UNIVERSITY OF NAIROBI

INCREASING THE CONSUMPTION OF ESSENTIAL LONG CHAIN FATTY ACIDS THROUGH FORTIFICATION OF YOGHURT WITH OMEGA-3 PUFA FROM NILE PERCH OIL

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DECLARATION

This thesis is my original work and has not been submitted for a degree award in any other University.

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This work is dedicated to Almighty God, the stronghold of my life in whom I trust and to my loving family for your inspiration and immense support.

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ABBREVIATIONS

ABS	Absorbance
AEAC	Ascorbic acid Equivalent Antioxidant Capacity
AI	Acceptable intake
AMDR	Acceptable macronutrient distribution range
ANOVA	Analysis of variance
AOCS	American Oil Chemists Society Official Methods
ALA	Alpha linoleic acid
AV	Anisidine value
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
TBHQ	Tert-Butylhydroquinone
CHD	Cardiovascular disease
DAGs	Diglycerides
DHA	Docosahexaenoic acid
DPA	Docosapentanoic acid
DPPH	2, 2-diphenyl-1- picrylhdrazyl
EPA	Ecosapentaenoic acid
FAME	Fatty acid methyl esters
FFA	Free fatty acids
GAE	Gallic acid equivalent
HUFAs	Highly unsaturated fatty acids
LA	Linoleic acid
MAGs	Monoacylglycerides

MOH	Ministry of Health
NaCl	Sodium chloride
NaOH	Sodium hydroxide
Na_2S_2O3	Sodium thiosulphate
NCD	Non-communicable diseases
PC	Phenolic content
PV	Peroxide value
PUFA	Poly-unsaturated fatty acids
RSA	Radical scavenging activity
RDA	Recommended dietary allowances
SPSS	Statistical package for social sciences
TAG	Triglycerides
TBA	Thiobarbituric Acid
TLC	Thin layer chromatography
TUK	Technical University of Kenya
UHT	ultra-heat temperature
WHC	Water Holding Capacity
WHO	World health organization

ABSTRACT

Increased incidence of Non communicable diseases (NCD) such as cardiovascular diseases, diabetes and cancer has been associated with decreased consumption of polyunsaturated fatty acids (PUFAs) of the omega-3 series, especially very long chain eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These fatty acids are majorly found in fish and their by-products. Nile perch (*Lates niloticus*) is one of the fish that dominates the commercial fish market in Eastern Africa and its processing results in generation of large amounts of by-products which are of high nutritional content.

This study aimed to develop functional yoghurts; YFH (yoghurt fortified with Omega-3 rich Nile perch fish oil and honey) and YFL (yoghurt fortified with Omega-3 rich Nile perch fish oil and lemon juice) and to evaluate their shelf life properties. Liberation of fish oil from Nile perch fat pads was done using a food grade enzyme Alcalase and in the absence of an exogenous enzyme. Characterization of the fish oil was done by thin layer chromatography and the quality parameters; PV (peroxide value), AV (anisidine value), TOTOX (total oxidation) and FFA (free fatty acids content) determined. Three honey samples and three citrus fruits were analyzed for their antioxidant activity and in both cases; the honey and citrus fruit with the highest antioxidant activity were used in development of the functional yoghurt. Four batches of yoghurt were produced, YFH, YFL, PY (plain yoghurt) and YF (yoghurt fortified with Omega-3 rich Nile perch fish oil only) where the latter two (PY and YF) were used as controls. Yoghurt samples were evaluated for sensory characteristics by a random panel of students. Quality parameters, pH, total acidity and ascorbic acid content were analyzed over one month storage period at one week's interval. Triplicate experiments were conducted and data analyzed using SPSS software.

Hydrolysis of the fish fat pads in the absence of an exogenous enzyme gave a better yield (60.67% wet weight) compared to Alcalase enzyme hydrolysis (48.33% wet weight). The quality parameters of the omega-3 rich fish oil were; Pv (meq/kg) = 4.83 ± 1.75, Av =16.50 ± 2.14, Totox= 26.16 ± 1.09 and FFA (%)

=2.16 ± 0.22. A commercial honey sample (Green forest) and lemon juice which had the highest Total Antioxidant content (AEAC) of 312± 2.34 and 197± 3.65 mg/L respectively were used for fortification. The sensory evaluation tests showed that the fortified yoghurt was acceptable to the tasting panel with the best two most preferred flavors being the strawberry plus honey and strawberry plus lemon juice samples with ranks of 4.80 and 4.825 respectively in a five point hedonic scale. During the last week of the one-month storage, the yoghurt honey sample gave the most reduced peroxide values (9.78± 2.65) followed by lemon juice (11.54± 2.59) and the sample without antioxidant giving the highest Pv values (15.54± 0.56). The Av values showed a similar trend. The pH decreased slightly over time in all the samples. The lemon juice fortified sample had the highest ascorbic acid content of 28 mg/100g, all the other samples had much reduced ascorbic acid values below 1 mg/100g.

The study indicates that enough fish oil can be obtained from Nile perch fish pads by hydrolysis without the need of using an enzyme since enzymatic hydrolysis decreases oil yield due to the formation of an emulsion. All the quality parameters of the omega-3 rich fish oil were in the acceptable range of 3-20 meq/kg for Pv and 4-60 for Av over the one month storage period. In addition, omega-3 rich fish oil–enriched yoghurt was highly stable to oxidation, with the addition of natural antioxidants such as honey and vitamin C evident by the low Pv and Av values in these samples over the one-month storage period.

In conclusion, fortification of yoghurt with the omega-3 rich Nile perch fish oils is therefore a practical approach towards ensuring a constant intake of EPA and DHA which is limited due to the low consumption of fish among many communities in Kenya as well as the high cost of ω-3 supplements present in the market.

CHAPTER 1

1.0 INTRODUCTION

Diet has a primary role of providing the required nutrients to meet the necessary metabolic requirements of the body. However, some foods have bioactive substances such as antioxidants, omega-3 fatty acids, minerals, vitamins etc. incorporated to modulate specific target functions in the body (Doyon & Labrecque, 2008). These types of foods are not only necessary for living but also contribute to the prevention and reduction of risk factors for several diseases or enhance certain physiological functions (Europe *et al*., 1999) and are referred to as functional foods. Increased market for functional foods has resulted from the progression of Nutritional science from the concept of 'adequate nutrition' to one of 'optimal nutrition' based on the potential of these foods in promoting health (Doyon & Labrecque, 2008).

Omega-3 fatty acids are examples of bioactive substances whose interest has increased over the years in scientific research due to their proven health benefits. They are essential fatty acids that the human body is not able to synthesize and hence have to be provided through the diet (Amegovu *et al*., 2014) or supplementation. In Kenya, Non Communicable diseases (NCD) such as Cardiovascular disease (CVD) have been shown to be on the rise (MOH, 2007). This has been associated with decreased consumption of polyunsaturated fatty acids of the omega-3 series, especially very long chain EPA and DHA (Vrablík *et*

al., 2009; Ruxton, 2009; Connor, 2000). Several prospective studies and clinical trials have shown that omega-3 fatty acids can reduce the risk of developing heart disease (Erkkilä *et al*., 2006; Tavazzi *et al.*, 2008). Various other studies have shown that consumption of omega-3 fatty acids is beneficial in treating disorders such as Alzheimer's disease (Morris *et al*., 2003), in reducing bone loss (Weiss *et al.*, 2005) in lowering incidence of depression and other related conditions (Lin, 2007; Griel *et al*., 2007). These and many other studies have proven the importance of the Omega-3 fatty acids in human health and hence the need to make them available in human diet.

Omega-3 fatty acids are majorly found in fatty fish, microalgae, krill and nut oils. There is reduced intake of long chain omega-3 PUFA in both developing and developed countries, which is far much below the recommended levels of 0.15g due to low fish consumption (Kris-Etherton *et al*., 2000). This therefore calls for suggestion of alternative ways to ensure increased consumption of the omega-3 PUFA. Fortification of foods with fish oils omega-3 PUFA has therefore been proposed as a practical approach towards increasing the consumption of these fatty acids (Metcalf *et al*., 2003). Kenya is one of the developing countries where low consumption of fish is evident. This has resulted from the high cost of fish and omega-3 supplements present in the market, and therefore it could be of great value to enrich regularly consumed foods with fish oil that are known to be a good source of essential ω-3 fatty acids.

Nile perch (*Lates niloticus*), is a warm water fatty fish found in East African Lakes and rivers and has been shown to contain considerable amounts of omega-3 PUFAs in the head and viscera which are by-products of Nile perch processing (Mbatia., 2011; Gumisiriza *et al*., 2009). A report by Brian Marshall (2008) from Lake Victoria Fisheries Organization shows that in Kenya, Nile Perch fishing is the second most sourced fish in the lake after Dagaa. In addition, Kenya borders the smallest part of the Lake but in contrast, most of the fish processing and exporting activities take place here. This therefore, generates large amounts of waste or residues of high nutritional content which, if not properly utilized or treated, is likely to be deposited in the environment creating pollution and health problems (Kotzamanis *et al.*, 2001; Hwang and Hansen, 1998; Gumisiriza *et al*., 2009).

Consumer interest in supplementation with healthy omega-3 fatty acids has led to increased research in fortification of popular foods with these healthy fats (Metcalf *et al*., 2003). Yoghurt is a good vehicle for incorporation of fish oil due to its healthy image and wide consumption. The incorporation of the fish oils, the processing and handling is associated with nutritional challenges for their healthy delivery (Huber *et al*., 2009). The extreme sensitivity of fish oils to oxidation by many factors such as heat, light and atmospheric oxygen can easily lead to the development of off-flavors and cause significant loss of product quality, stability, nutritional value, bio-availability and the overall acceptability of the food product (Jellinek, 1971) The high rate of oxidation of fish oils can be reduced by incorporation of synthetic or natural anti-oxidants (Huber *et al*., 2009). The

demand for natural antioxidants and their potential in increasing the lifetime (shelf life) of a product has increased due to health risks posed by use of synthetic antioxidants.

In the present study, knowledge gained on food fortification was utilised to fortify yoghurt with fish oils from Nile perch by-products and sensory evaluation conducted to evaluate the acceptability of the product. Honey and vitamin C obtained from citrus fruits were evaluated for their anti-oxidant properties and in prevention of fish oil oxidation. The effectiveness of the antioxidants in the storage capacity and preservation of the fortified yoghurt product was evaluated for a period of one month.

1.2 Problem statement and justification

In most developing countries, non-communicable disease (NCD) risk factor levels have increased during the past decade, portending an increase in the rate of such diseases in the next two decades (Beaglehole & Yach, 2003). Most of these countries have directed their attention towards control of infectious diseases and the growing burden of NCDs such as heart disease, stroke, cancer, diabetes and obesity have been neglected.

In Kenya for example, from 2005 - 2007, NCD contributed over half of the top twenty causes of morbidity and mortality and it is envisaged that by 2020, NCD will be the leading cause of death (MOH, 2007). Cardiovascular disease (CVD) is a non-communicable disease which has been associated with decreased consumption of polyunsaturated fatty acids of the omega-3 series, especially very long chain EPA and DHA (Vrablík *et al*., 2009).Several prospective studies and clinical trials have shown that omega-3 PUFA can reduce the risk of developing heart disease (Erkkilä *et al*., 2006; Tavazzi *et al*., 2008).

Nile perch is a warm water oily fish found in East African lakes and rivers (Gumisiriza, *et al*., 2009). Processing of fish fillets generates large amounts of waste or residues which are rich in Omega-3 fatty acids (Kotzamanis *et al.*, 2001; Hwang and Hansen, 1998; Gumisiriza *et al*., 2009). The oil is mainly present in the heads and viscera which are by-products of Nile perch processing (Gumisiriza *et al*., 2009).These oils have been shown to contain considerable amounts of omega-3 PUFAs (Mbatia, 2011). Utilization of Nile perch oil in fortification of

food products has not been reported in the world. Utilizing such oil by incorporating in commonly consumed foods such as yoghurt would result in increased consumption of omega-3 PUFA. This could therefore, be a long-term measure in reducing the increased incidence of CVD (Simopoulos, 1991) in Kenya. Value addition to the Nile perch by-products which have a low market value would result in generation of additional revenue, reduced disposal costs of these by-products and hence reduction of the environmental and health impacts of waste disposal (Arvanitoyannis & Kassaveti, 2008).

Fish oils are highly sensitive to oxidation. Prevention of oxidation has proven to be a great challenge in the food industry. Therefore, antioxidants are required to delay or inhibit the oxidation of the fish oil. The use of natural antioxidants compared to synthetic antioxidants is more preferred due to their implication in many health risks such as cancer (Stip & Belš, 2009). Honey and Vitamin C derived from citrus fruits are natural antioxidants that could be used to delay or inhibit oxidation of fish oil incorporated into yoghurt.

The use of honey as an antioxidant comes with added advantages such as antimicrobial properties (Postmes, 2010) which discourage the growth or persistence of many microorganisms and also offers anti-inflammatory effects in humans (Al-Waili, 2003) amongst many other advantages.

1.3 Objectives

1.3.1 Overall objective

To increase the consumption of essential long chain fatty acids through fortification of yoghurt with omega-3 PUFA from Nile perch oil.

1.3.2 Specific objectives

1. To compare the extraction of Nile perch oil using an enzyme and in the absence of an enzyme
2. To characterize extracted Nile Perch fish oil and determine its quality parameters
3. To evaluate antioxidative potential of natural honey visa viz Vitamin C derived from citrus fruits in the fortified yoghurt
4. To produce a fortified yoghurt flavored with a more readily accepted flavor and evaluate the storage capacity of the fortified yoghurt

1.7 Research Hypothesis

i. Nile perch processing by-products contains considerable amounts of fish oil that can be used in food fortification.

ii. Honey has antioxidant properties which prevents oxidation of fish oils in the fortified yoghurt.

iii. Citrus fruits are rich in Vitamin C, an anti-oxidant which prevents fish oil oxidation.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1General information on food fortification

The term 'functional food' was first coined in Japan. The Japanese observed that food could have a role beyond gastronomic pleasure, energy and nutrient supply to the human (Rekha *et al*., 2012). Functional foods are therefore used to enhance certain physiological functions, in order to prevent or even to cure diseases (Roberfroid, 1999). Therefore, the ultimate goal of the Scientific community and food industry should be to develop functional foods for improving quality of life (Doyon & Labrecque, 2008). A food is made functional by introducing a technological or biotechnological means to add, remove or modify a particular component in the food. The specific component must be proven to have a functional effect (Roberfroid, 1999). A number of health-related effects has been documented for functional foods and regular consumption of functional foods reduces the risk for several chronic diseases, such as cardio vascular disease, cancer, diabetes, hypertension and osteoporosis (Hornstra *et al*., 1998; Gogus& Smith, 2010). Thus, populations consuming a large proportion of plant-based foods, including fruits and vegetables, or those with high intake of seafood are known to have a lower incidence of cardiovascular diseases and certain types of cancer (Connor, 2000).

Omega-3 fatty acids have been proven to help control hypertension and lipid metabolism (Simopoulos, 1991). In the case of marine oils, their omega-3 fatty

acid constituents are known to influence health through their effect on eicosanoid metabolism as well as through other mechanisms. Although these fatty acids are naturally found in milk(Doyon & Labrecque, 2008), they are not in sufficient concentration to deliver the necessary health benefits that can help reduce the risk of associated diseases. Omega-3 fatty acids can therefore be enriched in various food products and this will ensure a constant intake of these fats hence delivering the required health benefits in the body(Metcalf *et al*., 2003).

Metcalf *et al*., 2003 evaluated the effects on the intake and on the blood and tissue proportions of omega-3 PUFA's either present naturally in foods or from foods fortified with omega-3 PUFA's. In the study, subjects were given a range of foods containing natural omega-3 PUFA (fresh fish, canned fish, flaxseed meal, rape-seed oil) and foods fortified with fish oil (spreadable fat, milk, sausages, luncheon meat, onion dip). The subjects were left to choose the food that they desired and their choices recorded in a diet diary. Results from the study showed that the consumption of omega-3 PUFA increased significantly: Alpha-linolenic acid (ALA) from 1.4 to 4.1 g per day, EPA from 0.03 to 0.51 g per day, and DHA from 0.09 to 1.01 g per day. The authors concluded that incorporation of fish oil into a range of commercial foods provided the opportunity for wider public consumption of omega-3 PUFA with its associated health benefits(Metcalf *et al*., 2003)

2.2 Nile perch

Nile perch (*Lates niloticus*) is a species of fresh water fish in family Latidae of order Perciformes ("perch-like" fishes). It is also one of the largest fresh water fish reaching a maximum length of two metres and can weigh up to 200 kg.

Figure1: Nile perch fish

(Adapted from http://www.fieldandstream.com/photos)

The fish is commonly found in River Nile and in the lakes and some rivers in Eastern, Central and Western Africa (Namulawa *et al*., 2011), occurs naturally in Lake Albert and the White Nile River in Uganda, and in Lake Turkana in Kenya. It was introduced into lakes Kyoga and Nabugabo from Lake Albert and into Lake Victoria from lakes Albert and Turkana (Mwanja, *et al*., 2012). It is a warm water fish that has been shown to contain considerable amounts of omega-3 PUFAs (Mbatia *et al.*, 2011).

2.2.1 Nile Perch production and processing in Kenya

Since the introduction of Nile Perch into lake Victoria, it has become a source of food for the local people, an important export species and hence a source of income for the fishers, processors and exporters of the three countries bordering

Lake Victoria (Matsuishi., 2006). Kenya borders the smallest part of the Lake but in contrast, most of the fish processing and exporting activities take place here. Some of the Nile Perch fish processing companies from both Uganda and Tanzania land their fish catches in Kenya for processing and export. Nile perch is one of the most valuable species in Lake Victoria and is therefore, subjected to much heavier fishing pressure. A report by Brian Marshall from Lake Victoria Fisheries Organization, 2008, shows that Nile Perch is the second most sourced fish in the lake after Dagaa (Marshall, 1992).

Table 1: Mean annual catch (tonnes) of fish from Lake Victoria, 2005-08 (Marshall, 2008)

	Nile Perch	**Dagaa**	**Tilapia**	**Haplo-chromines**	**Others**	**Total**
Kenya	40.1	73.0	12.7	3.3	4.1	133.3
Tanzania	133.1	342.3	23.6	98.3	3.0	600.4
Uganda	91.9	98.5	26.4	2.3	2.1	221.3
Total	265.1	513.8	62.7	103.9	9.2	955.0

The high Nile Perch Industrial fish processing therefore generates large amounts of waste or residues of high nutrient content which, if not properly utilized or treated, is likely to be deposited in the environment creating pollution and health problems (Kotzamanis *et al*., 2001).

