (5.23) of the antioxidants screened, confirming the reported potency of TBHQ relative to other antioxidants in the protection of highly unsaturated (i.e. fish) oils (Giese, 1996; Love, 1992). These results are similar to that obtained by Kaitaranta (1992), who reported that TBHQ (0.01%) offered the best protection against oxidation in a fish oil when compared to α-tocopherol acetate (0.03%), propyl gallate (0.01%), ascorbyl palmitate, ethoxyquin, BHT, BHA (all at 0.02%) and Anoxomer (a synthetic polymer with phenolic nature, included at 0.02-0.2% levels). The lower PF of the combination containing BHA and BHT commonly occurs in Rancimat evaluations of these antioxidants. This phenomenon has been attributed to the volatile nature of BHA and BHT, which allows the antioxidants to be swept out of the oil by the air stream at Rancimat test temperatures of 100°C.
Figure 5.1. The protection factors of selected antioxidants in cod liver oil as calculated from Rancimat induction times

(Rossell, 1994). The trials for the present study were, however, conducted at 80°C, which should eliminate this as a factor decreasing the efficiency of BHA and BHT.

Of the natural antioxidants tested, Biored Pure marginally gave the best PF. Flavonoids have previously been shown to effectively protect fish oil against oxidation (Nieto et al., 1993). However, the extraction of the active component of Biored Pure is tedious, while its’ use would also have been too expensive to justify the relatively small increase in PF obtained relative to the other natural antioxidants.

The tocopherols and ascorbic acid are the most important natural antioxidants used in commercial applications (Schuler, 1990) and their synergistic protective effect has been demonstrated (Mitsumoto et al., 1991). The widely reported synergistic nature of a ternary blend of lecithin, ascorbyl palmitate and tocopherols were confirmed as giving a greater protection against auto-oxidation in fish oil than when these components were used alone or in binary mixtures by Hamilton et al. (1998). In the present trial, RA and
WST, also ternary blends of the above-mentioned components, had a similar PF, which was much lower than that of the TBHQ-CA combination. Neither the addition of CA nor SMBS to RA or WST markedly improved the PF’s obtained.

The second Rancimat trial, conducted with WST\textsubscript{inc} and WST\textsubscript{acetate}, allowed evaluation of the effect of the increased level of the WST on the PF, and comparison of the effectiveness of the dl-\(\alpha\)-tocopherol and dl-\(\alpha\)-tocopherol-acetate respectively used in the two formulations. Tocopherol acetate does not normally function as an antioxidant since the active hydroxyl group is protected. A slow hydrolysis of the acetate form (e.g. in acidic aqueous systems) can, however, release the bound tocopherol to act as an antioxidant (Schuler, 1990). The acetate form has been used successfully in an antioxidant treatment for meat, with the treatment resulting in a significantly lower free FA content than in the untreated control (Sahoo & Anjaneyulu, 1997). Although less effective than the synthetic antioxidants tested, \(\alpha\)-tocopherol-acetate (0.03%) also, to some extent, slowed down oxidation in fish oil relative to an untreated control oil (Kaitaranta, 1992).

The increased concentration of the WST (WST\textsubscript{inc}) improved the PF to 9.32, a higher PF than what was recorded for the TBHQ-CA combination. This result, obtained with \(\alpha\)-tocopherol at a 1.8% level in the oil, is in contrast with other reports: Gunstone (1996) state that \(\alpha\)-tocopherol at levels higher than 0.1% has a pro-oxidant effect, while Hamilton \textit{et al.} (1998) report that, while 0.5% of \(\alpha\)-tocopherol successfully reduced fish oil oxidation, a 2% level had a pro-oxidant effect. The different result obtained in this study confirmed the need to evaluate the effectiveness of the increased level of WST in the flour.

The WST\textsubscript{acetate} gave an induction time greater than 48 h, the maximum measurable by the Rancimat. This finding was unexpected since the acetate form is very stable, and is therefore not the tocopherol form favoured for use as an antioxidant (Schuler, 1990). Another independent Rancimat run, however, confirmed the very effective use of
WST\textsubscript{acetate} at these levels as an antioxidant in CLO. Again this unexpected result had to be confirmed in the hake head flour.

