STUDIES ON RETENTION OF BETA-CAROTENE EXTRACTED FROM *AMARANTHUS* SPECIES PRESERVED IN VIRGIN COCONUT OIL AND UNADULTERATED HONEY

BY

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DECLARATIONS

I hereby declare that this is my original work and has not been presented for the award of a degree or any other award in any other University.

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We confirm that the work reported in this thesis was carried out by the candidate under our supervision as University Supervisors.

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DEDICATION

This thesis is dedicated to all those who are dealing with burden caused by cancer and to those who have battled the disease to be declared cancer free.

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ABBREVIATION AND ACRONYMS

AIV	African Indigenous vegetables
ANOVA	Analysis of variance
DNA	Deoxyribonucleic acid
DGLV	Dark green leafy vegetables
DPPH	2,2-diphenyl-1-picryl-hydrazyl
FT – IR	Fourier transformer – infra red
BHT	butylated-4-hydroxytoluene
HIV/AIDS	Human immune virus– acquired immune deficiency syndrome
HPLC	High performance liquid chromatography
HPLC-NMR	High performance liquid chromatography–nuclear molecular resonance
IACR	International Agency for Cancer Research
LC-MS	Liquid chromatography–mass spectrometer
LDL	Low–density lipoprotein
NCD	Non-communicable diseases
NP-HPLC	Normal phase – high performance liquid chromatography
OCC	Open column chromatography
RAE	Retinol activity equivalent
RDA	Recommended daily allowance
ROS	Reactive oxygen species
RP-HPLC	Reversed phase – high performance liquid chromatography

RSA	Radical scavenging activity
RSD	Relative standard deviation
SLAMENGHI	species, molecular linkage, amount, matrix, effective absorption, nutrient status, host-related factors, interactions
SNK	Student-Newman Keul test
TBARS	Thiobarbituric acid reactive substances
TLC	Thin layer chromatography
UV	Ultra-violet
UV-visible	Ultra-violet visible
VAD	Vitamin A deficiency
VCO	Virgin coconut oil
WHO	World Health Organization

ABSTRACT

Dark green leafy vegetables (DGLV) such as *Amaranthus* spp are known to be good sources of beta-carotene, a pro-vitamin A carotenoid and a highly potent anti-oxidant. Anti-oxidants terminate chain reactions, prevent recurrence and also prevent the formation of unstable oxygen which otherwise can initiates a chain reaction that propagates to cancerous cell. Cancer is strongly attributed to poor diet as well as lack of exercise. New cancer cases are diagnosed daily, leading to a projection of 22.2 million cases by 2030, with death tolls of up to 13.2 million. This has a huge economic burden especially to developing countries. As expected of all carotenoids, beta-carotene is highly degraded in the presence of light, heat and oxygen. Methods of its preservation are a current challenge. Preservation of powdered beta-carotene encapsulated in phospholipids, refrigerated in vacuum for a hundred days preserve 90 % of beta-carotene. Vacuum conditions require expensive instruments hence a need to explore locally available options to reduce and eradicate the menace. Moreover use of steel wool as an oxygen absorber can preserve up to 60 % beta-carotene from solar dried vegetables, however sanitation and health risk are issues of great concern. This calls for alternative methods that would ensure availability and stability of beta-carotene. In light of this, the study investigated the retention of beta-carotene extracted from *Amaranthus* spp bought from Githurai market (Nairobi county) and separately preserved in virgin coconut oil (VCO) extracted from coconut fruits bought from Kongowea market (Mombasa county) and unadulterated honey obtained from a farmer in Eldama Ravine (Koibatek county) as matrices for preservation. The antioxidant activity of the preservatives was determined using 2,2-diphenyl-1-picryl-hydrazyl (DPPH) assay method while, reversed phase HPLC was employed for beta-carotene analysis. Monitoring of beta-carotene was done at an interval of two weeks during the first month, followed by four weeks interval up to the sixth month. One way ANOVA was used for data analysis, with separation of mean using SNK. Coconut oil and honey gave anti-oxidant activities with % Radical Scavenging Activity (%RSA) of 65.12±0.70 and 81.51±1.39 ($p<0.001$) respectively. The concenteration of beta-carotene preserved in coconut oil and honey was 2.80±0.01 mg/100g (9.23 %) and 5.16±0.01 mg/100g (17.19 %) ($p<0.001$) respectively. Although there was over 80 % beta-carotene degradation the concentration of retained beta-carotene was 0.216±0.001 and 0.302±0.003 retinol activity equivalent (RAE) value higher than the recomended daily allowance (RDA). The RDA is 400 µg (4.0×10^{-4}mg) for infant while adult require 1,300 µg (1.3×10^{-3}mg). It is envisaged that data obtained from this study will be used a stepping stone on the improvement that can be done on the preservatives, hence provide basis for development of a local and cheaper method of beta-carotene availability and preservation hence contribute to reducing cancer cases in the world by availing the much needed anti-oxidants.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Dark green leafy vegetables (DGLV) are rich sources of pro-vitamin A carotenoids such as beta-carotene, vitamin C, minerals, protein and folate (Nyambaka and Ryley, 2001; Abukutsa-Onyango, 2002; Ojeniyi and Adejobi, 2002; Berganza *et al*., 2003; Makombo *et al*., 2010). In Kenya there are more than 210 known green leafy vegetable species (Ngugi *et al*., 2007). *Amaranthus* vegetables are a rich source of beta-carotene, a pro-vitamin A carotenoid and an anti-oxidant hence can scavenge for electrons, terminating the chain reaction which could otherwise be the genesis of myriad non-communicable diseases (NCD) (Devasagayam *et al*., 2004). Therefore a diet containing anti-oxidants coupled with physical activities can stop both the spread and initiation of degenerative diseases. This is a more favorable way to manage cancer compared to the burden brought about by surgery, radiation and chemotherapy which are the known methods of treatment.

There is an increasingly high mortality that is caused by NCD, where nearly 80 % of the people affected live in the middle and low income countries (Alwan *et al*., 2011). Among the NCD include cardiovascular diseases, respiratory diseases, diabetes and cancer (Boutayeb, 2006; Daar *et al*., 2007). Although the other NCD are equally fatal, facts on cancer are alarming and shocking hence the effective measures to curb the peril is needed. Deaths due to cancer are more than those caused by HIV/AIDS, tuberculosis and malaria combined (Daar *et al*., 2007). The International Agency for Cancer Research

(IACR) reported new cancer cases estimates at 14.1 million, while cancer-related human death toll estimated to be 8.2 million in the year 2012 (IACR, 2012). The report also estimated that 32.6 million people who are above 15 years of age showed prevalence to cancer diagnosis. Of great concern and saddening is the statistics that show 64.9 % of the mortality on cancer occurred in developing countries (IACR, 2013). Moreover, American cancer society indicated that 1 in every 7 deaths in the world will be caused by cancer by 2030, 21.7 million new cancer cases will be diagnosed and 13 million deaths reported (American cancer society, 2015).

Most cancer cases are attributed to malnutrition, physical inactivity, overweight and obesity (Kushi *et al*., 2012). Cancerous cells develop by free radical or reactive oxygen species (ROS) which are unstable, highly reactive and energized molecules (Lee *et al*., 2004). Unpaired electrons in radicals make them very unstable, such that body cells, deoxyribonucleic acid (DNA) and proteins can take up their unpaired electrons or donate some electrons during a chemical reaction that propagates to degenerative diseases. This reaction though, can be terminated by introduction of anti-oxidants such as beta-carotene into the body system. In the presence of radicals, anti-oxidants scavenge electrons forming radicals adducts which pose no danger to the body cells (Young and Lowe, 2001).

Statistics of vitamin A deficiency (VAD) in middle and low income countries are dis-heartening. Most expectant women, lactating mothers and children under the age of 5 from these regions suffer from VAD. A total of 190 million pre-school going children in

sub-Saharan Africa, west- Africa, East Asia and pacific suffer from VAD (WHO, 2009). It is estimated that 250,000 to 500,000 VAD children become blind every year, and half of them dying within 12 months of losing their sight (WHO, 2009).

Separation and quantification of carotenoids can be simultaneously performed by high performance liquid chromatography (HPLC) (Rouessac and Rouessac, 2007). However carotenoids are prone to isomerism and oxidation process that causes loss of anti-oxidant and pro-vitamin A properties. The characteristic conjugated double bond system produces the problems associated with work and manipulation on the carotenoids causing instability towards light, oxygen, heat, acid and alkaline conditions (Jordi and Andreu, 2000). However, encapsulating beta-carotene in lipid based matrices, under vacuum or in an inert medium such as nitrogen gas reduces degradation of the carotenoid hence preserving it (Nyambaka and Ryley, 2001; Moraes *et al*., 2013). These are rather expensive method of preservation since specialized instruments will be required. Yet owing to anti-oxidant properties and pro-vitamin A benefits of beta-carotene, it is necessary to find cheaper preservation method, its instability notwithstanding.

Honey is an anti-oxidant since it is rich in phenolic acids, carotenoids (lycopene and beta-carotene) and flavonoids. It also contains other anti-oxidants including glucose oxidase catalase, ascorbic acid, carotenoid derivatives, organic acids, amino acids and proteins (Ferreira *et al*., 2009; Khalil *et al*., 2010). Determination of anti-oxidant capacity can be done by determining loss of absorbance of a stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) or monitoring thiobarbituric acid reactive substances (TBARS) (McKibben and

Engeseth, 2002). Honey has preservative property which was demonstrated by prevention of lipid oxidation in ground poultry (McKibben and Engeseth, 2002). African traditional societies used honey in preservation of pre-cooked game meat by submerging it in honey which conserved it for several months.

While coconut oil is food, it is also an immune-enhancer, an anti-biotic and a drug that regulates body functions and its defense mechanism (Fife, 2005). Coconut oil has anti-oxidants properties and a longer shelf life that is attributed to phenolic compounds (Marina *et al*., 2008; Seneviratne *et al*., 2008). Coconut oil can increase the shelf-life of poultry meat when submerged in the oil which reduces moisture content and bacteria colony (Aritonang *et al*., 2009).

Therefore since both coconut oil and honey have good preservative properties by prevention of oxidation were therefore expected to prevent oxidative degradation of beta-carotene upon preservation. Furthermore presence of phenolic compounds which are the major contributor of anti-oxidants found in coconut oil and honey synergically increases the total anti-oxidants of preserved beta-carotene. This can therefore increase the shelf-life and avail pro-vitamin A carotenoid addressing VAD and radicals causing NCD.

1.2 Problem statement

Carotenoids readily found in green vegetables are able to address VAD and combat free radicals. Among the vegetables, *Amaranthus* spp has gained promotion for not only because vegetables contain carotenoids that have anti-oxidant property but that its carotenoid content is over 60 % (Nawiri *et al*., 2013). As such, this research attracts the promotion, availability and retention of beta-carotene, as it is a good radical scavenger that would reduce the cancer disease burden and with the highest pro-vitamin A potency. The major challenge to this field of research relates ease of beta-carotene to degradation. Consequently, a number of methods including refrigeration, canning, dehydrating and storage under oxygen absorbers have been employed to counter this although degradation of beta-carotene is inevitable. It is in this regard that this research was undertaken to provide more innovations in beta-carotene preservation using honey and lipid or lipophilic coconut oil matrices for preservation of the carotenoid extracted from *Amaranthus* spp.

The matrices carry advantages that over time it has been known to be good preservative and have anti-oxidants properties (McKibben and Engeseth, 2002; Seneviratne *et al*., 2008; Marina *et al*., 2008; Ferreira *et al*., 2009). These properties would give a synergic effect to anti-oxidant properties of beta-carotene. Further, they are locally available and hence pose for a cheaper method of preservation but most importantly that they are safe for human consumption.

1.3 Justification

This study is triggered by the daily increase in cancer related deaths and impact of vitamin A deficiency which has a direct implication on any nation's economy. The situation is particularly unfortunate for low income nations. In Kenya statistics report 28 000 new cancer cases yearly while for vitamin A deficiency, statistics stands at 80 % and 17 % for pre-school children and expectant women respectively (WHO, 2009). The conditions are aggravated by lifestyle patterns including consumption of otherwise nutritionally poor diets and physical inactivity. While a number of efforts including the high cost expenditure on drugs, supplementation and fortification are explored to correct the health situation, it is worth noting that a nutritional approach is cheaper and a more sustainable solution in the long term.