2.2.2 Nile perch Fish by-products

Nile perch accounts for 60% of the total commercial fish catch in East Africa and it is mainly processed into chilled fish fillet for export (Matsuishi., 2006).The processing leads to high volume of by-products, with an annual solid waste of 36,000 tones being generated by fish processing industries along Lake Victoria (Gumisiriza *et al*., 2009).

The amount of by-products from fish normally varies with fish species, size, season and fishing ground (Falch *et al*., 2006). These by-products are generated when the fish is gutted, headed and further processed into fillets. Depending on the efficiency of the production process, only 50-60% of total fish catch goes for human consumption (Falch *et al*., 2006). These by-products include heads, viscera, skin, scales and fats (Mbatia, 2011).

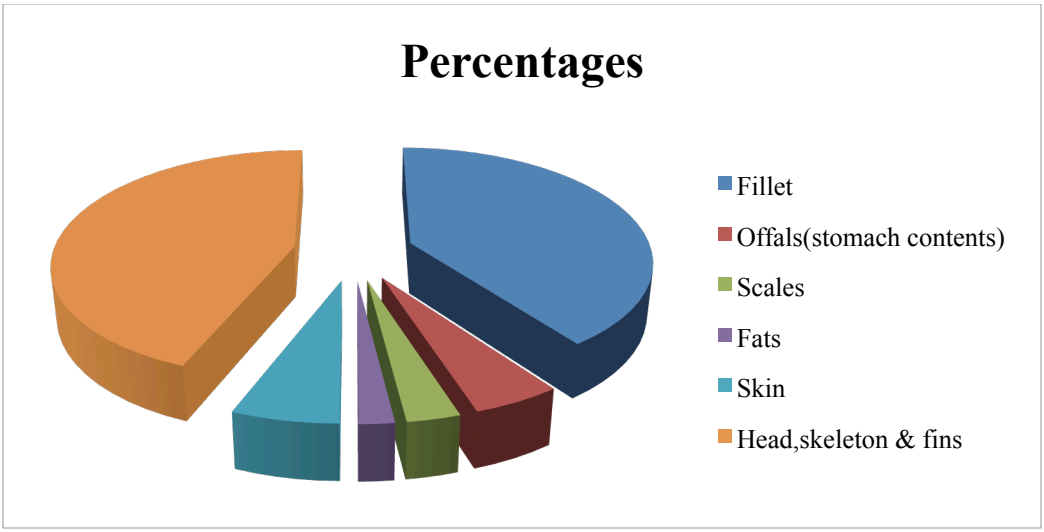


Figure 2: Products and by-products from Nile Perch processing. Head, skeleton and fins account for (44%) while Fillet (40%), skin (6%), Offals (5%), scales (3%) and Fats (2%) (Mbatia, 2011).

The utilization of fish by-products is considered an important clean production opportunity for the fish processing industry as it potentially generates additional revenue, reduces disposal costs of these materials and eliminates or reduces the environmental and health impacts of waste disposal (Arvanitoyannis & Kassaveti, 2008).

By-products from warm water fatty fish such as Nile perch, present in East African Lakes and rivers could also be used as a source of raw material for marine oils, enzymes, proteins and other valuable products (Arvanitoyannis & Kassaveti, 2008). The oil is mainly present in the heads and viscera which are by-products of Nile perch processing (Gumisiriza *et al*., 2009). Utilization of Nile perch oil in fortification of food products has not been reported. By utilizing such oil, it will add value to the by-products while increasing the consumption of omega-3 PUFA

among a larger Kenyan population. This could be a long-term measure in reducing the increased incidence of NCD among Kenyan population.

Table 2 Fatty acid composition of oil from Nile perch viscera oil (Adapted from Mbatia *et al* ., 2010).

Fatty acid	Fatty acid profile (%)
C14:0	3.4 ± 0.3
C15:0	0.5 ±0.01
C16:0	23.4 ± 0.1
C16:1	12.7 ± 0.1
C18:0	8.9 ± 0.01
C18:1	23.7 ±0.4
C18:2	2.2 ± 0.1
C18:3	2.3 ±0.1
C18:4	0.4 ± 0.03
C20:1	0.6 ± 0.1
C20:4	2.4 ±0.1
C20:5	3.0 ± 0.7
C22:4	1.1 ± 0.4
C22:5	6.2 ± 0.4
C22:6	9.0 ± 0.5

2.3 Omega-3 Poly Unsaturated Fatty Acids (PUFA's)

2.3.1 Synthesis of Omega-3 PUFA's in Human

Humans are able to synthesize most of the fatty acids in their bodies, but they are not able to synthesize these essential fatty acids (EFAs) of the omega-3 and omega-6 series. Consequently, these fatty acids must be made available in the diet or through supplementation. Alpha-linolenic acid (ALA), an omega-3 fatty acid, and linoleic acid (LA), an omega-6 fatty acid are the two essential fatty acids important in human nutrition. They are pre-cursors of the long chain fatty acids.

Table 3: Metabolic conversion of EPA to its active metabolites (omega-3 PUFA) in human (Russo, 2009)

The omega-3 PUFA's Synthesis
Alpha-linolenic acid (C18:3, n-3)
↓ Δ^6desaturase
Eicosapentaenoic acid (EPA) (C20:5, n-3)
↓ Δ^5desaturase
Decosapentaenoic acid (DPA) (C22:5, n-3)
↓ Δ^4desaturase
Decosahexaenoic acid (DHA) (C22:6, n-3)

Omega-3 and omega-6 fatty acids utilize the same enzymes for their metabolism. There is increased consumption of Omega-6 fatty acids due to their greater

availability and low cost in developing countries. These are present in cereals, legumes, vegetable oils, green leafy vegetables etc. This is unlike the omega-3 PUFA, which are majorly present in marine foods. This therefore results to an increased competition for the enzymes required for omega-3 PUFA's metabolism thus affecting the ideal ratio of 5-10:1 for optimal health benefits (Meharban Singh., 2005).

However, the conversion of ALA to EPA and DHA occurs only at a very low rate (Weiss *et al*., 2005). Such conversion is not sufficient to produce the required levels of EPA and DHA that are believed to have heart health benefits (Gogus & Smith, 2010). Therefore, the best approach to ensuring optimum intake of omega-3 fatty acids for health is to directly increase consumption of EPA and DHA via frequent fish consumption or supplementation (Metcalf *et al*., 2003).

2.3.2 Chemical Structure of Omega 3 fatty acids

Fatty acids are structured with a carboxylic acid group on one end, and a methyl group on the other end(Collins, 2010). Omega-3 fatty acids are polyunsaturated fats, meaning that they have less hydrogen molecules than saturated fats. In addition, they have their final double bond on the third carbon from the terminal (n) carbon bond. Therefore the omega-3 family of fatty acids has the first unsaturated bond, corresponding to a double chemical bond, always situated on the 3rd carbon from the metabolically non-reactive methyl end of the chain (Kapoor and Patil, 2011). EPA contains 5 double bonds in its carbon chain (Fig. 3) while DHA contains 6 double bonds (Fig. 4)**.**

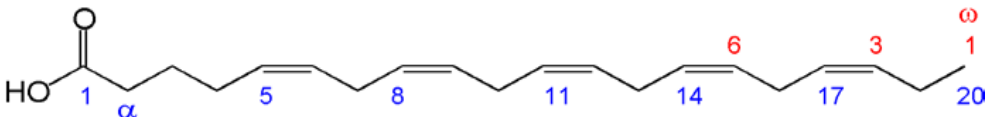


Figure 3: Chemical structure of Eicosapentanoic acid (*en.wikipedia.org/wiki/*)

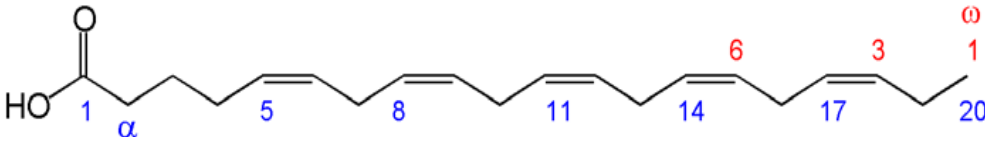


Figure 4: Chemical structure of Docosahexanoic acid (*en.wikipedia.org/wiki/*)

2.3.3 Dietary sources of Omega-3 fatty acids

Over the years people are being encouraged to reduce the consumption of saturated fats which are solid fats that are majorly found in junk foods and meat products such as beef, pork, poultry (Meharban, 2005). Increased intake of unsaturated fatty acids such as the omega-3 fatty acids is being encouraged due to their known health benefits. Alpha-linolenic acid (ALA) is present in various food sources, while the major sources of EPA and DHA is through dietary intake of fish and through supplementation.

Table 4: Dietary sources of omega-3 fatty acids (Collins, 2010).

Omega-3 fatty acids	Food source
ALA	Dark green leafy vegetables, certain nuts such as flax seed, walnut oil etc
EPA	Oily fish, fish supplements, food enriched with fish oil.
DHA	Oily fish, fish supplements, food enriched with fish oil.

2.3.4 Requirements of Omega-3 fatty acids

Due to the high intake of vegetable oils and processed foods the recommended omega-6: omega-3 ratio has been altered. Therefore people should be concerned on lowering the omega-6 intake and increasing the omega-3 intake by including oily fish in their diets and also by replacing commonly used vegetable oils with oils rich in omega-3 fatty acids (Mirajkar *et al*., 2011). Macronutrients have Acceptable Intake (AI) and Acceptable Macronutrient Distribution Range (AMDR) instead of Recommended Dietary allowances (RDA), (Mirajkar *et al*., 2011). Many studies have demonstrated the health benefits of omega-3 fatty acids but there is no consensus on the AI. Some of the recommended AIs are shown below:

Table 5: Recommended Acceptable intake of omega-3 PUFA (g/day) (European Food Safety Authority (EFSA) Panel on Dietetic Products, Nutrition and Allergies., 2012)

ORGANISATION	ALA	EPA+DHA
WHO	0.8-1.1	0.3-0.5
Group of experts	2.2	0.65
FDA (USA)	1.1	0.5
AFSSA (France)	2	0.12

2.3.5 Bioavailability of Omega-3 fatty acids

Bioavailability refers to the ability of the orally ingested omega-3 fatty acids to be digested in the gastrointestinal track (GIT) and the ability to cross the intestinal wall, thereby entering the blood stream and hence the other body tissues and organs (Holub *et al*, 2011).Different studies have been done to measure the bioavailability of both EPA and DHA in humans.

Higgins *et al*. (1999) evaluated the bioavailability of omega-3 PUFA from the intake of fish oil-enriched food compared with a fish oil capsules supplement. Twenty-eight healthy subjects took part in the randomized, controlled trial and were supplemented with 0.9 g of omega-3 PUFA per day for 4 weeks, which were delivered either as fish oil in a milkshake or as a fish oil capsules. Plasma fatty acids composition and plasma total cholesterol levels were measured at baseline

and after supplementation. Results obtained showed that Plasma omega-3 PUFA concentrations were raised significantly at a similar level by both fish oil supplements. Plasma total cholesterol levels were not altered by supplementation in either group. The results of this study therefore indicated that there was no significant difference in the bioavailability and health effect of omega-3 PUFA given as enriched milkshakes in comparison to omega-3 PUFA delivered as capsule supplements (Higgins *et al*., 1999). In the currently available published studies, the bio- availability of omega-3 PUFA from enriched food intake is demonstrated to be comparable with the bioavailability of the fatty acids from capsule supplements (Kolanowski *et al*., 2004).

Several factors influence the bioavailability of omega-3's in the body. These factors include the form in which the fatty acids are in; Triglyceride versus Ethyl ester form (Holub *et al*., 2011). Studies have shown that the Triglyceride form has a better human absorption compared to the Ethyl ester form (DHA, 2010). The omega-6/omega-3 ratio in each person's diet is also an important factor, whereby an excess of Omega-6 prevents a person's optimal use of the omega-3 through enzymatic competition. The metabolism of both omega-3's and omega-6 requires the same enzymes and also several common co-factors such as vitamin B, C, E, zinc and magnesium (Dybkowska *et al*., 2004).Therefore, the more omega-6 in one's diet, the more the person's use of omega-3 is inhibited. This imbalance therefore predisposes one to cardiovascular disease and inflammatory processes (Wijendran & Hayes, 2004).

Other minor factors that influence the bioavailability of omega-3 include; diseases such as diabetes as well as excess alcohol, tobacco or stress and these can influence the body's ability to transform ALA into EPA; and EPA into DHA (Kolanowski, 2004). Studies on Western dietary habits show that the current omega-6/omega-3 ratio is about 10-15:1 while it should ideally be a 4:1 ratio in favor of omega-6 (Meharban, 2005). Thus omega-6 intake should be limited and omega-3 intake increased without however, greatly increasing the total quantity of lipids absorbed daily (Hammock, 2011).

2.3.6 Health benefits of omega-3 fatty acids

Many clinical studies have been done to show the importance of omega-3 fatty acids in the treatment of a variety of health conditions (Mirajkar *et al*., 2011). High intake of EPA and DHA has been associated with the reduction of CVD risk (Kris-etherton *et al*., 2003; Wijendran & Hayes, 2004) where, they help to reduce the risk factors for heart disease such as high cholesterol and high blood pressure (Mirajkar *et al*., 2011). They are also associated with the reduction of inflammatory bowel disease (Belluzzi *et al*., 2000), in growth and development (Simopoulos, 1991), risk of certain cancer forms (Connor, 2000) and risk of developing depression (Lewin., 2005),symptoms of rheumatoid arthritis (Hammock, 2011), where it has been reported that reducing dietary intake of omega-6 fatty acids while increasing consumption of omega-3 fatty acids reduces the anti-inflammatory mediators of rheumatoid arthritis (Mantzioris *et al*., 2000). These fatty acids have also been shown to be highly concentrated in the brain and therefore important for cognitive and behavioral functions (Mirajkar *et al*., 2011).

Many scientific publications strongly suggest that regular consumption of significant amounts of PUFAs rich in ω-3 fatty acids can be highly effective in the prevention or treatment of CVD (Kris-etherton *et al*., 2003). The mechanisms responsible for the observed effects of ω-3 fatty acids on cardiovascular health are not known with confidence (Kris-etherton *et al*., 2002). The ones possibly involved are the decrease in serum triglyceride concentration, atherosclerotic plaque growth retardation and hypotensive, anti-thrombogenic and anti-inflammatory effects (Connor, 2000).

In addition, ω-3 fatty acids are essential to normal neuronal development and their depletion has been associated with neurodegenerative diseases such as Alzheimer's disease (Morris *et al*., 2003). PUFAs of the ω-3 series have very special roles in cell membranes, especially in the nervous system, all brain cells and organelles contain high levels of these fatty acids (Swanson *et al*., 2012).

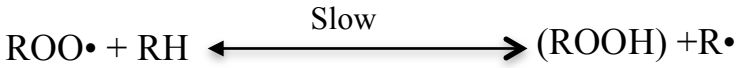
2.4 Susceptibility of Omega-3 fatty acids to Oxidation

2.4.1 The process of fish oil oxidation

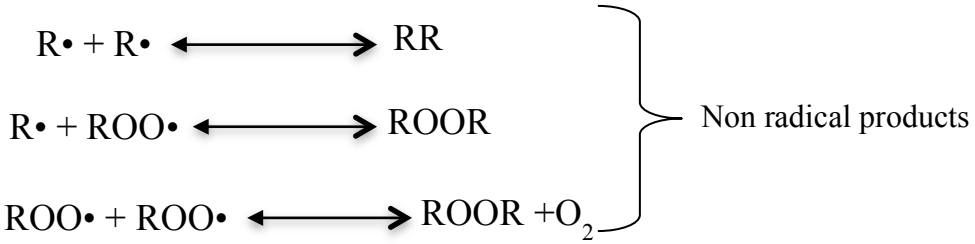
Fish oils are very susceptible to oxidation due to the high number of unsaturated fatty acids, which can be initiated by a number of chemical and physical phenomena (Antolovich *et al*., 2001). Oxidation results in deterioration of the oils hence, undesirable flavors, and decrease in the shelf life of the oils (Wsowicz *et al*., 2004). During oxidation, nutrients are lost and a fishy and rancid off flavor is developed from decomposition of hydro-peroxides (Keefe *et al*., 2012). Lipid oxidation occurs in three stages, which yield different oxidation products.

1) Initiation step- a homo-lytic hydrogen atom abstraction from a methylene group, this results to an alkyl radical (R•) formation.

2) Propagation step- peroxy radicals are formed (ROO•) which are able to react with unsaturated fatty acids. This results into the formation of hydro-peroxides.



3) Termination step-Formation of non-radical products by interaction of R• and ROO•



Where: R• - fatty acid radical

ROOH - fatty acid hydro peroxide oxide

ROO•- peroxy radical

2.4.1.1 Prevention of fish oil oxidation

Clinically, the most common intolerance to fish oils is a "fishy" smell and after-taste, as well as fishy eructation (Kris-Etherton *et al*., 2000). Fish oils are

naturally highly unstable and susceptible to oxidation, and this accounts for their rancid conversion and contributes to individual intolerance (Pak , 2005). Good-quality crude fish oil has a free fatty acid content of <4%, p-anisidine value (PAV) < 20, and peroxide value (PV) of < 5. In the presence of air, however, the unprotected oil develops a fishy and rancid flavor in a matter of hours or days (Swift & Dollear., 1950). Oxygen attacks the unsaturated portions of the fatty acids making the oils susceptible to oxidative spoilage (Mette *et al*., 2011). The oxidation of fish oil may increase the risk for toxicity (Huber *et al*., 2009), it also decreases the nutritional quality and safety by the formation of secondary products. Oxidized fish oils pose a health hazard to people when the oils become oxidized and free radicals are formed as by-products. These encourage inflammation and cancer and therefore it is of great importance to ensure that fish oil oxidation is minimized to maintain freshness of the fish oils (Hamilton *et al*., 1998).

A common way to reduce fish oil oxidation and thus reduce fish oil rancidity, maintain freshness, and increase shelf life is to add antioxidants. Antioxidants are substances which when incorporated into fish oils; inhibit their reaction with oxygen (Stip & Belš, 2009). The concurrent use of antioxidants with fish oil consumption also overcomes the oxidative stresses to the body that are observed when fish oils without antioxidants are consumed (Pritsche & Johnston, 1988). Synthetic antioxidants such as Butylated hydroxyanisole (BHA), Butylated hydroxytoluene (BHT) and Tert-Butylhydroquinone (TBHQ) have been used as

food additives, in order to overcome the stability problems of fish oil since they are less expensive than natural anti-oxidants. However these compounds have been implicated in many health risks such as in cancer and carcinogenesis (Stip & Belš, 2009). These compounds have been removed from the list of generally recognized as safe compounds (Stip & Belš, 2009). These safety concerns therefore encourage food scientists to replace synthetic antioxidants with natural ones which are safer (Al-Juhaimi & Ghafoor, 2013).The interest in natural antioxidants continues to grow as they are presumed to be safer (Farbstein *et al*., 2010). They naturally occur in foods and have been used for centuries.

