DETERMINATION OF THE MUTAGENICITY OF FOUR VEGETABLE OILS AFTER HEAT TREATMENT

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DECLARATION

I hereby declare that this is the result of my own work and that there has not been any previous submission for a Master of Science degree here or elsewhere. Also, work by others that served as sources of information have been duly acknowledged by making references to the authors.

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DEDICATION

This dissertation is dedicated to the memory of my late Father and Father - in- law for their encouragement and support, but who unfortunately did not live to see the completion of this work.

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ABSTRACT

Background: Vegetable oils are used either in the raw state or heated in the preparation of foods worldwide. Due to the nutrition transition, there is increased demand for fast cooked foods or convenience foods which involves the use of heated and repeatedly heated oils. Research has demonstrated that high temperature cooking with oils can result in the formation of mutagenic compounds that on consumption may induce the development of cancers. This study aimed at determining the mutagenic potential of red palm oil (salted and unsalted), *frytol* (refined bleached deodorized palm olein) and virgin coconut oil after heat treatment.

Method: Samples of the four vegetable oils were subjected to repeated heating in a convectional hot air oven at 180^{o}C for ten minutes holding time and at least five hours cooling intervals between heatings. The Muta-ChromoPlate™ test kit employing the bacteria test strains *Salmonella typhimurium* TA 100 and TA 98 was used in the determination of mutagenicity.

Results: In the *S. typhimurium* TA 100 experiments, virgin coconut oil and *Frytol* showed no mutagenic activity unheated. Repeatedly heated *Frytol* also showed no mutagenic activity. However, unheated salted and unsalted red palm oil samples showed significant mutagenic activity; (p=0.01) and (p=0.001) respectively, in the *S. typhimurium* TA 100 experiments. However, once heated and five times heated *Frytol,* and unsalted red palm oil showed significant mutagenic activity (all p-values <0.05) in the *S. typhimurium* TA 98 experiments.

Conclusions: Repeated heating had an effect on the mutagenicity of the vegetable oils in the study. *Frytol* and virgin coconut oil showed the least mutagenic activity comparatively. Unheated red palm oil (salted and unsalted) on the local market was highly mutagenic, and this has serious health implications.

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BHT	butylated hydroxy toluene
BMI	Body Mass Index
DMBA	7,12- dimethylbenz-α – anthracene
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
ECOWAS	Economic Community of West African States
FFA	Free fatty acid
FFB	Fresh fruit bunches
HPLC	High pressure-powered liquid chromatography
PO	*Frytol* (refined, bleached, deodorized palm olein)
PUFA	Polyunsaturated fatty acid
RBDCO	refined, bleached, deodorized coconut oil
RCO	Repeatedly heated coconut oil
RPO	*Zomi* Red Palm Oil (salted)
RRPO	Red Palm Oil (unsalted)
t-t-2,4-DDE	trans-trans-2,4-decadienal
VCO	Virgin Coconut Oil

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND

Edible vegetable oils form a component of the diet of many around the world. Eighty percent of the market share for vegetable oils is for food use with the rest being for industrial uses such as bioenergy production (Rosillo-Calle *et al*., 2009). Developing countries in particular China and other Asian countries continue to dominate the rise in vegetable oil consumption (OECD-FAO, 2011). The main vegetable oils commonly used in food preparation globally are palm oil, rapeseed oil, sunflower oil and soybean oil (Rosillo-Calle *et al*., 2009).

Oil is an excellent heating medium because it allows high rates of heat transfer into foods being cooked. However, the frying process also causes a number of chemical, physical, sensory and nutritional changes in the foods (Singh, 1995). Physico-chemical changes in the oil are due to oxidative changes through atmospheric oxygen entering the oil, hydrolytic changes due to the product being fried and thermal changes due to the oil being maintained at high temperatures (Singh, 1995). Over the years, research has demonstrated that the heating process renders some oils mutagenic resulting in changes in the DNA structure or chromosomal changes which may lead to carcinogenesis (Wu and Yen, 2004; Dung *et al*., 2006).

Non-communicable diseases including cancer are now a major public health burden in every region of the world (AICR, 2007). In Ghana, epidemiological studies show a steady increase in prevalence rates of non-communicable diseases since the 1950's (Agyei-Mensah and de-Graft Aikins, 2010). A number of cohort studies have linked diet to a higher risk of prostate, breast and colon cancers (Nelson *et al*., 2003). Research at the Sidney Kimmel Comprehensive Centre at John Hopkins School of

Medicine has led to discoveries that diet, inflammation and gene 'silencing' have roles to play in prostate cancer development. The Western diet of too high animal fats and insufficient fruits and vegetables as well as the type of food processing i.e. high energy heat from frying and grilling, induce the formation of heterocyclic amines and polycyclic aromatic hydrocarbons (Nelson *et al*., 2003). These compounds are the most mutagenic agents formed during cooking and high temperatures induce their formation. Data available indicate that cooking oil has little mutagenic potential when heated below 100^{o}C and high mutagenic potential above 230 ^{o}C (IARC, 2010).

The Shanghai Breast Cancer Study, a population based case-control study carried out between August 1996 to March 1998, evaluated the association of breast cancer risk with the consumption of animal foods and soybean cooking oils, according to cooking methods used and Body Mass Index (BMI) of participants. There was a positive association between intake of red meat and fresh water fish with breast cancer in those who ever used the deep fry method in cooking red meat (Dai *et al*., 2001). High consumption of soybean cooking oil was also associated with a reduced risk of breast cancer among those who did not use the deep fried cooking method (Dai *et al*., 2001). The positive association of breast cancer risk with well-done deep-fried red meat due to mutagens or carcinogens that originate from burnt meats and heated cooking oils was also observed (Dai *et al*., 2001).

Today, many food products are heat processed and the method of food preparation may have a significant impact on health. Modern man's demand for fast cooking methods has changed dietary habits to include more fried than boiled meat (Skog *et al*., 2003). The cost of cooking oils and the quest to increase profit margins by commercial food processors means that used oils are not discarded but are re-used several times.

There is no systematic way of disposal of used oils in Ghana as exists in developed countries. In Ireland for example, about 7,000 tonnes of recovered vegetable oil are collected each year (Fröhlich and Rice, 2005). The use of this material in animal feed rations has been banned under European Union legislations because of food safety and animal health concerns. Restrictions on their disposal in landfills are forcing more vegetable oil users to find other outlets for their waste oil such as biodiesel production (Fröhlich and Rice, 2005). In Ghana, some of these used vegetable cooking oils are finally converted to *shito,* a fried pepper sauce which is eaten as an accompaniment with other foods.

Illiteracy and ignorance on the part of food processors and consumers alike may increase the likelihood of exposure to the dangers posed by consuming such high temperature processed foods. Consumers who depend on such foods are often more interested in its convenience than in questions of its safety, quality and hygiene (Mensah *et al.*, 2002). According to the American Institute of Cancer Research (AICR, 2007) more than 30% of tumours are as a consequence of our diet. Every year, an estimated 11 million people worldwide are diagnosed with cancer (excluding skin cancers), and nearly seven million people are recorded as dying from cancer. Their projections for 2030 predict that these figures will double. Cancer is increasing at rates faster than the increase in global population (AICR, 2007). A retrospective review of autopsy cases from1991-2000 at Korle-Bu Teaching Hospital, Accra, showed that malignant neoplasms accounted for 914 (2.6%) of all 34,598 admissions, and 141 (5.6%) of all 2,501deaths in the year 1996 (Wiredu and Armah, 2006).

The need for tools able to predict chemical carcinogens in less time and at a lower cost in terms of animal lives and money is still a research priority. Inexpensive and fast tests such as the Ames mutagenicity test are able to identify up to 90% of

carcinogens, (Benigni and Bossa, 2011). Due to the common use of the frying method of cooking foods, determining the mutagenicity of these oils as they go through food processing would contribute to knowledge in the dietetic practice. This would help inform and influence best practices in the preparation of foods in order to guarantee safety from mutagens and thus reduce risks of developing non communicable diseases such as cancers through the promotion of healthy eating.

1.2 PROBLEM STATEMENT

Ghana is currently going through the nutrition transition resulting in changes in the traditional diet. The high fat diet typical of the Western diet is gradually infiltrating into the traditional Ghanaian diet. Due to industrialization and urbanization, more people eat out of home than before and as such rely on foods commercially produced by restaurants and food vendors (Agyei-Mensah and de-Graft Aikins, 2010). Most of these foods have been subjected to high temperature cooking such as frying, grilling and baking. Evidence from research indicates that the composition of vegetable oil changes with heat processing. Chemical compounds which are mutagenic are formed as these oils undergo heat treatment. These mutagens in food can lead to genetic mutations that can eventually lead to the development of cancers. Ghana like other African countries is going through an epidemiological transition and suffering from the 'double burden' of disease (communicable and non-communicable). Analyses of causes of death show that in 2001 neoplasms were the fourth leading cause of death in Accra (Agyei-Mensah and de-Graft Aikins, 2010). It is therefore important to identify risk factors of these diseases and through education help to improve the health status of the populace, and reduce the burden of disease to the economy and the individual.

1.3 JUSTIFICATION

Fats and Oils in our diet supply a major component (30%) of our daily energy requirements (Mahan *et al*., 2012). Their use enhances the nutritive value of food by making it edible and digestible. They also improve the texture and impart flavour to food. However, the method of cooking with oils may introduce harmful compounds (mutagens), developed in the cooking process.

Most cooking methods in Ghana use high temperatures such as frying, grilling and baking. Due to economic reasons, the oils are repeatedly heated in the preparation of foods. This is so especially for commercial food processors in order to increase profit margins. Industrialization and urbanization has resulted in a boom in fast food enterprises and food vendors, due to increased demand for out-of-home meals (Agyei-Mensah and de Graft Aikins, 2010). Fried foods are fast to prepare and easy to handle in the service of their clients. Poverty, illiteracy and ignorance on the part of food processors and consumers alike may increase the risk of exposure to mutagenic compounds. It is therefore of public health relevance and dietetic interest to identify these risk factors that may contribute to the increase in non-communicable diseases through current dietary practices, and thus inform health professionals in the education of the populace.

1.4 THE AIM OF THE STUDY

This study determined the mutagenic potential of red palm oil (unsalted), *zomi* red palm oil (salted), *frytol* (refined bleached and deodorized palm olein) and virgin coconut oil when subjected to repeated heating at optimal frying temperatures.

1.4.1 SPECIFIC OBJECTIVES

The specific objectives were;

1. To determine whether repeated heating had an effect on mutagenicity of the oils
2. To measure the degree of mutagenicity in relation to repeated heating of the oils.
3. To determine which oils had more mutagenic potential over the others.

1.5 HYPOTHESIS

Temperature and repeated heating has an effect on the mutagenic potential of the vegetable oils under study.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 VEGETABLE OILS

Edible vegetable oils are foodstuffs which are composed primarily of glycerides of fatty acids obtained only from vegetable sources. They may contain small amounts of other lipids such as phosphatides, unsaponifiable constituents and free fatty acids naturally present in the fat or oil (Codex Standards, 1999).

Virgin oils are obtained without altering the nature of the oil, by mechanical procedures such as expelling or pressing and the application of heat only. They may have been purified by washing with water, settling, filtering and centrifuging only. Cold pressed oils are obtained in a similar manner. They differ, in that there is no application of heat in the processing of cold pressed oils. Food additives are not permitted in virgin or cold pressed oils (Codex Standards, 1999). Vegetable oils come from oil bearing seeds such as palm nuts, corn, groundnuts, coconut, soybeans and olives.

The most common uses of edible vegetable oils include: as shortening for baked foods such as pastries and breads, to improve food texture, as a medium for cooking procedures such as frying, and as a base for flavoured content, marinades and salad dressings. Fats and oils are the most energy dense constituents of food supplies and diets. Their contribution to total dietary energy increases with industrialization and urbanization (AICR, 2007). The consumption of vegetable oils has significantly increased in all regions of the world; threefold in developing countries and twofold in the industrialized countries (Kearney, 2010).

Fats and oils are one of the most important constituents of food. It is recommended that between 15% -30% of our daily energy requirements come from fats (Mahan *et al*., 2012). They provide nine kilocalories per gramme of energy, and serve as carriers of the fat soluble vitamins A, D, E and K, as well as sources of the essential fatty acids, ω-6 linoleic acid and ω-3 alpha-linolenic acid (Mahan *et al*., 2012). Fats and oils contribute greatly to the feeling of satiety and make foods more palatable. High quality fats and oils are bland, odourless, free from impurities, and are oxidatively stable (Chow and Gupta, 1994).

The type of vegetable oil used for food production in Ghana varies according to residence (urban/rural), geographical region, and wealth quintile (Table 2.1). Every second household in Ghana (54%) uses red palm oil for cooking, 26.5% use *frytol* (refined bleached deodorized palm olein) / fortified vegetable oil, 9% use shea butter and 6.6% use other vegetable oils. Lard, suet and other oils high in saturated fat are used by less than 1% of the population (GDHS, 2008). Some easily available foods prepared domestically and commercially with these oils in Ghana are, *koose* (fried bean balls), plantain chips, *kelewele* (spiced fried ripe plantain), doughnuts, fried fish, meat and chicken, fried yam, *shito* (fried pepper sauce), fried rice and stews.

Saturated fat and partially hydrogenated oils have fewer oxygen binding sites and thereby have increased stability and a longer shelf life, however it is associated with a greater risk of cardiovascular diseases. On the other hand, too much polyunsaturated fatty acids (PUFAs) can also be dangerous, as the double bonds are highly reactive and bind oxygen to form peroxides when exposed to air or heat. When subjected to routine frying or cooking, PUFAs can generate high levels of toxic aldehydes that promote cardiovascular diseases and cancer (Mahan *et al*., 2012).

In recent decades, more attention has been given to issues concerning the quality of fats and oils, with distinctions made between saturated, hydrogenated, monounsaturated and polyunsaturated; from seeds, nuts and fish (AICR, 2007). Oils are complex mixtures of nutrients and other bioactive compounds, some of which may have harmful effects on cancer risk, and others beneficial effects (AICR, 2007). Fats and oils may become oxidized during processing, storage and usage. While oil processing is designed partly to remove or destroy oxidized products or factors that may initiate or enhance oxidative reactions, some processing steps, such as caustic refining and bleaching, may promote oxidation. Also, oxidative reactions may lead to the breakdown and formation of compounds that give the rancid odour and flavour during storage. Furthermore, products of hydrolysis, cleavage and polymerization may be formed when fats and oils are subjected to high temperature treatment, as in home and commercial culinary practices (Chow and Gupta, 1994).

Table 2.1: Types of Cooking Oil used in Ghana:

Percent distribution of households by type of oil used for cooking, according to background characteristics, Ghana 2008

Background characteristics	Palm oil	*Frytol* / Fortified veg.oil	Other veg.oil	Shea Butter	Lard, Suet, Butter, Margarine or other	Missing	No food cooked in household	Total	No of Households
Residence									
Urban	45.1	39.3	9.1	1.9	0.2	0.1	4.3	100.0	5,627
Rural	62.3	14.8	4.2	15.6	0.6	0.2	2.4	100.0	6,150
Region									
Western	71.1	21.5	4.4	0.0	0.1	0.0	2.8	100.0	1,184
Central	80.3	14.6	2.4	0.0	0.3	0.2	2.1	100.0	1,279
Gt.Accra	40.8	47.7	7.6	0.0	0.4	0.2	3.3	100.0	1,951
Volta	69.3	24.7	3.9	0.0	0.8	0.0	1.4	100.0	991
Eastern	78.3	18.7	1.0	0.0	0.1	0.4	1.5	100.0	1,260
Ashanti	55.4	31.9	5.8	0.1	0.1	0.1	6.5	100.0	2,263
Brong Ahafo	57.9	26.4	6.1	3.9	0.1	0.2	5.4	100.0	1,154
Northern	10.1	14.9	26.5	45.0	2.0	0.1	1.5	100.0	928
Upper East	0.6	15.3	3.7	78.5	0.3	0.2	1.5	100.0	540
Upper West	5.1	7.9	8.8	76.5	0.3	0.0	1.3	100.0	228
Wealth quintile									
Lowest									
Second	36.8	7.4	7.3	46.0	1.30	0.3	0.9	100.0	1,813
Middle	74.8	10.7	5.2	6.8	0.5	0.1	2.0	100.0	2,250
Fourth	66.9	19.4	6.3	2.1	0.3	0.2	4.8	100.0	2,548
Highest	51.8	35.8	5.8	0.7	0.1	0.2	5.7	100.0	2,646
	37.3	51.8	8.3	0.1	0.2	0.1	2.3	100.0	2,520
Total	54.0	26.5	6.6	9.0	0.4	0.2	3.3	100.0	11,777

Source – (GDHS, 2008)

2.2 EFFECT OF TEMPERATURE

Temperature is the most important factor to be considered in evaluating the oxidative stability of fats, especially unsaturated, because the mechanism of oxidation changes with temperature and different hydroperoxides of linoleate, acting as precursors of volatile flavours, decompose at different temperatures. Because the rate of oxidation

is exponentially related to temperature, the shelf life of a food lipid decreases logarithmically with increasing temperature (Frankel, 1998).