1.4 Hypothesis

Levels of extractable beta-carotene do not reduce significantly when preserved in virgin coconut oil and unadulterated honey

1.5 Objectives

1.5.1 General objective

To determine the anti-oxidant activity of coconut oil and honey, and monitor levels of beta-carotene extracted from *Amaranthus* spp and preserved in coconut oil and honey for 180 days.

1.5.2 Specific objectives

i. To determine the anti-oxidant activity of unadulterated honey and virgin coconut oil.

ii. To monitor levels of extracted beta-carotene from *Amaranthus* spp preserved in honey and coconut oil for 180 days.

1.6 Significance of study

The importance of this study was to provide information of cheap and locally available materials (coconut oil and honey) for preservation of beta-carotene. Beta-carotene is not only a potent anti-oxidant that is able to reduce oxidative stress that would otherwise be the genesis of degenerative diseases, but also a pro-vitamin A carotenoid that splits enzymatically to provide vitamin A. Virgin coconut oil and unadulterated honey were used to preserve beta-carotene from *Amaranthus* spp vegetables, which is a novel idea, compared to traditional use of the two in food preservation. The two preservative contain among others phenolic compounds that constitute to anti-oxidant properties, hence addressing oxidative stress. As such, the dissemination of this work play part in not only campaigns for promoting the consumption of *Amaranthus* spp vegetables but also seek to address solutions by providing readily available anti-oxidants needed for reduction of VAD and cancer incidences among other degenerative diseases of concern both in Kenya and the world at large.

1.7 Scope and limitation of the study

The chemistry of carotenoids indicates their susceptibility to degradation when exposed to conditions such as light, air, heat and acid. While this study didn't involve the direct

use of acids, it was inevitable to completely avoid the interaction of air, light and heat during the procedures of analysis. Therefore some degradation may have arisen as a result of these limitations. Beta-carotene was extracted from *Amaranthus* spp vegetables and preserved separately in suspension of honey and coconut oil whose source were not considered save their purity. While the chemical compositions of these matrices vary, this study determined only their anti-oxidant properties. Conversely, other compounds such as organic acids and reactions products during storage were not studied. Stability of beta-carotene was monitored in the two preservatives at interval of 14 days for the first month, and then monthly for six consecutive months. Any products of degradation that might have formed during laboratory procedure and during storage were not investigated.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

The spread of and deaths caused by non-communicable diseases are increasing especially in sub-Sahara Africa (Lee, 2003). A total of 7.9 million deaths, due to cancer were reported in 2009 which constitute 13 % of worldwide deaths. Of this, 70 % of the total deaths were in low and middle income countries. The burden caused by cancer will continue rising with a projection of 15.5 million people being diagnosed with the disease and 12 million deaths to be reported by 2030 as a result of cancer (WHO, 2009). On the other hand vitamin A deficiency (VAD) is also rampant in the under developed countries. It is estimated that 190 million of pre-school children and 17 % of expectant women mostly from developing and low income countries suffer from VAD (WHO, 2009). Globally night blindness affects 5.2 million pre-school age children and 9.8 million expectant women a condition also associated to VAD (WHO, 2009). The inception of these illness is based on poor diet and physical inactivity (Lee, 2003).

2.2 Dark green vegetables

With the increase of formal education in Kenya, indigenous feeding habits were abandoned as primitive and belonging to poor man's diet (Abukutsa-Onyango, 2002). Although Kenya is home to 210 known species of vegetables, most African indigenous vegetables (AIV) are still underutilized (Ngugi *et al*., 2007). The most abandoned AIV include kunde (cowpeas), miito (jute mallow), mrenda (African eggplant), managu (African nightshade), and mchicha (*Amaranthus*) (Abukutsa-Onyango, 2002). Dark green

leafy vegetables are rich sources of anti-oxidants, vitamin C, proteins and essential macro and micro nutrients such as Zn, Mg Fe, Ca (Abukutsa-Onyango, 2002; Negi and Roy, 2003; Singh *et al*., 2009; Amoussa-Hounkpatin *et al*., 2013). Presence and quantity of these nutrients is highly determined by the type of soil used for cultivation, climate of the location they are grown and the age in which the vegetables are harvested (Ojeniyi and Adejobi, 2002; Berganza *et al*., 2003; Makombo *et al*., 2010).

A study carried out to determine the chemical composition of common green leafy vegetables showed 29.5 mg protein, 13 mg vitamin C, 138 mg Ca, 58 mg P and 50 mg Mg was found in parsley leaf (*Petroselinum crispum*) while lettuce (*Lectuca sativa*) had 16.2 mg protein, 24 mg vitamin C, 36 mg Ca, 45 mg P and 6 mg Mg (Caunii *et al*., 2010). Studies show that DGLV including *Amaranthus* spp, broccoli, spinach and cowpeas are rich in beta-carotene. Dark green coloration on the leaves is an indication that these vegetables are rich in beta-carotene (Arab *et al*., 2001; Negi and Roy, 2003; Ndawula *et al*., 2004; Amoussa-Hounkpatin *et al*., 2013). Beta-carotene is one of the carotenoids which is bioactive components potent in scavenging radicals that cause degenerative diseases such as cardiovascular diseases, Alzheimer and cancer (Raju *et al*., 2007). The other nutrients are essential in body growth and development as well as regulating body processes.

2.3 Beta-carotene levels in *Amaranthus* spp vegetables

Beta-carotene in DGLV is mostly affected by post-harvest handling and processing which are key sources of loss of the carotenoid. Due to large surface area to volume ratio

of the leaves, water losses in vegetables are high which makes them perishable. This is evident on vegetable shrinkage and loss in chlorophyll (Negi and Roy, 2003). Polyethene bags containing *Amaranthus tricolor* were kept at low humidity and low temperatures reducing water loss to preserved the nutrients. Beta-carotene that was preserved this way ranged from 46.5 % to 85 % hence indicating that packaging of leaves in low density polyethene bags was beneficial in improving the shelf life and nutritive value (Negi and Roy, 2003). In two different studies *Amaranthus tricolor* indicated 8.17 mg/100g beta-carotene while *Amaranthus panicalatus* showed 15 mg/100g beta-carotene (Negi and Roy, 2003; Bhatia and Jain, 2003). Other studies aimed at monitoring changes of beta-carotene with storage of dried *A. Hybridus* at 22^{o}C, 28^{o}C and at room temperature (30^{o}-32^{o}C) showed retention of 32.2, 29.5 and 27.4 mg/100g of beta-carotene respectively (Mziray *et al*., 2000). This indicated that the vegetables can be dried to produce an acceptable product which maintained its quality for up to three months in storage (Mziray *et al*., 2000). Studies have indicated that temperature is a major contributor in beta-carotene loss and therefore it should be controlled (Mziray *et al*., 2000; Negi and Roy, 2003). Hence vegetables that were kept in perforated polyethene bags and preserved at low temperature, preserved the vegetables up to four days (Negi and Roy, 2003).

Heat processing duration and the method used for preparation of the vegetables determine the concentration and composition of the nutrients that will be present in the food matrix. It is therefore paramount to expose DGLV including *Amaranthus* spp to heat for shortest time possible, to make them palatable and preserve some beta-carotene. Due to seasonality, DGLV are plenty during wet season and scarce during drought. Sun drying

of vegetables therefore, is widely exercised in arid and semi-arid regions of the country as a preservation method. The process involves loss of water in the vegetable hence low moisture content slows down the perishability rate of the vegetables (Nyambaka and Ryley, 2001). Sun dried *Amaranthus* spp that were further cooked had a mean of 599 µg/g of dry matter. Sun drying of the *Amaranthus* spp indicated loss of beta-carotene retaining 66 %, and when the vegetables were cooked further resulted in further loss of beta-carotene was recorded. The amount of beta-carotene retained was 59% (Nawiri *et al*., 2013). Other contradictory studies aimed at comparing pro-vitamin A carotenes of sun-dried and solar dried DGLV showed that open sun-drying reduces pro-vitamin A carotenes (43 %) while solar dried DGLV increases the carotenes (120-275 %) (Mulokozi and Svanberg, 2003). This study emphasized consuming 100 g of solar dried vegetables daily to provide the required amounts of pro-vitamin A carotenoid (Mulokozi and Svanberg, 2003).

2.4 Carotenoids

2.4.1 Structures and chemistry of carotenoids

Carotenoids are phytochemical compounds called terpenes which are classified in two groups; carotenes and xanthophyls. Carotenes are hydrocarbons such as lycopene and beta-carotene while xanthophyls have oxygenated derivatives. Beta-cryptoxanthin has hydroxyl derivative, canthaxanthin have keto derivative while violaxanthin and beta-citraurin have epoxy and aldehyde derivatives respectively (Rodriquez-Amaya, 2001).

Figure 2.1 shows some carotenoids which include xanthophyls and carotenes which are acyclic, monocyclic or di-cyclic. Acyclic carotenoids include lycopene, gamma-carotene (γ -carotene) for monocyclic, alpha-carotene (α-carotene) and beta-carotene (β-carotene) are a di-cyclic. In nature carotenoids exist in stable all-trans form and in small quantities as cis-conformers (Rodriguez-Amaya, 2001).

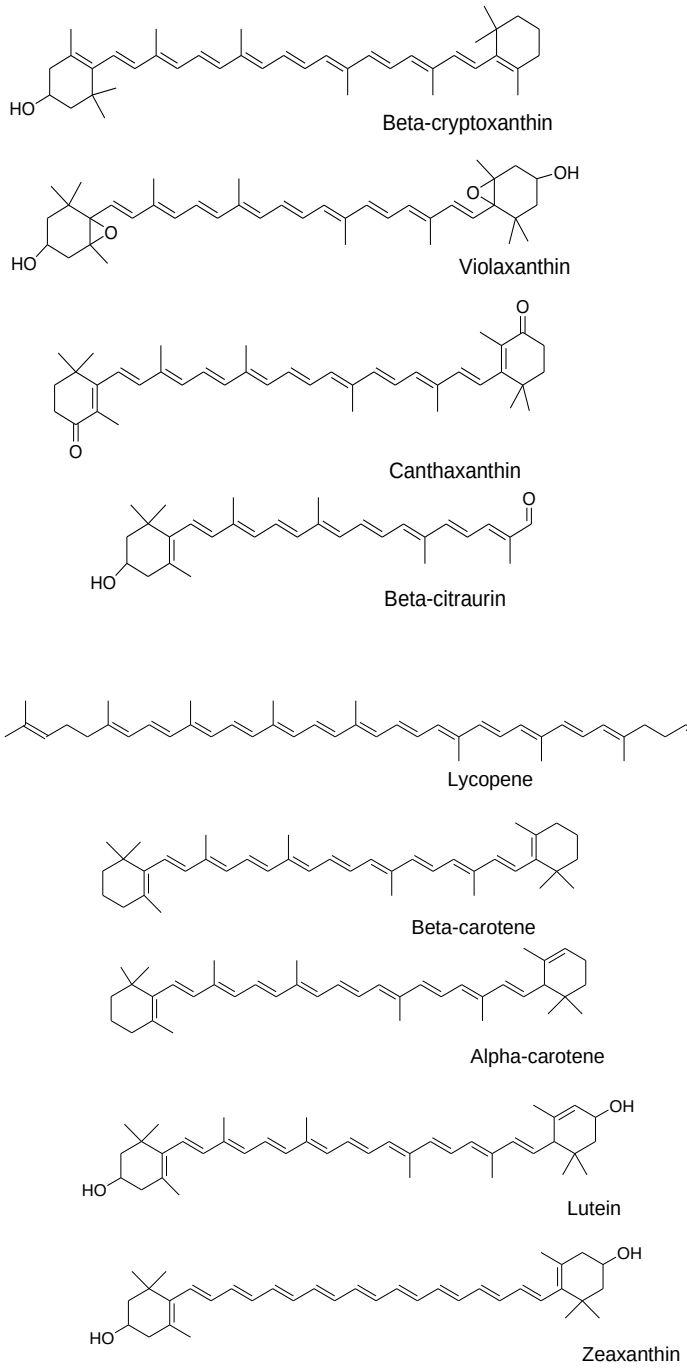


Figure 2.1: Structures of some carotenoids (Rodriquez-Amaya, 2001)

Carotenoids are lipophilic and have a highly conjugated system which makes them prone to isomerism and oxidation (Jordi *et al.*, 2000; Rodriquez-Amaya, 2001). During isomerism carotenoids change their isomerism from a more stable tran-form to cis. On

oxidation, carotenoids form epoxycarotenoids, apocarotenoids or hydroxycarotenoids depending on the parent carotenoid which eventually forms compounds of low molecular weight (Rodriquez-Amaya, 2001; Boon *et al*., 2010). Carotenoid degradation pathways are highly influenced by the agent involved in initiation of degradation. Possible degradation paths are represented in figure 2.2. Once oxidation is initiated by one of oxidizing agents, carotenoid may further react with themselves or other chemical species within the environment to form plethora of products (Boon *et al*., 2010).