2.5 Role of natural anti-oxidants in protection of oxidative deterioration of fish oil

Prevention of fish oil oxidation has been a challenge in the food industry. An anti-oxidant is a substance that when present in low concentration, compared to those of the oxidizable substrate, significantly delays or inhibits oxidation of the substrate (Antolovich *et al*., 2001). The use of natural oxidation instead of synthetic ones to improve stability and extend shelf life is of foods is of great interest to prevent oxidative deterioration (Keefe *et al*., 2012). The most important known natural antioxidants are tocopherols, spice extracts, ascorbic acid, citric acid, and their salts. Anti-oxidants can be classified into two based on their modes of actions. Primary anti-oxidants donate hydrogen to scavenge alkoxy and peroxy radicals and form stable anti-oxidant radical and these breaks the propagation cycle. Secondary anti-oxidants on the other hand prevent the formation of volatile and toxic compounds and convert hydro-peroxides to stable radical and to non-radical products (Keefe *et al*., 2012).

Antioxidants retard oxidation of fish oils and each anti-oxidant has a specific inhibitory mechanism to prevent oxidation. Tocopherols are some of the anti-oxidants that have been widely used to prevent lipid oxidation. Other natural products that have anti-oxidative properties can, otherwise be exploited to prevent fish oil oxidation (Keefe *et al*., 2012).

In the current study, the role of honey and vitamin C derived from citrus fruits such as lemon, orange and tangerine was evaluated in the prevention of oxidation of fish oils incorporated into yoghurt.

2.5.1 Honey: Nature's natural sweetener and antioxidant

2.5.1.1 Composition of honey

Honey is a natural sweet substance produced by honeybees. It is a natural product whose composition depends on the environment from which the original nectar was collected (Food and health Innovation, 2012). Honey is reported to contain about 200 substances and is considered as part of traditional medicine (Eteraf-oskouei & Najafi, 2012).

Honey is composed primarily of the glucose and fructose sugars and its third greatest component is water. It also contains other numerous types of sugars as well as acids, proteins and minerals (Ajibola *et al*., 2012).

Table 6: Typical carbohydrate composition of Honey (Al-qassemi & Robinson, 2003)

Carbohydrate	Average Content	Range
Fructose	38.38%	30.91-44.26
Glucose	30.31%	22.89-40.75
Reducing Sugars	76.65%	61.39-83.72
Sucrose	1.31%	0.25-7.57
Fructose/Glucose Ratio	1.23	0.76-1.86

2.5.1.2 Antioxidant activity of honey

Honey contains a variety of phyto-chemicals as well as other substances such as organic acids, vitamins, and enzymes that may serve as sources of dietary antioxidants and a source of free radical scavenging compounds (Alisi *et al*., 2012). There is evidence that honey contains a number of compounds shown to have antioxidant capacity (Ajibola *et al*., 2012; Alisi *et al*., 2012). The most important of these are the poly-phenols and there is evidence to suggest that the phenolic content of honey has been directly linked to its oxidative protection characteristics (Ferreira *et al*., 2007; Stip & Belš, 2009; Lachman *et al*., 2010). The amount and type of these antioxidant compounds depends largely upon the floral source/variety of the honey, climatic conditions and processing(Mohamed *et al*., 2010). In general, dark honey has better antioxidant activity than other

honey samples (amber and light) and the increase of color intensity seems to be related to an increase in the antioxidant properties and in the phenolic contents (Pérez-pérez *et al*., 2013).

A research conducted by Ferreira *et al*. (2007) showed good lipid per-oxidation inhibition in various Portuguese honey samples. The inhibition was measured by the (TBARS) assay. This indicates that honey is a good source of antioxidants, which can inhibit lipid oxidation in fish oils (Ferreira *et al*., 2007).

2.5.1.3 Other important health benefits of honey

In addition to its anti-oxidative properties, the use of honey to prevent fish oil oxidation comes with additional health benefits. It has long been regarded as having antimicrobial properties (Sherlock *et al*., 2010) and, in part, this activity is considered to be due to hydrogen peroxide liberated enzymatically in honey (Kumar *et al*., 2010). The use of honey as a traditional remedy against bacterial infections dates back to ancient times when the Ancient Egyptians are reported to have used honey to control eye infections (Mandal & Mandal, 2011). It enhances wound-healing in humans (Sherlock *et al*., 2010; Kumar *et al*., 2010) by restriction of wound pathogens like *Pseudomonas aeruginosa* (Molan, 1992). Alqurashi *et al.* (2013) evaluated the antibacterial activity of honey in Gram-negative pathogens like *E. coli, K. pneumoniae, P. aeruginosa and A. baumannii,* the study showed varying degrees of *in vitro* growth inhibition activity of honey against the tested organisms (Alqurashi *et al*., 2013).

Honey has been shown to have anti-inflammatory effects in humans consuming around 70g/day of honey (Bashkaran *et al*., 2011). In a recent study, it has been reported that honey reduces the activities of cyclooxygenase-1 and cyclooxygenase-2, thus showing anti-inflammatory effect (Vallianou *et al*., 2014). It has also been shown to provide an immediate anti-inflammatory treatment hence providing an antibacterial action and a barrier to further infection in wounds (Kumar *et al*., 2010).

2.5.2 Vitamin C (ascorbic acid) as an anti-oxidant

2.5.2.1 Sources, biochemistry and chemical structure of vitamin C

Vitamin C is mainly found in foods of plant origin, and is present mainly in fruits such as citrus fruits, papaya, berries and in vegetables such as cauliflower, sweet pepper, cabbage among others (Berenguer *et al*., 2004). Potatoes have also been reported to be a good source of vitamin C (Panel & Nda, 2013). A mean vitamin C intake of 90 mg/day is estimated to be required in healthy adults (Panel & Nda, 2013).

Vitamin C, also known as ascorbic acid is a water-soluble vitamin that is lost in large amounts during food processing (Rekha *et al*., 2012). Human, non-human primates and guinea pigs lack the enzyme gulonolactone oxidase, which is essential for the synthesis of Ascorbic acid (Padayatty *et al*., 2003). Consequently, humans require that vitamin C is included in their diet. Lack of vitamin C in human results into a deficiency state that presents with a wide spectrum of clinical manifestations referred to as scurvy (Padayatty *et al*., 2003). This is a lethal

condition unless it is appropriately treated and prevented by ensuring humans ingest adequate amounts of vitamin C in their diets.

Vitamin C is a six-carbon lactone, normally synthesized from glucose. It has a unique structure that includes two adjacent hydroxyl groups and a carbonyl group making it an excellent hydrogen or electron donor (Farbstein *et al*., 2010).

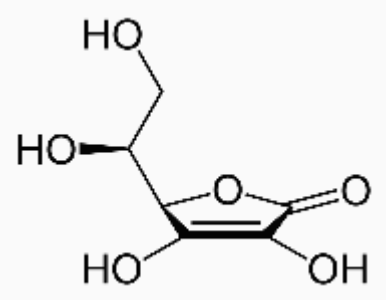


Figure 5: Structure of Vitamin C (http://en.wikipedia.org/wiki/Vitamin C)

All the known physiological and biochemical functions of vitamin C are due its role as an electron donor and it is therefore a reducing agent (Herbert & Mistry, 1998).

2.5.2.2 Role of vitamin C as an antioxidant

Depending on the conditions, ascorbic acid can act as an antioxidant, a pro-oxidant, a metal chelator, a reducing agent or as an oxygen scavenger (Montecinos *et al*., 2007). Vitamin C is therefore an antioxidant that is able to readily scavenge reactive oxygen and nitrogen species, such as super-oxide and hydroperoxyl radicals, aqueous peroxyl radicals, singlet oxygen, ozone,

peroxynitrite, nitrogen dioxide, nitroxide radicals, and hypochlorous acid (Hininger *et al*., 2005).

Vitamin C functions as an antioxidant by donating its electrons; it prevents other compounds from being oxidized. However, by the very nature of this reaction, vitamin C itself is oxidized in the process (Padayatty *et al*., 2003), it does so by donating two electrons from a double bond between the second and third carbons of the 6-carbon molecule. Vitamin C takes part as a co-factor in many enzymatic reactions, and acts as plasma localized anti-oxidant (Zalewska-korona, 2009). Once it has been oxidized, it is turned into ascorbate free radical (AFR), a molecule that is relatively stable due to electron delocalization (Farbstein *et al*., 2010b). AFR can donate another molecule, but it does not undergo further oxidation, rather, it is normally reduced back to ascorbate via NADH-dependent and independent mechanisms (Farbstein *et al*., 2010b).

Vitamin C can also act as a co-antioxidant, it does so by regenerating vitamin E from the tocopheroxyl radical, produced via scavenging of lipid-soluble radicals (Keefe *et al*., 2012). *In vitro* studies have shown that vitamin E can act as a pro-oxidant in the absence of co-antioxidants such as vitamin C (Morton & Valentine, 2003). However, this interaction between vitamin C and vitamin E is unclear (Carr & Frei, 1999).

Supplementation studies done using specific biomarkers of oxidative damage to lipids, DNA and proteins have given the most conclusive evidence of the role of vitamin C as a an antioxidant (Carr & Frei, 1999).

2.5.2.3 Role of Vitamin C in human metabolism and disease

During the various biological processes in the body, free radicals are produced. These are normally controlled by various enzymes and antioxidants in the body (Asđt & Bđr, 2009). Oxidative stress occurs when free radical formation exceeds the ability to protect against them, this normally results in tissue injury, inflammatory events and chronic conditions such as atherosclerosis, degenerative disease and cancer (Khassaf *et al*., 2003; Mikirova *et al*., 2007; Montecinos *et al*., 2007). Vitamins such as E, C, and β-carotene are often referred to as antioxidant vitamins. They have been suggested to limit oxidative damage in humans, thereby lowering the risk of certain chronic diseases (Asđt & Bđr, 2009; Yang *et al*., 1999).

Ascorbic acid is involved in a wide range of metabolic activities such as metabolism of several amino acids(Yang *et al*., 1999; Seyrek *et al*., 2010; Kuo, 2013) and it also regulates and participates in enzymatic reactions and transport of neurotransmitters and in hormone biosynthesis (Ko, 2006).

Decreased levels of plasma vitamin C have been associated to several conditions of increased with oxidative stress, such as cancer, cardiovascular diseases, diabetes mellitus, cataract, HIV infection, systemic lupus erythematosus (SLE)

and smoking habits (Collins *et al*., 1996; Smokers *et al*., 2007; Morton & Valentine, 2003). Several reports have shown that cancer cell lines are normally more sensitive to vitamin C than their non-malignant counterparts (Mikirova *et al*., 2007). Epidemiological evidence has also associated fruit and vegetable consumption with lower risk of cardiovascular diseases (Morton & Valentine, 2003; Ko, 2006).

2.5.2.4 Role of vitamin C in prevention of fish oil oxidation

Although, synthetic antioxidants are effective and less expensive than natural antioxidants there is an increasing demand for natural antioxidants because of the concern about the safety of synthetic antioxidants. The use of natural anti-oxidants not only prevents fish oil oxidation, but also enhance the health benefits of the foods by having additional health-promoting bioactivity from the food it is obtained from (Ozogul *et al*., 2010).Vitamin C is therefore, a natural anti-oxidant, which can be obtained from various fruits and vegetables.

2.6 Incorporation of fish oils into yoghurt

Yoghurt is a very nutritious food and its continued consumption in the Western world owes much to the development of its health food image (Allgeyer *et al*., 2010; Davis *et al*., 2007). It is an acidified, coagulated product, which is obtained from milk by fermentation with lactic acid producing bacteria. Of all cultured milk products, yoghurt is the most well known and most popular worldwide (Hassan & Amjad, 2010a).

Several problems such as variations in milk composition, irregular behavior of the starter organisms, faulty regulation of the incubation temperature, along with a number of other process variables, can give rise to a product that is deficient with respect to the overall quality of the yoghurt. A thorough understanding of the fermentation process is thus required so as to provide an operative with foresight to reduce risk of product failure (Gulzar *et al*., 2013).

2.6.1 Yoghurt manufacturing process

The main processing steps involved in yoghurt manufacture include the standardization of milk (fat and protein content), homogenization, milk heat treatment, incubation/fermentation, cooling, and storage (Lee & Lucey, 2010).

2.6.1.1 Milk standardization

Milk is often mixed with skim milk and cream to standardize the fat content to the desired level. Many commercial yoghurt products have milk solids contents of 14-15%. The minimum milk solids not-fat content required in standards or regulations in many countries ranges from 8.2 to 8.6% (Gulzar *et al*., 2013). Stabilizers, such as, pectin or gelatin, are often added to the milk base to enhance or maintain the appropriate yoghurt properties including texture, mouth feel, appearance, viscosity/consistency and to the prevention of whey separation (Sodini *et al*., 2005).

2.6.1.2 Homogenization

Homogenization is a step done for yoghurts containing fat. This prevents fat separation (creaming) during fermentation or storage, reduces whey separation, increases whiteness, and enhances consistency of yoghurts (Lee & Lucey, 2010).

When milk is homogenized, caseins and whey proteins form the new surface layer of fat globules, which increases the number of possible structure-building components in yoghurt made from homogenized milk (Sfakianakis & Tzia, 2014).

2.6.1.3 Heat treatment

Heating of milk is an important processing variable for the preparation of yoghurt since it greatly influences the physical properties and microstructure of yoghurt (Lee & Lucey, 2010). The temperature/time combinations for the batch heat treatments that are commonly used in the yoghurt industry include 85 °C for 30 min or 90-95 °C for 5 min (Gulzar *et al*., 2013). However, very high temperature short time (100°C to 130°C for 4 to 16 s) or ultra-heat temperature (UHT) (140 °C for 4 to 16 s) are also sometimes used (Lee & Lucey, 2010). The heat treatment of milk also destroys unwanted microorganisms, and this provides less competition for the starter culture (Allgeyer *et al*., 2010).

2.6.1.4 Fermentation process

After heat treatment, the milk base is cooled to the incubation temperature used for growth of the starter culture. The optimum temperature for the thermophilic lactic acid bacteria *Streptococcus subsp*, *thermophilus* and *Lactobacillus delbrueckii subsp bulgaricus*, is around 40-45 °C (Lee & Lucey, 2010; Hassan & Amjad, 2010a). Bacterial fermentation converts lactose into lactic acid, which reduces the pH of milk (Gorsek & Tramsek, 2006). During acidification of milk, the pH decreases from 6.7 to ≤4.6 (Lee & Lucey, 2010).

2.6.1.5 Cooling

When yoghurts have reached the desired pH (4.6), yoghurts are partially cooled (~20 °C) before fruit or flavoring ingredients are added (Gulzar *et al*., 2013). Yoghurt products are often blast chilled to <10 °C in the refrigerated cold store to reduce further acid development (Lee & Lucey, 2010).

2.7 Omega-3 fatty acids incorporation into yoghurt

Intake of dairy products such as yoghurt as part of our daily diet helps to improve on the quality of the diet and increase on the chances of achieving nutritional requirements (Allgeyer *et al*., 2010b). Research has shown that milk consumption is positively associated with the likelihood of children and teenagers achieving the recommended intakes for vitamin A, folate, vitamin B12, calcium and magnesium (Eyer, 2007). It is advantageous to use yoghurt as a vehicle for supplementing the diet with heart healthy nutrients (Tulk *et al*., 2013; Hassan & Amjad, 2010b; Hekmat & Mcmahon, 1997). Many efforts have been made by the industry to develop food products and nutritional preparations that contain appreciable amounts of omega 3 (Hilma Elma., 2012; Enrichment & Fish, 2005a). Yoghurt has been shown to be a good vehicle for fish oil enrichment, since the products are very stable towards oxidation (Eyer., 2007).

Estrada *et al*, (2011) developed a method of manufacturing yoghurt comprising of microencapsulated marine fish oil. Their objective was to provide a yoghurt product containing omega-3 and a vitamin E source that can easily be manufactured and the fish oils do not get oxidized during 4 weeks of refrigerated storage (Estrada *et al*., 2011). The production of yoghurt containing omega- 3

fatty acids with no fishy odor is done by masking using specific synthetic flavors (Estrada *et al*., 2011). Fish oil oxidation is reduced by use of natural products that have anti-oxidant capacity. Such products include honey; it is a natural anti-oxidant as well as a natural sweetener. Another natural anti-oxidant is vitamin C, obtained from fruits such as lemon, orange and tangerine.

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 Materials

Nile perch viscera were purchased from a local Nile perch processing plant in Nairobi (W.E. Tiley ltd, Kenya) located along Baba ndogo road in Nairobi County - Kenya. The Nile perch (*Late sp.*) were collected from Lake Victoria in Kenya (1◦S, 33◦E). Fresh milk, skimmed milk, sugar, citrus fruits were obtained from a local store (Tusky's Supermarket ltd). Starter cultures of *L. bulgaricus* and *S. thermophilus* were purchased locally (Pradip enterprises ltd). Natural honey was purchased from a local vendor from Baringo County - Kenya and the two processed honey samples (Amboseli and Green forest)were obtained from local stores (Tuskys and Nakumatt supermarkets ltd). All the solvents and chemicals used were of analytical grade.

3.2 Sample preparation and analysis

Five kg (5kg) of Nile perch viscera was ground and homogenized at 4^0C using a grinder (Summet research and Holdings PVT limited, Tamil NADU, India) to reduce the particle size. The homogenates were then preserved at −20 ^{0}C in a freezer (IGNIS, Italy) until further analysis. This was done so as to reduce oxidation of the long chain fatty acids and minimize endogenous lipolytic activity.

3.3 Enzymatic oil extraction from Nile perch fat pads

Enzymatic oil extraction was done by the method described by Mbatia *et al*. (2010) with a few modifications on the optimum temperature and the amount of enzyme added. Ground and homogenized frozen Nile perch fat pads were thawed

overnight at 4 ^{0}C. A 100 g of the homogenate was put in a 500 ml jacketed reactor (Schott, Germany) without adding water. The contents were heated to 55 ^{0}C from a re-circulating water bath (LKB, Bromma, Germany) with stirring at 200 revolutions per minute (rpm) using an electronic stirrer (Stuart, United Kingdom). Enzymatic hydrolysis was then initiated by the addition of 2 ml of a food grade enzyme alcalase 2.4 L (EMD, Inc. San Diego) per 100g of the homogenized fish pads. Hydrolysis was allowed to proceed for 1 h. The hydrolysates were placed at 100 ^{o}C for 20 minutes to inactivate the enzyme.