Yi \textit{et al.} (1991) report that the synergistic effect between a fixed level of ascorbic acid and varying levels of $\delta$-tocopherol was generally 100\% or more. Therefore, in order to test the strength of synergism as a possible explanation for the effective antioxidant use of the dl-\textalpha-tocopherol acetate, CLO was spiked with WST\textsubscript{inc} and WST\textsubscript{acetate} with the sodium ascorbate therein omitted. Much lower and similar PF's of respectively 1.07 and 1.13 were recorded. This firstly confirmed that the tocopherol and tocopherol acetate functioned equally well as antioxidants in CLO subjected to an ASLT (the Rancimat), and secondly that a highly synergistic interaction existed between the sodium ascorbate and both of the tocopherol forms. This synergistic action is attributed to the regeneration of the phenolic tocopherols by sodium ascorbate, which donates hydrogen atoms to spent antioxidants in a sparing activity (Gunstone, 1996; Love 1992). This is illustrated in a study on the inhibition of methyl linoleate oxidation by vitamins E and C (Niki \textit{et al.}, 1984). Initially, vitamin E remained almost unchanged while vitamin C decreased. Only once the vitamin C was exhausted did the vitamin E start to decrease. It appears that vitamin E scavenges the peroxy radical more effectively than vitamin C, and that vitamin C then regenerates the resulting vitamin E radical.

### 5.3.2 Results of antioxidant efficiency evaluations in hake head flour

All flours were tested the day after production (time 0 in terms of ASLT). The flours were then subjected to oven storage at $45^\circ C \pm 2^\circ C$ for T1 and T2 and at $60^\circ C \pm 2^\circ C$ for T3, with periodic determinations of peroxide, $A_{232nm}$ and $A_{268nm}$ values.

#### 5.3.2.1 Preliminary antioxidant screenings in hake head flour: Trials 1 and 2

A longer time to reach a specific PV indicates a better antioxidant activity (Shahidi, 1998), while a treatment resulting in consistently lower PV's than other treatments is considered as giving the better protection (Noor & Augustin, 1984). A PV of 5 meq/kg is
the point where obnoxious rancidity flavours start to develop (Stansby, 1982), while a fat is considered rancid at a PV of 10 meq/kg (Gunstone, 1996). Miller (1993) states that “fishy” or “paint” flavours are slight at PV’s below 5 meq/kg, but become pronounced at values of 5-20 meq/kg, and that rancid notes are perceived at PV’s higher than 20 meq/kg. Michelsen et al. (2001) recommend that fresh fish oil should preferably have a PV of 2-3 meq/kg, with a maximum PV of 5 meq/kg, and that while a guideline of a PV < 10 meq/kg may be sufficient for fish oils used as nutritional supplements, this value is too high for fish oils used for the enrichment of foods.

The freshly prepared control and TBHQ-CA treated flours had high starting PV’s of 74.06 and 42.87 meq/kg respectively, which dropped to ± 20 meq/kg after ± 24 h of storage at 45°C (results not shown). These high but decreasing PV’s suggest that oxidation was already past the induction period in both flours, and had reached the stage where the peroxides, which are intermediate oxidation products, were decomposing rapidly (Hamilton et al., 1997; Coppen, 1994). This lack of protection by the TBHQ-CA combination during the drying of the flour can possibly be attributed to the fat soluble nature of TBHQ, which, in spite of the inclusion of an emulsifying agent (EE), probably did not allow sufficient dispersion through the fish mince in order to protect the lipid component effectively.

The PV’s of the WSTacetate and control flours (T2) are illustrated in Figure 5.2. Results after 278 h of storage are not reported as the titration colour changes used in the peroxide test were masked by the darkening colour of the lipid extract. This can possibly be attributed to polymerisation of hydroperoxides, which leads to dark-coloured organic polymers (Khayat & Schwall, 1983).

The PV’s of the control and WSTacetate flours were respectively 33.88 and 0.79 meq/kg at time 0. The high PV in the freshly prepared control flour indicated that this flour suffered rapid oxidation during drying, in contrast to the WSTacetate flour, which had a very low PV at time 0. Since the WSTacetate was added just before drying to fish mince of the same batch as was used to prepare the control flour, the two treatments had a comparable level
of oxidation prior to drying. The protective capabilities of the \( WST_{acetate} \) were therefore already utilised during drying, resulting in a low level of oxidation (PV = 0.79 meq/kg) in the freshly prepared \( WST_{acetate} \) flour.