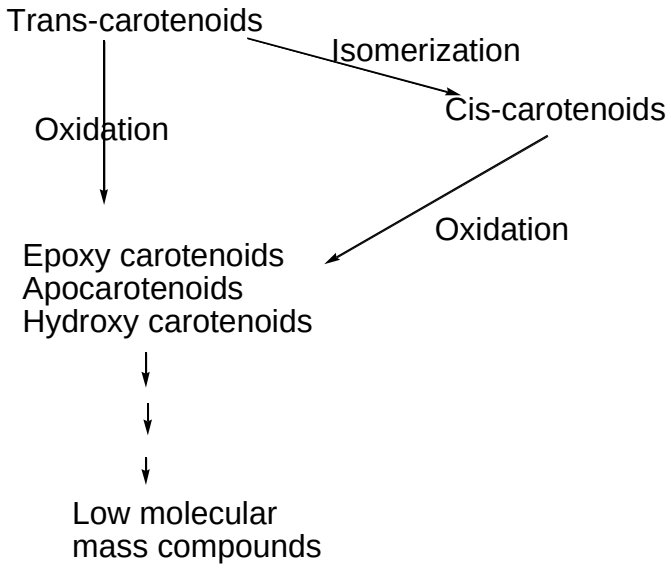


Figure 2.2: Possible path of carotenoids degradation (Rodriquez-Amaya, 2001)

Heat, light, acids and adsorption on an active surface such as alumina promote isomerism of trans-carotenoid to cis-carotenoids. This leads to loss of color and anti-oxidant activities (Rodriquez-Amaya, 2001). Oxidative degradation is the principal cause of extensive losses of carotenoids. Degradation occurs with availability of oxygen and stimulated by light, enzymes, metals and co-oxidation with lipids hydroperoxide (Preedy, 2012).

2.4.2 The chemistry and benefits of beta-carotene

Beta-carotene appears as a red or orange pigment found in green leafy vegetables and fruits. Alpha carotene, beta-carotene, delta carotene and gamma carotene are all various forms of beta-carotene with differences in isomerism (Rodriquez-Amaya, 2001). It is a conjugated hydrocarbon with the formula $C_{40}H_{56}$ with terminal double bonds not subjected to spatial changes because they are located to a ring (Rodriquez-Amaya, 2001). During thermo-degradation, beta-carotene maybe converted directly into dione, which may degrade to short chain species, or isomerize from trans– beta-carotene to cis– beta-carotene and epoxidize in the center to form apocarotenals. Epoxidation occurring near the ring may degrade to epoxy-apo-carotenals (Preedy, 2012).

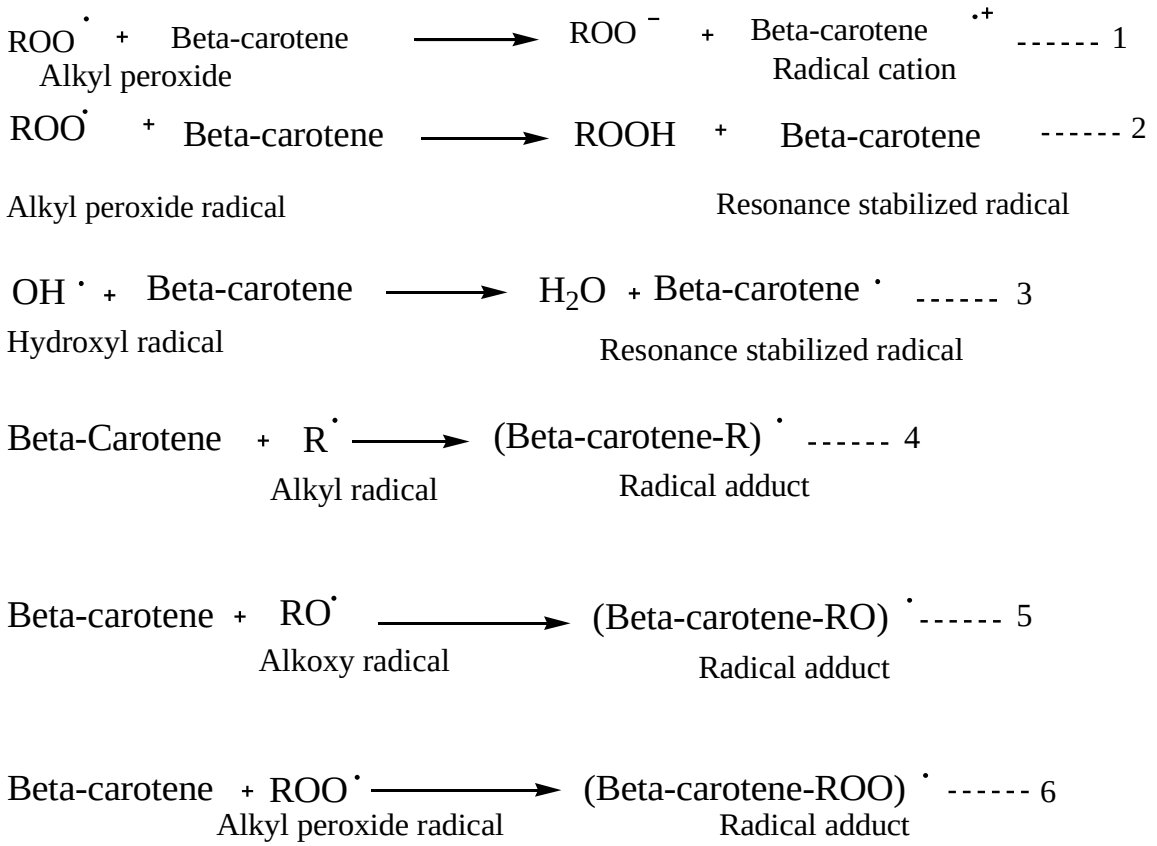
The ailments caused by radicals include NCD such as cardiovascular diseases, aging and cancer. Beta-carotene is able to fight these degenerative diseases due its potency as an anti-oxidant. A study aimed at investigating relative influence of nutritive and non-nutritive factors in fruits and vegetables on oxidative damage and enzymatic defense showed increased erythrocytes glutathione peroxide activity and resistance of plasma lipoproteins to oxidation. The study indicated fruits and vegetables gave better anti-oxidant activity than supplementation pill containing vitamins and minerals that are of equivalent quantities (Dragsted *et al*., 2004). In a different controversial study, that aimed at determining efficacy of a combination of anti-oxidants supplementation (6 mg beta-carotene along with vitamin E, selenium, zinc and ascorbic acid) was administered daily for 7 1/2 years to men and women. Supplementation lowered cancer incidences and all-

cause mortality in men but not in women. It was suggested that supplementation was effective in men due to their lower base line status of certain antioxidants especially beta-carotene, which is not found in women (Hercberg, 2004). Beta-carotene converted to Vitamin A is known in treating specific premalignant lesions and reducing prevalence of second primary tumors of cancer patients with prior head and neck, lung or liver cancer (Dragnev *et al*., 2000).

Carotenoids are the most efficient singlet oxygen quencher in biological systems. Singlet oxygen quenching mechanism by a carotenoid is physical quenching without generating oxidizing products. In high concentration of oxygen, beta-carotene acts as a pro-oxidant rather than anti-oxidant. Hence anti-oxidant activity of beta-carotene increases at low oxygen concentration (Lee *et al*., 2004). One mole of beta-carotene can quench 250 to 1000 molecules of singlet oxygen at a rate of 1.3×10^{10}/M/s. In addition pro-oxidant activities are dependent on the concentration of carotenoid (Lee *et al*., 2004). Free radicals or ROS in the body can cause lipid oxidation, protein oxidation, DNA strand break and base modification, and modulation of gene expression (Lee *et al*., 2004).

Beta-carotene has neutraceutical potential in fighting and preventing degenerative diseases that are caused by free radicals. Its high conjugated structure gives its potency as an anti-oxidant allowing radical scavenging. Electron transfer, hydrogen abstraction and adduct formation are three possible mechanisms of radical scavenging by beta-carotene (Young and Lowe, 2001; Boon *et al*., 2010). Electron transfer leads to formation of

radical cations, resonance stabilized radicals or radical adducts are formed when radical are quenched as indicated by the following equations 1,2,3,4,5 and 6.



(Young and Lowe, 2001; Boon *et al*., 2010)

On surprising findings beta-carotene supplementation was effective to some types of cancer but proved fatal to lung cancer patient and also to smokers. Asbestos workers and gastric cancer patients also were found to be at risk of beta-carotene supplements. The smokers and asbestos workers developed cancer while those who had it got worse (Bhatia and Jain, 2003; Tang, 2010; Druesne-Pecollo *et al*., 2010; Mishra *et al*., 2012; Moraes *et al*., 2013). There are no studies that support the reactions that take place between beta-

carotene and carcinogenic compounds present in cigarette but only speculations (Bhatia and Jain, 2003).

Effective functioning of any carotenoid depends on bioaccessibility and bioavailability. Bio-efficacy of carotenoid is influenced by acronym SLAMENGHI. Species of carotenoid, molecular linkage, the amount of carotenoid consumed in a meal, matrix in which the carotenoid is incorporated, effective absorption, the nutrient status of the host, genetic factors, host-related factors and interactions (Van Lieshout *et al*., 2001). Blanching showed an increase in bioaccessibility of beta-carotene which was indicated by increase in levels of beta-carotene (Addis *et al*., 2009). Carotenoid including beta-carotene are lipophilic and use of oil increases its bioaccessibility, most especially use of red palm oil which also is rich in beta-carotene (Edem, 2002; Hedrén *et al*., 2002; Amoussa-Hounkpatin *et al*., 2013). Turmeric, which is an anti-oxidant spice, and onions improve retention of beta-carotene hence increasing their bioaccessibility. a higher retention of beta-carotene was evident with use of acidulants such as lime and amchur (achari) - crisps of dried young mangoe fruits (Gayathri *et al*., 2004; Veda *et al*., 2008).

In the presence of the enzyme β,β-carotene-15,15-mono-oxygenase, beta-carotene obtained from plants is converted to vitamin A. This enzymatic process breaks down beta-carotene, forming two compounds that have retinal activities. The structure of beta-carotene makes it the most potent pro-vitamin a. the enzyme splits beta-carotene to form two molecules of vitamin A as shown in Figure 2.3 (Blomhoff, 2006; Biesalski *et al*., 2007).

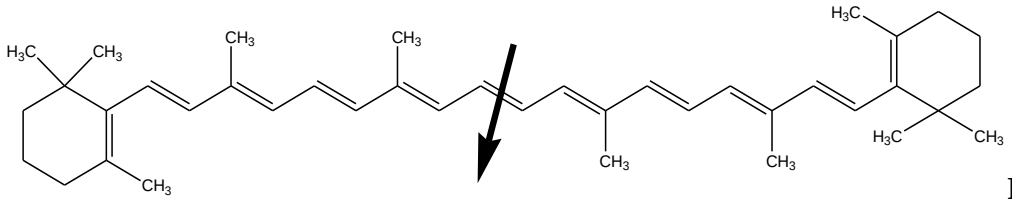


Figure 2.3: Enzymatic splitting of beta-carotene

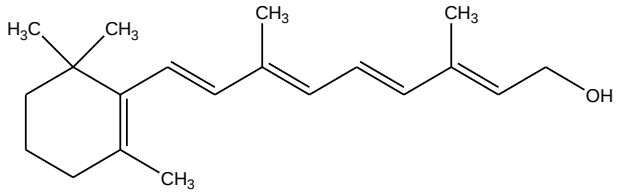


Figure 2.4: Structure of retinol (vitamin A)

Conversion of beta-carotene to vitamin A, depends on genetic variability in beta-carotene metabolism of a person hence not all beta-carotene is converted to Vitamin A (Tang, 2010). Table 2.1 gives dietary conversions of carotenoids to retinol activity (National Health Institute, 2013).