3.3.1 Oil extraction from Nile perch fat pads without addition of enzyme

This was done following the procedure described in section 3.3 but without addition of enzyme

3.3.2 Recovery of Omega-3 fatty acids rich oil

To recover Nile perch oil rich in Omega-3 fatty acids, the hydrolysate obtained as described in 3.3 and 3.3.1 was centrifuged at 1,000g at room temp to separate the oily fraction,, emulsion, aqueous and sludge phases. The wet weights of the different fractions were determined by placing the tubes in an upright position at -20 ^{o}C for 2 h. The fractions were then separated by cutting the frozen content in the tubes and their wet weights determined. The experiment was performed in triplicates and the mean ± {standard deviation (SD)} of the wet weights determined.

The oily fraction was decanted off into 50 ml centrifuge tubes (Thermo Fisher Scientific, Fair Lawn, NJ) and centrifuged at 1,000g in refrigerated centrifuge for

20 mins (Eppendorf, Pittsburgh,USA). A liquid oil (Omega-3 rich oil) and solid fat fraction (saturated fatty acids) was obtained. The weight of the two fractions obtained was then determined and the upper liquid layer rich in omega-3 fatty acids used in the fortification. The method by Mbatia *et al*. (2010) was used to determine the amounts of omega-3 fatty acids to be incorporated into the yoghurt. According to Mbatia *et al* 2010, 100 g of Nile perch oil contains 20.5% of omega-3 fatty acids which include 3% EPA, 6.2% DPA and 9.0% DHA. From this inference, 3.5 g of the upper omega-3 fatty acids rich oil phase obtained was incorporated into 150 g of yoghurt. This gave approximately 700 mg- 1 g of omega-3 fatty acids per serving of 150 g yoghurt which is far much more than the average recommended levels of 500 mg/day of omega-3 fatty acids (Kris-Etherton *et al*., 2000).

3.4 Thin layer chromatography analysis

Triglycerides (TAGs), diglycerides (DAGs), monoacylglycerides (MAGs), and free fatty acids (FFA) from the extracted lipid oils of fish (the entire homogenate before centrifugation, the oil phase,emulsion and corn oil as a standard),were separated on Silica gel 60 thin layer chromatography (TLC) plates (Merck, Darmstadt Germany) and eluted using a mobile phase that comprised of Hexane/diethylether/acetic acid (50:50:1,-v/v/v). Iodine vapour was used for visualization of the lipids and free fatty acids.

To identify the bands, the Retardation Factor (RF) was determined using the following fomular (Morris, 1962).

$$Rf = \frac{\text{distance starting line – middle of spot}}{\text{distance starting line – solvent front}}$$

3.5 Determination of the quality parameters of the Nile perch oil

3.5.1 Peroxide value (pv)

This is the measurement of primary oxidation products (Adios *et al*., 2001). It is the most widely used chemical test for the determination of fats and oil quality (O'Brien, 2009). The Pv (meq/ Kg) was determined following American Oil Chemists Society (AOCS) Official Methods (1997). The acetic acid-chloroform method (AOCS Cd 8-53) was used to determine the Pv. Briefly, 2 g of the homogenate, oil phase and fish protein hydrolysate was weighed into a 125 mL Erlenmeyer flask and 12 mL of 3:2 acetic acid-chloroform (Fisher Scientific, Fair Lawn, NJ) added in a hood. The flask was manually swirled until the sample was dissolved and 0.2 mL of a saturated Potassium iodide solution (Sigma Aldrich, Co., St. Louis, MO) added. The solution was then allowed to stand for 1 min with occasional swirling before addition of 12 mL of distilled water. One mL starch solution (Sigma Aldrich, Co. St. Louis, MO) was added and the resulting blue solution titrated with 0.01 N Sodium thiosulfate ($Na_2S_2O_3$) (Sigma Aldrich, Co., St. Louis, MO) added drop wise with constant shaking until the blue color disappeared. The same procedure was carried out for a blank solution. The PV of oils was calculated as indicated in equation 1.1 in which: *S* is the volume (mL) of $Na_2S_2O_3$ used to titrate the sample, *B* is the volume (mL) of $Na_2S_2O_3$ used to titrate the blank, *M* is the molar concentration of the $Na_2S_2O_3$ solution (N), and *W* is the weight of the sample in grams.

Equation 1.1

$$\mathbf{PV}=\frac{(\boldsymbol{S}-\boldsymbol{B})\times\boldsymbol{N}\times\mathbf{1000}}{\boldsymbol{W}}$$

3.5.2 Anisidine value (AV)

The anisidine value measures the secondary product of oxidation and determines the aldehydes in the lipid/oil. This was determined following American Oil Chemists Society (AOCS) Official Methods(1997). Official method AOCS Cd 18-90 was used to determine AV which is given by Eq.(1.2). Approximately 1 g of oil (*m*) was weighed into a 25 mL volumetric flask and dissolved to volume with anhydrous 99.8% 2, 2, 4 – trimethylpentane (isooctane). The absorbance of the solution (*Ab*) at 350 nm was measured with the solvent as a blank. Exactly 5 mL of the fat solution was pipetted into a test tube and exactly 5 mL of the solvent added into a second test tube. Into each tube, 1 mL of 99% *p*-anisidine reagent solution (0.25 g/100 mL glacial acetic acid) (Sigma Aldrich, Co. St. Louis, MO) was added followed by vigorous shaking. After exactly 10 min, the absorbance (*As*) of the solvent in the first test tube was measured at 350 nm (Thermo Scientific UV-Vis spectrophotometer, Germany), using the solution from the second test tube as a blank in the reference cuvette.

Equation 1.2

$$\mathbf{AV} = \frac{25\ (1.2As - Ab)}{M}$$

Total Oxidation (TOTOX) values were calculated as described by Wai *et al*., 2009 using Eq 1.3

Equation 1.3

TOTOX=2PA+AV

Where: PA = Peroxide value, AV = Anisidine value

3.5.3 Free fatty acid (FFA) content

FFA content (%) was determined using a titration method described by the AOCS Ca 5a-40 Official Method (1997), with slight modifications. Two grams of the oil samples was mixed with 10 ml of heated and neutralized ethanol solution and 0.4 ml of phenolphthalein in a 125 ml flask. The mixture was titrated using 0.1N sodium hydroxide (NaOH) until a faint pink color was retained. The % FFA (Eq. 1.4) expressed, as oleic acid was calculated as follows:

Equation 1.4

$$\boldsymbol{FFA}(\%) = \frac{(\boldsymbol{NaOH}(\boldsymbol{ml}) * \boldsymbol{N} * \mathbf{28.2})}{\boldsymbol{mass}(\boldsymbol{g})}$$

N = normality of the NaOH, Mass (g) = mass of sample used.

Table 7: Recommended quality parameters for crude fish oil

Recommended quality parameters for crude fish oil.	Recommended Value	Reference
Moisture	0.5%	Bimbo (1998)
Impurities	(1% maximum)	Bimbo (1998)
Free fatty acids	1-7%	Bimbo (1998)
Peroxide value (PV)	3-20 meq/kg	Bimbo (1998)
p-Anisidine value	4-60	Bimbo (1998)
Total oxidation value	10-60	Bimbo (1998)
Unsaponifiable matter	<2 (wt%)	Scrimgeour (2005)
Color (Gardner scale)	10.5-12.5	Bimbo (1998)

3.6 Collection of honey samples

Three honey samples were obtained as described in section 3.1. The samples were analyzed for quality and antioxidant potential.

3.6.1 Estimation of total phenolic content

The reaction of Folin-Ciocalteu reagent is a colorimetric method widely used for the determination of total phenolic contents in honey. This was determined by a method described by Singleton *et al*. (1999). Each honey sample (5 g) was diluted to 50 mL using distilled water. A 1 ml volume of this solution was then mixed with 2.5 ml of 0.2 N Folin–Ciocalteu reagents (Sigma– Aldrich Chemie, Steinheim, Germany) for 5 min after which, 2 mL of 75 g/l sodium carbonate (Na_2CO_3) was added. The reaction mixture was incubated for 2 h at room

temperature and the absorbance measured at 760 nm against a methanol blank. Gallic acid (0–200 mg/l) was used as standard to produce the calibration curve. The mean of three readings was used and the total phenolic content was expressed in mg of gallic acid equivalents (GAE)/100g of honey.

3.6.2 Determination of proline content

Proline is the most abundant amino acid in honey and hence proline content in honey is an indication of honey quality, where low proline content gives an indication of adulteration (Bogdanov*et al*., 2000). Proline content in the honey samples was determined using a method established by the International Honey Commission (IHC, 1999). An aliquot (0.5ml≈ 0.05mg) of honey solution was transferred to a test tube. A volume (0.5 mL) of water (blank test) was transferred to a second tube and 0.5 mL of (0.032 mg/ml) proline standard solution was dispensed into three other tubes. To each tube, 1 mL of formic acid and 1 mL of ninhydrin (Fisher Scientific, United Kingdom) solution was added. The tubes were capped carefully and shaken vigorously for 15 min. The tubes were then placed in a boiling water bath for 15 min and further transferred to another water bath and incubated at 70 °C for 10 min. A 5 mL solution of 2-propanol water solution was added to each tube followed by immediate capping. The tubes were left to cool for approximately 45 min and the absorbance values were measured at 510 nm. Proline concentration in mg/kg of honey was calculated as follows:

Proline (mg/kg) = (Es/Ea)x(E_1/E_2)x80,

Where: **Es** is the absorbance of the sample solution; **Ea** is the absorbance of the proline standard solution (average of three readings); $\mathbf{E_1}$is the mg of proline used

for standard solution; **E_2**is the weight of honey in grams; **80** is the dilution factor. The mean of three readings was used.

3.6.3 Determination of carotenoids content

Carotenoids contribute to the antioxidant properties of honey. Darker honey samples have been shown to contain higher levels of the pigment hence, higher antioxidant capacity (Ferreira *et al*., 2007). Carotenoid content in the honey samples was determined according to the method described by Ferreira *et al*. (2007). A sample of honey (100 mg) was vigorously shaken with 10 ml of acetone–hexane mixture (4:6) for 1 min and filtered through Whatman No. 4 filter paper. The absorbance of the filtrate was then measured at 453, 505 and 663 nm. Contents of β-carotene and lycopene were calculated according to the following equations:

Lycopene (mg/100 ml) = $-0.0458\ A_{663} + 0.372\ A_{505} - 0.0806\ A_{453}$;

β-carotene (mg/100 ml) = $0.216\ A_{663} - 0.304\ A_{505} + 0.452\ A_{453}$.

The results were expressed as mg of carotenoid/kg of honey

3.6.4 Color intensity (ABS_{450})

ABS_{450} is an index for measuring and confirming the presence of pigments with antioxidant activities, such as carotenoids (Islam *et al*., 2012). The mean absorbance of honey samples was determined using the method of Beretta *et al. (*2005). Briefly, honey samples were diluted to 50% (w/v) with warm (45 – 50 °C) distilled water, and the resulting solution was filtered to remove large particles. The absorbance was measured at 450 and 720 nm and the difference in absorbance was expressed as mAU.

mAU of honey =($ABS_{700} - ABS_{450}$)

3.6.5Antioxidant activity

3.6.5.1 DPPH radical scavenging activity

DPPH is a stable nitrogen centered radical that is widely used to test the free radical scavenging ability of various samples. The higher the DPPH scavenging activity the higher the antioxidant activity of the sample (Saxena *et al*., 2010). The radical scavenging ability of honey was tested based on the method proposed by Ferreira *et al*., 2007.. The honey samples (12.5 µL -100 µL/mL) were prepared in methanol. An aliquot (2 mL) of DPPH (Sigma -Aldrich, USA) solution (0.002 % in methanol) was added to 2 mL of the prepared samples. The samples were incubated at room temperature in dark for 30 minutes and the optical density was measured at 517 nm. The absorbance of the DPPH control was also noted. The scavenging activity of the samples was calculated using the formula:

Scavenging activity (%) = [(A – B) / A] x 100.

Where: **A** is absorbance of DPPH and **B** is absorbance of DPPH and honey sample.

3.6.5.2 Reducing power

The ferric reducing assay is a method widely used for antioxidant determination and assessment of the antioxidant and reducing power of honey. This was done according to the method described by Ferreira *et al*., (2007). Various concentrations of water honey solutions (2.5 mL) were mixed with 2.5 mL of 200 mmol/L sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was then incubated at 50 °C for 20 min. To the mixture,

2.5 ml of 10% trichloro acetic acid (w/v) (BDH Chemicals Ltd, Poole, - England) was added and the mixture centrifuged at 2000×g for 8 min. The upper layer (2 mL) was mixed with 5 mL of deionised water and 1 ml of 0.1% of ferric chloride, and the absorbance was measured at 700 nm. Higher absorbance indicates higher reducing power (Oyaizu, 1986).

3.6.5.3 Total antioxidant content of honey

The antioxidant content was determined by measuring Ascorbic acid Equivalent Antioxidant Capacity (AEAC) values using the method of Meda *et al.* (2005). Briefly, honey samples were dissolved in methanol to a final concentration of 0.03 g/mL. A 0.75 mL aliquot of the methanolic honey solution was then mixed with 1.50 mL of 0.02 mg/mL DPPH solution prepared in methanol. The mixture was then incubated at room temperature for 15 min, and the absorbance measured at 517 nm. The blank was composed of 0.75 mL of a methanolic honey solution mixed with 1.5 mL of methanol. Ascorbic acid standard solutions (2, 4, 6, 8 and 10µg/mL) prepared in distilled water were used to form a calibration curve. Measurements were performed in triplicate, and the mean value was expressed as mg of ascorbic acid equivalent antioxidant content per 100 g of honey.

3.7 Extraction of Citrus fruits juice

Ripe citrus fruits namely orange, lemon and tangerine were purchased from a local store (Muthurwa market, Nairobi County). The fruits were washed thoroughly in water and the juices extracted by cutting the fruits in half and carefully squeezing to extract juice. The collected juice was then filtered through and the pulp free juice collected in clean containers.

3.7.1 Determination of total acidity and pH

Total acidity of the juices was determined by titration method according to a method described by Rekha *et al*. (2012). A 10 % fruit juice was prepared by taking 10 mL juice and making up to 100 mL using distilled water in a volumetric flask. Each of the juice (10 mL) was titrated against standardized 0.1 N NaOH (Sodium hydroxide) using Phenolphthalein as an indicator. The end point was noted (color change from colorless to pale pink). Total acidity was calculated in terms of citric acid using formula:

Acidity (g/100 mL) = Normality of the juice x Equivalent weight of citric acid.

The pH of 10 % juice was determined using pH meter (Mettler Toledo, USA)

3.7.2 Estimation of Ascorbic acid content

Ascorbic acid content in fruit juices was estimated by titration method. Un-concentrated fruit juice (50 mL) was taken into a 100 mL volumetric flask and 25 mL of 20 % meta-phosphoric acid was added as a stabilizing agent and distilled water added up to the 100 mL mark. A 10 mL volume the solution was pipetted into a small flask and titrated using standard indophenols solution until a faint pink color persisted for 15 s. The mg of Ascorbic acid per mL of the sample was calculated as follows:

$$\textbf{Vitamin C content (mg/100g)} = (A-B)\times C \times 100/10 \times 1/S \times 100$$

Where: **A**= volume of the indophenols solution used to titrate the sample (mL)

B= volume of then indophenols solution used for the blank (mL)

C= mass in mg Ascorbic acid equivalent to 1.0mL of standard indophenols solution

S= volume of sample used

3.7.3 Antioxidant activity of the fruit juices

3.7.3.1 DPPH free radical scavenging assay

This was determined following the method proposed by Ferreira *et al.* (2007) as described in section 3.6.4.1.

3.7.3.2 Antioxidant activity of fruit juices by ferric reducing assay

This was determined following the method proposed by Ferreira *et al*. (2007) as described in section 3.6.4.2.

3.7.3.3 Total antioxidant content of fruit juices

The antioxidant content of the fruit juices was determined by measuring Ascorbic acid Equivalent Antioxidant Capacity (AEAC) values using the method of Meda *et al*. (2005) as described in section 3.6.4.3. From the three citrus fruits evaluated, the fruit with the highest ascorbic acid content and hence antioxidant activity was selected for use in the fish oil fortified yoghurt. The recommended dietary intake (RDI) of ascorbic acid (60mg/day) in the fruit juice with the highest antioxidant activity was used to determine the amount of the juice to be added into the yoghurt, and taking care that the pH of the yoghurt does not to go below the recommended pH of 4.5.

3.8 Production of functional Yoghurt fortified with Omega-3 fatty acids

Production of functional fortified yoghurt was done by a method developed at the Technical University of Kenya laboratory. Four types of stirred fortified yoghurts samples were produced at the Technical University of Kenya Food Science Laboratory.

The first yoghurt sample was fortified with Omega-3 fatty acids and honey. Green forest had the highest antioxidant activity was used in fortification. The amount of honey to be added was determined by preparing different proportions of the fortified yoghurt with different variations of the honey. The optimum level in which the yoghurt sample was well sweetened was used. Honey and the pre-determined amounts of omega-3 rich fish oil (section 3.3.2) were incorporated into the milk during the yoghurt mix formulation and the mixture homogenized to ensure a homogenous formulation was obtained (Table 7). Skimmed milk powder containing Soy lecithin was added as a thickener and to solubilize the fish oil in the yoghurt.

The second yoghurt sample was fortified with Omega-3 fatty acids and lemon juice which had the highest antioxidant activity among the three citrus fruits evaluated. The amounts of lemon juice to be added was determined by preparing different proportions of the omega-3 fortified yoghurt with different levels of lemon juice until an optimum sample was obtained in which the acidity was not below 4.5. The omega-3 fatty acids rich oil was incorporated into the milk (section 3.3.2), with sugar added as a sweetener and skimmed milk as a thickener

and solubilizer (Table 7). The determined amount of lemon juice (section 3.7.3.3) was added to the final yoghurt product prior to flavoring. The third yoghurt sample was a control, which contained only the oil sample without an antioxidant. A fourth control comprised of plain natural yoghurt (Table 7).

The yoghurt mix formulations for the samples were prepared in batches in glass containers. The homogenized mixes were batch pasteurized at 85 ± 1 ºC for 30 min and then rapidly cooled in an ice bath to 43 ± 1 ºC for inoculation with freshly thawed starter cultures. Incubation was carried out at 43 ± 1 ºC, until a pH of around 4.5 was attained. The yoghurts were then quickly cooled and stored overnight at 4 ºC. The finished plain stirred yoghurt batches were then packaged in tightly sealed containers and refrigerated at 4 ºC.

In order to perform sensory evaluation, aliquots of the different yoghurt types were flavored using four different flavors; Strawberry, Passion, Mango and Banana in order to mask the fishy flavor and taste. The amounts of flavors to be added were determined using specifications obtained from Pradip enterprises ltd (Nairobi, Kenya) where the flavors were purchased. The overall preferred yoghurt was prepared for shelf life analysis. Containers were well labeled: Initial, week 1, week 2, week 3 and week 4 and were removed from the refrigerator only at the corresponding time for analysis.