The low initial PV of the \( WST_{acetate} \) flour started to increase during oven storage, reaching 5 meq/kg after ± 20 h of storage, and 8.18 meq/kg after 38 h of storage. While the antioxidant function of the \( WST_{acetate} \) appeared to be depleted due to this initial rise in PV’s during storage, the PV’s then stabilised at less than 10 meq/kg until ± 140 h of storage, not following the typical peroxide curve of formation and decomposition (Hamilton et al., 1997). This could possibly be ascribed to the stable tocopherol-acetate used, i.e. the tocopherol acetate could still have retained bound active antioxidant. This possibly continued to gradually decompose and supply active antioxidant, which partially suppressed oxidation so that the rate of peroxide formation and decomposition reached a near equilibrium at the still relatively low PV of 10 meq/kg. The low starting PV in the freshly prepared \( WST_{acetate} \) treated flour, the extended storage period thereof before the PV’s increased above 10 meq/kg, and the relatively low PV’s reached during storage relative to that of the control flour all indicated that \( WST_{acetate} \) had effective antioxidant activity.
The $A_{232\text{nm}}$ and $A_{268\text{nm}}$ values recorded indicate the conjugated diene and triene contents (Yoon et al., 1985), which are respectively primary and secondary oxidation products (Noor & Augustin, 1984). The $A_{232\text{nm}}$ values for the WSTacetate treated flour (Figure 5.3) confirmed the oxidation pattern observed with the PV’s, with a higher absorbance for the control than for the WSTacetate treated flour at 0 h, indicating a higher level of oxidation in the control flour.

![Ultraviolet absorbance values (232 nm) of lipid fraction extracted from hake head control and antioxidant treated flours stored at 45°C](image)

**Figure 5.3. Ultraviolet absorbance values (232 nm) of lipid fraction extracted from hake head control and antioxidant treated flours stored at 45°C**

The $A_{232\text{nm}}$ values of the WSTacetate treated flour increased during the first 40 h of storage, then stabilised, and then increased again after ± 140 h of storage. This pattern corresponded with the increase in PV’s at these times. The correlation coefficient between PV’s and $A_{232\text{nm}}$ has indeed been reported as 0.993 (Hahm & Min, 1995). The $A_{268\text{nm}}$ values (not illustrated) confirmed the antioxidative behaviour of the WSTacetate during the drying of the flour observed with the peroxide and $A_{232\text{nm}}$ values since the WSTacetate treated flour had a lower absorbance than the control flour at time 0 (1.34 and 2.67 respectively). The WSTacetate treated flour was stored for ± 38 h at 45°C before equalling the starting $A_{268\text{nm}}$ value of the control flour.

Due to the susceptibility of unsaturated FA’s to oxidation, their rate of disappearance indicates the degree of oxidation (Noor & Augustin, 1984). Changes in the relative area
percentage of FA-ester peaks can therefore be used in antioxidant efficiency evaluation studies in that the higher the relative percentage of unsaturated FA’s that remain after a certain period, the better the antioxidant protected the unsaturated FA’s from thermal and oxidative breakdown (Duve & White, 1991). The change (decrease relative to initial value) in the ratio of the relative percentage of EPA to C16:0 after 374 h of storage (45°C) was 20.5% for the control and 15.3% for the WSTacetate treated flour, which confirmed that WSTacetate slowed down oxidation in hake head flour. This protection against a decrease of the n-3 FA’s is critical in a product intended to be a source thereof.

5.3.2.2 Comparison of the antioxidant efficiency of WSTinc and WSTacetate in hake head flour: Trial 3

In T3, the antioxidant efficiency of WSTinc and WSTacetate was compared in flour prepared from mince enriched with 4% hake liver. The liver was included to increase the flour lipid content, since the livers of lean fish are storage depots for triacylglycerides (The British Nutrition Foundation, 1992). The PV’s recorded during oven storage of these flours at 60°C are illustrated in Figure 5.4.

![Figure 5.4. Peroxide values of lipid fraction extracted from hake head control and antioxidant treated flours stored at 60°C](image-url)
The freshly prepared control flour again had a high starting PV (18 meq/kg), which decreased during storage, indicating, as in T1 and T2, that the control flour was already oxidised during production.