Katragadda *et al*. (2010) conducted a research on the emissions of volatile aldehydes from heated cooking oils, and concluded that the correct choice of oil for frying is important for several reasons. Firstly the oil is used as the heat transfer medium, and as such must be able to tolerate high temperatures and have high enough stability to allow its reuse. Secondly, products being fried take up some of the oil and therefore the oil needs to maintain a high oxidative stability during the life of the product and be both palatable and nutritious as similarly suggested by Andreu-Sevilla *et al*. (2009) and Talbot and Zand (2006). Finally, the oil should be as stable as possible during its heating and originate a low emission of potentially toxic volatile organic compounds. Furthermore, the generation of aldehydes after heating of coconut, safflower, canola and extra virgin olive oils to 210^{o}C was found to be constant with respect to time for all the four oils in that study (Katragadda *et al*., 2010). Additionally the total amounts of volatile compounds present in the fumes of cooking oils positively correlated with the frying temperature and the total concentrations of aldehydes, hydrocarbons, alcohols and ketones increased with increasing frying temperatures. Volatile aldehyde emissions from the four oils increased gradually with temperature until reaching their smoke points. From there on, emissions increased more sharply. It was concluded that the temperature of any oil used for deep frying operation should be established below its smoke point otherwise the emission of potentially toxic compounds will increase significantly. Reheating of oil was not recommended as used oil will contain a higher free fatty acid content and consequently drastically decrease its original smoke point resulting in higher emissions of volatiles at lower temperatures. Further conclusion was that coconut oil

should not be used as a deep frying medium because its smoke point of 175^{o}C is below the optimal temperature of 180^{o}C for frying operations.

Aqueous extracts from six cooking oils, soybean, extra virgin olive, peanut, sunflower, corn and various seed oils were studied by Isidori and Parella (2009) for their mutagenic and genotoxic effects. The oils were heated to their respective smoke points and the Ames and SOS Chromo tests were carried out for the evaluation of genotoxic and mutagenic compounds. The oils were tested again after repeated frying, simulating the domestic reuse process. The ability of different lactobacilli to reduce the potential genotoxic activity of the fried and re-fried oils was determined applying the SOS Chromo test, after co-incubation of the samples with lactobacilli. Results obtained showed that all the fried oils did not produce mutagenic effects. Double heat treatment caused an increase of the genotoxic activity until two times the first heating. The most susceptible oil to the re-frying procedure was sunflower oil.

2.3 PALM OIL

The oil palm, the highest oil producing plant is a monocotyledon belonging to the genus *Elaeis* (Sundram *et al.*, 2003). This genus comprises two species *Elaies guineensis* of West African origin and *Elaeis oleifera* of South American origin. The commercial planting material is mainly of the *E.guineensis* species (Sundram *et al*., 2003).

Red palm oil is natural orange red coloured semi-solid oil obtained from the fleshy mesocarp of the palm fruit. Indonesia is the world's largest exporter of palm oil with more than 70% of its production exported (OECD-FAO, 2012). Malaysia and Indonesia account for 90% of the palm oil world export trade (Palm Oil Facts, 2012).

Eighty percent of the palm oil produced globally is used as food (Palm Oil Facts, 2012).

In Africa, the main palm oil belt runs through the southern latitudes of Cameroun, Côte d'Ivoire, Ghana, Liberia, Nigeria, Sierra Leone, Togo and into the equatorial region of Angola and the Congo (Poku, 2002). The wild oil palm groves of Central and West Africa consist mainly of a thick shelled variety with a thin mesocarp called *Dura*. Through breeding work, crosses between *Dura* and *Pisifera* (a shell-less variety), has led to the development of a hybrid which has a much thicker mesocarp and thinner shell, termed *Tenera*, of a much higher oil content than the native *Dura* (Poku, 2002). Oil palm gives the highest yield of oil per unit area compared to any other crop, and produces two distinct oils, palm oil and palm kernel oil, both of which are important in world trade (Poku, 2002). Traditional farmers in Africa have not embraced the *Tenera* variety because consumers complained that the oil produced was so fatty that at ambient temperature it solidifies instead of remaining fluid and red, and that it has an unsuitable taste as oil or as a soup base. This negative perception of *Tenera* led to its slow adoption, and contributed to the failure of Africa to maintain its lead in palm oil production and was overtaken by Malaysia and Indonesia by 1966 (Poku, 2002).

An estimated land area of 285,000 hectares is under oil palm cultivation in Ghana, ranging from the Eastern to Ashanti, Central, Brong-Ahafo and Western Region (Poku and Asante, 2008). Ghanaian palm oil is mainly sold regionally within the ECOWAS zone (Vath and Kirk, 2011). The edible, highly flavoured oil is an essential ingredient in many of traditional West African cuisine.

2.3.1 Palm Oil Processing

The oil process in summary involves the reception of fresh fruit bunches, sterilizing and threshing of bunches to free the palm fruit, mashing the fruit, and pressing out the crude palm oil. The crude palm oil is further treated to purify and dry it for storage and for sale. In the edible oil refining industry, the most important quality criteria for crude oil are low content of free fatty acids (FFA), low content of products of oxidation and readily removable colour (Poku, 2002). Oil quality is maintained by careful harvesting of fruits at the optimum stage of ripeness, minimal handling of fruits during transportation, and proper processing conditions during oil extraction (Gunstone, 2002).

In order to meet these criteria, the most critical stages in the processing sequence are bunch sterilization as soon as possible after harvest, and effective clarification and drying of the crude oil after extraction (Poku, 2002). In contrast, for the domestic consumer of crude palm oil, flavour is the primary quality factor. Flavour is boosted by the fermentation that takes place within the fruit, when bunches are allowed to rest for three or more days after harvesting. Herbs and spices for flavour are introduced during the oil-drying phase of operations to mask off-flavours. Thus the quality requirements of one market leading to certain processing imperatives, may conflict with those of another market (Poku, 2002).

The village traditional processing method is simple but tedious and inefficient. It involves washing boiled pounded fruit mash in warm water, hand squeezing to separate fibre and nuts from the oil / water mixture, and straining out fibre and nuts with a colander, basket or vessel with fine perforated holes (Poku, 2002). The wet mixture is then boiled vigorously for one to two hours depending on the volume of material. It is then allowed to cool, and herbs may be added to the mixture before

cooling to body temperature. A calabash or shallow bowl is then used to skim off the palm oil. This processing method is called the wet method, because of the large quantities of water used in washing the pulp (Poku, 2002).

A mechanical improvement based on the traditional wet method process combines digestion, pressing and hot water dilution into one mechanical unit operation. Processing plants handling more than six tonnes fresh fruit bunches (FFB) per hour prefer the dry method of oil extraction. This method uses a digester to pound the boiled fruit which is a considerable labour-saving device. The oil in the digested or pounded pulp is separated in a press that may be a manual, mechanical or motorized mechanical press of hydraulic or screw type (Poku, 2002).

Zu *et al*. (2012) carried out a study among ten communities and 62 palm oil mills in the Kwaebibirem District of the Eastern Region of Ghana, on the type of processing equipment and practices among small scale palm oil processors and their impact on the yield and quality of palm oil produced. The major processing practice identified as having a major effect on quality was the duration of storage of palm fruits before processing. Both processing equipment and storage durations had significant ($p<0.05$) effect on the resultant quality of crude palm oil processed.

A previous study by Vissoh *et al*. (2010) also reported that the crude palm oil produced by the small-scale processors in Ghana was of poor quality due to high FFA, high moisture content and impurities in the oil. This was due to the low level of technology used in production. To produce palm oil with low FFA, Poku (2002) recommends that palm fruits should be processed within 48 hours of harvesting.

Approximately 80% of the quantity of crude palm oil produced in Ghana is by small scale processors (Adjei-Nsiah *et al*., 2012). Within the small scale processing industries, fruits are processed within a period varying from six to twenty-eight days

after fruit harvesting (Taiwo *et al*., 2000). The main reason for the delay in processing is the removal of the palm fruits from spikelets by hand, which is time consuming, tedious and labour intensive and depends on the availability of labour and quantity of fruits to be processed. The delay in processing may lead to the production of crude palm oil with high FFA content (Taiwo *et al*., 2000). The crude palm oil produced by these processors cannot be utilized by major refineries in Ghana due to its quality (Adjei-Nsiah *et al*., 2012; Vissoh *et al*., 2010). This grade of palm oil cannot be utilized by the local refineries because it will unduly increase their cost of production and also result in wastage of oil.

The quality of crude palm oil affects the efficiency and yield of refining and the quality of the fully processed product (Gibon *et al*., 2007). In the study by Zu *et al*. (2012), none of the palm oil processed had FFA content below 5% as required by Codex standards. About 90% of the palm oil processors in the Kwaebibirem District indicated that they had not received any formal training in good processing practices from any organization (Adjei-Nsiah *et al*., 2012). The lack of skills and knowledge in good processing practices often results in the production of crude palm oil of poor quality which impedes their entry into the industrial and export markets. Additionally, the absence of a regulatory body in the small scale palm oil processing industry to regulate the activities of producers often results in the use of environmentally unfriendly and unhealthy practices, such as the use of vehicle tyres in place of fuel wood, disposal of effluent into water bodies, as well as the use of contaminated water for processing (Adjei-Nsiah *et al*., 2012). In most places, because of the scarcity of firewood, waste vehicle tyres and/or mesocarp fibre are used instead, thereby generating a lot of smoke with serious health consequences. Most producers prefer vehicle tyres because it burns for a longer period and produces a lot of heat. Steam

boilers have been designed by the Ghana Regional Appropriate Technology Industrial Services (GRATIS) foundation; however this equipment is not being widely used by the producers because of cost (Adjei-Nsiah *et al*., 2012).

Generally, processing units handling up to two tonnes fresh fruit bunches (FFB) per hour are considered small scale, those that process between three and eight tonnes FFB per hour are termed medium scale, while large scale refers to mills that process more than ten tonnes FFB per hour (Poku, 2002). The five big palm oil investors in Ghana are; Ghana Oil Palm Development Company (60 tonnes per hour), Twifo Oil Palm Plantation (30 tonnes per hour), Norpalm (22 tonnes per hour) Benso Oil Palm Plantations (20 tonnes per hour), and Juaben Oil Mills (10 tonnes per hour) (Vath and Kirk, 2011).

Ghana is unable to compete on the world market with Indonesia and Malaysia. As Ghanaian local palm oil producers are unable to deliver the quality requested by industry, large investors dominate the sub-sector, and are seen as the engine for growth and development. However, these investors are unable to produce the locally demanded *zomi* for cooking and thus they are unable to dominate the local producers' market. *Zomi* sells at a higher price (approximately twice the price) than ordinary crude palm oil (Adjei-Nsiah *et al*., 2012). The difference between *zomi* and crude palm oil is the addition of salt and spices to flavour the oil and improve its taste. Processors who produce *zomi*, which has a relatively better quality than the crude palm oil, do not store their palm fruits beyond one week after harvesting (Adjei-Nsiah *et al*., 2012). Although both small and large scale industries serve different sales markets, they use the same FFB, and thus compete on the commodity market (Vath and Kirk, 2011).

2.3.2 Composition and Products of Palm Oil

Palm oil is a well-balanced edible vegetable oil of approximately 50% saturation, yet does not contribute to coronary heart disease, atherosclerosis and arterial thrombosis as was previously thought (Edem, 2002; McNamara, 2010). Palm oil is trans - fat free, cholesterol free, with a rich content of vitamins and antioxidants. Its moderate content of linoleic acid and high levels of antioxidants makes palm oil less prone to oxidation and deterioration (Edem, 2002). Red palm oil by virtue of its beta-carotene content may protect against vitamin A deficiency and certain forms of cancer (Edem, 2002). Palm oil is widely used by the commercial food industry because of its high oxidative stability and low cost (Fan and Eskin, 2012).

The oils preferred for frying have changed considerably over the years and reflect flavour preferences, nutritional requirements, oil stability and economic factors. For example, animal fats have now been widely replaced by vegetable oils. This change has been mainly on the grounds of nutritional requirements, animal fats being rich in saturated fat, despite the good flavour they impart to the food (Talbot and Zand, 2006). Semi-solid fats, or frying shortenings, are however still sometimes used to prevent an oily texture being formed in products such as french fries and doughnuts. Partially hydrogenated vegetable oils were once widely used in industrial frying because of their high oxidative stability. Over the past few years, the use of such oils has decreased significantly because of the effects of trans fatty acids in such oils on cardiovascular health. They were initially replaced by oils such as rapeseed oil and soya bean oil, but the oxidative stability of these oils is quite low, especially when being used repeatedly at frying temperatures and so, more and more, the oil of choice for industrial frying is palm oil based (Talbot and Zand, 2006).

Crude palm oil can be refined and used in the industrial production of non-dairy creams, ice cream powder, confectioneries, salad additives, fat spread, margarine and baking fats as well as soaps and detergents (Kyei-Baffour and Manu, 2008). Palm oil is fractionated into a liquid olein and solid stearin to increase its versatility in food processing (Palm Oil Facts, 2012). Red palm oil is refined, bleached and deodorized to produce bright golden oil (olein), which is mostly used as cooking and frying oil. During refining of crude palm oil, impurities in the oil are removed and the carotenoids present are thermally destroyed to produce the desired colour for a refined bleached deodorized palm oil. Palm oil can be fractionated by thermo-mechanical processes to produce refined bleached deodorized palm olein used mainly as cooking oil, and refined bleached deodorized palm stearin used mainly in margarines and shortenings (Chong, 1993). There is a general preference for the use of palm olein, the low-melting fraction of palm oil. The reasons for this are two-fold. Firstly the melting point of palm olein (15 - 25°C) is much lower than that of palm oil, making it much easier to handle, and secondly the degree of unsaturation in palm olein is higher than that in palm oil, giving it better nutritional characteristics. This means that there is a slight trade-off in oxidative stability, but the stability of palm olein is still much higher than that of other liquid oils such as rapeseed oil (Talbot and Zand, 2006).

Like all oils, triacylglycerols (95%) are the major constituents of palm oil (Sundram *et al*., 2003). The other components in palm oil are the metabolites in the biosynthesis of triacyglycerols and products from lipolytic activity. These include the monoacylglycerols, diacylglycerols and free fatty acids (Sundram *et al*., 2003). The fatty acids belong to the class of aliphatic acids. The triacylglycerols in palm oil partially define most of the physical characteristics of the palm oil such as its melting point and crystallization behaviour (Sundram *et al*., 2003). The major fatty acids in

palm oil are myristic (14:0), palmitic (16:0), stearic (18:0), oleic (18:1) and linoleic (18:2) as seen in Table 2.2. Palm oil has saturated and unsaturated fatty acids in approximately equal amounts. It contains mainly 44% saturated palmitic (C16:0) and 39% mono-unsaturated oleic (C18:1) and 10% poly-unsaturated linoleic fatty acids (McNamara, 2010). Several minor non-glyceride compounds are found in palm oil. These consist of sterols, triterpene alcohols, tocopherols, phospholipids, chlorophylls, carotenoids and volatile flavour components such as aldehydes and ketones. Sterols, tetracyclic compounds with generally 27, 28 or 29 carbon atoms, make up a sizeable portion of the unsaponifiable matter in oil. The content of sterols in palm oil is about 0.03% of its total composition (Sundram *et al*., 2003). Tocotrienols are rarely seen in vegetable oils, with the exception of palm and rice bran oils. Oils such as corn, soya and sunflower are good sources of the tocopherols but they do not contain tocotrienols. Crude palm oil contains 600 – 1000 ppm of tocopherols and tocotrienols. Refining reduces the level down to 350 – 630 ppm (Sundram *et al*., 2003).

Table 2.2: Fatty acid composition of vegetable oils as determined by gas liquid chromatography from authentic samples expressed as percentage of total fatty acids.