Table 8: Ingredients used to prepare the fish oil fortified yoghurt

Ingredient	YF	YFH	YFL	PY
Milk (L)	2	2	2	1
Skimmed milk powder(g)	80	90	120	20
Sugar(g)	-	-	110	-
Omega-3 fatty acids rich oil(g)	53.3	53.3	53.3	-
Honey(g)	-	190	-	-
Lemon juice (ml)	-	-	66.6	-
S. *thermophiles*(g)	0.5	0.5	0.5	0.25

YFH= yoghurt fortified with fish oil and honey, **YF**= yoghurt fortified with fish oil, **YFL**= yoghurt fortified with fish oil and lemon juice, **PL**=plain natural yoghurt (control)

3.9 Sensory evaluation Analysis

3.9.1 Selection criteria of the panelist

- The study was a descriptive type of study.
- Technical University of Kenya (TUK) students from the Departments of Food Science and Nutrition and Dietetics were selected for sensory evaluation.
- Students with prior knowledge on aspects of sensory evaluation was critical

3.9.2 Sensory evaluation of the flavored fortified yoghurt samples

A panel of 40 TUK students, consisting of 22 males and 18 females, participated in sensory evaluation of the flavored yoghurt samples enriched with omega-3

fatty acids. The panel was trained in descriptive sensory analysis. Before sensory profiling descriptors for taste, aroma, color, flavor, mouth feel and after-taste were developed. The descriptors were evaluated on a structured five-point hedonic scale and the samples labeled using a three-digit code obtained from the table of random numbers. Potable water was used to rinse the mouth between samples. Data was collected using questionnaires (Appendix 1) for the various samples. Sensory evaluation was performed at two levels, level one to determine the most preferred flavor (either strawberry, mango, passion or banana) and level two to determine the preferred natural antioxidant (either honey or vitamin C from lemon juice). The role of anti-oxidants and their effect on the fish oil quality parameters was evaluated four times during the one-month storage period. The evaluations were performed at initial, 1, 2, 3 and 4 weeks of storage. The evaluations were performed at initial, 1, 2, 3 and 4 weeks of storage.

3.10 Evaluation of the effect of storage on quality parameters of the fortified yoghurt

3.10.1.1 Extraction of lipids in fortified yoghurt.

This was done by the Method described by Bligh & Dyer, 1959. Five gram of the yoghurt sample was weighed, 5 ml of distilled water and 30 ml 1:2 (v/v) $CHCl_3$: MeOH solution was added and vortexed. To this, 10 ml each of $CHCl_3$ and dH_2O was added and vortexed. Centrifugation at 1000g for 5 min at room temperature was done to give a two-phase system (aqueous top, organic bottom). The organic phase at the bottom was recovered by inserting a Pasteur pipette through the upper phase with gentle positive-pressure (i.e., gentle bubbling) so that the upper

phase did not get into the pipette tip. The bottom phase was carefully withdrawn avoiding the interface or upper face. The extracted lipids were analyzed using TLC as described in section 3.4.

3.10.2 Determination of quality parameters of the omega-3 fortified yoghurt

3.10.2.1 Peroxide value, Anisidine value, Total oxidation and free fatty acids content.

The PV, AV, and FFA content were assayed to determine the lipid oxidation of the fish oils during the one-month storage period at one-week intervals. The lipid samples used were obtained from the oil extracted by the method of Bligh & Dyer. (1959) as described in section 3.10.1.1. The PV (meq/ Kg) was determined by the acetic acid-chloroform method (AOCS Cd 8-53) as described in section 3.5.1, and AV was determined following Official Methods (AOCS 1997) as described in section 3.5.2. Totox value was calculated as described by Wai *et al*. (2009) using Eq 1.3 outlined in section 3.5.2. Free fatty acid content was determined using a titration method described by the AOCS Ca 5a-40 Official Method (1997) as described in section 3.5.3.

3.10.3 pH, total acidity and vitamin C content over four weeks storage period

3.10.3.1 Total acidity

The acid content of the omega-3 fortified Yoghurt samples was determined by titration over the four week storage period using a titration method described by Rekha *et al*, (2012). Where, 10 ml of the yoghurt samples was discharged into 50 mL beakers and 40 mL of distilled water added. Ten mL of the diluted samples

was drawn and discharged into a separate beaker. Three drops of phenolphthalein was added into each of the samples and titrated using 0.1M sodium hydroxide (NaOH) already poured into a burette. The point of neutrality was achieved when the indicator changed from colorless to pink and the amount of NaOH used recorded. Percentage (%) acid was determined by the equation below:

$$\textbf{\% Acid} = \text{Titre} \times \frac{\text{acid factor} \times 100}{10 \text{ (ml of yoghurt)}}$$

3.10.3.2 pH determination

The pH of the omega-3 fortified yoghurt samples was measured directly using pH meter (Mettler Toledo, USA).The yoghurt samples were stirred before pH measurements were done.

3.10.3.3 Vitamin C (ascorbic acid) content determination

Ascorbic acid content in the omega-3 fortified yoghurt samples was estimated by titration method. A volume of 50 ml diluted yoghurt samples was taken into a 100 mL volumetric flask. Into it, 25 mL of 20 % meta-phosphoric acid was added as a stabilizing agent and distilled water added up to the 100 mL mark. From the solution, 10 mL was pipetted into a small flask and titrated using standard indophenols solution until a faint pink color persisted for 15 s. Then mg of Ascorbic acid per ml of the sample was calculated as follows:

$$\textbf{Vitamin C content (mg/100g)} = \textbf{(A-B) } \times \textbf{C } \times \textbf{100/10} \times \textbf{ 1/S } \times \textbf{100}$$

Where: A= volume of the indophenols solution used to titrate the sample (mL)

B= volume of then indophenols solution used for the blank (mL)

C= mass in mg Ascorbic acid equivalent to 1.0mL of standard indophenols solution

S= volume of sample used.

3.11 Statistical analysis

The entire experiment was replicated three times and the means and standard deviations reported. The SPSS software (IBM SPSS statistic 19) was used to conduct analyses of variance (ANOVA) to determine the differences among treatment means in the various weeks and the *post hoc* Tukey's test was used. Correlation analyses were done using the SPSS software.

CHAPTER 4

4.0 RESULTS

4.1 Distribution of the different phases after hydrolysis

Enzymatic hydrolysis resulted in formation of four phases (oily phase, emulsion phase, aqueous phase and sludge). The oil was recovered as the top layer (fig. 6A). Two phases were formed in the absence of an exogenous enzyme (fig. 6B).

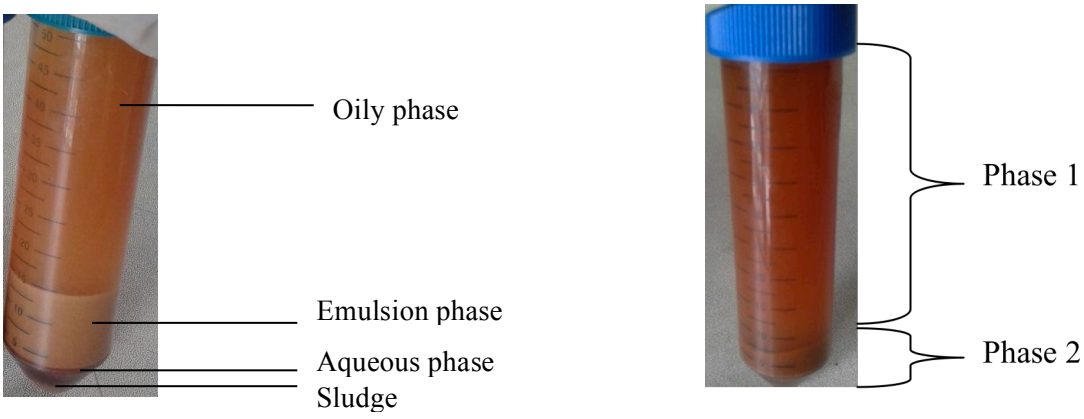


Figure 6: Phases formed after hydrolysis of Nile perch viscera A. The four phases formed after centrifugation of the enzymatic hydrolysis product and B Two phases formed after centrifugation of the product in the absence of an exogenous enzyme , phase 1=Oily phase and phase 2= Sludge.

4.2 Wet weight distribution of the different fractions after hydrolysis

The mean wet weight distribution of the different phases is shown in Table 9. An oil yield of 48.33 ± 1.15 g was obtained without addition of water from 100 g of the raw material in enzymatic hydrolysis. However oil recovery without enzyme addition was higher at 60.67± 3.78 g. Mbatia *et al*. (2010) reported that maximum enzymatic hydrolysis occurred without addition of water during hydrolysis of

salmon heads. Nile Perch homogenate (100 g) hydrolyzed for 1 h with 2% (w/v) Alcalase without addition of water and the experiment without an exogenous enzyme.

Table 9: The wet weights of the different hydrolysis fractions

	Sample (with enzyme) in grammes	**Control (g) without exogenous enzyme**
Oil	48.33± 1.15	60.67± 3.78
Emulsion	10.33± 0.58	-
Aqueous phase	2.67± 0.58	-
Sludge	2.00± 1.0	4.76± 1.96

Values are means ± standard deviation of triplicate determinations.

4.3 Omega-3 fatty acids recovery from the Oily phase

During enzymatic hydrolysis 100 g of Nile perch fat pads yielded 48.33 g of the oily phase. After centrifugation of the oil phase in a refrigerated centrifuge 31.33 g of the liquid fraction rich in omega-3 fatty acids was obtained and 10.12 g of saturated fatty acids. From 60.67 g of the oily phase in the absence of an exogenous enzyme 48.42 g of the liquid fraction and obtained and 5.76 g of the solid fraction rich in saturated fatty acids were obtained.

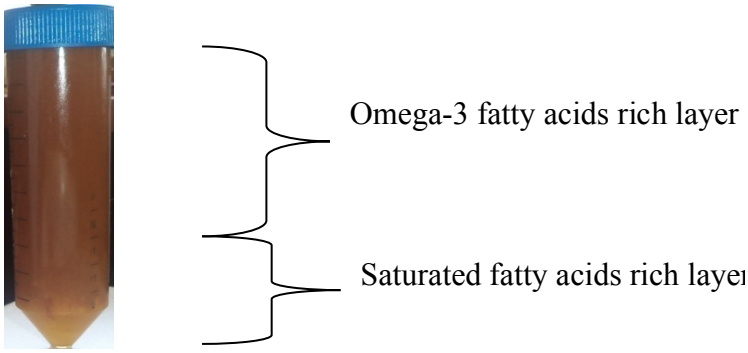


Figure 7: Omega-3 fatty acids recovered from the oil phase

4.4 Thin Layer Chromatograms of the different phases of fish oils

The bands from the TLC separation showed that the different fish oils fractions(the entire homogenate before centrifugation (**H**), the oil phase (**Op**), emulsion (**Ep**) and corn oil (**C**) as a standard) comprised of Triglycerides (TAG), Diglycerides (DAG), Monoglycerides (MAG) and free fatty acids (FFA) (Fig 8). Similar bands were obtained from all the three fractions analyzed with Corn oil was used as a reference standard. Table 10 shows the retardation factor (RF) of the different bands. The dominant component in the fish oils was Triglycerides.

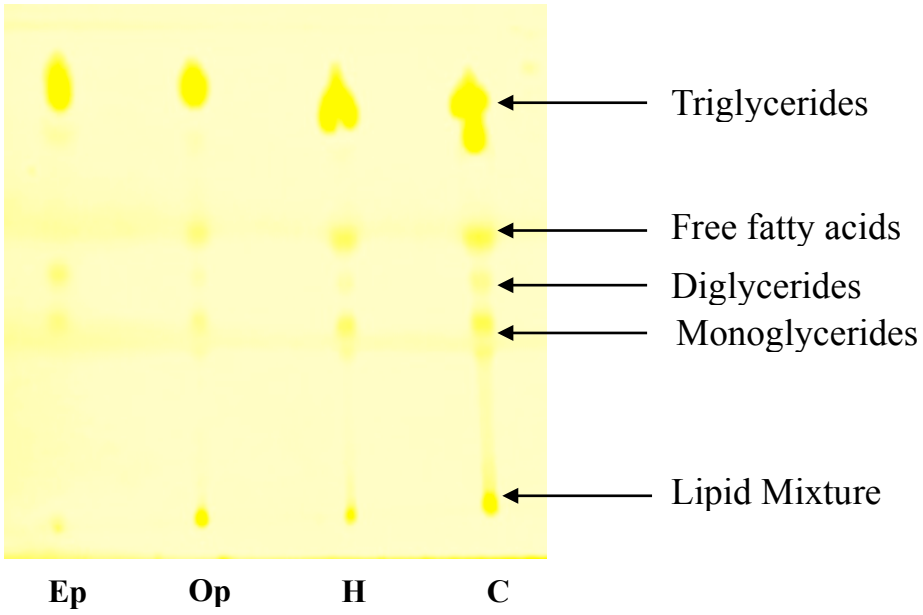


Figure 8: Thin Layer Chromatography analysis of different phases of fish oils. H=the entire homogenate before centrifugation, OP= oil phase, EP=emulsion and C=corn oil

Table 10: Rf values of the different lipid components in the various fish oil fractions

Band	**Retardation factor**
Triglycerides	0.41
Free fatty acids	0.34
Diacylglycerides	0.29
Monoacyl glycerides	0.25

4.5 Fish oil quality parameters (PV, A V, TOTOX and FFA)

The quality parameters are indicators of initial lipid oxidation. The PV's, AV, TOTOX and FFA values of the Oil phase, Emulsion phase and the homogenate (before centrifugation) determined are presented in Table 11. All the quality parameters of the omega-3 rich fish oil were in the acceptable range.

Table 11: Quality properties of the three different fractions of extracted fish oils

Parameter	Oil phase	Emulsion phase	Homogenate
PV (meq/kg)	4.83 ± 1.75	4.67 ± 1.26	5.67 ± 0.76
AV	16.50 ± 2.14	18.17 ± 1.88	20.81 ± 1.27
TOTOX	26.16 ± 1.09	27.51 ± 0.95	32.15 ± 1.42
FFA%	2.16 ± 0.22	27.51 ± 0.95	2.71 ± 0.43

Values presented are mean ± SD of three determinations. **PV**= peroxide value, **AV**= anisidine value, **TOTOX**= total oxidation, **FFA**= free fatty acids.

4.6 Honey sample quality parameters (Phenolic, Proline, carotenoids and Color intensity contents)

Since the standard curve produced a good correlation (R^2= 0.993) between concentration and its absorbance at 765 nm, the equation for this graph: y = 0.007x + 0.043 was used to calculate the gallic acid equivalent (GAE) of the honey samples (fig 9).

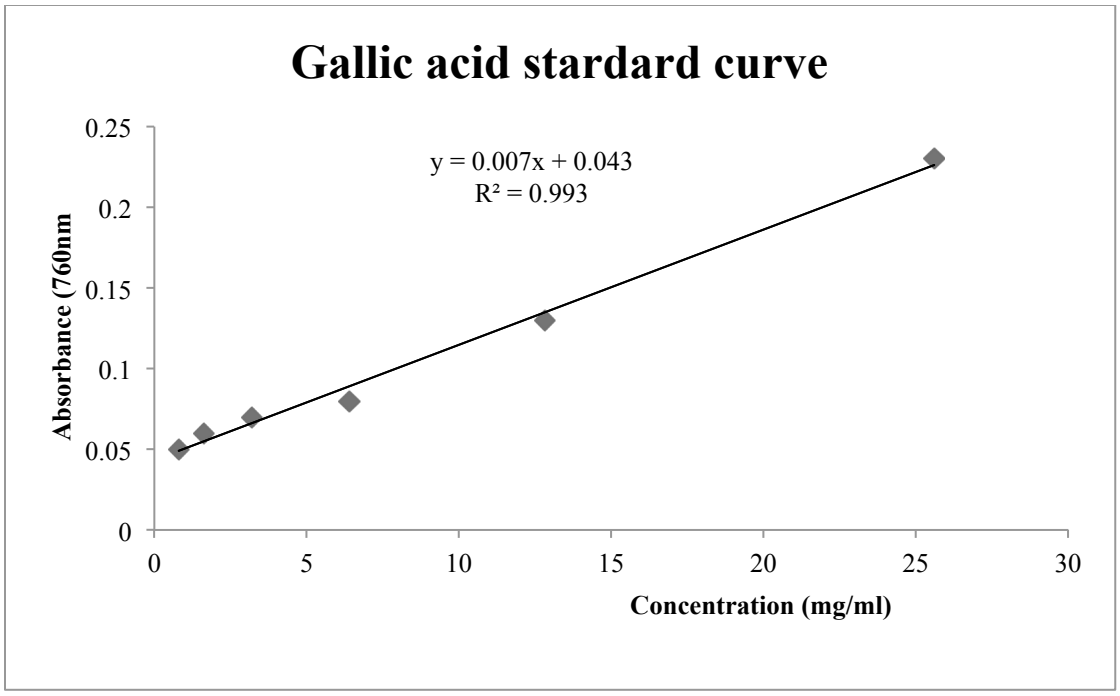


Figure 9: Gallic acid standard curve for determination of the phenol content of honey samples in mg/100g of honey.

Proline is the most abundant amino acid in honey and is therefore used as a standard to measure amino acid content as shown in Table 12. Carotenoids such as β-carotene and lycopene account for the appearance of honey samples.

The color intensity of the three honey samples is indicated by $ABS_{450,}$ which is also an indicator of the presence of pigments such as carotenoids that are known to have antioxidant properties.

Table 12: Biochemical and physical parameters of the honey samples

Sample	**Phenol content (mg/100g)**	**Proline content (mg/g)**	**β-Carotene (mg/kg)**	**Lycopene(mg/kg)**	**Color intensity (mAU)**
Baringo(Natural)	58.56± 1.78	2.41± 0.24	7.23 ± 0.92	5.31 ± 0.52	219± 23.43
Amboseli	67.25± 0.89	1.62± 0.53	8.31 ± 1.02	6.71 ± 0.02	406± 12.34
Green forest	71.56± 2.34	1.72± 1.02	8.92 ± 0.32	6.81 ± 0.01	394± 11.68

Values presented are mean ± SD of three determinations.

4.6.1 Antioxidative properties of honey samples {Free radical scavenging activity (RSA), Reducing power and antioxidant content}

The RSA of honey was determined in the DPPH reaction system (Table 13). The total antioxidant content was measured as mg of antioxidant equivalent ascorbic acid content per kilogram of honey (AEAC/kg of honey) using an ascorbic acid standard curve (R^2= 0.985, fig 10). The reducing power was measured by the ferric reducing assay.