In comparison, the freshly prepared WST\textsubscript{inc} treated flour had a low PV of 3.03 meq/kg, which indicated that the WST\textsubscript{inc} protected the lipid fraction from oxidation during flour production. The PV's of the WST\textsubscript{inc} treated flour, however, increased sharply during the first 24 h of storage, reaching a PV of 10 meq/kg after only $\pm$ 10 h. This rapid increase in the oxidation level indicates that the antioxidant activity of the WST\textsubscript{inc} was quickly depleted during flour storage. Due to this unexpected rapid change, the frequency of testing was increased. A very typical peroxide curve, indicating hydroperoxide formation and decomposition (Hamilton \textit{et al.}, 1997; Coppen, 1994), was then recorded during the remainder of the storage period.

The freshly prepared WST\textsubscript{acetate} treated flour had the lowest starting PV (1.73 meq/kg), again indicating a good protection against oxidation by this treatment during the production of the flour. The PV's only reached 5 meq/kg after 24 h of storage, and only increased to above 10 meq/kg after $\pm$ 35 h. The PV's then, as in T2, remained relatively constant, and lower than the other treatments, during storage. This confirmed the results obtained with the WST\textsubscript{acetate} treated flour at 45°C, except that the increase in PV's above 10 meq/kg was more rapid at the higher temperature storage used in T3.

The $A_{232\text{nm}}$ values are illustrated in Figure 5.5. The $A_{232\text{nm}}$ values at time 0 support the conclusions made from the PV's, since the freshly prepared control flour had a higher value (10.21) than the WST\textsubscript{inc} and WST\textsubscript{acetate} treated flours, which had similar values (6.63 and 6.64 respectively), confirming a higher level of oxidation in the control flour. The values of the WST\textsubscript{inc} treated flour increased rapidly during the first 24 h, confirming the increasing PV's at this time. The WST\textsubscript{acetate} treated flour's values increased notably
Figure 5.5. Ultraviolet absorbance values (232 nm) of lipid fraction extracted from hake head control and antioxidant treated flours stored at 60°C after ± 35 h, which paralleled the increase in PV’s at this time, and remained consistently lower than the A$_{232\text{nm}}$ values of the control throughout storage.

An increase in the A$_{268\text{nm}}$ values during the flour storage can also be observed (Table 5.2).

Table 5.2. Ultraviolet absorbance values at 268 nm of lipid fraction extracted from hake head control and antioxidant treated flours stored at 60°C

<table>
<thead>
<tr>
<th>Storage (hours)</th>
<th>Ultraviolet absorbance at 268 nm</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (no antioxidants)</td>
<td>WST$_{\text{inc}}$</td>
</tr>
<tr>
<td>0</td>
<td>2.721</td>
<td>1.595</td>
</tr>
<tr>
<td>24</td>
<td>3.427</td>
<td>4.528</td>
</tr>
<tr>
<td>35</td>
<td>3.694</td>
<td>4.887</td>
</tr>
<tr>
<td>45</td>
<td>4.711</td>
<td>6.120</td>
</tr>
<tr>
<td>54</td>
<td>4.609</td>
<td>6.270</td>
</tr>
<tr>
<td>67</td>
<td>5.061</td>
<td>7.542</td>
</tr>
<tr>
<td>79</td>
<td>5.201</td>
<td>7.037</td>
</tr>
<tr>
<td>125</td>
<td>7.856</td>
<td>7.049</td>
</tr>
<tr>
<td>149</td>
<td>7.456</td>
<td>7.681</td>
</tr>
</tbody>
</table>
The control flour had a higher value at time 0 than the treated flours, again confirming a more advanced state of oxidation. The antioxidant treated flours had comparable starting values, but the increase in values during storage of the WST\textsubscript{acetate} treated flour was relatively slow compared to the other flours, suggesting a better protective effect. The relatively rapid increase in secondary oxidation products (conjugated trienes) observed in the flours so early during the storage test could possibly be attributed to the nature of polyenoic acids, as they can form a considerable amount of secondary oxidation products during the early stage of oxidation (Cho \textit{et al.}, 1987).

The relative percentage change (decrease) in the n-3 PUFA to C16:0 ratios after 149 hours of storage (60°C) was greatest for the control and least for the WST\textsubscript{acetate} treated flour (Table 5.3), a final affirmation that the WST\textsubscript{inc} and WST\textsubscript{acetate} slowed down oxidation in hake head flour relative to a control, and that WST\textsubscript{acetate} was the most effective formulation of the two.

Table 5.3. Relative percentage change (decrease) in the C20:5 or C22:6/C16:0 ratios of lipid fraction extracted from hake head control and antioxidant treated flours (60°C storage)

<table>
<thead>
<tr>
<th>60°C storage (hours)</th>
<th>Control C20:5/C16:0</th>
<th>Control C22:6/C16:0</th>
<th>WST\textsubscript{inc} C20:5/C16:0</th>
<th>WST\textsubscript{inc} C22:6/C16:0</th>
<th>WST\textsubscript{acetate} C20:5/C16:0</th>
<th>WST\textsubscript{acetate} C22:6/C16:0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.32</td>
<td>1.05</td>
<td>0.31</td>
<td>1.06</td>
<td>0.26</td>
<td>0.96</td>
</tr>
<tr>
<td>45</td>
<td>0.30</td>
<td>1.00</td>
<td>0.26</td>
<td>0.87</td>
<td>0.23</td>
<td>0.84</td>
</tr>
<tr>
<td>97</td>
<td>0.27</td>
<td>0.82</td>
<td>0.25</td>
<td>0.85</td>
<td>0.23</td>
<td>0.87</td>
</tr>
<tr>
<td>149</td>
<td>0.18</td>
<td>0.54</td>
<td>0.23</td>
<td>0.71</td>
<td>0.23</td>
<td>0.77</td>
</tr>
<tr>
<td>% Change*</td>
<td>43.75</td>
<td>48.57</td>
<td>25.81</td>
<td>33.02</td>
<td>11.54</td>
<td>19.79</td>
</tr>
</tbody>
</table>

*Change in ratio relative to initial value

The relatively brief protection against oxidation gained by the addition of antioxidants throughout this study should be considered in the light of the PUFA’s present in the flour, which contain EPA and DHA. Cho \textit{et al.} (1987) compared the extent of oxidation of the methyl esters of EPA, DHA, ethyl linoleate (Lo), and ethyl linolenate (Ln) during storage in the dark at 5°C. The esters of EPA and DHA oxidised rapidly with an induction period of 3-4 days, while the Ln- and Lo-esters had induction periods of 20 and more than 60 days respectively. Therefore the rapid rate of oxidation in the EPA and DHA rich lipid
component of the hake head flour, produced with a process that would accelerate oxidation through heat and air exposure, was to be expected.

5.4 CONCLUSION

Of the antioxidants used to treat hake head mince during the production of hake head flour, $WST_{acetate}$ best protected the flour against oxidation as evaluated by induction times yielded during oven storage at 60°C. This protection, clearly reflected in the comparatively small relative percentage decrease over time in the n-3 PUFA to C16:0 ratios in hake head flour treated with $WST_{acetate}$, is critical in a product intended to enrich a staple food with n-3 FA’s. The effectiveness of the $WST_{acetate}$ was unexpected since it contained dl-$\alpha$-tocopherol acetate, the bound, stable form of tocopherol, which does not usually function as an antioxidant. The ASLT conditions employed in the antioxidant evaluations may, however, have enhanced the effectiveness of the acetate form as the heat employed in the ASLT could have contributed to the release of the bound antioxidant. The results generated with ASLT of hake head flour treated with $WST_{acetate}$ should ideally be confirmed in ambient shelf-life tests of the final product in which the flour is to be included.

5.5 ACKNOWLEDGEMENTS

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CHAPTER 6
Conclusions and recommendations

6.1 PRODUCTION PROCESS

A production process to produce a dehydrated fish flour from hake heads has been developed to the point where the large-scale production of a consistent product is viable.

The hake head flour production process had to be simple and efficient, and had to minimise factors that could promote oxidation of the raw material. Different equipment and processing techniques were evaluated to determine their contribution to obtaining a fish flour production process that met these criteria. As an example, one of the main production challenges was to process the fish heads into a suitable form for quick drying. A micro-cutter, which could reduce the particle size of coarsely ground heads very efficiently, was therefore designed and built. This micro-cutter eliminated the need for the extended step-wise particle reduction mincing steps initially used, all of which were time consuming and increased the handling of the product. The reduced mincing time also minimised heat build-up, and therefore oxidation, in the raw material.

The procedure for preparing the mince for drying was also much improved over time by M. Melnyczuk. The initial strip production procedure was labour intensive, and the strips were thick, requiring an extended drying period, which increased the risk of oxidation. A process that was both easier to perform and which produced a thinner product requiring a shorter drying time had to be developed. The final procedure implemented resulted in thin pellets that dried very quickly and evenly, reducing the risk of oxidation.

During this product development phase, the researcher placed much emphasis on standardisation of the production process in order to ensure that different treatments of the hake head mince could be compared on the basis of the variable introduced. This required developing a process standardised in terms of:
• the raw material used within a trial: i.e. the source, age since harvesting, and size of the heads had to be constant

• the production process: the same equipment had to be used for the same duration within a trial, i.e. variations in mixing times would introduce varying amounts of oxygen and varying temperature increases, both of which would influence oxidation

• temperature control: i.e. within a trial, the same weight of mince was defrosted for a similar time under the same conditions before using the mince for flour preparation

• the drying procedure: in order to increase experimental control, this was changed from an open system, utilising ambient air, to a closed system. This closed system continuously circulated the same air, which was dried over a silica wheel.

• temperature control: control of the air temperature in the drying cabinet was obtained by installing thermostatically controlled heaters.

Due to financial constraints, a controlled temperature factory environment could not be obtained, which is especially critical when working with a high spoilage risk product such as fish.

6.2 HAKE HEAD FLOUR OXIDATION AS A FUNCTION OF THE STORAGE AGE OF BOTH THE HEADS AND THE FLOUR

The oxidation level of the hake head flour produced was found to be influenced by both the experimental storage age of the hake heads used to prepare the flour and the storage age of the flour. These two factors had a significant, interactive influence on the flour oxidation level, and an increase in either led to an increased level of flour oxidation. Consequently, in fish flour oxidation studies, the storage age of the fish (i.e. days after harvesting) used to produce the flour, as well as the storage age of the flour must be considered.
The 2-thiobarbituric acid (TBA) test proved largely ineffective in monitoring oxidation in
the hake head flour since the main oxidation product measured, malonaldehyde (MDA),
appeared to escape from the dehydrated flour, possibly due to being present in a volatile,
chelated form. Further research on the rate of MDA escape, its' relationship with
peroxide formation and decomposition, and with the level of oxidation in the product, as
pertaining to a dehydrated product, is required to determine whether the TBA test can be
used to assess the extent of lipid oxidation in a dehydrated fish flour.

The determination of peroxide values gave a useful comparative picture of the oxidation
levels in different hake head flours. Peroxide values should, however, due to their fast
decomposition rate, which could lead to misinterpretation of the level of oxidation, be
interpreted in combination with another indicator of oxidation (preferably of a secondary
oxidation product).

6.3 PROTECTION OF HAKE HEAD FLOUR AGAINST LIPID OXIDATION

The efficiency of several antioxidants in protecting the lipid fraction of the hake head
flour against oxidation was compared, with particular emphasis on the preservation of the
n-3 polyunsaturated fatty acids (PUFA's), docosahexanoic and eicosapentanoic acid.
Due to practical constraints, only a limited number of antioxidants could be evaluated in
the flour, and therefore the efficiency of commonly used antioxidants was first screened
in fish oil in the Rancimat.

The Rancimat is a commercial instrument used in the oil stability index (OSI) test, which
accelerates oxidation by exposing oils to higher than ambient temperatures and a constant
airflow (§ 2, section 2.5.1.1). The Rancimat records the induction time of each oil tested,
which can be used to calculate the protection factor (PF) of each treatment. The use of
the Rancimat, however, posed several problems. Firstly, the type of oil used as a carrier
for the antioxidants to be evaluated proved critical in obtaining interpretable results. Two
types of chemically extracted fish oils (hake liver and anchovy oil) did not give clear
induction curves, in spite of various adaptations to the Rancimat’s settings, possibly
because of the slow release of volatiles from some remaining traces of the extraction chemicals during the Rancimat test. The cod liver oil (CLO) finally selected as carrier oil was found to give clear induction periods at very specific Rancimat settings. This indicated that the use of each type of oil in the Rancimat first requires determining the exact temperature and conductivity settings that yields clear induction periods for that oil.