Fatty acid		Palm oil	Palm olein	Palm Stearin	Coconut oil
C6:0	Caproic	ND	ND	ND	ND-0.7
C8:0	Caprylic	ND	ND	ND	4.6-10.0
C10:0	Capric	ND	ND	ND	5.0-8.0
C12:0	Lauric	ND-0.5	0.1-0.5	0.1-0.5	45.1-53.2
C14:0	Myristic	0.5-2.0	0.5-1.5	1.0-2.0	16.8-21.0
C16:0	Palmitic	39.3-47.5	38.0-43.5	48.0-74.0	7.5-10.2
C16:1	Palmitoleic	ND-0.6	ND-0.6	ND-0.2	ND
C17:0	Magaric	ND-0.2	ND-0.2	ND-0.2	ND
C17:1	Myristoleic	ND	ND-0.1	ND-0.1	ND
C18:0	Stearic	3.5-6.0	3.5-5.0	3.9-6.0	2.0-4.0
C18:1	Oleic	36.0-44.0	39.8-46.0	15.5-36.0	5.0-10.0
C18:2	Linoleic	9.0-12.0	10.0-13.5	3.0-10.0	1.0-2.5
C18:3	Linolenic	ND-0.5	ND-0.6	ND-0.5	ND-0.2
C20:0	Arachidoic	ND-1.0	ND-0.6	ND-1.0	ND-0.2
C20:1	Gadoleic	ND-0.4	ND-0.4	ND-0.4	ND-0.2
C20:2	Eicosadienoic	ND	ND	ND	ND
C22:0	Behenic	ND-0.2	ND-0.2	ND-0.2	ND
C22:1	Erucic	ND	ND	ND	ND
C22:2	Docosodienoic	ND	ND	ND	ND
C24:0	Lignoceric	ND	ND	ND	ND
C24:1	Nervonic	ND	ND		ND

ND –non detectable, defined as ≤ 0.05%

(Source - Codex standards 210-1999)

2.3.3 Studies on the Beneficial Effects of Palm Oil

The technology to isolate and concentrate the minor components in palm oil has led to their use in several studies aimed at understanding their physiological effects. Accordingly, the emphasis has been on the cholesterol lowering effects of palm oil tocotrienols, the pro-vitamin A activity of red palm oil and palm carotene concentrates, and the antioxidant and anti-cancer properties of palm vitamin E, carotenoids and the phenolic flavonoid complex (Sundram *et al*., 2003).

Palm tocotrienols may have potential anti-cancer properties. Sundram *et al.* (1989) suggested that crude palm oil was more effective than refined palm oil in increasing the tumour latency period in 7,12-dimethylbenz-α-anthracene (DMBA) treated rats. This was attributed to the presence of tocotrienols and carotenoids in the crude oil. When the vitamin E content in palm oil was removed, significantly more tumours became apparent (Nesaretnam *et al*., 1992). Addition of palm vitamin E to corn oil (500 or 1000 ppm) resulted in a lower tumour incidence and occurrence compared to rats fed corn oil alone. A series of studies also investigated the *in vitro* effects of tocotrienols on human breast cancer cells. Compared to α-tocopherol (500 µg/ml concentration), which had no growth inhibition of human breast cancer cells, palm tocotrienols inhibited the incorporation of (3H) thymidine into human breast cancer cells by 50% (at a concentration of 180 µg/ml) (Nesaretnam *et al*., 1995).

In another similar study, oestrogen-receptor negative and positive human breast cancer cells were used to test the efficacy of individual palm tocotrienols at varying concentrations. These individual tocotrienols showed even greater inhibitory effects on these cells and at much lower concentrations than palm tocotrienols (Guthrie *et al*., 1997). There also appears to be a synergy in the inhibition of human

cancer cells between palm tocotrienols and flavonoids. Combinations of tocotrienols, flavonoids and tamoxifen proved to be even more effective than the individual components (Guthrie *et al*., 1997). Palm tocotrienols have also been reported to be effective against transplantable mice tumours (Sundram *et al*., 2003).

Red palm oil is the richest dietary source of β-carotene (pro-vitamin A) and antioxidant nutrients such as carotene, tocotrienols and tocopherols which have the capacity to retard peroxidation and scavenge free radicals, besides having anti-mutagenic and hypo-cholesterolaemic potential and is nutritionally safe and wholesome (Rukmini,1994). Palm oil also provides energy density to the diet. Palm oil is also the richest natural source of the vitamin E, α – tocotrienol which is one of the eight naturally occurring and chemically distinct vitamin E analogs (Sen *et al*., 2000). Both tocopherols and tocotrienols are antioxidants which contribute to the stability of palm oil. Tocopherols can interrupt lipid oxidation by inhibiting hydroperoxide formation in the chain-propagation step or the decomposition process by inhibiting aldehyde formation. Besides its free-radical scavenging activity, α-tocopherol is highly reactive towards singlet oxygen and protects the oil against photo sensitized oxidation (Sundram *et al*., 2003).

The pigmentation of palm fruits is related to their stage of maturity. Two classes of natural pigments occurring in crude palm oil are the carotenoids and the chlorophylls. Palm oil from young fruits contains more chlorophyll and less carotenoids than oil from mature or ripe fruits. The pigments in palm oil are involved in the mechanisms of autoxidation, photoxidation and antioxidation within the plant. Carotenes are sensitive to oxygen and light (Sundram *et al*., 2003).

The rich orange red colour of crude palm oil is due to its high content of carotene (700 - 800 ppm). The major carotenoids in palm oil are β and α-carotene

which account for 90% of the total carotenoids (Yap *et al*., 1997) (Table 2.3). Crude palm oil contains about eleven different kinds of carotenoids. Yap *et al*. (1997) undertook a quantitative analysis of the carotene composition of different species of oil palm, and found no significant difference in the types of carotenoids found in the oils of *E.oleifera* and *E.guineensis* and their hybrids. The study also showed that *E.guineensis* contained a higher level of lycopene compared to *E.oleifera* and its hybrids with *E.guineensis*.

Almost 90% of the world's palm oil production is used as food (Sundram *et al*., 2003). Palm oil has 15 times more Retinol Equivalents than carrot, and 300 times more than that of tomatoes (Sundram *et al*., 2003). For most practical purposes, palm oil does not need hydrogenation; however hydrogenation of palm oil has been explored to maximize the utilization of palm oil and its fractions in edible food products. Cake shortenings made from palm oil products such as hydrogenated or interesterified palm oil, in combination with butterfat, produce cakes with better baking properties than cakes made with 100% butterfat. The palm products enhanced the baking performance. Some hydrogenated palm oil products are suitable for application in a number of high premium specialty products such as toffee and confectionery fats (Sundram *et al*., 2003).

The inhibition of chemical carcinogenesis by palm oil carotenoids with reference to benzo (a) - pyrene metabolites *in vivo* and *in vitro* in rat hepatic cells has been reported by Tan and Chu (1991). It has also been reported that palm carotenoids exhibit an inhibitory effect on the proliferation of a number of human cancer cells. These include the neuroblastoma, GOTO, pancreatic cancer PANC-1, glio-blastoma A172 and gastric cancer HGC-27 (Murakoshi *et al*., 1989). Of significant interest from these studies was the observation that palm α-carotene and a palm carotene

concentrate were protective, whereas synthetic β-carotene was tumour promoting. Murakoshi *et al.* (1992) isolated palm α-carotene and a palm carotene concentrate and showed its ability to inhibit liver, lung and skin tumours in mice. The same effect could not however be attributed to synthetic β-carotene. Similar superior inhibitory effects for α-carotene were apparent in a chemically induced skin tumour progression model. These results led Sundram *et al*. (2003) to the conclusion that the natural bouquet of carotenoids in palm oil has promising chemopreventive activities against cancer.

Table 2.3: Content of various components in the unsaponifiable fraction of palm oil.

Component		%	Mg/Kg (in palm oil)
Carotenoids	α-carotene	36.2	
	β-carotene	54.4	
	γ-carotene	3.3	500 – 700
	Lycopene	3.8	
	Xanthophylls	2.2	
Vitamin E	α-tocopherol	28	
	α-tocotrienol	29	500 - 800
	γ-tocotrienol	28	
	δ-tocotrienol	14	
Sterols	Cholesterol	4	
	Campesterol	21	- 300
	Stigmasterols	21	
	-sitosterol	63	
Phosphatides			500 - 1000
Total alcohols	Triterpenic alcohol	80	- 800
	Aliphatic alcohol	20	

Source- Sundram *et al*., 2003

2.3.4 Differences between Red Palm Oil and Palm Olein

Conversion of red palm oil to refined oil involves removal of the products of hydrolysis and oxidation, colour and flavour. After refining, the oil may be separated (fractionated) into liquid and solid phases by thermo-mechanical means (controlled cooling, crystallization, and filtering), and the liquid fraction (olein) is used extensively as a liquid cooking oil in tropical climates, competing successfully with the more expensive groundnut, corn, and sunflower oils (Poku, 2002).

2.4 VIRGIN COCONUT OIL

Virgin coconut oil (VCO), is produced from the coconut palm, *Cocos nucifera*. The coconut palm is widely distributed throughout Asia, Africa, Latin America, the Caribbean and the Pacific region. Coconut palm is not grown in Europe and Australia. The three leading producers of coconut oil are Philippines, Indonesia and India (USDA, 2011). Virgin coconut oil is officially defined by the Philippine National Standard for virgin coconut oil as the oil obtained from the fresh, mature kernel (meat) of the coconut by mechanical or natural means with or without the use of heat, without undergoing chemical refining, bleaching or deodorizing and which does not lead to the alteration of the nature of the oil (Bawalan and Chapman, 2006). Coconut oil accounts for less than 2% of global edible oil consumption (UNCTAD, 2012).

VCO production is dominated by micro-scale and village-scale enterprises. Micro-scale enterprises include those with coconut processing capacities below 1,000 nuts per day and village-scale enterprises have capacities to process between 1,000 to 5, 000 nuts per day (Bawalan and Chapman, 2006). Coconut oil has been widely used throughout history for its medicinal value and has served man as important food for thousands of years. It consists of a mixture of triglycerides containing only short and medium chain saturated fatty acids (92%) and unsaturated fatty acids (8%) and exhibits good digestibility (Reynolds, 1982; Dayrit, 2003; Che Man and Marina, 2006; Marina *et al.*, 2009). Refined bleached deodorized coconut oil (RBDCO), is made from copra, is yellow in colour, odourless, tasteless and does not contain natural vitamin E, as this is destroyed when the oil is subjected to high temperature and various chemical processes (Bawalan and Chapman, 2006).

2.4.1 Virgin Coconut Oil Processing

Various methods have been developed to extract coconut oil, either through dry or wet processing. Dry processing is the most widely used form of extraction. Clean, ground and steamed copra is pressed by wedge press, screw press or hydraulic press to obtain coconut oil, which then goes through the refining, bleaching, and deodorizing processes to produce refined bleached and deodorized coconut oil (RBDCO). During the refining, bleaching, deodorizing, and processing of oils, heat is applied especially during the deodorization process, which is carried out at high temperatures between 204^{o}C and 245^{o}C (O'Brien, 2004). Bawalan and Chapman (2006) have described eight different techniques for producing VCO. These are traditional fresh-dry process (wet milling route), fresh-dry process (desiccated coconut route), fresh-dry process (grated coconut route), low pressure method, traditional wet or modified kitchen method, modified natural fermentation method, the centrifuge process and the Bawalan-Masa process (Bawalan and Chapman, 2006).

Unlike refined coconut oil which is produced through the dry method from copra, VCO is produced through the wet method of processing, via coconut milk. The wet processing method entails the extraction of the cream from the fresh coconut milk and consequently breaking the cream emulsion. This process is more desirable as no chemical or high heat treatment is imposed on the oil (Marina *et al*., 2009). There are several other techniques of processing VCO such as the chilling, freezing and thawing techniques, fermentation technique using pure culture of *Lactobacillus plantarum* strain 1041 IAM, and enzymatic extraction techniques (Marina *et al*., 2009).

The physico-chemical properties of VCO do not vary much from RBDCO. Samples of VCO available on the Malaysian and Indonesian market examined were found to have iodine, peroxide, saponification and FFA values well within the specification limit of Codex standards for refined bleached coconut oil (Dia *et al*., 2005; Marina *et al*., 2009). Descriptive sensory analysis provides important criteria to better differentiate VCO from RBDCO (Villarino *et al*., 2007). Dia *et al*. (2005) also compared the physicochemical properties of VCO produced by different methods. Their results revealed that α tocopherol was actually found in the coconut testa, the thin brown layer next to the white coconut meat. Only trace amounts of α tocopherol was detected in the VCO samples since the brown testa is removed during VCO processing. Most processors remove the testa because of the general belief that it causes discoloration of the oil. However, this is not true as proven by various production trial runs (Bawalan and Chapman, 2006).

2.4.2 Virgin Coconut Oil Composition and Uses

Coconut is currently considered a healthy food, therefore there is a shift in the market towards high-valued food uses for the coconut fruit such as tender coconut water, coconut milk, spray dried coconut milk, coconut vinegar and virgin coconut oil (UNCTAD, 2012). The kernel (endosperm) is eaten fresh, green or dry and used for making value added products such as coconut oil, milk, cake and copra. The three most important forms of consumption of coconuts are fresh (coconut water), coconut oil and desiccated coconut (UNCTAD, 2012).

Virgin coconut oil is extracted directly from fresh coconut meat. VCO is the purest form of coconut oil, essentially colourless, contains natural vitamin E and has not undergone any hydrolytic and atmospheric oxidation as demonstrated by its very

low FFA content even without refining and has a low peroxide value (Table 2.4). The fatty acid composition of VCO is predominated by medium chain fatty acids which are resistant to peroxidation (Table 2.2). VCO behaves and metabolizes differently in the human body to other saturated and unsaturated fats or oils (Bawalan and Chapman, 2006). Medium chain fatty acids in coconut oil are about 64%, with lauric fatty acid (C:12) as the highest, ranging from 47% to 53% depending on the coconut variety (Bawalan and Chapman, 2006). Coconut oil is generally used as frying and cooking oil because of its excellent resistance to rancidity development. Coconut oil is used as a substitute for expensive butterfat in filled milk, filled cheese, ice cream fat, in confectionaries, as fat in infant formulas and baby foods because of its easy digestibility and absorbability. When hydrogenated it is used as margarine, shortening and baking fat (Bawalan and Chapman, 2006).

To safeguard the quality of VCO, it is important that moisture levels are kept at the minimum i.e. 0.2% maximum according to the Philippine National Standard for VCO (Carandang, 2008) (Table 2.4). VCO has a fresh coconut aroma that can be mild to intense, depending on the oil extraction process used (Bawalan and Chapman, 2006). VCO is considered a nutraceutical and functional food because the medium chain (C8-C12) fats in coconut oil are similar in structure to the fats in breast milk that gives babies immunity to diseases.

Table 2.4: Virgin coconut oil specifications.

Property	Specification
Moisture and volatile content	0.20% max
Free fatty acids (expressed as lauric acid)	0.20% max
Peroxide value	3.0meq/kg oil max.
Food additives	None permitted
Contaminants	0.20% max
Matter volatile at 105^{o}C	
Heavy metal	mg/ kg max.
Iron(Fe)	5.0
Copper (Cu)	0.40
Lead (Pb)	0.10
Arsenic (As)	0.10

(Source - Bawalan and Chapman, 2006)

There are also similar beneficial effects in adults (Kabara, 2000). Among the many beneficial effects of VCO are that it inhibits the action of cancer forming substances (Lim-Sylianco, 1987). Both positive and negative health effects of coconut oil have been reported. Formation of some pro mutagenic DNA adducts is reported to be lower in rats fed with a coconut oil supplemented diet, compared with the formation of such adducts in rats fed with linoleic acid-rich diets (Eder *et al*., 2006).

The extraction of VCO from the fresh endosperm of coconut is believed to be more beneficial than usually prepared or regular coconut oil because its mode of extraction retains more biologically active components such as vitamin E and polyphenols (Nevin and Rajamohan, 2004). The antioxidant properties of VCO produced through chilling and fermentation were investigated and compared with RBDCO. VCO showed better antioxidant capacity than RBDCO. The VCO produced through the fermentation method had the strongest scavenging effect on 1, 1-diphenyl-2-picrylhydrazyl and the highest antioxidant activity based on the β-carotene–linoleate bleaching method (Marina *et al*., 2009). However, VCO obtained through the chilling method had the highest reducing power. The major phenolic acids detected were ferulic acid and *p*-coumaric acid. A very high correlation was found between the total phenolic content and scavenging activity (r=0.91), and between the total phenolic content and reducing power (r=0.96) (Marina *et al*., 2009). There was also a high correlation between total phenolic acids and β-carotene bleaching activity. The study indicated that the contribution of antioxidant capacity in VCO oil could be due to phenolic compounds (Marina *et al*., 2009).

2.5 DIET, COOKING METHODS AND CANCER RISK

Dietary fat, both in terms of quantity and quality has been implicated in cancer development either positively or negatively (Psaltopoulou *et al*., 2011). Studies suggest that dietary intake of added fats and oils from both animal and vegetable sources may also influence the risk of cancer (Kotsopoulous *et al*., 2006). Doll and Peto (1981) identified diet as one of the major factors in the aetiology of cancer. Cancer epidemiological studies have provided evidence that cancer chemo-preventive

agents exist naturally in our diets. Although over 1000 compounds have been tested, the retinoids and carotenoids have received the most attention.

A number of epidemiological studies have demonstrated an inverse correlation between dietary intake or blood level of vitamin A/carotenoids and cancer risk, as well as an anti-carcinogenic effect for these compounds. The data further indicate that a wide range of cancer sites may be influenced by these carotenoids (Doll and Peto,1981).

Frying, grilling (broiling) and barbecuing (charbroiling) can generate temperatures up to 400°C. These cooking methods create high levels of carcinogenic compounds (AICR, 2007). For any cooking involving wood fires, the type of wood used is an important factor in determining chemical contaminants (AICR, 2007). Frying is a process of immersing food in hot oil with a contact among oil, air and food at a high temperature of between 150^{o}C to 190^{o}C (Choe and Min, 2007).