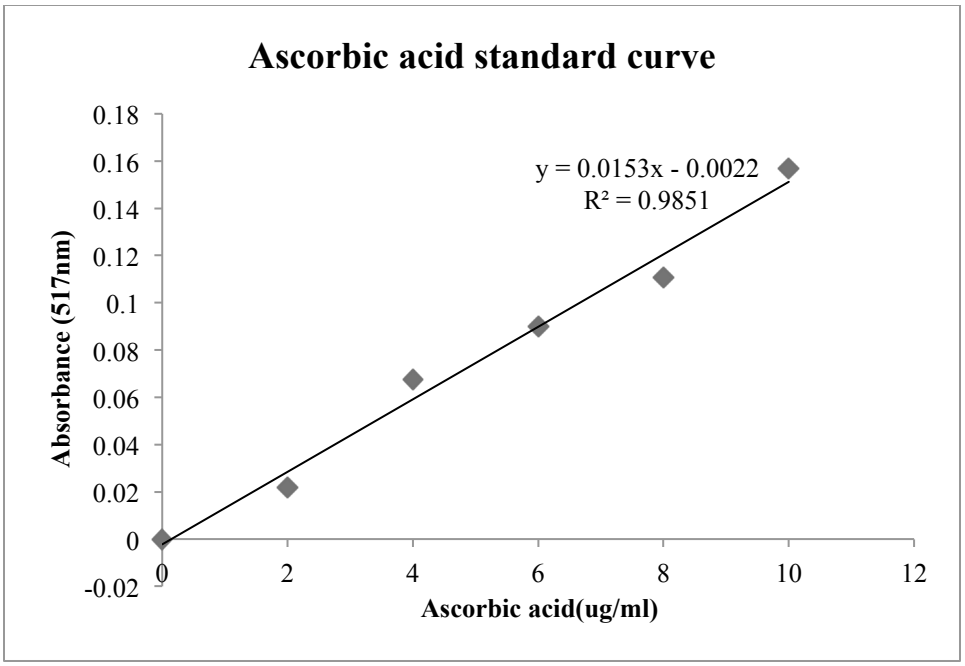


Figure 10: Ascorbic acid standard curves for determining the AEAC of the honey samples.

Table 13: Free radical scavenging activity and antioxidant activity of the honey samples.

Sample	DPPH Scavenging activity (%)	Reducing power Abs at 700nm	Antioxidant content (mg of AEAC/kg of honey)
Baringo	65.86± 2.94	0.98± 2.54	280± 0.56
Amboseli	84.47± 2.58	2.56± 1.23	305± 1.23
Green forest	86.16± 1.43	2.78± 1.69	312± 2.34

Values shown are means± SD of three replicate experiments

6.2 Correlations amongst biochemical parameters and antioxidant potentials

Several strong correlations were established amongst different biochemical and antioxidant parameters using SPSS software (IBM SPSS statistic 19) (Table 14).

Table 14: Correlation matrix showing the interrelation among Proline, B-carotene, lycopene, ABS450, DPPH scavenging, reducing power and AEAC

	Proline	**βcarotene**	**Lycopene**	**ABS_{450}**	**DPPH**	**AEAC**
Proline	1	-0.887	-0.985	-0.998*	-0.982	-0.947
β-carotene	-0.887	1	0.954	0.912	0.958	0.988
Lycopene	-0.985	0.954	1	0.993	1.000**	0.989
ABS_{450}	-0.998*	0.912	0.993	1	0.991	0.965
DPPH	-0.982	0.958	1.000**	0.991	1	0.991

*Correlation is significant at 0.05 level (2-tailed)

**Correlation is significant at 0.01 level (2-tailed)

4.7 Total acidity and pH of Citrus fruits

Citrus fruits contain high levels of citric and ascorbic acid. In this study ripe fruits were used since unripe fruits contain lower pH than ripe fruits (Rekha *et al*., 2012) which would further reduce the pH of the fortified yoghurt below unacceptable levels.

Table 15: Total acidity and pH of citrus fruit juices

Citrus fruit	pH	Total acidity (Citric acid g/100 Ml)
Orange juice	3.8± 0.01	0.8± 0.01
Lemon juice	2.9± 0.02	3.7± 0.01
Tangerine juice	4.1± 0.01	0.6± 0.02

Values shown are means± SD of three replicate experiments

4.7.1 Anti-oxidative properties of Citrus fruit juices

The ascorbic acid content in the fruit juices was estimated by volumetric method, free radical scavenging activity of citrus fruits was determined by DPPH free radical scavenging assay are as shown in Table 16. Ferric reducing assay was used to determine the reducing potential of the fruit juices.

Table 16: Ascorbic acid content, DPPH free radical scavenging activity, Ferric reducing activity and total antioxidant content (AEAC) of three citrus fruit

Citrus fruit	Ascorbic acid(mg/100 ml)	DPPH (%)	Ferric reducing activity (Abs 700nm)	AEAC (mg/L)
Orange	55.25± 0.34	62.06± 2.56	0.98± 1.54	178± 2.49
Lemon	62.82± 0.48	71.29± 3.52	1.34± 0.65	197± 3.65
Tangerine	57.34± 1.54	59.02± 1.67	0.78± 1.92	169± 1.35

Values shown are means± SD of three replicate experiments

4.7.2 Correlation matrix showing the relationship between Ascorbic acid content and Antioxidant potential of Citrus fruits

All the parameters evaluated to show the antioxidant potential of citrus fruit juices showed strong positive correlation (Table 17).

Table 17: Correlation matrix showing the relationship among Ascorbic acid content, DPPH free radical scavenging activity, Ferric-reducing activity and Total Antioxidant content (AEAC) of Citrus fruits.

	Ascorbic acid	**DPPH**	**Ferric reducing activity**	**AEAC**
Ascorbic acid	1	0.872	0.808	0.830
DPPH	0.872	1	0.993	0.997
Ferric reducing activity	0.808	0.993	1	0.999*
AEAC	0.830	0.997	0.999*	1

*Correlation is significant at 0.05 level (2-tailed)

4.8 Overall acceptance of differently flavored fish oil fortified yoghurts

Forty Technical University of Kenya (TUK) students participated in the sensory evaluation of the four types of stirred fortified yoghurts produced.

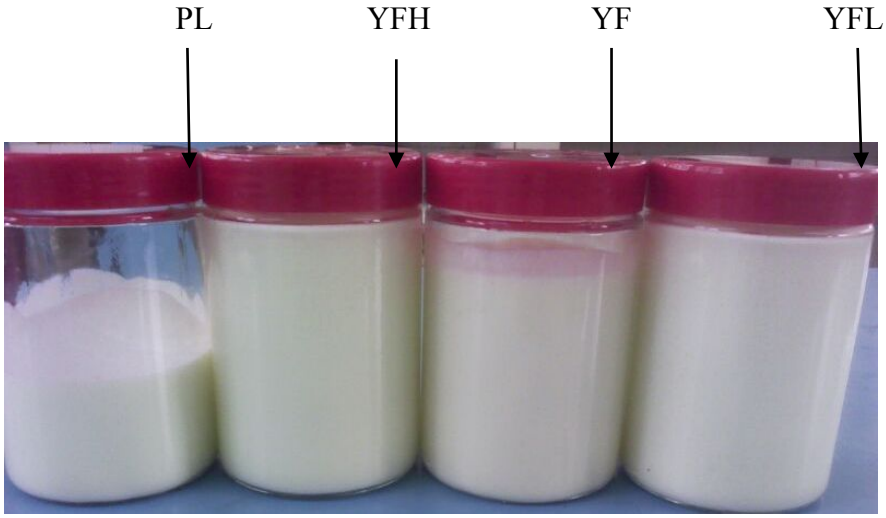


Figure 11: The four types of Plain stirred fortified Yoghurt. YFH= yoghurt fortified with fish oil and honey (Green forest), YF= yoghurt fortified with fish oil, YFL= yoghurt fortified with fish oil and lemon juice, PL=plain natural yoghurt

The evaluation done using a five scale hedonic scale and the means of the attributes evaluated are shown in the mean plot below (Fig. 12). Passion and strawberry flavors were the most liked flavors with mean values of 4.65 and 4.625, respectively (Fig. 12).

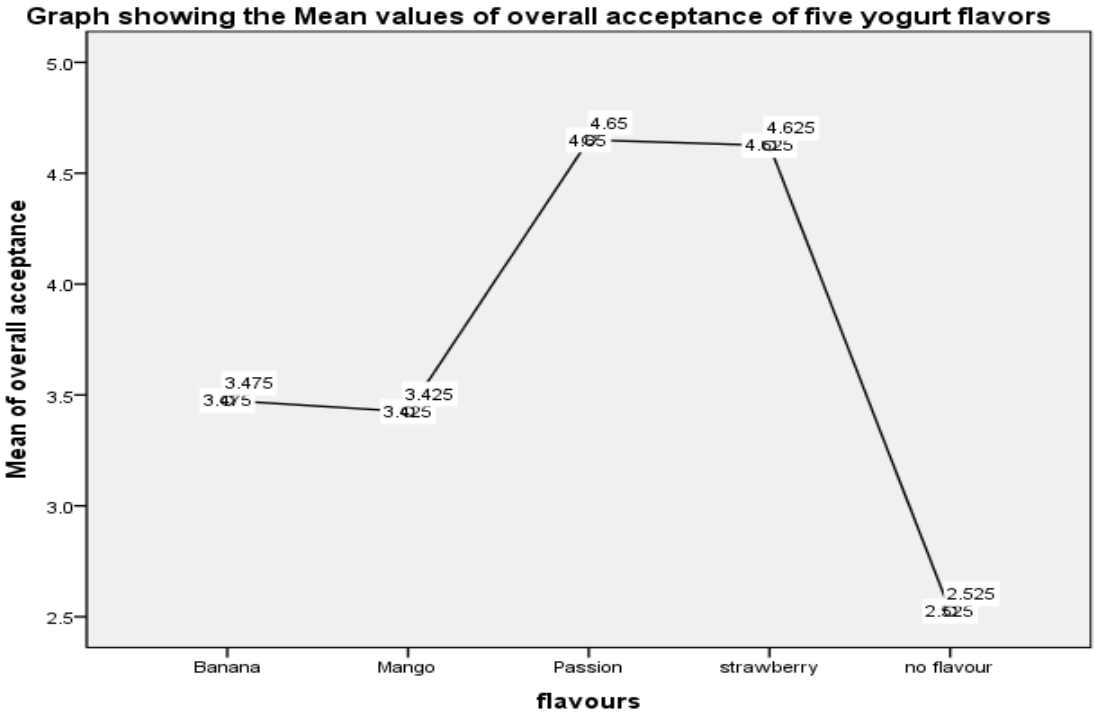


Figure 12: Mean Plot showing mean of overall acceptance of four flavors

The means of the attributes for the four flavors analyzed using one way Anova and Post Hoc Multiple Comparisons (Tukey test) in SPSS software (IBM SPSS statistic 19) showed that the unflavored yoghurt sample was most disliked and was in subset 1 (Table 18). There was no significant difference in the overall acceptance of Mango and Banana flavors as well as passion and strawberry flavors as they were found in the same subsets (Table 18). Overall, the panelist preferred Strawberry and passion flavors whose scores were higher than those of the other flavors. The scores were strawberry (4.63) and passion (4.65). From the hedonic scale these two flavors were liked very much (Appendix 1).

Table 18: Homogeneous subsets of the different flavors

overall acceptance

		Subset for alpha = 0.05		
Flavors	N	1	2	3
no flavor	40	2.53		
Mango	40		3.43	
Banana	40		3.48	
strawberry	40			4.63
Passion	40			4.65
Sig.		1.000	.998	1.000

Means for groups in homogeneous subsets are displayed.

4.8.1 Overall acceptance of fish oil fortified yoghurt with antioxidants (honey and lemon juice)

From the second sensory evaluation, omega-3 fortified yoghurt spiked with strawberry and passion flavors with both antioxidants (ascorbic acid from lemon and honey) scored highly. However, strawberry flavors with both honey and lemon juice were the most liked flavors with mean values of 4.80 and 4.83 respectively (Fig. 13). Yoghurt samples fortified with lemon juice as antioxidant

were more liked compared to those fortified with honey.

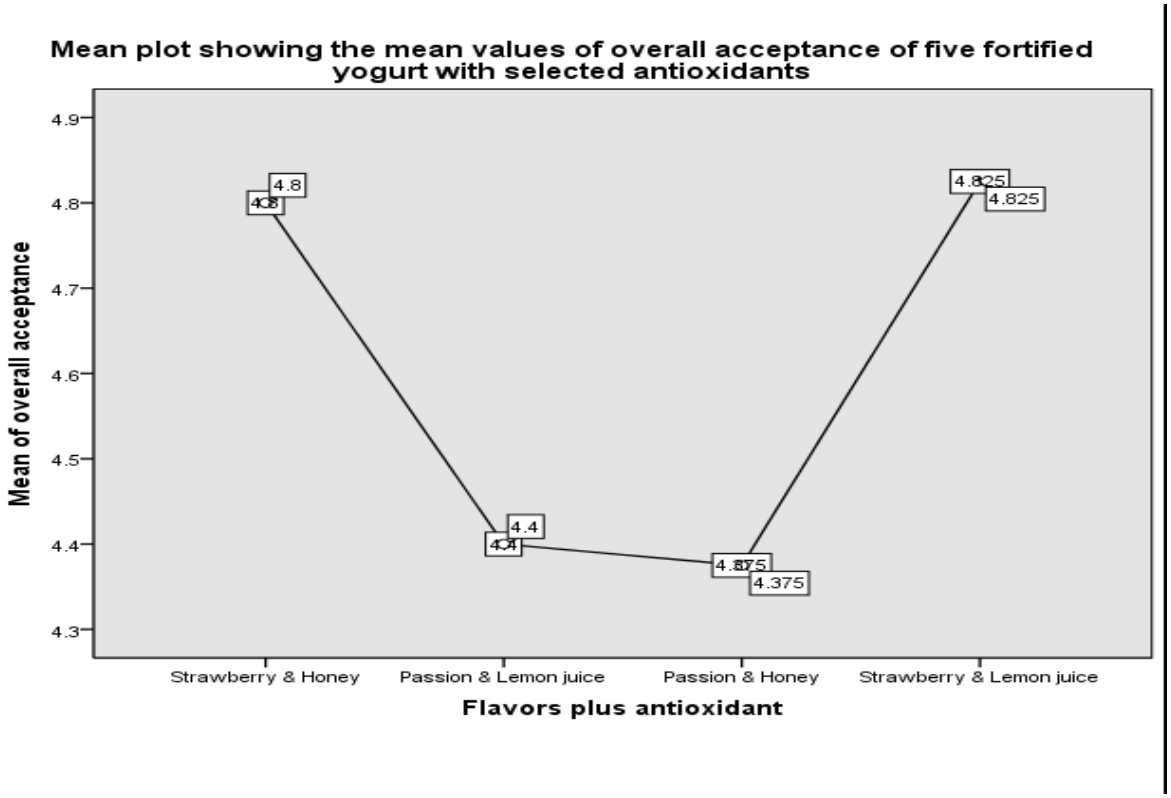


Figure 13: Mean Plot of overall acceptance of four fortified yoghurt samples with antioxidant (honey & lemon juice).

There was no significant difference in the overall acceptance between passion plus Honey/Passion plus Lemon juice fortified yoghurts as well strawberry and honey/strawberry and lemon juice (vitamin C) fortified yoghurts as the two sets were found in the same subset (Table 19). Overall, the panelists preferred strawberry flavors both with lemon juice and honey. From the hedonic scale the strawberry fortified yoghurt samples were liked very much while the passion flavored yoghurt samples were less liked.

Table 19: Homogeneous subsets of Strawberry and Passion flavors with antioxidants

overall acceptance

Flavors plus antioxidant	N	Subset for alpha= 0.05	
		1	2
Passion plus Honey	40	4.38	
Passion plus Lemon juice	40	4.40	
Strawberry plus Honey	40		4.80
Strawberry plus Lemon juice	40		4.83
Sig.		.994	.994

Means for groups in homogeneous subsets are displayed.

4.9 Storage Capacity of fish oil fortified yoghurt with antioxidants

4.9.1 Analysis of lipids extracted from the fortified yoghurt samples

The bands from the TLC separation of the lipids extracted by Dyer & Bligh method showed that the fish oils were present in the fortified yoghurt (Fig. 14). The extracted lipids from the fortified yoghurt were used for analysis during the four weeks of storage.

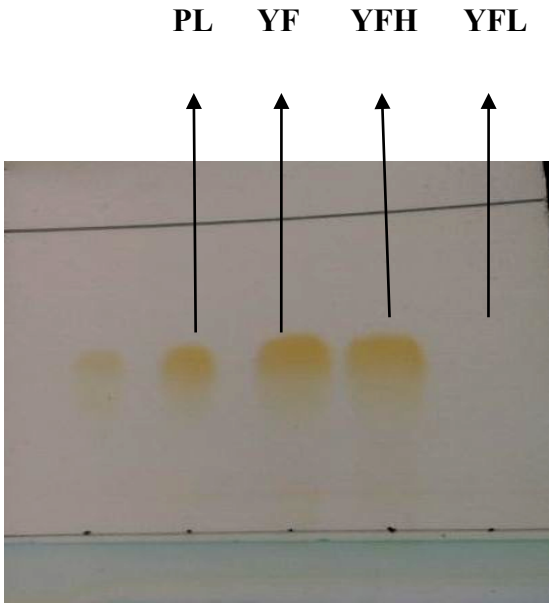


Figure 14: Thin Layer Chromatography (TLC) of lipids extracted by Dyer & Bligh, 1958 method. YFH= yoghurt fortified with fish oil and honey, YF= yoghurt fortified with fish oil, YFL= yoghurt fortified with fish oil and lemon juice, PL=plain natural yoghurt

4.9.2 Changes in Peroxide, Anisidine, Totox and FFA of the fish oil fortified yoghurt over four weeks storage period

All the parameters used to assess the quality of fish oils increased over time (Fig 15, 16, 17 and 18). Significant changes were observed between the sample with no antioxidant and those with the antioxidant.