During the evaluation of antioxidants in the Rancimat, it was found that greatly increasing the levels of the \( \alpha \)-tocopherol containing, partially water soluble treatment (WST) increased the antioxidant efficiency of this treatment noticeably, in contrast to reports of a pro-oxidant effect by \( \alpha \)-tocopherol at such levels (# 5, section 5.3.1). The WST consisted of 5\% dl-\( \alpha \)-tocopherol, 25\% sodium ascorbate and 70\% Emulfluide E, although the levels of EE was much reduced for the Rancimat trials in order to allow the formation of an emulsion in the oil. This effective increased level of the WST (WST\textsubscript{inc}) was also investigated with the dl-\( \alpha \)-tocopherol replaced with the same quantity of dl-\( \alpha \)-tocopherol acetate (WST\textsubscript{acetate}). The WST\textsubscript{acetate} gave a comparable PF to the WST\textsubscript{inc}, which was unexpected, since \( \alpha \)-tocopherol-acetate does not normally function as an antioxidant.

Selected antioxidants were applied to hake head mince to compare the induction times obtained during accelerated shelf-life tests (ASLT) of flours produced from antioxidant-treated mince with that prepared from untreated mince (i.e. a control flour). The more natural antioxidants were preferred to the synthetics due to the intended use of the final product for children. The WST\textsubscript{inc} and WST\textsubscript{acetate} were selected for further flour trials because of the good protection obtained with these combinations during the Rancimat screenings, their mostly natural compositions, and the presence of both water- and fat-soluble components in them. Because of the latter characteristic, it was postulated that these combinations would distribute better in the water-laden fish mince than Ronoxan A, which is fat-soluble. This theory was supported by the suprising inefficiency observed for the fat-soluble tertiary butyl hydroquinone (TBHQ) during the flour trials, in spite of the extremely good PF obtained with TBHQ during the screenings in the CLO.
Periodic determinations of PV's, ultraviolet (UV) absorbance by the conjugated dienes and trienes and the relative decreases in the ratios of the relative percentages of DHA and EPA to C16:0 yielded sufficient data to clearly interpret the state of oxidation in hake head flour over time. Of the antioxidants tested, the WST\textsubscript{acetate} best protected the flour against oxidation and a resulting decrease in the n-3 PUFA's. This effectiveness of the WST\textsubscript{acetate} in both the CLO and the flour was unexpected since the dl-\(\alpha\)-tocopherol acetate it contained is the bound, stable form of tocopherol, which does not usually function as an antioxidant.

6.4 RECOMMENDATIONS

It is possible that the ASLT conditions employed in the antioxidant evaluations enhanced the effectiveness of the dl-\(\alpha\)-tocopherol acetate form present in the WST\textsubscript{acetate} treatment, as the heat employed in the ASLT could possibly have contributed to the release of the bound antioxidant. This hypothesis should be confirmed with ambient shelf-life tests of the efficiency of the WST\textsubscript{acetate} in hake head flour. If the flour is included in a carrier food that requires heat processing, i.e. in bread, which is baked, this postulated occurrence of a slow release of active antioxidant under heat conditions may be an advantage, since the heat processing could possibly have a similar effect on the antioxidant function of the WST\textsubscript{acetate} as was postulated as occurring during the ASLT.

It is recommended that the effectiveness of the WST\textsubscript{inc} is investigated with the dl-\(\alpha\)-tocopherol therein replaced with \(\delta\)-tocopherol. Various sources state that \(\delta\)-tocopherol is the most effective antioxidant form of the tocopherols (#2, section 2.4.2.2.3.1). However, in the current study, dl-\(\alpha\)-tocopherol was used in the WST\textsubscript{inc} since the commercial formula tested, Ronoxan A (RA), on which the WST was modeled, contained \(\alpha\)-tocopherol. The protection against oxidation achieved in this study with RA, WST\textsubscript{inc} and WST\textsubscript{acetate}, in spite of using the weaker antioxidant form of tocopherol, could possibly be ascribed to the synergistic effect obtained between the ingredients in these formulations. However, it would be of interest to determine the induction time obtained in hake head flour treated with WST\textsubscript{inc} containing \(\delta\)-tocopherol.
The effectiveness of the final antioxidant mix selected should be confirmed in ambient shelf-life tests, preferably including sensory profiling of the final product in which the flour is included.