Deep-fat frying produces desirable or undesirable flavour compounds, changes the flavour, stability and quality, colour and texture of fried foods and the nutritional quality of foods. Volatile and non-volatile compounds are produced and these can be absorbed into the fried foods. The frying temperature, time, type of frying oil, presence of antioxidants and the type of fryer, affect the hydrolysis, oxidation and polymerization of the oil during frying (Choe and Min, 2007). Deep-fat frying is an important, ubiquitous and highly versatile process which has been used since antiquity to cook a wide variety of products.

Despite its considerable fat content and intensified consumer awareness of the relationships between food, nutrition and health, frying remains a principal cooking method (Saguy and Dana, 2003). Fried foods despite their high caloric value can be nutritious and favourably compared with other cooking methods such as baking and

boiling. Fried foods are popular due to their taste, distinctive flavour, aroma and crunchy texture. Misconceptions about frying extend beyond nutrition to the fundamental aspects of the process such as the role of water and oil quality during frying. The water released during frying enhances heat transfer, may cause oil deterioration and can also prevent oxidation (Saguy and Dana, 2003).

Deep-fat frying is widely used both domestically and commercially. During deep frying, fats and oils are repeatedly used at elevated temperatures between 160^{o}C and 240^{o}C with an optimal value of 180^{o}C, in the presence of atmospheric oxygen and receive maximum oxidative and thermal abuse (Katragadda *et al*., 2010). Studies on the chemical compounds detected in the emission or fumes resulting from heating edible oils such as rapeseed oil, soybean oil, peanut oil and lard to high temperatures exhibited mutagenicity and genetic toxicity (Katragadda *et al*., 2010). Cooking methods that produce high levels of mutagens are broiling, grilling and pan-frying with pan-frying yielding higher mutagenic activity when compared to grilling at similar temperatures (Joshi *et al*., 2012).

Temperature is the most important factor to be considered in evaluating the oxidative stability of fats (Frankel, 1998). The effect of different cooking methods like baking, seasoning, deep frying and shallow frying on retention of β-carotene was studied and it was observed that 70% to 80% of it was retained in the cooked foods. Repeated deep frying using the oil five times consecutively resulted in a total loss of β-carotene by the fourth frying stage and alteration of its organoleptic, physical and chemical properties (Manorama and Rukmini, 1991).

Reports indicate that oil fumes resulting from heating edible oils such as rapeseed, soybean and peanut oils to high temperatures exhibit mutagenicity and genetic toxicity (Chiang *et al*., 1997; Qu *et al*., 1992). Some of the commonly used

oils in industrial applications involving food frying are coconut oil, corn oil, palm oil, palm olein, rapeseed oil, soybean oil and sunflower seed oil (Singh, 1995). The genotoxic and carcinogenic risks associated with the consumption of repeatedly heated coconut oil was studied by Srivastarva *et al*. (2010). In their study, the polycyclic aromatic hydrocarbons generated in the oil samples were analysed using high pressure-powered liquid chromatography (HPLC) and their genotoxic effects in mammalian cells also examined. Fresh coconut oil, single heated coconut oil and repeatedly heated coconut oil were administered to Wistar rats by gavage. Administration of repeatedly heated coconut oil (RCO) caused a higher incidence of micronuclei and chromosomal aberrations in the rats. RCO was found to enhance the incidence of aberrant cells including breaks, fragments, exchanges and multiple chromosomal damages and micronuclei in a dose-dependent manner (Srivastarva *et al*., 2010).

A case-control study in Manila, Philippines, examined the association between methods of cooking and the risk of breast cancer (Kotsopoulos *et al*., 2006). It was observed that boiling food in coconut milk was associated with a significantly increased risk of breast cancer (OR=2.2; 95% CI 1.3-3.8). Also a positive association between frying food and breast cancer risk was restricted to women whose household fried food at 12 years of age (OR= 1.89, 95% CI 1.1-3.4). Their results suggested that various cooking methods during adolescence and possibly in adulthood may be associated with an increased risk of breast cancer. They also found a positive association between high temperature cooking particularly of meat products and cancer risk (Kotsopoulos *et al*., 2006).

Results from a multi ethnic case-control study showed that white fish intake was associated with increased risk of advanced prostate cancer among men who

cooked with high temperature methods (pan-frying, oven-broiling and grilling) until fish was well done (P_{trend} = 0.001) (Joshi *et al*., 2012). No associations were found among men who cooked fish at low temperature and or just until done ($P_{interaction}$= 0.040). Their results indicated that consideration of fish type (oily vs. lean), specific fish cooking practices and levels of doneness of cooked fish helps elucidate the association between fish intake and prostate cancer risk, and they suggested that avoiding high temperature cooking methods for white fish may lower prostate cancer risk (Joshi *et al*., 2012).

2.6 MODULATORS OF MUTAGENICITY

Human beings are exposed to a host of environmental chemicals which may be introduced into the body through lifestyle: alcohol, smoking, food additives, cosmetics, pesticides, insecticides, drugs or occupational exposure e.g. various organic solvents, vinyl chloride, epichlorohydrine (Madle and Obe, 1980). Many organisms including man have detoxifying systems which transform foreign compounds metabolically (Madle and Obe, 1980). There are substances that can reduce the activity of genotoxins known as antigenotoxins. Examples are vitamins, mineral ions, amino acids and plant foods (Sylianco and Guevara, 1989; Sylianco, 1991).

Many studies have indicated that tocotrienols exhibit superior anti-oxidative and anticancer properties. Palm tocotrienols have been products of interest in recent years for applications in various industries such as nutraceuticals, pharmaceuticals and cosmeceuticals (Nesaretnam *et al*, 2007). *In-vitro* studies as well as studies in animals have shown the anti-carcinogenic potential of tocotrienols (Nesaretnam *et al*., 2004), but their relevance and applicability to humans is as yet unclear. Studies in cell

culture and in rodents have provided some insights into the potential therapeutic value of tocotrienols in protecting against neuro-degeneration (Sen *et al*., 2000).

A research conducted by Azuine *et al.* (1992) examined the anti-mutagenic and anti-carcinogenic effects of carotenoids in dietary palm oil. They tested four carotenoids, canthaxanthin, β-carotene, 8'-apo-B-carotenal, and 8'- apo-β-carotene methylester for their ability to suppress the mutagenicity of 1-methyl-3-nitro-1-nitroguanidine and benzo (a) pyrene in *Salmonella typhimurium* tester strain TA100. The four carotenoids showed a dose dependent decrease in the mutagenicity and also significant anti-carcinogenic effect. Dietary administration of palm oil showed a dose-dependent anti-tumour activity in the female Swiss mice used in their experiments. A study by Lim-Sylianco *et al.* (1991) revealed the antigenotoxic activity of dietary coconut oil against known mutagens and genotoxins benzo (a) pyrene, dimethyl nitrosamine, methylmethanesulfonate and tetracycline. These compounds were administered to mice fed with coconut oil, soya bean oil and fat free diet. Mice fed with diets containing 18% coconut oil for 23 days showed great reduction in the formation of micro-nucleated polychromatic erythrocytes and also had an increased fertility index.

The chromosome breaking potential of fresh coconut oil, refined coconut oil and soya bean oil have been compared (Sevanian and Hochstein, 1985). Coconut oil whether fresh or refined did not possess chromosome breaking effects. Soya bean oil on the other hand exhibited chromosome breaking potential. This could be attributed to the high content of linoleic acid, a polyunsaturated fatty acid, which can readily form free radicals and peroxides which are very reactive with bases of DNA (Sevanian and Hochstein, 1985). The fat free diet induced more chromosome breaking effects than those with either coconut oil or soya bean oil, suggesting that

the presence of lipids in some ways reduced the chromosome breaking effects of genotoxins (Lim-Sylianco *et al*., 1991).

The relationship between FFA content of vegetable oils and mutagenicity is demonstrated in a study carried out on peanut oil. The influence of a degumming treatment of peanut oil on the content of mutagenic compounds in fumes from heated peanut oil was investigated by Yen and Wu (2003). Results obtained indicated that peanut oil which underwent degumming treatment had a lower FFA content, a higher smoke point, was clearer in colour and produced less fumes when heated at smoke point. When compared to untreated peanut oil, the mutagenicity of oil fumes of degummed peanut oil toward *Salmonella typhimurium* TA 98 and TA 100 was reduced to 81% and 73% ($p<0.05$), respectively. The degummed peanut oil which was obtained by adding 3% water and heating at 60^{o}C for 20 minutes produced the least amount of mutagenic fumes. The content of four mutagenic compounds in the fumes was drastically reduced, especially amount of trans-trans-2, 4-decadienal (t-t-2, 4-DDE). Further results indicated that FFA content had a high linear correlation with mutagenicity ($r^2 = 0.9978$) and content of t-t-2, 4-DDE. The four mutagens detected in the study were isolated from the peanut oil fumes at a much lower temperature of 97.5^{o}C.

2.6.1 Antioxidant Additives

The main improvements that can be made to frying oils are in their oxidative stability as well as the antioxidants naturally present in oils. It is permissible under certain circumstances to add either natural or synthetic antioxidants to frying oils. It is important to check legislation, however, before doing this as regulations vary from country to country (Talbot and Zand, 2006).

There is growing interest in formulations of medicinal plant origin and other plant additives as antioxidants. This is as a result of the realization that synthetic antioxidants can participate as mutagenic and genotoxic agents (Dzomba *et al*., 2012). The use of synthetic antioxidants in lipid based foods to maintain oxidative stability is not a first choice anymore because they are considered to be carcinogenic (Bianco and Uccella, 2000; Lapornik *et al*., 2005; Lafka *et al*., 2007; Rankovic *et al*., 2010). In the study by Dzomba *et al*. (2012), the efficiency of plant extracts of *Temnocalyx obovatus* as an alternative natural antioxidant on the improvement of sunflower and soya bean oil oxidative stability was evaluated. It was concluded that the plant extracts from *T. obovatus* contained potent antiperoxidants for sunflower and soya bean oil and recommended their application in the food industry as natural antioxidants to increase the shelf life by preventing lipid oxidation.

Phenolic compounds act as antioxidants for a number of potential factors. The most important is by free radical scavenging in which the phenol can break the free radical chain reaction. The presence of different substituents in the phenol backbone structures is responsible for their different antioxidant properties, in particular their hydrogen-donating capacities (Mahamadi *et al*., 2011; Dzomba *et al*., 2012). With the trend away from synthetic food additives to more natural equivalents, there is an increasing move towards the use of natural antioxidants such as herb extracts. Rosemary and sage extracts are particularly beneficial in this, with rosemary being found to be more effective than the synthetic antioxidant BHT (butylated hydroxy toluene), in prolonging the life of snacks fried in palm olein (Che Man and Tan, 1999).

2.7 GENOTOXICITY, MUTAGENS AND CANCER

Genotoxicity is a broad term that refers to any deleterious change in the genetic material regardless of the mechanism by which the change is induced (ICH, 2012). Genotoxic effects can be manifested through two mechanisms; induction of gene mutations and / or chromosomal aberrations. Gene mutations can be considered to be permanent single mutations involving only one gene, while chromosomal aberrations are considered to be changes in the structure of the chromosome or changes in chromosome number.

Generally chromosomal aberrations affect more than one gene. A mutagenic effect can be manifested through one or both of these mechanisms (OECD, 1997). Genotoxic substances are known to be potentially mutagenic or carcinogenic specifically those capable of causing genetic mutation and contributing to the development of tumours. Mutagens are physical or chemical agents that change the genetic material, usually DNA, of an organism and thus increase the frequency of mutations above the natural background level. As many mutations cause cancer, mutagens are typically also carcinogens (Sen *et al*., 2011). Cancer development occurs in response to the successive accumulation of mutations that eventually target key regulators of cell proliferation. To develop one oncogenic mutation, it is likely that several others have to occur before or simultaneously (Bhadury *et al*., 2013).

2.8 MUTAGENICITY TESTS

In vitro assays commonly used in genetic toxicity testing include the Ames bacterial reverse mutation assay. The Ames test is a biological assay used to assess the mutagenic potential of chemical compounds (a bacterial reverse mutation assay). It has been widely used as an early detecting system for potential genotoxicity. This assay was designed by Ames *et al*. (1975) to identify genetic damage caused by chemicals in bacterial cells. The test detects chemicals that cause point mutations or frameshift mutations in histidine auxotrophic strains of *Salmonella typhimurium*, for example, TA 100, TA 98 and TA 102 (OECD, 1997). The *S. typhimurium* strains carry mutations in genes involved in histidine synthesis. Such a mutant is referred to as auxotrophic, and they require histidine for growth. The variable being tested is the mutagen's ability to cause a reversion to growth on a histidine-free medium. The bacteria tester strains are specially constructed to have both frameshift and point mutations in the genes required to synthesize histidine. Rat liver extract is optionally added to simulate the effect of metabolism as some compounds for example benzo (a) pyrene are not mutagenic themselves but their metabolic products are. The bacteria are spread on an agar plate with a small amount of histidine in the growth medium to allow the bacteria to grow for an initial time and have the opportunity to mutate. When the histidine is depleted, only bacteria that have mutated to regain the ability to produce its own histidine will survive (Sen *et al*., 2011).

The bacterial strains of *S. typhimurium* TA 98 and TA 100 are two common strains used in the Ames test. The strain TA 98 is capable of detecting frameshift mutations and the strain TA 100 detects base-pair substitution or point mutations

(Figure 2.1 – Figure 2.6). A frameshift mutation is a change in the genetic code in which one base or two adjacent bases are added to (inserted), or deleted from the nucleotide sequence of a gene (Figure 2.1). This can lead to an altered or truncated protein. A point mutation occurs when there is a change in the genetic code usually confined to a single DNA base pair (ICH, 2012). Both TA 98 and TA100 *Salmonella typhimurium* strains have *rfa* mutations resulting in a defective lipopolysaccharide layer which makes the bacteria cell wall more permeable to larger molecules, *uvrB* mutations which eliminate excision repair of DNA damage and the *pKM101* plasmid which increases error-prone repair of DNA damage (Sen *et al*., 2011; Cyprotex, 2013).

The Ames test is used worldwide as an initial screen to determine the mutagenic potential of new chemicals and drugs. Over the years its value has been recognized by the scientific community, government agencies and corporations (Mortelmans and Zeiger, 2000).

Extensive reviews have shown that many compounds that are mutagenic in the Ames test are rodent carcinogens (ICH, 2012). *In vitro* assays offer advantages in that they are relatively inexpensive and easy to conduct and do not directly involve the use of animals, but usually require supplementation with exogenous metabolic activation enzymes in order to simulate mammalian metabolism. *In vitro* assays are typically used to provide an initial indication of the genotoxicity of a chemical and the results often serve as a guide to subsequent *in vivo* studies (OECD, 1997). Experience with genetic toxicology testing over several decades has demonstrated that no single assay is capable of detecting all genotoxic effects.

The genotoxicity of a chemical compound is determined through a battery of tests, both short and long term and both *in vitro* and *in vivo* (OECD, 1997; ICH,

2012). An example of the battery approach involves the initial assessment of mutagenicity in a bacterial reverse gene mutation test which has been shown to detect relevant genetic changes and the majority of genotoxic rodent and human carcinogens. This should be followed by the evaluation of genotoxicity in mammalian cells *in vitro* and *in vivo*.

In vivo tests are included in the test battery because some agents are mutagenic *in vivo* but not *in vitro* and because it is desirable to include assays that account for such factors as absorption, distribution, metabolism and excretion (ICH, 2012). A single Ames test is considered sufficient when it is clearly negative or positive and is carried out with a fully adequate protocol, including all strains with or without metabolic activation, a suitable dose range that fulfils criteria for top dose selection and appropriate positive and negative controls. Weak positive results might indicate that it would be appropriate to repeat the test, possibly with a modified protocol such as appropriate spacing of dose levels (ICH, 2012). Certain cases exist where positive results in bacterial mutation assays might be shown not to indicate genotoxic potential *in vivo* in humans, for example when bacterial specific metabolism occurs, such as activation by bacterial nitroreductases (ICH, 2012).