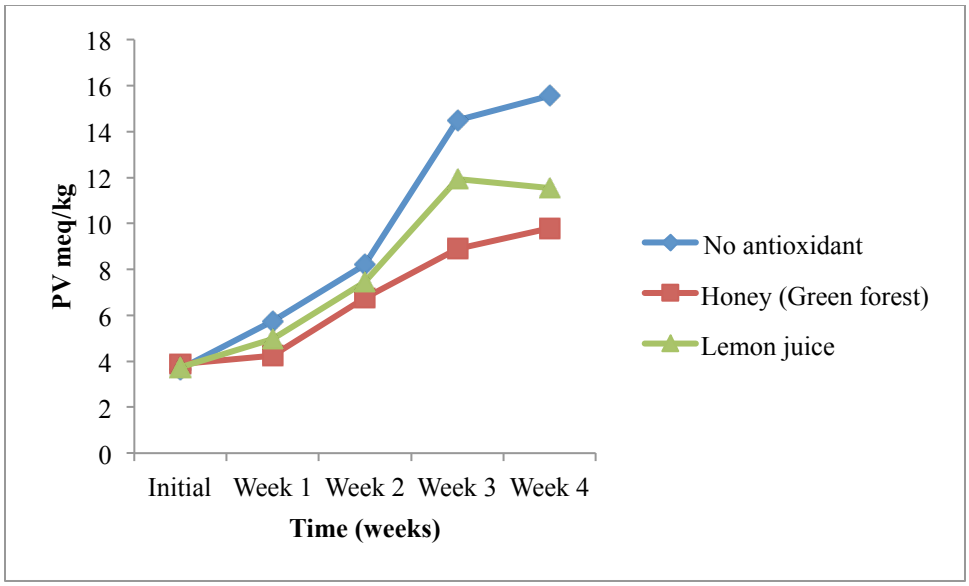


Figure 15: Changes in the Peroxide values of the fortified yoghurt over a four weeks storage period. The honey sample gave the most reduced peroxide values followed by lemon juice and the sample with no antioxidant added giving the highest peroxide values.

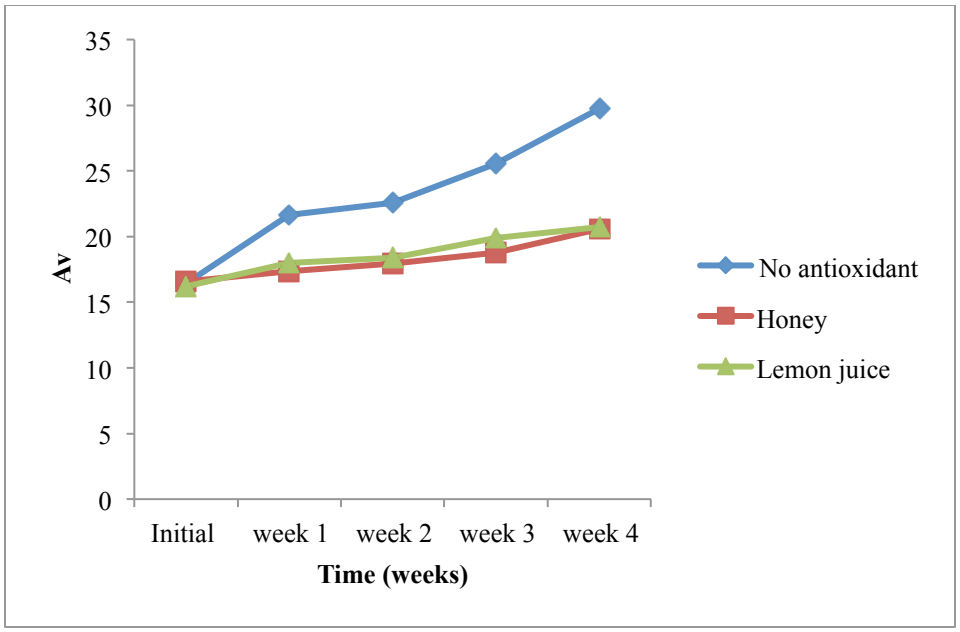


Figure 16: Changes in AV values over time. The Av values increased slightly over time in all the samples meaning there was much reduced secondary oxidation of the fish oils. The samples with antioxidants had the least values compared to the sample with no antioxidant added.

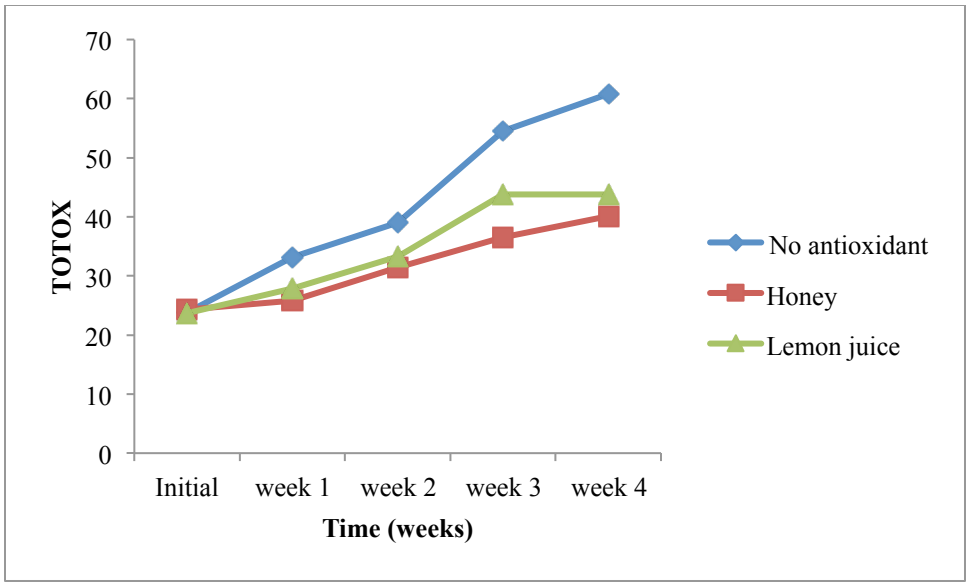


Figure 17: Changes in TOTOX values over time. The TOTOX values increased slightly over time with the highest values in the yoghurt sample without antioxidant added. The sample with honey and lemon juice added as antioxidants differed slightly**.**

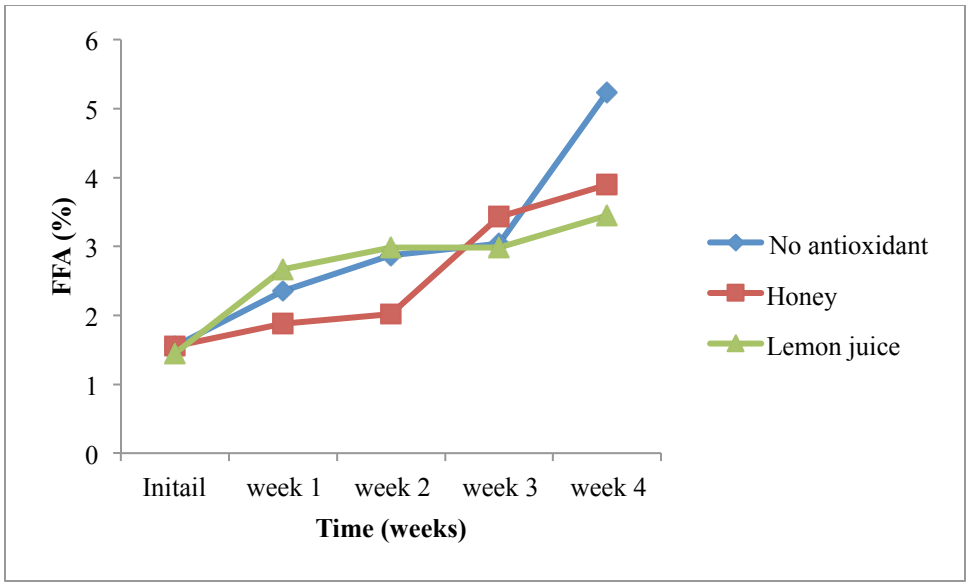


Figure 18: Changes in FFA values (%) over time. There were very little changes observed in all the samples.

4.9.3 Changes in pH, Total acidity and Ascorbic acid content over four weeks storage period

Overall, the pH values in all the samples decreased slightly over time (Fig. 19). The total acidity increased slightly in all the samples with the highest % in the lemon juice fortified yoghurt (Fig. 20). The lemon juice fortified yoghurt sample had the highest vitamin C content compared to all other samples (Fig. 21).

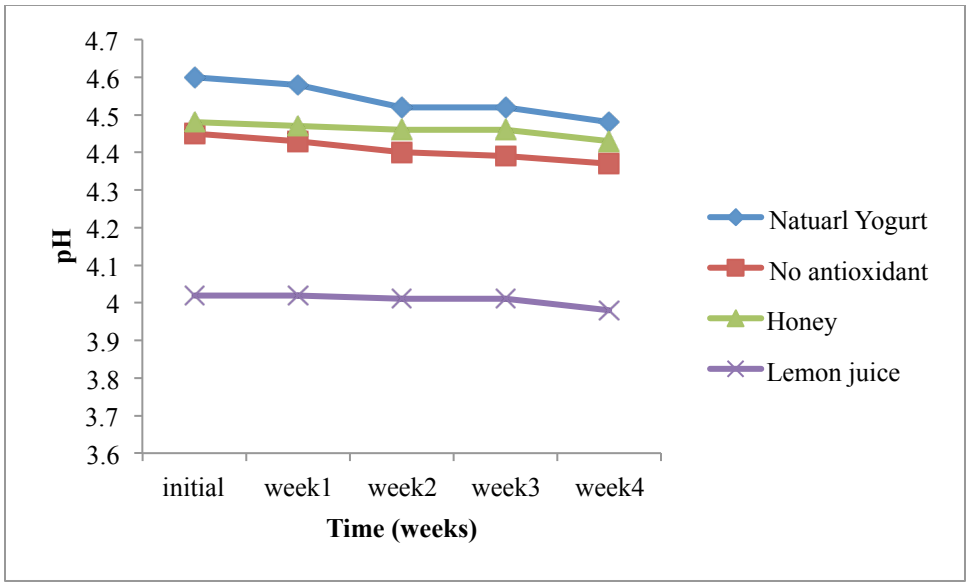


Figure 19 Changes in pH of the different yoghurt samples over four weeks storage period.

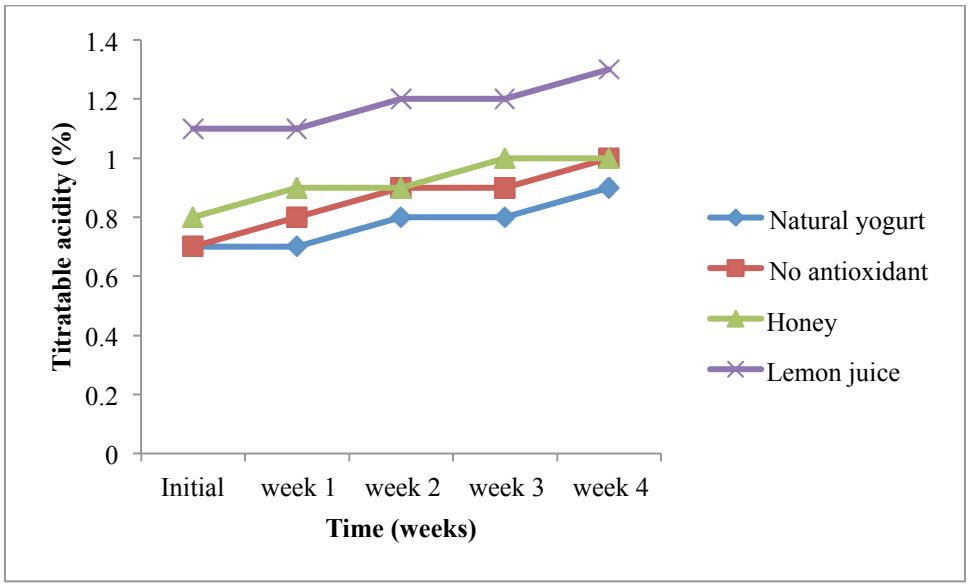


Figure 20: Total acidity (%) of the yoghurt samples over four weeks storage period.

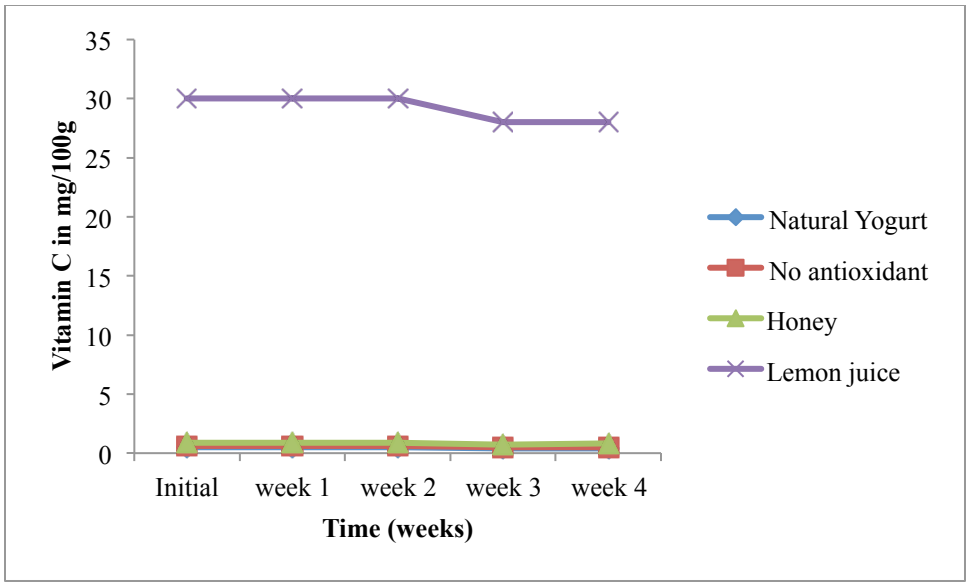


Figure 21: Changes in vitamin C content in the yoghurt samples over time.

CHAPTER 5

5.0 DISCUSSION

5.1 Nile perch fat pads as a rich source of omega-3 fatty acids

Nile perch was chosen in this study as the source of fish oil because it is the major raw material for fish filleting factories in Kenya and thus the biggest contributor of by-products which, if not well utilized poses a great risk to the environment (Kabahenda *et al*., 2009). Nile perch fish fillet constitutes about 37-40% of the total fish and the remaining 60-63% is basically by-products (frames) which in most cases have low commercial value (Kabahenda *et al*., 2009). Frames comprises of the head, skeleton, fins, tail, and guts (Gibbon., 1997). The guts, fat pads and scales are often discarded and the rest of the frames sold to the local people where they are used in making stews (FRRI, 2003).

Previous studies have shown that the fat pads from viscera and belly flaps of Nile perch contained on average 750 mg of oil rich in omega 3 fatty acids from 100 gram of perch (UIAa., 2008). Another report also indicated that oil extracted from the belly flaps of Lake Victoria Nile perch had significant amounts of PUFAs (Ogwok *et al*. 2008). There are many health promoting effects which have been associated with these polyunsaturated fatty acids (PUFAs) which are concentrated in the fish oils (Connor, 2000). Unfortunately, the Nile perch fat remains an underexploited resource (Kabahenda *et al*., 2009). Local women normally purchase these fat pads and use them to deep fry fish and fish by-products because they are cheaper than other commercial fats and oils, and also due to the lack of knowledge that these oils are rich in omega 3 fatty acids with

health promoting effects. However, excessive exposure of these oils to air increase spoilage (lipid oxidation) therefore reducing the functionality of the essential fatty acids.

A study done in Uganda indicated that the people in Uganda were aware that cod liver oil improves children's health and has benefits for the elderly; however, none of them felt that oil from local fish such as Nile perch had health-promoting properties (Kabahenda *et al*., 2009). This therefore calls for the need to sensitize people about the health benefits of fish oil from fish harvested in the region. Nile perch oil is therefore a cheaper source of PUFAs which reduce the risk of many chronic diseases and improve general human health.

5.2 Hydrolysis of the Nile perch fat pads

Hydrolysis without exogenous enzyme gave better oil yield; this is because the fat pads have limited muscles and hence the oils are easily released by heating. Enzymatic extraction of fish oil was done using Alcalase as the enzyme of choice because it has a broad substrate specificity whereby it hydrolyses both native and denatured proteins (Enzim & Ekstrak., 2013; Dauksas *et al*., 2005; Linder *et al*., 2005).This therefore explained the low oil yield obtained due to an emulsion phase formed when the enzyme breaks down peptides present in the fat pads. The emulsion traps some of the oil evident by the thin layer chromatography analysis hence reducing the amount of oil recovered. This study therefore suggested that enough fish oil can be obtained from Nile fat pads by hydrolysis with exogenous enzymes which is cost effective.

Several studies have been done on various fish oil extraction processes and enzymatic extraction of fish oil shown to have better yield as compared to other chemical methods (Rodriguez *et al*., 2010). Some of the advantages include non-use of solvents that are harmful to human health and less energy is required for the extraction process. However no study was found to have been done on the hydrolysis of fish fat pads in the absence of an exogenous enzyme. This is therefore a cost effective and efficient way of extracting fish oils from fish fat pads.

5.3 Wet weight distribution of the different fractions of the extracted fish oil

Hydrolysis with endogenous enzyme gave two phases; an upper oil phase and sludge. The average weight of the oil phase was 60.67 g and 4.76 g sludge. This gave 60.67% oil recovered from 100 g of fat pads. No data was available from other studies to be able to compare the oil recovery without enzyme hydrolysis.

Enzymatic hydrolysis resulted in four layers an upper oil, emulsion, aqueous and sludge phases. Similar observation was reported by Mbatia *et al*., 2010. The amount of oil recovered depends on the amount of protein present in the raw material (Batista *et al*., 2009). The results from wet weight distribution indicated that the average weight was 48.33 g oil, 10.33 g emulsion, 2.67 g aqueous phase and 2.00 g Sludge. This gave 48.33% oil recovery; this reduced recovery could have resulted from entrapped oil in the emulsion phase and loss of much of the oil during the steps following hydrolysis. A study done by Dauskas *et al*. (2005) in cod viscera indicated that lipids were formed in three forms: free oil, emulsion

and sludge with a maximum oil recovery of 82.8% using flavorzyme enzyme and a recovery of 36.4% using Neutrase enzyme. The formation of emulsion is not desirable as it decreases the amount of free oil produced (Batista *et al*., 2009).

5.4 Composition of the Nile perch fish oil

Separation of the fish oils by TLC was achieved; this allowed an insight into the composition of the Nile perch fish oil. Based on the TLC separation, the fish oil comprised of TAGs, DAGs, MAGs, and FFA. Similar results were reported by Mbatia *et al. (*2010). The dominant component of fish oils comprised of the TAGs, meaning that hydrolysis of the fish oils did not occur. Other separation techniques that are more sensitive have been used to fractionate fish oil and also the compositon of the different fractions. Silver ion- TLC has been widely used in the fractionation of tri- glycerides in fish oil according to their degrees of unsaturation (Morris, 1962). Reversed-phase TLC has been used to effectively fractionate cod-liver oil (Kaufmann *et al*., 1964).