Table 2.1: Retinol activities equivalence

1µg of a Compound	**Retinol activity equivalent**
Vitamin A	1
Beta-carotene	0.5
Other carotenoids	0.25

International units (IUs) are used to give µg RAE conversions.

- 1 IU retinol = 0.3 µg RAE
- 1 IU beta-carotene from dietary supplements = 0.15 µg RAE
- 1 IU beta-carotene from food = 0.05 µg RAE (National Health Institute, 2012).

The daily amount of retinol activity equivalence (RAE) required in terms of gender and age-brackets are given by table 2.2.

Table 2.2: Recommended daily allowance for vitamin A

Age	Male	Female	Pregnancy	Lactation
0–6 months	400 µg RAE	400 µg RAE	N/A	N/A
7–12 months	500 µg RAE	500 µg RAE	N/A	N/A
1–3 years	300 µg RAE	300 µg RAE	N/A	N/A
4–8 years	400 µg RAE	400 µg RAE	N/A	N/A
9–13 years	600 µg RAE	600 µg RAE	N/A	N/A
14–18 years	900 µg RAE	700 µg RAE	750 µg RAE	1,200 µg RAE
19–50 years	900 µg RAE	700 µg RAE	770 µg RAE	1,300 µg RAE
51+ years	900 µg RAE	700 µg RAE	N/A	N/A

(National Health Institute, 2012)

Table 2.2 indicates that lactating mothers requires highest amounts of vitamin A than the other entire group.

Human beings can also obtain vitamin A from animal products such as eggs, liver, milk and cod liver oil. Statistics show that children and lactating mothers suffer from VAD in the sub-Sahara Africa (WHO, 2009). Moreover beta-carotene converted to vitamin A is known in treating specific premalignant lesions and reducing prevalence of second primary tumors of cancer patients with prior head and neck, lung or liver cancer (Dragnev *et al.*, 2000). Weak teeth and bones, poor eyesight and cataract, glaucoma and age related ocular are all associated to VAD (Tang, 2010).

Toxicity of vitamin A occurs by ingestion of higher levels of preformed vitamin A obtained from animal products such as liver, milk and fish oil. Since vitamin A is fat

soluble, its disposition is hard. A patient with vitamin A toxicity was diagnosed with liver disease with obscured origin and other odd symptoms which included thinning eye brows, sparse and coarse hair, cheilosis and bulging eyes. Liver biopsy showed hepatic congestion and fibrosis around the central vein (Russell, 2000).

Ingestion of more beta-carotene than the daily allowances causes yellowing of the skin, a condition known as carotenemia. This is a skin color disorder characterized by yellow-orange pigmentation seen mostly on palms and soles. Carotenemia is also seen in patients with hyperlidermia associated with diabetes and myxedema. This condition is quite harmless and its therapy is regulation of dietary habits, which involves reducing beta-carotene supplementation or the amount of beta-carotene intake in diet and reducing excessive ingestion of beta-carotene rich food, yellow or orange-colored foods such as carrots and oranges (Takita *et al*., 2006).

2.5 Variation of beta-carotene levels based on various preservation methods

Losses of anti-oxidative properties occur easily to beta-carotene due to their susceptibility to isomerism and degradation caused by light, heat, oxygen, acids, pro-oxidant metals and active surfaces (Maiani *et al*., 2009; Boon *et al*., 2010). The structural attributes of carotenoids that are thought to impact health benefits also make these compounds highly susceptible to oxidation. Conjugated polyene chain is characteristic of carotenoids that make the compound susceptible to degradation from a number of agents (Boon *et al*., 2010).

Moraes *et al*. (2013) reported 10 % loss of powdered beta-carotene encapsulated in dry phospholipid particles which were then preserved in refrigerated vacuum conditions and 30 % loss in normal atmosphere. Degradation was high within the first 60 days, minimal degradation thereafter up to 100 days. Nyambaka *et al*. (2012) using steel wool reported 19.5-37.6 % beta-carotene retention for 168 days, compared to 47-72 % beta-carotene losses of solar dried vegetables kept for the same period under normal conditions. Fresh vegetables indicated 781.94-1047.42 µg/g dry weight beta-carotene which was then reduced to 653.61-712.99 µg/g dry weight (Nyambaka *et al*., 2012). It is evident that there is need to develop methods of preserving beta-carotene that is hygienically safe, make it bioaccessible and with an increased shelf life.

With an aim to determine stability of encapsulated beta-carotene and its degradation kinetics in maltodextrin/gum arabic and maltodextrin/gelatin matrices, in relation to the physical properties and state of dehydrated matrix Ramoneda (2011) reported degradation of beta-carotene at relative humidities above the glass transition temperature (T_g) of the system, where the matrices were fully plasticized and collapsed at 75 and 92 % relative humidities. Best retention of beta-carotene at these relative humidities was obtained with gum Arabic (Ramoneda, 2011).

2.6 Properties of honey

Traditionally, honey has been used as remedy especially in healing of wounds and burns (Molan 2001; Ingle *et al.*, 2006; Ferreira *et al.*, 2009). Honey promotes tissue generation through stimulation of angiogenesis and the growth of fibroblasts and epithelial cells,

thereby hastening healing and minimizing the need for skin grafting (Molan, 2001). Honey has different properties depending on the species in which the bee belongs to and the source in which nectar is collected from. Different sources of nectar used by the bees to make honey determine the quantity of anti-oxidants present (McKibben and Engeseth, 2002). Anti-oxidants found in honey include flavonoids, ascorbic acid, lycopene, reducing sugars and beta-carotene (Ferreira *et al*., 2009). Suarez-Luque *et al*. (2002) indicated that the organic acids that are found in honey include malic, maleic, citric, succinic and fumaric acids (Suarez-Luque *et al*., 2002).

No universal method can be used to measure anti-oxidant capacity of all samples accurately and quantitatively but some chemical and bio-chemical assays using animal cells can be employed. These include loss of absorbance at 517 nm of a 2,2-diphenyl-1-picryl-hydrazyl (DPPH) which is a stable radical in spectrophotometric assay, reducing power, beta-carotene bleaching inhibition and thiobarbituric acid reactive substances (TBARS) assay (Ferreira *et al*., 2009). Studies done by Ferreira *et al*. (2009) reported that dark honey showed better anti-oxidant properties compared to the amber and light colored honey. Further entire sample had more anti-oxidant capacity (mean values that ranged from 106.67-168.94 DPPH scavenging activity) than the phenolic extract values were much more reduced and ranged from 84.98-90.78 scavenging activities. Other studies have given a mean ranging from 84.98 - 90.78 mg/ cm^3 of methanolic extract of honey while entire honey had a mean of 106.67 - 168.94 mg/ cm^3 (Ferreira *et al*., 2009). The variations were attributed to interference caused by non-phenolic compounds such as ascorbic acid, reducing sugars and amino acids which are present in the honey. This

could have given a positive error leading to overvaluation of the phenolic contents. Beta-carotene and lycopene are other anti-oxidants present in the honeys which could have led to increased absorbance (Ferreira *et al*., 2009). Presence of flavonoids, ascorbic acid, beta-carotene and lycopene gave synergic effect on anti-oxidant properties in honey (Ferreira *et al*., 2009). Ferreira *et al*. (2009) reported that quantities of beta-carotene in honey varied from 8.64-9.49 mg/kg with dark honey having the highest quantities. Worth noting is that the honey samples were stored in the dark at 21^{o}C and analyzed within two months upon collection (Ferreira *et al*., 2009). The current study fortified honey with beta-carotene with an aim to increase the carotenoid shelf life and hence provide a cheaper method of preservation.

Honey with highest anti-oxidant property (buckwheat honey) was found to be the best in prevention of lipid oxidation (McKibben and Engeseth, 2002). Lipid oxidation is the major factor for deterioration in meat and poultry meat. Effectiveness of honey to reduce poultry oxidation was determined by monitoring thiobarbituric acid reactive substances (McKibben and Engeseth, 2002). Honey has anti-oxidants potential that is effective in healing of wounds (Molan, 2001; Ingle *et al.*, 2006; Ferreira *et al*., 2009). Glucose oxidase which is also found in honey converts to hydrogen peroxide that has antibacterial activities that are responsible to healing of wounds (Molan, 2001). Honey has a mechanism that controls formation and removal of ROS (hydroxyl radical) formed by hydrogen peroxide as well as minerals such as iron and copper present in honey (Ferreira *et al*., 2009) . These metals, just like hydroxyl radical compounds are capable of initiating a chain reaction due to the presence of unpaired electrons (Ferreira *et al*., 2009).

Deterioration of lipids caused by oxygen is prevented by honey as demonstrated using poultry meat (McKibben and Engeseth, 2002). Studies aimed at improving quality of carrot fortified milk stored at 30^{o}C for 10 days. The quality of the milk was evaluated on the basis of changes in acidity, pH, free fatty acids and sensory analysis of the samples. Equal proportions of beet and honey had reduced acidity and free fatty formation and carotene degradation which was described to have synergic role of compounds in the two mixture (Bandyopadhyay *et al.*, 2008).

2.7 Properties of virgin coconut oil

Coconut fruit has many health benefits that include anti-bacterial, anti-fungal, anti-viral, anti-parasitic, anti-dermatophytic, anti-oxidant, hypoglycemic, hepatoprotective and immune-stimulant (DebMandal and Mandal, 2011). Coconut oil contains about 60 % - 65 % of the oil. 92 % saturated fatty acid in the form of triglycerides, where about 70 % are medium chain saturated fatty acids (Krishna *et al*., 2010). Extraction method of virgin coconut oil (VCO) therefore is mild and less mechanical which allows retention of anti-oxidant properties. Extraction of VCO from coconut milk allows the oil to retain phenolic compounds and flavonoids which contribute to anti-oxidant properties allowing the oil to retain its many health benefits (Marina *et al*., 2008). The anti-oxidants dissolve in the aqueous phase of coconut milk, such that in order to obtain the oil, water is evaporated and incorporating these compounds in the VCO. The resulting oil contains a complex composition of flavonoids and phenolic compounds which include cafeic acid, *p*-coumaric acids, ferulic acids, and other unidentified phenolic compounds and flavonoids (Seneviratne *et al*., 2008). Ferulic acid and *p*-coumaric acids are the major phenolic contents detected in VCO. It was indicated there was a correlation between total phenolic

content and scavenging power, reducing power and beta-carotene bleaching, which are all determinants of anti-oxidant capacity (Marina *et al*., 2008). Seneviratne *et al*. (2008) attributed boiling of coconut milk above 100^{o}C incorporated phenolic compounds in the oil which contribute to anti-oxidant properties. The total phenol content of VCO amounts was found to be 618 ±46 mg/kg and 7.78-29.18 mg garlic acid equivalent/100g oil (Seneviratne *et al*., 2008; Marina *et al*., 2008).

Virgin coconut oil prevents oxidation of lipids and therefore used as a preservative which was demonstrated using poultry meat submerged in the oil for two hours at room temperature. The meat had an increased shelf life and was possible to preserve it for 10 days (Aritonang *et al*., 2009).

2.8 Methods of analysis

Carotenoids analysis is mostly done by HPLC, UV-visible spectroscopy and although open column chromatography (OCC) which is followed by thin layer chromatography (TLC) can also be used in analysis of carotenoids (Rodriguez-Amaya, 2001). Though a tedious process, OCC has a capacity to identify and quantify a wide range of carotenoids in a sample. The method is especially good in identification pro-vitamin A and a non-pro-vitamin A carotenoids. It is efficient for isolating carotenoids that are not available synthetically or commercially to serve as standards for HPLC. Efficiency of OCC is improved by varying polarity, ionic strength or pH of the solvent. Variation of eluting solvent in the column makes it possible to obtain the best separation in the shortest time

possible. It can also separate large amounts of materials. Rechromatography is done after the first OCC to achieve better separation (Rodriguez-Amaya, 2001).

Since carotenoids are prone to oxidation and degradation caused by light and heat, they should not be kept for a long time, but a rapid analysis is needed. The TLC is a good tool for it gives quantitative and qualitative analyses of carotenoids for a very short time. The analyses methods are simple, accurate and reproducible. Unlike other chromatographic processes, TLC is capable of separating a large number of samples in single run. High performance-TLC is more efficient in separation and has a greater sensitivity of most of the carotenoids (Preedy, 2012).

More accurate carotenoids analysis is achieved by reversed phase-high performance liquid chromatography (RP-HPLC) (Rodriguez-Amaya, 2001). Normal phase-high performance liquid chromatography has a polar stationery phase and non-polar, non-aqueous mobile phase. It has poor reproducibility of retention time (R_t) due to the presence of water or protic organic solvent layer (Rouessac and Rouessac, 2007). On the other hand, RP-HPLC has a non-polar stationery phase and an aqueous, moderate polar mobile phase. The RP-HPLC can have isocratic elution or gradient elution of mobile phase. Isocratic elution is when the composition of mobile phase remains constant throughout the analysis. Changing the polarity of the mobile solvent in gradient elution method can vary R_t. Flow rate of the mobile phase is determined and maintained by pump (Rouessac and Rouessac, 2007).

Separation of all analytes of analysis occurs in the column of HPLC instrument. Columns are straight stainless steel tubes that range from 3 cm to 15 cm. Columns are packed with silica gel of the formula $SiO_2(H_2O)_n$ (where n is very close to 0). Silica gel is in the form of spherical particles, sometimes porous, with a diameter of 2 μm to 5 μm. The size assures a homogenous packing and allows for a regular flow of mobile phase. Quality of separation of analytes is affected by temperature and should therefore be thermostatically controlled. It is argued separation occurs by adsorption of the mobile phase and stationery phase or by a continuous slowing down at the mobile phase/stationery phase interphase (Rouessac and Rouessac, 2007).

An effective detector should be sensitive, have a wide linear range and capable of filtering background noise. Detectors are based on optical properties of the analyte, which include absorption, fluorescence and refractive index (Rouessac and Rouessac, 2007). Detectors used for HPLC include ultraviolet (UV) absorption detectors with filters, florescence, refractive index, evaporative light scattering, electrochemical, mass spectrometry, fourier transformer- infrared (FT-IR) (Skoog *et al.*, 1997).

High performance liquid chromatography is capable of separating, identifying and quantifying carotenoids simultaneously (Rouessac and Rouessac, 2007). This process has made the major advances in the study of carotenoids. Positive identification of carotenoids is done by comparison of the retention time of the carotenoid and that of the standard. Quantification of carotenoids is determined by peak areas or heights upon calibration of the standard (Rouessac and Rouessac, 2007). Quantification of carotenoids

however, maybe difficult if the peaks are not well resolved or are overlapped (Skoog *et al*., 1997). Analysis of carotenoids is made more efficient if HPLC is coupled with a detector which is also an analytical instrument, making it quantifying bi-dimensional. This is because a chromatogram from HPLC a spectrum from separated species is also obtained. Tandem instruments include high performance liquid chromatography-nuclear magnetic resonance (HPLC-NMR ^{1} H) or liquid chromatography-mass spectrometer (LC-MS) (Rouessac and Rouessac, 2007).

The efficiency of HPLC in carotenoid analysis is evident as many researchers use it. Since reversed phase-HPLC is accurate and reproducible hence more effective in carotenoid analysis. The instrument is also capable of analyzing numerous carotenoids and several other compounds in real time. In a bid to determine the carotenoid contents in DGLV, RP–HPLC was used to determine three classes of pigments. They include xanthophyls (lutein), carotenes (α- and β- carotene) and chlorophyll (Žnidarčič *et al*., 2011). Other studies that analyzed role of palm oil in bioaccessibility of beta-carotene (among other carotenoids) from *Amaranthus* spp used HPLC in analysis.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Research design

This study involved determining levels of beta-carotene extracted from *Amaranthus* species that was preserved in unadulterated honey and virgin coconut oil (VCO) for 180 days of storage. The study also determined anti-oxidant activities of the oil and honey which were used for preservation of beta-carotene.

3.2 Sampling procedures

Seasonality and the source of the vegetables weren't considered, but the vegetables were collected during the wet season. Three different vendors selling *Amaranthus* spp were randomly selected at Githurai market, Nairobi County. The vegetables were bought in bundles and transported to Kenyatta University laboratories for further procedure. Thirty bundles of the *Amaranthus* spp were obtained from each vendor to make approximately 500 g of the vegetables. The vegetables bundles were thoroughly mixed up to form homogenized sample which was used for analysis. Honey in honey-combs was bought from a farmer in Eldama-Ravine (Koibatek County) whereas coconut fruits were bought from three coconut vendors in Kongowea market (Mombasa County).

3.3 Chemical and reagents

All solvents used during extraction of carotenoid from *Amaranthus* spp were analytical grade. Acetone, celite, petroleum ether, anhydrous sodium sulphate, butylated hydrotoluene (BHT), potassium hydroxide, ethanol, DPPH and beta-carotene standard (type 1, sigma chemicals) were obtained from Kobian Kenya limited. The mobile phase

comprised of HPLC grade acetonitrile, dichloromethane and methanol which were obtained from sigma chemicals company St Louis USA.

3.4 The HPLC instrument and its operating parameters

Reverse phase-HPLC instrument used was manufactured from Shimadzu-Japan which had a column of model prominence LC-20AD operated at 25-30 kgF and its oven (CTO-10ASVP model) had a temperature of 40^{o}C and used isocratic elution. Deuterium lamp (SPD-20A model) was used as the detector to obtain UV spectra at 450 nm wavelength. Column of 250 × 4.6 mm internal diameter and 5µm particle size was used as the stationary phase while the mobile phase comprised of Acetonitrile: Methanol: Dichloromethane in the ratio of 70:10:20. The mobile phase was allowed to run through HPLC instrument for 20 minutes before injecting 1µL volume of the sample allowing a flow rate of 0.8 cm^{3}/min.

3.5 Cleaning of apparatus

To ensure that all apparatus used for this procedure were not contaminated, the glass and plastic ware were soaked overnight in 2 M nitric acid and then washed with detergent and rinsed with distilled water. Drying of glassware was done in an oven at 105^{o}C while plastics were dried in an open rack. The vials were soaked in dichloromethane and then washed with distilled water.

3.6 Preparation of virgin coconut oil and unadulterated honey

Virgin coconut oil was prepared by wet process to retain anti-oxidant properties of the oil (Marina *et al*., 2008). Extraction of VCO was done as described by Seneviratne and Dissanayake (2008) and Mansor *et al.* (2012) but with slight modification. Fresh coconuts were mechanically grated from the kernel and the milk squeezed out using a clean piece of cloth. Coconut milk obtained was then boiled above 100^{o}C for an hour to allow water to evaporate and obtain the oil. A portion of the oil obtained was run in HPLC to determine presence of beta-carotene in the matrix while the rest was stored in air-tight bottle to be used as preservative in beta-carotene formulation.

The unadulterated honey was extracted from honeycombs by squeezing it out with a clean piece of cloth. The semi-solid honey wax was placed in a hot water bath to enable squeezing out of honey. Slight warming increased the viscosity of honey and enabled storage in air tight container. The extracted honey was then used as a preservative for extracted beta-carotene. Presence of beta-carotene in honey was determined using HPLC. This value was subtracted from the total amount of beta-carotene that was preserved in honey.

3.7 Preparation of beta-carotene standard

One mg of beta-carotene standard was dissolved in 2 cm^3 of analytical grade methanol to make a stock solution with a concentration of 0.5 mg/ cm^3. Further dilution was done to obtain 0.025 mg/ cm^3 from 0.5 mg/ cm^3 which represent 100 %. Consequently more dilution was done to obtain 0.02 mg/ cm^3 which is 80 % (given by 8/10 × 0.5). Further

dilutions were done to obtain 60 %, 40 % and 20 %. These concentrations were run in HPLC to give chromatograms that were for confirmatory determination of extracted beta-carotene from *Amaranthus* spp by comparing retention time of the analyte peaks. Their peak areas were used to obtain calibration curve, limit of detection and coefficient of determination (R^2). The calibration curve obtained was used to determine the concentration of extracted beta-carotene using the equation below;

$$C_{\text{beta-carotene}} = \left\{\frac{P-C}{S}\right\} \times 100g \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (10)$$

Where,

$C_{\text{beta-carotene}}$ = Beta-carotene concentration of the analyte in mg/100g
P = Peak area of analyte,
C = y-intercept
S = slope

3.8 Method validation

(i) Anti-oxidant

Absorbance of the blank which was DPPH in HPLC grade methanol was determined using UV-Visible. This was prepared by dissolving 0.002 g DPPH in 100 cm^3 HPLC grade methanol. Methanolic extract of 3 samples of VCO were separately prepared by placing 5g VCO in 20 cm^3 methanol of HPLC grade and then concentrating it using a rotatory evaporator. 2 cm^3 of the extract was placed in a test tube and 4 cm^3 DPPH added. The mixture was allowed to stay for 30 minutes in the dark to allow decolorization. The absorbance of the sample was then determined by UV visible. %RSA was determined as shown in equation 7

$$\% \text{ RSA} = [(A_{\text{DPPH}} - A_s) / A_{\text{DPPH}})] \times 100 \ldots\ldots\ldots\ldots\ldots\ldots (7)$$

Where A_{DPPH} is absorption of the blank and A_s is the absorption of the sample (Eurachem, 1998).

(ii) Beta-carotene determination

Repeatability was calculated for 5 measurements where beta-carotene from 5.0 g of *Amaranthus* spp was used. The extraction of beta-carotene was followed as described in extraction procedure (Page 36). Peak areas obtained were used to determine the concentration of beta-carotene from the standard calibration curve. Using the mean concentration, precision was evaluated by relative standard deviation (%RSD), according to the equations 8 (Eurachem guide, 1998).

$$\%RSD = \frac{s}{\bar{x}} \times 100 \quad \text{.....(8)}$$

Accuracy of the study was determined by recovery studies where beta-carotene extracted from 5 samples of *Amaranthus* spp was spiked with 1 ppm of beta-carotene standard. Peak areas from the chromatograms were used to determine the concentration of beta-carotene. Accuracy was evaluated by recovery test, according to equation 9 (Eurachem guide, 1998).

$$\%\ \text{Recovery} = \frac{C_F - C_U}{C_A} \times 100 \quad \text{.....(9)}$$

Where C_u is the concentration in unspiked sample; C_A is the concentration of spike (added solution); C_F is the concentration determined in the spiked sample.

3.9 Lab procedure

3.9.1 Determination of anti-oxidant activities in the standard, synthetic anti-oxidants and preservation matrices

A measurement of 0.002 g of DPPH was dissolved in methanol in a 100 cm^3 volumetric flask to the mark to make 0.02 g/L solution. Absorbance of this solution was determined in triplicates and recorded. The value obtained was used as the standard value for anti-

oxidant properties. Five grams of honey was placed in a conical flask and 20 cm^3 of HPLC grade methanol added. The mixture was stirred, filtered using Buchner funnel and then concentrated in a rotatory evaporator at 40^oC. A volume of 2 cm^3 of the concentrate was placed in a test tube and 4 cm^3 of DPPH solution added. The reagents were kept in the dark for 30 min to allow electron scavenging by DPPH. Absorption was determined by UV-visible where test tube contents was taken in triplicates and recorded. The above procedure was repeated using 5 g VCO and their absorbance recorded. Absorbance of both matrices (honey and coconut oil) was compared with synthetic antioxidants. A measure of 5 g of ascorbic acid and BHT were separately added to 4 cm^3 DPPH. Then the mixtures were kept in darkness for 30 minutes. Absorbance of these synthetic anti-oxidants were determined and the value obtained compared with the sample matrixes.

3.9.2 Extraction of beta-carotene from *Amaranthus*

Approximately 500 g of homogenized *Amaranthus* spp vegetables were thoroughly washed with distilled water. Clean vegetables were blanched in water boiling at 90^oC for two minutes. The leaves were then plucked and placed in a blender. Celite powder was added to the vegetables and blended to make a smooth paste.

About 15 g of homogenous paste was placed in a conical flask and 50 cm^3 of acetone which was refrigerated for 2 hours was then added. The mixture was sonicated for about 10 minutes and the extract filtered using a Buchner funnel. The extraction was repeated until there was no more color change to the residue. Then partitioning was done by placing 25 cm^3 of petroleum ether in a separating funnel and 10 g of butylated hydro toluene (BHT) added. Small portions of acetone extract were added at a time until all

acetone extract was over. The mixture was stirred thoroughly on each addition to allow carotenoid to partition in petroleum ether. The lower solvent that contains majorly chlorophyll was allowed to run out of the separating funnel.

Saponification is an important step in eliminating interferences from the sample. It was done by washing the sample with potassium hydroxide in ethanol (0.1 % potassium hydroxide in ethanol) to the sample containing petroleum ether. The mixture was allowed to stand for about 15 minutes to allow separation of the carotenoid from chlorophyll. Washing (up to 5 times) was done using distilled water to remove impurities and allow carotenoids to be collected which appeared yellow in petroleum ether. The carotenoid collected was dried using anhydrous sodium sulphate then concentrated using a rotatory evaporator at 30^{o}C.

3.9.3 Test of beta-carotene from the blank

10 cm^3 of acetone was placed in a conical flask and a one spatula of celite added. The mixture was placed in a separating funnel and 20 cm^3 of petroleum ether (boiling point 40-60^{o}C) added. Aqueous mixture was washed out using distilled water for about five times. Petroleum ether layer was placed in a round bottom flask through a filter funnel that contained anhydrous sodium sulphate. The layer was concentrated using rotatory evaporator. Reconstitution was done using 5 cm^3 methanol and the mixture run in HPLC.

3.9 Preparation of preservative samples

Upon extraction, carotenoid layer was preserved in VCO by placing 445 mg of extracted beta-carotene in 15 cm^3 VCO. A homogenized mixture was made using a magnetic stirrer

in a stream of nitrogen gas. Using a syringe the mixture was placed eight 1.5 cm^3 brown air-tight vial and kept at room temperature. The levels of beta-carotene was determined on the first day, after two weeks in the first month and then monthly for the months that followed for six months using RP-HPLC.

Beta-carotene was preserved in unadulterated honey by placing 442 mg of beta-carotene from carotenoid extract in 15 cm^3 of unadulterated honey to form a homogenous mixture using a magnetic stirrer under a stream of nitrogen gas. The mixture was separately placed in eight 1.5 cm^3 brown air-tight vials using a syringe. The mixture were kept at room temperature and beta-carotene levels determined on the very day of storage, in every two weeks for the first month and then monthly for the consecutive six months.

Dry nitrogen was blown in an 8 empty 2 cm^3 dark bottles to drive out air. Then 10.0 mg of beta-carotene was placed in each bottle and nitrogen blow again into the bottle. The quantity of beta-carotene was determined using HPLC on the first day, 14^{th}, 28^{th} days and monthly for consecutive six months.

Nitrogen gas was blown into the vials before storage of beta-carotene incorporated in carotenoid layer in the oil and honey to drive out air which would otherwise cause oxidation of the carotenoid. Their preservative properties were expected to play an important role in the retention of beta-carotene. Concentrations of beta-carotene in the matrices were subtracted so as to determine the actual levels of beta-carotene in the

formulations. This was done by running the sample of each matrix separately in HPLC and the equations below used to determine levels of retained beta-carotene;

$$HBC = BC(f) - BC(m) \ldots\ldots\ldots\ldots\ldots (11)$$

Where, HBC--------retained beta-carotene in honey
BC(f)------ beta-carotene in the formulation
BC(m)------ beta-carotene in the matrix

3.10 Analysis of beta-carotene from formulations

To determine the concentration of beta-carotene from contents in the brown vial, 1 mg of the formulation was reconstituted in 1cm^3 of HPLC grade methanol and run on HPLC to obtain peak areas from chromatograms. Due to sticky nature of honey centrifuging was done to allow for separation of the constituents before reconstitution.

Beta-carotene is a pro-vitamin A carotenoid which in the presence of enzyme oxygenase, splits into to two molecules to form retinol (vitamin A). Retinol activity equivalent of beta-carotene preserved in coconut oil, honey and nitrogen were calculated at every intervals of beta-carotene determination using the ratio of 1:12 (1 RAE = 12 μg beta-carotene) using the equation below.

$$1\ RAE = x_s \times \frac{1}{13} \ldots\ldots\ldots\ldots\ldots\ldots\ldots (12)$$

Where X_s = concentration of the sample

3.11 Data analysis

Data obtained was analyzed by one way ANOVA (analysis of variance) where significant difference between means of concentration levels of beta-carotene was found, separation of means using multiple range tests (Student Newman Keul Test) was employed. The level of confidence (α) for all the values analyzed was all performed at 95 %.

CHAPTER FOUR

RESULTS AND DISCUSIONS

4.1 Method validation

This section gives the results of methods performance parameters such as accuracy (recovery test), precision (repeatability) and limit of detection (LOD) that were used to determine the validity of the method used for beta-carotene quantification and radical scavenging activities of unadulterated honey and virgin coconut oil (VCO).

4.2. Calibration curve from beta-carotene standard

Well resolved chromatograms were obtained from running different concentrations of beta-carotene standard in HPLC instrument. Figure 4.1 shows a sample of such chromatogram.

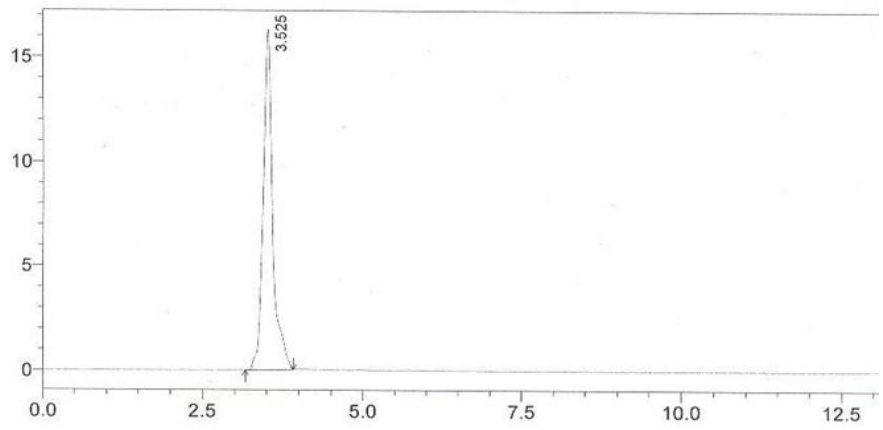


Figure 4.1: Chromatogram of beta-carotene standard

The chromatogram of beta-carotene gave a peak at about 3.5 retention time. At different concentration at the same retention time, different peak areas obtained. These areas

obtained from chromatograms were plotted against the concentration to obtain calibration curve which gave a linear relationship. Figure 4.2 shows calibration curve of beta-carotene standards, equations and determination coefficient.

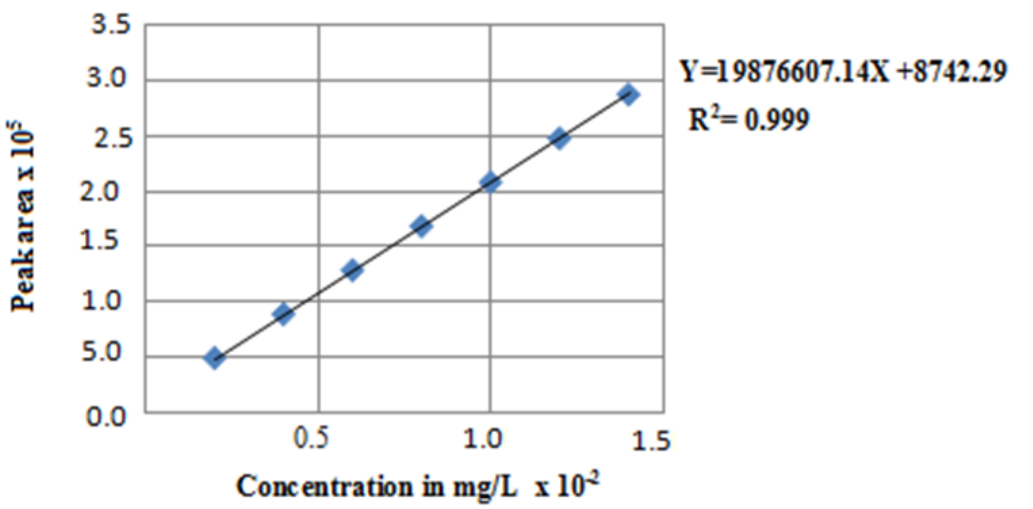


Figure 4.2: Calibration curve for beta-carotene

The linear relationship given by the equation, $y = 19876607.14x + 8742.29$, similar to that reported by Barba *et al*. (2006), is indicating the reliability of the curve. The linear relationship indicates the suitability of the deuterium lump detector in beta-carotene analysis (Khachik *et al.*, 1992). The equation, $y = 19876607.14x + 8742.29$ was used to determine the concentration of extracted beta-carotene that was preserved in VCO and unadulterated honey. Table 4.1 gives parameters used to determine suitability of the method used.

Table 4.1: Method validation parameters

Parameter	Value
Slope	19876607.14 ± 111.74
Intercept	8742±412.10
LOD (mg/L)	1.084mg/L
R^2	0.999
Equation	$y = 19876607.14x + 8742.29$
%RSD	3.22

Coefficients of determination (R^2) obtained was 0.999 which is a value that is approaching unity. This shows the method is valid and peak areas obtained can be used on calibration curves to give concentration at their respective range. The method detection limits (LOD) for the beta-carotene, both from the standard and extracted from the vegetable was 1.084 mg/L. This is the least concentration of beta-carotene that can be detected during analysis. The sensitivity of the method is given by the slope which was 19876607.14 ± 111.74. The accuracy of the method was determined by recovery test. The mean % recovery obtained was 104.30±1.21 which is within range of 100-109 % obtained by Barba *et al*. (2006). The method employed therefore, expected to give accurate results. Precision of radical scavenging activity was determined by evaluating percentage Relative Standard Deviation (%RSD). The value obtained was 3.22 %, a value less than 10 %, indicating the methods for determining anti-oxidants were reliable (Eurachem, 1998).

4.3 Anti-oxidant activities of virgin coconut oil and honey

The antioxidant activity of honey and coconut oil was determined by loss of absorbance of 2,2-diphenyl-1-picryl-hydrazyl (DPPH). The values obtained were expressed as percentage radical scavenging activity (% RSA) as indicated in table 4.2.

Table 4.2: Anti-oxidant activities in honey and coconut oil

	% RSA (Mean ± SD), n = 3
Sample	% RSA
VCO	65.12±0.70
Honey	81.51±1.39
BHT	77.47 ± 0.10
Ascorbic Acid	70± 0.17
p-value	<0.001

*VCO = virgin coconut oil
*BHT= butylated 4-hydroxytoluene

From the results, honey and VCO exhibited anti-oxidant activities and there were no significant variations ($p<0.05$) between the two preservation matrices. Comparing %RSA of VCO and honey with the synthetic anti-oxidants indicates that the two matrices have sufficient anti-oxidants which can scavenge radicals hence reduce oxidative stress, hence they can address reduction of degenerative diseases.

Seneviratne *et al*. (2008) attributed boiling of coconut milk above 100^0C incorporated phenolic compounds in the oil which contribute to anti-oxidant properties. A study done by Marina *et al*. (2008) on VCO indicated a correlation between phenolic compounds and scavenging activity ($r=0.91$) and reducing power ($r=0.96$). It was concluded that anti-oxidant capacity in VCO could be due to phenolic compounds (Marina *et al*., 2008).

Current study used wet process of extraction of VCO. This therefore can be concluded was the reason for presence of anti-oxidant properties.

Ferreira *et al*. (2009) on the other hand attributed anti-oxidant properties in honey to presence of carotenoids and phenolic compounds. Anti-oxidant properties due to phenolic compounds indicate the matrices are good lipid peroxidation inhibitor indicating a good preservative (Molan, 2001; McKibben and Engeseth, 2002; Marina *et al*., 2008; Ferreira *et al*., 2009). It can be concluded from the current study that presence of beta-carotene among other carotenoids and phenolic compounds were the reason for anti-oxidant properties.

4.4 Beta-carotene levels in preservatives

Levels of beta-carotene in the matrices were determined prior to preservation in VCO and unadulterated honey. Figures 4.3 and 4.4 give the chromatograms obtained.

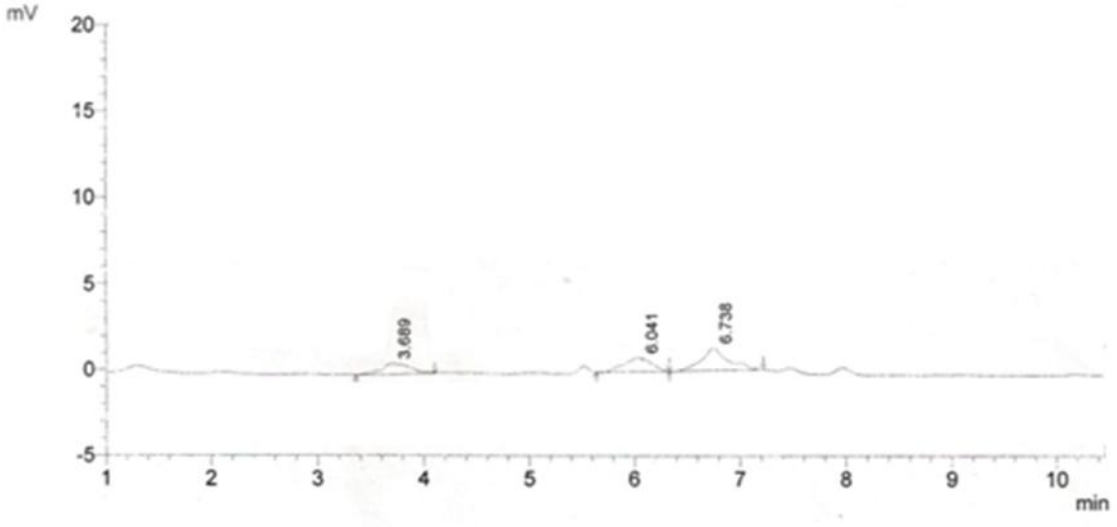


Figure 4.3: Chromatogram of virgin coconut oil

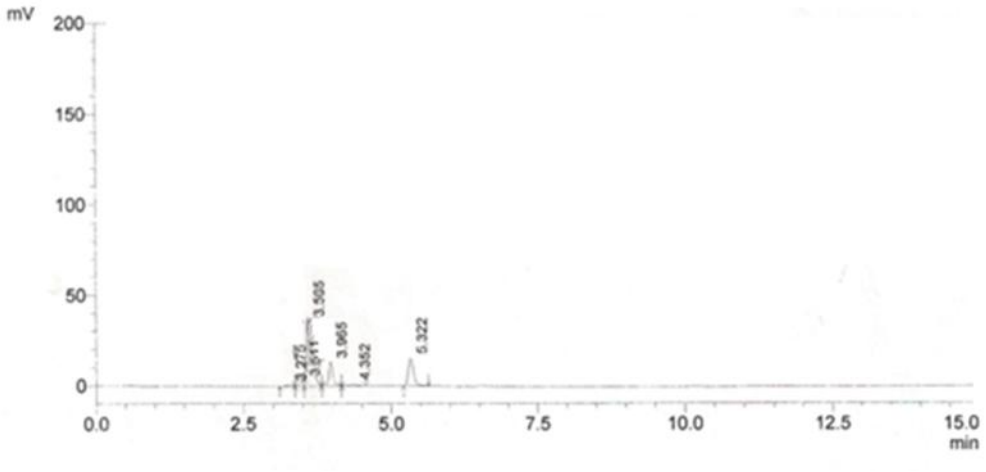


Figure 4.4: Chromatogram of unadulterated honey

Figure 4.3 shows a chromatogram for VCO. A 3.505 retention time can be estimated to be that of beta-carotene. The peak area obtained at this point was below detectable limits hence the levels of beta-carotene levels were negligible. Honey being an organic compound contained several peaks at different retention time. As shown on figure 4.4. Beta-carotene was detected at 3.585 with significant peak areas. Levels of beta-carotene in the matrices were determined so as to subtract from the total beta-carotene preserved in the formulations. Table 4.3 gives a summary of the mean values of beta-carotene in the preservative matrices.

Table 4.3: Beta-carotene levels in coconut oil and honey

Preservative matrix	**Concentration (mg/100g) DM** **Mean** ± SD n=3
Virgin coconut oil	Below detectable limits
Unadulterated honey	11.6± 0.07

The results indicate that beta-carotene levels were below detectable limits (1.084 mg/L) in VCO while honey had 11.6± 0.07 mg/100g (1.16 mg/kg). The variations can be attributed to post harvest handling since storage of honey was done at room temperature.

4.5 Concentration of beta-carotene in *Amaranthus* spp vegetables

Beta-carotene that was extracted from the fresh *Amaranthus* spp. Figure 4.5 shows the chromatogram obtained when levels of beta-carotene was determined from the vegetables

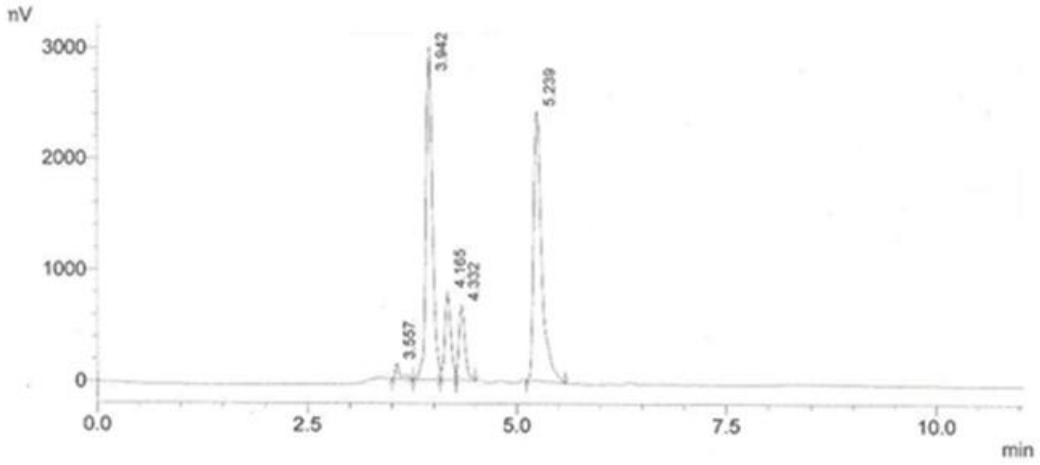


Figure 4.5 Chromatogram of fresh vegetables

Amaranthus spp is a natural product and hence several chromatograms were obtained as indicated by figure 4.5 significant levels of beta-carotene were detected at 3.542 retention time. By calculations 59.97±0.04 mg/100g DM of beta-carotene was recorded from fresh vegetable of *Amaranthus* spp.

4.5 Preservation of beta-carotene in the preservatives

Extracted beta-carotene was separately preserved in unadulterated honey and VCO. Figure 4.6 shows a sample of chromatograms of beta-carotene preserved in the matrices.

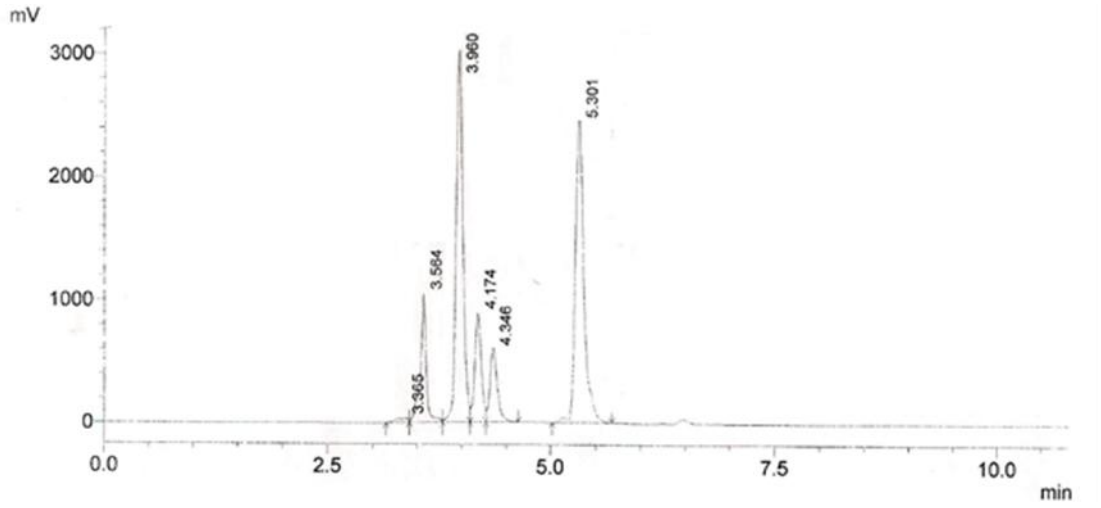


Figure 4.6: Chromatogram of beta-carotene preserved in VCO

Several other compounds were detected when carotenoid layer was preserved in unadulterated honey which is shown by several peaks on figure 4.6. Beta-carotene using this method of preservation was detected at 3.564 retention time. To obtain the amount of extracted beta-carotene retained upon preservation the levels of beta-carotene in honey were subtracted from the total concentration of beta-carotene. The levels of beta-carotene in the VCO and honey with storage were determined and results obtained presented in table 4.4.

Table 4.4: Mean levels of beta-carotene preserved in nitrogen, honey and coconut oil for 180 days

	Concentration mg/100g DM (Mean±SD), n=3		
Days	**NBC**	**VCOBC**	**HBC**
0	9.48±0.02[a]	30.33±0.08[a]	29.67±0.04[a]
14	9.67±0.17[a]	25.00±0.04[b]	28.76±0.12[b]
28	9.38±0.13[a]	17.00±0.02[c]	14.78±0.06[c]
60	9.41±0.01[a]	9.00±0.03[d]	8.74±0.03[d]
90	9.50±0.03[a]	9.00±0.01[e]	7.88±0.01[e]
120	9.48±0.02[a]	8.00±0.01[f]	6.27±0.01[f]
150	9.65±0.20[a]	3.00±0.02[g]	5.22±0.02[g]
180	9.45±0.08[a]	3.00±0.01[h]	5.10±0.02[h]
p-values	>0.05	<0.001	<0.001

*Mean values followed by different small letter in the same column are significantly different (α=0.05, One-way ANOVA, SNK-test)
*VCOBC = beta-carotene in virgin coconut oil
*HBC = beta-carotene in honey
*NBC= beta-carotene in nitrogen

Concentration levels of beta-carotene preserved in nitrogen ranged from 9.67-9.38 mg/100g dry matter during the 180 days of storage, indicating no major degradation of beta-carotene preserved in nitrogen (93.8 % - 96.7 %) hence no signifcant difference ($p>0.05$). The concentration in levels of beta-carotene preserved in nitrogen did not significantly differ, however the differences in the mean of beta-carotene at different intervals could have risen from presence of trapped air in the vial, or by inability to avoid air while passing nitrogen gas when opening and clossing the vial. However, there were no significant variations on the level of beta-carotene preserved. Similar reports were reported by Nyambaka *et al*. (2012) where 93.8 % of beta-carotene in plant matrix was preserved for 180 days.

Beta-carotene levels in VCO ranged from 30.33±0.08 mg/100g to 3.00±0.01 mg/100g dry matter implying 9.89 % beta-carotene was preserved in coconut oil. On the other hand, 17.19 % beta-carotene was retained in honey with mean values varying from 29.67±0.04 to 5.10±0.02 mg/100g dry matter. There were significant reduction in the levels of beta-carotene retained in the matrices ($p<0.5$) that varied as indicated by the small letters.

The current study recorded degradation which could be attributed to reaction that occurred between added beta-carotene and acids contained in VCO which include lauric as indicated by Marina *et al.* (2008) and cafeic acid, *p*-coumaric acids, ferulic acids as reported by Seneviratne *et al*. (2008).

The reduction in the levels of beta-carotene within the 180 days storage period undertaken can be attributed to the degradation chemistry of beta-carotene, but retained some levels of beta-carotene after the period. The current study recorded a degradation of beta-carotene preserved in honey over the six months of storage. Organic acids that are found in honey include malic, maleic, citric, succinic and fumaric acid (Suarez-Luque, *et al*., 2002). In addition to the organic acids, honey contains hydrogen peroxide which is a strong oxidizing agent (Bang *et al*., 2003). Presence of acids and oxidizing agent causes degradation of all carotenoids. Though the concentrations of these compounds were not determined, it is believed they caused degradation of beta-carotene preserved in honey.

Comparison of the amount of degradation beta-carotene in the formulation at the different intervals was determine to investigate if degradation was uniform through the storage season and also between the matrices as shown on figure 4.6.

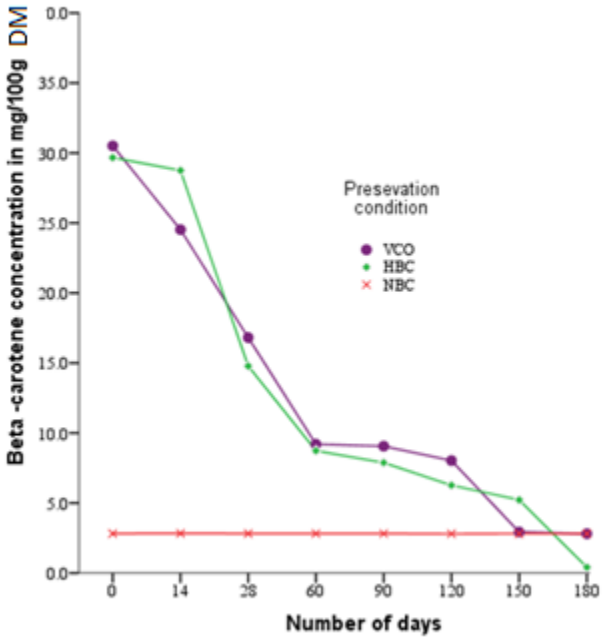


Figure 4.6: Beta-carotene degradation with storage

The general trend of beta-carotene reduction with storage indicates that reduction is not uniform. Within the first 3 months of storage 22.46 mg (70.4 %) beta-carotene had degraded whereas minimal degradation of beta-carotene was recorded between 60-180 days (28.1 %). Similarly 21.32 mg (65.1 %) degraded within the first three months compared to 6.39 mg (21.5 %) in the subsequent months. Although the general degradation is attributed to the chemisrty of beta-carotene and the chemical composition

of the matrix, the non-uniform degradation can be attributed to raddom error during measurements

In the presence of enzyme oxygenase, beta-carotene splits to form two molecules of retinol also known as vitamin A. Table 4.5 gives calculated retinol activity at each stage of storage.

Table 4.5: Retinol activity equivalent from beta-carotene levels preserved in coconut oil and honey with storage

Days	**Calculated**	**RAE**
	VCOBC	HBC
0	2.347 ± 0.006^{a}	2.282 ± 0.003^{a}
14	1.887 ± 0.001^{b}	2.212 ± 0.009^{b}
28	1.294 ± 0.001^{c}	1.137 ± 0.005^{c}
60	0.708 ± 0.003^{d}	0.672 ± 0.002^{d}
90	0.697 ± 0.002^{e}	0.606 ± 0.001^{e}
120	0.618 ± 0.002^{f}	0.482 ± 0.001^{f}
150	0.226 ± 0.001^{g}	0.402 ± 0.002^{g}
180	0.216 ± 0.001^{h}	0.302 ± 0.003^{h}

*VCOBC = Calculated retinol activity equivalent of beta-carotene in honey
* HBC = calculated retinol activity of beta-carotene in honey

The calculated retinol activity of beta-carotene that was retained in VCO and unadulterated honey varied significantly. The values ranged from 2.347±0.006 to 0.216±0.001 of beta-carotene in VCO. Whereas levels of calculated RAE that ranged from 2.282±0.003 to 0.302±0.003 in unadulterated honey. Calculated RAE reduced significantly through the 180 days of preservation ($p<0.05$).

Test to determine by comparison of which of the two matrices is a better preservative is given by Table 4.6.

Table 4.6: Differences in preservatve properties of coconut oil and honey

Matrix	**Mean (mg/100g)DM**
Virgin Coconut Oil	12.99 ±1.97
Unadulterated Honey	12.72 ±2.13
P-value = 0.556	

Table 4.6 indicates there are no significance difference ($p>0.05$) between the two matrices used as preservative. It implies that the two matrices have similar abilities as preservative. The similarities could have been caused by presences of compounds with similar chemical properties namely phenolic compounds and organic acids.

From the current study it show that retention of up to 10 % beta-carotene in the preservation matrices, a value that would still significantly contribute sufficient RDA of vitamin A which is 400 μg for infants and 1300 μg for adult (Canada health, 2014). Bio-availability and bio-accessibility of body nutrients is affected by species, molecular linkage, amount, matrix, effective absorption, nutrient status, host-related factors, interactions (SLAMENGHI) (Van Lieshout *et al.*, 2001). Moreover the RDA of beta-carotene also depends on age and gender. The amount of beta-carotene intake for infants and adults is a minimum of 180 μg (1.8×10^{-4} mg) and 390 μg (3.9×10^{-4} mg) per day respectively. However, the daily intakes shouldn't exceed 3600 μg (3.6×10^{-3} mg) and 18,000 μg (1.8×10^{-2} mg) for infant and adult respectively (Canada health, 2014). Required daily allowance for infant is 1320 IU for infants, 1650 IU (82.5μg) for children

between 4-8yrs, while those between 9-13 years require 2000 IU (100 µg). Male and female adult need 3000IU (15 µg) and 2300 IU (11.5 µg) respectively (National Institute of Health, 2002)

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Carotenoids are highly conjugated, the property that makes them good anti-oxidants. Unadulterated honey and VCO were found to have high levels of anti-oxidants majorly due to presence of phenolic compounds. Moreover unadulterated honey contains carotenoids such as beta-carotene among other anti-oxidants. Hence preserving beta-carotene extracted from *Amaranthus* spp vegetables will have a high level of anti-oxidants. Formulations of beta-carotene in the two matrices are good in scavenging free radicals, hence preventing the genesis and/or development of degenerative diseases.

Amaranthus spp were found to have sufficient amount of beta-carotene hence provide enough RDA for beta-carotene and vitamin A. Unadulterated honey and VCO were found to preserve some levels of beta-carotene, with degradation of carotenoid occurring. The remaining quantities of beta-carotene provide sufficient RDA of pro-vitamin A carotenoid. Therefore addition beta-carotene in VCO and adulterated honey for preservative purposes, have ability to scavenge ROS, hence boosting the body's ability to reduce oxidative stress.

5.2 Recommendations

5.2.1 Recommendations from the study

From the study it was evident that VCO and unadulterated honey are preservatives and are capable of preserving some beta-carotene. Due to presence of anti-oxidants in honey

and VCO, formulating beta-carotene in the two matrices works in synergy providing radical scavenging ability. Consuming this formulation of beta-carotene in these matrices will reduce imbalance of ROS that causes oxidative stress

5.2.2 Areas of further study

The study has pointed out that degradation of beta-carotene was attributed to the chemical composition. While this study did not determine the chemical composition of these preservative matrices, previous determination of the chemical composition can address the one of limitations of this study. Moreover determination of organic acid present in the preservative matrices would enable possibility of reducing or immobilizing labile hydrogen ion capable of degrading beta-carotene. Chemical composition of honey is determined by the type of bee and the source of nectar. Furthermore uses of all types of honey are recommended to be used so as to determine the best preservative. It is also recommended to study/determine the degradation products after storage. Furthermore monitoring degradation process daily would give an elaborate degradation curve hence determine the highest degradation interval.

It is projected that adoption, with little modification of preservative matrices can go a long way of formulation of capsules of beta-carotene in coconut oil and honey. This can be possible since some beta-carotene was preserved in the matrices. Therefore, development of encapsulated beta-carotene in honey and coconut oil is recommended. The capsules would eliminate air which would otherwise cause degradation. Furthermore the capsules allow accurate measurements of beta-carotene to provide enough RDA.

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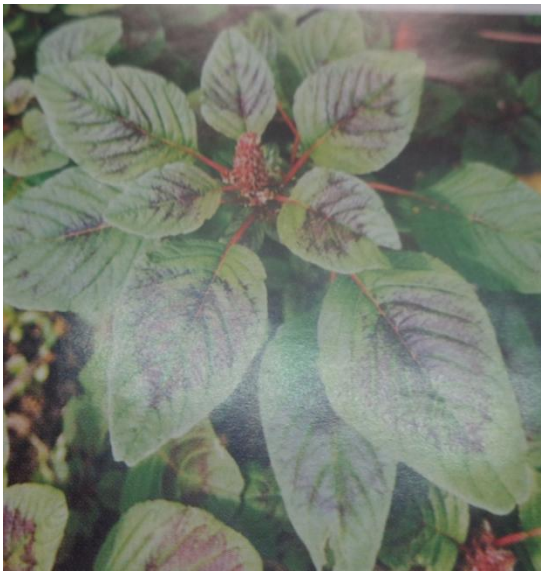
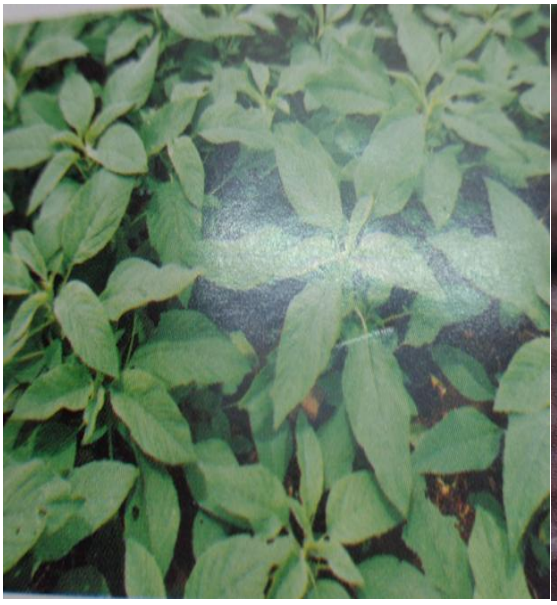
APPENDIX

Appendix I: Species of *Amaranthus*

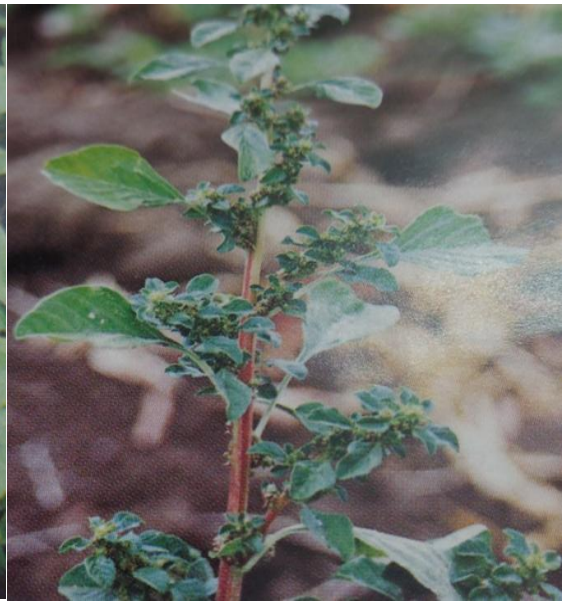


Amaranthus blitum

Amarantus dubius



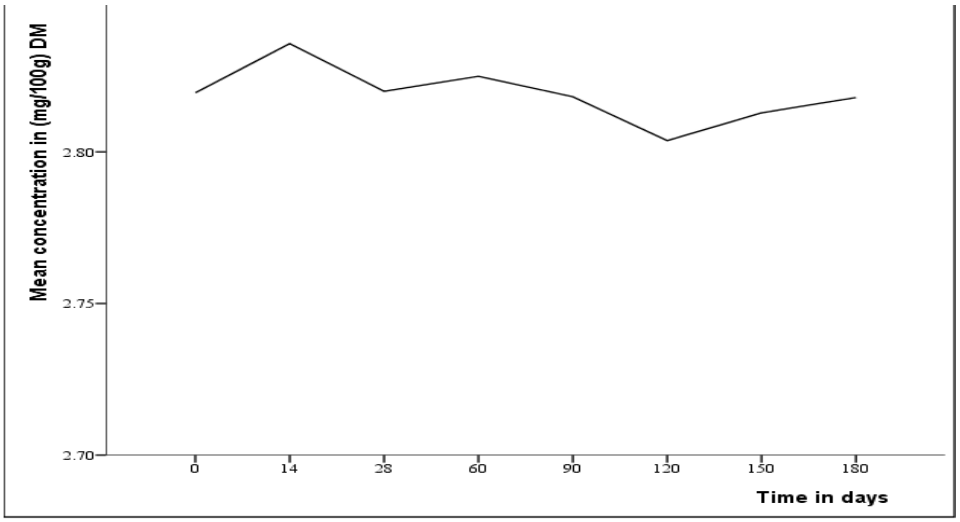
Amaranthus creutus



Amaranthus graecizans

Appendix ii: Photos of carotenoid extraction and preservation

Appendix iii: Curve of beta-carotene preserved in nitrogen (control)



Appendix iv: Degradation of beta-carotene in VCO and unadulterated honey