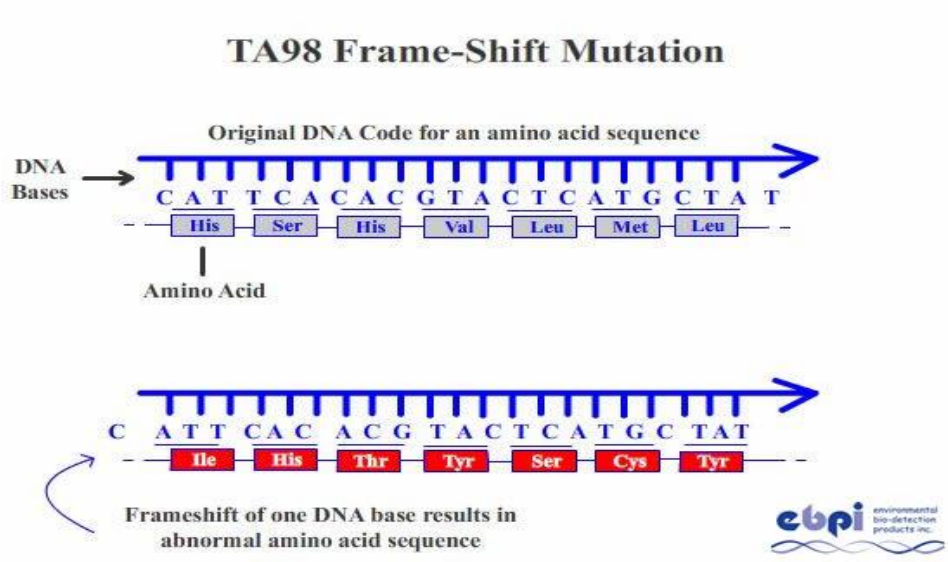

Figure 2.1: Diagram showing a frame shift mutation (Source - Environmental Bio-detection Products Incorporated, Canada EBPI, 2012)

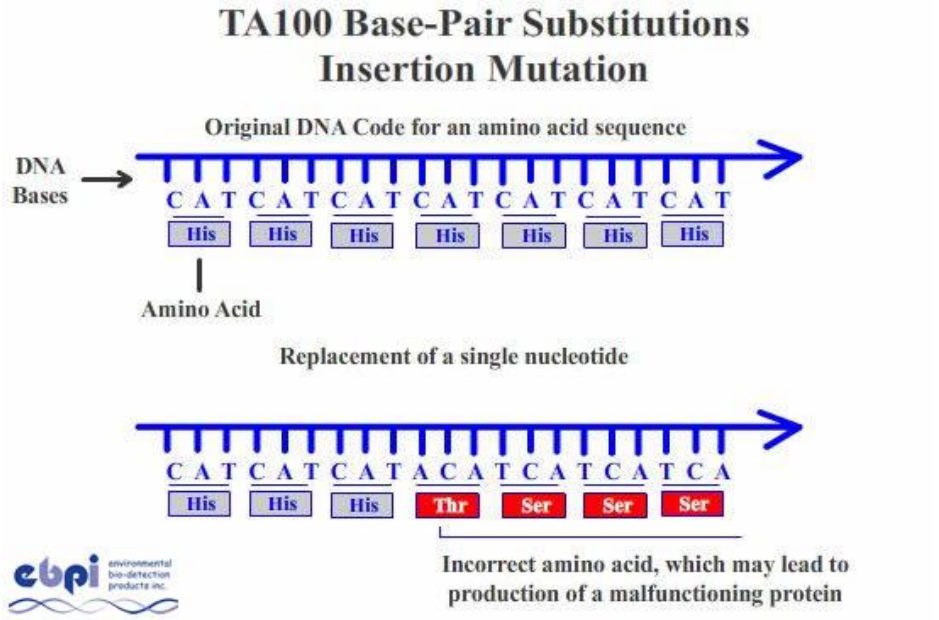

Figure 2.2: Diagram showing a point mutation or Base-Pair substitution by insertion of a single nucleotide base. (Source - Environmental Bio-detection Products Incorporated, Canada EBPI, 2012)

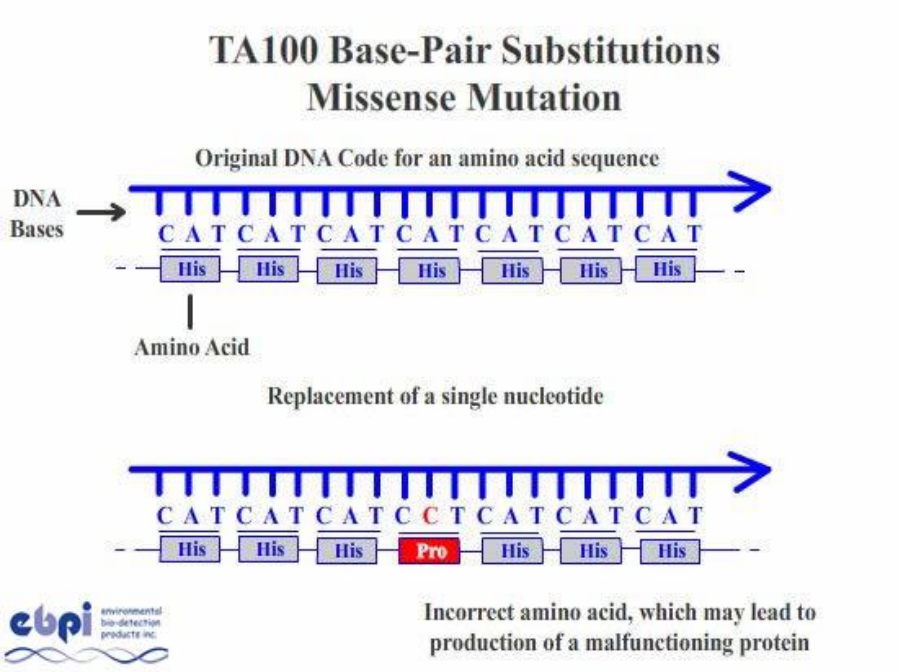

Figure 2.3: An example of a missense mutation (Source - Environmental Bio-detection Products Incorporated, Canada EBPI, 2012)

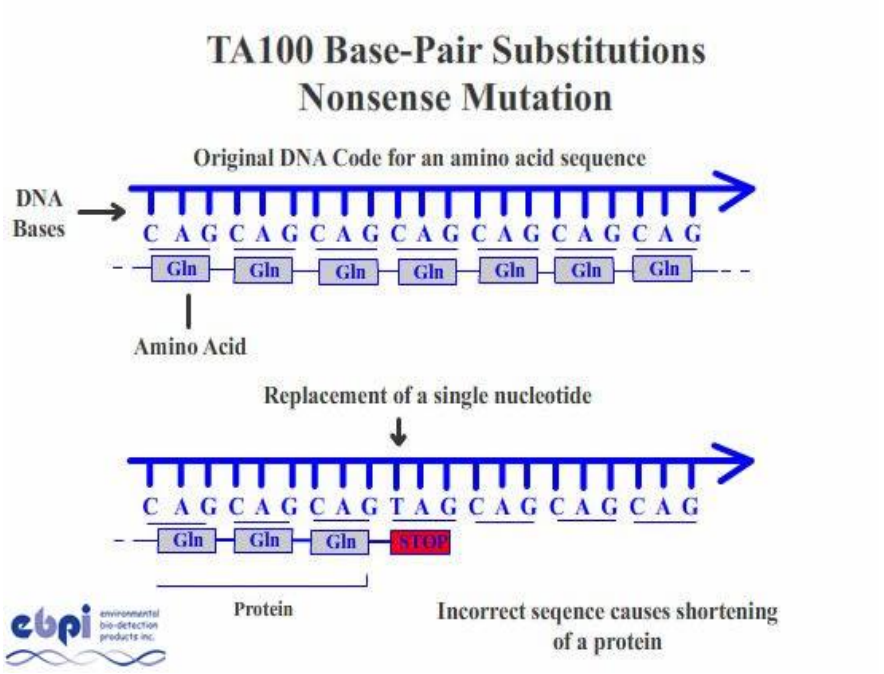

Figure 2.4: An example of a nonsense mutation (Source - Environmental Bio-detection Products Incorporated, Canada EBPI, 2012)

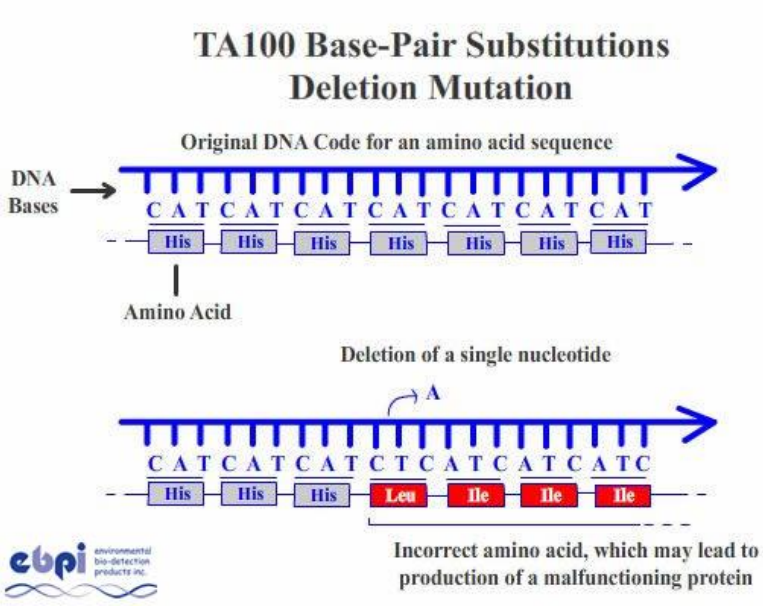

Figure 2.5: A diagram showing an example of a deletion mutation (Source - Environmental Bio-detection Products Incorporated, Canada EBPI, 2012)

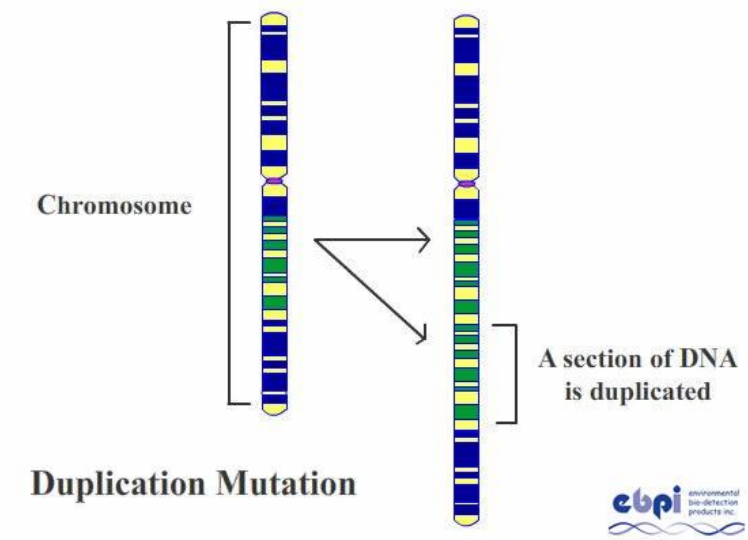

Figure 2.6: A diagram showing an example of a duplication mutation (Source - Environmental Bio-detection Products Incorporated, Canada EBPI, 2012)

2.8.1 The Muta-ChromoPlate™ test

It is a 96-well micro-plate version of the *Salmonella typhimurium* Ames test used for the detection of mutagenic activity. It has been developed to test mutagenic materials in water, soluble extracts of sediment, air, chemicals, food components, cosmetics, waste water, potable water and any other material that can be solubilised or placed into micro-suspension in water such that the material being tested can be taken up by the test strain (EBPI, 2012). Just as in the Ames test, it employs a mutant strain of *Salmonella typhimurium*, carrying mutations in the operon coding for histidine biosynthesis. When these bacteria are exposed to mutagenic agents, under certain conditions, reverse mutations from amino acid (histidine) auxotrophy to prototrophy occurs, allowing growth and turning the wells on the microtitre plate from purple to yellow. The clear yellow colour endpoint signifies the presence of mutagens. The Muta-ChromoPlate™ kit is generally more sensitive than the Ames pour-plate assay, because it allows testing of higher concentrations of sample (up to 75% v/v) (EBPI, 2012). The assay procedure is simple and requires minimal training. The test kit comes with reagents, cultures and other consumable components, supplied ready-to-use in a non-specialized laboratory. The advantage is that there is no need for preparation of growth media for cultures and time consuming dilutions which may lead to contamination (EBPI, 2012).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 OILS AND MUTAGENICITY TEST KIT

Unsalted red palm oil (RRPO), *zom*i or salted red palm oil (RPO), and *frytol* (PO) were purchased from a local supermarket in Tema. The virgin coconut oil (VCO) was purchased from a local health shop in Accra. The oils were kept at room temperature, 28^{o}C. The mutagenicity test kit was procured from Environmental Bio-detection Products Incorporated, Canada (Appendix I). The kit contained all reagents and chemicals needed for the assay (Appendix II). On arrival the vial containing the lyophilized bacteria and growth media were kept frozen at -20^{o}C and the other chemicals and reagents were kept refrigerated at 4^{o}C till needed.

3.2 OIL SAMPLE PREPARATION

The oil samples were prepared according to the protocol of Owu *et al*. (1998) with some modifications. Each of the four oils (250 ml) was measured into conical flasks and their corresponding weights taken. Unheated samples of the four oils were labelled accordingly. To prepare repeatedly heated oils, samples of the oils were then heated in a convectional hot air oven at 180^{o}C for 10 minutes (Appendix III). After completion of the heating process, single heated oil (X1) was obtained. The process was repeated four times to obtain five times heated oil with a cooling interval of at least 5 hours between each heating process. The weights and the volumes of the various oils were taken after each heat treatment and recorded to the nearest two decimal places. The final volume of each oil after the final heat treatment was also recorded. After the heating process 30 ml volume samples of each of the different

heat treated oils were taken and kept frozen at -20^{o}C to await the commencement of the assay procedure.

3.3 THE ASSAY PROCEDURE

3.3.1 Handling the Muta-ChromoPlate™ Kit

The test kit was handled with strict safety precautions as it contained bio-hazardous materials such as known mutagens (positive controls) which are possible carcinogenic agents. All used components were collected into the biohazard bag included in the kit and sterilized for safe disposal.

3.3.2 Protocol

On the evening prior to the assay, the vial of *Salmonella typhimurium* bacterial test strain TA 100 supplied in the kit and kept in the freezer and the vial of growth media (G) from the fridge were removed and allowed to thaw to room temperature (25^{o}C). Face masks and sterile gloves were worn and changed intermittently and 70% alcohol was sprayed onto laboratory work benches, packages and the immediate atmosphere frequently, to maintain aseptic conditions.

Using aseptic techniques, the contents from the growth media G was added to the vial that contained the bacteria strain. The rubber stopper was used to cover the vial that contained the bacteria and growth media and the vial was shaken to ensure that the bacteria and growth media were well mixed. The vial was then incubated at 37^{o}C for 18 hours and examined visually for turbidity (an indication of growth).

Reagents A through E (Appendix II) were removed from the refrigerator and thawed to room temperature (25^{o}C) and pipetted [21.62 ml (A) + 4.75 ml (B) + 2.38 ml (C)

+1.19 ml (D) + 0.06 ml (E)] with a sterile micropipette to make a total of 30.00 ml into a labelled sterile reaction mixture bottle.

Each oil sample was thawed at room temperature and 200 µl of each thawed oil sample was then pipetted into sterile 50 ml corning tubes and labelled accordingly. To each oil sample tube was next added 100 µl Tween 20 (polysorbate 20) and 200 µl DMSO (dimethylsulphoxide) to solubilise the oil samples. The tubes were then agitated to ensure thorough mixing. The volume of each tube containing the oil samples and organic solvents were made up to 5 ml with sterile distilled water provided in the kit, and each was filter sterilized using sterile 0.22 µm membrane filters into labelled fresh sterile corning tubes. The resulting sterile filtrates were then topped up to 17.5 ml with the sterile distilled water provided and 2.5 ml each of the reaction mixture prepared earlier were dispensed into each labelled oil sample tube, making a total volume of 20 ml in each tube. The contents were then mixed thoroughly.

The positive control and blank tubes were also labelled accordingly. For the positive control, 100 µl of the standard mutagen was pipetted into a sterile corning tube and 2.5 ml of the reaction mixture was added and made up to 20 ml volume with the sterile distilled water provided. The blank tube contained 2.5 ml reaction mixture and 17.5 ml sterile distilled water making up a total volume of 20 ml. To each labelled tube (i.e. oil samples and positive control only), was added 5 µl of the cultured bacteria with the exception of the blank tube and the tubes were then vortexed for 15 sec to ensure thorough mixing. Then, 200 µl of the contents of each tube were then pipetted into labelled sterile 96-well micro-titration plates provided and thereafter, each plate was covered with its lid and sealed in sterile plastic Ziploc bags to prevent evaporation. The plates were then incubated at 37^{o}C for 3 to 6 days.

The same procedure was repeated using the bacterial strain *Salmonella typhimurium* TA 98, to test the *frytol* oil and unsalted red palm oil samples.

3.3.3 Scoring of Plates

The plates were visually observed and scored from day 3 to 6 though final readings were made on day 5. After 6 days the plates were removed from the incubator. Each well of the 96 well plates was considered a colony. Plates were scored visually in the following manner; all yellow, partially yellow or turbid wells were counted and scored as positives and all purple wells were scored as negatives by comparing to negative control plates.

The blank (sterility check) plate was observed and scoring of treatment plate was done.

3.3.4 Interpretation of Data

Interpretation of the results was done by the use of statistical tables provided by the manufacturer (Appendix XV).

CHAPTER FOUR

4.0 RESULTS

4.1 WEIGHT AND VOLUME CHANGES IN THE VEGETABLE OIL SAMPLES AFTER HEAT TREATMENTS.

Table 4.1 shows the weight (at 25^{o}C) of the vegetable oil samples before heating and after each heating process. The weights of the various oils across the various treatments were not significantly different ($p>0.05$).

Table 4.2 shows the volume (at 25^{o}C) of the vegetable oil samples before heating and after the final heating process. Virgin coconut oil had the highest volume loss after final heating and *zomi* palm oil had the least volume loss. The change in volumes of the various oils before and after heat treatments was however, not significantly different ($p>0.05$).

4.2 EFFECT OF REPEATED HEATING ON MUTAGENICITY

In the *S. typhimurium* TA 100 bioassays (Table 4.3), unheated VCO did not show the presence of mutagenic activity (0/96 wells) as seen in Figure 4.2. However with single heating, 3/96 positive wells were recorded, which indicates the presence of significant mutagenic activity ($p=0.05$) as seen in Figure 4.2B. Five times repeatedly heated VCO recorded 1/96 positive wells, showing insignificant mutagenic activity. Unheated RPO recorded 9/96 positive wells, showing a significantly higher mutagenic activity ($p=0.01$) than VCO unheated and heated. Once heated RPO recorded a positive well count of 8/96 ($p=0.01$) as seen in Figure 4.3B. Five times repeatedly heated RPO showed a significant reduction in the number of positive wells counted, 3/96 ($p=0.05$).

PO showed no mutagenic activity in the unheated, once heated and repeatedly heated oil recording all negative wells, 0/96 in the *S. typhimurium* TA100 bioassays as seen in Figure 4.4. RRPO unheated recorded a significantly high presence of mutagens, 58/96 wells (p=0.001) as seen in Figure 4.3A. Once heated and 5 times repeatedly heated showed progressive increase in the number of positive wells counted 65/96 and 82/96 respectively, showing significantly higher mutagenic activity (p=0.001) than all the other vegetable oils tested with the *S. typhimurium* TA 100 strain.

In the *S. typhimurium* TA 98 bioassays, (Table 4.4) only PO and RRPO were tested. PO remained negative unheated, 0/96 wells. However with single and repeated heating, PO recorded significant mutagenic activity, 3/96 positive wells (p=0.05). Unheated RRPO recorded insignificant mutagenic activity, 2/96 positive wells. Once heated and repeatedly heated RRPO recorded significant mutagenic activity, 4/96 and 5/96 positive wells respectively (p=0.05).

Table 4.1: Weight of vegetable oil samples (at 25°C) before heating and after each heating process

Vegetable oil sample[a]	Weight of vegetable oil samples (g)					
	X0[b]	X1[c]	X2[c]	X3[c]	X4[c]	X5[c]
VCO	225.35	225.44	225.24	225.20	225.17	225.15
RPO	222.74	222.87	222.65	222.60	222.57	222.54
PO	223.80	223.94	221.66	221.64	221.64	221.67
RRPO	225.14	225.09	224.91	225.89	225.81	225.80

[a]VCO- virgin coconut oil
[a]RPO - *zomi* palm oil (salted)
[a]PO – *frytol*
[a] RRPO – red palm oil (unsalted)
[b]unheated oil
[c]number of times heated e.g.X1 means single heated oil

Table 4.2: Volume of oil samples taken at room temperature (25°C) during the heating process.

Veg. Oil sample	Initial (ml)	Final (ml)	Loss(ml)
VCO	250	245	5.0
RPO	250	247	3.0
PO	250	246	4.0
RRPO	250	246	4.0

[a]VCO - virgin coconut oil
[a]RPO - *zomi* red palm oil (salted)
[a]PO – *frytol*
[a]RRPO - red palm oil (unsalted)

Table 4.3: Muta-ChromoPlate™ Assay Report of the various oils for *Salmonella typhimurium* - TA 100

Samples[a]	[b]Number of times	Number of positive (yellow) wells in plate after				*p*-value	Interpretation
		Day 3	Day 4	Day 5	Day 6		
Blank	NA	0	0	0	0	-	No mutagens
VCO	X0	0	0	0	0	-	No mutagens
VCO	X1	2	3	3	3	0.05*	Weak mutagens
VCO	X5	1	1	1	1	-	No mutagens
RPO	X0	8	8	9	9	0.01**	Moderately mutagenic
RPO	X1	8	8	8	8	0.01**	Moderately mutagenic
RPO	X5	2	3	3	3	0.05*	Weak mutagens
PO	X0	0	0	0	0	-	No mutagens
PO	X1	0	0	0	0	-	No mutagens
PO	X5	0	0	0	0	-	No mutagens
RRPO	X0	50	56	58	58	0.001***	Highly mutagenic
RRPO	X1	63	63	65	65	0.001***	Highly mutagenic
RRPO	X5	79	80	80	82	0.001***	Highly mutagenic

[a]VCO - virgin coconut oil
[a]RPO - *zomi* red palm oil (salted)
[a] PO - *frytol*
[a] RRPO – red palm oil (unsalted)

[b] Number of times heated e.g. X1 means once heated oil

NA – not applicable
* Significant at 5%
**Significant at 1%

*** Significant at 0.1%

Table 4.4: Muta-ChromoPlate™ Assay Report of the various oils for *Salmonella typhimurium* - TA 98

Samples[a]	[b]Number of times	Number of positive (yellow) wells in plate after				*p*-value	Interpretation
		Day 3	Day 4	Day 5	Day 6		
Blank	NA	0	0	0	0	-	No mutagens
PO	X0	1	2	2	2	-	No mutagens
PO	X1	2	3	3	3	0.05*	Weak mutagens
PO	X5	2	3	3	3	0.05*	Weak mutagens
RRPO	X0	0	1	2	2	-	No mutagens
RRPO	X1	2	3	3	4	0.05*	Weak mutagens
RRPO	X5	2	2	5	5	0.05*	Weak mutagens

[a]PO - *Frytol*

[a]RRPO - Red Palm Oil (unsalted)

[b] Number of times heated e.g. X1 means once heated oil

NA – not applicable

*Significant at 5%

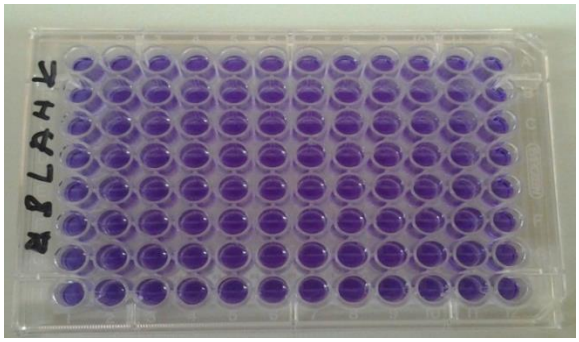

Figure 4.1: Blank plate on day 6 of assay - *S. typhimurium* TA 100 All wells remained purple signifying that there was no contamination of the assay. If yellow or turbid wells were observed in the blank plate, the assay would have been contaminated rendering the results invalid.

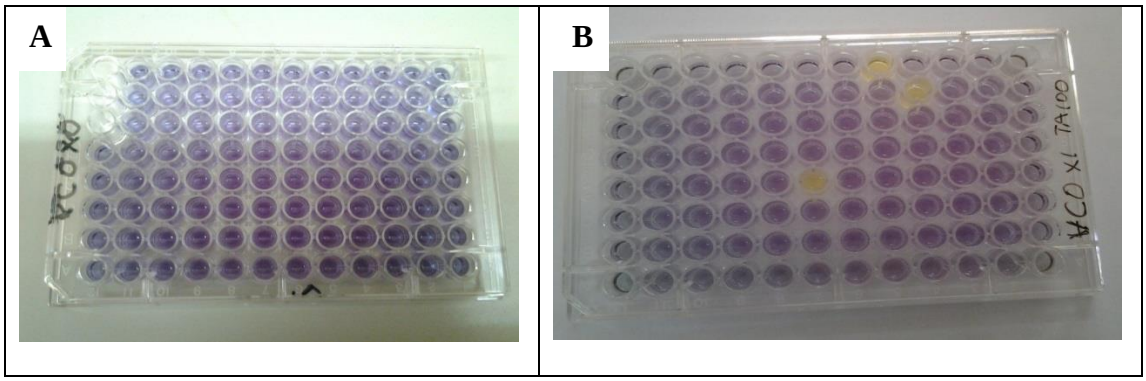

Figure 4.2: (A) Virgin coconut oil unheated showing all negative wells on day 6 using *S. typhimurium* TA100 (B) Once heated virgin coconut showing three positive wells on day 6 using *S. typhimurium* TA 100

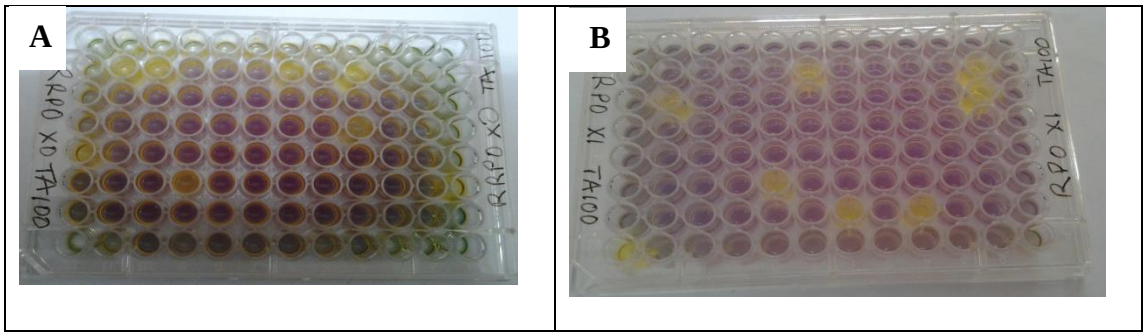

Figure 4.3: (A) Unheated red palm oil (unsalted) showing positive wells using *S. typhimurium* TA 100 (B) Once heated z*omi* palm oil showing positive wells day 6 using *S. typhimurium* TA 100

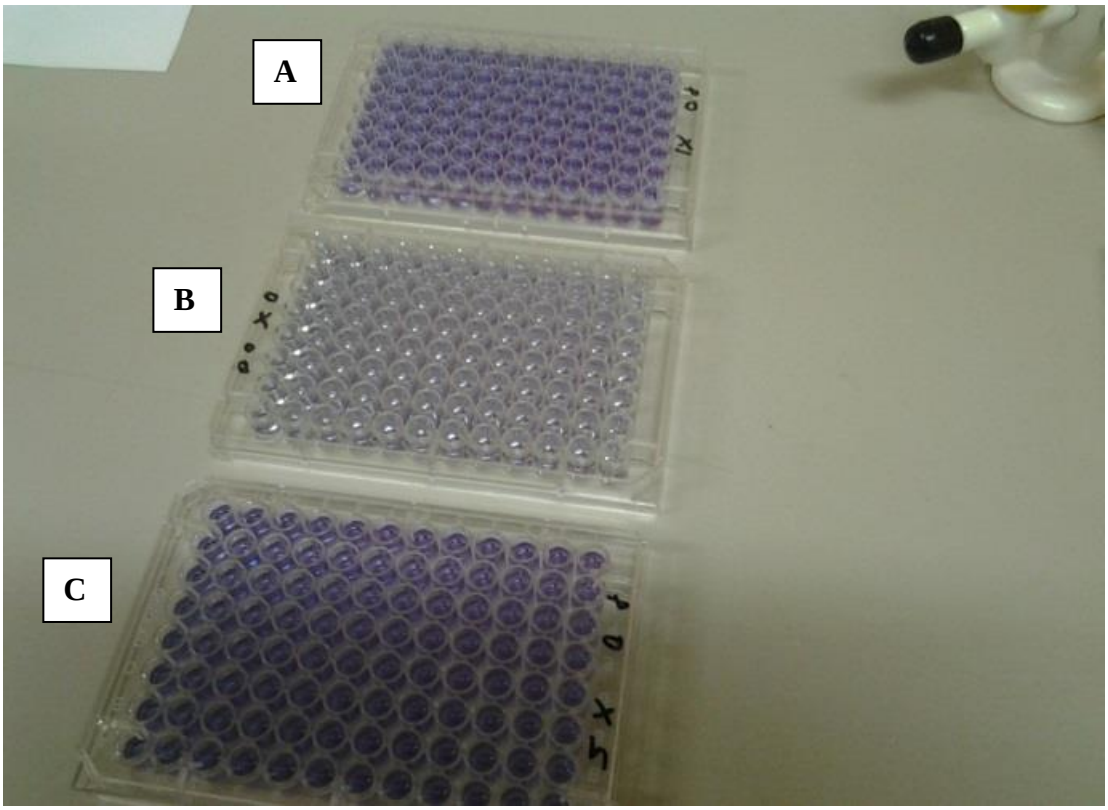

Figure 4.4: *Frytol* oil showing no mutagenic activity (all purple wells) on day 6 of assay using *S. typhimurium* TA 100.

A. Once heated *frytol* oil
B. Unheated *frytol* oil
C. Five times heated *fryto*l oil

CHAPTER FIVE

5.0 DISCUSSION AND CONCLUSIONS

5.1 DISCUSSION

Vegetable oils represent an indispensable ingredient in most Ghanaian cuisines. Palm oil (crude and refined) is the oil most used by Ghanaian households (GDHS, 2008). Studies indicate that heating generates mutagenic compounds which can lead to the development of cancer. This study sought to determine the presence of these compounds in the selected oils.

The single heating of virgin coconut oil at 180^{o}C, was above its smoke point (175^{o}C) which caused the significant generation of mutagens. This agrees with the research findings by Katragadda *et al*. (2010) who recommended that the temperature of any frying oil should be established below its smoke point and that coconut oil should not be used as a deep frying medium. Repeated heating increases the FFA content and consequently reduces drastically its original smoke point. The FFA content of oils has a high linear correlation with mutagenicity (Yen and Wu, 2003). It was expected that with repeated heating of VCO, more mutagenic substances would have been generated as in literature; repeated heat treatment causes increase in genotoxic activity (Isidori and Parella, 2009). The experiments however did not test virgin coconut oil with *Salmonella typhimurium* TA98; which detects frameshift mutations (because of limited reagents). There is the possibility that frameshift mutations would have occurred with the repeated heating of the virgin coconut oil, which the *Salmonella typhimurium* strain TA100 could not detect as it detects only point mutations. The presence of antioxidants in the oil could also reduce

genotoxicity. Virgin coconut oil is known to contain natural vitamin E which is a modulator of genotoxicity.

With respect to the salted palm oil or zomi, strict aseptic techniques were employed in conducting the experiments and thus the presence of mutagens in the unheated oil, was probably due to contaminants in the oil sample itself, prior to the mutagenicity test. A study conducted by Adjei-Nsiah *et al*. (2012) attests to the low quality of palm oil on the Ghanaian market due to the processing methods used. Their study attributed the poor quality of locally produced red palm oil to the late processing of the palm fruit bunches after harvesting, contaminated water used, the use of vehicle tyres as fuel wood and the low level of knowledge of the small and village scale palm oil processors. *Zomi red* palm oil is produced only by the small and village scale processors.

Successive heat treatments of *zomi* red palm oil showed progressive reduction in mutagenic activity in the *Salmonella typhimurium* TA100 experiments. Reduction in mutagenic activity with the repeated heating was not expected. This could be attributed to the presence of antioxidants in the *zomi* red palm oil. It is a crude unrefined palm oil which contains natural antioxidants such as carotenoids, vitamin E (tocopherols and tocotrienols) and polyphenols (Table 2.3). The inhibition of chemical carcinogenesis by palm oil carotenoids both *in vitro* and *in vivo* has been documented by Tan and Chu (1991). Sundram *et al*. (2003) also concluded from their study on palm oils that, the natural bouquet of carotenoids in palm oil has promising chemopreventive activities against cancer. The *zomi* red palm oil was however not tested with TA 98 to ascertain whether frameshift mutations had also occurred with the heat treatments.

Frytol oil was tested with both strains of *Salmonella typhimurium*. In the *Salmonella typhimurium* TA100 experiments, *frytol* showed no mutagenic activity in all the heat treatments. This signified that no point mutations occurred in the *fryto*l oil with repeated heat treatments. The process of refining oils removes the products of oxidation, hydrolysis and reduces the FFA content making the oils more stable at high temperatures.

Heating *frytol* oil once in the *Salmonella typhimurium* TA 98 experiments, caused frameshift mutations which the *Salmonella typhimurium* TA 100 bacterial assay could not detect. Heating the oil five times repeatedly did not cause further increases in mutagenic activity. The *fryto*l oil is refined and had been fortified with vitamin A as indicated on its label. These could have contributed to its stability to genotoxicity as refining removes products of oxidation and hydrolysis and antioxidants (both synthetic and natural) have a modulatory effect on genotoxicity and mutagenicity (Dzomba *et al*., 2012). Testing with both strains of bacteria showed that heating caused frame shift mutations but no point mutations in *frytol*.

Red palm oil (unsalted) was also tested with both bacterial strains. In the *Salmonella typhimurium* TA 100 experiments, the unheated and repeatedly heated oil showed the highest mutagenicity compared with the other oils in the study. This could be due to the poor quality of the unsalted red palm oil as seen with the *zomi* palm oil. The unsalted red palm oil sample was also processed by village and small scale processors. The same reasons could therefore be attributed to its quality prior to the experiments. Comparatively, red palm oil (unsalted) had a higher mutagenicity than *zomi* red palm oil (salted) and this agrees with studies by Adjei-Nsiah *et al*. (2012) on *zomi* and red palm oil quality.

Red palm oil (unsalted) showed progressive increase in mutagenic activity with repeated heating. This indicated that the red palm oil (unsalted) showed several point mutations which progressively increased with repeated heat treatments.

In the *Salmonella typhimurium* TA 98 experiments, red palm oil (unsalted) showed insignificant mutagenic activity when unheated. However, with single heating significant mutagenic activity was recorded. This could indicate that the heat treatment induced frameshift mutations as the *frytol* oil was also not mutagenic unheated but mutagenic on single heating in the *Salmonella typhimurium* TA 98 experiments. Repeatedly heated red palm oil (unsalted) showed significant mutagenic activity. This demonstrated that repeated heating induced mutagenic activity in the oil.

This study however had limitations such as the limited number of vegetable oil samples that could be tested with the Muta-ChromoPlate™ test kit due to cost and procurement procedures. This resulted in the inability to repeat assays where there was lack of consistency in the results. Virgin coconut oil and *zomi* palm oil could not be tested at all with *Salmonella typhimurium* TA 98 strain due to this limitation.
There is also limited relevant literature on *zomi* palm oil.

5.2 CONCLUSIONS

From the results of the experiments, it can be concluded that heating has an effect on the mutagenicity of the vegetable oils studied. From both bacterial strain experiments, *frytol* oil showed that repeated heating of vegetable oils causes progressive mutagenic activity. Vegetable oils used in food preparation should not be heated above their respective smoke points as shown in the virgin coconut oil experiments. VCO is best used unheated or at temperatures lower than its smoke point. Firm conclusions can be

made from these experiments, that the red palm oils are mutagenic. Both salted and unsalted samples demonstrated point mutations. Frameshift mutations were further demonstrated in unsalted red palm oil. Both unsalted and salted palm oil recorded the highest mutagenic activity. This finding brings to the conclusion that the safety in terms of mutagenicity of the red palm oils on the local market cannot be guaranteed.

Recommendations made from this study are;

1. As no single test is capable of detecting all genotypic mechanisms relevant in the development of tumours (ICH, 2012), it would be ideal for this experiment to be repeated on the vegetable oils using the S9 rat liver enzyme to observe the effect of metabolism on the mutagenicity of the oils. Further tests can be carried out by evaluation in mammalian cells *in vivo. In vivo* assays are required components of any thorough genetic toxicity testing programme (OECD, 1997). Unlike *in vitro* assays, *in vivo* assays are capable of accounting for toxicokinetic factors and DNA repair processes that may in some cases modulate genotoxicity within the whole animal (OECD, 1997).

2. Chemical analyses of the red palm oils need to be done on various samples of the red palm oils to ascertain their contents, which may have contributed to the results observed in the experiments.

3. The number of vegetable oil samples should be reduced in order to compensate for the limited number of kit contents, and also make room for extensive testing of the selected oils.

4. Palm oil production needs to be looked at with concern. Approximately 54 % of the populace use palm oil in their households as documented by the Ghana Demographic Health Survey (2008). A monitoring agency or task force could be set up to monitor the activities of palm oil processors in the country, and equip them with

knowledge on good manufacturing practices in palm oil processing especially the small and village scale processors. The Ministry of Health, Ministry of Food and Agriculture, Food and Drugs Board, Palm oil processing organizations and other stakeholders in the country should collaborate to devise ways of improving on the quality and safety of palm oil on the local market. The public needs to be educated to appreciate the importance of food safety with regard to taste and flavour of food.

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APPENDICES

Appendix I: The Muta-ChromoPlate™ test kit components

Appendix II: The Muta-ChromoPlate ™ test kit contents

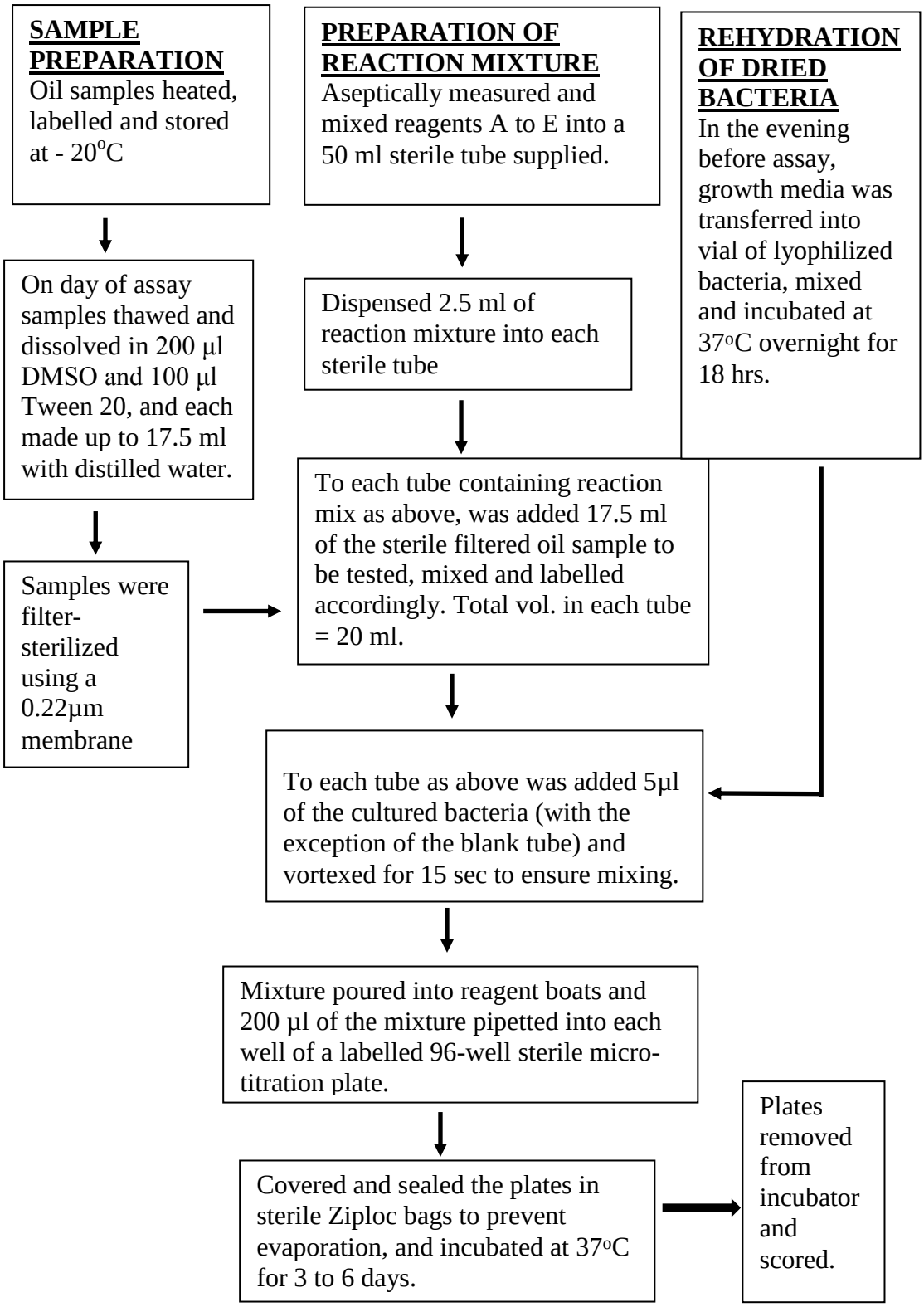
- 25 Sterile 50 ml tubes
- 20 Sterile Reagent Boats
- 24 Sterile micro-plates with lids
- 2 Sterile 0.22 um Filter
- Ziploc bags
- Sterile reaction mixture bottle
- Biohazard disposable bags
- Bacterial Strain (TA 100)
- Bacterial Strain (TA 98)
- Standard mutagens (sodium azide,2-Nitrofluorene)

Reagents A - G

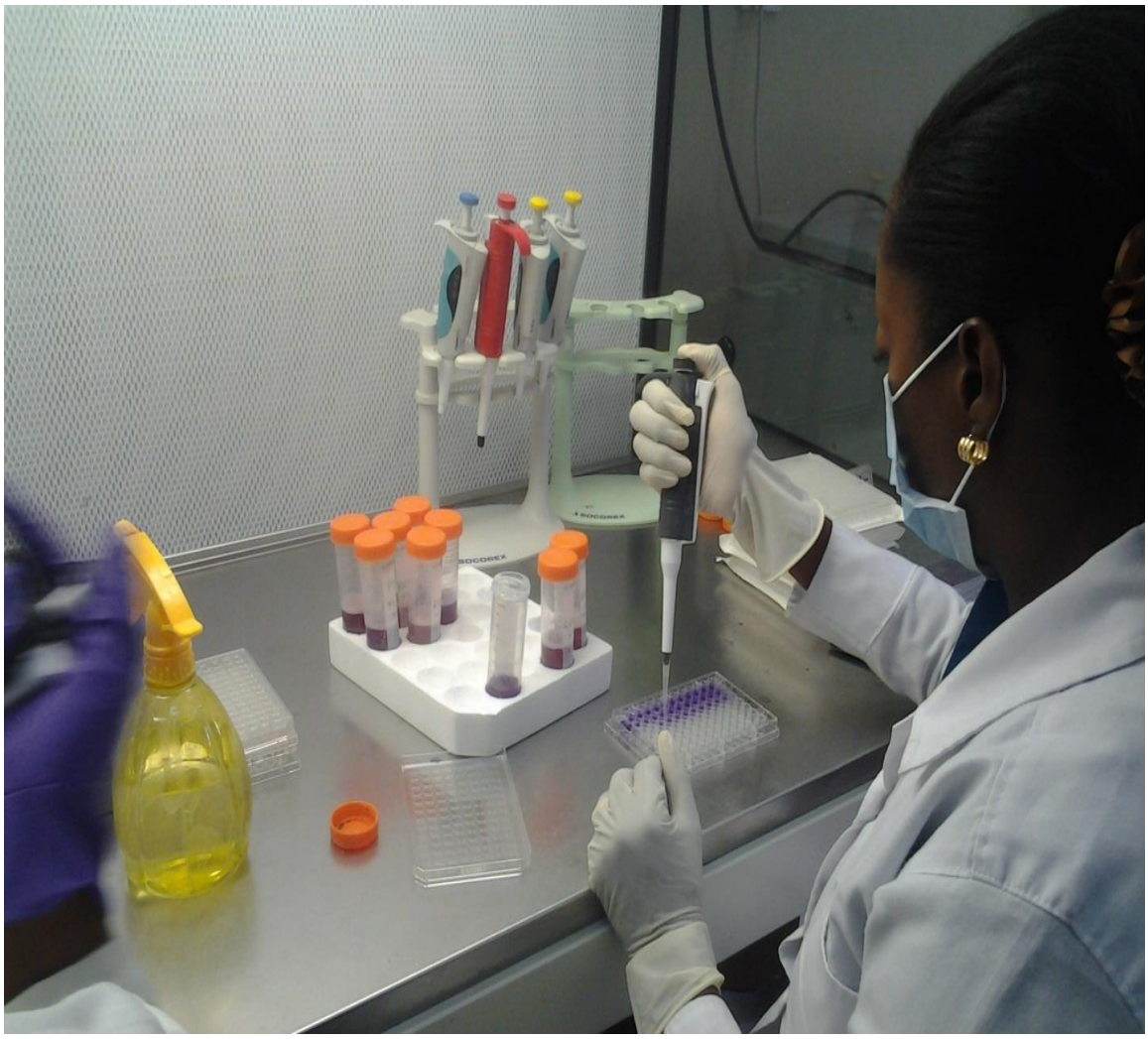
- A: Davis-Mingioli salts
- B: D-glucose,10 ml
- C: Bromocresol Purple,5 ml
- D: D-Biotin,3 ml
- E: L-Histidine,200 µl
- F: Sterile distilled water 4 x 100 ml
- G: Growth Medium,5 ml
- DMSO

Appendix III: Sample preparation – repeated heating of oil samples in a hot air oven

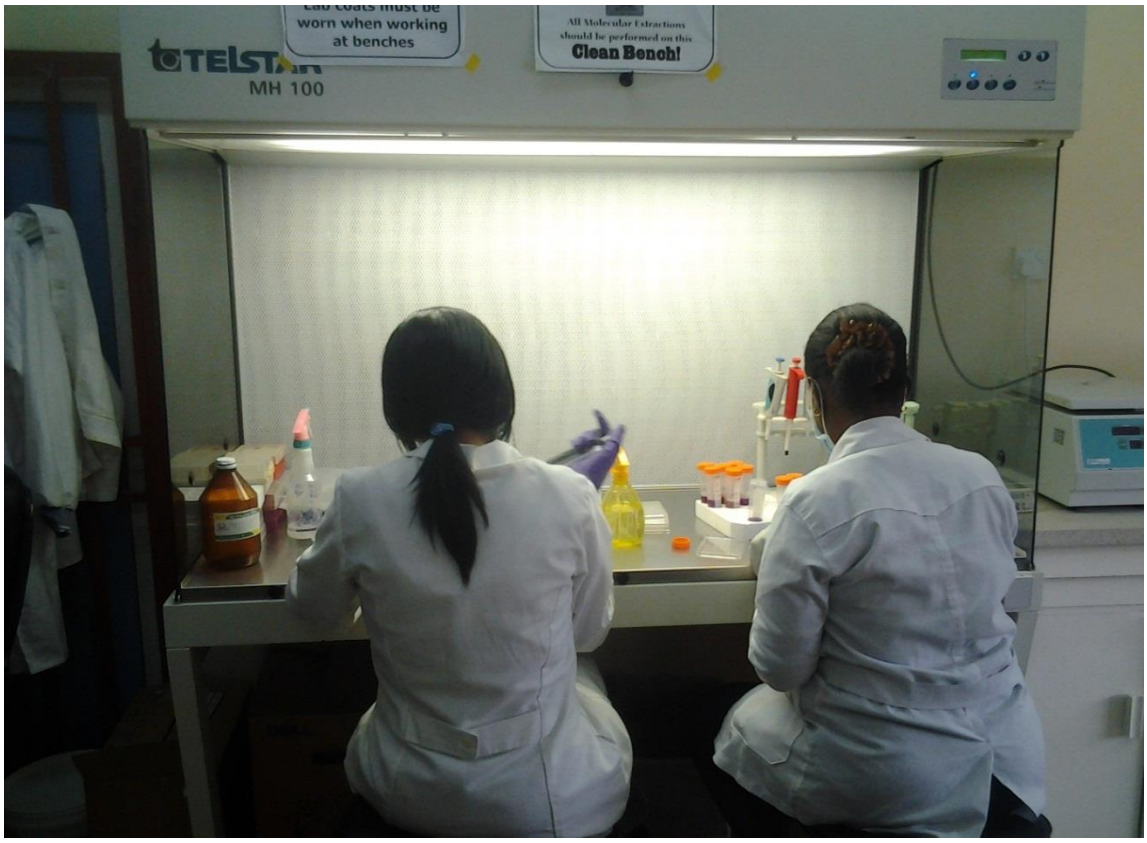
Appendix IV: Mutagenicity experiment flow process



Appendix V: Preparation of the assay- pipetting final mixtures into microtitre plates

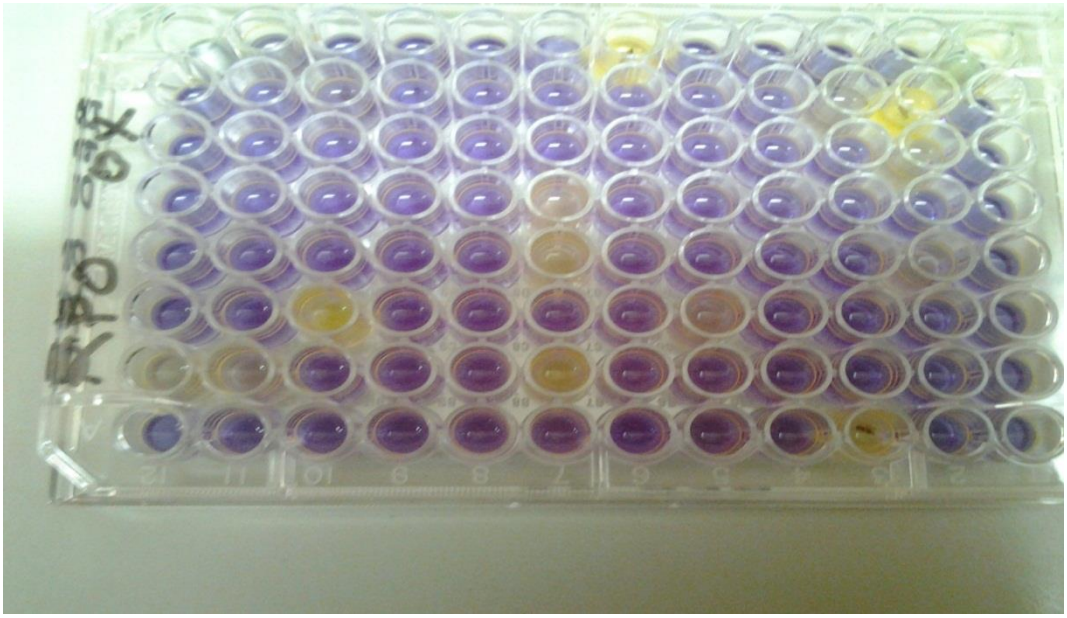


Appendix VI: Preparation of the assay- pipetting final mixtures into microtitre plates