5.5 Quality parameters of fish oil

The primary oxidation products of fish oils are measured as the hydro-peroxides which are presented as peroxide value (Pv) (Jacobsen 1999). Fish oil is rich in polyunsaturated fatty acids and hence susceptible to oxidation. Lipid oxidation results from a number of factors such as endogeneous enzymes present in the fish tissue (Lubis *et al*., 1990). The initial PV for all the samples was quite low, indicating that it was not oxidized. The hydrolysate had the highest Pv value of 5.67±0.76 meq/ kg oil, whereas the oily phase and the emulsion phase had a Pv of 4.83±1.75, 4.67±1.26 meq/ kg oil respectively. Similar Pv values are reported in

previous studies for herring oil (Aidos., 2002) and those of Cuban shark liver oil (Garcia *et al*., 2005). According to Gracey *et al*. (1999), fish oil with a Pv of 5 meq/ kg and below can be considered fresh oil or one in which hydro-peroxides have degraded into secondary oxidation products, like ketones and aldehydes. Bimbo, (1998) showed recommended Pv for crude fish oil to be between 3-20 meq/kg.

The Anisidine value measures the secondary oxidation products such as aldehydes. The Council for Responsible Nutrition (2006) set a fish oil quality standard of an Av less than or equal to 20, while Bimbo, (1998) showed the recommended Av to be 4 - 60. The Av was determined to be 16.50 ± 2.14, 18.17 ± 1.88 and 20.81 ± 1.27 for oil phase, fish protein hydrolysate and the homogenate respectively. All the samples had acceptable levels (Table 5) with the homogenate having the highest Av value. The Av values were higher than the Pv values. This results from rapid decomposition of hydroperoxides into secondary oxidation products which are highly unstable (Frankel *et al*., 1998). These secondary oxidation products are usually determined using Av; this therefore results in decreased Pv values and increased Av values (Frankel *et al*., 1998).

The homogenate had higher TOTOX values of 32.15 ± 1.42 compared to the oil phase and Emulsion phase that had values of 26.16 ± 1.09 and 32.15 ± 1.42 respectively. All the samples had TOTOX values below the recommended values. The FFA values for the three fractions were 2.16 ± 0.22, 1.55 ± 0.14 and 2.71 ± 0.43 for the oil phase, emulsion phase and the homogenate respectively. The FFA

content was well below the recommended values of 3% in all the samples (Gracey *et al*., 1999). Due to the polar nature of FFA, they are normally accumulated preferentially in the protein fraction rather than in the oil fraction and hence the reduced FFA content in the oil fraction (Addison *et al*., 1969). FFA is an important quality parameter because they are more susceptible to oxidation compared to esterified fatty acids thus resulting in reduction of the shelf-life of the oil. They are generated as a result of hydrolysis of fish oils due to high moisture content present in fish oil (Addison *et al*., 1969).

5.6 Biochemical parameters of the honey samples

Phenolic compounds influence not only the appearance but also the functional properties of honey (Cimpoiu *et al*., 2013). The honey samples analyzed contained significant levels of phenolic compounds as shown in Table 11. The Phenol content per 100 g of honey ranged from 58.56 mg gallic acid Equivalent (GAE) to 71.56 mg GAE. The commercial honey samples had higher phenolic content compared to the natural honey sample. Phenoilc compounds are known to be the greatest contributors to the anti-oxidative potential of honey (Meda *et al*., 2005; Alisi *et al*., 2011).The phenolic content of the samples is in agreement with phenolic content of honey reported in literature (Beretta *et al*., 2005; Gheldof *et al*., 2002). A general observation can be made that dark honeys (Amboseli and Green forest) were characterized by considerably higher phenolic content than the natural honey samples. This trend is similar to the relationship found in previous studies done on Burkina Faso and Italian honeys (Blasa *et al*., 2006; Meda *et al*., 2005).

Proline is the most abundant amino acid in honey and it is produced from the salivary secretions of bees during the conversion of nectar into honey (Hermosin *et al*., 2003). The content of proline in honey is used to assess the quality of honey (Meda*et al*., 2005). Very reduced proline content in honey shows adulteration (Bogdanov *et al*., 2000). The natural honey sample had good proline content of 2.41± 0.24 (mg/g), whereas the Amboseli and Green forest honey samples appeared adulterated evidenced by the low proline. The higher amount of proline in a honey sample indicates the honey's ripeness and that there is less probability for sugar adulteration. Our results indicate that the proline content in our honey samples is similar to those reported for Bangladeshi honey (1.06–6.81 mg/g) **(**Alvarez-Suarez *et al*., 2009)**.**

Carotenoid such as β-carotene and lycopene were more in the processed honey samples than in the natural honey (Table 6). The higher β-carotene content in the processed honey samples accounted for their darker appearance compared to natural honey samples. The ABS_{450} defines the color intensity of honey and also indicates the presence of pigments such as carotenoids which are known to have antioxidant properties (Terrab *et al*., 2002). The ABS_{450} of the Natural honey (219 ± 23.43 **mAU)** was lower than that of the processed honey samples with Amboseli honey having highest ABS (Table 11). This suggested a lower antioxidant activity in the natural honey compared to processed honey.

5.6.1 Honey as a natural antioxidant

The antioxidant activity of honey varied from 65.7 % to 86.2 % in the DPPH reaction system (Table 7). The results of the DPPH radical scavenging activity showed that the processed honey (Amboseli and Green forest) tended to be highly active in the reaction with DPPH, while Natural honey (Baringo) had a lower radical scavenging activity. The higher antioxidant activity in processed honey could be due to the high proline content as shown in Table 12. The DPPH reducing activity of both natural and processed honey was however lower than that of commercial antioxidants. Ascorbic acid was shown to have a higher DPPH radical scavenging activity (96.56± 1.09) compared to α-tocopherol (93.93± 0.84).

The Baringo sample had lower AEAC (280 ± 0.56) compared to the Amboseli (305 ± 1.23) and Green forest (312 ± 2.34) mg of AEAC/kg of honey (Table 12). Green forest honey had higher reducing activity (2.78 ± 1.69) compared to Amboseli (2.56 ± 1.23) and Baringo (0.98 ± 2.54) (Table 12).These results indicated that all the honey samples had high antioxidant potential with the Green forest honey sample possessing the highest antioxidant acidity and was therefore used for the fortification process.

5.6.2 Interrelation between biochemical parameters and anti-oxidative properties of the honey samples

Several strong correlations were established amongst the biochemical and antioxidant parameters of the honey samples evaluated. A strong correlation was

found between the color intensity of honey samples and antioxidant parameters (DPPH and AEAC) (Table 13). Strong correlations between the β-carotene, lycopene and AEAC suggest that these components contribute to antioxidative capacity of honey (Ferreira, *et al.*, 2007). These findings suggest that honey color pigments such as β-carotene and lycopene may have a role in the observed antioxidant activities of honey samples. Similar to our findings, a strong correlation between the antioxidant capacity and ABS_{450} was reported by Bertoncelj *et al*. (2007) and Beretta *et al*. (2005) indicating that honey color intensity may be treated as a good initial indicator of its antioxidant capacity.

The correlation between phenolic content and antioxidant activity of the honey samples examined is presented in Table 13. Statistical analysis showed that there was a strong positive correlation between the antioxidant activity and total phenolic content (PC) (R^2=0.967 for PC/DPPH•, R^2=0.976 for PC/Ferric reducing assay, R^2=0.993 for PC/AEAC. This means that phenolic compounds are one of the main components responsible for the antioxidant activity of honeys. This correlation was in agreement with the findings of other authors such as the high correlation between radical scavenging activity and the total phenolic content at a level of p=0.5, (Meda *et al*., 2005). The positive correlations between DPPH and total phenolic compounds suggest that they are the strongest contributing factor to the radical scavenging activity of the honey samples. Overall, the strong positive correlations suggest that the honey samples had a strong antioxidant potential that could be utilized to prevent fish oil oxidation in the omega-3 fortified yoghurt.

Negative correlation was evident between proline content and all other parameters. This suggests that proline content is a measure of the quality of honey and does not contribute to the anti-oxidative potential of honey.

5.7 Vitamin C (ascorbic acid) from citrus fruits as an antioxidant

Citrus fruits belong to the family rutaceae and comprises of 40 species (Bocco *et al*., 1998). They are one of the most important fruits being consumed fresh or as juice due to high nutritional value and flavor and contain a range of key nutrients including high levels of vitamin C (Bocco *et al*., 1998). Ascorbic acid is highly bio-available and is consequently the most important water soluble antioxidant vitamin in cells, effectively scavenging reactive oxygen species (ROS) (Ebrahimzadeh *et al*., 2004). When relating the antioxidant activities of fruit juices to health and disease risk, it is important to consider the contribution of ascorbic acid in addition to that of phenolic compounds with antioxidant activity (Gardner *et al*., 2000). All fruit juices tested in this study were found to contain high levels of ascorbic acid. Antioxidant activity was found to be higher in lemon juice which also had the highest level of ascorbic acid. This is in accordance with results obtained in previous studies by (Gardner *et al*., 2000). Gardner *et al*, (2000) also showed that vitamin C is the main antioxidant in most of the citrus fruits.

The pH was lowest in lemon juice (2.9± 0.02) because lemon juice contains higher amounts of ascorbic acid in addition to citric acid. Orange and tangerine juice had a pH of 3.8 ± 0.01 and 4.1 ± 0.01, respectively. The total acidity was

higher in lemon juice followed by orange juice and then Tangerine (Table 14). pH and total acidity are important in determining the level of acidity of the juices which may affect the acidity of the yoghurt once added. The ascorbic acid content in the fruit juices ranged from 55.25 ± 0.34 to 62.82 ± 0.48 mg/100 ml (Table 15). Highest ascorbic acid content was observed in lemon juice followed by orange and tangerine.

Radical scavenging activities of citrus juices investigated by DPPH radical scavenging assay showed that lemon juice had the strongest scavenging activity (71.29 ± 3.52 %) compared to orange (55.06 ± 2.56) and tangerine (51.02 ± 1.67). Ferric reducing assay showed that lemon juice had the strongest activity. This was in agreement to a study done by Ali *et al*. (2011) which showed that citrus fruits had a high DPPH radical scavenging and ferric reducing antioxidant potential. Although the antioxidant capacity evaluated by the DPPH method was higher than that evaluated by the Ferric reducing assay method, the correlations were good for both methods. This indicated that ascorbic acid contributed to the antioxidant activity of the citrus fruits. The total antioxidant activity was highest in lemon juice compared to orange and tangerine juice. Total acidity was high in the lemon juice which also had high antioxidant potential hence, positive relationship between acidity and antioxidant potential.

Other than the antioxidant potential of citrus fruits, a recent study by the World Health Organization showed convincing evidence of positive effects obtained

from dietary intake of citrus fruits on cardiovascular disease (WHO, 2003). They have also been shown to possess anti-inflammatory, antioxidant, antitumor and antifungal activities (Ghafar *et al*., 2010).

5.7.1 Interrelation between biochemical parameters and anti-oxidative properties of the Citrus fruit juices

A strong positive correlation was evident between ascorbic acid content and total antioxidant activity ($R^2 = 0.997$). Citrus fruits are a good source of vitamin C and they also possess good antioxidant activity (Berenguer *et al*., 2004). Lemon juice was the best in terms of ascorbic acid content and hence highest antioxidant capacity, it is available every season and very economical compared to the other studied fruits. Therefore, lemon juice was chosen for the fortification process of the yoghurt.

5.8 Overall acceptability of the fortified yoghurt

The most accurate way to do sensory evaluation of quality parameters in fish oil is to use a taste panel, as humans can be trained to be able to detect very low levels of volatile components that traditional tests of oxidation cannot (Carlson *et al*., 2010). Unfortunately, these panels are expensive to establish and maintain and this therefore, makes it difficult for them to be used (Carlson *et al*., 2010). To determination the oxidative stability, shelf life and consumer acceptance of such fortified products, methods for the determination of lipid oxidation can be ranked in the following order: sensory analysis > Pv > Av (Frankel, 1998).

Fruit flavors are very well accepted in yoghurt. In this study the panel was not made aware of the flavor system in the yoghurt so as to avoid a pre-conceived

bias against the flavors. Both overall acceptability and flavor acceptability scores for the fortified yoghurt with fish oil are presented (Figure 12 and 13).This study suggests that fortification of yoghurt with omega-3 rich fish oil could be acceptable to the greater general population and hence have a potential market among health- and nutrition-conscious consumers. In addition, fish oil–enriched yoghurt is highly stable to oxidation, with the addition of natural antioxidants such as honey and vitamin C.

5.9 Storage capacity of the fortified yoghurt

5.9.1 Changes in Peroxide, Anisidine, Totox and FFA values of the fish oil fortified yoghurt over four week storage period

Fish oils are highly susceptible to oxidation due to the high concentration of long-chain polyunsaturated fatty acids (eicosapentaenoic acid and docosahexaenoic acid) (Antolovich *et al*., 2001). Apart from sensory evaluation, the formation of lipid oxidation compounds is evaluated by measuring the PV and AV of fish oils (Carlson *et al*., 2010). This is because it is has been studied that both of these measures of oxidation have little relationship to the sensory properties of fish oil (Frankel, 2005; Jacobsen, 1999).

The PV, AV, and TOTOX values of the omega-3 fortified yoghurt increased slightly over time in all the samples with the honey having the least values followed by and lemon juice and the highest values seen in the sample without antioxidant added. The FFA value in the lemon juice fortified sample was higher than the sample with no antioxidant up to the third week. This is because FFA

estimation is a titration method and thus the increased acidity in this sample gave the high FFA content. In the fourth week a gradual increase in FFA in all the samples was observed overtime with the control (the sample with no antioxidant) having the highest FFA values. When comparing samples with added antioxidants with those without anti-oxidants, it was found that these samples had lower values of these quality parameters. The antioxidants therefore, helped to reduce lipid per-oxidation of the fish oils. Honey was a better antioxidant compared to lemon juice. This is because honey has several compounds that play a critical role in antioxidant activity such as phenols, flavonoids, carotenoids (Ferreira *et al*., 2007) compared to lemon juice which has only ascorbic and citric acid as the major compounds enhancing its antioxidant activities.

The addition of natural antioxidant to the fortified yoghurt reduced lipid per-oxidation as compared with the control sample with no antioxidants. Nevertheless, formation of oxidation products such as peroxides still occurred even in the presence of antioxidants, this is because peroxides manifest a transitory nature, and over time they are converted to secondary products of oxidation (Pritsche *et al*., 1988). Secondary products of lipid per-oxidation are measured by AV which also increased over time. In contrast to PV, secondary products formation depends on specific site oxidation accompanied by serial decomposition (Dahle *et al*. 1962). This therefore, suggests the possibility of subsequent conversion of the secondary products of lipid per-oxidation to other related compounds, such as aldehydes and alk-2-enals (Kosugi *et al*. 1987). From

this study, honey and vitamin C can be used as natural antioxidants in yoghurt fortified with omega-3 rich fish oil thereby extending its shelf life.

5.9.2 Changes in pH, total acidity and ascorbic acid content over four weeks storage period

Overall, the pH values in all the samples decreased slightly over time (Fig. 19). The sample with lemon juice as antioxidant had very low pH due to the presence of citric acid in addition to Ascorbic acid in the juice. The other three samples (Natural yoghurt, YF, YFH) had close pH values with the plain natural yoghurt having the highest pH values. The total acidity increased slightly in all the samples with the highest percentage in the lemon juice fortified yoghurt (Fig. 20). Starter cultures usually transform lactose in milk into lactic acid which is responsible for the initial acidification which coagulates the milk at pH 4.5 and also the post acidification during storage. The changes in pH and total acidity results from persistent metabolic activity of starter cultures which become reduced over time but not completely stopped (Rasic & Kurmann, 1978). The lemon juice fortified yoghurt sample had the highest vitamin C content compared to all other samples (Fig. 21). Milk has very little vitamin C, honey and lemon juice have considerable amounts of vitamin C and therefore in addition to offering anti-oxidative properties to the fish oils, they also increase the daily intake of vitamin C.

CHAPTER 6

6.0 CONCLUSION & RECOMMENDATIONS

6.1 Conclusion

In recent years, research has been pursued towards the fortification of commonly consumed foods such as yoghurts with micronutrients and functional food ingredients so as to enhance their nutritional quality. The production of nutritionally superior quality yoghurt enriched with omega-3 fatty acids rich oil as functional food lipids can be successfully achieved. This kind of yoghurt is desired for general body health function and in protection against Cardio-Vascular Diseases (Erkkilä *et al*., 2006; Tavazzi *et al.*, 2008). In addition, incorporation of natural antioxidants in the fortified yoghurt helps in fighting free radicals in the body.

Fortification of yoghurt with omega-3 rich fish oil delivers sufficient heart-healthy omega-3 fatty acids so as to achieve the suggested daily intake of 150 mg/day (Kris-Etherton *et al*., 2000) and this can be accomplished in a single serving (150 g) of yoghurt. This provides an alternative and easily incorporated source of omega-3 fatty acids. This innovation of omega-3 fortified yoghurt is of great interest to a large group of nutrition and health cautious population and hence potential market for this product exists.

Nile perch by-products if well stored have good storage stability. Omega-3 rich oil with low oxidation products can be produced from the stored by products. The greatest challenge in the addition of these omega-3 rich oils to yoghurt as a source of n-3 FA is the potential “fishy” flavor, yet the goal is to add a sufficient level of

oil that can provide a significant contribution of omega-3 fatty acids into the daily diet while minimizing the fishy flavor. This therefore calls for the need to add natural antioxidants and flavors to reduce the fishy flavor.

Several natural antioxidants can be exploited to help delay oxidation of the fish oils in the fortified yoghurt. In our study honey and lemon juice were shown to have good anti-oxidative properties and this helped delay oxidation of the fish oils in the fortified yoghurt. Citrus fruits, which are consumed for their nutritional value, have also great antioxidant activity since they are rich in ascorbic acid.

6.2 Recommendations

1. Establishment of the proximate composition of the fortified yoghurt sample in comparison to natural yoghurt.
2. The use of Gas chromatography tool to establish the levels of Omega-3 fatty acids in the fortified yoghurt sample over one month storage period.
3. The use of a consumer panel for sensory evaluation of the fortified yoghurt should be done to establish the acceptance of the product in the general population.
4. Studies on the effectiveness of synthetic antioxidants in comparison to natural antioxidants on the fortified sample.
5. Microencapsulation of omega-3 fatty acids before fortification to mask fish flavor

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APPENDICES

Appendix 1: Questionnaire used in sensory evaluation tests.

Code: ……... **Date:** ……

You have been provided with 5 different flavored yoghurt samples for sensory evaluation. Please rank them in order of preference as follows:-

Dislike very much ……**1**

Dislike a little……**2**

Not sure……**3**

Like a little……....**4**

Like very much……**5**

Attribute	**Sample no**	**331**	**171**	**204**	**131**	**442**
Taste						
Aroma						
Color						
Flavor						
Mouth feel						
After taste						

Comments: