The Role of Antioxidants in Enhancing the Vitamin A Value of Plant Foods in Child Nutrition

A dissertation submitted by

TAWANDA MUZHINGI

In partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in

Biochemical and Molecular Nutrition

TUFTS UNIVERSITY

March 2014

Advisor

Dr. Guangwen Tang

Gerald J. and Dorothy R. Friedman School of Nutrition Science and Policy

Carotenoids and Health Laboratory

Jean Mayer ARS USDA Human Nutrition Research Center on Aging at Tufts University

ABSTRACT

Vitamin A deficiency is a public health problem among children aged 6-36 months old in Zimbabwe. The children are vitamin A deficient partly because the complementary diets are starchy white gruels, devoid of vitamin A or provitamin A carotenoids. Green leafy vegetables of *Brassica oleracea family* such as kale are rich in provitamin A carotenoids. Despite the widespread consumption of kale, it is not a common complementary food. A kale complementary food cooked with peanut butter is not only nutrient and energy dense, but may also increase the bioavailability of β-carotene. Kale is consumed as relish to staple maize porridge. Therefore, it is important to also optimize the vitamin A value of maize based complementary foods. Our previous studies show that α-tocopherol, an antioxidant found abundantly in maize promotes the exclusive central cleavage of β-carotene by the BCMO1 enzyme to vitamin A. However, the genetic variation of vitamin E and antioxidants in biofortified yellow maize and their effects on BCMO1 enzyme is unknown. Currently, there is also a lack of information on the genetic characterization of carotenoids in *Brassica oleracea* green vegetables consumed in Zimbabwe, and human studies showing their vitamin A value. The primary objective of this thesis was to demonstrate that kale and biofortified maize can improve the vitamin A value of complementary foods in Zimbabwe. The following studies were pursued to address the primary objective.

The first study determined the genetic variation of carotenoids, vitamin E and phenolic compounds in biofortified maize. HPLC analysis of 20 genotypes of biofortified maize showed β-cryptoxanthin and β-carotene as the main provitamin A carotenoids. Biofortified maize was also high in vitamin E, γ-oryzanol, ferulic acid and p-coumaric acid. Our study showed that genotype was a significant determinant of provitamin A carotenoids and vitamin E variation in maize ($p<0.01$). The second study characterized carotenoid profiles of *brassica oleracea var. acephala* vegetables varieties commonly consumed in Zimbabwe. HPLC analysis showed significant differences in the lutein and β-carotene contents among the six *brassica oleracea* vegetables varieties ($p<0.05$). Our study showed that the Zimbabwean *brassica oleracea var. acephala* vegetables are a very good source of provitamin A carotenoids. Our third study determined the effect of antioxidants on the enzymatic cleavage of β-carotene *in vitro*. Extracts of kale and biofortified maize were incubated with rat intestinal mucosal homogenate for an hour at 37°C. This study showed that vitamin E and γ-oryzanol promote central cleavage of β-carotene to form vitamin A. The fourth study determined the effect of peanut butter on the bioconversion of deuterium labeled kale [$^{2}H_{9}$] β-carotene to vitamin A. Preschool children were randomly assigned to ingest 1 mg [$^{13}C_{10}$] retinyl acetate reference dose and 50 g cooked kale (1.5 mg β-carotene) with either 33 g peanut butter (PBG) or 16 g lard (LG) on d1. Serum samples were analyzed by NCI-GC/MS for the enrichments of labeled [^{2}H] retinol from kale [$^{2}H_{9}$] β-carotene and [$^{13}C_{10}$] retinol from reference dose. The area under the curve (AUCs) of molar retinol enrichments at days 1, 2, 3, 6, 15, and 21 after the labeled doses showed the calculated conversion factors of kale β-carotene to vitamin A to be 13.4 ± 3.1 and 11.0 ± 3.9 to 1 by weight for LG and PBG respectively. This showed that kale is a good source of vitamin A. In summary, our research studies showed that kale and biofortified maize can improve the vitamin A value of complementary diets of children aged 6-59 months who are vulnerable to vitamin A deficiency.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude, special appreciation and thanks to my supervisor and thesis advisor Dr. Guangwen Tang. I have no words that can express the wonderful things you did for me since my arrival at Tufts University. I would like to thank you for creating an environment that enabled me to flourish professionally and academically. You taught me to be detailed, honest and never to give up. Thank you and God bless you. I would also like to thank my committee members, Dr. Kyung-Jin Yeum, Dr. Odilia Bermudez, and Dr. Andrew H. Siwela for taking time from your busy schedules to serve as my thesis committee members, even at weird time-zones. I also want to thank you for making my thesis such an enjoyable journey through your brilliant comments and suggestions. I would like to particularly thank Dr. Kyung-Jin Yeum, my academic advisor for believing in me and for pushing me to go the extra mile. I would especially like to thank current and past members of the Carotenoids and Health Laboratory, Dr. Elizabeth Johnson, Dr. Robert M. Russell, Dr. Rohini Vishwanathan, Dr. Julie Evans, Emily Mohn, Jian Qin, and Jun Zhong for the wonderful support. I also would like to thank students, faculty and staff at the Friedman School of Nutrition Science and Policy for making my time at Tufts University a worthwhile experience. I would like to thank my immediate family for being there for me. Words cannot express how grateful I am to my wife Lucy Rudo, my sons Ivainashe and Tadiwanashe for their sacrifices. I cannot be who I am without my mom, thank you mom, your prayers sustained me. To my father even though you are gone, you inspired me, rest in peace dad. Last but not least, I want to thank all my friends from Tufts University community, my church fellowships and fellow Zimbabweans.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AAHP	2, 2'-AZOBIS (2-AMIDINOPROPANE) DIHYDROCHLORIDE
AMD	AGE RELATED MACULAR DEGENERATION
ANOVA	ANALYSIS OF VARIANCE
APCI	ATOMSPHERIC PRESSURE CHEMICAL IONIZATION
ARS	AGRICULTURAL RESEARCH SERVICES

ART	AGRICULTURAL RESEARCH TRUST
AUC	AREA UNDER THE CURVE
BCA	BICINCHONINIC ACID
BCMO1	BETA-CAROTENE 15’ 15 MONOXYGENASE 1
BODIPY	BORON-DIPYRROMETHENE
CIMMYT	INTERNATIONAL MAIZE AND WHEAT IMPROVEMENT CENTER (IN SPANISH)
DDT	DITHIOTHREITOL
ECNCI	ELECTRON CAPTURE NEGATIVE ION CHEMICAL IONIZATION
EDTA	ETHYLENEDIAMINE-TETRAACETIC ACID
FRAP	FERIC REDUCING ANTIOXIDANT POWER
GC/MS	GAS CHROMATOGRAPHY MASS SPECTROMETRY
GLM	GENERALIZED LINEAR MODEL
GOZ	GOVERNMENT OF ZIMBABWE
HCL	HYDROCHLORIC ACID
HEPES	4-(2-HYDROXYETHYL)-1-PIPERAZINEETHANESULFONIC ACID
HNRCA	HUMAN NUTRITION RESEARCH CENTER ON AGING
HPLC	HIGH PERFORMANCE LIQUID CHROMATOGRAPHY
IAEA	INTERNATIONAL ATOMIC ENERGY AGENCY
ICAM	INTERCELLULAR ADHESION MOLECULE-1
IGA	IMMUNOGLOBIN A

IOM	INSTITUTE OF MEDICINE
IRB	INSTITUTIONAL REVIEW BOARD
KCL	POTASSIUM CHLORIDE
KOH	POTASSIUM HYDROXIDE
LC/MS	LIQUID CHROMATOGRAPHY MASS SPECTROMETRY
LG	LARD GROUP
MA	MASSACHUSETTS
MBTE	METHYL-TERT BUTYL ETHER
MEO-AMVN	2, 2′-AZOBIS (4-METHOXY-2, 4-DIMETHYLVALERONITRILE
MRCZ	MEDICAL RESEARCH COUNCIL OF ZIMBABWE
MRU	MEDICAL RESEARCH UNIT
N-EVAP	NITROGEN EVAPORATION
NACL	SODIUM CHLORIDE
NC	NORTH CAROLINA
NCI	NEGATIVE CAPTURE IONIZATION
NS	NOT SIGNIFICANT
NUST	NATIONAL UNIVERSITY OF SCIENCE AND TECHNOLOGY
NY	NEW YORK
ORAC	OXYGEN RADICAL ABSORBANCE CAPACITY
PBG	PEANUT BUTTER GROUP
PBS	PHOSPHATE BUFFERED SALINE
PDA	PHOTO DIODE ARAY

RAR	RETINOIC ACID RECEPTOR
RDA	RECOMMENDED DAILY ALLOWANCE
RXR	RETINOIC X RECEPTOR
TAP	TOTAL ANTIOXIDANT PERFORMANCE
TEAC	TROLOX EQUIVALENT ANTIOXIDANT CAPACITY
THF	TETRAHYDROFURAN
TRL	TRIGLYCERIDE RICH LIPOPROTEIN
SAS	STATISTICAL ANALYSIS SOFTWARE
UNICEF	UNITED NATIONS CHILDREN'S FUND
UPLC	ULTRA PERFORMANCE LIQIUD CHROMATOGRAPHY
US	UNITED STATES
USA	UNITED STATES OF AMERICA
USDA	UNITED STATES DEPARTMENT OF AGRICULTURE
VAD	VITAMIN A DEFICIENCY
VAS	VITAMIN A SUPPLEMENTATION
W.H.O	WORLD HEALTH ORGANIZATION

LIST OF FIGURES

CHAPTER 4

CHAPTER 5

CHAPTER 6

- Figure 4a: Calculated labeled retinol in the circulation of pooled serum time-points after consumption of kale [$^{2}H_{9}$] β-carotene with 16g lard (animal fat) and a reference dose of [$^{13}C_{10}$] retinyl acetate on day 1. The continuous line and solid-circle data points show the serum [^{2}H] retinol response after consumption of kale [$^{2}H_{9}$] β-carotene and the dashed line data points show serum [$^{13}C_{10}$] retinol after consumption of a labeled reference dose of [$^{13}C_{10}$] retinyl acetate on day 1 of the study. The retinol in circulation measured in nanomoles is shown on the y axis, and time in days is shown on the x axis.
- Figure 4b: Calculated labeled retinol in the circulation of pooled serum time-points after consumption of kale [$^{2}H_{9}$] β-carotene with 33g peanut butter and a reference dose of [$^{13}C_{10}$] retinyl acetate on day 1. The continuous line and solid-circle data points show the serum [^{2}H] retinol response after consumption of kale [$^{2}H_{9}$] β-carotene and the dashed line data points show serum [$^{13}C_{10}$] retinol after consumption of a labeled reference dose of [$^{13}C_{10}$] retinyl acetate on day 1 of the study. The retinol in circulation measured in nanomoles is shown on the y axis, and time in days is shown on the x axis.

LIST OF TABLES

CHAPTER 3

CHAPTER 4

CHAPTER 6

CHAPTER 1

INTRODUCTION

BACKGROUND

Introduction of solid foods is associated with stunting and vitamin A deficiency

Children aged 6-36 months are at higher risk of vitamin A deficiency (VAD) because of cessation of exclusive breast-feeding, introduction of solid foods and rapid growth induced increased vitamin A requirements [1, 2]. Even though breastfeeding is nearly universal in Zimbabwe, exclusive breastfeeding rates are relatively low [3]. Complementary feeding which should be started at age of 6 months is introduced very early, with 63% of children aged 4 -5 months, 41% aged 2 - 3 months and 12% aged 0 - 1 month introduced to solids in 2009 [4]. The World Health Organization (WHO) recommends that children begin complementary feeding, in addition to breast milk, between four and six months of age, in order to ensure adequate growth and nourishment [5]. In Zimbabwe, traditional complementary foods are gruels that are based on starchy staple cereals such as maize, millet and sorghum [6]. Since young children have small gastric capacities, they are unable to meet their energy requirements and consequently may become malnourished because of the low energy and nutrient densities of these complementary foods [7]. Children consuming these foods grow poorly and have higher mortality rates [8]. Early introduction of solid foods exposes children to gastrointestinal diseases resulting in micronutrient deficiencies such as VAD and growth retardation (stunting). This consequence is clearly shown by data from Zimbabwe where 35% percent of the children aged 6-71 months were stunted [3], and also 35% of children aged 6-71 months had VAD indicated by serum retinol levels below 20 μg/dL [9]. It is obvious

that lack vitamin A in the complementary diets of children will lead to VAD and stunting as it is essential for growth, development and immune function. *Increasing the nutrient density of complementary foods is a strategy commonly recommended for improving child growth and survival.*

Food based strategies are the most appropriate VAD strategy in poor countries

Poor people in developing countries depend on plant food carotenoids as their main source of vitamin A because animal products such as dairy, poultry, red meats and fish that are rich in preformed vitamin A are very expensive [10]. Therefore, the Government of Zimbabwe (GOZ) and international organizations embarked on vitamin A supplementation (VAS) programs for all children under 5 years old with vitamin A capsules as a strategy to control VAD. However, while VAS is effective, the coverage of children under 5 years old ranges between 20 and 80% in Zimbabwe [3, 4]. As a result, a majority of vulnerable children in hard to reach remote rural areas are usually left uncovered by VAS. International organizations and private companies also coordinate vitamin A fortification of foods like maize meal, cooking oil and sugar. Fortification of staple foods like maize flour and sugar has the potential of reaching many people. Fortification has been very successful where it was supported by government legislation, for example the universal salt iodation in Zimbabwe [11]. Unfortunately there is no mandatory vitamin A fortification of food in Zimbabwe. Therefore, the effectiveness, coverage and impact of vitamin A fortification of edible oils, breads and dairy products by private companies cannot be adequately assessed. Also in a poor country like

Zimbabwe, where more than 90% of the population lives on less than US$2.00 per day, the consumption of vitamin A fortified commercial foods by the vulnerable groups in rural areas is limited by high costs and access to markets [12]. Therefore, a majority of infants and toddlers in Zimbabwe are fed maize based starchy complementary foods that have little or no vitamin A.

VAS and vitamin A fortification require donor funding and huge capital investments. It is therefore important to address VAD sustainably by empowering rural communities in Zimbabwe with knowledge of local plant foods available to them. As a typical developing country, agriculture is the main economic activity in Zimbabwe and plant foods dominate the diet. Plant foods provide between 70 to 90% of total vitamin A intake in developing countries [10, 13]. Food based dietary approaches such as home-gardens, community gardens and biofortification are increasingly being emphasized because they are effective, sustainable sources of vitamin A, provide other nutrients and are capable of reaching undernourished populations in relatively remote rural areas [14, 15].

Food based vitamin A dietary interventions should focus on staple food crops such as green vegetables and maize. Currently, food based strategies are not aggressively pursued by developmental organizations in Zimbabwe and around the world, partly because they are considered long-term intervention strategies whose impact takes time to show, compared to VAS and fortification. One additional setback for food-based strategies in Zimbabwe is the dearth of nutrition research that limits the availability and access to information on the nutritional value of local plants foods to promote their consumption by the vulnerable groups. *Therefore, the success of food-based strategies to*

address VAD among children depends on increasing the knowledge about the nutritional value of local foods through research and nutrition education.

Enhancing the vitamin A value biofortified yellow maize as a complementary food

Studies in Zimbabwe show that the type of complementary foods is mainly dependent on household supplies, as infant and family meals are prepared together [16]. Maize is used as the main complementary food because, as a staple crop, it is grown widely and therefore easily accessed by the poor [16-19]. Regular white maize is mainly starch and devoid of other important nutrients. However, maize can be nutritionally enhanced by biofortification to provide essential micronutrients such as vitamin A. Biofortified yellow maize is rich in provitamin A carotenoids such as β-carotene that can be converted to vitamin A by the body. Bioefficacy studies showed that biofortified yellow maize is an effective source of vitamin A in humans [20, 21]. Biofortified yellow maize complementary foods would be effective in countries like Zimbabwe because more than 80% of households consume maize porridge at least once a day, and about 56% consume maize with green vegetables at least once a day, making green vegetables such as kale and maize the most frequently consumed food crops [22, 23]. Biofortified yellow maize can then substitute white maize to prepare the maize based complementary foods for children. White maize is nutritionally inferior to yellow maize and biofortified yellow maize, because the latter contains provitamin A carotenoids, vitamin E and oils. Studies in east and southern Africa showed that biofortified yellow maize acceptance is not a big issue among the poor who are vulnerable to VAD [23-26]. Yellow maize porridge can be

cooked with peanut butter to enhance the palatability and nutrient density. However, the acceptability by toddlers of peanut butter cooked yellow maize porridge in Zimbabwe needs to be established, to ensure the success of biofortified yellow maize as a sustainable source of vitamin A.

Despite the scientific evidence that show that biofortified yellow maize is a very good source of vitamin A, the bioconversion of provitamin A carotenoids to vitamin A is very variable [27, 28]. Therefore, it is imperative to find ways to enhance the vitamin A value of biofortified yellow maize, especially if it will be used as a complementary food for children under different conditions. The vitamin A value of biofortified yellow maize can be enhanced by the presence of antioxidants. Antioxidants such as α-tocopherol have been shown to promote the exclusive central cleavage of β-carotene to vitamin A but in their absence, β-carotene was excentrically cleaved to produce apo-carotenoids [29] **(Figure 1)**. Therefore, the presence of antioxidants influences the activity of the enzyme β-carotene 15, 15′-monoxygenase (BCMO1). This enzyme cleaves β-carotene by central cleavage, which involves the metabolism of one β-carotene at the central double bond to produce two retinals. The random cleavage or excentric cleavage of one β-carotene produces retinal and several products such as β-apo-carotenoids. Some studies reported that the presence of lipoxygenase inhibitors and antioxidants such as α-tocopherol prevent random cleavage of β-carotene [30]. This is important because the vitamin A activity of β-carotene, even when measured under controlled conditions, is highly variable and sometimes low [27, 28].

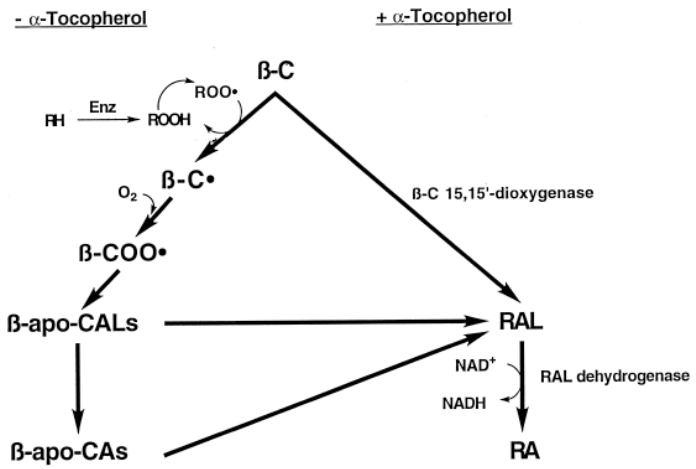

Figure 1: Possible mechanisms of β-carotene cleavage in a purified post-mitochondrial fraction with and without vitamin E [29].

Currently, there are no studies showing the effect of other vitamin E forms (γ-tocopherol, α-tocotrienol and γ-tocotrienol) on the bioconversion of β-carotene to vitamin A. Biofortified yellow maize in addition to provitamin A carotenoids also contains antioxidants such as vitamin E (α-tocopherol, γ-tocopherol, α-tocotrienol and γ-tocotrienol) and γ-oryzanol that may enhance the bioconversion of β-carotene to vitamin A. Regular yellow maize vitamin E forms contents range from 67-230 mg α-tocopherol, 5.8-10.4 mg γ-tocopherol, 4.6-8.9 mg α-tocotrienol and 9.9-23.0 mg γ-tocotrienol per 100 g of dry weight maize [31, 32]. Tocotrienols are considered to be more potent antioxidants than tocopherols [33]. The levels of tocopherols and tocotrienols can be manipulated through biofortification [32]. Regular yellow maize is also rich in antioxidant γ-oryzanol. It will be interesting to determine whether γ-oryzanol can have a similar effect on BCMO1 as α-tocopherol. In order to increase the nutrition knowledge of local foods in Zimbabwe, this study determined the sources of variation and genetic

variation of carotenoids, α-tocotrienol, γ-tocopherol, α-tocotrienol, γ-tocotrienol and γ-oryzanol and antioxidant activity in kale and several varieties of biofortified yellow maize targeted for production in Zimbabwe.

Rat intestinal mucosa homogenate contains the BCMO1 enzyme and has been used to study the activity of the BCMO1 enzyme [29, 34]. The amino acid comparison of human BCMO1 and the homologous enzyme in mice and rats was shown to be around 85% [35], thus giving us the ability to study the enzyme activity as it may perform in humans. In this study the effects of different vitamin E forms (α-tocotrienol, γ-tocopherol, α-tocotrienol and γ-tocotrienol), γ-oryzanol and antioxidants from kale and biofortified yellow maize extracts were incubated with rat intestinal BCMO1 *in vitro. Results from this study showed how these antioxidant promoted increased enzymatic vitamin A production from kale and biofortified yellow maize β-carotene. This information can therefore lead to incorporation of biofortified yellow maize as the complementary feeding options is important towards increasing vitamin A intakes by children 6-36 months in Zimbabwe.*

Kale as a complementary food for infant children in Zimbabwe

Factors such as nutrient profile, availability, accessibility, and filling effect determine a mother's choice of types of complementary foods [19]. This can explain in part why complementary diets in Zimbabwe are mainly maize based and contain little to no fruit and vegetables despite their widespread availability [19]. Fruits and vegetables are rich in provitamin A carotenoids which are converted to vitamin A by the body.

Incorporating provitamin A carotenoid rich fruits and vegetables in the complementary diets of infants in Zimbabwe may lead to improvements in the vitamin A status of children.

Green leafy vegetables such as kale are a common sight in Zimbabwe. Nearly every household in Zimbabwe has a vegetable garden in which at least one of the following *brassica oleracia* var. *acephala, brassica juncea, brassica carinata, brassica nepus, tronchuda Portuguesa var*., and *brassica* var. *capitata* are grown [36-38]. Kale *(brassica acephala)* is locally known in Zimbabwe as *rugare, covo, viscose* and *Chou Moellier* are the most widely grown and consumed perennial green vegetables [38]. Kale is consumed sautéed as a side dish to the thick porridge (sadza). It is also a traditional practice in Zimbabwe to add peanut butter to food while cooking, especially green vegetables and maize porridge to increase palatability, filling effect and nutrient density. Despite the nutrient superiority, neither sautéed nor peanut butter cooked kale is used as a complementary food in Zimbabwe. This can be a result of cultural beliefs or ignorance of the mothers and care givers on the nutritional importance of kale. The provitamin A carotenoid contents of *Brassica oleracea* green vegetables grown and consumed in Zimbabwe is currently unknown. In the US, *Brassica oleracea* var *acephala* (kale) was shown to be rich in provitamin A carotenoid β-carotene [39-41]. The β-carotene content of kale is one of the highest in plant foods, ranging from 3-15 mg/100g fresh weight [42]. Kale β-carotene was shown to be very bioavailable in humans, and the kale β-carotene was shown to be effectively converted to vitamin A [41]. However, what is yet to be determined is the amount of retinol (vitamin A) that can be form from kale β-carotene, especially in children.

The vitamin A value of green vegetables

The bioavailability of green leafy vegetable β-carotene and bioconversion to vitamin A varies widely [43-46]. Studies comparing the bioavailability of β-carotene from green leafy vegetables to purified β-carotene found that it ranges between 3 and 6% for green leafy vegetables, 19 and 34% for carrots and 22 and 24% for broccoli [47-50]. In some cases, β-carotene from fruits was found to be 2.6-6 times as effective in increasing plasma concentrations of retinol and β-carotene as green leafy vegetables [47]. These differences may result from differences in intracellular location of carotenoids. It is also possible that other factors such as the fat content of the meal, food matrix, food processing and amount and type of fiber in the food sample played a role [51]. Most β-carotene bioavailability studies have focused mainly on carrot, spinach, broccoli, peas, sweet potato, and tomato because these foods are important in the western diet [27, 51-52]. Very little is known about the bioavailability of β-carotene from *Brassica oleracea* vegetables that are important in diets of poor people in east and southern Africa. However, one study in US adults showed the bioavailability of [^{13}C] labeled kale β-carotene after consuming 400 g of cooked kale (20 mg β-carotene) with 30 g of peanut oil [41]. Labeled [^{13}C] β-carotene (**Figure 2**) from the kale was detectable in the subject's plasma for the full 46-day sampling period [41]. Labeled retinol [^{13}C] retinol detected throughout the entire 46-day sampling period was derived from [^{13}C] β-carotene in the kale dose, since β-carotene was the only provitamin A carotenoid present in the kale. Interestingly in this study the kale was consumed with 30 g of peanut oil that is rich

in vitamin E. Maybe the peanut oil vitamin E resulted in the observed β-carotene bioavailability and retinol formation. Peanut butter is rich in oils, vitamin E and protein, and these factors have been known to affect bioavailability of β-carotene from plant foods [27, 53-54]. Therefore, one of the aims of this study was to determine the effect of vitamin E rich peanut butter cooked kale on the bioavailability of kale β-carotene and it's bioconversion to vitamin A in Zimbabwean toddlers aged 12-36 months.

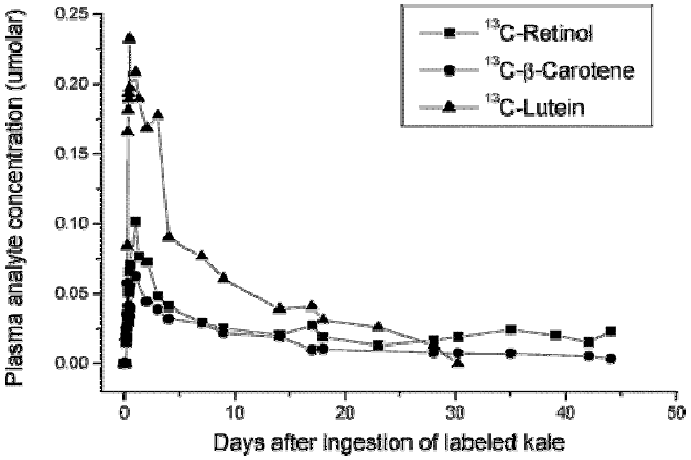

Figure 2: Plasma lutein, β-carotene and ^{13}C-retinol after consumption of a 400 g-cooked kale [49].

Determining the vitamin A value of kale in children

Other techniques have been employed to assess the bioavailability of β-carotene from plant foods such as postprandial chylomicron, and separation triacylglycerol-rich lipoproteins (TRL) [55-57]. These procedures have several pitfalls for obtaining accurate data due to variability in the recovery of TRL. Stable isotope labeling has been used safely in humans to assess nutritional status. Vegetables grown hydroponically in 15-30%

atom-% D_2O have been developed for nutritional studies in humans. Kale has been successfully labeled with deuterium to assess the vitamin K bioavailability in humans [58]. Kale labeled with [^{13}C] β-carotene was used to evaluate the bioavailability of β-carotene in humans [41]. In order to determine the bioconversion of kale β-carotene to vitamin A and quantitate the vitamin A value, kale plant material in which the carotenoids have been endogenously or intrinsically labeled with a low abundance deuterium stable isotope were used in this study. The deuterium isotopic tag allows the separate identification of serum carotenoids from labeled kale and from other dietary sources. The deuterium labeled kale β-carotene that is derived from the labeled kale can be traced after being eaten by a human subject. In this way it was possible to determine absorption of β-carotene from the food matrices and the subsequent conversion of the β-carotene to vitamin A [59]. A reference dose of a known amount of [$^{13}C_{10}$] retinyl acetate was ingested by subject, which allowed the amount of labeled retinol from the kale to be calculated. The vitamin A value of kale was quantitatively determined by comparing the areas under the curve (AUC) of a 1 mg quantity of [^{13} C_{10}] retinyl actate reference dose in serum to that of deuterium labeled retinol from labeled kale β-carotene .

SIGNIFICANCE

Poor people in developing countries eat diets composed mainly of plant foods which are their staple foods. Adding variety to their diets through the promotion of household production and consumption of diverse locally available foods rich in provitamin A carotenoids will significantly contribute to controlling and eliminating VAD. This thesis addressed the most important food crops in the diets of vitamin A deficient populations in east and southern Africa. Improving the vitamin A value of staple foods ensures that people are getting a daily dose of vitamin A from their diets. Maize and kale are consumed daily by millions of people in east and southern Africa. Results from this study increase the knowledge and access to provitamin A rich foods such as *Brassica oleracea* vegetables, which in turn may result in improved vitamin A intake by children. Other programs such as vitamin A supplementation and food fortification while effective and cost effective, are not sustainable in many resource poor Africa countries where the majority of vitamin A deficient populations live in remote rural areas. Food based strategies such as home gardens, dietary diversification and biofortification which are just as effective as VAS and fortification, are sustainable and can research everyone in the community are currently being promoted for controlling VAD. This study utilized kale a *Brassica oleracea* vegetable that is already consumed in Zimbabwe to determine its vitamin A value and potential use as vitamin A rich complementary food. Kale is grown widely in home-gardens in both rural and urban areas. It grows rapidly and grows for many seasons, allowing families to have constant daily supply of vitamin A. Maize porridges that are the main complementary food for children aged 6-36 months can also

be optimized to provide vitamin A through biofortification. This study showed that biofortified yellow maize rich in provitamin A carotenoids can provide vitamin A to children if used as complementary food. Genetic characterization of different biofortified yellow maize hybrids for carotenoids, vitamin E and phenolic compounds and antioxidant activity showed that biofortified yellow maize rich in provitamin A carotenoids can be grown in different regions of the world with very little change in the provitamin A carotenoid contents. This study demonstrated that antioxidants present in kale and biofortified yellow maize such as vitamin E when consumed with β-carotene resulted in increased vitamin A production by the BCMO1 enzyme through inhibition of the excentric cleavage pathway.

The availability of information on the vitamin A value of kale and provitamin A carotenoids enriched biofortified yellow maize allows nutritionists working in Zimbabwe to develop nutrition education tools to encourage mothers to feed their children these vitamin A rich foods. This empowers rural communities to address VAD sustainably and effectively. It is envisioned that results from this study will influence the development of effective and sustainable dietary guidelines for infant and young children nutrition in Zimbabwe and neighboring countries in east and southern Africa.

HYPOTHESIS

The main objective of this study was to determine the role of antioxidants in enhancing kale and biofortified yellow maize as rich sources of vitamin A in the complementary diets of children aged 6-36 months in Zimbabwe. *We hypothesized that provitamin A rich kale and provitamin A enriched yellow maize will improve the vitamin A value of complementary foods in Zimbabwe*. The following specific aims were pursued to address our main objective:

SPECIFIC AIMS

Specific aim 1(a): Determine the carotenoid, vitamin E, γ-oryzanol contents and antioxidant capacity of biofortified yellow maize varieties for Zimbabwe.

The hypothesis tested was that there are associations between carotenoids, vitamin E and γ-oryzanol in biofortified yellow maize with their antioxidant capacity. To test this hypothesis 30 provitamin A enriched yellow maize hybrids varieties developed by the International Maize and Wheat Improvement Center (CIMMYT) in Mexico were analyzed by HPLC for carotenoids, vitamin E, γ-oryzanol, ferulic acid and p-coumaric acid. The antioxidant activity was determined using the Total Antioxidant Performance (TAP) assay.

Specific aim 1(b): Determine the effect of vitamin E and γ-oryzanol in kale and biofortified yellow maize on the cleavage of their β-carotene to vitamin A in vitro.

We tested the hypothesis that antioxidants in biofortified yellow maize and kale (vitamin E and γ-oryzanol) will promote the central cleavage of β-carotene to vitamin A. To test this hypothesis, extracts of biofortified yellow maize and kale were incubated with rat intestinal mucosal homogenate at 37°C for an hour. Pure standards of β-carotene were also incubated with rat intestinal homogenate in the presence of either α-tocopherol, γ-tocopherol, α-tocotrineol, γ-tocotrienol and γ-oryzanol. The β-carotene cleavage products were extracted and analyzed and identified by HPLC.

Specific aim 2: To genetically profile the provitamin A carotenoids in brassica oleracea green vegetables commonly consumed in Zimbabwe.

The hypothesis tested was that there were no differences in the carotenoid contents of different *Brassica oleracea* vegetables commonly grown and consumed in Zimbabwe. To test this hypothesis, *Brassica oleracea* vegetables commonly grown and consumed in Zimbabwe were identified, samples and analyzed for carotenoid contents by HPLC.

Specific aim 3: Determine the effect of vitamin E rich peanut butter on the bioconversion of kale β-carotene to vitamin A in humans.

The hypothesis tested was that peanut butter cooked kale β-carotene will be better absorbed and easily converted to vitamin A compared to the lard (animal fat) cooked kale β-carotene in preschool children aged 12-36 month. Intrinsically labeled kale was grown in Houston, Texas. Forty children were recruited and on day 1 of the study and were randomly assigned to either 50 g cooked kale (1.5 mg β-carotene content) with 33 g of peanut butter or with 16 g of lard. All subject ingested a capsule with 1mg [$^{13}C_{10}$] retinyl acetate reference dose. Baseline blood draw was collected before ingestion of the labeled doses, and blood was collected on day 1, 2, 3, 6, 15 and 21 after ingestion of the labeled doses from 5 children per time-point per group. Serum was them processed and analyzed by HPLC for carotenoids, retinol and tocopherols. GC/MS was used to analyze the serum enrichment of [^{2}H] retinol from [$^{2}H_{9}$] β-carotene from kale and [$^{13}C_{10}$] retinol from 1 mg reference dose. Molar enrichments of [^{2}H] retinol from kale [$^{2}H_{9}$] β-carotene and [$^{13}C_{10}$] retinol were used to compute AUCs. The AUCs for [$^{13}C_{10}$] retinol and [^{2}H] retinol were used to calculate the vitamin A equivalence and the conversion factors of kale [$^{2}H_{9}$] β-carotene from the peanut butter group and the lard group.

REFERENCES:

1. World Health Organization (WHO). Global prevalence of vitamin A deficiency. The Micronutrient deficiency information system. WHO. Geneva. 1995
2. Malaba, L.C et al. Effect of postpartum maternal or neonatal vitamin A supplementation on infant mortality among infants born to HIV-negative mothers in Zimbabwe. *Am J Clin Nutr*, 2005; 81(2): p.454-60
3. Zimbabwe National Statistic Agency (ZIMSTAT) and ICF International. 2012. Zimbabwe Demographic and Health Survey 2010-11. Calverton, Maryland: ZIMSTAT and ICF International Inc.
4. Central statistical office Zimbabwe, United Nations Children's fund (UNICEF) and MICS. Multiple Indicator Monitoring indicator survey (MIMS) 2009. Preliminary report November 2009.
5. Hellstrom A, Hermansson A, Karlsson A, Ljungqvist B, Mellander O, Svanberg U. Dietary bulk as a limiting factor for nutrient intake - with special reference to the feeding of pre-school children II. Consistency as related to dietary bulk - a model study. *J Trop Paediatr* 1981; 27: 127-35.
6. Food and Agricultural Organization of the United Nations (FAO0. Cereal Fermentation in African Countries. Fermented Cereal: A Global Perspective; Chapter 2. FAO, Rome, 1999. http://www.fao.org/docrep/x2184E/x2184E00.html (accessed 01/20/2013)
7. Ljungqvist BG, Mellander O, Svanberg U. Dietary bulk as a limiting factor for nutrient intake in pre-school children. I.A problem description. *J Trop Paediatr* 1981; 27: 68-73.

8. Pelletier DL, Frongillo EA, Schroeder DG, Habicht J-P. The effects of malnutrition on child mortality in developing countries. *Bull WHO* 1995; 7: 443-8.
9. Ministry of Health and Child Welfare, Nutrition Unit. Zimbabwe National Micronutrient Survey, 1999. Harare. Ministry of Health and Child Welfare, 2001
10. Codjia, G. Food sources of vitamin A and the provitamin A specific to Africa. An FAO perspective. *Food Nutr Bull*. 2001, 22 (4): p 357-60
11. United Nations Children's Fund (UNICEF). Sustainable Elimination of Iodine Deficiency. Progress Since 1990 World Summit for Children. UNICEF. New York, 2008. http://www.childinfo.org/files/idd_sustainable_elimination.pdf (accessed 01/20/2013)
12. The World Bank. World Development Indicators for Zimbabwe 2011. World Bank, Washington D.C.
13. de Pee, S and West CE. Dietary carotenoids and their role in combating vitamin A deficiency. A review of literature. Eur J Clin Nutr 1996; 50 suppl 3; 538-53
14. Ruel, M. Can food-based strategies help reduce vitamin A and iron deficiency? 2001. The International Food Policy Research Insistitute (IFPRI). Washington D.C 2001
15. Mayer JE et al. Biofortified crops to alleviate micronutrient malnutrition. *Curr Opin Plant Biol* 2008, 11 (2):166-70
16. Orne-Gliemann J at al. Community based assessment of infant feeding practices within a programme for preventation of mother to child HIV transmission in rural Zimbabwe. *Public health Nutr* 2006; 9: 563-69

17. Smale M et al. Maize Revolutions in Sub-Saharan Africa. Policy Research Working Paper 5659. The World Bank Development Research Group, Agricultural and Rural Development Team. 2011. The World Bank. Washington D.C
18. Paul, K. H. et al. J. Complementary feeding messages that target cultural barriers enhance both the use of lipid-based nutrient supplements and underlying feeding practices to improve infant diets in rural Zimbabwe. *Matern Child Health J*. 2012, 8 (2), 225-238
19. Maclaine A. Infant feeding Practices in Binga and Nyaminyami. Save the Children UK. 2006. http://www.ennonline.net/pool/files/ife/save-the-children-justification-final.pdf (accessed 02/19/2014)
20. Muzhingi, T., Gadaga, T.H., Siwela, A.H., Grusak, M.A., Russell, R.M., Tang, G. Yellow maize with high beta-carotene is an effective source of vitamin A in healthy Zimbabwean men. *Am J Clin Nutr* 2011, 94 (2):510-17
21. Li, Shanshan, et al. Vitamin A equivalence of the β-carotene in β-carotene–biofortified maize porridge consumed by women. *Am J Clin Nutr* 2010, 92 (5): 1105-1112.
22. Merchant AT, Dehghan M, Chifamba J, Terera G, Yusuf S. Nutrient estimation from an FFQ developed for a Black Zimbabwean population. *Nutr J* 2005,4: 37-41.
23. Muzhingi, Tawanda, et al. Consumer acceptability of yellow maize products in Zimbabwe. *Food Policy* 2008, 33 (4): 352-361.

24. De Groote, Hugo, and Simon Chege Kimenju. Comparing consumer preferences for color and nutritional quality in maize: Application of a semi-double-bound logistic model on urban consumers in Kenya. *Food Policy* 2008, 33 (4): 362-370.
25. Nuss, Emily T., et al. Comparative intake of white-versus orange-colored maize by Zambian children in the context of promotion of biofortified maize. *Food Nutr Bull* 2012, 33 (1): 63-71.
26. Pillay, Kirthee, et al. Consumer acceptance of yellow, provitamin A-biofortified maize in KwaZulu-Natal. *South Afr J Clin Nutr* 2011, 24 (41): 186-191.
27. Yeum KJ, and Russell R Carotenoid bioavailability and bioconversion. *Annu Rev Nutr* 2002; 22:483-504.
28. Borel, Patrick, et al. Chylomicron β-carotene and retinyl palmitate responses are dramatically diminished when men ingest β-carotene with medium-chain rather than long-chain triglycerides. *J. Nutr* 1998, 128 (8): 1361-1367.
29. Yeum, Kyung-Jin, et al. The effect of α-tocopherol on the oxidative cleavage of β-carotene *Free Radic Biol Med* 2000**,** *29* (2), 105-114.
30. Yeum, Kyung-Jin, et al. Similar Metabolites Formed from β-Carotene by Human Gastric-Mucosal Homogenates, Lipoxygenase, or Linoleic-Acid Hydroperoxide. *Arch Biochem Biophys* 1995, 321 (1): 167-174.
31. Goffman, Fernando D., and Timo Böhme. Relationship between fatty acid profile and vitamin E content in maize hybrids (Zea mays L.). *J Agric Food Chem* 2001, 49 (10): 4990-4994.
32. Rocheford, Torbert R., et al. Enhancement of vitamin E levels in corn. *J Am Coll Nutr* 2002, 21.suppl 3: 191S-198S.

33. Sen, Chadan, Sashwati Roy, and Savita Khanna. Protective and therapeutic uses for tocotrienols. U.S. Patent Application 10/914,339.
34. Lietz, Georg, Jennifer Lange, and Gerald Rimbach. Molecular and dietary regulation of β, β-carotene 15, 15′-monooxygenase 1 (BCMO1). *Arch Biochem Biophys* 2010, 502 (1): 8-16.
35. Shmarakov I, et al. Carotenoid Metabolism and Enzymology, In Carotenoids and Health, Nutrition and Health, 2013.p29-56. Editor Sherry A. Tanumihardjo. Humana Press.
36. Karavina, C et al. Biofumigation for crop protection; potential for adoption in Zimbabwe. *J Anim Plant Sci* 2012. 14 (3):p 1996-05
37. Mariga I et al. Nutritional assessment of traditional local vegetables (brassica oleracia var. acephala). *J Med Pl Res* 2012. 6 (5):784-9.
38. Grubben GJH and Denton OA. Plant resources of tropical Africa, volume 2. Vegetables. Plant Reseources of Tropical Africa (PROTA) 2004. ISBN: 9057821486. The Netherlands.
39. Lefsrud M et al. Changes in kale (Brassica oleracea L. var. acephala) carotenoid and chlorophyll pigment concentrations during leaf ontogeny. *doi: 10.1016/j.scienta.2006.12.026*
40. Kurilich AC, Britz SJ, Clevidence BA, Novotny JA. Isotopic labeling and LC-APCI-MS quantification for investigating absorption of carotenoids and phylloquinone from kale (*Brassica oleracea*). *J Agric Food Chem* 2003; 51:4877-83.

41. Novotny JA, Kurilich AC, Britz SJ, Clevidence BA. Plasma appearance of labeled β-carotene, lutein, and retinol in humans after consumption of isotopically labeled kale. *J Lipid Res*. 2005; 46:1896-903.
42. Kurilich, Anne C., et al. Isotopic labeling and LC-APCI-MS quantification for investigating absorption of carotenoids and phylloquinone from kale (Brassica oleracea). *J Agric Food Chem* 2003, 51 (17): 4877-4883.
43. De Pee S., West C. E., Muhila, Daryadi D., Hautvast J.G.AJ. Lack of improvement in vitamin A status with increased consumption of dark-green leafy vegetables. *Lancet* 1995;346:75-81
44. van het Hof K. H., Tijburg L.B.M., Pietrzik K., Weststrate J. A. Bioavailability of carotenoids and folate from different vegetables. Effect of disruption of the vegetable matrix. *Br. J. Nutr*. 1999;82:203-212
45. van Lieshout M. Bioavailability and bioefficacy of β-carotene measured using ^{13}C-labeled β-carotene and retinol: studies in Indonesian children. *PhD thesis.* Wageningen, Netherlands: Wageningen University, 2001.
46. Khan NC, et al. The contribution of plant foods to the vitamin A supply of lactating women in Vietnam: a randomized controlled trial. *Am J Clin Nutr* 2007; 85:1112-20.
47. Haskell, Marjorie J. The challenge to reach nutritional adequacy for vitamin A: β-carotene bioavailability and conversion-evidence in humans. *Am J Clin Nutr* 2012, 96 (5): 1193S-1203S.

48. Van Loo-Bouwman CA, et al. Vitamin A equivalency and apparent absorption of β-carotene in ileostomy subjects using a dual-isotope dilution technique. *Br J Nutr* 2010*;* 103:1836-43.

49. Van Loo-Bouwman CA, et al. Vitamin A equivalency of β-carotene in healthy adults: limitation of the extrinsic dual-isotope dilution technique to measure matrix effect. *Br J Nutr*2009*;* 101:1837-45.

50. Edwards AJ et al. A novel extrinsic reference method for assessing the vitamin A value of plant foods. *Am J Clin Nutr* 2001*;* 74:348-55.

51. Pellegrini et al. Effect of Different Cooking Methods on Color, Phytochemical Concentration, and Antioxidant Capacity of Raw and Frozen Brassica Vegetables. *J Agric Food Chem* 2010, *58* (7), 4310-4321

52. Tang G, et al. Spinach or carrots can supply significant amounts of vitamin A as assessed by feeding with intrinsically deuterated vegetables. *Am J Clin Nutr* 2005; 82:821-8.

53. Castenmiller JJ, et al. The food matrix of spinach is a limiting factor in determining the bioavailability of β-carotene and to a lesser extent of lutein in humans. *J Nutr* 1999; 129:349-55.

54. Dimitrov NV, Meyer C, Ullrey DE, Chenoweth W, Michelakis A, et al. 1988. Bioavailability of beta-carotene in humans. *Am. J. Clin. Nutr*. 48:298-304

55. Johnson EJ and Russell RM. Distribution of orally administered beta-carotene among lipoproteins in health men. *Am J Clin Nutri* 1992; 56:128-35

56. van Vliet, T., Schreurs, W. H., & van den Berg, H. Intestinal beta-carotene absorption and cleavage in men: response of beta-carotene and retinyl esters in the

triglyceride-rich lipoprotein fraction after a single oral dose of beta-carotene. *Am J Clin Nutri* 1995, 62 (1), 110-116.

57. O'Neil and Thurnham DI. Intestinal absorption of β-carotene, lycopene and lutein in men and women following a standard meal: response curves in the triacyglycerol-rich liopoprotein fraction. *British J Nutr* 1998; 79: 149-59
58. Erkkila A et al. Plasma transport of vitamin K in men using deuterium-labeled collard greens. *Metabolism* 2004; 53(2);215-21
59. Tang G. Techniques for measuring vitamin A activity from β-carotene. *Am J Clin Nutr* 2012; 95: 1185S-88S.

CHAPTER 2

LITERATURE REVIEW

What is vitamin A and where is it found?

Vitamin A is an essential nutrient required by the human body for normal growth and development. The term vitamin A refers to all compounds that qualitatively exhibit the biological activity of retinol [1]. In almost all tissues the active form of vitamin A responsible for metabolic functions is retinoic acid [2]. The retinoic acid nuclear receptors (RAR and RXR) have greater affinity specifically for all-trans-retinoic acid form, but recently other functional retinoic acids such as 9 cis retinoic acid have emerged [3]. The most effective dietary sources of vitamin A are foods of animal origin such as dairy products, poultry, fish and meat. These foods contain preformed vitamin A in the form of retinol, which is very bioavailable. Plant foods such as fruits, green leafy vegetables, tubers and flowers contain precursors of vitamin A called carotenoids [4]. There are more than 600 carotenoids in nature and of these only 50 have provitamin A carotenoid activity [5]. Of all the provitamin A carotenoids, all-trans β-carotene is the largest contributor to vitamin A activity. Other provitamin A carotenoids such as 9 cis-β-carotene, 13 cis-β-carotene, α-carotene, β-cryptoxanthin and γ-carotene have half the vitamin A activity of all trans β-carotene [6]. Provitamin A carotenoids when consumed by humans can be converted to vitamin A by enzymes such as the β-carotene monoxygenase cleavage enzyme (BCMO1) [7]. Vitamin A has been added to a wide variety of foods in order to improve their nutritional value through large scale fortification of commonly consumed foods such as milk in the (US), cooking oil (Bangladesh), sugar (Guatemala), rice (India) and maize flours (South Africa) [8]. *In nature provitamin A carotenoids provide all the vitamin A in the food chain, animals convert them to retinol esters which are then consumed by humans in animal products.*

Vitamin A functions important to child development and survival

Vitamin A and vision

Vitamin A deficiency (VAD) is the leading cause of preventable blindness in children and pregnant women in developing countries [9]. Untreated VAD leads to night blindness and eventual blindness because vitamin A is required for vision. In the retina of the eye, *trans*-retinol released from retinyl esters is enzymatically isomerized to 11-*cis*-retinol, which can be oxidized to form 11-*cis*-retinal. The 11 *cis*-retinal interacts with a protein called opsin to form rhodopsin a visual pigment which upon absorption of a photon of light catalyzes the isomerization of 11-*cis*-retinal to all-*trans*-retinal [11]. This conformational change results in the generation of an electrical signal to the optic nerve, which is transmitted to the brain and interpreted as vision [11]. In marginal VAD (serum retinol levels of 0.35 to 0.70 µmol/L), night blindness is caused by the rod cells of the retina that become very sensitive to VAD [12]. Structural damage of the rod cells called xerophthalmic fundus as of result of VAD have been reported [13]. The highest proportion of preschool-age children affected by night blindness resides in Africa with 2.55 million affected, representing almost half of the children affected globally [14, 15]. The highest proportion of pregnant women affected by night blindness is also in Africa with 9.8% of the global prevalence rate [14]. VAD women in turn give birth to children at risk of developing VAD [15]. *Blindness caused by VAD is unacceptable because VAD can be prevented and treated cost effectively through a variety of intervention strategies.*

Vitamin A and immune function

Vitamin A plays an important role in the regulation of immune function. It is known that vitamin A metabolites such as all-trans retinoic acid and 9-cis retinoic acid act through RAR and RXR to regulate gene transcription [16]. Vitamin A influences many aspects of immunity through regulation of gene function and gene expression of immune cells such as neutrophils, natural killer cells, macrophages, T and B lymphocytes, immunoglobulin production, and expression of cytokines and adhesion molecules such as ICAM-1 [17]. VAD compromises immunity, resulting in major morbidity and mortality. VAD compromises mucosal immunity by altering the integrity of mucosal epithelia in the eye, respiratory, gastrointestinal, and genitourinary tracts [18]. It has been shown that VAD causes pathologic alterations in the gastrointestinal tract that may contribute to increased severity of diarrheal disease in VAD children [19]. Studies show that VAD affects the genitourinary tract by the replacement of normal transitional epithelium with stratified squamous epithelium thus increasing the risk of VAD children to increased urinary tract infections [20]. VAD also affects the gastrointestinal mucin, a first line of defense for cells lining the gut by impairment of the production and secretion of IgA in responses to various pathogens [21, 22]. Loss of microvilli, goblet cells, and mucin in the small intestine were also observed in VAD cases [23] VAD impairs the expression and functions of lactoferrin an iron-binding glycoprotein involved in immunity to bacteria, viruses, and fungi found in mucosal secretions [21]. *It is therefore important to supply adequate vitamin A to all children at risk of VAD for their survival and also to improve their*

access to adequate health care to treat repeated infections that cause and exacerbate the consequences of VAD.

Children require adequate vitamin A for growth and development

Retinoic acid an active form of vitamin A is known to have hormone like functions in controlling growth and development of tissues in the musculo-skeletal system [24]. Retinoic acid is an enhancer of skeletal myogenesis in stem cells, regulating muscle growth and development by interacting with Wnt and BMP4 signaling pathways during cell differentiation [25]. Retinoic acid activates RARs bound directly to mesoderm and skeletal muscle progenitor genes enhancing skeletal myogenesis [25]. Retinoic acid regulates expression of the genes responsible for growth hormones. Research shows that both retinol and retinoic acid produce rapid release of cyclic AMP and human growth hormone secretion influencing growth and development [26]. Retinol and retinoic acid are essential for embryonic development, particularly during fetal development of limb, heart, eyes, and ears [27]. VAD is closely associated with growth retardation and stunting in children. Observational studies have shown strong associations between VAD and the risk of being stunted [28-29]. One study showed that children with mild xerophthalmia were more likely to be stunted that those without [30]. Animal studies with vitamin A as the only growth stimulating factor manipulated showed growth stopped when liver stores were exhausted and growth resumed when vitamin A was re-established [31-34]. Evidence from clinical trials shows vitamin A supplementation on children with mild to severe VAD in South East Asia, South Asia and Africa resulted in increased linear growth [35-36]. *Children under the age of 5 years have rapid growth and increased*

vitamin A requirements. It is therefore important to ensure adequate supply of vitamin A to vulnerable children in a manner that is sustainable, effective and cost effective so they can reach their full growth potential.

Strategies for controlling VAD among children

VAD is defined by the World Health Organization (WHO) as severe public health problem when more than 20% of children under the age of 5 year have serum retinol levels less than 20 µg/dL (0.70 µmol) [37]. The control of VAD is important to child survival in many poor countries. The immediate causes of VAD are inadequate intake of preformed retinol from foods of animal origin. In many poor countries, access to animal source foods rich in bioavailable preformed vitamin A is limited because these foods are very expensive. If children do not consume these vitamin A rich foods, their liver vitamin A stores will be depleted which eventually leads to VAD. Diseases and infections also cause VAD by increasing utilization, requirements, and excretion of vitamin A. VAD increases the risk and severity of infections and diseases [38, 39]. Several strategies have been pursued in different countries to control VAD and its effects on the health of children and long-term impacts on economic development. The three most common intervention strategies for controlling VAD are vitamin A supplementation (VAS), food fortification with vitamin A, and food-based approaches that aim to increase access to and intake of vitamin A-rich foods.

Vitamin A supplementation (VAS)

One of the Millennium Development Goals (MDGs) is to reduce by two thirds the mortality rate among children under five years old by 2015 [40]. VAS is an important component of the strategies required to reach this goal. In countries where VAD is a public health problem, it is recommended to implement VAS for infants and children 6-59 months of age to reduce child morbidity and mortality. This involves the administration of vitamin A capsules or supplements (100000-200000 IUs dose) every four to six months. VAS is an inexpensive, quick, and effective way to improve vitamin A status and reduce child morbidity and mortality in the long term [41]. The mega doses of vitamin A are meant to boost liver vitamin A stores every 4-6 months. In many countries, combining the vitamin A supplementation with immunizations enhances the delivery and effectiveness of VAS programs [41]. According to the World Bank, the VAS coverage rates in Africa ranged from 51-82% from 2001-2013 [42]. The VAS coverage rates vary greatly by country and by regions within each country. Unfortunately, VAS is not ideal in poor countries Africa countries where road and healthcare infrastructure are very poor. Therefore, a lot of children in remote hard to reach areas will not be covered by VAS programs. Many poor countries in Africa cannot afford to implement VAS on their own resources, hence VAS programs are often implemented by United Nations agencies such UNICEF and WHO with donor funding from developed countries [43]. *Donor funded VAS is vulnerable to fluctuations in the funding received making it an unsustainable option. In order to completely eliminate VAD, local sustainable and effective strategies should be pursued.*

Food fortification

Food fortification with vitamin A is one strategy that has proven to be an effective and cost effective way to increase the vitamin A supply, reducing the consequences of VAD in developed countries. Food fortification was successful in the US and other western countries because they had well educated consumers and advanced manufacturing and distribution networks that facilitated monitoring and enforcement [44]. These condition are absent in many developing countries, making food fortification a challenge. The advantage of food fortification is that it requires minimal changes in food habits, it usually costs less than 2% of the cost of the unfortified food and its delivery system is already in place [45]. In the Philippines margarine was successfully fortified with vitamin A and pilot studies showed that vitamin A fortified margarine (25 mg/kg) was able to decrease the prevalence of low serum retinol (<0.70 μmol/L) in preschool-aged children from 25.7 to 10.1 % after 6 months of consumption [46]. Venezuela used vitamin A maize flour fortification (2.7 mg/kg) aimed at providing about 30% of the vitamin A RDA based on the national consumption per capita of maize [47]. In Africa, with the exception of successful iodine salt fortification, many countries do not have mandatory food fortification with vitamin A. In West Africa, international organizations such as the Helen Keller International are advocating fortification of cooking oil with vitamin A through the *Tache D'Huile,* a partnership between public and private party stakeholders in eight francophone countries [48]. Also pursued are home-food fortification initiatives also called point of use fortification where a fortificant premix in a sachet is added to food during or after processing. *While food fortification is effective at reducing VAD, large-scale food fortification with vitamin A requires*

government legislation, advanced food manufacturing and distribution and adequate quality control. The use of in-house fortification is also effective but it is not sustainable because it requires households to purchase the fortificants or at least receive them for free from a donor funded program.

Food based vitamin A intervention strategies

Food based VAD intervention strategies are aimed at increasing the production, accesses and availability of vitamin A rich foods through promotion of home production, increasing the intake of provitamin A rich plant foods through nutrition education, increasing the bioavailability of provitamin A carotenoids and increasing the vitamin A content of foods through plant breeding strategies [49]. Food based strategies have been described as sustainable because they focus on individual households or communities by empowering them through nutrition education to take responsibility over their vitamin A status by growing and consuming provitamin A rich foods. Food-based approaches have the potential to be effective and sustainable in addressing VAD, but many questions remain on their effectiveness, reviving the need for more well-designed studies on efficacy, effectiveness, cost effectiveness, and sustainability.

Several studies show the efficacy and effectiveness of plant based foods as sources of vitamin A in humans. Some controlled feeding trials showed improvements in vitamin A status after three weeks to four months of feeding β-carotene-rich plant foods [50-54]. In some of these trials the β-carotene-rich plant foods was consumed together with fat to enhance the bioavailability, and in some studies subjects were administered

deworming tablets to ensure the efficient uptake of the provitamin A carotenoids. These controlled feeding trials were essential in establishing the bioefficacy of β-carotene-rich plant foods as good source of vitamin A. The major limitations of well controlled feeding bioefficacy studies are that their results cannot be generalized to the entire populations, because people live freely and consume their food under different conditions. Therefore, effectiveness studies of β-carotene-rich plant foods provide more useful information for designing intervention programs towards alleviation of VAD in poor countries. However, these effectiveness studies or community trials are challenging to design, hence only a few well-designed studies have been reported [55]. One study in Bangladesh showed that vitamin A intake was derived almost entirely from the consumption of fruits and vegetables obtained from home gardens, indicating that traditional production of provitamin A-rich fruits and vegetables in the homesteads may provide a valuable contribution to vitamin A intake in communities where alternative dietary sources of vitamin A are scarce [56]. In South Africa, frequent consumption of yellow and dark green leafy vegetables resulted in significantly higher serum retinol concentrations in intervention children compared to children in control villages [57]. This again showed that home-gardening programs focused on the production of yellow and dark-green leafy vegetables can significantly improve the vitamin A status of children aged 2-5 years. Orange-flesh sweet potato effectiveness studies with children under 5 years old in Mozambique, South Africa and Uganda showed that children's vitamin A intakes were much higher in intervention children than those of control children [58-60]. Also, vitamin A intakes from orange flesh sweet potato were also positively associated with vitamin A status. *Therefore, bioefficacy and effectiveness studies showed that plant foods are good*

source of vitamin A, more specifically that homestead food production can contribute to combating VAD directly, by increasing intake of vitamin A-rich foods.

Can plant foods provide enough vitamin A in humans?

Quantitating the β-carotene to vitamin A conversion factors for green vegetables

The zeal for promoting plant foods and nutrition gardens as effective strategies for controlling VAD in poor countries faced significant challenges over the past decades. In the late 1990s, conversion factors for estimating vitamin A obtained from plant foods were revised from 6 to 1 to 12 to 1 (μg β-carotene: retinol activity equivalent) by the U.S. Institute of Medicine [61]. West and Casternmiller found β-carotene to vitamin A conversion factors of 21:1 for a mixed diet (12:1 for fruits and 26:1 for vegetables) [62]. One study in Indonesia investigating, the effect of an additional daily portion of dark-green leafy vegetables consumption every day for 12 weeks showed very little to no effect on the serum retinol levels [63]. These studies highlighted the role that food matrix plays in determining the bioavailability and bioconversion of plant food β-carotene to vitamin A. It is now known that factors that influence the bioavailability of carotenoids and their bioconversion to retinol include species of carotenoids, molecular linkages, amount of carotenoids consumed in a meal, matrix in which the carotenoid is incorporated, effectors of absorption and bioconversion, nutrient status of the host, genetic factors, host-related factors, method of food preparation, and the fat content of a meal [64, 65]. However, it is a challenge to study the conversion of β-carotene to vitamin A at physiologic doses and even more of a challenge to study this conversion at dietary

intake amounts because of the inability to distinguish newly formed retinol from body reserves [66]. Therefore, some investigators question the poor conversion factors of dark green vegetable β-carotene to retinol as low as 26 to 1 [53]. Instead they decry the use of inferior analytical techniques employed in those studies [67]. One study using stable isotope dilution techniques to evaluate whether plant carotenoids can sustain or improve vitamin A nutrition during the fall season in kindergarten children in the Shandong province of China, estimated a conversion factor of 27 to 1 of β-carotene to vitamin A. However, this study did not use intrinsically labeled plant food β-carotene to calculate the conversion factors but rather estimated liver stores [53].

Studies using intrinsically labeled β-carotene from plant foods, the isotopic tag enable the identification of serum carotenoids and derived retinol which comes from the specific food being tested [67]. Recently, several studies using intrinsically labeled β-carotene in rice and biofortified yellow maize had β-carotene to retinol conversion factors of 3.8 to 1 and 3.2 to 1 by weight [68, 69]. One US study using intrinsically labeled kale showed that [^{13}C] labeled kale β-carotene was very bioavailable in humans, and also the [^{13}C] labeled kale β-carotene was converted to vitamin A as shown by the plasma appearance of [^{13}C] labeled retinol [70, 71]. This is important because kale is one of the highest sources of β-carotene among green vegetables with contents ranging from 2-15 mg/100g of fresh weight [72, 73]. *It is therefore important to evaluate the vitamin A value of kale in children who are vulnerable to VAD in Zimbabwe using intrinsically labeled kale.*

Dark green vegetables as vitamin A rich complementary foods in Africa

Dark green leafy vegetables such as kale are staple foods in parts of VAD ravaged Sub Saharan Africa [74]. The *brassica oleracea* vegetables are consumed daily by the poor in Africa because they are easy to grow, are perennial and they grow very fast unlike the traditional, seasonal, indigenous vegetables [75]. A conservative estimation for the area planted with *brassica oleracea* vegetables in Zimbabwe is 2500 ha for commercial crops and 2500 ha for subsistence crops, as most rural household grow leaf cabbage for family use [74]. In Zimbabwe the most important *brassica oleracea* types are called '*rugare', 'viscose', covo,* and '*Chou Moellier'* [74]. However, there is a dearth of information on the provitamin A contents of kale (*brassica oleracea*) varieties grown and consumed in Zimbabwe. While research shows that kale is very rich in provitamin A carotenoids, there are no studies showing the vitamin A value of kale in humans. Provitamin A carotenoid rich kale can be used as a complementary food in Africa providing pre-school children daily doses of vitamin A required for their growth and development. Given the severity of consequences and high prevalence rates of VAD in Sub Saharan Africa, it is important to promote the use of kale as a complementary food for children aged 6-36 months in order to reduce the risk of VAD. Nutrition education can help mothers appreciate the nutritional importance of kale to their children, and various kale processing methods such as pureeing that makes it easy for children to eat kale easily. In Zimbabwe, the major complementary foods given to children aged 6-59 months are starchy foods which often lack essential nutrients like vitamin A [76]. Studies in rural districts of Zimbabwe showed that the diet of infants lacked fruits and vegetables high in provitamin A carotenoids [77, 78]. Kale is widely consumed in Zimbabwe by

adults. However, it is not used in infant feeding as complementary food, probably because of cultural and safety concerns. Currently, it is difficult to promote kale as a complementary food in Zimbabwe because there are few reports showing the provitamin A contents of *brassica oleracea* varieties currently grown and consumed in Zimbabwe. Also, very little is known about the bioavailability of kale provitamin A carotenoids and their conversion to vitamin A in children. Given the importance of kale in the Zimbabwean diet, demonstrating the superiority of kale as a better source of vitamin A will strengthen existing efforts to reduce infant and child morbidity and mortality due to VAD. To maximize the impact of kale as a complementary food, kale can be cooked with peanut butter to increase its energy and nutrient density. Groundnuts which are used to make peanut butter are grown by rural farmers in most parts of Zimbabwe. The combination of a kale cooked in peanut butter sauce will increase the absorption and bioavailability of kale β-carotene because of the proteins, oils and vitamin E [65]. *Determining the vitamin A value of kale will motivate mothers to maintain and improve their vegetable gardens. The increased intake of brassica oleracea vegetables rich in β-carotene will result in improved vitamin A intake by pre-school children. Home grown kale can become an important part of the complementary feeding strategy for infants and toddlers in Zimbabwe.*

Biofortified maize and kale as vitamin A rich complementary foods for children in Africa

One study in Zimbabwe showed that the first food given to infants aged 3 months and above was a gruel or porridge most commonly made from maize flour [79]. Maize is the staple food crop for many countries in east and southern Africa and is consumed as a flour used to make porridge for breakfast and a thicker porridge for lunch and dinner. The thick porridge also known as *ugali, sadza, isitshwala, nsima, nshima, polenta and pap* in east and southern Africa is served almost always with a kale or related green vegetables. In Zimbabwe it was found that people consumed maize and green vegetables together in a meal at least twice a day [80]. Therefore, it makes sense to promote the consumption of provitamin A rich kale and maize especially in the preparation of complementary foods. However, the preferred choice of maize in Africa is white which is devoid of provitamin A carotenoids. Currently efforts to increase the provitamin A content of maize through conventional plant breeding techniques called biofortification are at advanced stages. Human and animal bioefficacy studies showed that biofortified yellow maize is a very good source of vitamin A [68, 81 and 82]. However, effectiveness studies in Zambia with biofortified yellow maize are showing no effect [unpublished data]; therefore more research is required to optimize the vitamin A supply to children who have greater needs for vitamin A using biofortified yellow maize. In order to design good effectiveness studies for biofortified yellow maize, it is important to look at other dietary factors that can optimize the bioconversion of plant food β-carotene to vitamin A

Increasing the provitamin A contents of yellow maize is one way to optimize the vitamin A intake among children in maize based poor countries where VAD is a public health problem. While it is established that in the bioconversion of dietary β-carotene to

vitamin A is affected by the major factors such as food matrix, food processing, and fat in the diet. Other factors not considered important yet are dietary factors that affect the activity of the key enzyme responsible for β-carotene conversion into vitamin A, the β-carotene 15, 15′-monoxygenase (BCMO1). In the intestinal cells, the enzyme determines whether provitamin A carotenoids are converted to vitamin A, apo-carotenoids or delivered through lymph to tissues as intact carotenoids [83]. The central cleavage pathway involves the metabolism of one β-carotene at the central double bond to produce two retinals by β-carotene 15, 15-monoxygenase (BCMO1) [84]. The random cleavage or excentric cleavage of one β-carotene produces retinal and several products such as β-apo-carotenoids [85]. The cleavage of β-carotene by the BCMO1 enzyme has been extensively studied *in vitro* using intestinal homogenates from different animal models [86-90]. The existence of excentric cleavage pathway was initially confirmed with the formation of β-apo-13-carotene and β-apo-14-carotenal after the incubation of β-carotene with rat intestinal mucosal homogenate [91]. Prior, it was previously shown that β-carotene cleavage takes place via both central and excentric cleavage producing retinal, retinoic acid and apo-carotenoids [88]. We showed that the presence of antioxidants such as α-tocopherol promotes the central cleavage of β-carotene to produce retinal exclusively, but in the absence of antioxidants excentric cleavage products were forms [92]. These findings are significant because yellow maize is naturally rich in α-tocopherol, other forms of vitamin E and γ-oryzanol [93]. Studies show wide variation of vitamin E contents of yellow maize [94]. However, the genetic variation of all forms of vitamin E, γ-oryzanol and carotenoids have not yet been fully identified in biofortified yellow maize hybrids. There are no studies to date that report effect of plant food extracts

rich in antioxidants and β-carotene on BCMO1 activity to producing vitamin A. *Therefore, it is important to investigate the effects of vitamin E compounds and antioxidants found in biofortified yellow maize and kale on the cleavage of the β-carotene. If these antioxidants promote central cleavage of β-carotene to produce vitamin A, recommendations can then be suggested to biofortify crops with antioxidants or promote consumption of provitamin A rich plant food together with antioxidant rich food for optimal bioconversion of plant food β-carotene to vitamin A.*

References

1. Soprano, Dianne Robert, and Kenneth J. Soprano. Retinoids as teratogens. *Annu Rev Nutr* 1995;15:111-132
2. Blomhoff, Rune, Michael H. Green, Trond Berg, and Kaare R. Norum. Transport and storage of vitamin A. *Science* 1990. 250 (4979)
3. Heyman, Richard A., David J. Mangelsdorf, Jacqueline A. Dyck, Robert B. Stein, Gregor Eichele, Ronald M. Evans, and Christina Thaller. 9-cis retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell* 1992, 68 (2): 397-406.
4. Bouis, Howarth E., Christine Hotz, Bonnie McClafferty, J. V. Meenakshi, and Wolfgang H. Pfeiffer. Biofortification: a new tool to reduce micronutrient malnutrition. *Nutr Bull* 2011, (32) 1: 31S-40S
5. Krinsky, Norman I. The biological properties of carotenoids. *Pure Appl. Chem* 1994, 66 (5): 1003-1010.
6. Haskell, Marjorie J. The challenge to reach nutritional adequacy for vitamin A: β-carotene bioavailability and conversion-evidence in humans. *Am J Clin Nutr* 2012, 96 (5): 1193S-1203S.
7. Lietz, Georg, Jennifer Lange, and Gerald Rimbach. Molecular and dietary regulation of β, β-carotene 15, 15′-monooxygenase 1 (BCMO1). *Arch Biochem Biophys* 2010. 502 (1): 8-16.
8. Allen, Lindsay H., Bruno De Benoist, Omar Dary, and Richard Hurrell. Guidelines on food fortification with micronutrients/edited by Lindsay Allen et al.

World Health Organization (WHO) and Food and Agriculture Organization (FAO) of the United Nations. 2006.

9. West, Keith P. Vitamin A deficiency disorders in children and women. *Food Nutr Bull* 2003 (24); 2: 78-90.
10. Bridges, C. D. B., R. A. Alvarez, S-L. Fong, F. Gonzalez-Fernandez, D. M. K. Lam, and G. I. Liou. Visual cycle in the mammalian eye: retinoid-binding proteins and the distribution of 11-cis retinoids. *J Vis* 1984 (24); 11: 1581-1594.
11. Ma, J-X., Y. Chen, Y. Takahashi, and G. Moiseyev. RPE65 is the isomerohydrolase in the retinoid visual cycle. *Invest Ophthalmol Vis Sci* 2005 (46); 5: 1057.
12. Rushton, W. A. H. Rhodopsin measurement and dark-adaptation in a subject deficient in cone vision. *J Physiol* 1961, 156 (1); 193-205.
13. Sommer, Alfred. Xerophthalmia, keratomalacia and nutritional blindness. *J Physiol* 1990, 14 (3): 195-199.
14. WHO. Global prevalence of vitamin A deficiency in populations at risk 1995–2005. WHO Global Database on Vitamin A Deficiency. Geneva, World Health Organization, 2009. World Health Organization (WHO), Geneva, Switzerland. http://whqlibdoc.who.int/publications/2009/9789241598019_eng.pdf (accessed 01/16/2014).
15. Christian P et al. Night blindness of pregnancy in rural Nepal- nutritional and health risks. *Int J Epidemiol* 1998, 27:231-237.

16. Lampen, A., Meyer, S., Arnhold, T., & Nau, H. Metabolism of vitamin A and its active metabolite all-trans-retinoic acid in small intestinal enterocytes. *Pharmacol Rev* 2000, *295* (3), 979-985.
17. Semba, R. D. The role of vitamin A and related retinoids in immune function. *Nutr Rev* 1998, 56 (1), S38-S48.
18. Stephensen, Charles B. Vitamin A, infection, and immune function. *Annu Rev Nutr* 2001, 21 (1): 167-192.
19. Sommer, Alfred. Vitamin A: its effect on childhood sight and life. *Nutr Rev* 1994, 52 (2): S60-S66.
20. Brown, Kenneth H., Abdul Gaffar, and Sharif M. Alamgir. Xerophthalmia, protein-calorie malnutrition, and infections in children. *J Pediatr* 1979 (4): 651-656.
21. Semba, Richard D. The role of vitamin A and related retinoids in immune function. *Nutr Rev* 1998, 56 (1): S38-S48.
22. Sommer, Alfred, Edi Djunaedi, A. A. Loeden, Ignatius Tarwotjo, KeithP West, Robert Tilden, and Lisa Mele. Impact of vitamin A supplementation on childhood mortality: a randomised controlled community trial. *Lancet* 1986, 327 (8491):1169-1173.
23. Rojanapo, Wannee, Adrian J. Lamb, and James A. Olson. The prevalence, metabolism and migration of goblet cells in rat intestine following the induction of rapid, synchronous vitamin A deficiency. *J Nutr* 1980, 110 (1): 178-188.
24. Mangelsdorf, David J., Uwe Borgmeyer, Richard A. Heyman, J. Yang Zhou, Estelita S. Ong, Anthony E. Oro, Akira Kakizuka, and Ronald M. Evans.

Characterization of three RXR genes that mediate the action of 9-cis retinoic acid. *Genes Dev* 1992, 6 (3): 329-344.

25. Kennedy, Karen, Tammy Porter, Virja Mehta, Scott Ryan, Feodor Price, Vian Peshdary, Christina Karamboulas et al. Retinoic acid enhances skeletal muscle progenitor formation and bypasses inhibition by bone morphogenetic protein 4 but not dominant negative β-catenin. *BMC Biol* 2009, 7 (1): 67-75.
26. McLaren, Donald Stewart, and Martin Frigg. *Sight and life manual on vitamin A deficiency disorders (VADD).* Switzerland: Task Force Sight and Life, 2001.
27. Niederreither, Karen, Vemparala Subbarayan, Pascal Dollé, and Pierre Chambon. Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. *Nat Genet* 1999, 21 (4): 444-448.
28. Sommer A. Nutritional blindness: xerophthalmia and keratomalacia. New York: Oxford University Press, 1982.
29. Fuchs GJ, Ausayakhun S, Ruckphaopunt S, Tansuhaj A, Suskind RM. Relationship between vitamin A deficiency, malnutrition, and conjunctival impression cytology. *Am J Clin Nutr* 1994; 60:293-8.
30. Arwotjo I, Katz J, West KP, Tielsch JM, Sommer A. Xerophthalmia and growth in preschool Indonesian children. *Am J Clin Nutr* 1992; 55:1142-6.
31. McCollum EV, Davis M. The necessity of certain lipids in the diet during growth. *J Biol Chem* 1913; 15:167-75.
32. Wolbach SB, Hegsted DM. Vitamin A deficiency and excess in relation to skeletal growth. *J Bone Joint Surg* 1947; 29:171-92.

33. Lewis JM, Bodansky O, Falk KG, McGuire G. Vitamin A requirements in the rat, the relation of vitamin A intake to growth and to concentration of vitamin A in the blood plasma, liver and retina. *J Nutr* 1942; 23:351-63.
34. Gil A, Briggs GM, Typpo J, Mackinney G. Vitamin A requirement of the guinea pig. *J Nutr* 1968; 96:359-62.
35. Hadi, Hamam, Rebecca J. Stoltzfus, Michael J. Dibley, Lawrence H. Moulton, Keith P. West, Chris L. Kjolhede, and Tonny Sadjimin. Vitamin A supplementation selectively improves the linear growth of Indonesian preschool children: results from a randomized controlled trial. *Am J Clin Nutr* 2000; 71 (2): 507-513.
36. West, Keith P., Steven C. LeClerq, Sharada R. Shrestha, Lee S-F. Wu, Elizabeth K. Pradhan, Subarna K. Khatry, Joanne Katz, Ramesh Adhikari, and Alfred Sommer. Effects of vitamin A on growth of vitamin A-deficient children: field studies in Nepal. *J Nutr* 1997; 127 (10):1957-1965.
37. Büyükgebiz, B., I. Özalp, and O. Oran. Investigation of serum vitamin A levels of children who had a history of recurrent diarrhoea and acute respiratory infections in Ankara. *J Trop Pediatr* 1990, 36 (5): 251-255.
38. Villamor, Eduardo, and Wafaie W. Fawzi. Effects of vitamin A supplementation on immune responses and correlation with clinical outcomes. *Clin Microbiol Rev* 2005, 18 (3): 446-464.
39. Semba, R. D. Vitamin A and immunity to viral, bacterial and protozoan infections. *Proc Nutr Soc* 1999, 58 (3): 719.

40. United Nations Children's Fund and the (UNICEF). *Progress for children*. Vol. 1. UNICEF, 2004.

41. Goodman, T., Dalmiya, N., de Benoist, B. & Schultink, W. Polio as a platform: using national immunization days to deliver vitamin A supplements. *Bull. WHO* 2000, 78:305-314

42. World Bank. Vitamin A supplementation coverage rate (% of children aged 6-59 months) http://data.worldbank.org/indicator/SN.ITK.VITA.ZS

43. Vitamin A Supplementation: A decade of progress. © The United Nations Children's Fund (UNICEF), 2007. ISBN: 978-92-806-4150-9

44. Dary, Omar, and Jose O. Mora. Food fortification to reduce vitamin A deficiency: International Vitamin A Consultative Group recommendations. *J. Nutr* 2002, 132 (9): 2927S-2933S.

45. Klemm, Rolf DW, Jr West, P. Keith, Amanda C. Palmer, Quentin Johnson, Philip Randall, Peter Ranum, and Christine Northrop-Clewes. Vitamin A fortification of wheat flour: considerations and current recommendations *Food Nutr Bull* 2010, 31, Supplement 1: 47S-61S.

46. Solon, Florentino S., Rolf DW Klemm, Liza Sanchez, Ian Darnton-Hill, Neal E. Craft, Parul Christian, and Keith P. West. Efficacy of a vitamin A–fortified wheat-flour bun on the vitamin A status of Filipino schoolchildren. *Am J Clin Nutr* 2000, 72 (3): 738-744.

47. Chavez, J. F. Enrichment of precooked corn flour and wheat flour in Venezuela: a successful experience. *Food Fortification to End Micronutrient Malnutrition.*

State of the Art: 62-65. The Micronutrient Initiative, International Development Research Centre Ottawa, Canada. 1997.

48. Jarvis, M. Faire Tache d'Huile: cooking oil fortification in West Africa.*Case Study prepared for Business Innovation to Combat Malnutrition. World Bank Institute. Washington, DC: World Bank*. 2009.
49. Nestel, P., Bouis, H. E., Meenakshi, J. V., & Pfeiffer, W. Biofortification of staple food crops. *J Nutr* 2006, 136 (4); 1064-1067.
50. Drammeh BS, Marquis GS, Funkhouser E, Bates C, Eto I, Stephensen CB. A randomized, 4-month mango and fat supplementation trial improved vitamin A status among young Gambian children. *J Nutr* 2002;132:3693-9
51. Jalal F, Nesheim MC, Agus Z, Sanjur D, Habicht JP. Serum retinol concentrations in children are affected by food sources of beta-carotene, fat intake, and anthelmintic drug treatment. *Am J Clin Nutr* 1998; 68:623-9.
52. Takyi EE. Children's consumption of dark green, leafy vegetables with added fat enhances serum retinol. *J Nutr* 1999;129 (8):1549-5
53. Tang G, Gu X, Hu S, Xu Q, Qin J, Dolnikowsk GG, Fjeld CR, Gao X, Russell RM, et al. Green and yellow vegetables can maintain stores of vitamin A in Chinese children. *Am J Clin Nutr* 1999;70:1069-76
54. Takyi, Etor EK. Children′ s Consumption of Dark Green, Leafy Vegetables with Added Fat Enhances Serum Retinol. *J. Nutr* 1999, 129 (8): 1549-1554.
55. Berti PR, Krasevec J, FitzGerald S. A review of the effectiveness of agriculture interventions in improving nutrition outcomes. *Public Health Nutr* 2004; 7:599-609.

56. Bloem, M. W., N. Huq, J. Gorstein, S. Burger, T. Kahn, N. Islam, S. Baker, and F. Davidson. Production of fruits and vegetables at the homestead is an important source of vitamin A among women in rural Bangladesh. *E J Clin Nutr* 1996, 50: S62-7.

57. Faber, Mieke, Michael AS Phungula, Sonja L. Venter, Muhammad A. Dhansay, and AJ Spinnler Benadé. Home gardens focusing on the production of yellow and dark-green leafy vegetables increase the serum retinol concentrations of 2-5-y old children in South Africa. *Am J Clin Nutr* 2002, 76 (5): 1048-1054.

58. Low, Jan W., Mary Arimond, Nadia Osman, Benedito Cunguara, Filipe Zano, and David Tschirley. A food-based approach introducing orange-fleshed sweet potatoes increased vitamin A intake and serum retinol concentrations in young children in rural Mozambique. *J. Nutr* 2007, 137 (5): 1320-1327.

59. Hotz, Christine, Cornelia Loechl, Abdelrahman Lubowa, James K. Tumwine, Grace Ndeezi, Agnes Nandutu Masawi, Rhona Baingana et al. Introduction of β-Carotene–Rich Orange Sweet Potato in Rural Uganda Resulted in Increased Vitamin A Intakes among Children and Women and Improved Vitamin A Status among Children. *J. Nutr* 2012, 142 (10): 1871-1880.

60. van Jaarsveld, Paul J., Mieke Faber, Sherry A. Tanumihardjo, Penelope Nestel, Carl J. Lombard, and Ambrose J. Spinnler Benadé. β-Carotene-rich orange-fleshed sweet potato improves the vitamin A status of primary school children assessed with the modified-relative-dose-response test. *Am J Clin Nutr* 2005, 81 (5): 1080-1087.

61. Food and Nutrition Board, Institute of Medicine. Dietary Reference Intakes for vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc. A report of the Panel on Micronutrients, Subcommittees on Upper Reference Levels of Nutrients and of Interpretation and Use of Dietary Reference Intakes, and the Standing Committee on the Scientific Evaluation of Dietary Reference Intakes. Washington, DC: National Academy Press, 2001.
62. West, C. E., & Castenmiller, J. J. Quantification of the" SLAMENGHI" factors for carotenoid bioavailability and bioconversion. *Int J Vitam Nutr Res* 1997 68 (6), 371-377.
63. De Pee, S., West, C. E., Permaesih, D., Martuti, S., & Hautvast, J. G. Orange fruit is more effective than are dark-green, leafy vegetables in increasing serum concentrations of retinol and beta-carotene in schoolchildren in Indonesia. *Am J Clin Nutr* 1998, 68 (5), 1058-1067.
64. de Pee S, West CE, Muhilal S, Karyadi D, Hautvast JGAJ. Lack of improvement in vitamin A status with increased consumption of dark-green leafy vegetables. *Lancet* 1995; 346:75-81.
65. Yeum KJ, Russell RM. Carotenoids bioavailability and bioconversion. *Annu Rev Nutr* 2002; 22:483-504
66. Tang, G. Techniques for measuring vitamin A activity from β-carotene. *Am J Clin Nutr* 2012, 96 (5), 1185S-1188S.
67. Tang, G. Bioconversion of dietary provitamin A carotenoids to vitamin A in humans. *Am J Clin Nutr* 2010, 91 (5), 1468S-1473S.

68. Muzhingi T, Gadaga HT, Siwela A, Grusak MA, Russell RM, Tang G. Yellow maize with high β-carotene is an effective source of vitamin A in healthy Zimbabwean men. *Am J Clin Nutr* 2011; 94:510-9

69. Tang, Guangwen, Yuming Hu, Shi-an Yin, Yin Wang, Gerard E. Dallal, Michael A. Grusak, and Robert M. Russell. β-Carotene in Golden Rice is as good as β-carotene in oil at providing vitamin A to children. *Am J Clin Nutr* 2012, 96 (3): 658-664.

70. Novotny, Janet A., Anne C. Kurilich, Steven J. Britz, and Beverly A. Clevidence. Plasma appearance of labeled β-carotene, lutein, and retinol in humans after consumption of isotopically labeled kale. *J Lipid Res* 2005, 46 (9): 1896-1903.

71. Kurilich, Anne C., Steven J. Britz, Beverly A. Clevidence, and Janet A. Novotny. Isotopic labeling and LC-APCI-MS quantification for investigating absorption of carotenoids and phylloquinone from kale (Brassica oleracea). *J Agric Food Chem* 2003, 51 (17): 4877-4883.

72. Reif, C., Arrigoni, E., Berger, F., Baumgartner, D., & Nyström, L. Lutein and β-carotene content of green leafy< i> Brassica</i> species grown under different conditions. *LWT-Food Science and Technology*, 2013, 53 (1), 378-381.

73. Kurilich, Anne C., Grace J. Tsau, Allan Brown, Lenora Howard, Barbara P. Klein, Elizabeth H. Jeffery, Mosbah Kushad, Mathew A. Wallig, and John A. Juvik. Carotene, tocopherol, and ascorbate contents in subspecies of Brassica oleracea. *J Agric Food Chem* 1999, 47 (4): 1576-1581.

74. Grubben, G. J. H., Denton, O. A., Messiaen, C. M., Schippers, R. R., Lemmens, R. H. M. J., Oyen, L. P. A., & Plant Resources of Tropical Africa. *Vegetables*. Wageningen: Backhuys Publishers. 2004.

75. Nekesa, P., and B. Meso. Traditional African vegetables in Kenya: production, marketing and utilization. In *Guarino eds, Traditional African Vegetables: proceedings of the IPGRI International Workshop on Genetic Resources of Traditional Vegetables in Africa: Conservation and use held on*, pp. 29-31. 1995.

76. Gibson RS, Ferguson EL, Lehrfeld J. Complementary foods for infant feeding in developing countries: their nutrient adequacy and improvement. *Eur J Clin Nutr* 1998; 52:764-70.

77. Cosminsky S, Mhloyi M, Ewbank D. Child feeding practices in a rural area of Zimbabwe. *Soc Sci Med* 1993; 36:937-47.

78. Paul, K. H., Muti, M., Chasekwa, B., Mbuya, M. N., Madzima, R. C., Humphrey, J. H., & Stoltzfus, R. J. Complementary feeding messages that target cultural barriers enhance both the use of lipid-based nutrient supplements and underlying feeding practices to improve infant diets in rural Zimbabwe. *Matern Child Health J*. 2012, 8 (2), 225-238.

79. Paul, K. H., Muti, M., Khalfan, S. S., Humphrey, J. H., Caffarella, R., & Stoltzfus, R. J. Beyond food insecurity: how context can improve complementary feeding interventions. *Food Nutr Bull* 2011, 32 (3), 244-253.

80. Muzhingi T, Langyintuo AS, Malaba LC, Banziger M. Consumer acceptability of yellow maize products in Zimbabwe. *Food Policy* 2008; 33:352-61.

81. Li S, Nugroho N, Rocheford T, White S.Vitamin A equivalence of β-carotene in β-carotene-biofortified maize porridge consumed by women. *Am J Clin Nutr* 2010; 92:1105-12

82. Howe JA, Tanumihardjo SA. Carotenoid-biofortified maize maintains adequate vitamin A status in Mongolian gerbils. *J Nutr* 2006; 136:2562-7.

83. Harrison, E. H. Mechanisms involved in the intestinal absorption of dietary vitamin A and provitamin A carotenoids. *Biochim Biophys Acta* 2012, 1821 (1), 70-77.

84. Lietz, G., Oxley, A., Boesch-Saadatmandi, C., & Kobayashi, D. (2012). Importance of β, β-carotene 15, 15′-monooxygenase 1 (BCMO1) and β, β-carotene 9′, 10′-dioxygenase 2 (BCDO2) in nutrition and health. *Mol Nutr Food Res*, 2012, 56 (2), 241-250.

85. Kim, Y. S., Yeom, S. J., & Oh, D. K. Production of β-apo-10′-carotenal from β-carotene by human β-carotene-9′, 10′-oxygenase expressed in E. coli. *Biotechnol Lett*, 2011, 33 (6), 1195-1200.

86. Mein, J. R., Dolnikowski, G. G., Ernst, H., Russell, R. M., & Wang, X. D. (2011). Enzymatic formation of apo-carotenoids from the xanthophyll carotenoids lutein, zeaxanthin and β-cryptoxanthin by ferret carotene-9′, 10′-monooxygenase. *Arch Biochem Biophys* 2011, 506 (1), 109-121.

87. Sharma, R. V., Mathur, S. N., Dmitrovskii, A. A., Das, R. C., & Ganguly, J. Studies on the metabolism of β-carotene and apo-β-carotenoids in rats and chickens. *Biochim Biophys Acta* 486 1977, (1), 183-194.

88. Wang, X. D., Tang, G. W., Fox, J. G., Krinsky, N. I., & Russell, R. M. Enzymatic conversion of β-carotene into β-apo-carotenals and retinoids by human, monkey, ferret, and rat tissues. *Arch Biochem Biophys* 1991, 285 (1), 8-16.

89. Napoli, J. L., & Race, K. R. Biogenesis of retinoic acid from beta-carotene. Differences between the metabolism of beta-carotene and retinal. *J Biol Chem*, 1988, 263 (33), 17372-17377.

90. Hansen, S., & Maret, W. Retinal is not formed in vitro by enzymic central cleavage of beta.-carotene. *Biochem* 1988, 27 (1), 200-206.

91. Tang, G., Wang, X. D., Russell, R. M., & Krinsky, N. I. Characterization of beta.-apo-13-carotenone and beta.-apo-14'-carotenal as enzymic products of the excentric cleavage of beta-carotene. *Biochem* 1991, 30 (41), 9829-9834.

92. Yeum, K. J., Smith, A. F., Krinsky, N. I., & Russell, R. M. The effect of α-tocopherol on the oxidative cleavage of β-carotene. *Free Radic Biol Med* 2000**,** 29 (2), 105-114.

93. Tadmor, Y., Larkov, O., Meir, A., Minkoff, M., Lastochkin, E., Edelstein, M., & Lewinsohn, E. Reversed-phase high performance liquid chromatographic determination of vitamin E components in maize kernels. *Phytochem Anal* 2000, 11 (6), 370-374.

CHAPTER 3

Genetic variation of carotenoids, vitamin E and phenolic compounds in biofortified maize

Genetic variation of carotenoids, vitamin E and phenolic compounds in biofortified maize

Tawanda Muzhingi [1, 2], Natalia Palacios-Rojas[3], Alejandra Miranda[3], Octavio Custodio[3], Kyung-Jin Yeum [2, 4] and Guangwen Tang [1, 2]

[1]Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, 711 Washington Street, Boston, MA, 02111.

[2]Gerald J. and Dorothy R. Friedman School of Nutrition Science and Policy, Tufts University, 150 Harrison Avenue, Boston, MA, 02111.

[3]Global Maize Program, International Maize and Wheat Improvement Center (CIMMYT), CIMMYT Research Station, Km. 45 Carretera Mexico-Veracruz, El Batan, Texcoco, 56130, Mexico 00174.

[4]Division of Food Bioscience, College of Biomedical and Health Sciences, Konkuk University, Glocal Campus, Chungju-Si, Chungcheongbuk-do, 380-701, South Korea

Corresponding author

Natalia Palacios Rojas

[3]Global Maize Program, International Maize and Wheat Improvement Center (CIMMYT), CIMMYT Research Station, Km. 45 Carretera Mexico-Veracruz, El Batan, Texcoco, 56130, Mexico 00174.

Email: n.palacios@cgiar.org

Abstract

Background. Biofortified maize can be a vehicle for delivering micronutrients such as carotenoids, vitamin E and phenolic compounds in provitamin A carotenoids enriched maize for prevention of micronutrient deficiencies and maintenance of optimal health. **Results**. The ranges of major carotenoids in provitamin A carotenoids enriched maize were zeaxanthin (1.2-13.2 µ/g), β-cryptoxanthin (1.3-8.8 µg/g) and β-carotene (1.3-8.0 µg/g dry weight). The ranges of vitamin E compounds identified in provitamin A carotenoids enriched maize were α-tocopherol (3.4-34.3 µg/g), γ-tocopherol (5.9-54.4 µg/g), α-tocotrienol (2.6-19.5 µg/g), and γ-tocotrienol (45.4 µg/g dry weight). The ranges of phenolic compounds were γ-oryzanol (0.0-0.83 mg/g), ferulic acid (0.4-3.6 mg/g) and p-coumaric acid (0.1-0.45 mg/g dry weight). There was significant correlation between α-tocopherol and cis isomers of β-carotene ($p<0.01$). Tocotrienols were correlated with α-tocopherol and γ-oryzanol ($p<0.01$). Genotype was significant in determining the variation in β-cryptoxanthin, β-carotene contents, α-tocopherol and γ-tocopherol contents ($p<0.01$). Genotype by Environment (G x E) interaction was observed in γ-tocopherol contents ($p<0.01$).

Conclusion. Provitamin A carotenoids enriched maize is a good source of provitamin A carotenoids that can increase vitamin A intake in developing countries, while its antioxidants can contribute to the maintenance of optimal health.

Keywords: biofortification, maize, carotenoids, tocopherols, tocotrienols, phenolics, antioxidants, vitamin A

Introduction

Maize in Europe, North America and some parts of South America and Asia, is a cash crop grown for animal feed, industrial purposes (source of sugar, oil, starch and ethanol) and to a lesser extend use for human nutrition. In Africa, Central America and some parts of Asia, maize is a staple food of hundreds of millions of people [1].

The diversity of maize has been the base for the breeding programs that have generated much of the higher-yielding maize used worldwide [2]. Historically, this effort has primarily focused on increasing stability and grain yield potential under abiotic and biotic stresses [3]. In the last decade many efforts have been placed in evaluating and using the diversity of maize for animal feed and human nutrition [4-6]. Biofortification is focused on increasing the micronutrient content of staple crops [7]. Development of provitamin A enriched maize is a strategy to alleviate vitamin A deficiency (VAD), because such maize contains higher quantities of β-carotene, α-carotene and β-cryptoxanthin carotenoids. There is already provitamin A carotenoids enriched germplasm available, developed by conventional breeding [8-9]. Bioefficacy studies in animal and human models have shown that such biofortified maize is a good source of vitamin A [8, 10-11]. Successful biofortified varieties offer improved yield stability and potential in addition to nutritional quality, thereby enhancing household income, food security, and overall livelihoods. The higher micronutrient content of biofortified crops needs to be retained after storage and processing the food [12]. Carotenoid losses occur during storage and food processing and the mechanisms of catabolism and/or degradation of these compounds is not well understood. Antioxidant environment in the maize kernels contributes to the stability of

polyunsaturated fatty acids [13] and such scenarios may also contribute to the retention of carotenoids [14].

Maize kernels have a large diversity of antioxidant compounds found in different grain parts and cell compartments [15]. Such compounds play an important role in the metabolism of the grains as well as give to the grain many health benefits. In addition to the provitamin A carotenoids biofortified yellow maize also contains lutein and zeaxanthin both of which serve as antioxidants in the grain endosperm [16]. Colored and white maize also contains tocopherols and tocotrienols which are also antioxidants. Vitamin E is the common name that describes eight naturally occurring compounds possessing α-tocopherol activity. The eight compounds are lipid-soluble antioxidants with two distinct groups, tocopherols and tocotrienols [17]. They can contribute to the protection of other compounds within the grain or to their bioavailability in the human body. Research shows that vitamin E promotes the centric cleavage of β-carotene to vitamin A by the β-carotene cleavage monooxygenase 1 (BCMO1) enzyme [18].

Other antioxidants found in whole maize kernels are the phenolic acids, which are present as soluble free and conjugated or insoluble bound forms [19]. The bound forms are the major phenolic acid forms in maize kernels, up to 91% of the total ferulic and p-coumaric acid is in the bound form. Both ferulic and p-coumaric acid can act as antioxidants directly scavenging reactive oxygen species (ROS). One study showed that p-coumaric minimizes the oxidation of low-density lipoprotein (LDL) [20]. Ferulic acid was shown to improve cardiovascular and kidney functions through endothelium-dependent relaxation in isolated thoracic aortic rings and antioxidant status by increasing superoxide dismutase (SOD) and catalase (CAT) in the heart and kidneys [21]. Total γ-oryzanol, a

mixture of ferulic acid esters with phytosterols possesses a variety of biologic properties such as cholesterol lowering, anti-inflammatory, anticancer, anti-diabetic, and antioxidant activities [22-25]. Most literature on γ-oryzanol is on rice, wheat and barley, with very little on maize. Given the body of evidence on the importance of γ-oryzanol, ferulic acid and *p-coumaric* acid on human health, it is important to determine their variation in provitamin A carotenoids enriched maize and compare values with reported data on regular maize. The objectives of this study were: 1) to assess the diversity of carotenoids, vitamin E, γ-oryzanol and phenolic compounds (ferulic acid and *p-coumaric* acid) as well as the antioxidant activity in provitamin A carotenoids enriched maize; 2) to investigate any association among the antioxidants evaluated in the maize kernels.

Materials and Methods

Plant materials

Thirty-seven provitamin A carotenoids enriched experimental maize hybrids from the International Center for maize and wheat improvement (CIMMYT) were grown at two locations in Mexico during the mean season in 2011 in an alpha-lattice design with two replications. One location at Tlaltizapan, Morelos (18°41´ N, 99°07´ W; 945 m above the sea level; average annual temperature 23.5°C; average annual precipitation 840 mm) and the other one at Agua Fria, Puebla (AF) (20°32´N, 97°28´ W; 110 m above sea level (masl); average annual temperature 22°C; average annual precipitation 1200 mm). F1 ears from self-pollinated plants were harvested, dried and grain was stored at -20 ºC

before using for laboratory analysis. Hybrids were selected based on the carotenoid content and profile.

Reagents and chemicals

All reagents and chemical used were of HPLC and analytical grade. All *trans*-β-carotene, 9 *cis*-β-carotene, 13 cis-β-carotene, β-cryptoxanthin, lutein, zeaxanthin were purchased from CaroteneNature (Lusingen, Switzerland). Internal standard of β-apo-8'-carotenal was purchased from Fluka (Sigma-Aldrich, St Louis, MO). Standards of α-tocopherol, γ-tocopherol, *p-coumaric acid* acid, trans ferulic acid, and gallic acid were obtained from Merck. Methyl-*tert*-butyl ether (MTBE), tetrahydrofuran (THF), methanol, acetonitrile, ethy alcohol, sodium hydroxide, was also obtained from Merk KGaH (Darmstadt, Germany). Sodium chloride, sodium phosphate mono-basic, sodium phosphate dibasic, sodium carbonate, Folin-Ciocalteu phenol reagent, potassium phosphate dibasic, Trolox, hydrocholiric acid, and sodium salt fluorescein were obtained from Sigma Co. (St Louis, MO, USA). Azo-compounds 2, 2′-azobis (2-amidinopropane) dihydrochloride (AAPH), 2, 2′-azobis (4-methoxy-2, 4-dimethyl-valeronitrile) (MeO-AMVN) and γ-oryzanol standards were purchased from Wako (Richmond, VA, USA). 4, 4-Difluoro-5-(4-phenyl-1, 3-butadienyl)-4-bora-3*a*, 4*a*- diaza-*s*-indacene-3-undecanoic acid (BODIPY 581/ 591) was obtained from Molecular Probes (Eugene, OR, USA). A-tocotrienol and γ-tocotrienol standards were purchased from Cayman Chemical Company (Ann Arbor, Michigan, USA).

Determination of carotenoids and vitamin E by UPLC

Carotenoid and vitamin E extraction was done as previously described [26]. Briefly, provitamin A carotenoids enriched maize kernel were ground into a fine powder, and samples of 600 mg underwent a 5 min 6 mL ethanol (with 0.1% butylated hydroxytoluene) precipitation in an 85°C water bath before being subjected to a 10 min saponification with 120 µL of 80 % w/v KOH in water. After saponification, samples were immediately placed in ice, and 3 mL cold deionized water was added. Two hundred micro-liters of the internal standard (β-apo-8'-carotenal) were added and vortexed. Carotenoids were extracted 3 times with 3 mL of hexanes by centrifugation at 800 x *g* and the hexane fraction was extracted. The combined extracted hexane layers were dried under nitrogen and reconstituted in 500 µL 50:50 (v: v) methanol: dichloroethane. All carotenoid extraction procedures and analysis was conducted under yellow light. Two microliter of the sample was injected in to the Acquity UPLC Water equipment. Separation was performed using an Acquity UPLC BEH C8, 1.7 µm 2.1 x 100 mm column and an Acquity col. in-line filter. For better peak resolution of vitamin E, samples were separated using an Acquity UPLC BEH C18, 1.7 µm 2.1 x 100 mm and the same inline filter.

Determination of bound ferulic and *p-coumaric* acid

The determination of ferulic acid and p-coumaric acid was done as previously described with minor modifications [27]. Briefly, extraction of bound phenolics was started by adding 10 mL of 2 M sodium hydroxide to 0.1 mg of the pellet previously extracted with 80 % ethanol and mixing thoroughly. The sample was incubated in a shaking water

bath at 50˚C for 45 min. After hydrolysis the contents were left to equilibrate to room temperature. The hydrolyzed phase was transferred to a 50 mL conical flask. Then 10 ml of 2 M HCL solution was then added to adjust pH of 2.0 ± 0.5. A 6 mL hexane extraction was conducted by centrifugation at *800 x g*, and the hexane fraction was discarded. This was followed by further three 3 mL more extraction of the residue with ethyl acetate (1:1) by vortexing and centrifuging at *800 x g*. The supernatants were collected and dried under nitrogen and re-constituted with 5 mL of 50% methanol. A standard of gallic acid was used as an internal standard. A ten microliter volume was injected into an HPLC system with a Zorbax SB-Aq column (4.6 x 150mm 3.5µ) using gradient elution profile as previously published [27]. The HPLC consisted of a Waters 2695 pump, Waters 2996 Photo Diode Array (PDA) detector and Water Empower-Pro software. The UV-Vis detector was set at 280 nm wavelength. The detection limit for both ferulic acid and *p-coumaric* acid was 0.01 µg/mL.

Determination of total gamma-oryzanol

Extraction of total γ-oryzanol was conducted using a previously described method [28]. Briefly, 600 mg of the maize powder were weighed into 50 mL glass test-tubes. Ten mL of methanol was added and the contents were homogenized for 30s using a Polytron 1600E homogenizer. The test-tubes were incubated in a water-bath at 70˚C for 2 hours. After incubation, the flask was vortexed and centrifuged at *800 x g* for 10 min. The methanol layer was extracted into a 50 mL volumetric flask. Ten mL of THF were added to the maize residue and vortexed for 1 min. The flasks were centrifuged again at *800 x g* for 10 min. After that the THF layer was extracted and combined with the first methanol

extract. The THF extraction was repeated 3 more times. The volume in the volumetric flask was topped to the 50 mL mark with THF. The contents of the 50 mL volumetric flask were shaken and 1 mL was extracted into a 3 mL test tube, and dried under a gentle stream of nitrogen. The dried residue was reconstituted in 1 mL of ethanol, vortexed and sonicated for 30s. Twenty microliters were injected into the HPLC system. The HPLC system consisted of a C30 carotenoid column (3mm, 150mm x 3.0 µm, YMC, Wilmington, NC), Waters 2695 LC pump and autosampler (Milford, MA), Waters 2996 programmable Photodiode Array Detector (PDA), and Empower2 Pro software. The HPLC gradient elution profile was previously published [29]. The PDA detector was set at 325 nm wavelength for γ-oryzanol.

Determination of total antioxidant performance (TAP)

A wide range of methods has been described in the literature for assessing the antioxidant activity of fruits and vegetables and most of these methods focus on determining only the hydrophilic antioxidant components such as phenols and vitamin C [30-32]. Oxygen radical absorbance capacity (ORAC), ferric reducing antioxidant power (FRAP) and Trolox equivalent antioxidant capacity (TEAC) are the most popular assay for determining the antioxidant capacity of fruits and vegetables. These assays are limited because they rely on the hydrophilic antioxidants, while the contributions of lipophilic components such as carotenoids and tocopherols, which are important plant antioxidants, are not assayed. Yellow maize is rich not only in water-soluble but also fat-soluble antioxidants including tocopherols and carotenoids. This study was focused on determining the antioxidant activity of the lipophilic and hydrophilic components in the

provitamin A carotenoids enriched maize, therefore we used the Total Antioxidant Performance (TAP) assay. The determination of TAP was conducted using previously published methods with minor modifications [33, 34]. The methanol-THF extracts used for γ-oryzanol HPLC analysis were also used for the total antioxidant performance (TAP) assay. Sample (2 mL) was dried under a gentle nitrogen stream, rolling the vial to deposit a thin film on the walls. After drying, 2 mL of Tween 40/4.5% acetone was added to the sample vials and dried again to complete dryness under a gentle stream of nitrogen gas. Control vials only contained 2 mL of Tween 40/4.5% acetone. The vials were then maintained under a nitrogen stream for the dried tubes and vortexed and dried again under a gentle nitrogen stream. The resulting film was then rehydrated with 1 mL phosphate-buffered saline (PBS) buffer (40 mmol L^{-1}, pH 7.4) and sonicated in a water/ice-bath sonicator. Aliquots of 300 µL PBS and 100 µL BODIPY solution were then added to 100 µL Tween 40/extract suspensions, vortexed for 20s at low speed and then incubated under aerobic conditions for 10 min at 37 °C. Then 485 µL of PBS was added to the test-tubes to adjust the final volume to 1mL. MeO-AMVN was added to the sample at a final concentration of 2 mmol L. Aliquots of 200 µL were transferred to a 96-microwell plate, and the lipid oxidation kinetics was monitored by measuring the green fluorescence (λ_{ex} = 500, λ_{em} = 520 nm) of the oxidation product of BODIPY. The results were expressed as percent (%) TAP values, representing the percentage of inhibition of BODIPY oxidation in food samples with respect to that occurring in a control sample (Tween 40/4.5% acetone).

Equation 1:

TAP= [{Area Under the Curve (AUC) $_{control}$-AUC_{sample}}/$AUC_{control}$] ×100,

Where $AUC_{control}$ and AUC_{sample} represented the AUC of BODIPY oxidation kinetics in the control and food samples, respectively.

Statistical Analysis

All data were analyzed using SAS Inc. (Cary, NC). Analysis of Variance (ANOVA) was performed using General Liner Model procedure with genotypes (hybrids) as fixed factors. Pearson phenotypic correlation was calculated among all compounds using measured compound contents. Of the thirty-seven provitamin A carotenoids enriched experimental maize hybrids from CIMMYT, only 20 hybrids were had complete data analyzed for individual carotenoids, vitamin E compounds, phenolic compounds and TAP values and were used for statistical analysis.

Results and Discussion

Maize carotenoids

The major carotenoids found in all provitamin A carotenoid enriched maize analyzed were lutein, zeaxanthin, β-cryptoxanthin, and β-carotene (**Figure 1)**. There was a positive correlation between β-cryptoxanthin and cis isomers of-β-carotene ($p<0.001$) (**Table 1**). As expected there was a significant positive correlation between trans β-carotene and its cis isomers ($p<0.001$). The importance of correlation between carotenoids in maize is due to their common biosynthetic pathways, with some compounds being precursors to other compounds [35]. The carotenoids, vitamin E and γ-

oryzanol profile of provitamin A enriched yellow was similar to that of normal yellow maize (represented by the letter U) (Figure 1). However, provitamin A enriched yellow maize was previously shown to be superior to regular yellow maize in terms of its vitamin A value [26, 28, 36]. Genotype effects were significant in determining the variation in cis β-carotene isomers, trans β-carotene and β-cryptoxanthin contents ($p<0.001$) (**Table 2a)**. Genotype played a major role in the variation in the contents of these carotenoids in maize. This is important from a plant breeding perspective because such diversity can be exploited to breed for provitamin A enriched maize [16]. Provitamin A concentration is controlled primarily by additive gene action and there is usually significant environment effect [37].

Maize vitamin E, phenolics and antioxidant activity

The major forms of vitamin E in the provitamin A carotenoids enriched maize hybrids analyzed were α-tocopherol, γ-tocopherol, α-tocotrienol and γ-tocotrienol (**Figure 2**). All provitamin A carotenoids enriched maize samples contained γ-tocopherol and α-tocopherol. The most predominant and ubiquitous vitamin E form in the provitamin A carotenoids enriched maize was γ-tocopherol with a range of 5.9 to 54.4 µg/g dry weight. The α-tocopherol contents ranged from 3.4 to 34.3 µg/g dry weight. These values are consistent with other studies on maize vitamin E analysis [13, 38-40]. It is generally accepted that α-tocopherol has more antioxidant activity than γ-tocopherol [41]. Recent studies show that γ-tocopherol may be important to human health and that it possesses unique features such as the ability to be a more effective trap for lipophilic electrophiles than α -tocopherol [41]. In our study we detected α-tocotrienol and γ-

tocotrienols in maize, with γ-tocotrienol as the second dominant vitamin E compound with a range of 36 µg/g dry weight. Tocotrienols are important to human health because they have been shown to lower the risk of lipid related LDL cholesterol associated with cardiovascular disease [43]. The significant correlation between α-tocopherol with α-tocotrienol (0.60) ($p<0.0001$) and γ tocotrienol (0.27) and ($p<0.05$) (Table 1) was also observed in two separate studies in maize and rice [13, 44]. There was an interesting significant positive correlation between α-tocopherol and cis isomers of β-carotene ($p<0.001$) (Table 1). This correlation was also reported in carrots [45]. The association of α-tocopherol with other fat soluble nutrients such as fatty acids was also observed in maize [13], it was suggested that α-tocopherol protects fatty acids from peroxidation. We can also speculate that α-tocopherol may protect carotenoids from oxidation, hence enhancing their storage stability. There were significant differences in the α-tocopherol and γ-tocopherol content among the maize hybrids (Table 2b), showing the role of genotype in the tocopherol contents. This was consistent with findings from other studies using genome wide association (GWAS) analysis in maize [46, 47]. In our study G x E interaction was significant in the γ-tocopherol contents, showing the significant roles that genes and the environment of the variation. Our study also found significant positive correlations between α-tocopherol, α-tocotrienol and γ-tocotrienol with total γ-oryzanol (Table 1). These correlations were also observed in rice [44]. This is the first study to report a very strong association of γ-oryzanol with α-tocotrienol (0.48) and γ-tocotrienol (0.33) $p<0.001$ (Table 1). Provitamin A enriched maize has similar levels of γ-oryzanol, ferulic acid and p-coumaric acid as compared to regular yellow maize represented by letter U (Figure 3). Environmental and genetic (G x E) effects were not significant for the

phenolic contents. This may suggest a homeostatic control of these nutrients by the plants. Ferulic acid, γ-oryzanol and p-coumaric acid are antioxidants that may play a role in defending the human body against oxidative stress associated free radicals [48].

The antioxidant activity of plant foods has been traditionally determined mainly using the hydrophilic based assays such as TEAC, FRAP and ORAC which are mainly influenced by water soluble antioxidants [49]. The TAP assay has the ability to measure the antioxidant activity of plant food extracts which contains both the lipophilic and water soluble antioxidants [50]. The TAP assay used in this study showed wide variation in the antioxidant activity of various maize hybrids (Figure 4). There were no significant correlations observed between TAP values and other antioxidants in maize analyzed. There was a trend observed showing that hybrids with high vitamin E contents and γ-oryzanol tended to have higher TAP values, thus demonstrating the great antioxidant power of vitamin E. This is not surprising because the TAP value represents the antioxidant activity of both the lipophilic and water soluble antioxidants.

Conclusion

Provitamin A carotenoids enriched maize is a good source of antioxidants such as carotenoids, vitamin E and phenolic compounds as compared to regular maize. Provitamin A enriched yellow maize contains higher contents of provitamin A carotenoids compared to regular yellow maize. Associations between carotenoids and vitamin E may be utilized in plant breeding to develop nutritious maize that can adapt to different agronomic environments. The combined concentration of carotenoids, vitamin E

and phenolic compounds in maize makes it an important source of antioxidants and vitamin A.

Acknowledgements

Funding for this study was provided by HarvestPlus through a cooperative agreement with the International Maize and Wheat Improvement Center (CIMMYT). Tawanda Muzhingi was funded by United States Department of Agriculture (USDA) Agricultural Research Service (ARS), and the Gerald J. and Dorothy R. Friedman School of Nutrition Science and Policy's James Sadowski Memorial Internship fellowship at Tufts University. I would like to thank Dr. Odilia Bermudez and Dr. Andrew H. Siwela for technical comments during the development of the manuscript.

Author contributions

Tawanda Muzhingi conducted sample and data analysis. Guangwen Tang, Kyung Jin Yeum, Natalia Palacios-Rojas assisted with the study design and revised the manuscript. Alejandra Miranda and Octavio Custidio helped with provitamin A carotenoids enriched maize sample analysis for carotenoids, vitamin E, and phenolic compounds.

References:

1. Atlin, G. N., Cairns, J., Breseghello, F., Leal-Bertioli, S. D. M., Magalhães, J. D., Carneiro, N. P., & Ulian, E. C. Breeding for improved drought tolerance in maize: CIMMYT's experience. In *Simpósio sobre tolerância à deficiência hídrica em plantas: adaptando as culturas ao clima do futuro, Goiânia, 19-21 October 2010*. Embrapa Arroz e Feijão, No. 265, pp. 117-122. (2010)
2. Keleman, A., Hellin, J., & Bellon, M. R. Maize diversity, rural development policy, and farmers' practices: lessons from Chiapas, Mexico. *Geogr J*. ***175***: 52-70.(2009)
3. Rosales, A., Galicia, L., Oviedo, E., Islas, C., & Palacios-Rojas, N. Near-infrared reflectance spectroscopy (NIRS) for protein, tryptophan, and lysine evaluation in quality protein maize (QPM) breeding programs. *J Agric Food Chem 59****:*** 10781-10786. (2011).
4. Muttoni, G., Foerster, J. M., Johnson, J. M., Haase, N. J., Beissinger, T. M., Stelpflug, S. C., & de Leon, N. Phenotypic and Genetic Dissection of Maize Internode Length. In *Plant and Animal Genome XXI Conference*. Plant and Animal Genome. 2013.
5. Pixley, K., Rojas, N. P., Babu, R., Mutale, R., Surles, R., & Simpungwe, E. Biofortification of maize with provitamin A carotenoids. In *Carotenoids and Human Health*. Humana Press. pp. 271-292. (2013)
6. Yan, J., Kandianis, C. B., Harjes, C. E., Bai, L., Kim, E. H., Yang, X., & Rocheford, T. Rare genetic variation at Zea mays crtRB1 increases β-carotene in maize grain. *Nat Genet **42**:*322-327.(2010)

7. Saltzman, A., Birol, E., Bouis, H. E., Boy, E., De Moura, F. F., Islam, Y., & Pfeiffer, W. H. Biofortification: progress toward a more nourishing future. *Global Food Security.2013*.
8. Pixley, K., Rojas, N. P., Babu, R., Mutale, R., Surles, R., & Simpungwe, E. Biofortification of maize with provitamin A carotenoids. In *Carotenoids and Human Health.2013,* (pp. 271-292). Humana Press.
9. Babu, R., Rojas, N. P., Gao, S., Yan, J., & Pixley, K. Validation of the effects of molecular marker polymorphisms in LcyE and CrtRB1 on provitamin A concentrations for 26 tropical maize populations. *Theor Appl Genet **126**:* 389-399. (2013)
10. Muzhingi, T., Gadaga, T. H., Siwela, A. H., Grusak, M. A., Russell, R. M., & Tang, G. Yellow maize with high β-carotene is an effective source of vitamin A in healthy Zimbabwean men. *Am J Clin Nutr **94**:* 510-519. (2011).
11. Li, S., Nugroho, A., Rocheford, T., & White, W. S. Vitamin A equivalence of the β-carotene in β-carotene biofortified maize porridge consumed by women. *Am J Clin Nutr **92**:* 1105-1112. (2010).
12. Ceballos, H., Luna, J., Escobar, A. F., Ortiz, D., Pérez, J. C., Sánchez, T., & Dufour, D. Spatial distribution of dry matter in yellow fleshed cassava roots and its influence on carotenoid retention upon boiling. *Food Research International **45**:* 52-59. (2012)
13. Goffman, F. D., & Böhme, T. Relationship between fatty acid profile and vitamin E content in maize hybrids (Zea mays L.). *J Agric Food Chem* 49, 4990-4994. (2001)

14. De Moura, F. F., Miloff, A., & Boy, E. Retention of Provitamin A Carotenoids in Staple Crops Targeted for Biofortification in Africa: Cassava, Maize and Sweet Potato. *Crit Rev Food Sci Nutr:* (In Press) (2014).
15. Bailly, C. Active oxygen species and antioxidants in seed biology. *Seed Science Research* ***14****:* 93-107. (2004)
16. Ortiz-Monasterio, J. I., Palacios-Rojas, N., Meng, E., Pixley, K., Trethowan, R., & Pena, R. J. Enhancing the mineral and vitamin content of wheat and maize through plant breeding. *J Cereal Sci* ***46****:* 293-307. (2007).
17. Shutu, X., Dalong, Z., Ye, C., Yi, Z., Shah, T., Ali, F., & Jianbing, Y. Dissecting tocopherols content in maize (Zea mays L.), using two segregating populations and high-density single nucleotide polymorphism markers. *BMC Plant Biol* ***12****:* 201-210. (2011).
18. Yeum, K. J., Smith, A. F., Krinsky, N. I., & Russell, R. M. The effect of α-tocopherol on the oxidative cleavage of β-carotene. *Free Radic Biol Med*, ***29****:* 105-114. (2000).
19. Z□ilić, S., Serpen, A., Akıllıog□lu, G., Go□kmen, V., & Vanc□etović, J. Phenolic compounds, carotenoids, anthocyanins, and antioxidant capacity of colored maize (Zea mays L.) kernels. *J Agric Food Chem* 60:1224-1231. (2012).
20. Cheng, H. H., Ma, C. Y., Chou, T. W., Chen, Y. Y., & Lai, M. H. Gamma-oryzanol ameliorates insulin resistance and hyperlipidemia in rats with streptozotocin/nicotinamide-induced type 2 diabetes. *Int J Vitam Nutr Res* ***80***: 45-50. (2010)

21. Cicero, A. F. G., & Gaddi, A. Rice Bran Oil and γ-Oryzanol in the Treatment of Hyperlipoproteinaemias and Other Conditions. *Phytother Res **15***: 277-289. (2001).

22. Verschoyle, R. D., Greaves, P., Cai, H., Edwards, R. E., Steward, W. P., & Gescher, A. J. Evaluation of the cancer chemopreventive efficacy of rice bran in genetic mouse models of breast, prostate and intestinal carcinogenesis. *Br J Cancer **96***: 248-254. (2007).

23. Akihisa, T., Yasukawa, K., Yamaura, M., Ukiya, M., Kimura, Y., Shimizu, N., & Arai, K. Triterpene alcohol and sterol ferulates from rice bran and their anti-inflammatory effects. *J Agric Food Chem* 48:2313-2319. (2000).

24. Zang, Lun-Yi, Greg Cosma, Henry Gardner, Xianglin Shi, Vince Castranova, and Val Vallyathan. Effect of antioxidant protection by p-coumaric acid on low-density lipoprotein cholesterol oxidation. *Am J Physiol Cell Physiol **279***: C954-C960. (2000)

25. Alam, Md Ashraful, Conrad Sernia, and Lindsay Brown. Ferulic acid improves cardiovascular and kidney structure and function in hypertensive rats. *J Cardiovasc Pharmacol **61:*** 240-249. (2013).

26. Kurilich, A. C., & Juvik, J. A. Quantification of Carotenoid and Tocopherol Antioxidants in Zea m ays. *J Agric Food Chem **47***:1948-1955. (1999).

27. Gutiérrez-Uribe, J. A., Rojas-García, C., García-Lara, S., & Serna-Saldivar, S. O. Phytochemical analysis of wastewater (nejayote) obtained after lime-cooking of different types of maize kernels processed into masa for tortillas. *J Cereal Sci **52:*** 410-416. (2010).

28. Muzhingi, T., Yeum, K. J., Russell, R. M., Johnson, E. J., Qin, J., & Tang, G. Determination of carotenoids in yellow maize, the effects of saponification and food preparations. *Int J Vitam Nutr Res* ***78***: 112-120. (2008).

29. Yeum, K. J., Booth, S. L., Sadowski, J. A., Liu, C., Tang, G., Krinsky, N. I., & Russell, R. M. Human plasma carotenoid response to the ingestion of controlled diets high in fruits and vegetables. *Am J Clin Nutr* ***64***:594-602. (1996).

30. Kähkönen, M. P., Hopia, A. I., Vuorela, H. J., Rauha, J. P., Pihlaja, K., Kujala, T. S., & Heinonen, M. Antioxidant activity of plant extracts containing phenolic compounds. *J Agric Food Chem* ***47****:* 3954-3962. (1999).

31. Vinson, J. A. Black and green tea and heart disease: a review. *Biofactors* ***13****:* 127-132. (2000).

32. Snapp, S. S., Swinton, S. M., Labarta, R., Mutch, D., Black, J. R., Leep, R., ... & O'Neil, K. Evaluating cover crops for benefits, costs and performance within cropping system niches. *Agronomy* ***97****:* 322-332. (2005).

33. Beretta, G., Aldini, G., Facino, R. M., Russell, R. M., Krinsky, N. I., & Yeum, K. J. Total antioxidant performance: a validated fluorescence assay for the measurement of plasma oxidizability. *Anal Biochem* ***354***: 290-298. (2006).

34. Cho, Y. S., Yeum, K. J., Chen, C. Y., Beretta, G., Tang, G., Krinsky, N. I., & Russell, R. M. (2007). Phytonutrients affecting hydrophilic and lipophilic antioxidant activities in fruits, vegetables and legumes. *J Sci Food Agric* ***104***: 1096-1107. (2007).

35. Wong, J. C., Lambert, R. J., Tadmor, Y., & Rocheford, T. R. QTL associated with accumulation of tocopherols in maize. *Crop Sci* ***43***: 2257-2266. (2003).

36. Menkir, A., Liu, W., White, W. S., Maziya-Dixon, B., & Rocheford, T. Carotenoid diversity in tropical-adapted yellow maize inbred lines. *Food Chem* ***109****:* 521-529. (2008)

37. Suwarno, W. B., Pixley, K. V., Palacios-Rojas, N., Kaeppler, S. M., & Babu, R. Formation of Heterotic Groups and Understanding Genetic Effects in a Provitamin A Biofortified Maize Breeding Program. *Crop Sci* ***54*****:** 14-24. (2014).

38. Picot, A., Atanasova-Pénichon, V., Pons, S., Marchegay, G., Barreau, C., Pinson-Gadais, L., & Richard-Forget, F. Maize Kernel Antioxidants and Their Potential Involvement in Fusarium Ear Rot Resistance. *J Agric Food Chem chemistry* ***61****:* 3389-3395. (2013).

39. Zhang, L., Luo, Y., Zhu, Y., Zhang, L., Zhang, W., Chen, R., & Wang, L. GmTMT2a from soybean elevates the α-tocopherol content in corn and Arabidopsis. *Transgenic Res*, 1-8. (2013).

40. Rocheford, T. R., Wong, J. C., Egesel, C. O., & Lambert, R. J. Enhancement of vitamin E levels in corn. *J Am Coll Nutr* ***21***:(suppl 3), 191S-198S.(2002)

41. Qing J, and Ames B. γ-Tocopherol, but not α-tocopherol, decreases proinflammatory eicosanoids and inflammation damage in rats. *FASEB* ***17.8***: 816-822. (2003).

42. Jiang, Qing, et al. γ-Tocopherol, the major form of vitamin E in the US diet, deserves more attention. *Am J Clin Nutr* **74**: 714-72. (2001).

43. Theriault, Andre, et al. Tocotrienol: a review of its therapeutic potential. *Clin Biochem* ***32.5***: 309-319. (1999)

44. Bergman, C. J., & Xu, Z. Genotype and environment effects on tocopherol, tocotrienol, and γ-oryzanol contents of southern US rice. *Cereal Chem* ***80:*** 446-449. (2003).
45. Koch, T. C., & Goldman, I. L. Relationship of carotenoids and tocopherols in a sample of carrot root-color accessions and carrot germplasm carrying Rp and rp alleles. *J Agric Food Chem chemistry* ***53****:*325-331. (2005).
46. Li, Q., Yang, X., Xu, S., Cai, Y., Zhang, D., Han, Y., & Yan, J. (2012). Genome-wide association studies identified three independent polymorphisms associated with α-tocopherol content in maize kernels. *PloS one 7:* e36807. (2012).
47. Lipka, A. E., Gore, M. A., Magallanes-Lundback, M., Mesberg, A., Lin, H., Tiede, T., & DellaPenna, D. Genome-Wide Association Study and Pathway Level Analysis of Tocochromanol Levels in Maize Grain. *G3* ***3***:1287-99. (2013)
48. Graf, Ernst. Antioxidant potential of ferulic acid. *Free Radic Biol Med* ***13***: 435-448. (1992).
49. Prior, R. L., Hoang, H., Gu, L., Wu, X., Bacchiocca, M., Howard, L., & Jacob, R. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity (ORACFL)) of plasma and other biological and food samples. *J Agric Food Chem chemistry* ***51****:*3273-3279. (2003).
50. Cho, Y. S., Yeum, K. J., Chen, C. Y., Beretta, G., Tang, G., Krinsky, N. I., & Russell, R. Phytonutrients affecting hydrophilic and lipophilic antioxidant activities in fruits, vegetables and legumes. *J Sci Food Agric* ***104***: 1096-1107. (2007).

Table 1: Correlations between carotenoids, vitamin E and phenolic compounds in provitamin A carotenoids enriched maize

Phytonutrient	**Lutein**	**Zeax**	**β-Cry**	**13-cis-βC**	**trans βC**	**9-cis-βC**	**α-TT**	**γ-TT**	**γ-TP**	**α-TP**	**pcoumaric Acid**	**Ferulic Acid**	**γ-oryzanol**
Lutein	1.00	0.97***	0.52***	0.01	-0.01	0.04	-0.17	-0.09	-0.01	-0.22	0.34**	0.25	0.22
Zeaxanthin		1.00	0.56***	0.06	0.05	0.07	-0.21	-0.12	0.06	-0.21	0.29***	0.19	0.24
β-Cryptoxanthin			1.00	0.26**	0.20	0.24**	-0.20	-0.19	0.10	0.01	0.01	0.05	0.16
13-cis-β-carotene				1.00	0.97***	0.89***	0.17	0.04	-0.12	0.37***	-0.05	0.09	0.08
trans β-carotene					1.00	0.83***	0.14	0.03	-0.19	0.33	-0.09	0.06	0.13
9-cis- β-carotene						1.00	0.17	0.12	0.07	0.46***	0.03	0.09	0.15
α-tocotrienol							1.00	0.37***	-0.02	0.60***	0.15	-0.07	0.48***
γ-tocotrienol								1.00	0.12	0.27**	0.02	0.00	0.33***
γ-tocopherol									1.00	0.08	0.05	-0.02	-0.03
α-tocopherol										1.00	-0.02	-0.18	0.27**
p-coumaric Acid											1.00	0.67***	0.30**
Ferulic Acid												1.00	0.05
γ-oryzanol													1.00

*** Statistical significance at p <0.001,

**Statistical significance at p<0.05,

Abbreviations: βC = β-carotene, TT = tocotrienol, TP = tocopherol, β-Cry = β-Cryptoxanthin, zeax =zeaxanthin

Table 2a: Type III Sum Squares for the Analysis of Variance for Carotenoids in provitamin A carotenoids enriched maize hybrids

Source	**df**	**Lutein**	**Zeaxanthin**	**β-Crypt**	**13 cis β-Carotene**	**trans β-Carotene**	**9 cis β-Carotene**	**Provitamin A**
Environment (E)	1	0.02	0.67	14.08**	0.10	2.39	0.23**	0.27
Genotype (G)	20	10.61	225.42	73.10**	3.89***	83.72***	2.62***	208.11***
G x E	17	4.86	119.65	22.48	0.35	6.29	0.50	21.64
Error	30	7.45	198.27	49.36	0.80	17.92	0.53	48.31

*** Statistical significance at p <0.001

**Statistical significance at p<0.05

β-Crypt = β-Cryptoxanthin

Table 2b: Type III Sum of Squares for Analysis of Variance for vitamin E and phenolics in provitamin A carotenoids enriched maize

Source	**df**	**α-Tocopherol**	**α-Tocotrienol**	**γ-Tocopherol**	**γ-Tocotrienol**	**γ-Oryzanol**	**Ferulic acid**	**p-coumaric acid**
Environment (E)	1	29.39	1.74	170.21	262.98	0.01	0.25	0.00
Genotype (G)	20	1006.71	176.35	93.40***	756.49	0.34	6.19**	0.09
G x E	17	307.80	142.69	263.62**	594.81	0.31	1.24	0.04
Error	30	441.15	175.95	1119.96	2496.95	0.58	4.56	0.01

*** Statistical significance at $p < 0.001$

**Statistical significance at $p < 0.05$

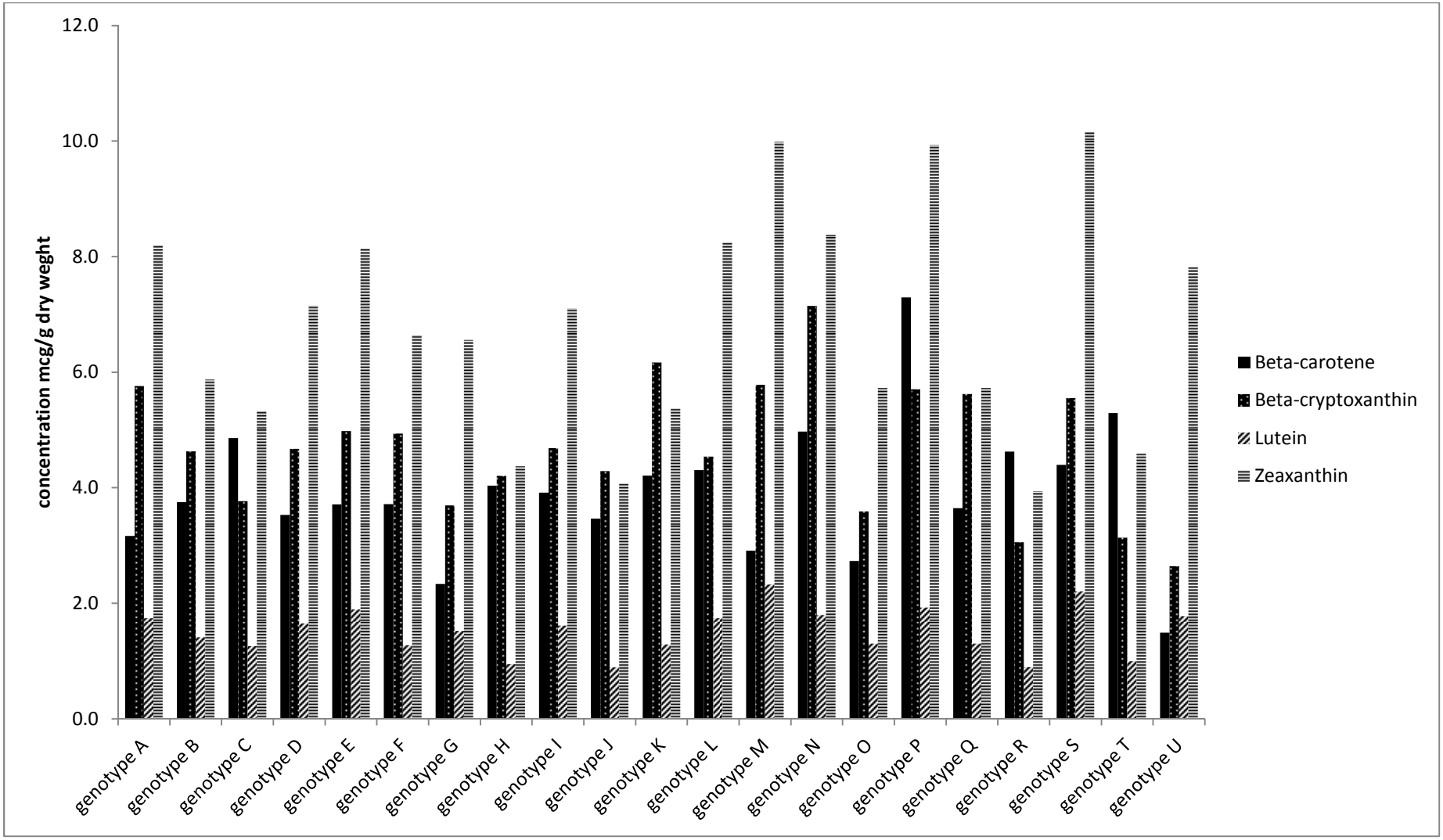

Figure 1: Carotenoid distribution in provitamin A carotenoids enriched maize by genotype

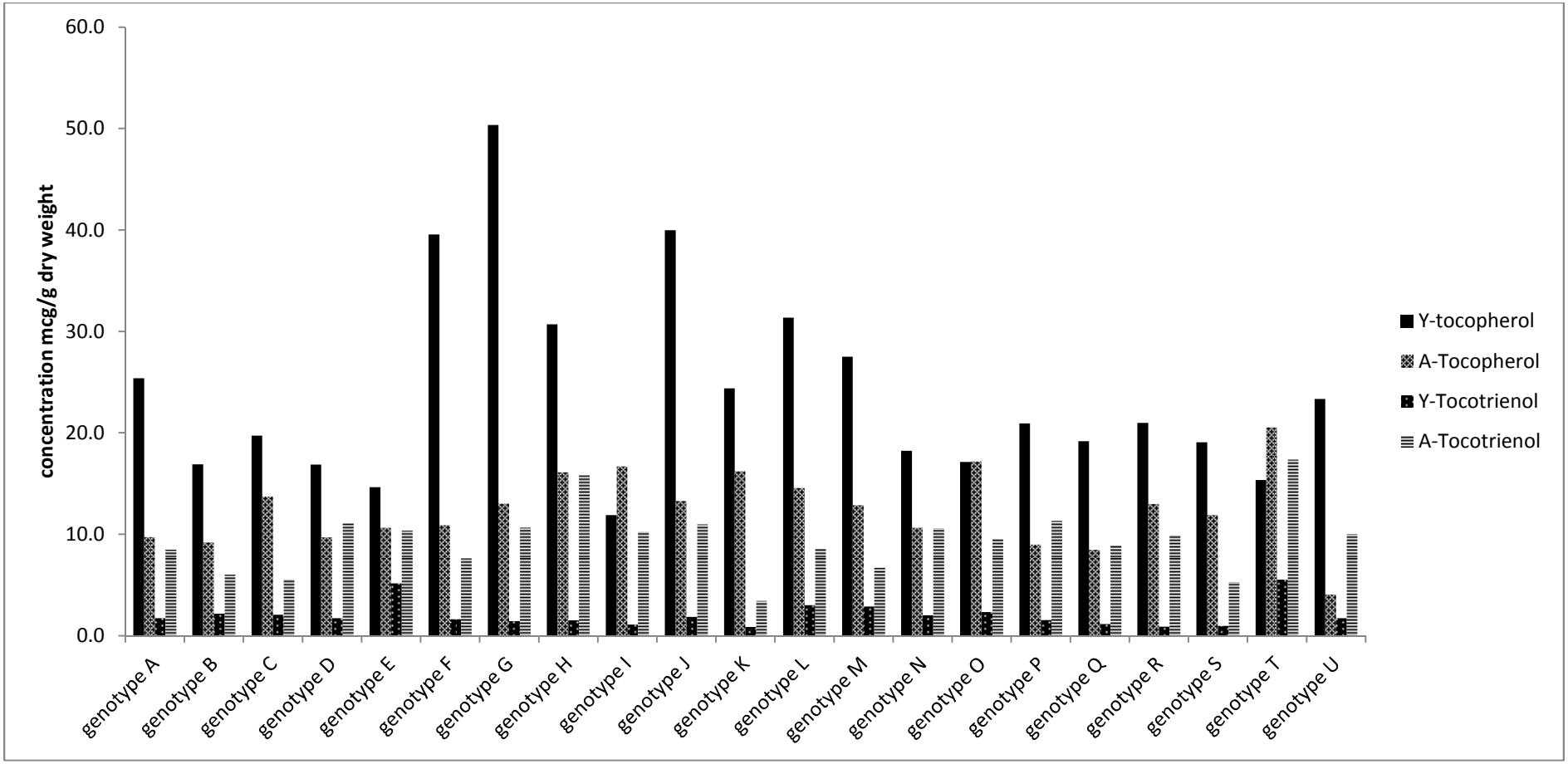

Figure 2: Distribution of vitamin E in provitamin A carotenoids enriched maize by genotype

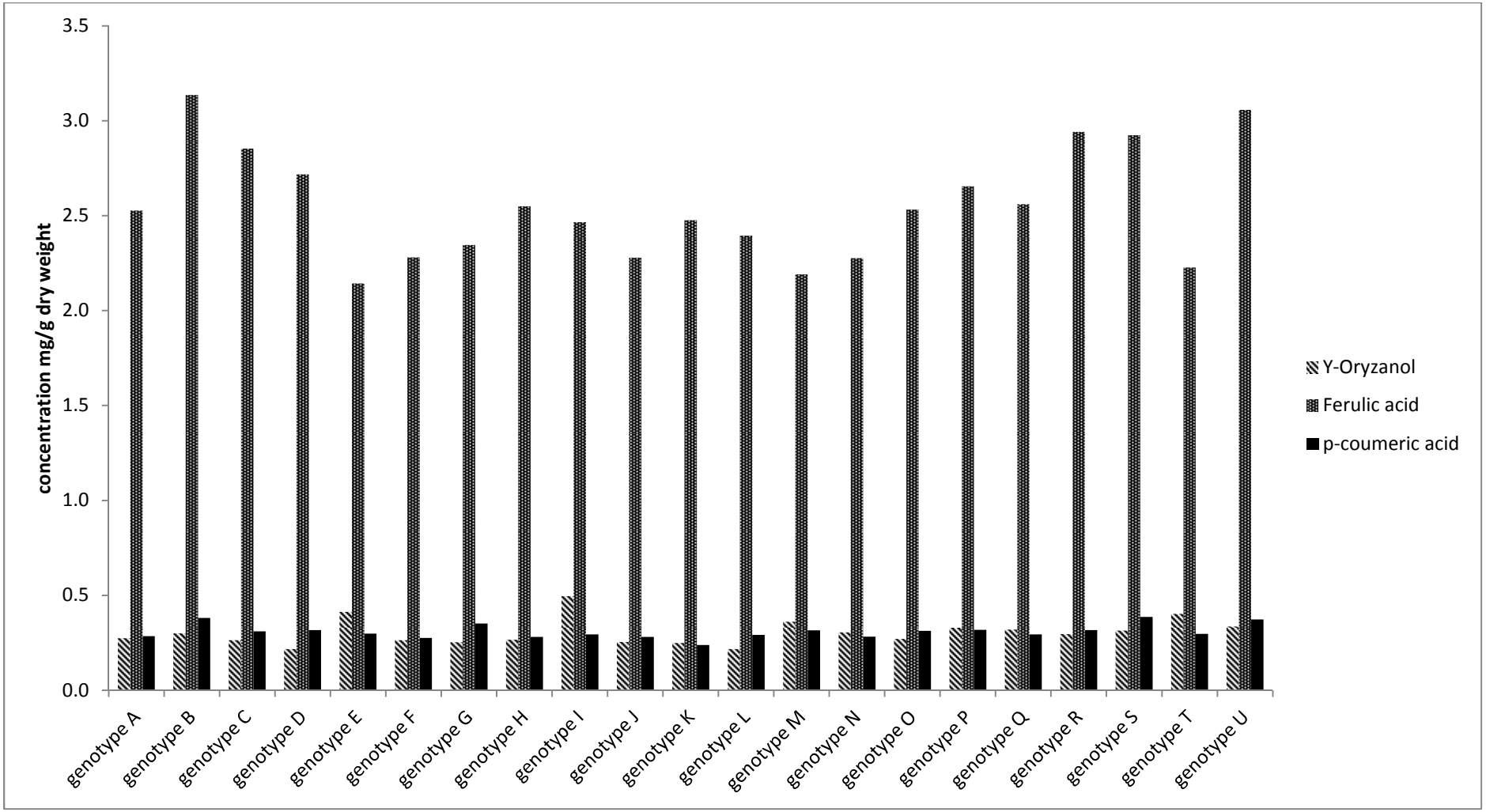

Figure 3: Distribution of phenolic compounds analyzed in provitamin A carotenoids enriched maize by genotype

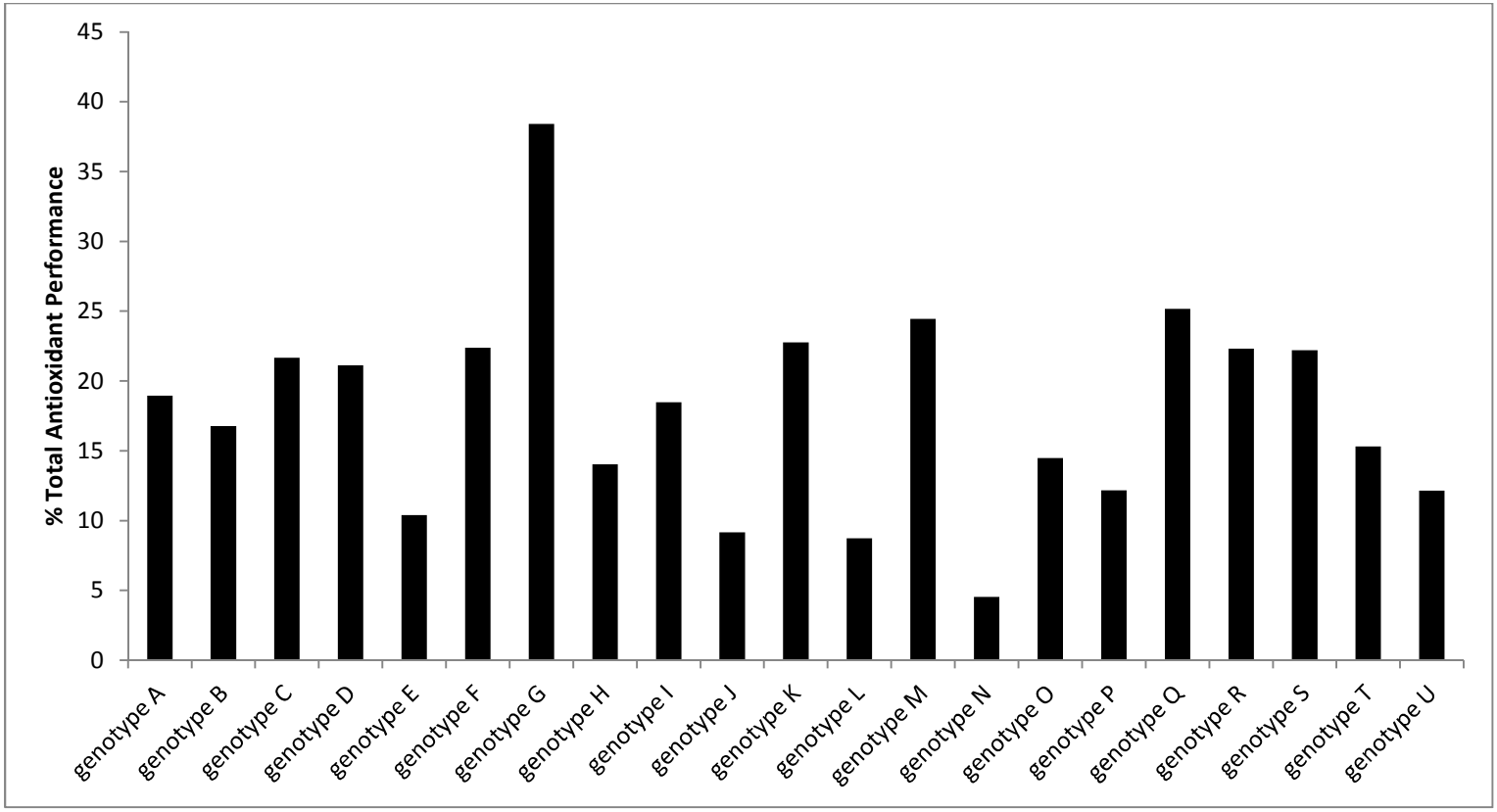

Figure 4: Percent Total Antioxidant Performance (TAP) distribution in provitamin A arotenoids enriched maize by genotype

Table 3: Codes for provitamin A carotenoids enriched maize hybrids

Pedigree (Genotype)	**Code**
(([CML197/N3//CML206]-X-32-1-4-B*5/[BETASYN]BC1-4-4-4-1-B-B-B-B-B//MAS[206/312]-23-2-1-1-B-B-B/[BETASYN]BC1-4-1-1-1-B-B-B-B-B-B))/(CML297)-B	A
((CML488/[BETASYN]BC1-15-5-B-B-B//MAS[206/312]-23-2-1-1-B-B-B/[BETASYN]BC1-10-3-#-B-B-B-B))/(CML297)-B	B
((CML488/[BETASYN]BC1-15-5-B-B-B//MAS[206/312]-23-2-1-1-B-B-B/[BETASYN]BC1-10-3-#-B-B-B-B))/(CML304)-B	C
((CML488/[BETASYN]BC1-15-5-B-B-B//MAS[206/312]-23-2-1-1-B-B-B/[BETASYN]BC1-6-1-1-1-B-B-B-B-B))/(CML297)-B	D
((CML488/[BETASYN]BC1-15-7-1-1-1-B-B//CML489/[BETASYN]BC1-2-#-B-B-B-B))/([SAM4/BETASYN]BC2FS1-1-1-1-B-B-B-B-B-B-B-B-B)-B	E
((CML488/[BETASYN]BC1-15-7-1-1-1-B-B//CML489/[BETASYN]BC1-2-#-B-B-B-B))/(CML300)-B	F
((CML488/[BETASYN]BC1-15-7-1-1-1-B-B//CML489/[BETASYN]BC1-2-#-B-B-B-B))/(CML496)-B	G

((CML488/[BETASYN]BC1-15-7-1-1-1-B-B//CML489/[BETASYN]BC1-2-#-B-B-B-B))/(MAS[206/312]-23-2-1-1-B-B-B/[BETASYN]BC1-10-2-1-#-B-B-B-B-B-B-B-B)-B	H
((CML488/[BETASYN]BC1-15-7-1-1-1-B-B//CML489/[BETASYN]BC1-2-#-B-B-B-B))/(MAS[206/312]-23-2-1-1-B-B-B/[BETASYN]BC1-11-3-1-#-B-B-B-B-B-B-B)-B	I
((CML488/[BETASYN]BC1-15-7-1-1-1-B-B//MAS[206/312]-23-2-1-1-B-B-B/[BETASYN]BC1-11-3-1-#-B-B-B-B))/(CML300)-B	J
((CML488/[BETASYN]BC1-15-7-1-1-1-B-B//MAS[206/312]-23-2-1-1-B-B-B/[BETASYN]BC1-11-3-1-#-B-B-B-B))/(CML304)-B	K
((CML488/[BETASYN]BC1-15-7-1-1-1-B-B//MAS[206/312]-23-2-1-1-B-B-B/[BETASYN]BC1-11-3-1-#-B-B-B-B))/(KUI carotenoid syn-FS17-3-2-B-B-B-B-B-B-B-B)-B	L
((CML489/[BETASYN]BC1-2-#-B-B-B-B//Ac8730SR-##-124-1-5-B-1-#/[BETASYN]BC1-5-#-B-B-B-B))/([SAM4/BETASYN]BC2FS1-1-1-1-B-B-B-B-B-B-B-B)-B	M
((CML489/[BETASYN]BC1-2-#-B-B-B-B//Ac8730SR-##-124-1-5-B-1-#/[BETASYN]BC1-5-#-B-B-B-B))/(CML297)-B	N
((CML489/[BETASYN]BC1-2-#-B-B-B-B//Ac8730SR-##-124-1-5-B-1-#/[BETASYN]BC1-5-#-B-B-B-B))/(MAS[206/312]-23-2-1-1-B-B-B/[BETASYN]BC1-11-3-1-#-B-B-B-B-B-B-B)-B	O

((MAS[206/312]-23-2-1-1-B-B-B/[BETASYN]BC1-10-2-1-#-B-B-B-B-B//MAS[206/312]-23-2-1-1-B-B-B/[BETASYN]BC1-6-1-1-1-B-B-B-B-B))/(CML297)-B	P
((MAS[206/312]-23-2-1-1-B-B-B/[BETASYN]BC1-10-3-#-B-B-B-B//MAS[206/312]-23-2-1-1-B-B-B/[BETASYN]BC1-11-3-1-#-B-B-B-B))/(CML297)-B	Q
((CML489/[BETASYN]BC1-2-#-B-B-B-B//Ac8730SR-##-124-1-5-B-1-#/[BETASYN]BC1-5-#-B-B-B-B))/(MAS[206/312]-23-2-1-1-B-B-B/[BETASYN]BC1-11-3-1-#-B-B-B-B-B-B-B)-B	R
((MAS[206/312]-23-2-1-1-B-B-B/[BETASYN]BC1-10-3-#-B-B-B-B//MAS[206/312]-23-2-1-1-B-B-B/[BETASYN]BC1-11-3-1-#-B-B-B-B))/(KUI carotenoid syn-FS17-3-2-B-B-B-B-B-B-B-B)-B	S
(MAS[206/312]-23-2-1-1-B-B-B/[BETASYN]BC1-10-2-1-#-B//MAS[206/312]-23-2-1-1-B-B-B/[BETASYN]BC1-11-3-1-#-B)/(CML489/[BETASYN]BC1-2-#-B-B-B-B-B-B-B)-B	T
Check1: CML451/CML486-B	U

CHAPTER 4

Enzymatic conversion of β-carotene from biofortified yellow maize and kale into retinoids and apocarotenoids

Enzymatic conversion of β-carotene from biofortified yellow maize and kale into retinoids and apocarotenoids

[1, 2] Tawanda Muzhingi, [1],[3]Kyung-Jin Yeum, [1, 4]Odilia Bermudez, [5]Andrew H. Siwela and [1, 2] Guangwen Tang

[1] Gerald J. and Dorothy R. Friedman School of Nutrition Science and Policy, Tufts University, 150 Harrison Avenue, Boston, MA, 02111.

[2] Jean Mayer United Stated Department of Agriculture (USDA), Agricultural Research Services (ARS), Human Nutrition Research Center on Aging at Tufts University. 711 Washington Street, Boston, MA, 02111.

[3] Division of Food Bioscience, College of Biomedical and Health Sciences, Konkuk University, Glocal Campus, Chungju-Si, Chungcheongbuk-do, 380-701, South Korea.

[4] Department of Public Health and Community Medicine, Tufts School of Medicine, 136 Harrison Avenue, Boston, MA.

[5]Department of Applied Biology and Biochemistry, National University of Science and Technology, Gwanda Road, Bulawayo, Zimbabwe.

Corresponding Author

Guangwen Tang. PhD

Jean Mayer USDA ARS HNRCA at Tufts University

Boston, MA, 02111

Email guangwen.tang@tufts.edu

Tel: +1(617)5563226

Abstract

Provitamin A carotenoids in plant foods provide more than 80% of vitamin A intake for people in developing countries. As such, the conversion efficiency of β-carotene to vitamin A is important in determining the effectiveness of plant foods as sources of vitamin A in humans. This study examined the role that α-tocopherol, γ-tocopherol, α-tocotrienol, γ-tocotrienol and total γ-oryzanol antioxidants found in plant foods play on the cleavage of β-carotene. Rat intestinal mucosa homogenate (post mitochondrial fraction) was incubated with β-carotene-rich extracts of kale and biofortified yellow maize for an hour at 37°C. Rat intestinal mucosa homogenate was also incubated with β-carotene in presence or absence of either α-tocopherol, γ-tocopherol, α-tocotrienol, γ-tocotrienol or γ-oryzanol for 60 min at 37°C. The β-carotene cleavage products were extracted and analyzed by an HPLC equipped with a C18 column. No β-carotene cleavage products were detected in control incubations done without intestinal mucosa homogenate or β-carotene. In the absence of antioxidants, β-carotene cleavage produced mainly excentric cleavage products, β-apo-13-carotenone and β-apo-14-carotenal, in addition to retinal, retinol and retinoic acid. In the presence of either α-tocopherol, γ-tocopherol or α-tocotrienol the formation of excentric cleavage products β-apo-14-carotenal and β-apo-13-carotenone was inhibited by more than 10 fold. Beta-carotene-rich extracts of kale incubated with rat intestinal mucosa homogenate produced twice as much β-apo-13-carotenone compared to biofortified maize extracts. These results suggest that antioxidants inhibit excentric cleavage of β-carotene and the formation of additional excentric cleavage products.

Introduction

Currently it is estimated that close to 190 million pre-school children mostly in south Asia and sub Saharan Africa are affected by vitamin A deficiency (VAD) [1]. Vitamin A is an essential nutrient required by the body for cell differentiation, embryonic development, immune function, growth and vision [2]. VAD is characterized by increased risk of blindness, morbidity and mortality [3]. VAD is caused mainly by inadequate dietary intake of foods of animal origin (rich in preformed vitamin A) because they are expensive [4]. As a result, poor people in developing countries obtain more than 80 % of their vitamin A intake from plant based foods (provitamin A carotenoids) which are easy to grow and are readily available. However, this plant food vitamin A intake is not enough to meet their Recommended Daily Allowance (RDA) [5, 6]. It is therefore important to understand factors that can increase the effectiveness of plant food provitamin A carotenoids as sources of vitamin A in humans.

It is known that the bioconversion of β-carotene (in oil) to vitamin A varies from 2 to 1 to 16 to 1 by weight in humans [7]. Human studies have also shown wide variations in the bioconversion of vegetable and fruit β-carotene to vitamin A. The conversion factors observed for fruits were 12 to 1 and 26 to 1 by weight in green vegetables [8, 9]. In grains, the bioconversion factors of β-carotene to vitamin A were much better than in fruits and vegetables ranging from 3.2 to 1 by weight in biofortified maize and 3.8 to 1 by weight in golden rice [10, 11]. While it is established that bioavailability of plant food provitamin A carotenoids and their bioconversion to vitamin A in humans is affected by factors such as food matrices, food preparation, and the fat content of a meal [12], the effect of other dietary factors present in the diet such as

antioxidants on the extent of the conversion of β-carotene into vitamin A is still unclear and needs to be determined in order to improve the effectiveness of plant foods as vitamin A sources.

The mechanisms of provitamin A carotenoids conversion into vitamin A is of prime importance in public health. It is known that in humans the bioconversion of β-carotene into vitamin A takes place mainly in the intestine [13]. The central cleavage pathway involves the metabolism of β-carotene at the central double bond (15, 15′) by β-carotene 15, 15′-dioxygenase (BCMO1) to produce retinal [14]. Several studies have reported exclusive central cleavage of β-carotene to produce retinal, which can be converted to retinol and retinoic acid respectively [15-18]. There are conflicting reports on origins and fate of retinal in central cleavage of β-carotene with some studies reporting that it is converted to retinoic acid, thereby acting as an intermediate β-carotene cleavage product [19, 20]. The excentric cleavage of β-carotene is known to produces mostly β-apo-carotenoids which may be converted to retinoic acid but mostly are devoid of vitamin A activity [21, 22]. The formation of excentric cleavage products such as β-apo-13-carotenone and β-apo-14-caronal from β-carotene incubation with intestinal mucosa homogenates from human, monkey, ferret and rat was established [23, 24]. It was previously thought that apocarotenoids were formed exclusively *in vitro* from β-carotene auto-oxidation [25]. However, these β-carotene excentric cleavage products have been detected in humans [26]. The conclusive evidence for excentric cleavage pathway was provided by the discovery of an enzyme that cleaved β-carotene at 9, 10 double bonds to produce β-apo-10-carotenal and β-ionone [27]. Observations from *in vitro* studies suggest that excentric cleavage occurs mostly under oxidative conditions especially when

antioxidants are limiting as in smoking and oxidative stress conditions [28]. Other *in vitro* studies showed that radicals from lipoxygenase enzymes attack β-carotene excentrically forming excentric cleavage products [9, 29 and 30]. Our previous study demonstrated that β-carotene cleavage pathway depended on the presence of an antioxidant, such that in the presence of α-tocopherol, β-carotene was converted exclusively to retinal by the 15, 15-dioxygenase enzyme and in the absence of α-tocopherol, β-apocarotenoids were formed [14]. This is significant because plant food β-carotene is often consumed with several dietary antioxidants such as tocopherols, tocotrienols and γ-oryzanol in a meal. Staple foods such as yellow maize, rice and green vegetables that are rich in β-carotene are also rich in vitamin E, and γ-oryzanol [30-33]. Some green vegetables such as kale, collard greens and cabbage consumed with maize in sub Saharan African dishes are rich in provitamin A carotenoids and antioxidants [34]. Currently, there is a dearth of information on the effect of these antioxidants on the cleavage of their β-carotene to vitamin A. It was worth determining how these antioxidants inherent in biofortified yellow maize and kale affect the cleavage of their β-carotene to vitamin A. These foods can be targeted for interventions because they are staple foods in sub-Saharan Africa consumed daily by millions of people at risk of VAD. This study tested the hypothesis that γ-tocopherol, α-tocotrienol, γ-tocotrienol and γ-oryzanol could enhance the central cleavage of β-carotene to vitamin A *in vitro* using rat intestinal mucosa homogenate. Understanding factors that affect the efficiency of bioconversion of provitamin A carotenoids into vitamin A is important for improving the effectiveness of plant food based VAD intervention strategies in poor countries where people depend on them for their vitamin A intake.

Materials and Methods

Chemicals

All HPLC solvents were HPLC grade and were obtained from Sigma Aldrich (St. Louis, MO, USA). All-trans- β-carotene (≥ 97% purity), all-trans-retinoic acid, all-trans-retinal, dithiothreitol (DTT), Hepes, Tricine, EDTA (ethylenediamine-tetraacetic acid), Tris buffer, sodium taurocholate, α-tocopherol and γ-tocopherol were purchased from Sigma Aldrich (St. Louis, MO, USA). Alpha-tocotrienol and γ-tocotrienol standards were purchased from Cayman Chemicals (Ann Arbor, Michigan). Gamma-oryzanol standards were purchased from Wako Chemicals (Richmond, VA, USA). The standards of β-apo-13-carotenone, β-apo-12-carotenal and β-apo-14'-carotenal were gifts from Hoffman-La Roche Inc. (Basel, Switzerland).

Tissue preparation

The use of rat models in this study protocol was approved by the Animal Care and Use Committee at Tufts University. The preparation of intestinal mucosa homogenate and incubation with β-carotene were described previously [14, 17]. Briefly, the upper half of the intestine was washed with ice-cold isotonic saline (0.85% NaCl), and the intestinal mucosa was then gently scraped off with a glass cover and homogenized on ice in a test-tube with a Brinkmann Polytron homogenizer (Westbury, NY, USA) with 50 mM HEPES buffer (weight: volume 5 1:4), pH 7.4, containing 1.15% KCl, 1 mM EDTA, and 0.1 mM DTT. A post nuclear fraction was prepared by centrifuging the intestinal homogenate at *800 x g* for 30 min. The post nuclear fraction was centrifuged further at

10,000 x g for 1 h at 4°C to remove particulate matter and some organelles to obtain the post-mitochondrial fractions which were used in the experiments. The resulting supernatant solution's protein concentrations of the protein fractions were determined using the BCA (bicinchoninic acid) Protein Assay (Pierce Co.; Rockford, IL, USA).

Beta-carotene incubation with post-mitochondrial fractions of rat intestinal mucosa homogenate

The β-carotene standard was purified by eluting it in an open column with aluminum oxide with hexane as previously published [35], the peak purity was confirmed by HPLC and it was used for the incubations immediately. The β-carotene incubation with intestinal homogenate procedure was as previously described [14]. Briefly, the standard incubation mixture contained 1 mg protein, 15 µM of β-carotene, 0.1 M Tricine buffer, pH 8.0, 6 mM sodium taurocholate, and 0.5 mM DTT in a total volume of 400 µL. The protein fraction was pre-incubated with cofactors at 37°C in a shaking water bath for 5 min. After the pre-incubation, the enzyme reaction was started by adding 80 µL of β-carotene solubilized in aqueous Tween 40 to 320 µL of the incubation mixture containing 0.1 mM of either α-tocopherol, γ-tocopherol, α-tocotrienol, γ-tocotrienol and γ-oryzanol standards. Fresh kale and biofortified yellow maize samples were extracted for carotenoids and other fat soluble components such as vitamin E and γ-oryzanol using methanol and Tetrahydrofuran (THF) as previously described [25-26]. The biofortified yellow maize extract contained 100 µg of β-carotene, 60 µg α-tocopherol and 130 µg γ-tocopherol and 3 mg total γ-oryzanol content. The kale extract contained 100 µg of β-carotene and no detectable vitamin E and γ-oryzanol compounds. The β-carotene-rich

extracts of kale and biofortified yellow maize was dried under a gentle stream of nitrogen and was reconstituted in Tween 40. After the pre-incubation, the enzyme reaction was started by adding 80 µL extracts of kale β-carotene and biofortified β-carotene. Control vials were run lacking either β-carotene or the protein fraction. Incubations of β-carotene with the rat intestinal mucosa homogenate homogenates were conducted in triplicates. All experimental procedures were carried out under red light.

HPLC Analysis

The HPLC analysis, mobile phase and gradient elution program were as previously published [14]. Retinyl acetate was added as internal standard and the incubation mixture was extracted with 3 mL of chloroform: methanol (2:1, v/v), followed by 3 mL of hexane. The mixture was centrifuged for 10 min at *800 x g* at 4°C. The chloroform and hexane layers were evaporated to dryness under a gentle stream of nitrogen, and the residue was reconstituted by addition of 100 µL of ethanol which was then transferred to an HPLC vial. A 70 µL volume of the reconstituted sample was injected into the HPLC system. The HPLC system consisted of a Waters Corporation (Milford, MA, USA) 2596 pump, Waters 2996 Photodiode Array Detector (PDA), Waters Empower2 data acquisition and analysis software. The HPLC system was equipped with a Pecosphere-3 C18 4.6mm x 83 mm cartridge column (Perkin-Elmer, Inc.). Carotenoids, retinoids, vitamin E and γ-oryzanol were detected at 450, 340, 292 and 325 nm wavelengths respectively. The standards of β-apo-14-carotenal, β-apo-12-carotenal, β-apo-13-carotenone, retinol, retinoic acid, and retinal, retinyl acetate were adequately separated using this method. Beta-carotene, β-apo-carotenal, β-apo-13-

carotenone, retinol, retinoic acid and retinal were quantified by determining peak areas in the HPLC chromatograms calibrated against known amounts of standards. Concentrations were corrected for extraction and handling losses by monitoring the recovery of the internal standards.

Statistical Analysis

All statistical analysis was conducted using Statistical Analysis Software (SAS) Inc. (North Carolina) version 9.3. Descriptive statistics were conducted and difference between treatments means were analyzed by ANOVA with the level of significance set at 0.05.

Results

Cleavage products of kale and biofortified yellow extracts

Biofortified yellow maize extract (containing 100 µg of β-carotene, 60 µg α-tocopherol and 130 µg γ-tocopherol equivalence) were incubated with rat intestinal mucosa homogenate the cleavage products formed were retinoic acid, β-apo-13-carotenone, retinal and β-apo-12-carotenal (**Figure 1**). Extracts of kale containing 100 µg β-carotene equivalence were also incubated with rat intestinal mucosa homogenate and the β-carotene cleavage products were found to be retinoic acid and β-apo-13-carotenone (Figure 1). There was no significant difference in the amount of retinoic acid formed from kale and biofortified yellow maize. However, there was more β-apo-13-carotenone

produced in the kale incubation mixture as compared to the biofortified yellow maize mixture.

Characteristics of the β-carotene cleavage products in the absence of antioxidants

In this study 15 µM β-carotene was incubated with post mitochondrial fraction of rat intestinal mucosa homogenate in the absence of antioxidants. The incubation of rat intestinal mucosa homogenate without β-carotene did not produce any detectable retinoid peaks, and β-carotene incubated without rat intestinal mucosa homogenate did not produce any detectable retinoid peaks. In both controls no β-carotene cleavage products were detected (**Figure 2**). Rat intestinal mucosa homogenate incubated with β-carotene in the absence of antioxidants produced several peaks that were identified as β-apo-13-caroteneone, β-apo-14-carotenal, retinal, retinol, and retinoic acid according to the retention times and spectra of pure standards (**Table 1**). This shows that in the absence of antioxidants, the major β-carotene cleavage product formed were excentric cleavage products β-apo-13-carotenone and β-apo-14-carotenal (**Figure 2**) (**Table 1**).

Effect of antioxidants on β-carotene cleavage by rat intestinal mucosa homogenate

The effect of antioxidants on β-carotene cleavage was investigated using rat intestinal mucosa homogenate post mitochondrial fraction incubated with 15 µM β-carotene in the presence of either (0.1 mM) α-tocopherol, γ-tocopherol, α-tocotrienol, γ-tocotrienol or total γ-oryzanol. When β-carotene was incubated with rat intestinal mucosa homogenate in the presence of 0.1 mM total γ-oryzanol, the pattern of β-carotene cleavage products detected were similar to that of β-carotene incubation without

antioxidants (Table 1). The major cleavage products of β-carotene incubation with rat intestinal homogenate in the presence of γ-oryzanol were excentric cleavage products β-apo-13-carotenone and β-apo-14-carotenal. Also detected were other cleavage products retinol, retinal and retinoic acid formed (Table 1).

The incubation of β-carotene with rat intestinal mucosa homogenate in the presence of α-tocopherol, γ-tocopherol or α-tocotrienol produced mainly retinoic acid, β-apo-13-carotenone and retinol (Table 1). The amounts of β-apo-13-carotenone formed in the presence of α-tocopherol, γ-tocopherol or α-tocotrienol was at almost 10 fold lower than that of incubation of β-carotene without antioxidant (Table 1). In the absence of antioxidants 30.7 ng of β-apo-13-carotenone was formed as compared to 2.1 ng in the presence of α-tocopherol, 3.1 ng in the presence of γ-tocopherol and 3.9 ng in the presence of α-tocotrienol, showing significant inhibition of β-apo-13-carotenone formation ($p<0.05$) (Table 1). Retinal was only detected in β-carotene incubation in the presence of total γ-oryzanol, γ-tocotrienol antioxidants and when no antioxidants were present (Table 1). There was no significant difference in the amount of retinol formed between all the groups (Table 1).

Discussion

Incubation of plant food lipid soluble extracts with rat intestinal mucosa homogenate

A review of published literature shows that this is the first study in which extracts of plant foods have been incubated with intestinal homogenate to study β-carotene cleavage products. Methanol and THF solvent system was used to extract both

amphiphilic and lipophilic antioxidants from the kale and biofortified yellow maize. When kale extracts were incubated with rat intestinal mucosa homogenate the β-carotene cleavage products detected were retinoic acid and β-apo-13-carotenone (Figure 1). The kale extracts did not contain any detectable levels of vitamin E and γ-oryzanol. This may explain why more excentric cleavage product β-apo-13-carotenone was the major product formed. When biofortified yellow maize extracts which contained significant contents of α-tocopherol, γ-tocopherol and γ-oryzanol were incubated with rat intestinal mucosal homogenate the β-carotene cleavage products detected were retinol, retinoic acid, retinal and β-apo-13-carotenone and β-apo-12-carotenal (Table 1). The excentric cleavage product β-apo-13-carotenone formed from maize extracts was less than that from kale (Figure 1). This may suggest that antioxidants in maize extract were able to inhibit excentric cleavage of β-carotene. This study finding suggests that antioxidants rich foods when consumed with β-carotene may be enough to prevent the excessive formation of excentric cleavage products. However, efforts such as biofortification may be required to boost the antioxidants in provitamin A carotenoid rich plant foods.

The β-carotene cleavage products by rat intestinal mucosa homogenate

When β-carotene standard was incubated with rat intestinal homogenate without any antioxidant, the major cleavage products were from excentric cleavage pathway (Table 1 and Figure 2). These products were β-apo-13-carotenone and β-apo-14-carotenal as observed in other studies [14, 23-18]. These excentric cleavage products were confirmed *in vitro* from β-carotene incubation with intestinal mucosa homogenates from rat, ferret, monkey and humans [23]. Our previous studies show that in the absence of

antioxidants, β-carotene is attacked randomly by free radicals formed by lipoxygenase enzymes to form β-apo-carotenoids [14, 29-30]. Studies that used pure BCMO1 enzyme or purified post-mitochondrial fractions of intestinal homogenate for β-carotene incubation reported retinal as the only β-carotene cleavage product [15-18]. When crude intestinal homogenates were used as in this study, retinal, retinol and retinoic acids were detected (Table 1) [20, 22-23]. This is not surprising because crude intestinal homogenate still contained enzymes such as aldehyde dehydrogenases, NAD^+ and alcohol dehydrogenases that convert the retinal to retinol and retinoic acid [15-17]. Our study using crude rat intestinal mucosa homogenate is important because it may closely resemble the intestinal cell cytosolic conditions where β-carotene cleavage take place in the presence of these redox compounds. We can therefore imply that under cytosolic conditions the cleavage of β-carotene produces a variety of products which may include retinol, retinal, retinoic acid, and apocarotenoids. Increasing the physiological concentration of antioxidants such as α-tocopherol may lead to more central cleavage products of β-carotene.

Effect of antioxidants on β-carotene cleavage

When β-carotene was incubated with rat intestinal mucosa homogenate without antioxidants, more cleavage products were formed and in higher quantities (Table 1) (Figure 2). When β-carotene was incubated with rat intestinal mucosa homogenate in the presence of antioxidants such as α-tocopherol, γ-tocopherol, α-tocotrienol, γ-tocotrienol and γ-oryzanol standards, the amounts of excentric cleavage products (β-apo-13-carotenone and β-apo-14-carotenal) formed was significantly reduced compared to the β-

carotene incubation without antioxidants. This result confirms what we previously reported that the efficiency of the conversion of β-carotene to retinal was reduced 10 fold by omitting α-tocopherol in the β-carotene incubation mixture [14]. We also observed that the amounts and variety of β-carotene cleavage product was dependent on the type of antioxidant (Table 1). This suggests that the inhibition of β-carotene cleavage was by antioxidant protection from free radical attack. Alpha-tocopherol followed by γ-tocopherol and α-tocotrienol offered the greatest β-carotene from excentric cleavage. These observations maybe significant for human health, because *in vitro* incubations of β-carotene with post-nuclear fractions of lung tissue from cigarette smoke exposed-ferret led to more excentric cleavage of β-carotene forming an abundance of β-apo-carotenals, as a result of the free radical rich atmosphere in the lungs [36]. A diet rich in antioxidants typical of high fruit and vegetable intake may reduce β-carotene cleavage and β-apo-carotenoids formation among smokers [37-38]. Recent research suggests that β-apo-carotenoids may exert powerful biological effects on mammalian cells by a possible disruption of normal signaling through multiple ligand-activated nuclear receptors [39, 40]. Some research suggests that β-apo-carotenoids maybe involved in the development of lung cancer [38]. However, more research is required because the biological and metabolic implications of these signaling disruptions are not yet clear.

There was no significant difference in the amount of retinol formed between different β-carotene incubations with or without antioxidants ($p<0.05$) (Table 1). This showed that the amount of retinol formed was homeostatically controlled. This may also suggest that the amount of substrate (β-carotene) was not a limiting factor during the enzymatic cleavage of β-carotene and that the BCMO1 enyzme has a higher affinity for

retinol formation. Also retinal was not detected in β-carotene incubation with rat intestinal mucosa homogenate in the presence of α-tocopherol, γ-tocopherol and α-tocotrienol. This suggests that retinal an intermediate product of β-carotene cleavage was converted to retinoic acid faster in the presence of α-tocopherol, γ-tocopherol and α-tocotrienol. Also we observed that in the absence of α-tocopherol, γ-tocopherol and α-tocotrienol, β-apo-14-carotenal was detected. This may suggest that β-apo-14-carotenal is an intermediate product of excentric β-carotene cleavage. In the presence of α-tocopherol, γ-tocopherol and α-tocotrienol, the amount of β-apo-13-carotenone was significantly lowered, while the amounts of retinol was not affected (Table 1). This shows that these antioxidants promote central cleavage of β-carotene, and they protect β-carotene from oxidative cleavage. These findings are in agreement with our previous study which showed that in the presence of α-tocopherol β-carotene was exclusively cleaved to retinal; while in the absence β-apo-carotenoids were formed [14].

Conclusion

This study found that extracts of biofortified yellow maize containing antioxidants inhibit excentric cleavage of β-carotene. Our study also showed that antioxidants α-tocopherol, γ-tocopherol and α-tocotrienol promote central cleavage of β-carotene by inhibiting excentric cleavage of β-carotene to form β-apo-13-carotenone and β-apo-14-carotenal. This finding is of significance to people in developing countries who depend on plant food β-carotene for their vitamin A requirements. Dietary messages can be

tailored to promote intake of antioxidant rich foods such as nuts, peanut butters together with provitamin A carotenoid rich foods such as kale or biofortified maize.

Acknowledgements

Authors would like to thank Dr. Natalia Palacios at the International Maize and Wheat Improvement Center (CIMMYT) in El Batan, Mexico for generously providing samples of biofortified yellow maize. We also would like to thank Dr. Oliver Chen in the Antioxidant Research Laboratory at the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University for providing rat intestines. Funding for this research was made possible through USDA CRIS grant # 58-1950-0-014.

References

1. Gerster, H. Vitamin A-functions, dietary requirements and safety in humans. *Int. J. Vitamin. Nutr. Res.* 1997, 67 (2), 71-90.
2. West, K. P. Extent of vitamin A deficiency among preschool children and women of reproductive age. *J. Nutr.* 2002, 132 (9), 2857S-2866S.
3. Humphrey, J. H., West Jr, K. P., & Sommer, A. Vitamin A deficiency and attributable mortality among under-5-year-olds. *WHO Bull*. 1992, 70 (2), 225.
4. West, C. E., Eilander, A., & van Lieshout, M. Consequences of revised estimates of carotenoid bioefficacy for dietary control of vitamin A deficiency in developing countries. *J. Nutr.* 2002, 132 (9), 2920S-2926S.
5. Khan, N. C., West, C. E., de Pee, S., Bosch, D., Do Phuong, H., Hulshof, P. J., ... & Hautvast, J. G. The contribution of plant foods to the vitamin A supply of lactating women in Vietnam: a excentricized controlled trial. *Am. J. Clin. Nutr.* 2007, 85 (4), 1112-1120.
6. Ruel, M. T. Can food-based strategies help reduce vitamin A and iron deficiencies? A review of recent evidence. 2001, *Intl Food Policy Res Inst*. (Vol. 5).
7. Tang, G. Bioconversion of dietary provitamin A carotenoids to vitamin A in humans. *Am. J. Clin. Nutr.* 2010, 91 (5), 1468S-1473S.
8. De Pee, S., West, C. E., Permaesih, D., Martuti, S., & Hautvast, J. G. Orange fruit is more effective than are dark-green, leafy vegetables in increasing serum concentrations of retinol and beta-carotene in schoolchildren in Indonesia. *Am. J. Clin. Nutr*. 1998, 68 (5), 1058-1067.

9. Tanumihardjo, S. A. Food-based approaches for ensuring adequate vitamin A nutrition. *Comp Rev Food Sci Food Safety*. 2008, 7, 373-81.
10. Muzhingi, T., Gadaga, T. H., Siwela, A. H., Grusak, M. A., Russell, R. M., & Tang, G. Yellow maize with high β-carotene is an effective source of vitamin A in healthy Zimbabwean men. *Am. J. Clin. Nutr*. 2011, 94 (2), 510-519.
11. Tang, G., Qin, J., Dolnikowski, G. G., Russell, R. M., & Grusak, M. A. Golden Rice is an effective source of vitamin A. *Am. J. Clin. Nutr*. 2009, 89 (6), 1776-1783.
12. Yeum, K. J., & Russell, R. M. Carotenoid bioavailability and bioconversion. *Annu. Rev. Nutr.* 2002, 22 (1), 483-504.
13. Goodman, D. S., Blomstrand, R., Werner, B., Huang, H., & Shiratori, T. The intestinal absorption and metabolism of vitamin A and beta-carotene in man. *J* Clin Invest. 1966, 45 (10), 1615.
14. Yeum, K. J., Smith, A. F., Krinsky, N. I., & Russell, R. M. The effect of α-tocopherol on the oxidative cleavage of β-carotene. *Free Radical Biol. &. Med.* 2000, 29 (2), 105-114.
15. Olson, J. A., & Hayaishi, O. The enzymatic cleavage of beta-carotene into vitamin A by soluble enzymes of rat liver and intestine. *Proceedings of the National Academy of Sciences of the United States of America.*1965, 54 (5), 1364.
16. Goodman, D. S., Huang, H. S., Kanai, M., & Shiratori, T. The enzymatic conversion of all-trans β-carotene into retinal. *J. Biol. Chem.* 1967, 242 (15), 3543-3554.

17. Nagao, A., During, A., Hoshino, C., Terao, J., & Olson, J. A. Stoichiometric Conversion of< i> all trans</i>-β-Carotene to Retinal by Pig Intestinal Extract. *Arch. Biochem. Biophys.* 1996, 328 (1), 57-63.
18. Kim, Y. S., & Oh, D. K. Substrate specificity of a recombinant chicken β-carotene 15, 15′-monooxygenase that converts β-carotene into retinal. *Biotechnol Lett.* 2009, 31 (3), 403-408.
19. Napoli, J. L., & Race, K. R. Biogenesis of retinoic acid from beta-carotene. Differences between the metabolism of beta-carotene and retinal. *J. Biol. Chem.* 1988, 263 (33), 17372-17377.
20. Wang, X. D., Krinsky, N. I., Tang, G., & Russell, R. M. Retinoic acid can be produced from excentric cleavage of β-carotene in human intestinal mucosa. *Arch. Biochem. Biophys.*1992, 293 (2), 298-304.
21. Russell, R. M. The enigma of β-carotene in carcinogenesis: what can be learned from animal studies. *J. Nutr.*2004, 134 (1), 262S-268S.
22. Krinsky, N. I., Wang, X. D., Tang, G., & Russell, R. M. Conversion of carotenoids to retinoids. *Am J Clin Dermato.* 1993, 5, 1-1.
23. Tang, G., Wang, X. D., Russell, R. M., & Krinsky, N. I. Characterization of beta-apo-13-carotenone and, beta.-apo-14'-carotenal as enzymic products of the excentric cleavage of beta-carotene. *Biochem.* 1991, 30 (41), 9829-9834.
24. Wang, X. D., Tang, G. W., Fox, J. G., Krinsky, N. I., & Russell, R. M. Enzymatic conversion of β-carotene into β-apo-carotenals and retinoids by human, monkey, ferret, and rat tissues. *Arch. Biochem. Biophys.* 1991, 285 (1), 8-16.

25. During, A., & Harrison, E. H. Intestinal absorption and metabolism of carotenoids: insights from cell culture. *Arch. Biochem. Biophys.* 2004, 430 (1), 77-88.
26. Ho, C. C., de Moura, F. F., Kim, S. H., & Clifford, A. J. Excentral cleavage of β-carotene in vivo in a healthy man. *Am. J. Clin. Nutr*. 2007, 85 (3), 770-777.
27. Hu, K. Q., Liu, C., Ernst, H., Krinsky, N. I., Russell, R. M., & Wang, X. D. The biochemical characterization of ferret carotene-9′, 10′-monooxygenase catalyzing cleavage of carotenoids in vitro and in vivo. *J. Biol. Chem.* 2006, 281 (28), 19327-19338.
28. Wang, X. D. Carotenoid oxidative/degradative products and their biological activities. *Carotenoids in health and disease*, 2004, 313-335.
29. Yeum, K.-J.; Lee-Kim, Y. C.; Yoon, S.; Lee, K. Y.; Park, I. S.; Lee, K. S.; Kim, B. S.; Tang, G.; Russell, R. M. Similar metabolites formed from β-carotene by human gastric mucosal homogenates, lipoxygenase, or linoleic acid hydroperoxide. *Arch. Biochem. Biophys.* 1995, 321:167-174.
30. Handelman, G. J.; van Kuijk, F. J. G. M.; Chatterjee, A.; Krinsky, N. I. Characterization of products formed during the autoxidation of b-carotene *Free Radical Biol. &. Med*. 1991, 10:427-437; 1991.
31. Rocheford, T. R., Wong, J. C., Egesel, C. O., & Lambert, R. J. Enhancement of vitamin E levels in corn. *J Am Coll Nutr.* 2002, 21(sup3), 191S-198S.
32. Cao, G., Sofic, E., & Prior, R. L. Antioxidant capacity of tea and common vegetables. *J. Agric. Food Chem. 1996*, 44 (11), 3426-3431.

33. Graham, R. D., Welch, R. M., & Bouis, H. E. Addressing micronutrient malnutrition through enhancing the nutritional quality of staple foods: principles, perspectives and knowledge gaps. *Adv Agron.* 2001, 70, 77-142.
34. Novotny, J. A., Kurilich, A. C., Britz, S. J., & Clevidence, B. A. Plasma appearance of labeled β-carotene, lutein, and retinol in humans after consumption of isotopically labeled kale. *J. Lipid Res.* 2005, 46 (9), 1896-1903.
35. N.I. Krinsky, D.G. Cornwell, J.L. Oncley, The transport of vitamin A and carotenoids in human plasma. *Arch. Biochem. Biophys.* 1958, 73 (1), 233-246 (http://www.sciencedirect.com/science/article/pii/0003986158902595)
36. Liu, C., Wang, X. D., Bronson, R. T., Smith, D. E., Krinsky, N. I., & Russell, R. M. Effects of physiological versus pharmacological β-carotene supplementation on cell proliferation and histopathological changes in the lungs of cigarette smoke-exposed ferrets. *Carcinogenesis.* 2000, 21 (12), 2245-2253.
37. Liu, C., Russell, R. M., & Wang, X. D. α-Tocopherol and Ascorbic Acid Decrease the Production of β-Apo-carotenals and Increase the Formation of Retinoids from β-Carotene in the Lung Tissues of Cigarette Smoke–Exposed Ferrets In Vitro. *J. Nutr*, 2004, 134 (2), 426-430.
38. Wang, X. D., Marini, R. P., Hebuterne, X., Fox, J. G., Krinsky, N. I., & Russell, R. M. Vitamin E enhances the lymphatic transport of β-carotene and its conversion to vitamin A in the ferret. *Gastroenterology.* 1995, 108 (3), 719-726.
39. Harrison, E. H., dela Sena, C., Eroglu, A., & Fleshman, M. K. The formation, occurrence, and function of β-apocarotenoids: β-carotene metabolites that may

modulate nuclear receptor signaling. *Am. J. Clin. Nutr*. 2012, 96 (5), 1189S-1192S.

40. Eroglu, A., Hruszkewycz, D. P., dela Sena, C., Narayanasamy, S., Riedl, K. M., Kopec, R. E., & Harrison, E. H. Naturally occurring excentric cleavage products of provitamin A β-carotene function as antagonists of retinoic acid receptors. *J. Biol. Chem.* 2012, 287 (19), 15886-15895.

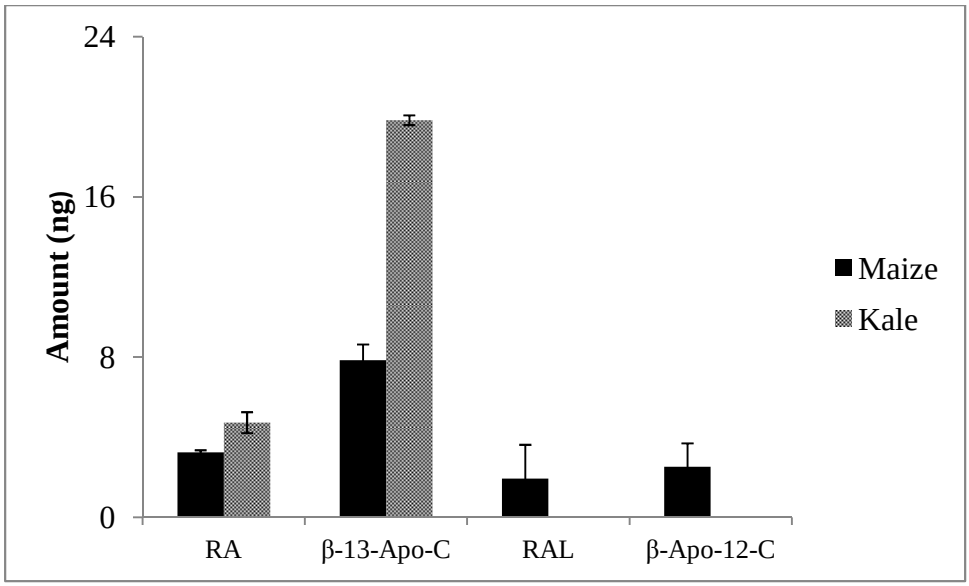

Figure 1: The cleavage products formed from β-carotene-rich extracts of kale and biofortified yellow maize incubated with rat intestinal mucosa homogenate. [RA (retinoic acid), β-apo-13-car (β-apo-13-carotenone), and β-apo-12-car (β-apo-12-carotenal)]

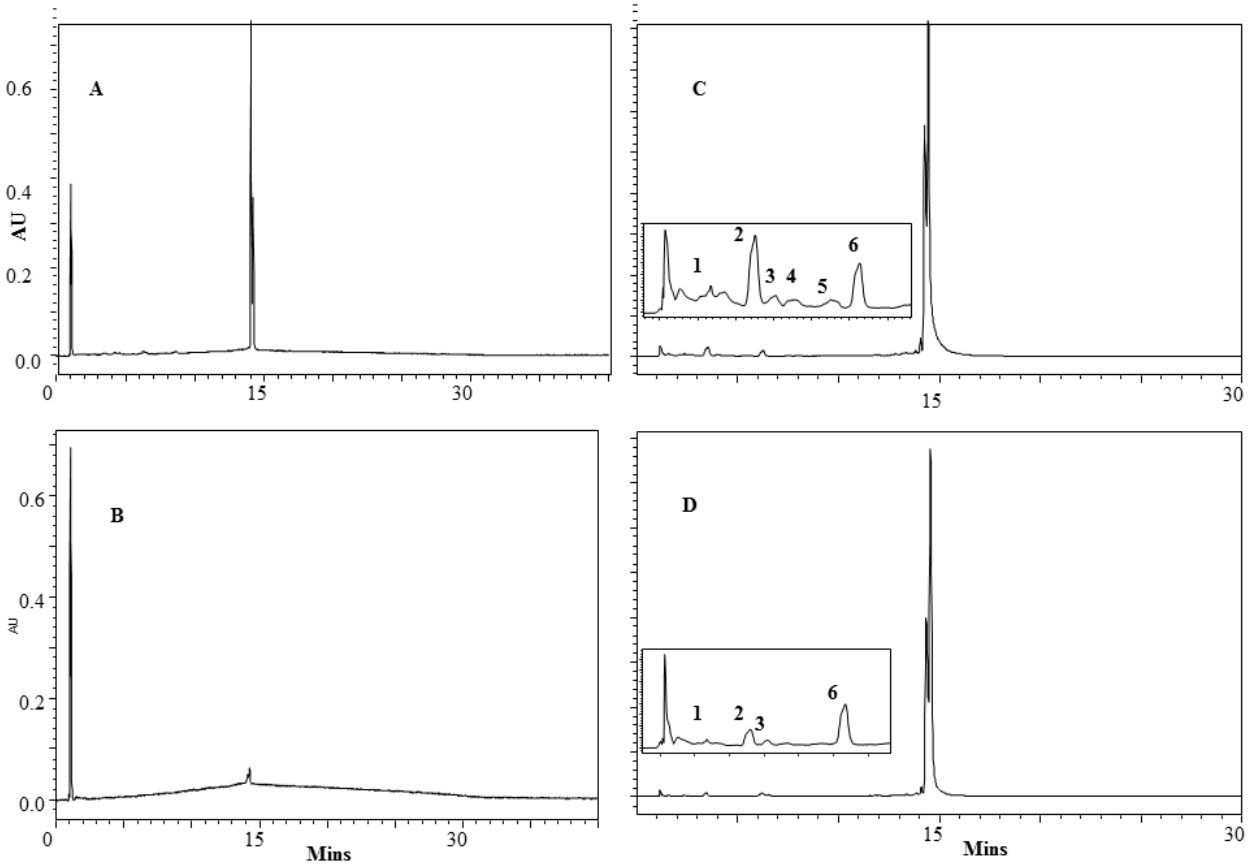

Figure 2: Panel A shows control A β-carotene incubated without rat intestinal homogenate with and Panel B control B rat intestinal homogenate without β-carotene. Panel C shows β-carotene incubation products with rat intestinal mucosal homogenate in the absence of an antioxidant. Insert C shows the peaks 1(retinoic acid), 2 (β-apo-13-carotenone), 3 (retinol), 5 (β-apo14-carotenal). Panel D shows cleavage products from β-carotene incubated with rat intestinal homogenate in the presence of an antioxidant (γ-tocopherol). Insert D shows the peaks 1 (retinoic acid), 2 (β-apo-13-carotenone) and 3 (retinol).

Table 1: Cleavage products formed from β-carotene incubated with rat intestinal mucosa homogenate with or without an antioxidant

Treatment/	β-carotene incubation cleavage products (ng)				
	Retinoic acid	β-apo-13-carotenone	Retinol	Retinal	β-apo-14-carotenal
β-carotene*	0.55 (0.03) ^{B}	30.7 (1.77) ^{C}	3.85 (0.17) ^{G}	7.48 (2.06) ^{H}	10.59 (2.01) ^{J}
BC + α-TP	0.43 (0.17) ^{B}	2.09 (0.10) ^{D}	3.56 (0.70) ^{G}		
βC+ γ-TP	0.72 (0.32) ^{AB}	3.05 (0.52) ^{D}	4.02 (1.39) ^{G}		
βC+ α-TT	0.79 (0.20) ^{AB}	3.9 (1.24) ^{DE}	3.27 (0.16) ^{G}		
βC + γ-TT	1.15 (0.02) ^{A}	6.0 (0.40) ^{E}	4.59 (0.51) ^{G}	3.54 (0.72) ^{I}	
βC + γ-Oryzanol	0.40 (0.16) ^{B}	16.5 (0.54) ^{F}	4.31 (0.31) ^{G}	4.55 (0.89) ^{IH}	5.28 (0.35) ^{K}

Letters in superscript show statistical differences between treatments for each cleavage product, with the same letters showing no significant differences ($p>0.05$), and different letters showing significant difference ($p<0.05$).

β-carotene* (no antioxidant added)

CHAPTER 5

Carotenoid characterization of *Brassica oleracea var. acephala* varieties in Zimbabwe

Carotenoid characterization of *Brassica oleracea var. acephala* varieties in Zimbabwe

Tawanda Muzhingi [1, 2], Andrew H. Siwela[3], Kyung-Jin Yeum [4], Odilia Bermudez [5] and Guangwen Tang [1, 2]

1. Carotenoids and Health Laboratory, Jean Mayer USDA ARS Human Nutrition Research Center on Aging at Tufts University, 711 Washington street, Boston, MA, 02111
2. Gerald J. and Dorothy R. Friedman School of Nutrition Science and Policy, 150 Harrison Avenue, Boston, MA, 02111
3. Department of Applied Biology and Biochemistry, Faculty of Applied Sciences, National University of Science and Technology, Ascot, Bulawayo, Zimbabwe
4. Division of Food Bioscience, College of Biomedical and Health Sciences. Konkuk University, Glocal Campus, Chungju-Si, Chungcheongbuk-do, 380-701, South Korea
5. Department of Public Health and Community Medicine, Tufts School of Medicine, 136 Harrison Avenue, Boston, MA, 02111

Corresponding author

Tawanda Muzhingi

Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University

711 Washington Street, Boston, MA, 02111.

Email: tawanda.muzhingi@tufts.edu

ABSTRACT

Green vegetables of the brassica oleracea family are an important part of Zimbabwean diet, consumed daily as casseroles together with the maize based staple food. These green vegetables are rich in carotenoids such as lutein and β-carotene which are important for eye health and are precursors of vitamin A. In this study five different brassica oleracea vegetable commonly grown and consumed in Zimbabwe were identified as *Chou Moellier, covo, rugare, viscose* and drum head cabbage. The green leafy vegetables were cut into pieces and were pureed; the carotenoids were extracted with methanol and THF. The carotenoid contents and profile were compared to kale grown and consumed in the US. Statistical Analysis was conducted with SAS using descriptive statistics and ANOVA. All vegetables analyzed contained lutein, trans β-carotene and 9 cis β-carotene with the contents being significantly different among the 6 varieties for lutein and trans β-carotene. Kale grown in the USA had the highest content of lutein (12.5 ± 1.3) and β-carotene (8.5 ± 0.8) mg/100g fresh weight respectively. Among the Zimbabwean green vegetables, covo variety had the highest contents of lutein (10.4 ± 0.5), viscose variety had highest β-carotene 7.8 ± 0.3 mg/100g fresh weight content, while rugare variety (5.2 ± 0.3) had the lowest trans β-carotene content among the local varieties. Zimbabwe green vegetables had a 13 cis β-carotene isomer as opposed to the US kale. Zimbabwe Viscose variety also had phytoene and phytofluene carotenoid compounds. The Zimbabwean *Brassica oleracia* vegetables are a very good source of β-carotene and lutein. The provitamin A carotenoids content is sufficient enough to justify the promotion of these vegetables as part of a diet to combat vitamin A deficiency which is a public health problem in Zimbabwe.

INTRODUCTION

Food based dietary strategies are gaining popularity as effective and sustainable strategies to address vitamin A deficiency (VAD) in developing countries [1]. Promoting the consumption of locally available and underutilized food crops is sustainable and ideal because it does not require complex behavior change and affects people even in remote areas [3]. VAD is a public health problem health problem among children under 5 years in poor countries of east and southern Africa [4]. Green vegetables such as kale rich in provitamin A carotenoids are widely grown and consumed by adults. The provitamin A carotenoids (α-carotene, β-cryptoxanthin and β-carotene) are precursors of vitamin A, an essential nutrient required for vision, gene regulation, growth, immune function and skin health [5, 6]. Kale (*Brassica oleracea* var. *acephala*) ranks highest among all leafy vegetables for lutein and β-carotene content [7-9]. The contents of lutein have been shown to range from 8-39.5 mg/100 g fresh weight, and 2.8-14.5 mg/100 g fresh weight for β-carotene [9, 10]. Epidemiological studies also show additional benefits to the consumption of vegetables from Brassica oleracea such as reduced risk of cancer, and protection against degenerative diseases [11, 12]. However, the consumption of green vegetables among children under 5 years old is very low in Zimbabwe [13].

Brassica oleracea var. *acephala* (kale) is a cool weather vegetable that is grown and harvested for its edible leaves [14]. Kale is genetically similar to spring greens and collard greens, cabbage, Brussels sprouts, broccoli, cauliflower and kohlrabi [15]. Kale is propagated vegetatively in Zimbabwe and it is popular among the poor because it grows fast, allows repeated pickings and the plants can grow for years reaching a height of 2-3

meters [16]. In Zimbabwe, kale varieties are classified by various local names such as *rugare, viscose, Chou Moellier and covo* [16]. Viscose and Chou Moellier are varieties developed from rugare [15]. *Chou Moellier* is popular for commercial productions because of its leaves are darker green and more pronouncedly curled than rugare and viscose [16]. *Chou Moellier* is propagated by seed and the plants are comparatively short with short internodes and very thick stems, with large, dark green, somewhat curly leaves. *Covo* is similar to Portuguese kale. *Sukuma wiki* is grown widely in Kenya is mostly propagated by seed, sometimes vegetatively and is rarely found in Zimbabwe [17]. In Kenya households purchased and consumed about 13 kg of kale per month [18].

Although the carotenoid contents of *brassica oleracea* vegetables have been quantitated previously, the carotenoid profile and contents of *brassica oleracea* sub species developed and adapted for Zimbabwe has not been reported. Because of the reported high β-carotene contents of *brassica oleracea* vegetables and the additional health benefits of vegetables consumption, the objective of this study was to characterize the carotenoids in kale varieties commonly grown and consumed in Zimbabwe. In this study we assayed the edible portions of five kale varieties *rugare, covo, viscose, Chou Moellier.* Zimbabwean Cabbage (brassica oleracea var. capitata) green leaves were collected for analysis because when in season cabbages are used as kale substitutes because they become cheaper. US grown kale was also analyzed and compared to the carotenoid profile and contents of its Zimbabwean counterparts.

METHODS AND MATERIALS

Plant Material

Kale and cabbage varieties grown and consumed in Zimbabwe were identified by agricultural extension officers in the Ministry of Agriculture, Government of Zimbabwe (GOZ) and vegetables breeders at the Agricultural Research Trust (ART) in Zimbabwe. The vegetable samples were harvested from gardens in Harare, and shipped under ice to Tufts University in Boston, MA. Approximately equal amounts of tissue were collected from three plants. Kale leaves and green leaves of the cabbage were collected for analysis. Kale samples grown in the US were shipped from USDA ARS Children's Nutrition Research Center at Baylor College of Medicine in Houston, TX. The kale samples were stored fresh at 4ºC on arrival in the laboratory and were analyzed within 24 hours of receipt. The vegetables samples were analyzed fresh to determine the natural carotenoid profile without adjustments for moisture contents. To achieve this vegetables sample were kept fresh at 4°C and were analyzed immediately without further storage.

Carotenoid Extraction and Analysis

All experimental procedures were conducted under red-yellow lights. Carotenoids were extracted from leaves of kale and cabbages using a modified procedure published by Riso and Porrini [19]. Briefly, leaves of vegetables were cut into small pieces and weighed into 1 g samples of three replicated per variety. Extraction was performed by homogenizing with a Polytron PT1600E homogenizer 1 g of green vegetable leaves cuts in 10 mL methanol, incubating for 2 hours at room temperature and vortexing at 30 min intervals. The mixture was centrifuged at *800 x g* for 10 min at 4ºC. The methanol layer

was transferred into a 50 mL volumetric flask and the extraction repeated four times with 10 mL of Tetrahydrofuran (THF), followed by vortexing and centrifugation. The THF layers were combined with the methanol layer and the volume brought up to 50 mL. One mL of the extract was taken, dried under nitrogen, and re-suspended in 1 mL of ethanol and 20 μL were injected into an HPLC.

The HPLC gradient profile and solvent system were as described in previously published method [20]. Briefly, the extracted sample was analyzed for carotenoids using a reverse-phase, gradient HPLC system (14). The HPLC system consisted of a Waters Alliance 2695 Separation Module LC pump, autosampler, Waters 2996 Photo Array Detector (Millipore, Milford, MA), and a C30 column (3 μm, 150 x 4.6 mm, YMC, Germany). The chromatographic separations were performed on a Waters Alliance 2695 HPLC (Waters, Milford, MA) system using a UV detector and Waters Empower2 software. The carotenoids were separated at a flow rate of 0.4 mL/min and by a gradient elution with two mixtures of methanol, tert-butyl methyl ether, and water [mixture A: 83/15/2 (v/v/v), mixture B: 8/90/2 (v/v/v); gradient procedures were: 0 to 1 min 100% A, 1 to 8 min linear gradient to 70% A, 8 to 13 min 70% A, 13 to 22 min linear gradient to 45% A, 22 to 24 min 45% A, 24 to 34 min linear gradient to 5% A, 34 to 38 min 5% A, 38 to 40 min linear gradient to 100% A, and 40 to 50 min 100% A]. Carotenoids were quantified by determining the peak area at 450 nm wavelength in the HPLC chromatogram calibrated against known amount of standards. The lower limit of detection for this method was 0.2 pmol for carotenoids.

Statistical Analysis

All statistical analysis was performed using Statistical Analysis Software (SAS) Inc. NC version 9.3. Descriptive statistics were performed for each carotenoid variable, and Proc GLM procedure was used to determine the differences in the carotenoid contents among the six vegetables varieties. Tukey post hoc procedure was used to perform multiple comparisons of carotenoids the different *Brassica oleracea* vegetables varieties.

RESULTS

The major carotenoids found in all the *Brassica oleracea* vegetables were lutein, all trans β-carotene and lutein. The lutein content was significantly different among the six vegetable varieties analyzed ($p<0.01$) (Figure 1). US kale had the highest lutein content with 12.5 ± 1.3 mg/100 g fresh weight. Rugare variety which had the lowest lutein content of 7.3 ± 0.5 mg/100 g fresh weight was significantly different from the *Chou Moellier* and US kale ($p<0.01$). The trans β-carotene content was higher in the US kale. There was a significant difference in the β-carotene contents of rugare to that of US kale and *Chou Moellier* ($p<0.01$) (Figure 2). Among the Zimbabwean brassica oleracea vegetables the *viscose* variety had the highest β-carotene content of 7.6 ± 0.3 mg/100g. All the vegetable varieties analyzed had 9 cis β-carotene and a content of 1.2 ± 0.2 mg/100g fresh weight and there were no significant difference among all the varieties ($p>0.05$) (Figure 3). Only the Zimbabwean brassica vegetables had 13 cis β-carotene isomer, with mean contents of 0.5 ± 0.1 mg/100g fresh weight, no significantly

differences were detected among the varieties ($p>0.01$). Only the viscose variety had detectable peaks of phytoene and phytofluene.

DISCUSSION

This study showed that kale is a good source of lutein as previously shown by other investigators [21]. However, the lutein content of the Zimbabwean kale varieties was not known. Lutein is an important nutrient for eye health. Inverse associations have been reported between the incidence of advanced, neovascular, age-related macular degeneration (AMD) and the combined lutein and zeaxanthin intake in the diet [22]. Recent studies also show that higher dietary intake of lutein was associated with a significantly decreased risk of cataract formation [23]. Cataracts are major cause of blindness in Zimbabwe [24]. Studies show that daily intake for lutein of 10 mg/day show a health benefit [25-27]. This study shows that Zimbabwean brassica oleracea vegetables have lutein contents of about 10 mg/100g fresh weight. This showed that Zimbabwean kale varieties very good sources of lutein. One cup of the *Brassica oleracea* var. *acephala* vegetable would meet this beneficial 10 mg/day which may offer protection against cataracts among the Zimbabwean elderly population.

Kale (*Brassica oleracea* var. *acephala*) ranks highest among all leafy vegetable crops for β-carotene content which ranges from 2.8-14.5 mg/100g fresh weight [28, 29]. Our study showed an average β-carotene content of 6.9 ± 1.4 mg/100g fresh weight. This is important because β-carotene is a provitamin A carotenoid, a precursor of vitamin A.

In developing countries people obtain more the 80% of the total vitamin A intake from plant food source, but vitamin A deficiency is still a public health problem [30-32]. In east and southern Africa *Brassica oleracea* var. *acephala* vegetables are a staple food, consumed everyday as relish to the maize thick porridges. However, the high β-carotene content of Zimbabwean kale makes it ideal as a complementary food for children aged 6-36 months who are at risk of VAD. Kale β-carotene was shown to be very bioavailable and easily converted to vitamin A in humans [33]. However, as this study was conducted in US male adults, it will be important to determine kale β-carotene vitamin A equivalency in Zimbabwean preschool children. Besides the vitamin A, a diet high in carotenoids has been associated with significant reductions in the risk of degenerative diseases.

There were no major significant differences in the lutein and β-carotene contents of the Zimbabwe brassica oleracea vegetable varieties and the US kale. No significant differences were observed also between kale (*Brassica oleracea* var *acephala*) and green leaves from cabbage (*Brassica acephala* var. *capitata*). This can be explained by the fact that the vegetables varieties analyzed belong to the same species and sub-family. The differences observed between the rugare, *Chou Moellier* and the US kale can be explained by differences due to season and maturity. Studies have shown that the carotenoid contents in vegetables of the same genotype will vary by season and maturity [29, 34 and 35].

CONCLUSION

This study showed that *Brassica oleracea* var. *acephala* vegetables grown and consumed in Zimbabwe are a good source of lutein and provitamin A carotenoids. The study also showed that there were no significant differences between brassica sub species of kale and cabbage *(Brassica oleracea var. capitata)* green leaves in their lutein and β-carotene content. This study shows that the β-carotene contents of kale can justify the promotion of kale as a vitamin A rich complementary food in east and southern Africa.

ACKNOWLEDGEMENTS

The authors would like to thank agricultural extension officers from Agritex and ART who helped identify the different brassica oleracea vegetables and cabbages grown and consumed in Zimbabwe. Funding support was from International Atomic Agency and USDA Financial support USDA contract #58-1950-0-014

REFERENCES

1. Greiner, T. Vitamin A: Moving the Food Based Approach Forward. http://www.fao.org/fileadmin/user_upload/agn/pdf/Greiner_VITAMIN_A_Final.pdf. *(Accessed 02/19/2014).* 2013.
2. Bruins, M., Kraemer, K., & Hopkins, J. Public health programmes for vitamin A deficiency control. *Do vitamin A deficiency and undernutrition still matter?*2013. http://www.cehjournal.org/wp-content/uploads/Do-vitamin-a-deficiency-and-undernutrition-still-matter.pdf#page=9 *(accessed 02/14/2014)*
3. Gibson, R. S., Hotz, C., Temple, L., Yeudall, F., Mtitimuni, B., & Ferguson, E. Dietary strategies to combat deficiencies of iron, zinc, and vitamin A in developing countries: development, implementation, monitoring, and evaluation. *Food Nutr Bull* 2000, 21 (2), 219-231.
4. Ministry of Health and Child Welfare. Nutrition Unit. Zimbabwe national micronutrient survey: 1999. Harare, Zimbabwe: Ministry of Health and Child Welfare, 2001.
5. Stewart, G. F., Schweigert, B. S., Hawthorn, J., & Bauernfeind, J. C. *Carotenoids as Colorants and Vitamin A Precursors: Technological and Nutritional Applications*. *Edited by J. Christopher Bauernfeind, George F. Stewart, Bernard S. Schweigert and John Hawthorn*, 2012. ISBN: 978-0-12-082850-0 Elsevier.
6. Fujita, M., & Rendon, M. The Role of Vitamin A in Health of Infants and Vitamin A Status Assessment Methods. In *Nutrition in Infancy,* 2013. (pp. 441-455). Humana Press.

7. Kopsell DA, Kopsell DE, Lefsrud MG, Curran-Celentano J, Dukach LE. Variation in lutein, β-carotene, and chlorophyll concentrations among *Brassica oleracea* cultigens and seasons. *HortScience* 2004, 39a: 361–364
8. Kurilich, A. C., Tsau, G. J., Brown, A., Howard, L., Klein, B. P., Jeffery, E. H., & Juvik, J. A. Carotene, tocopherol, and ascorbate contents in subspecies of Brassica oleracea. *J Agric Food Chem* 1999, 47 (4), 1576-1581.
9. Sommerburg, O., Keunen, J. E., Bird, A. C., & van Kuijk, F. J. Fruits and vegetables that are sources for lutein and zeaxanthin: the macular pigment in human eyes. *Br J Ophthalmol* 1998, 82 (8), 907-910.
10. Khachik, F., Beecher, G. R., & Whittaker, N. F. Separation, identification, and quantification of the major carotenoid and chlorophyll constituents in extracts of several green vegetables by liquid chromatography. *J Agric Food Chem* 1986, 34 (4), 603-616.
11. Stoewsand, G. S. Bioactive organosulfur phytochemicals in< i> Brassica oleracea</i> vegetables-A review. *Food Chem Toxicol* 1995, 33 (6), 537-543.
12. van Leeuwen, R., Boekhoorn, S., Vingerling, J. R., Witteman, J. C., Klaver, C. C., Hofman, A., & de Jong, P. T. Dietary intake of antioxidants and risk of age-related macular degeneration. *Jama*, 2005, 294 (24), 3101-3107.
13. Ruel, M. T., Minot, N., & Smith, L. Patterns and determinants of fruit and vegetable consumption in sub-Saharan Africa: a multicountry comparison. Geneva: *WHO*. 2005
14. Splittstoesser, W. E. *Vegetable growing handbook*. Springer. 1990

15. Kushad, M. M., Brown, A. F., Kurilich, A. C., Juvik, J. A., Klein, B. P., Wallig, M. A., & Jeffery, E. H. Variation of Glucosinolates in Vegetable Crops of Brassica o leracea. *J Agric Food Chem* 1999, 47 (4), 1541-1548.
16. Grubben, G. J. (Ed.). *Vegetables* (Vol. 2). Prota. 2004.
17. Nekesa, P., & Meso, B. Traditional African vegetables in Kenya: production, marketing and utilization. In *Workshop report. IPGRI*. 1997
18. van der Lans, C., Snoek, H., de Boer, F., & Elings, A. Vegetable chains in Kenya. (2012). http://edepot.wur.nl/216710 *(Accessed 02/18/14)*
19. Riso, P., & Porrini, M. Determination of carotenoids in vegetable foods and plasma. *Int J Vitam Nutr Res* 1996, 67(1), 47-54.
20. Yeum, K. J., Booth, S. L., Sadowski, J. A., Liu, C., Tang, G., Krinsky, N. I., & Russell, R. M. Human plasma carotenoid response to the ingestion of controlled diets high in fruits and vegetables. *Am J Clin Nutr* 1996, 64 (4), 594-602.
21. Mangels, A. R., Holden, J. M., Beecher, G. R., Forman, M. R., & Lanza, E. Carotenoid content of fruits and vegetables: an evaluation of analytic data. *J Am Diet Assoc* 1993, 93 (3), 284-296.
22. Landrum, J. T., BONE, R. A., JOA, H., KILBURN, M. D., MOORE, L. L., & SPRAGUE, K. E. A one year study of the macular pigment: the effect of 140 days of a lutein supplement. *Exp Eye Res*, 1997, 65 (1), 57-62.
23. Brown, L., Rimm, E. B., Seddon, J. M., Giovannucci, E. L., Chasan-Taber, L., Spiegelman, D., & Hankinson, S. E. A prospective study of carotenoid intake and risk of cataract extraction in US men. *Am J Clin Nutr* 1999, 70 (4), 517-524.

24. Chipendo, G. N., January, J., Tapera, R., & Dube, B. Community perceptions of eye diseases among 14-40 year olds in Chiota, Zimbabwe. *Educational Research* 2012: 3 (10); 2141-5161.
25. Maci, S. The Role of Lutein in Eye Health. 2010
26. Koh, H. H., Murray, I. J., Nolan, D., Carden, D., Feather, J., & Beatty, S. Plasma and macular responses to lutein supplement in subjects with and without age-related maculopathy: a pilot study. *Exp Eye Res* 2004, 79 (1), 21-27.
27. Dagnelie, G., Zorge, I. S., & McDonald, T. M. Lutein improves visual function in some patients with retinal degeneration: a pilot study via the Internet. *Optometry* 2000, 71 (3), 147-164.
28. Sommerburg, O., Keunen, J. E., Bird, A. C., & van Kuijk, F. J. Fruits and vegetables that are sources for lutein and zeaxanthin: the macular pigment in human eyes. *Br J Ophthalmol* 1998, 82 (8), 907-910.
29. Kopsell, D. A., Kopsell, D. E., Lefsrud, M. G., Curran-Celentano, J., & Dukach, L. E. Variation in lutein, β-carotene, and chlorophyll concentrations among Brassica oleracea cultigens and seasons. *HortScience* 2004, 39 (2), 361-364.
30. de Pee, S.,Bloem, M.W.,Gorstein, J.,Sari, M.,Satoto,Yip, R.,Shrimpton, R.&Muhilal. 1998a. Reappraisal of the role of vegetables in the vitamin A status of mothers in Central Java, Indonesia. *Am J Clin Nutr* 1998, 68 (5a): 1068-1074.
31. Codjia, G. Food sources of vitamin A and provitamin A specific to Africa: An FAO perspective. *Food Nutr Bull* 2001, 22 (4): 357-360.
32. Kurilich, A. C., Britz, S. J., Clevidence, B. A., & Novotny, J. A. Isotopic labeling and LC-APCI-MS quantification for investigating absorption of carotenoids and

phylloquinone from kale (Brassica oleracea). *J Agric Food Chem* 2003, 51 (17), 4877-4883.

33. Novotny, J. A., Kurilich, A. C., Britz, S. J., & Clevidence, B. A. Plasma appearance of labeled β-carotene, lutein, and retinol in humans after consumption of isotopically labeled kale. *J Lipid Res* 2005, 46 (9), 1896-1903.
34. Kurilich, A. C., Tsau, G. J., Brown, A., Howard, L., Klein, B. P., Jeffery, E. H.,. & Juvik, J. A. Carotene, tocopherol, and ascorbate contents in subspecies of Brassica oleracea. *J Agric Food Chem* 1999, 47 (4), 1576-1581.
35. Mercadante, A. Z., & Rodriguez-Amaya, D. B. Carotenoid composition of a leafy vegetable in relation to some agricultural variables. *J Agric Food Chem* 1991, 39 (6), 1094-1097.

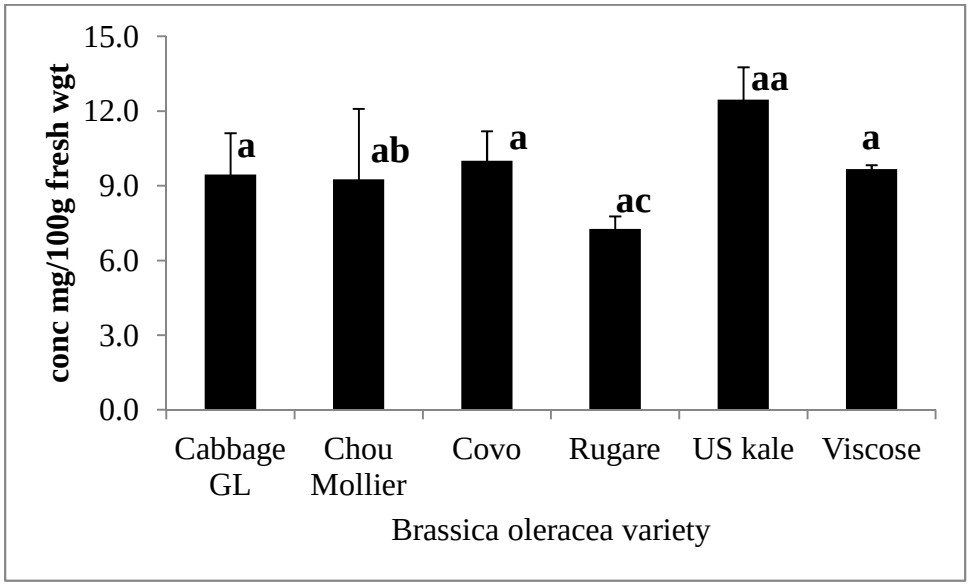

Figure 1: Lutein distribution among the Zimbabwean *Brassica oleracea* vegetable varieties. Different letters signify significant statistical differences ($p<0.05$).

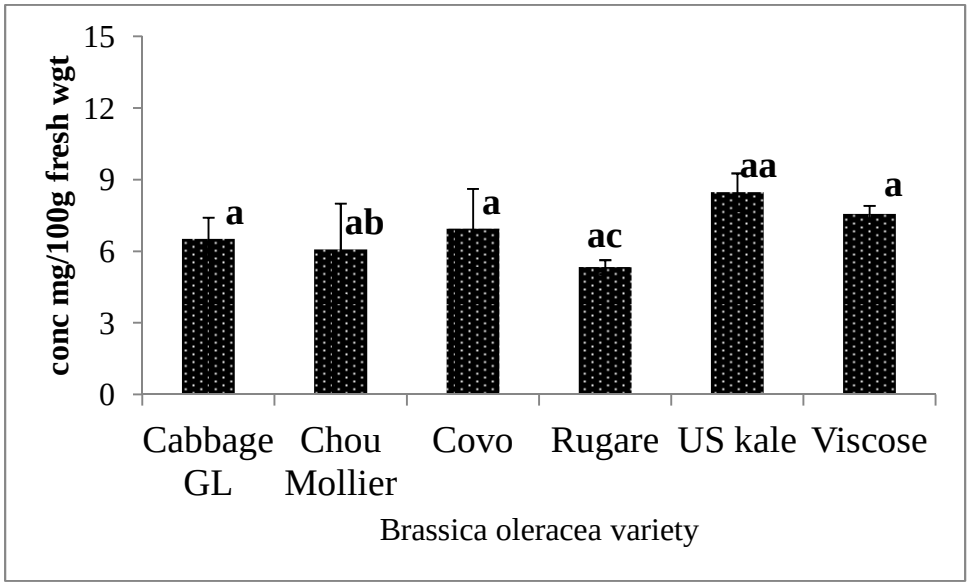

Figure 2: Distribution of trans β-carotene in the *Brassica oleracea* green vegetables. Different letters signify significant statistical differences ($p<0.05$).

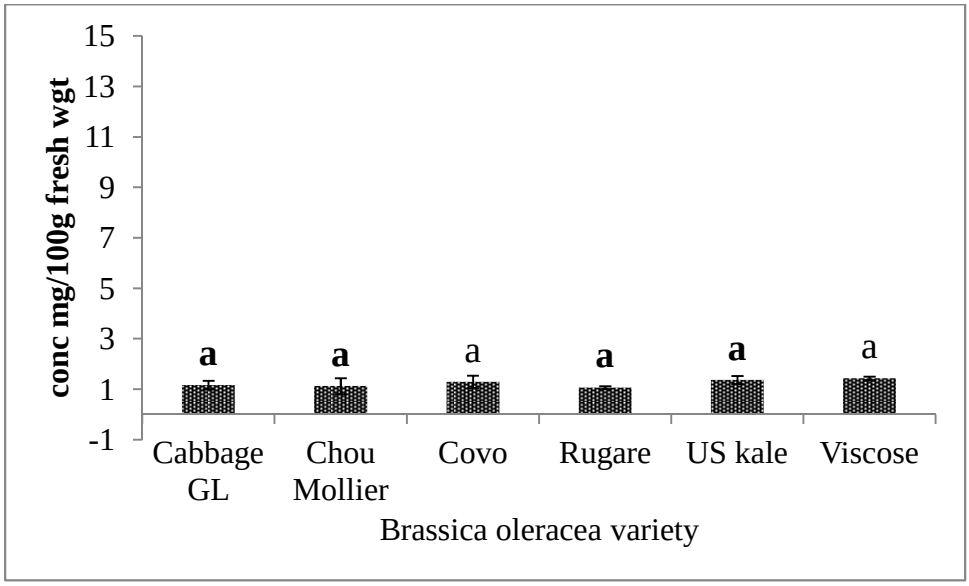

Figure 3: The distribution of 9 cis β-carotene among the six *Brassica oleracea* vegetables analyzed. Different letters signify significant statistical differences ($p<0.05$).

CHAPTER 6

Beta-carotene from peanut butter cooked kale is efficiently absorbed and converted to vitamin A in pre-school children

Beta-carotene from peanut butter cooked kale is efficiently absorbed and converted to vitamin A in pre-school children

Tawanda Muzhingi[1, 2], Andrew H. Siwela[3], Kyung-Jin Yeum[1, 4], Odilia Bermudez [5], Monica Muti [6], MGuangwen Tang [1, 2]

1. Carotenoids and Health Laboratory, Jean Mayer USDA ARS Human Nutrition Research Center on Aging at Tufts University, 711 Washington street, Boston, MA, 02111
2. Gerald J. and Dorothy R. Friedman School of Nutrition Science and Policy, 150 Harrison Avenue, Boston, MA, 02111
3. Department of Applied Biology and Biochemistry, Faculty of Applied Sciences, National University of Science and Technology, Gwanda Road, Bulawayo, Zimbabwe
4. Division of Food Bioscience, College of Biomedical and Health Sciences. Konkuk University, Glocal Campus, Chungju-Si, Chungcheongbuk-do, 380-701, South Korea
5. Department of Public Health and Community Medicine, Tufts School of Medicine, 136 Harrison Avenue, Boston, MA, 02111
6. Ministry of Health, National Nutrition Unit, Kagubi Building, Causeway, Harare, Zimbabwe.

Corresponding author

Guangwen Tang, PhD

guangwen.tang@tufts.edu

Abstract

Brassica oleracea var. *acephala* (kale) is a rich source of β-carotene that can be used to provide vitamin A to vitamin A deficient preschool children in Africa. This study was designed to determine the effect of peanut butter on the vitamin A equivalence and bioconversion efficiency of kale β-carotene to vitamin A in preschool children. Deuterium labeled kale was grown in a hydroponic medium with 23 atom % $^{2}H_2O$ during development. The intrinsically labeled kale β-carotene showed the highest abundance of enrichment as [$^{2}H_9$] β-carotene. Preschool children (*n* = 37; age 12-36 mo) were randomly assigned to consume 50 g cooked kale (1.5 mg β-carotene content) with either 33 g peanut butter (PBG) or with 16 g lard (LG). On day 1 after a baseline blood draw; all subjects consumed the labeled kale doses and 1 mg [$^{13}C_{10}$] retinyl acetate capsule. Blood samples were collected from five subjects per-time point per group over 21 d. Processed serum samples were analyzed by using NCI-GC/MS for the enrichments of labeled [^{2}H] retinol from kale [$^{2}H_9$] β-carotene and [$^{13}C_{10}$] retinol from reference dose. The area under the curve (AUCs) of molar enrichment at days 1, 2, 3, 6, 15, and 21 after the labeled doses was 56.3 ± 10.5 and 84.8 ± 16.2 (nmole) for [^{2}H] retinol from LG and PBG kale [$^{2}H_9$] β-carotene respectively. The AUC of [$^{13}C_{10}$] retinol from reference dose was 432.6 ± 54.9 (LG) and 560.3 ± 156.7 (nmole) (PBG) respectively. The calculated conversion factors were 13.4 ± 3.1 and 11.0 ± 3.9 to 1 ($p>0.05$) by weight for LG and PBG respectively. Peanut butter had higher retinol absorption response than the lard group. This study showed that kale β-carotene was efficiently converted to vitamin A in in children. Kale should therefore be promoted as a complementary food for children in developing countries who are vulnerable to VAD and general malnutrition.

Background

Children under the age of 5 years in sub Saharan Africa are fed starchy complementary foods that often lack essential nutrients such as vitamin A [1]. As a result, these children are at risk of developing vitamin A deficiency (VAD). According to the World Health Organization, an estimated 250 million preschool children are affected by VAD worldwide [2]. Providing vitamin A to those children could prevent about a third of all under-five deaths, which amounts to up to 2.7 million children that could be saved from dying unnecessarily [3]. Night blindness, a consequence of VAD, is estimated to affect 5.2 million preschool-age children worldwide [3]. Most of these children affected by VAD are in sub-Saharan Africa [2]. In Zimbabwe, VAD is a public health problem affecting more 40 % of children under the age of 5 years [4]. VAD compromises the immune systems of approximately 40 percent of children under five in the developing world, greatly increasing the severity of common childhood infections, often leading to deadly outcomes [5].

Plant based foods provide more than 80 % of the total vitamin A intake in developing countries [6]. This is because poor people in developing countries have limited or no access to vitamin A rich animal food products such as dairy, meats and poultry [7]. However, these people have easy access to fruits and vegetables they grow or buy cheaply from the markets. Fruits and vegetables are rich in provitamin A carotenoids such as β-carotene, β-cryptoxanthin and α-carotene [8-10]. Humans have the ability to convert these provitamin A carotenoids into vitamin A [11, 12]. In east and southern Africa, green vegetables are a staple food crop consumed daily [13, 14]. These green vegetables are either seasonal traditional (indigenous) vegetables or perennial

domesticated (foreign) vegetables. The most common domesticated vegetables are of the *Brassica oleracea var. acephala* family such as kale, collard green, cabbage and broccoli [15]. In east Africa kale and collard greens are known as *sukumi wiki*, while in southern Africa they are known as *covo, rugare, viscose, and Chou Moellier* [15, 16]. These *Brassica olerecea* vegetables are popular because they are easy to grow; they grow fast and are perennial [15]. They are found in almost every household garden in Zimbabwe and on street market stalls [13-16].

Kale has been shown to be very rich in β-carotene with contents ranging from 3-15 mg/100 g fresh weight [17, 18]. The β-carotene content is even higher than in some carrot varieties and other common green vegetables such as spinach [17, 19]. In one study to determine the bioavailability of [^{13}C] labeled kale β-carotene in humans showed that kale β-carotene was very bioavailable and was also efficiently converted to [^{13}C] retinol (vitamin A) in the body [20, 21]. However, this study was not designed to determine the conversion factor and vitamin A equivalence of kale β-carotene to vitamin A; as a result the amount of vitamin A formed was not quantitated. In the US, kale is used in some baby foods [22]. In southern Africa, particularly in Zimbabwe the diet of pre-school children is deficient in vegetables [1, 22]. Studies show that most common complementary foods given to children in Africa are starchy gruels made from maize, millet, sorghum or cassava whose nutrient density is very low [24, 25]. Some of these infant feeding practices are a result of lack of nutrition education, food availability and cultural practices [23].

Despite the ubiquitous presence of kale and daily consumption by adults in Zimbabwe, this provitamin A carotenoid rich green vegetable is not used as a

complementary food. There is a lack of information on the carotenoid contents of kale varieties grown in Zimbabwe, and also there are no human studies to date that show the vitamin A value of kale in humans especially in children. Promotion of kale as a complementary food in Zimbabwe will increase the vitamin A intake of children leading to a reduction of VAD. In Zimbabwe kale is prepared by boiling or sautéing. In some cases peanut butter is added to boiled kale to increase its palatability and nutrient density [26]. Peanut butter cooked kale can be a very nutritious complementary food, providing children with vitamin A, vitamin E, proteins and oils. These nutrients are important to child growth and development. The vitamin E and oil content of peanut can enhance the bioavailability of bioconversion of kale β-carotene to vitamin A [27].

The determination of the absorption and conversion of kale β-carotene to vitamin A is important for designing infant and young child feeding programs in many kale consuming regions of the world, where VAD is prevalent. In order to accurately assess the vitamin A value from a plant food source, researchers are utilizing intrinsically labeled plant material in which the β-carotene is labeled with a low abundance stable isotope. In this way it was possible to determine absorption of β-carotene from the food matrices and the subsequent conversion of the β-carotene to vitamin A and allowing the quantitative determination of the vitamin A equivalence of β-carotene as shown in previous studies [9, 12 and 28]. In this study in order to quantitatively evaluate the absorption and intestinal conversion of kale β-carotene, deuterium labeled kale and [$^{13}C_{10}$] labeled retinyl acetate reference dose were used.

Methods

Production of deuterium labeled kale and preparation of labeled kale doses

Intrinsically labeled kale was grown and harvested from a hydroponic plant system by growing the plants with heavy water (deuterium oxide, D_2O) at the USDA-Agriculture Research Service Children's Nutrition Research Center in Houston, TX. The procedures of growing the intrinsically deuterated plants are described in previous publications [9, 29]. One batch (2.7 kg) of freshly harvested deuterium labeled kale was shipped overnight under ice packs from the USDA/ARS Children's Nutrition Research Center, Houston, Texas to the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University in March 2012. Samples of the labeled kale were immediately analyzed for carotenoid contents and deuterium enrichment profile by HPLC and LC/MS (**Figure 1 and 3**). The rest of the labeled kale was weighed, vacuum packed and stored at -80°C.

One week after receiving the kale from Houston Texas, the labeled kale was cooked in the Metabolic Research Unit Kitchen (MRU) at the Jean Mayer USDA Human Nutrition Research on Aging at Tufts University using a previously published protocol for green vegetables [9]. Briefly, kale leaves were steamed for 10 mins. After steaming the leaves were pureed by homogenizing using a food processor. After homogenization, the cooked labeled kale was immediately analyzed for carotenoid contents by HPLC **(Figure 2)**. Fifty cooked kale doses weighing 50 g each (containing 1.52 mg of β-carotene content) were vacuum packed and stored at -80ºC for 16 months. In June of 2013, cooked labeled kale samples were analyzed for carotenoid contents before

shipment under ice-packs for 20 hours (h) to Bulawayo, Zimbabwe. The kale doses with ice-packs were still frozen upon arrival at the National University of Science and Technology (NUST), Bulawayo, Zimbabwe. The labeled kale doses were then stored at -80ºC. On day one of the study, the kale doses were thawed at room temperature. After thawing, 20 x 50 g of the cooked kale doses were each mixed 33 g of peanut butter and was microwaved for 1 min. Another 20 x 50 g cooked kale dose were each mixed with 16 g of lard and microwaved for 1 min. The 33 g of peanut butter contained the same amount of fat as 16 g of lard.

Human Subjects

Subjects were recruited from day care centers operated by the City of Bulawayo. Forty children aged between 12-36 months of age were screened and enrolled in the study. Thirty seven subjects successfully completed the study. Inclusion criteria was as follows; children not taking vitamin A and carotenoids supplements, who are generally healthy, with no history of liver, gastrointestinal disease, cancers and cardiovascular diseases that would interfere with vitamin A absorption and metabolism. The following situations excluded potential subjects from the study: severe and symptomatic cardiac disease, a history of bleeding disorders; chronic history of gastric, intestinal, liver, pancreatic, or renal disease; any portion of the stomach or the intestine removed; history of intestinal obstruction or malabsorption. Parental written informed consent was obtained from all subjects before enrolling in the study. Ethical approval for the study was obtained from Tufts Medical Center Institutional Review Board (IRB) and the Medical Research Council of Zimbabwe (MRCZ) in 2013 before enrollment of subjects.

Study design and procedures

In order to compute the power and sample size we used changes in serum retinol and standard deviations from our previous studies with dark green vegetables in children [30]. The required sample size was 5 children per group. However, given difficulty of drawing seven blood draws per child over a 21 d period, we designed the study using the published "Super Child Model" such that each time-point per group will have five randomly assigned subjects [31]. Each subject contributing a total of 3 (3-5 mL) blood draws for the 21 day duration of the study.

Six day care centers selected to participate in the study were randomly assigned to either Peanut Butter Group (PBG) or the Lard Group (LG). On day one of the study, subjects were assembled at their respective centers and a baseline 3-5 mL of blood (time = 0 h) was withdrawn into a no-additive Vacutainer™ from a forearm vein by a registered nurse from all subjects. The cooked labeled kale doses were thawed and mixed with 33 g (one teaspoon) peanut for the PBG and 16 g lard i.e. animal fat for LG and microwaved for 1 min. After cooling, subjects in each group consumed the labeled kale doses under the supervision of research staff to make sure everything was consumed. After consuming the kale doses subjects immediately ingested 1 mg [$^{13}C_{10}$] retinyl acetate (synthesized by the Cambridge Isotope Laboratory, Andover, MA) in 0.5 g corn oil capsule with a glass of water to wash down their mouths. The [$^{13}C_{10}$] retinyl acetate, which converts to [$^{13}C_{10}$] retinol once in circulation, was used as a reference dose to assess the conversion efficiency of the labeled kale β-carotene to retinol. Three to five mL of blood were withdrawn (an intravenous line was inserted for drawing these

samples) at 24, 48, 72 hours, days 6, 15, and 21 after the consumption of labeled kale and labeled retinyl acetate doses from 5 subjects per time point per group. Subjects consumed their normal diets the entire duration of the study.

Serum processing

After blood draws, blood samples were kept in a cooler box, and were then transported by car to National University of Science and Technology (NUST), Department of Applied Biology and Biochemistry Laboratory for processing. The blood samples were centrifuged at *800 x g* for 30 min at 4°C. The processed serum collected into 2 mL cryogenic vials, labeled and stored at -80°C until shipment under icepacks by air to the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University in Boston, MA, US. After an 18 h trip the serum vials were still frozen upon arrival in Boston in September, 2013.

Biochemical analysis of serum samples

The extraction of serum retinol, carotenoids, and tocopherols was conducted using a previously published method [32]. Briefly, three mL of chloroform: methanol (2:1, v/v) extraction of 100 µL serum sample fat soluble components was then followed by a 2 mL hexane extraction of the residue. The combined chloroform: methanol and hexane fraction were evaporated under N_2 gas on an N-EVAP (Organomation Associates Inc, South Berlin, MA). After complete dryness the residue was dissolved in 100 µL ethanol forming a clear solution, and 20 µL was injected into an HPLC system equipped with a YMC Carotenoid S C_{30} column (3.0 x 150mm). The HPLC equipment, gradient profile and quality assurance was as described in our previously published work [32].

LC-APCI-MS analysis of deuterium labeled kale

In order to determine the percentage enrichment of labeled β-carotene, 1 g of kale was weighed into a 50mL test-tube and extracted for carotenoids as described in published methods [33]. An LC-APCI-MS analysis of labeled kale β-carotene mass-to-charge ratios (*m/z*) was conducted as previously described [34]. Our kale β-carotene had the highest enrichment abundance of [$^{2}H_{9}$] β-carotene as 546 (M+ H^{+}+9) (Figure 3), and the deuterium enrichment was randomly distributed to all possible positions of the β-carotene molecule as determined by NMR [35].

NCI-GC/MS analysis of [^{2}H] retinol from deuterated kale and [$^{13}C_{10}$] retinyl acetate dose

In order to analyze the enrichment of labeled retinol from kale [$^{2}H_{9}$] β-carotene and labeled retinol from [$^{13}C_{10}$] retinyl acetate reference dose, serum samples from all subjects were extracted for retinol as described in published methods [36]. Briefly, 400 µL of the serum sample was extracted for retinol, and the reconstituted concentrated extract (50 µL) was injected into an HPLC apparatus equipped with a C_{18} column (Perkin-Elmer Inc, Norwalk, CT). Retinol fractions were collected using a Gilson Fraction collector FC203B model. The collected retinol fractions were dried under a gentle stream of nitrogen gas to complete dryness. To the dried residue 10 µL *N,O-bis* (trimethylsilyl) trifluoroacetamide containing 10% trimethylchlorosilane (Pierce, Rockford, IL) was added and heated for 30 minutes at 70° C to form retinyl trimethylsilyl ether [36]. After cooling, 3 µL was injected into an Agilent 6890 GC instrument equipped with a GC column was a Zebron ZB-1MS capillary (Phenomenex Inc, Torrance, CA) and 5973 Network Mass Selective Detector. The mass spectrometer was

set at a mass to charge ratio (*m/z*) of 260 to 280. The linearity of the GC-MS response and the detection limit of the gas chromatography/electron capture negative ionization-mass spectrometry (GC–ECNCI-MS) system have been previously addressed [37].

The kale β-carotene enrichment was fifty percent (the *m/z* 544 -548), hence an adjustment factor of 0.5 was used for the total enrichment of retinol formed from the labeled maize ß-carotene (**Equation 1**). The cleavage of [$^{2}H_{9}$] β-carotene as 546 (M+ H^{+}+9) produced labeled $M_{retinol}$ + 4 and $M_{retinol}$ + 5 ($^{2}H_{4}$- and $^{2}H_{5}$- retinol). The percentage enrichments measured by GC-MS and the concentration of retinol in serum were used to calculate the concentration of labeled retinol in the circulation. The mass to charge ratio of $M_{retinol}$ + 4 and $M_{retinol}$ + 5 = *m/z* 272 and 273 could be clearly detected and integrated to represent the formation of retinol from the labeled kale.

Equation 1:

Enrichment of labeled retinol from kale β-carotene:

= (∑ areas of *m*/*z* 272-273 / 50% / ∑ areas of *m*/*z* 268–280) [38]

In order to quantitate deuterium labeled vitamin A formed from kale [$^{2}H_{9}$] β-carotene, a reference dose differently labeled retinol was used. In this study the reference dose was given as 1 mg of [$^{13}C_{10}$] retinyl acetate capsule that was consumed on day 1 of the study just together with the labeled kale dose. During the NECI-GC/MS analysis the retinol *m/z* values are reduced by the mass of H_2O, which is removed from retinol during ionization in the mass spectrometer [37]. Therefore, $M_{retinol}$ -H_2O equals *m/z* 268 and *M*

$[^{13}C_{10}]_{retinol}$ equals *m/z* 268+10 = *m/z* 278 [28]. The total enrichment of labeled retinol was determined by evaluating the negative ions at $M_{retinol}$ [*m/z* 268-270 ($^{13}C_0$-$^{13}C_2$)], $M_{retinol}$ + 4 and $M_{retinol}$ +5 [*m/z* 272-273 ($^{2}H_4$-$^{2}H_5$)] and $M_{retinol}$ +10 [*m/z* 278-280 ($^{13}C_0$-$^{13}C_2$] [28]. The percentage enrichment of labeled retinol derived from $[^{13}C_{10}]$ retinyl acetate was calculated by integrating the peak area under the reconstructed mass chromatogram of the negative ions at *m/z*, 278, 279, and 280, divided by the total area response of labeled and unlabeled retinol fragment ions as shown by,

Equation 2:

Enrichment of ^{13}C retinol from $[^{13}C_{10}]$ retinyl acetate:

= ($\sum$ areas of *m*/*z* 278–280 / $\sum$ areas of *m*/*z* 268–280) (38)

Retinol equivalence calculations

The area under the curve (AUC) of the total serum $[^{2}H]$ retinol response from kale $[^{2}H_9]$ β-carotene (mmol vs time) was compared with the AUC of the vitamin A reference dose (1mg $[^{13}C_{10}]$ retinyl acetate; molecular mass = 336) were calculated using the KaleidaGraph Software, Synergy version 4.1.0 Reading, PA) and by SAS software using proc mixed procedure. The labeled $[^{2}H]$ retinol derived from kale $[^{2}H_9]$ β-carotene and $[^{13}C_{10}]$ retinyl acetate reference dose was calculated as enrichment multiplied (x) by retinol concentration x body weight x 0.0497, where body weight 3 0.0497 L/kg (for children of this age group according to the Blood Transfusion guidelines) was used to determine the total-body serum volume [39]. The retinol equivalence was calculated by

comparing the AUC of serum [^{2}H] retinol response from kale [$^{2}H_{9}$] β-carotene (nmole) to the AUC of 1 mg [$^{13}C_{10}$] serum response using Equation 3:

Equation 3:

[^{2}H] retinol from [^{2}H] kale β-carotene (nmole):

= (AUC of [^{2}H] retinol /AUC of [$^{13}C_{10}$] retinol) x [$^{13}C_{10}$] retinyl acetate/336* [38]

(*The molecular mass of [$^{13}C_{10}$] retinol = 336)

Conversion factor calculations

Equation 4:

The conversion factor of ß-carotene to retinol by weight [38]

= ß-carotene dose in kale (nmole) x (536)/

[^{2}H] retinol from ß-carotene dose (nmole) x (286)

Statistical Analysis

Statistical analyses were conducted using Statistical Analysis Software (SAS) Inc. (Cary, NC) version 9.3. Descriptive statistics were used to describe the distribution of the variables such as age, weight, height and BMI. Proc t-test was used to determine differences between baseline characteristics of subjects in PBG and LG. Area under the curve (AUC) response for [^{2}H] retinol from labeled kale β-carotene and [$^{13}C_{10}$] retinol from reference dose was calculated using KaleigdaGraph software (Synagey, PA). The

Proc MIXED Procedure of SAS was used to estimate the mean AUC and its standard error (SE) for kale [^{2}H] retinol, and [$^{13}C_{10}$] retinol enrichments. The delta method was used to estimate the SE of the conversion factors. A total of 37 subjects completed the study and each time-point analyzed per group had a minimum of three subjects of the required 5 subjects.

Results

There were no significant differences in the distribution of age, sex, weight, height, baseline serum retinol, tocopherols and carotenoids between the PBG and LG as shown in Table 1. The mean serum retinol levels were 44.7 ± 16.3 and 44.0 ± 19.8 µg/dL for the PBG and LG respectively. The mean serum β-carotene was 18.2 ± 8.1 and 18.6 ± 10.4 µg/dL for the PBG and LG respectively. All subjects had detectable levels of α-tocopherol and γ-tocopherol with the former being more predominant 1309 ± 640.9 and 1241 ± 731 µg/dL in the LG and PBG compared to 192.4 ± 142.1 and 218 ± 146.2 µg/dL for γ-tocopherol. The baseline serum vitamin A for all the subjects ranged from 9.5-90.5 µg/dL, three subjects were vitamin A deficient with serum retinol less than 20 µg/dL, 32 subjects had serum retinol between 20-90 µg/dL, and 2 subjects' serum retinol level was above 90 µg/dL (**Table 1**).

The major carotenoids found in kale were lutein, trans β-carotene and 9 cis β-carotene (**Figure 1**). The trans-β-carotene content of kale before cooking was 4.6 ± 0.4 mg/g fresh weight, and 1.5 ± 0.1 mg/g wet weight after cooking (**Figure 2**). The β-carotene content in the final cooked labeled kale was 1.52 mg/50 g wet weight. APCI-

LC/MS analysis of deuterium labeled kale β-carotene showed the most abundant enrichment peak at molecular mass+9 (**Figure 3**).

Enrichment peaks of retinol were detected in all blood samples after the ingestion of the [$^{2}H_{9}$] β-carotene and labeled reference dose of [$^{13}C_{10}$] retinyl acetate on day one of the study. Blood samples collected at 24, 48, 72 hr, days 6, 15 and 21 after ingestion of labeled kale doses and labeled reference dose were processed into serum and analyzed by GC/MS. The enrichment responses measured in AUCs of the labeled [^{2}H] retinol from labeled kale dose and for the [$^{13}C_{10}$] retinol were determined for LG and PBG as shown by Figure 4A and B. The calculations of the retinol equivalence of peanut butter cooked kale and lard cooked kale were performed by comparing the AUCs of labeled [^{2}H] retinol formed from the labeled kale β-carotene, to that of 1mg [$^{13}C_{10}$] retinol (Equation 1 and 2). The labeled β-carotene conversion factors of the LG and PBG kale β-carotene to vitamin A were then determined (Equation 1 and 2). Our study shows that the conversions of LG labeled kale β-carotene (1.52 mg) and PBG labeled kale β-carotene (1.52 mg) to retinol were 13.4 ± 3.1 and 11.0 ± 3.9 to 1 by weight and were not statisitically significant ($p>0.05$) (Table 2).

Discussion

VAD is a public health problem in Zimbabwe, where more than 40% of the children under 6-59 months had serum retinol levels <20 μg/dL [4]. In this study only 2 subjects representing 7.4% had VAD. This is not surprising because the subjects were recruited from day care centers in the city of Bulawayo. Children who attend day care

come from working families who can afford to buy vitamin A rich foods as compared to children who do not attend day care. In Zimbabwe, children in city day care centers are also fed nutritious foods compared to those at home. Also children in urban centers are exposed to a variety of foods purchased from a variety of sources as opposed to children in rural areas. The baseline serum carotenoids in the subjects are similar to those in western subjects [32]. Serum carotenoids are used as a biomarker of fruit and vegetable intake [40]. Higher serum carotenoids reflect intakes of fruits and vegetables. The subjects also had higher serum levels of α-tocopherol, γ-tocopherols similar to those observed in US subjects, which reflects diets that are rich in vegetables, cereals and nuts [40]. This confirms the fact that these urban children are exposed to a varieity of foods because of their high income families they come from.

Some studies have used intrinsically labeled plant foods to accurately assess carotenoid absorption and conversion to vitamin A, thus determining the vitamin A value of a food source [9, 12, 28 and 38]. Plant carotenoids are intrinsically labeled with the addition of a hydrogen-stable isotope presented to the roots in the form of heavy water, [^2H_2O] via hydroponic growth on a nutrient solution composed of a fixed ^2H_2O percentage [35]. The isotopic label enables identification and separation of test food carotenoids and their metabolites in serum carotenoids from other serum carotenoids and retinoids. The kale used in this study had [$^2H_9$] β-carotene as the most abundant isotopomer (Figure 3), and this level of enrichment was found to be adequate to detect the labeled retinol metabolites formed from enzymatic cleavage of β-carotene in the body [28].

Previous studies in the US with adults using [^{13}C] labeled kale showed that kale β-carotene was bioavailable and was converted to vitamin A [21]. This study was aimed only at determining the bioavailability of kale carotenoids. Our study was designed to determine the vitamin A value of provitamin A rich kale, and to determine whether cooking it with peanut butter or lard (animal fat) had an effect on the absorption and bioconversion of the β-carotene to vitamin A in children. This study showed that peanut butter increased the absorption of kale vitamin A [^{2}H] retinol and [$^{13}C_{10}$] retinol compared to the lard (Table 2). In Zimbabwe, for adults, kale is cooked by sautéing or boiling with peanut butter. Unfortunately traditional child feeding practices do not promote the consumption of green vegetables by children aged 6-36 months. This group is particularly vulnerable to VAD. Our study showed that the kale β-carotene conversion factor to vitamin A was 13.4 ± 3.1 and 11.0 ± 3.9 to 1 by weight for lard and peanut butter cooked kale respectively. There was no significant difference between the total [^{2}H] retinol enrichment (nmole) of the LG and PBG and the conversion factors ($p>0.05$). The lack of significant differences in the conversion factors between the lard and peanut butter groups observed maybe was because the fat or oil content was matched between the groups 33 g (16 g fat) in peanut butter and 16 g in lard. Other explanation could be the smaller sample size of the study, high variability between subject retinol response kinetics and the better vitamin A status of the subjects. This study shows that the type of fat does not affect the vitamin A equivalency of a plant food. However, as mentioned earlier, it is interesting to note that the PBG had higher response for both [^{2}H] retinol and [$^{13}C_{10}$] retinol as compared to the LG (Table 3) though there was no statistical significance. This showed that peanut butter components increased the bioavailability of

vitamin A compared to lard which is mostly saturated fat. Maybe the type of fat (unsaturated fats in peanut butter) affects the bioavailability of vitamin A. More research is required in this area to fully understand the mechanisms. The β-carotene conversion factors of 13.4 ± 3.1 and 11.0 ± 3.9 to 1 by weight in kale are better than what has been observed in other studies. One study with Chinese pre-school children fed 275 mg β-carotene from green-yellow vegetables showed that 27 μg β-carotene from vegetables was equivalent to 1 μg retinol [30]. This conversion factor is similar to that reported in another study in Vietnam where subject ingested 5.6 mg β-carotene/d from green leafy vegetables, in this study 1 μg retinol was found to equivalent to 28 μg β-carotene [41]. Other studies also showed the conversion factors of 26 to 1, and 28: 1 in green vegetables [8]. Our study shows better conversion factors because we used advanced techniques of stable isotope labeled kale which are accurate in determining sources of vitamin A in serum and was conducted in children who have been shown to convert β-carotene to vitamin A better than adults. It is known that the food matrix play an important role in the bioavailability and bioconversion of β-carotene to vitamin A in humans [27]. The green leafy vegetables have thus been shown to have a food matrix that hinders the efficiently utilization of β-carotene to vitamin A [8-10]. Another important factor to consider is the vitamin A status of the subjects; subjects with adequate vitamin A status showing low conversion of β-carotene to vitamin A [42]. In our study 86% of the subject had marginal VAD, which might have contributed to the moderate β-carotene to vitamin A conversion factors of 13.4 ± 3.1 and 11.0 ± 3.9 to 1 by weight. Kale β-carotene to vitamin A conversion factors observed in this study are important for the development of complementary feeding guidelines in east and southern Africa where kale is a staple food.

This study showed that cooked pureed kale is a good source of vitamin A in children. When kale is cooked with peanut butter, it will not only provide vitamin A, but lutein, protein, vitamin E and fatty acids that are required by growing children.

The main limitation of this study is that we used super-child model approach. This approach has been used in other studies successfully [31, 39]. This study design was necessitated by an inability to collect multiple blood samples from an individual child for ethical reasons. Therefore, we constructed one AUC for each grouping, with SDs around the various points on the curve. These single group curves provide estimations for conversion efficiency for a group as a whole, but individually they are not suitable for the statistical analysis of differences in conversion efficiencies between the groups.

Conclusion

This the first study to show the conversion of kale β-carotene to vitamin A in children. We showed that kale β-carotene is a good source of vitamin A in pre-school children when consumed with peanut butter and lard. The conversion factors of 13.4 ± 3.1 and 11.0 ± 3.9 to 1 by weight show that kale can be promoted as complementary food for infant and toddlers who are often vulnerable to VAD. However, kale β-carotene cannot be depended on as the sole source of vitamin A, but can be used as part of a dietary diversification strategy to complement vitamin A intake from other source.

Acknowledgements

The authors would like thank all the subjects who volunteered to be in the study. We also acknowledge the logistical assistance in conducting the feeding trial in Bulawayo, Zimbabwe from the Bulawayo City Council especially the Department of Housing and Community Services. We also acknowledge the assistance from the teachers and directors of day care centers involved in the study. We also acknowledge Research Assistants who helped with the data collection process. The study was made possible with funding from the International Agency for Atomic Energy (IAEA) contract #CR 16989 and the United State Department of Agriculture (USDA) contract #58-1950-0-014.

References

1. Michaelsen, Kim Fleischer, and Henrik Friis. Complementary feeding: a global perspective. *J. Nutr 1998* 14 (10): 763-766.
2. Goodman, Tracey, Nita Dalmiya, Bruno de Benoist, and Werner Schultink. Polio as a platform: using national immunization days to deliver vitamin A supplements. *Bull. WHO* 2000, 78 (3): 305-314.
3. World Health Organization. Global prevalence of vitamin A deficiency in populations at risk 1995-2005. WHO Global Database on Vitamin A Deficiency. World Health Organization (WHO), Geneva
4. Ministry of Health and Child Welfare. Nutrition Unit. Zimbabwe national micronutrient survey: 1999. Harare, Zimbabwe: Ministry of Health and Child Welfare, 2001.
5. Beaton, George H., Reynaldo Martorell, Kristan A. Aronson, Barry Edmonston, George McCabe, A. Catharine Ross, and Bart Harvey. "Vitamin A supplementation and child morbidity and mortality in developing countries." *Food Nutr Bull* 1994, 15: 282-289.
6. Khan NC, West CE, de Pee S, et al. The contribution of plant foods to the vitamin A supply of lactating women in Vietnam: a randomized controlled trial. *Am J Clin Nutr* 2007;85:1112-20
7. Swift, Jeremy. Why are rural people vulnerable to famine? *IDS bulletin* 1989, 20, (2): 8-15.
8. de Pee S, West CE, Permaesih D, Martuti S, Muhilal Y, Hautvast JG. Orange fruit is more effective than are dark-green, leafy vegetables in increasing serum

concentrations of retinol and beta-carotene in schoolchildren in Indonesia. *Am J Clin Nutr* 1998*;* 68:1058-67.

9. Tang G, Qin J, Dolnikowski GG, Russell RM, Grusak MG. Spinach or carrot can supply significant amounts of vitamin A as assessed by feeding with intrinsically deuterium-labeled vegetables. *Am J Clin Nutr* 2005;82:821-8
10. Tang G, Gu X, Xu Q, et al. Green and yellow vegetables can maintain vitamin A nutrition of Chinese children. *Am J Clin Nutr* 1999; 70:1069-76.
11. Lietz, Georg, Jennifer Lange, and Gerald Rimbach. Molecular and dietary regulation of β, β-carotene 15, 15′-monooxygenase 1 (BCMO1). *Arch Biochem Biophys* 2010, 502 (10): 8-16.
12. Tang G, Qin J, Dolnikowski GG, Russell RM. Short-term (intestinal) and long-term (whole-body) conversion of *β*-carotene to vitamin A in adults as accessed by a stable isotope reference method. *Am J Clin Nutr* 2003; 78:259-66.
13. Muchuweti, M., A. Kasiamhuru, M. A. N. Benhura, B. Chipurura, P. Amuna, F. Zotor, and W. Parawira. Assessment of the nutritional value of wild leafy vegetables consumed in the Buhera District of Zimbabwe: a preliminary study. In *International Symposium on Underutilized Plants for Food Security, Nutrition, Income and Sustainable Development 806*, pp. 323-330. 2008.
14. Moustier, Paule. Urban horticulture in Africa and Asia, an efficient corner food supplier. In *XXVII International Horticultural Congress-IHC2006: International Symposium on Horticultural Plants in Urban and Peri-Urban 762*, pp. 145-158. 2006.

15. G J H Grubben; O A Denton; C -M Messiaen; R R Schippers; R H M J Lemmens. Vegetables. In Plant Resources of Tropical Africa, Volume 2: *Vegetables.*

16. Mariga, I. K., Lutendo Mativha, and D. Maposa. Nutritional assessment of a traditional local vegetable (Brassica oleracea var. acephala). *DOI: 10.5897/JMPR11.1426*

17. Mangels, Ann Reed, Joanne M. Holden, Gary R. Beecher, Michele R. Forman, and Elaine Lanza. Carotenoid content of fruits and vegetables: an evaluation of analytic data. *J Am Diet Assoc* 1993, (3): 284-296.

18. de Azevedo, Cristiane H., and Delia B. Rodriguez-Amaya. Carotenoid composition of kale as influenced by maturity, season and minimal processing. *J Agric Food Chem* 2005, 85 (4): 591-597.

19. Rock, Cheryl L., Jennifer L. Lovalvo, Curt Emenhiser, Mack T. Ruffin, Shirley W. Flatt, and Steven J. Schwartz. Bioavailability of β-Carotene Is Lower in Raw than in Processed Carrots and Spinach in Women. *J Nutr* 1998, 128 (5): 913-916.

20. Novotny, J. A., Kurilich, A. C., Britz, S. J., & Clevidence, B. A. Plasma appearance of labeled β-carotene, lutein, and retinol in humans after consumption of isotopically labeled kale. *J Lipid Res* 2005, 46 (9), 1896-1903.

21. Kurilich, A. C., Britz, S. J., Clevidence, B. A., & Novotny, J. A. Isotopic labeling and LC-APCI-MS quantification for investigating absorption of carotenoids and phylloquinone from kale (Brassica oleracea). *J Agric Food Chem* 2003, 51 (17), 4877-4883.

22. Mennella, J. A., Ziegler, P., Briefel, R., & Novak, T. Feeding Infants and Toddlers Study: the types of foods fed to Hispanic infants and toddlers. *J Am Diet Assoc*, 2006, 106 (1), 96-106.
23. Paul, K. H., Muti, M., Chasekwa, B., Mbuya, M. N., Madzima, R. C., Humphrey, J. H., & Stoltzfus, R. J. (2012). Complementary feeding messages that target cultural barriers enhance both the use of lipid-based nutrient supplements and underlying feeding practices to improve infant diets in rural Zimbabwe. *Matern Child Health J*. 2012, 8 (2), 225-238.
24. Mensah, P., & Tomkins, A. Household-level technologies to improve the availability and preparation of adequate and safe complementary foods. *Food Nutr Bull* 2003, 24 (1), 104-125.
25. Mosha, A. C., & Svanberg, U. Preparation of weaning foods with high nutrient density using flour of germinated cereals. *Food Nutr Bull*, 1983, 5 (2), 10-14.
26. Robertson, R. *Peanut Butter Planet: Around the World in 80 Recipes, from Starters to Main Dishes to Desserts*. 2006. Rodale.
27. Yeum, K. J., & Russell, R. M. Carotenoid bioavailability and bioconversion. *Annu Rev Nutr* 2002, 22 (1), 483-504.
28. Muzhingi T, Gadaga HT, Siwela A, Grusak MA, Russell RM, Tang G. Yellow maize with high β-carotene is an effective source of vitamin A in healthy Zimbabwean men. *Am J Clin Nutr* 2011; 94:510-19.
29. Erkkilä, A. T., Lichtenstein, A. H., Dolnikowski, G. G., Grusak, M. A., Jalbert, S. M., Aquino, K. A., & Booth, S. L. Plasma transport of vitamin K in men using deuterium-labeled collard greens. *Metabolism*, 2004, 53 (2), 215-221.

30. Tang G, Gu X, Xu Q, Hu S, Xu Q, Jian Q, Dolnikowski G, Fjel CR, Gao X, Russel RM, et al. Green and yellow vegetables can maintain vitamin A nutrition of Chinese children. *Am J Clin Nutr* 1999;70:1069-76

31. Haskell MJ, Lembcke JL, Salazar M,

Green MH, Peerson JM, Brown KH.Population-based plasma kinetics of an oral dose of [$^2H_4$] retinyl acetate among preschool-aged, Peruvian children. *Am J Clin Nutr* 2003; 77:681-686.

32. Yeum KJ, Booth SL, Sadowski JA, et al. Human plasma carotenoid response to the ingestions of controlled diets in fruits and vegetables. *Am J Clin Nutr* 1996; 64:594-602.

33. Riso, P., & Porrini, M. Determination of carotenoids in vegetable foods and plasma. *Int J Vitam Nutr Res* 1996, 67 (1), 47-54.

34. Tang G, Andrien BA Jr., Dolnikowski G, Russell RM. Determination of the β-carotene-d8 to retinol-d4 conversion in humans using APCI- and ECNCI-MS. *Methods Enzymol* 1997; 282:140-54

35. Putzbach K, Krucker M, Albert K, Tang G, Grusak MA, Dolnikowski G.Structur e determination of partially deuterated carotenoids from intrinsically labeled vegetables by HPLC-MS and ^{1}H NMR. *J Agric Food Chem* 2005; 53:671-7.

36. Tang G, Qin J, Dolnikowski G, Russell RM. Short-term (intestinal) and long-term (whole-body) conversion of β-carotene to vitamin a in adults as accessed by a stable isotope reference method. *Am J Clin Nutr* 2003; 78:259-66.

37. Tang, G., Qin, J., & Dolnikowski, G. G. Deuterium enrichment of retinol in humans determined by gas chromatography electron capture negative chemical ionization mass spectrometry. *J Nutr Biochem* 1998, 9 (7), 408-414.

38. Wang J, Wang Y, Zhixu Wang Z, et al. Vitamin A equivalence of spirulina β-carotene in Chinese adults as assessed by using a stable-isotope reference method. *Am J Clin Nutr* 2008; 87:1730-7.

39. Tang, G., Hu, Y., Yin, S. A., Wang, Y., Dallal, G. E., Grusak, M. A., & Russell, R. M. (2012). β-Carotene in Golden Rice is as good as β-carotene in oil at providing vitamin A to children. *Am J Clin Nutr* 2012, 96 (3), 658-664.

40. Van Kappel, Anne Linda, et al. Serum carotenoids as biomarkers of fruit and vegetable consumption in the New York Women's Health Study. *J Public Health* 2001**,** 4 (3): 829-835.

41. Khan NC, West CE, dePee S, Bosch D, Phuong HD, Hulshof PJM, Khoi HH,Ver hoef H, Hautvast JGAJ. The contribution of plant foods to the vitamin A supply of lactating women in Vietnam: a randomized controlled trial. *Am J Clin Nutr 2007*; 85:1112-20.

42. Haskell, Marjorie J. The challenge to reach nutritional adequacy for vitamin A: β-carotene bioavailability and conversion evidence in humans *Am J Clin Nutr* 2012, 96 (5): 1193S-1203S.

Table 1: Baseline characteristics of the subjects

	Lard	**Peanut Butter**	***p-value (0.05)***
N	20	17	NS
Girls	12	9	NS
Age (months)	29.7 (6.5)	32 (5.7)	NS
Weight (kg)	11.9 (1.8)	12.1 (2.0)	NS
Height (cm)	79.4 (11.0)	82.1 (11.7)	NS
cis Lutein	5.5 (2.4)	5.9(3.5)	NS
Lutein	35.3	36.7 (24.8)	NS
Zeaxanthin	6.5(2.9)	6.6 (4.8)	NS
Cryptoxanthin	17.3 (18.0)	14.9 (16.1)	NS
α-carotene	4.5 (2.9)	4.1 (1.7)	NS
Trans β-carotene	18.2 (8.1)	18.6 (10.4)	NS
Lycopene	31.0 (20.2)	38.1 (32.3)	NS
Vitamin A (Retinol)	44.7 (16.3)	44.0 (19.8)	NS
γ-Tocopherol	192.4 (142.1)	218.2 (146.3)	NS
α-Tocopherol	1309.6 (640.9)	1241.3 (731.9)	NS

Concentrations of serum carotenoids, vitamin A and vitamin E in mcg/dL

In parenthesis are standard deviations

NS presents $p>0.05$

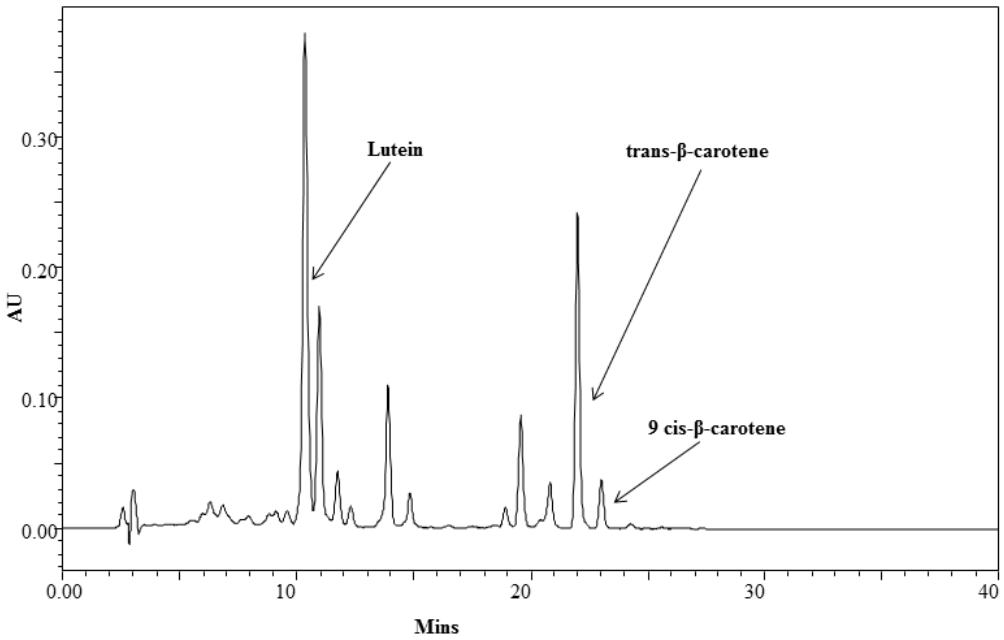

Figure 1: Carotenoid profile of *Brassica oleracea var acephala* (kale) using C30 HPLC column at 450nm wavelength. The first arrow to the left is pointing at lutein, the second and third arrow to the right are pointing at all-trans β-carotene and 9 cis β-carotene.

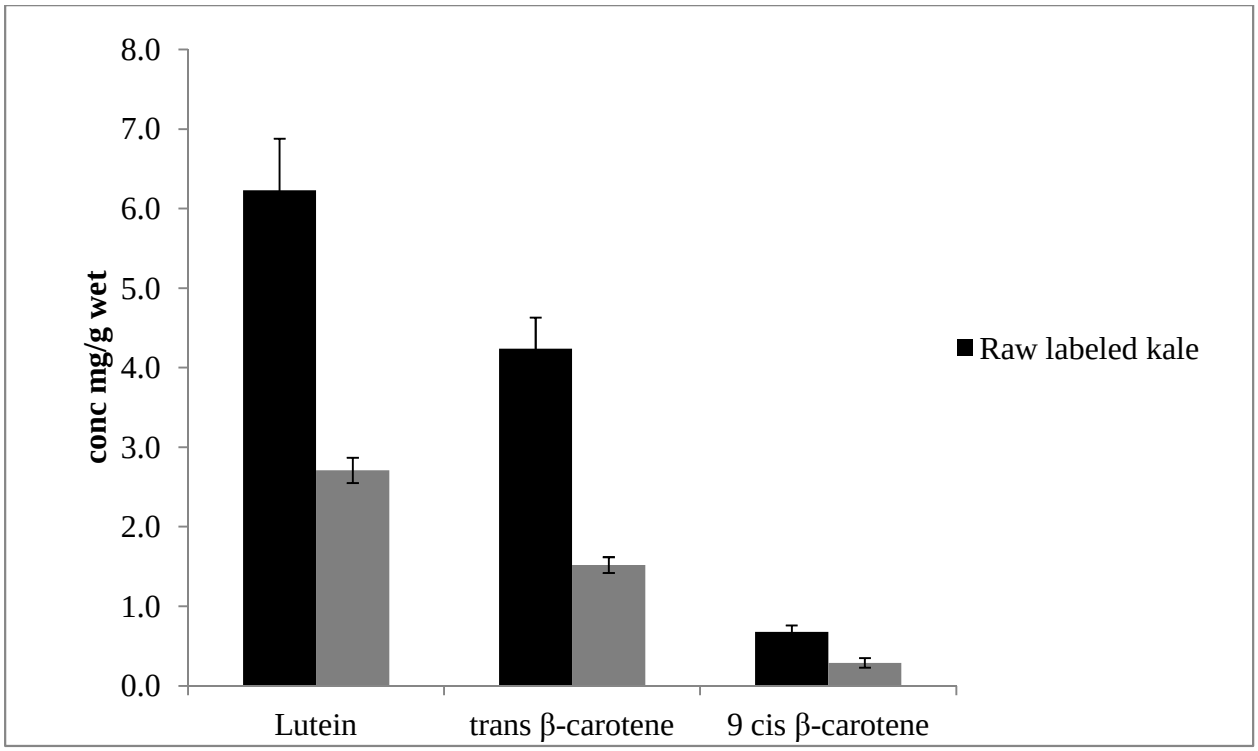

Figure 2: The carotenoid contents of raw labeled kale before cooking (raw) in black and cooked labeled kale in gray color. Values are means of three independent analysis and concentration are mg/g of fresh weight for raw kale and mg/g wet weight for cooked kale.

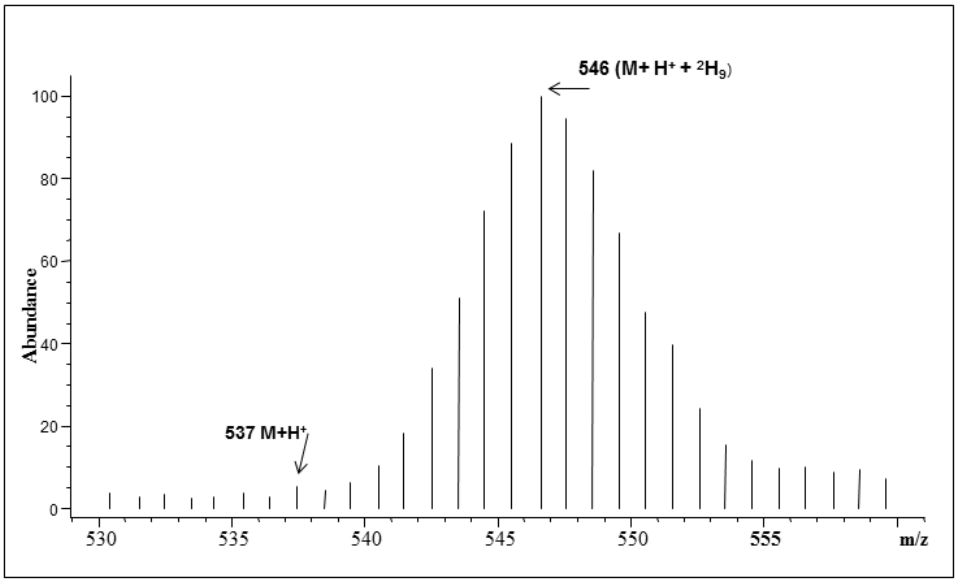

Figure 3**:** Deuterium enrichment profiles of kale by liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry (positive ion mode). The most abundant isotopomer of labeled β-carotene with 9 deuterium atoms is represented by a mass-to-charge ratio (m/z) of 546 ($M+H++^{2}H_{9}$). The first arrow on each profile points to the 537 peak, showing that the molecular mass of unlabeled β-carotene is 537 (M+H+). The second arrow on each profile points to peak 546 ($M+H++^{2}H_{9}$), showing the highest abundance of enrichment.

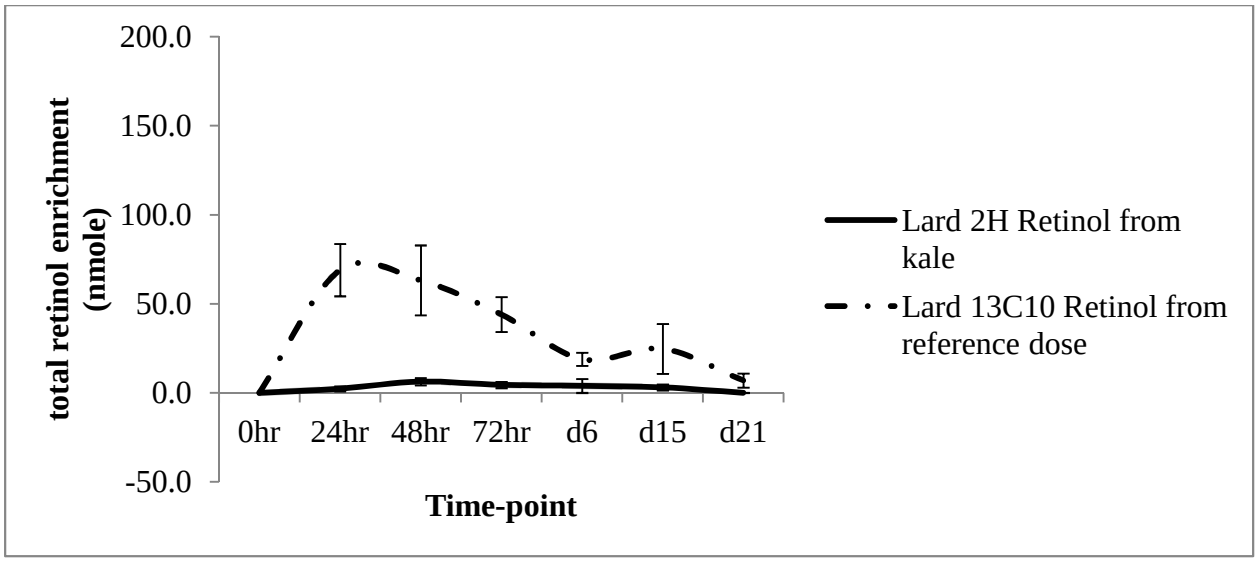

Figure 4a: Calculated labeled retinol in the circulation of pooled serum time-points after consumption of kale [$^{2}H_{9}$] β-carotene with 16g lard (animal fat) and a reference dose of [$^{13}C_{10}$] retinyl acetate on day 1. The continuous line and solid-circle data points show the serum [^{2}H] retinol response after consumption of kale [$^{2}H_{9}$] β-carotene and the dashed line data points show serum [$^{13}C_{10}$] retinol after consumption of a labeled reference dose of [$^{13}C_{10}$] retinyl acetate on day 1of the study. The retinol in circulation measured in nanomoles is shown on the y axis, and time in days is shown on the x axis.

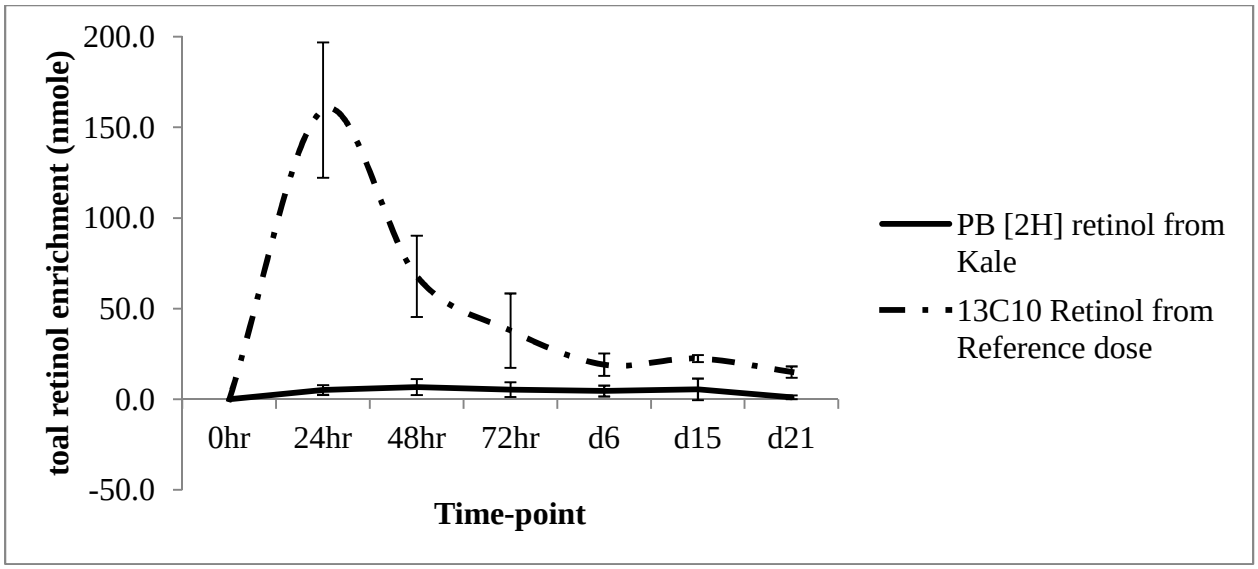

Figure 4b: Calculated labeled retinol in the circulation of pooled serum time-points after consumption of kale [$^{2}H_{9}$] β-carotene with 33g peanut butter and a reference dose of [$^{13}C_{10}$] retinyl acetate on day 1. The continuous line and solid-circle data points show the serum [^{2}H] retinol response after consumption of kale [$^{2}H_{9}$] β-carotene and the dashed line data points show serum [$^{13}C_{10}$] retinol after consumption of a labeled reference dose of [$^{13}C_{10}$] retinyl acetate on day 1of the study. The retinol in circulation measured in nanomoles is shown on the y axis, and time in days is shown on the x axis.

Table 2: The calculated AUC for kale [^{2}H] retinol, [$^{13}C_{10}$] retinol for LG and PBG and β-carotene to vitamin A conversion factors by weight

	AUC [^{2}H] retinol	Standard error	AUC [$^{13}C_{10}$] retinol	Standard error	Conversion factors by weight	Standard errors
Lard	56.19	10.53	432.63	54.88	13.4	3.06
Peanut butter	84.83	16.18	560.26	156.73	11.0	3.90
P-value	>0.05		>0.05		>0.05	

CHAPTER 7

SUMMARY

Discussion

The main aim of this thesis was to determine the role that antioxidants play in the vitamin A value of plant foods in child nutrition. We pursued three specific aims to address our general aim. The first aim was aimed at determining the carotenoid, vitamin E and γ-oryzanol contents of provitamin A enriched yellow maize and determining the antioxidant activity. The hypothesis was that provitamin A enriched yellow maize was a good source of carotenoids and antioxidants. The rationale was that children in east and southern Africa are fed starchy maize based complementary foods, the maize they consume is mainly white maize lacking provitamin A carotenoids. Therefore, the promotion of provitamin A rich biofortified yellow maize as a complementary food will increase the vitamin A intake, which may improve their vitamin A status. Determining the contents of antioxidants such as vitamin E and γ-oryzanol in biofortified yellow maize may improve the production of vitamin A from the enzymatic cleavage of β-carotene in the body as some research suggests. Our study found that provitamin A enriched yellow maize is very rich in provitamin A carotenoids, β-carotene and β-cryptoxanthin together with vitamin E, ferulic acid and γ-oryzanol. Analysis of variance and genetic by environment (G x E) tests showed that genotype was more important in determining the variation of provitamin A carotenoids. This is important because it shows that biofortified yellow maize hybrids developed at CIMMYT in Mexico can be planted in other environments and still maintains their provitamin A carotenoid contents. Therefore, when provitamin A enriched yellow maize is planted in different parts of Africa, the carotenoid content will not vary so much. This study was the first to genetically characterize γ-oryzanol, and tocotrienols in provitamin A enriched yellow

maize. These are important antioxidants in human health that may protect the body against free radical attack during disease or inflammatory states.

The second specific aim determined the genetic characterization of carotenoids in *Brassica oleracea* green vegetables grown and consumed in Zimbabwe. The hypothesis was that there were no significant differences in the provitamin A carotenoids of kale varieties grown in Zimbabwe and also those grown in the US. Kale grown in the US was found to be very high in provitamin A carotenoid β-carotene and lutein. Kale is a staple vegetable in Zimbabwe, but the vitamin A potential of kale is not being tapped in child nutrition. This may be due to lack of knowledge about the vitamin A value of kale by nutrition educators and mothers. This study showed that kale varieties grown and consumed in Zimbabwe such as *rugare, viscose, Chou Moellier* and *covo* are just as high in β-carotene contents just like the US grown kale. Therefore, the Zimbabwean kale should be promoted for consumption by children as a source of vitamin A in their complementary diets. However, it was important to prove that kale β-carotene was converted to vitamin A efficiently in Zimbabwean children.

The third was aimed at addressing the effects of antioxidants on the cleavage of β-carotene to vitamin A by the β-carotene monoxygenase enzyme (BCMO1). Since we want to promote provitamin A carotenoid rich kale as complementary food for children in Zimbabwe, it was important to show that kale β-carotene was converted to vitamin A and not other metabolites without vitamin A value. Since white maize is the currently the preferred complementary food for children, it was important to show that antioxidants in provitamin A enriched maize can promote the central cleavage of β-carotene to form exclusively vitamin A. By incubating kale and biofortified yellow maize extracts with rat

intestinal mucosal homogenates we showed that antioxidants such as vitamin E and γ-oryzanol inhibit the excentric cleavage of β-carotene, thus promoting the formation of vitamin A.

The fourth and final aim was to determine the effect of peanut butter on the conversion of kale β-carotene to vitamin A in children aged 12-36 months. The hypothesis tested was the kale cooked in peanut butter was effectively absorbed and converted to vitamin A. Intrinsically labeled kale grown hydroponically with heavy water produced deuterium labeled [$^{2}H^{9}$] β-carotene. Subjects consumed the deuterium labeled kale 50 g (1.5 mg β-carotene equivalency) with either 33 g of peanut butter or 16 g of lard and 1mg of [$^{13}C_{10}$] retinyl acetate reference dose. Area under the curve calculations of molar enrichments of serum retinol [^{2}H] from label kale β-carotene and [$^{13}C_{10}$] retinol from both the peanut butter and lard group showed that the conversion factors of kale [$^{2}H^{9}$] β-carotene was 13.4 ± 3.2 and 11.0 ± 3.9 to 1 by weight respectively. Our study was the first one to show the conversion factors of kale β-carotene to vitamin A in children. This study showed that kale β-carotene is effectively converted to vitamin A in the presence of 16 g of fat. However, using peanut butter as a source of fat can be used as complementary food because peanut butter increases the energy and nutrient density of the food.

In conclusion, kale and maize are staple foods in east and southern Africa. Provitamin A enriched maize should be promoted for complementary foods for children aged 6-36 months to provide vitamin A in their diets in the mornings. Feeding the children kale cooked with peanut butter for lunch or dinner will complement the vitamin A from other sources. These foods because they are widely available and consumed daily

will lead to improvements in the vitamin A intake by these vulnerable children. This in turn can lead to reduction in the prevalence of VAD and its devastating consequences on child health and development.

Strengths

1. Our study determining the genetic variation of carotenoids, vitamin E and phenolic compounds in provitamin A rich maize is the first to document the genetic variation of γ-oryzanol in biofortified yellow maize. A lot of research on γ-oryzanol has been conducted in rice and wheat bran but very little to no focus on maize as a source of γ-oryzanol. In rice bran the γ-oryzanol ranges from 2.5 - 6.7 mg/g dry weight while in biofortified yellow maize grains we found a range of 0.1 to 1 mg/g. This study also generated new data on the genetic variation of tocotrienols in biofortified yellow maize. Like γ-oryzanol, most research on cereal tocotrienols has been focused on rice and wheat germs, but very little research efforts on maize.
2. This study used HPLC to characterize for the first time the carotenoid profiles of kale grown and consumed in Zimbabwe. The carotenoid contents values of kale grown in Zimbabwe published in literature showed crude analysis which reported total carotenoids instead of individual carotenoid contents. This is because most research centers in Zimbabwe lack advanced chromatography equipment to individually analyze carotenoids in foods. Results from this thesis will increase

people's nutrition knowledge on the potential vitamin A value and lutein contents of Zimbabwean kale.

3. Our study was the first to use extracts of plant foods in incubations with rat intestinal mucosal homogenate to study the mechanism of β-carotene cleavage by the BCMO1 enzyme. Traditionally people have used purified standards of β-carotene. Our study is also the first the report the effects of γ-tocopherol, α-tocotrienol, γ-tocotrienol and γ-oryzanol on the cleavage of β-carotene. Our previous study showed the effects of α-tocopherol and this study went a step further.
4. There is one study published showing the bioavailability of intrinsically labeled kale β-carotene and its bioconversion to vitamin A in humans. This study was conducted with 8 US adults and it did not show the vitamin A equivalency of kale β-carotene. Our study is the first to show the bioconversion efficiency of kale β-carotene conversion to vitamin A in children. Our study is novel in that we tested a nutrient (β-carotene) and its meal components (peanut butter) to determine the vitamin A equivalency. This important because nutrients in the diet are consumed and absorbed together with other dietary components that may or may not affect their metabolism.

Limitations

1. We conducted in vitro studies with kale and biofortified maize with rat intestinal homogenate to study the effects of antioxidants on the β-carotene by the BCMO1

enzyme. In vitro studies are limited by their nature of being conducted in a test-tube and environment in the test tube is not similar to that in the body. It was going to be more appropriate to conduct an animal model using a Mongolian gerbil model which unlike rats and mice absorbs and cleaves β-carotene like humans. A study design like this would have been implemented. *Mongolian gerbils will be fed a powdered diet containing adequate vitamin A for 1 week. After a week adaptation, six randomly selected gerbils will be sacrificed and the remainder fed the diet without vitamin A for 6 weeks to produce marginal vitamin A status. After this depletion period, six randomly selected gerbils will be sacrificed, and the remaining gerbils will be randomly divided into six groups of 12 gerbils each and fed two genotypes of biofortified yellow maize as the only source of vitamin A: 1) Biofortified yellow maize genotype 1 alone, 2) Biofortified yellow maize genotype 1 plus additional α-tocopherol and γ-tocopherol, 3) Biofortified yellow maize genotype 1 plus additional α-tocotrienol and γ-tocotrienol, 4) Biofortified yellow maize genotype 2 alone, 5) Biofortified yellow maize genotype 2 plus additional α-tocopherol and γ-tocopherol, 6) Biofortified yellow maize genotype 2 plus additional α-tocotrienol and γ-tocotrienol. Six gerbils from each of the groups will be sacrificed after 3 week of consuming these diets, and the other six after 6 weeks.*

2. The second limitation of the study was super child model we used to determine the bioconversion of kale β-carotene to vitamin A. This was done because it is hard to collect multiple blood draws from a child required to plot the individual person β-carotene absorption and conversion kinetics. In the end average molar

enrichment of retinol at each time-point was used to estimate the vitamin A conversion factors. This data is useful at population level application but can be used on an individual basis.

Future Research Directions

1. It is important to conduct effectiveness studies of biofortified yellow maize porridge as vitamin A rich source complementary food in children. Effectiveness studies are conducted under real life settings unlike bioefficacy studies that are well controlled. The changes in vitamin A status in effectiveness studies will be evaluated by utilizing stable isotope dilution techniques to accurately estimate changes in body liver stores due to the intervention.
2. Effectiveness studies are also required to prove that high β-carotene kale is a good vitamin A complementary food for children. Changes in liver stores can be accurately be determined by use of stable isotope dilution techniques. Effectiveness studies allow program managers of nutrition interventions to develop targeted and appropriate messages to teach mothers and caregiver about kale vitamin A nutrition.
3. The key enzyme responsible for β-carotene conversion into retinal is β-carotene 15, 15′-monoxygenase (BCMO1). Some studies in western countries show that there is genetic variability in β-carotene metabolism. It is now known that the conversion of β-carotene into vitamin A is highly variable in up to 45% of healthy individuals, studies that show that genetic polymorphisms in the *BCMO1* gene

could contribute to the occurrence of the poor converter phenotype are required. It is important to characterize the genetic profiles of Zimbabwean subjects or African subject to determine the prevalence of no responders. This will help nutrition program manager to target plant food based vitamin A interventions to appropriate population where it will have an impact.

THE END