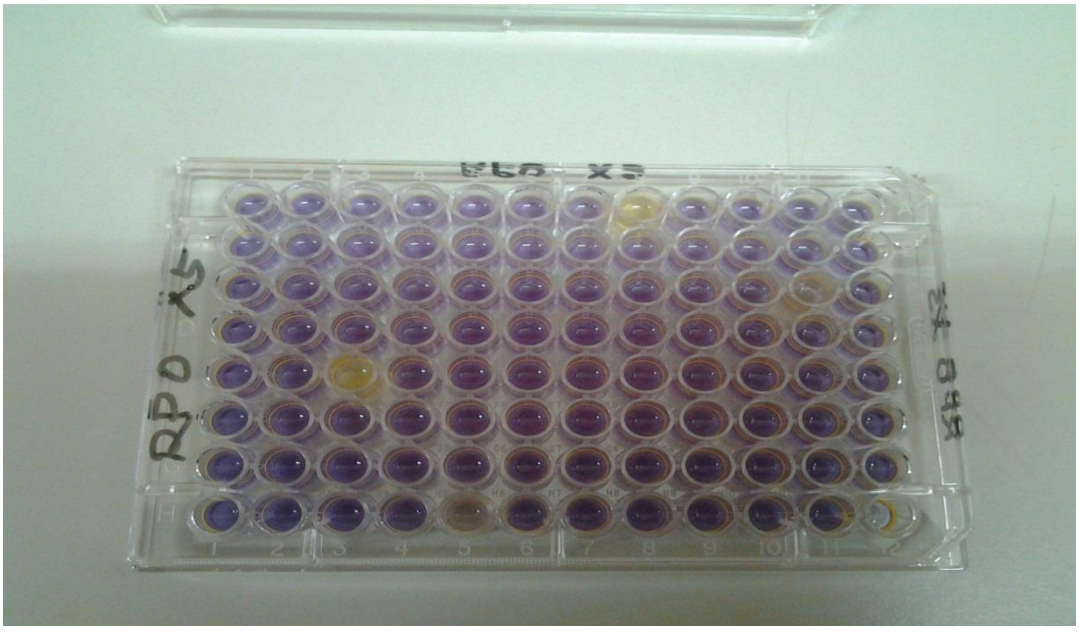


Appendix VII: Incubation of plates in incubator at 37^{o}C for 6 days

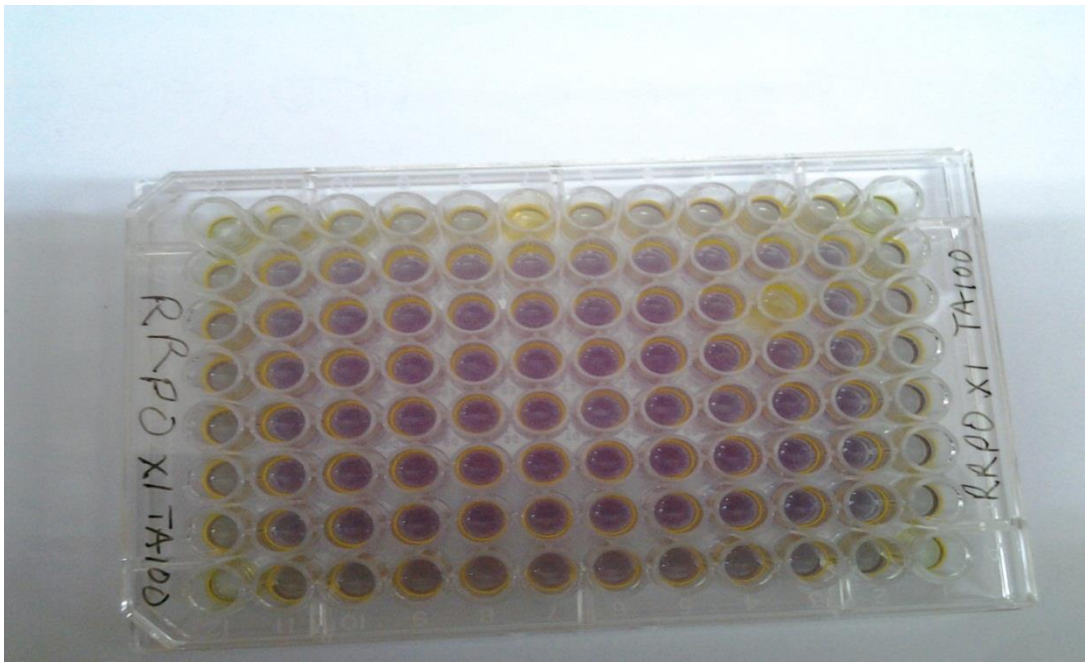
Appendix VIII: Unheated *Zomi* palm oil - *S.typhimurium* TA 100



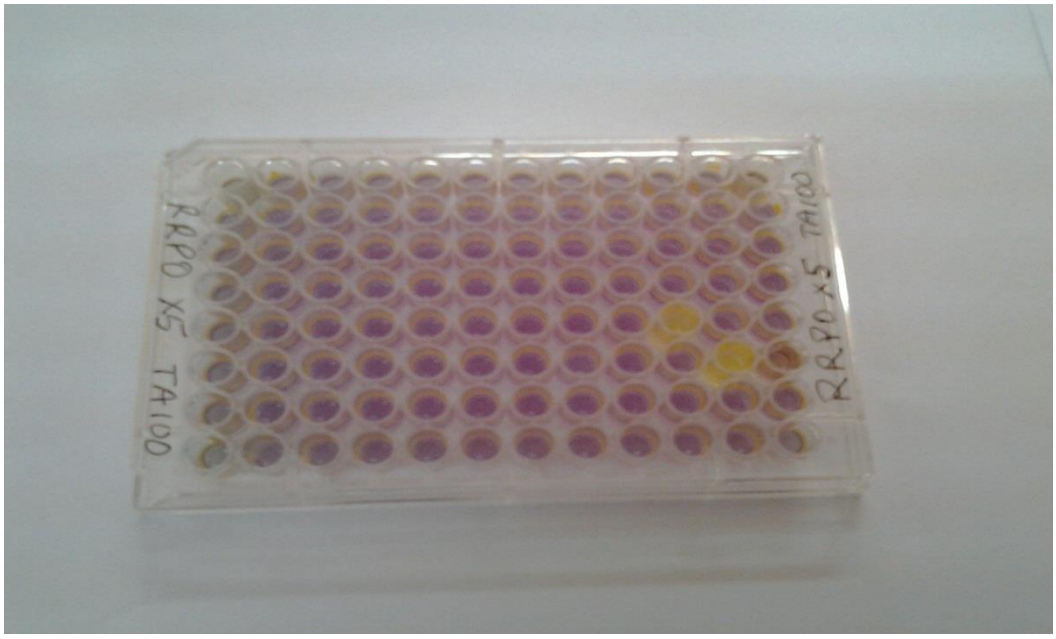
Appendix IX: Five times heated *zomi* oil on day 6 – *S.typhimurium* TA 100



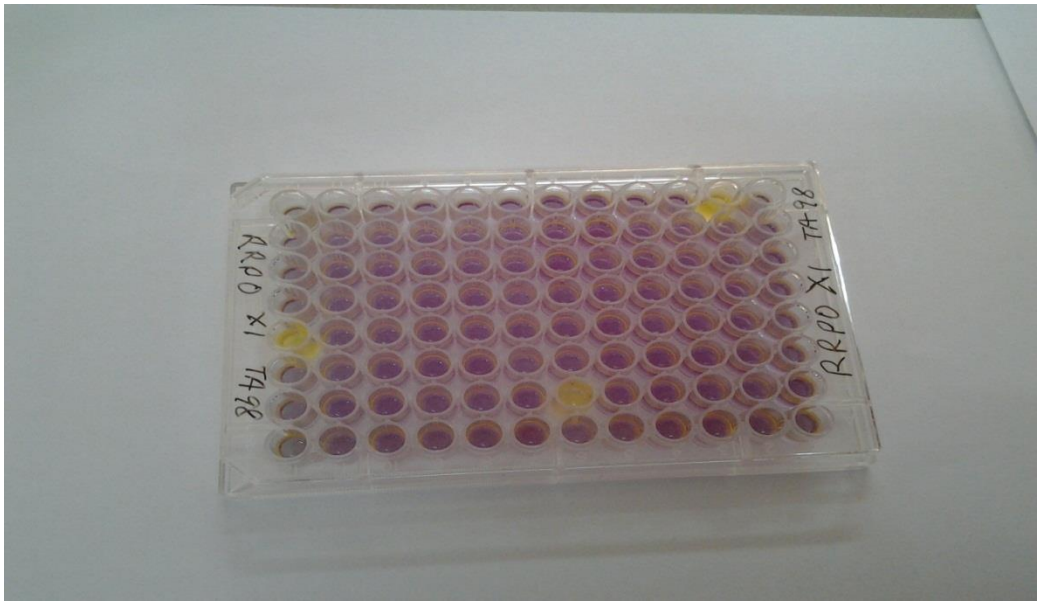
Appendix X: Once heated red palm oil (unsalted) on day 6 – *S.typhimurium* TA 100



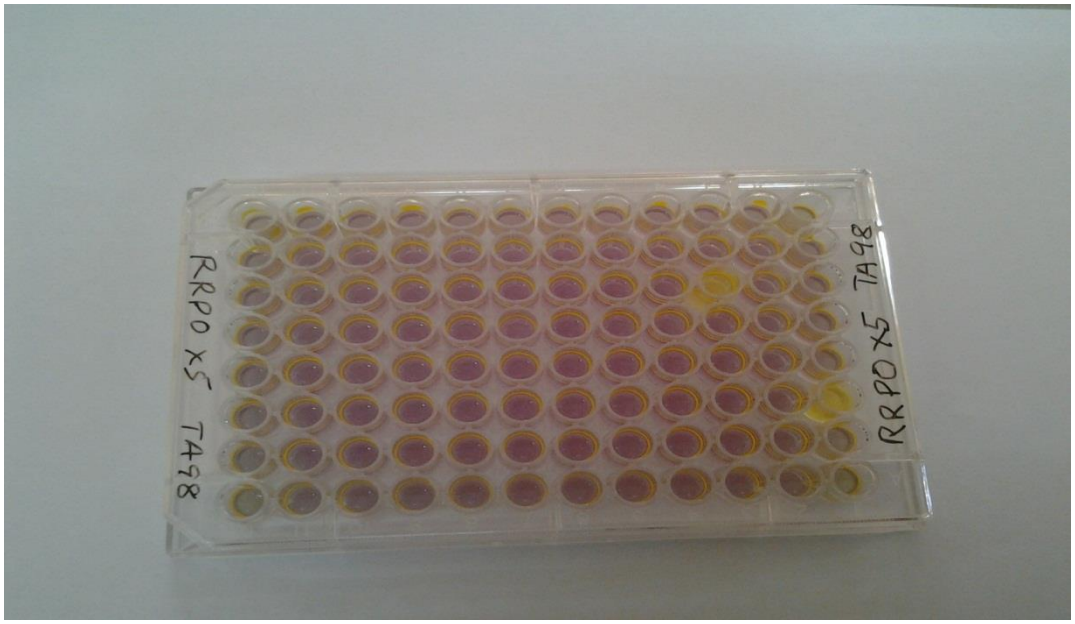
Appendix XI : 5 times heated red palm oil (unsalted) on day 3 - *S.typhimurium* TA100



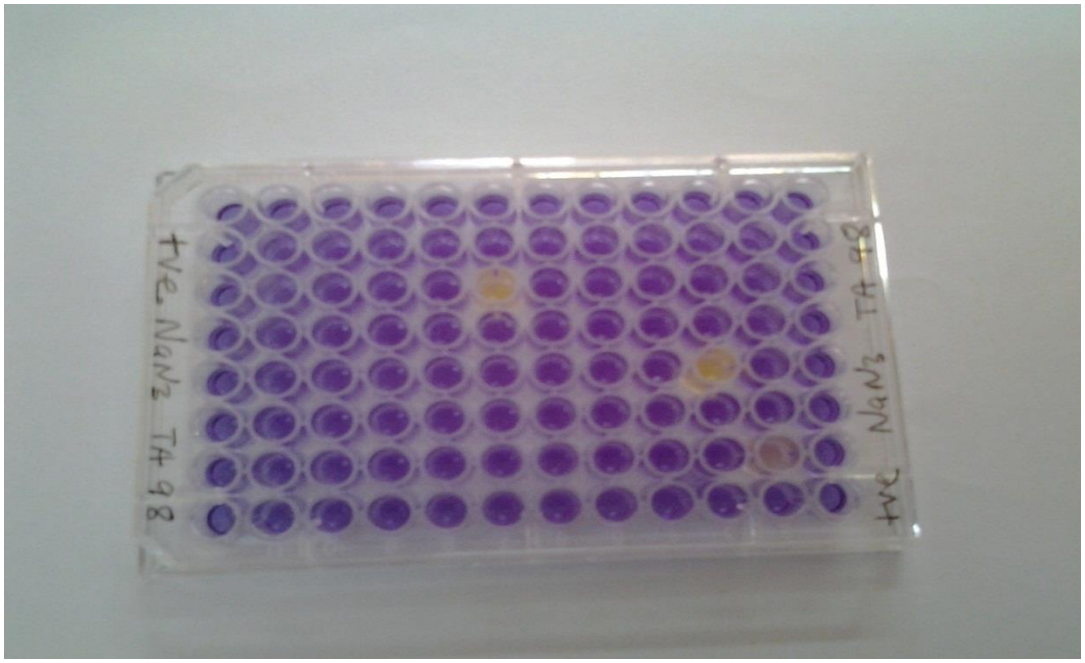
AppendixXII: Once heated red palm oil (unsalted) on day 6 –*S.typhimurium* - TA98



Appendix XIII: 5 times heated red palm oil (unsalted) on day 6 – *S.typhimurium* TA98



AppendixXIV: Sodium azide positive control on day 3 of assay – *S.typhimurium* TA98



Appendix XV: The number of positive wells scored in a 96-well microplate leading to clear significance in the fluctuation test.

No. wells positive in Background plate	No. wells positive in treatment plate			No. wells positive in background plate	No. wells positive in treatment plate		
	0.05	0.01	0.001		0.05	0.01	0.001
0	3	6	10	36	48	53	58
1	5	8	12	37	49	54	59
2	7	10	14	38	50	55	60
3	9	12	16	39	51	56	61
4	10	14	19	40	52	57	62
5	12	15	20	41	53	58	63
6	13	17	21	42	54	59	64
7	15	18	23	43	55	60	65
8	16	20	25	44	56	61	66
9	17	21	26	45	57	62	67
10	19	23	27	46	58	63	68
11	20	24	29	47	59	64	69
12	21	25	30	48	60	63	70
13	22	27	32	49	61	66	70
14	24	28	33	50	62	67	71
15	25	29	34	51	63	67	72
16	26	30	36	52	64	68	73
17	27	32	37	53	65	69	74
18	28	33	38	54	66	70	75
19	30	34	39	55	67	71	76
20	31	35	40	56	68	72	77
21	32	36	42	57	68	72	77
22	33	38	43	58	69	74	78
23	34	39	44	59	70	75	79

Appendix XV: Continued

No. wells positive in Background plate	No. wells positive in treatment plate			No. wells positive in background plate	No. wells positive in treatment plate		
	0.05	0.01	0.001		0.05	0.01	0.001
24	35	40	45	60	71	75	80
25	36	41	46	61	72	76	81
26	37	42	47	62	73	77	71
27	39	43	49	63	74	78	82
28	40	44	50	64	75	79	83
29	41	45	51	65	76	80	84
30	42	47	52	66	77	80	84
31	43	48	53	67	78	81	85
32	44	49	54	68	78	82	86
33	45	50	55	69	79	83	87
34	46	51	56	70	80	84	87
35	47	52	57	71	81	84	88
72	82	85	89	84	91	94	95
73	83	86	89	85	92	94	96
74	83	87	90	86	93	94	96
75	84	87	90	87	93	95	-
76	85	88	91	88	94	95	-
77	86	89	92	89	94	96	-
78	87	89	92	90	95	96	-
79	87	90	93	91	96	-	-
80	88	91	93	92	96	-	-
81	89	91	94	93	96	-	-
82	90	92	94	-	-	-	-
83	90	93	95	-	-	-	-

Source – (Gilbert 1980; EBPI,2012)